Handbook of indices of food quality and authenticity

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Preface

Assessment of food quality has all along been in terms of wholesomeness, acceptability and adulteration. Many ingenious approaches have been worked out and subjected to comparative investigations. Their reproducibility, sensitivity, simplicity and feasibility for routine application have been critically evaluated in collaborative studies and the methods approved have been selected as official. Several of these methods have withstood the test of time. The history of the Kjeldahl method for the determination of nitrogen and proteins in foods is unique as it has survived over a hundred years. During the recent past however several new challenges have been posed to the food scientist and the food analyst. Factors such as identification of botanical source and of geographical origin, the diverse and varying contaminations that enter through the environment including agrochemical residues and radioactive isotopes, the discovery of new health hazards, the manufacture and marketing of new formulations and blends, the chemical, biochemical and biologically induced changes in composition, chemical changes associated with processing including thermal, radiation, fermentation and such other treatments – all have come to the attention of food manufacturers as well as the enlightened consumers who have become aware of the implications of many of these factors in the success of the food processing operation, maintenance of quality and uniformity of the product and above all the wholesomeness of the food articles that reach the consumer. The traditional methodology for food analysis has begun to reveal inadequacy in tackling these and other such problems that have arisen continually.

At the same time since the mid-1970s there has been brisk activity in biological research from which novel analytical approaches have been emerging. The work on chemotaxonomy that began in a faltering manner a few decades earlier, has gained momentum and several new observations of far reaching importance to analysts have emerged. New molecules in the plant kingdom have been discovered as antinutritional or toxic phytoalexins, substances imparting resistance to pests. Many of these may be species specific or even variety specific. Most notable, however, is knowledge of molecular biology and genetics which has enabled the development of novel analytical applications based on the unique properties of proteins and nucleic acids.

It is therefore considered opportune to survey these trends and highlight the novel approaches that are opening up to the analyst and that are sure to help in solving the new problems that the food scientist is likely to face in the future. Problems arising in the area of major food commodities, the application of the new approaches and the direction of future work are indicated. It is hoped that this discussion will be fruitful in

inducing analytical researchers to reorient themselves to the new and varied parameters of food quality that are gaining prominence.

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Chapter 1

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The Development of the Concept of Food Quality, Safety and Authenticity

1.1 Diversity of composition

Since man began his experiments with cultivation of plants, over a thousand species of food plants have been intensively and extensively grown, studied, adapted, trained, varied, hybridized, grafted and mutated with a view to obtaining commercial crops with the desired appearance, size, composition, taste, flavour, functional attributes and adaptation to farming advances. This has resulted in literally hundreds of variants, cultivars of each species which are now being produced the world over. This entails a fairly wide range of composition and characteristics, too wide to define standards of quality. With the development and application of genetic engineering techniques the diversity in quality characteristics is being extended further. The creation of glandless gossypol-free cottonseed and low-erucic rapeseed are examples of such variants with drastically altered characteristics. No doubt the government agencies concerned have been trying to grade and classify each agrohorticultural produce so as to help in the trade and pricing of these commodities. Yet the task remains daunting. The need for such grading is being felt acutely with growing international trade and consumer awareness.

1.2 Food contaminants

During their journey from farm to consumer food commodities are likely to be exposed to a multitude of hazards that may lead to contamination by dust, dirt, weeds, mechanical injury, physicochemical changes accelerated by heat, light, metal ions, contamination or spoilage due to microorganisms, insects and rodents, or biochemical changes brought about by enzymes that may be endogenous or contributed by the invading biological agents. Food commodities are thus likely to undergo significant alterations. Even though the consumer preference is undoubtedly for farm fresh foods and farmers and traders have been striving to keep up the farm fresh image of food commodities, the question remains, how fresh? Amongst food grains, particularly oilseeds, which are seeds high in essential oils, the entry of weed seeds at harvest, especially if harvesting is mechanical, is a serious contamination if the weed seeds harbour toxicants like *Crotolaria, Datura* and *Argemone* for instance. Not only fruits

and vegetables but even seeds may undergo mechanical damage. In the case of high moisture commodities, this will most likely be followed by microbial infections and spoilage. In fat rich commodities such as oilseeds and nuts, oxidative chemical changes are most likely to be catalysed by exposure to air, elevated temperature, humidity, light and metal salt contaminants leading to rancidity. Such oxidative reactions affect essential oils and oil bearing materials adversely. Microbial spoilage of foods and health hazards to consumers through bacterial and fungal toxins and enteric diseases are especially associated with high moisture foods, animal foods in particular. Moisture pick up or loss depending on the relative humidity (RH) is another change that significantly affects the quality. Many foods undergo staling on storage: bread and coffee are good examples. In many countries, where feasible, specifications have been laid down for food commodities indicating the tolerances with respect to changes to their quality.

1.3 Food quality

Consumer preferences for foods have been known for ages. These are determined by geographic/regional, ethnoreligious, palatability and cost considerations. There are cases where preferences are modified by sociopolitical, cultural dominations causing taste transfer under the garb of modernism. Preferences are thus created for a food material from a particular region or particular brand. Yet, by and large, the consumer prefers to have good quality, pure, safe, authentic food. For the manufacturer, quality means a composite of attributes which are important to the commercial success of a food product. The manufacturers therefore prefer quality raw materials and their objective is to produce products of superior quality and uniformity employing a process standardized on the basis of a particular quality commodity. In fact, often the most suited raw material may be a particular variety grown in a particular region, harvested at a desired maturity. High-grown tea and coffee are universally preferred. Cocoa grown in Ghana and Nigeria is known to be superior. The origin of a food commodity has thus become greatly significant in determining its quality, its applicability and its price.

In spite of the several factors that influence the preferences of the consumer for food products enumerated above, one can single out the important consideration that 'the consumer likes it', and this is determined entirely on the basis of sensory perception. The sensory assessment depends on three principal considerations. First are appearance characteristics including colour, form, size, shape, integrity, transparency or opacity, gloss or shine, viscosity or consistency. Second are textural characteristics which may include handfeel, mouthfeel, bite, chewability, smoothness, body, juiciness, softness, stiffness, crispness. The third of the principal considerations includes flavour factors such as taste, odour, off-flavour. The quantitative assessment of these sensory attributes requires trained panels of judges who can minimise subjectivity and in this they can be aided by appropriate statistical methodologies. In some traditional family

enterprises such as wineries, breweries, tea plantations, cheese making units, expert tasters do the evaluation with due authority and with a considerable degree of objectivity. The plan and procedures for sensory evaluation of food products have been worked out in minute detail and methods for training judges have been developed.

1.4 Nutritive quality

Since food is needed by humans for the maintenance of normal health in adults and supporting standard growth in children, the nutritive quality of foods is an important aspect in evaluating foods. With the growing knowledge of human nutrition and its dissemination amongst educated consumers, demands have been made by consumers for nutrient details to be included on labels of marketed food products. Any nutritional claims made on the label need to be substantiated by data on nutrient content. The nutritive quality can be measured in terms of the content of the nutrients, proteins, calorigenic components, vitamins and minerals. Of late, the importance of nutritional fibre content has also been recognized. In the case of several nutrients, it is not merely the content but the bioavailability that is of prime importance and this may have to be estimated by bioassays with laboratory animals. Since proteins vary in their nutritive value in any food commodity the protein estimation has to be supplemented with data on protein quality. The nutrients differ in their stability to processing and storage conditions so that the consumer needs nutritional information about the final ready-to-eat food product. While formulating a food product and designing its label, therefore, all such relevant considerations have to be attended to. Methods have been developed for the measurement of the nutritive quality of foods in all these aspects.

1.5 Food safety

With the advancement in organic chemistry during the latter half of the nineteenth century hundreds of new compounds were being made synthetically and tested for possible applications. Saccharine was one such that proved to be a very active sweetener. Several synthetic dyes were invented. At the same time, the aetiology of several human diseases was elucidated to be due to bacterial infection and a search started for antimicrobial agents from amongst these synthetic compounds. With the knowledge that food spoilage is mainly caused by microorganisms, a logical corollary was to use such antimicrobial agents as food preservatives. Thus by 1833, creosote was recommended for preservation of meat. Boric acid was recognized as an antimicrobial in 1858 and salicylic acid by 1874. The toxicological implications of such chemical additives in foods were only gradually recognized. In fact, so many diverse preservatives and dyes were used during these times in food products that medical practitioners started expressing objections and demands were made to enact laws to prevent indiscriminate use of chemical additives in foods. This was the beginning of the realization of the hazards to consumer health from foods.

Advances in microbiology during this era, notably the observation of John Snow in 1840 (Boyd and Hoerl, 1977) that drinking water spreads cholera and William Budd's finding in 1856 that typhoid fever was spread by milk and water polluted with excretions of an infected person clearly established the role played by food and water in the spread of epidemics of enteric diseases. Food poisoning by *Salmonella* was discovered by Gaertner in 1888 and the cause of botulism as *Clostridium botulinum* was reported by Van Ermengem by 1896. The hazards in foods were manifested in cases of food poisoning and epidemics of enteric fever, dysentery, cholera and diarrhoea. In the course of the next few decades, other food borne infections were characterized as caused by staphylococci, *Clostridium perfringens*, *Bacillus cereus*, *Vibrio parahemolyticus*, *Aeromonas hydrophila*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Listeria monocytogenes*, serotypes of *Escherichia coli*, enteric viruses and parasites. Several bacterial enterotoxins causing acute effects and mycotoxins causing chronic toxicity including possible carcinogenic manifestations have been recognized as foodborne. These serious hazards from consumption of food products have necessitated strict control of microbiological quality of foods. Routine monitoring of total bacterial load, coliform counts and presence of staphylococci have become a necessity in food analysis.

1.6 Natural toxicants

Consumer organizations have been vocal about the hazards from synthetic additives in processed foods and health authorities have made it mandatory to screen thoroughly every contemplated chemical additive and to determine the acceptable daily intake (ADI).

However, during the period 1930–1960, the occurrence of several endogenous toxicants and antinutritional substances in native plant food commodities was discovered. These include proteinaceous protease inhibitors, hemaglutinins or lectins, vitamin and mineral binding macromolecules as well as non-protein small molecules such as cyanogenic glycosides, goitrogenic glucosinolates, favism-inducing pyrimidine derivatives, estrogenic isoflavones and coumestans, solanin, tomatin, gossypol. Even in marine foods toxicants such as the paralytic toxin in shellfish are occasionally encountered. Several compounds so far considered as innocuous such as phytates, oxalates, tannins and saponins, have been suspected of antinutritional action.

1.7 Problem of chemical residues

A variety of chemicals have been in use in modern agrohorticultural and animal husbandary practices and these chemicals may remain in the plant crops or animal foods at concentrations that may be hazardous to the consumer. Thus excess use of inorganic nitrogenous fertilizers in the soil may cause a rise in the level of nitrite or nitrate in the vegetative plant portions especially in the leafy vegetables. Weedicides,

insecticides, fungicides, rodenticides and sprouting inhibitors of diverse chemical nature are used in farm practices and pesticides and fumigants in storage warehouses. Residues of these compounds or their metabolites may survive in the foods. Anabolic steroids or their analogues and antibiotics have been used for fattening meat animals and poultry and milk releasing hormones have been used in dairy animals. Such treatments may leave residues in the flesh or milk. A close monitoring of the residue levels has now become necessary in view of the liberal usage of these treatments. In many countries, tolerances have been laid down for these chemicals in specific foods.

1.8 Problem of food adulteration

Food commodities have always been vulnerable to fraudulent admixture or adulteration with cheaper inferior materials. Such practices are revealed within countries when food materials are transported from the countryside to the urban centres. In international trade, such practices were noted in the eighteenth century when the UK and other European nations were importing spices, oils, oilseeds, honey, tea, coffee and such other materials from their colonies. Since wide variations in quality were suspected, the customs and excise department in England established analytical laboratories to check the purity of these commodities. Valuable research work was carried out by these laboratories to investigate the problem of adulteration, to lay down standard specifications and to devise analytical methods to detect and quantitate adulteration. Food adulteration during the nineteenth century was so rampant in the UK that it prompted Frederick Accum to write in 1820 that 'Indeed it would be difficult to mention a single article of food which is not to be met with an adulterated state and there are some substances which are scarcely ever to be procured genuine'. Accum and Fibly (see Fennema, 1985) have cited common cases of adulteration which are revealing. These included black pepper with gravel, leaves, twigs, paper dust, linseed meal, pea flour, sago, rice flour; cayenne pepper with vermillion (mercury sulphide), ochre (earthy mixture of metallic oxides and clay), turmeric; essential oil with oil of turpentine, other oils, alcohol; vinegar and lime juice with sulphuric acid; coffee with roasted grains, occasionally roasted carrots or scorched beans and peas, baked horse liver. A similar situation existed in other countries as well. At times, the adulterants were toxic as in the cases of mercuric sulphide, ochre, sulphuric acid mentioned above or the presence of lead chromate in turmeric and dimethylamino azobenzene or butter yellow, a hepatocarcinogen, in butter. Even now, the situation may not be any better in certain countries. The adulterators are quite innovative although unscrupulous.

1.9 Changes associated with processing

The epoch-making discovery of fire by humans unleashed their innovative capabilities in many directions both beneficial and destructive, yet the most important application

that revolutionized the lifestyle of human beings and in no small measure contributed to their health and welfare is the thermal treatment of food. Humans have learnt to cook, to broil, to steam, to bake, to toast, to roast, to fry, to smoke and to barbecue food on burning coal, wood or oil, transmitting heat through metal surface contact, by convection, by radiation, using steam or gas or fluids. To these can be added in modern times, to pasteurize, to sterilize, to use high temperature, short time (HTST) and ultra high temperature (UHT) treatments heating with infrared, microwave and ohmic heating. Each of these treatments can transform raw food commodities into speciality appetizing products with distinctive texture, taste, flavour and aroma.

Along with these culinary inputs the food could be decontaminated of biohazards, such as pathogens, toxins, endogenous toxicants and antinutritional substances. Its chewability and digestibility could be improved, and unpalatable off-taste and odour eliminated or minimized.

Yet gradually some ill effects of processing on food composition and wholesomeness started to come to light, for example the Maillard interaction between carbonyl and amino compounds and their subsequent cyclization and polymerization; caramelization reactions that lead to the formation of heterocyclic compounds, polycyclic aromatic molecules, nitrosamines and such other toxic, carcinogenic and mutagenic molecules during processing. Although these are known to be produced in trace quantities, the consumer has become aware of the lurking danger. Possible chronic cumulative effects are yet to be assessed. New analytical techniques with increased sensitivity and specificity are needed to monitor these molecules.

1.10 Conservation of excess produce

The revolution in methods of farming including animal husbandry, dairy farming, fishery and aquaculture along with modern techniques of food conservation has now been rewarded by occasional seasonal excess food production. This necessitates application of established and novel technologies for the preservation of this excess food. The following ways have emerged for conserving such glut production for later use, by reconstitution if necessary storage of seeds after drying; insect disinfestation by conventional methods or by irradiation and packaging; use of low temperatures, of freezing, of controlled gaseous atmosphere for storage of high moisture foods, and of dehydration of milk, fruit and vegetables by fluidized bed, roller, spray or freeze-drying methods; separation of cream and skim milk and stabilizing these separately; and bulk preservation with chemicals, by thermal processing, by salting, pickling, by aseptic packaging or by use of low dose radiation. From using only farm fresh raw materials, the food industry has strayed far, learning to conserve food and use it in food products after appropriate treatment. No doubt, this is the only logical way of using precious food material, with nutrients retained as far as possible. Can food materials that have undergone preprocessing be identified? Do handling, preprocessing and storage treatments leave fingerprints in the food materials? This is yet another

challenge that the food analyst has to accept. The exposure of milk to excess microbial load before it is pasteurized is known to be reflected in the high pyruvate concentration. The use of skimmed milk powder and butter oil to reconstitute liquid milk may be traced to the high level of free ammonia.

1.11 Evolution of food legislation

The wide prevalence of deceitful practices in the trade of food articles with respect to their quality attracted the attention of the excise department in the UK during the early eighteenth century when statutes were introduced for tea and coffee and which linked the tariff with the alcohol content of beer, wine and other alcoholic beverages with the sole objective of protecting the revenue. However, the possible health implications of the indiscriminate use of chemicals as preservatives, colourants and improvers for food products being suggested by clinical practioners with increasing frequency prompted Wakley, the editor of Lancet, to establish the Lancet Sanitary Commission to survey frauds in the food supply. The reports of the first ever scientific enquiry of this nature under Dr. A. H. Hassal published in the Lancet during 1851–1854, created a sensation in England and caused the government to set up The Select Commmitee on Adulteration of Food in 1855. The world's first Food and Drink Act, passed in 1860, was the outcome. This was revised in 1875 as the Sale of Food and Drugs Act along with the foundation of The Society of Public Analysts in 1874 with the onerous responsibility of laying down standards for foods and developing methods of analysis. The responsibility for monitoring the safety of food supply in the UK was entrusted to a steering group of food surveillance in the early 1980s linked to Ministry of Agriculture, Fisheries and Food (MAFF) in conjunction with the Department of Health, Department of Trade and Industry and recently the Department of Environment.

This movement gradually spread to other countries. New Zealand passed the Pure Food Legislation in 1866 and Canada the Food and Drug Law in 1874. The Food Act in 1884 in the UK provided legal control over food manufacture. In 1902, Dr. H. Wiley of the United States Department of Agriculture (USDA) set up a 'Poison Squad' to evaluate the safety of common food preservatives and ingredients. These efforts led to the passing of the Pure Food and Drug Act of 1906. By 1931, the Food and Drug Administration (FDA) was formed within the USDA and in 1938, the Federal Foods, Drugs and Cosmetics Act was passed in the USA. FDA in the USA is responsible for the wholesomeness of foods on the market. In this they are assisted by other agencies like the Department of Health and Human Services, the Department of Agriculture Inspection, the Department of Commerce and the Environmental Protection Agency (EPA). The Delaney amendment brought a severe curb on the use of additives. The addition of any substance that exhibits carcinogenicity in any animal species at any dose is not to be permitted in foods according to this amendment. Prohibitions came accordingly on the use of such long accepted substances as saccharine and cyclamate.

Several synthetic colours were omitted from the permitted list of colours. Systematic work on re-evaluating the toxicity of accepted additives was initiated.

These mandatory requirements on the safety of additives also necessitated studies on the methodology of testing safety. The failure of the then current procedures for screening drugs for hazards in the case of the tranquillizer, thalidomide, which proved to cause teratogenic effects on foetuses, and attempts to employ new high energy technologies for food processing, in particular radiation processing, raised the need to evolve a new foolproof schedule of methods for screening drugs and food additives. Thus, apart from assessing acute and chronic toxicity, methods were developed to chart out complete pharmacological action, to test for carcinogenicity in animals, mutagenicity or induced aberrations in the chromosomes in tissue culture systems and microorganisms as in the Ame's test, and for teratogenicity and effects on the reproductive system as well as foetal development in multigeneration screening studies.

In 1962, the Codex Alimentarius Commission was established for implementation of the Joint FAO/WHO standards programme. The aims of Codex Alimentarius include protecting the health of the consumer, ensuring fair practices in the food trade, coordination of all food standards work, publishing regional and world standards, recommending international standards for individual foods and making provision with respect to food hygiene, contaminants, additives, labelling and so on. The work is done by committees run by several member countries. The Codex recommendations are often used by bodies like the European Union (EU) to formulate their standards.

According to the pure food legislation in many countries, food is considered adulterated when the food article: consists of any filthy, putrid, decomposed or diseased animal or vegetable material; is insect infested or unfit for human consumption; is prepared, packed or stored under insanitary conditions; contains any poisonous ingredients; has been substituted by any inferior or cheaper substance; has had any constituent abstracted; is packed in a container of any poisonous or deleterious substance; has any unpermitted additive or has a permitted additive present in an amount exceeding the prescribed limit; consists of a quality falling below the prescribed standard; or is not as purported or claimed.

1.12 Current methods of food analysis

Since the establishment of analytical laboratories by the customs and excise authorities in the UK to check the quality of imported food commodities and especially after the foundation of the Society of Public Analysts in 1953, over the last century and a half, pioneering work has been carried out by these and other such organizations worldwide. The Association of Official Analytical Chemists (AOAC) particularly has developed standard specifications for food commodities and manufactured products and methods for evaluating food samples to enable comparison with such standards. These methods, now accepted as official or standard, may be categorized as physical,

instrumental, chemical, nutritional, microbiological and sensory analytical methods.

The physical methods include microscopy for histological examination of plant or animal tissues, starch granules, pollen grains and crystal structures to determine the species of origin of the source material and applied to, for example, spices, grains, animal flesh or honey. Other physical methods determine specific gravity, optical rotation and refractive index in the case of liquid foods such as edible oils, fats, syrups, honey and essential oils and the freezing point in the case of milk and fruit juices.

Although a very wide variation has been observed in the gross chemical composition of food samples over the large number of varieties, types and cultivars of a single species cultivated in different regions and seasons, with different farming practices, the proximate composition gives an indication of possible adulteration and a rough idea about the nutritive quality in terms of caloric value and protein content. In some foods such as tea, coffee, cocoa, alcoholic beverages, spices and condiments, the active principles can be characterized and quantified by physicochemical methods. The colour produced in a specific chemical reaction by an active ingredient measured on a colorimeter, or better by a spectrophotometer, gives a quantitative measure. For chemical, biochemical, microbial or insect spoilage of foods, chemical methods have been developed. Similar microcolorimetric or fluorimetric methods are available for the estimation of vitamins, antinutritional agents and minerals. The latter can also be determined by gravimetry, titrimetry and complexometry procedures. The estimation of toxic metals including lead, arsenic, antimony, cadmium and mercury and contaminants such as tin, zinc, aluminium, chromium is an important application. Advances in biochemical analysis of body fluids and tissues using techniques such as chromatography of all types, electrophoresis, enzyme assays, UV, IR and NMR spectroscopy and atomic absorption spectroscopy have been applied to food analysis.

Apart from chemical methods, vitamins and amino acids in foods can be estimated by microbiological methods. Vitamins occur in foods partly in bound form and have to be liberated by treatment with certain enzymes before such assays. In the case of vitamins and minerals, it is not merely the total content that is important nutritionally, but also their availability. Bioassays with experimental animals or human volunteers can only give a good estimation of their nutritive value. In the case of proteins, an estimation of nutritive value by bioassay is conducted and translated into protein efficiency ratio (PER), net protein utilization (NPU), and biological value (BV). Similarly the nutritive value of fats is measured not only in terms of the energy that they provide, but also on the basis of their content of essential fatty acids, linoleic, linolenic, arachidonic and other polyunsaturated fatty acids.

Many of these traditional physicochemical methods have been automated, for example, the Kjeldahl method for protein, autoanalysers for chemical colorimetric analyses, automated proximate analyses based on near infrared (NIR) spectroscopy for routine analyses of specific commodities like wheat and milk and automated microbial cell counting.

1.13 New techniques for food analysis

Since the mid-1970s rapid advances have taken place in molecular biology and genetics and applications of these to clinical analysis for rapid diagnosis, which was treated as a priority, have led to the development of simplified, rapid and, where possible, automated methods and kits. With the success in the application of these approaches in clinical diagnostics, their use has been applied actively in food analysis to the identification of species, varieties, geographical origin, admixtures and adulterations, microbial pathogens and contaminants of starter cultures and so on. The new techniques of genetic engineering, of gene isolation, splicing, introduction into recipient bacteria, cloning, the hybridoma technique, and use of recombinant DNA, have facilitated this approach greatly. The result is success in the application of immunochemical techniques, biosensors, DNA probes and the polymerase chain reaction for rapid and foolproof analysis of foods.

Chemical methods for the determination of trace molecules in foods have now been strengthened with the introduction of more specific enzymic methods suitable for automation. At the same time the association of distinctive enzymes with different species or varieties and stability differences amongst enzymes of different origins are under investigation for possible use as indices of authenticity.

Study of plant intermediary metabolism has brought to light C_3 and C_4 pathways of carbon utilization which predominate in different plant species. An ingenious use of knowledge of these pathways has been made in tracking the origin of sugars by the use of radioactive carbon labels, since the ^{13}C content of sugars formed by the C_4 pathway is significantly higher than those formed by the C_3 pathway in plants. Similarly the origin of ethanol whether natural or synthetic can be ascertained by isotope ratio mass spectrometry (IRMS) and site specific natural isotope fractionation measured by NMR (SNIF–NMR). These techniques can give valuable information about the origin of compounds and hence about possible adulteration.

The traditional parameters of quality such as content of sugars, organic acids, amino acids and mineral elements in foods determined by statistical analysis such as principal discriminant analysis (PDA), response surface methodology and nearest neighbour analysis (KNN) and so on, have been proving relevant in predicting the quality, authenticity and degree of adulteration in many foods. Some of these new techniques are described briefly here along with their possible applications. Applications of some of these novel procedures for the identification of fish species serve as an illustration.

1.13.1 Enzymes as indicators of food quality

Enzymes are not inactivated after harvest or slaughter and act as factors detrimental to food quality as assessed in terms of colour, flavour, aroma, texture and nutritional value. Superior quality food can be obtained when heat treatment is applied just sufficient to inactivate the most crucial enzymes responsible for deterioration. For each

product, specific changes are responsible for deterioration in its quality. Generally, polyphenoloxidase, chlorophyllase, lipoxygenase, lipase, esterase and protease may be responsible for colour, flavour and aroma changes. Pectic enzymes, cellulase and hemicellulase may be responsible for texture changes in plant materials. Thiaminase, ascorbic acid oxidase or other oxidoreductases may cause a loss of nutritional quality (Whitaker, 1991) in terms of some vitamins, or essential fatty acids. These can act as indicator enzymes. Among these, in the food industry, peroxidase and alkaline phosphatase, the most heat stable enzymes, found in raw fruits and vegetables and milk respectively, have been used for testing the efficacy of blanching and pasteurization. In fact heat treatment sufficient for their inactivation can lead to overheating of the sample, leading to loss of quality. Williams *et al.* (1986) have pointed out that neither enzyme is directly involved in detrimental changes and the only reason the test works is that they are the most heat stable enzymes. It has been proved that best quality frozen, stored peas, green beans, cauliflower and Brussels sprouts can be obtained when 6.0–6.3%, 0.7–3.2%, 2.9–8.2% and 7.5–11.5% respectively, of peroxidase activity remains at the end of blanching (Bottcher, 1975). Off-flavour development and colour loss in green beans, peas and corn is caused by lipoxygenase. Aroma deterioration in broccoli and cauliflower is by cystine lyase (Velasco *et al.*, 1989; Whitaker, 1991). Textural changes and loss of cloud in citrus juice is caused by one or more of the pectic enzymes. Polyphenol oxidase is responsible for brown and black colour development in peach, apricot, apple and so on, while lipoxygenase is responsible for green and yellow colour losses in vegetables (Whitaker, 1994).

Thus there is a need to pinpoint the indicator enzymes in each food and develop simple sensitive methods for their assay to be adaptable by the industry for routine on-line quality assessment.

A simple method of monitoring lipoxygenase activity by the use of filter paper strips can detect as little as 1–2% residual enzyme in 1 to 5 min (Adams *et al.*,1989). Attempts are also being made to develop an assay method for cystine lyase in broccoli and cauliflower.

Fully automated enzyme assay systems based on modern techniques have great potential (Whitaker, 1991) and can therefore assist in monitoring the quality of foods. It is expected that in coming years, the food industry will adopt more specific enzyme indicators of quality, if assay methods are available. Biosensors could be developed for this purpose as a more acceptable alternative.

1.13.2 Biosensors in food analysis

Biosensors are analytical devices containing a biological recognition element like an enzyme, antibody or microbe coupled to a chemical or physical transducer including an electrochemical (electrode), mass (piezoelectric crystals or acoustic wave devices), optical (optrodes) and thermal (thermistors or heat sensitive sensors) detector. They can be designed to match individual analytical requirements for any organic or

inorganic molecule which can interact in any way with a biological system. Several biosensors based on fluorimetric and luminometric fibre optic detection and in the form of whole cell and tissue systems, enzyme electrodes, piezoelectrode immunosensors and enzyme thermistors have been prepared and studied by researchers. Biosensors can have applications in proximate analysis and analysis of pesticide residues, naturally occurring toxins and antinutrients, processing changes, microbial contamination and in monitoring enzyme inactivation.

Wagner (1994) has discussed the use of biosensors in food analysis. Besides analysis of food constituents like carbohydrates, peptides, amino acids, vitamin C, ethanol and lactate, biosensor applications have been reported for analysis of contaminants like fluorine and penicillin, and for complex parameters like fish freshness using xanthine oxidase to measure IMP (inosine monophosphate) (Watanabe *et al.*, 1988) or xanthine/hypoxanthine ratio (Thomas, 1988) or biogenic amines (Suzuki *et al.*, 1992), and taste measurements based on IMP, *l*-glutamate, *l*-lactate, odour, Brix and *l*-lactate oxidase, glutamate oxidase and xanthine oxidase (Asano *et al.*, 1992). A survey of commercially available biosensors and new trends suggests that the existing shortcomings of biosensors will soon be overcome effectively. With the availability of standardized methods for sample preparation these inexpensive, rapid and simple tools of analysis are no doubt going to gain popular application in quality control in the food industry.

1.13.3 Immunochemical techniques

An immunoassay utilizes for analytical purposes the reaction between an antibody or fragment thereof produced in response to a characteristic antigen and the biological material containing the antigen. Application of immunoassays to foods or food components has been comparatively delayed, though in recent years great interest has been generated in this area. A number of immunoassay kits are now available for food applications. The tests are semiquantitative or quantitative. They have been in use for low molecular weight analytes like mycotoxins, pesticides, natural toxicants like solanin (Morgan *et al.*, 1985) or proteins like gluten, allergens (Allmann *et al.*, 1993), staphylococcal enterotoxins or pathogens like *Salmonella* and *Listeria*. In fact the applications can be unlimited. Many immunoassays have been validated through internationally recognized collaborative testing programmes. The reviews by Lee and Morgan (1993) and Paraf (1992) detail these assays.

Investigations on bioavailability and bioactivity of different vitamins using procedures based on antibody probes with high sensitivity, is an area which needs to be studied carefully. Immunoassays for pesticide detection and quantitation have started to emerge. A new development in the form of recombinant antibodies offers good potential. It has been successfully used in pesticide analysis and offers opportunities in food analysis.

There is no doubt that most chemical assessment of food quality is going to be

replaced by immunochemical techniques in the near future. Monoclonal antibody (MAb) technology is becoming readily available which provides the benefit of substituting the need for using live animals to raise the antisera. Monoclonal antibodies can be useful in detecting structural modifications of food proteins in the presence of components of a complex food matrix. Specific applications in quality control of foods include authenticity testing, detection of adulteration and modification of protein structure during thermal processing. There is a need within the food industry to control the fraudulent substitution of less expensive proteins for higher priced declared ingredients. Specific monoclonal antibodies (MAbs) raised against the suspected fraudulent additive can be useful for such testing.

Ideally the target molecule or epitope chosen for use in developing MAbs for food applications should be thermostable. Thus thermostable muscle antigens (Kangethe *et al.*, 1984) may be the most appropriate regions to target when raising MAbs for speciation of heat processed meat products. Also MAbs raised against milk protein, k-casein, which is thermoresistant would be useful in analysis of milk products. Two MAbs have been successfully isolated that are specific for soya proteins, useful for identifying the soy proteins in meat products (Carter *et al.*, 1992).

MAbs used in indirect enzyme linked immunosorbent assays (ELISA) tests have been found to identify polymerized heat denatured ovalbumin better than native ovalbumin while some of those used in sandwich ELISAs are suitable only for native ovalbumin (Varshney *et al.*, 1991). A set of MAbs with overlapping specificities for native and denatured ovalbumin has been identified that is capable of determining the approximate temperature to which the protein has been exposed (Paraf and Mahana, 1990). The AOAC has adopted some of the immunoassay techniques or test kits as official analytical methods. These include Agriscreen ELISA for detection of aflatoxin B_1 in corn and roasted peanuts and *Salmonella* TEK, a colorimetric enzyme-linked MAb immunoassay screening method and a fluorescent antibody screening method for detection of *Salmonella* in foods. There is a great promise for the development of monoclonal antibodies against antigens of special interest in food quality control in the coming years.

1.13.4 DNA probes

A DNA probe is a fragment of DNA. Each probe can be used to reveal the presence of a specific 'target' DNA sequence within the rest of the organism's DNA. The complementarity or base pairing between polynucleotides is the basis of hybridization (recognition and binding) of the probe and its target (complementary) DNA. Probes are labelled to allow the investigator to determine when they have hybridized with the target sequence. Labels include radioactive ^{32}P, enzymes and molecules such as biotin which are easy to detect. The ability of DNA probes to detect and characterize specific organisms and materials derived from them is being applied to problems in the food industry. These include the detection and assessment of foodborne pathogens, and

authenticity testing of meat products, plant materials and so on. The application of DNA probes in food analysis has been adequately reviewed (Leighton, 1991). Probe-based methods have been developed for detection and enumeration of foodborne pathogens like *Salmonella* (Fitts, 1985), *Staphylococcus* species (Notermans *et al.*, 1988), *Listeria* spp. (Herman and Ridder, 1993; Klinger *et al.*, 1988) and hepatitis A virus (Metcalf and Jiang, 1988). Lactic bacteria in wines and grape must (Lonvaud-Funel *et al.*, 1991) and *Vibrio vulnificus* in oysters (Wright *et al.*, 1993) have been detected by using DNA probes. *L. monocytogenes* in artificially inoculated soft cheese and ground chicken have been successfully detected using a hydrophobic grid membrane filter DNA probe (Peterkin *et al.*, 1992).

Probes for typical plant pathogens like avocado sun blotch viroid, potato tuber spindle viroid (Mcinnes *et al.*, 1989) and *Erwinia amylovora* (Falkenstein *et al.*, 1988) can replace lengthy bioassays and give a clearcut diagnosis of these plant diseases. Probes for livestock pathogens like virus of poultry (Cavanagh, 1989) have been produced and used. All these have great potential for incorporation into kits which will replace the existing methods for pathogen analysis.

The laborious practices of plant and animal breeding aimed at incorporating desirable characteristics are now being assisted by DNA probes to detect molecular markers called restriction fragment length polymorphisms (RFLPs) which can be used by breeders as guides to the selection of appropriate organisms. Breeding of plants (Melchinger, 1990), animals (Hope, 1989) and edible fungi (Castle *et al.*, 1987) is being tried using RFLPs.

Identification of a particular species or cultivar has been possible using DNA probes. The dot blot meat speciation test based on a DNA probe can identify the origin of meat (Chikuni *et al.*, 1990). Typing of yeasts relevant to wine and baking industry (Walmsley *et al.*, 1989), identifying species of edible mushrooms (Castle *et al.*, 1987) and individual cultivars of rice (Dallas, 1988) have also been reported using DNA probes. All these studies suggest that many more applications of DNA probe assays will be possible in the food area. However, developing means of extracting recognizable target DNAs from different types of foods in raw and processed forms is essential for using probes in detection of genetically engineered material in foods, the sex determination in meat and assessment of DNA fragmentation as a possible marker of food irradiation (Leighton, 1991).

1.13.5 Polymerase chain reaction

Methods based on the polymerase chain reaction (PCR) have also proved to be very efficient and applicable in foods. Rapid and direct determination of $>10^2$ cfu g^{-1} (colony forming units per gram) of *Brochothrix* spp. in meat samples was possible using a DNA-based PCR assay within one working day (Grant *et al.*, 1993). Detection and identification of pathogens like *Shigella sonnei*, *S. flexneri*, *S. boydii*, *S. dysenteriae*, *Salmonella paratyphi* A & B, *Aeromonas hydrophila*, *Staphylococcus aureus*, *Clostridium*

perfringens (Sawada *et al.*, 1992), type A *Clostridium botulinum* in canned green peas, corn and lima beans (Ferreira *et al.*, 1993), *Vibrio cholerae* in seafoods (Koch *et al.*, 1993), enteric virus in oysters (Atmar *et al.*, 1993), toxigenic *S. aureus* in beef, pork, cheese and milk (Tsen and Chen, 1992), enteroinvasive *E. coli* in raw milk (Keasler and Hill, 1992) and *Listeria monocytogenes* in poultry (Wang *et al.*, 1992; Rossen *et al.*, 1991) have been successful using PCR. A detailed review on detection of foodborne pathogens by PCR is available (Harris and Griffins, 1992). The minimum detectable levels of organisms, type of interferences associated with each food sample, precautions to be taken in sample preparation, need for pre-enrichment and correlation of the results with the conventional methods are all important considerations in standardization of the methods.

Undeclared wheat addition to cereal food products can pose serious health problems to people suffering from wheat allergies. A rapid sensitive analysis of food samples determining wheat contamination has been established using PCR methodology. It can support and confirm the analysis and characterization by immunoenzymatic methods (Allmann *et al.*, 1993).

1.13.6 Rapid methods for microbiological analysis of foods

Isolation, early detection, enumeration and characterization of microorganisms and their metabolites in different types of samples including foods have been improved with the development of rapid methods and automation. Extensive and rapid progress has been made in improving the sampling and sample preparation techniques and detection procedures. For enumeration of microorganisms, radiometry, microcalorimetry, ATP (adenosine triphosphate) measurement, limulus amebocyte lysate (LAL) test or use of the direct epifluorescent filtrate technique have also been available. For identification, use of techniques like enzyme linked immunosorbent assays (ELISA), polymerase chain reaction, use of DNA probes and magnetic beads or commercially available kits have been practised. These methods have an important application in food quality control. The newer developments in this field have been reviewed by Fung (1992). These include a 'gravimetric diluter' which automatically prepares accurate dilutions and delivers required volumes to obtain the desired dilution (Manninen and Fung, 1992). The automatic spiral plating system and laser colony scanner have been tried on different samples successfully. The double tube method has been shown to be very useful for enumeration of *Clostridium perfringens* in ground beef and turkey (Ali and Fung, 1991).

Using selective dyes, identification and enumeration of particular species are shown to be possible (Goldschmidt *et al.*, 1991). An enzyme patented under the name 'oxyrase' has been reported to stimulate rapid growth of facultative anaerobic food pathogens like *Listeria monocytogenes*, *Salmonella typhimurium* and *Staphylococcus aureus* after which they can be detected by rapid diagnostic methods (Yu and Fung, 1991a, 1991b, 1992).

The possibility of enhancing the speed of detection of other microbes is also being studied. The colorimetric detection technique employed by the 'Omnispec Bioactivity Monitor System' (Wescor Inc., UT, USA) is proved to simplify the analysis by saving labour, materials and has high sampling capacity.

Rapid methods are now a part of quality control programmes. An automated conductance method for detection of *Salmonella* in coconut, fish meal, prawns, non-fat dry milk, liquid egg and minced beef has been found to be equal to the AOAC official BAM (Bacteriological Analytical Manual)/AOAC method (Gibson *et al.*, 1992). A unique enzyme, deoxyguanosine 5′-triphosphate (dGTP)–triphosphohydrolase (EC 3.1.5.1), seems to be confined to members of the family Enterobacteriaceae and can act as its indicator (Quirk and Bessman, 1991). In spices and nuts latex agglutination assay has been found to be reliable for rapid detection of moulds (Kamphuis *et al.*, 1989).

Such methods are being adapted slowly for research and quality programmes. With the development and availability of kits for such analysis these methods have a bright future in microbial quality assessment at the raw material stage at a rapid rate for on-line process control as well as finished product quality control.

The minimum detection level of organisms, types of interferences associated with each food sample, precautions to be taken in sample preparation, need for pre-enrichment and correlation of the results with conventional methods are all important considerations in standardization of these methods.

1.13.7 Authentication of foods using isotopic methods

The isotopic content and distribution in molecules from plants and animals are influenced by climate, the isotopic distribution in the nutrients absorbed and the metabolic pathways involving the molecules. It is therefore well recognized that as regards the products synthesized in natural conditions, stable isotopes present in natural abundance are an important source of information about the history of each chemical species.

Methods for detecting 'synthetic' or 'natural' origin of a chemical species are therefore based on isotopic analysis. Isotope ratio mass spectrometry provides the overall molecular isotope content, but can mislead the analyst in the case of appropriate enrichment. However, the SNIF–NMR method makes it possible to measure directly the isotopic ratios at several positions in a given molecule. This improves the performance of isotopic methods and supplies genuine proof of the 'natural' or 'synthetic' origin of a molecule. The specificity of SNIF–NMR depends on ^{2}H concentration at specific sites, on the test molecule, for instance, in the case of ethanol, of the possible sites, determination of ^{2}H concentration of methyl or methylene groups can be used as a 'fingerprint' of the origin of the molecule (O'Brien, 1992). A combination of determination of ^{2}H concentration on the C1 and C2 groups together with measurement of overall ^{13}C content can easily discriminate ethanol from different sources like barley, beet, cane or whey (Martin *et al.*, 1991). The

SNIF–NMR method was officially adopted in 1987 by the International Office of Vine and Wine and by the Commisions of the European Community as a means of detecting the chapatalization (adding extra sugar before fermentation) of wine with beet sugar. In some parts of the world chapatalization is permitted while in others it is illegal. Data on reference wines from EC member states is being collected to create a European isotopic data bank. Thus the SNIF–NMR method can provide an isotopic fingerprint characterizing the origin of a wide variety of food products enumerated as follows (Martin *et al.*,1993):

- Wines: detection and quantification of chapatalization; confirmation of origin; detection and quantification of edulcoration (sweetening) of sweet wines.
- Vinegars: origin, identification (maize, other grains, beet, grape, cider, synthetic).
- Beers: origin, identification (grains other than malt).
- Fruit juices: detection and quantification of added sugar (beet or cane).
- Honey and jams: detection and quantification of sucrose addition (beet or cane).
- Lipids, fatty acids, amino acids, oils, fats and milk: guarantee of natural origin as opposed to synthetic or semi-synthetic identicals, distinction between plant and animal origin.
- Flavours: guarantee of natural origin as against synthetic or semi-synthetic substitutes of all aromatic terpenic molecules (vanilla, benzaldehyde, cinnamate, limonin, menthol, etc.).

In the plants, the dark reactions of photosynthesis take place by two possible metabolic pathways. In C_3 plants, for example, apple, grape, barley and wheat, the first product of carbon dioxide reduction is 3-phosphoglycerate, a C_3 compound. This on reduction yields glyceraldehyde-3-phosphate which undergoes the Calvin cycle or carbon reduction cycle. This results in the formation of hexoses such as glucose and fructose. In C_4 plants, for example, maize, sorghum and sugarcane, the first products of carbon dioxide reduction are four carbon (C_4) dicarboxylic acids which undergo Hatch–Slack metabolism. The difference between C_3 and C_4 plants is due to the contribution of metabolic pathways to the isotopic composition of plant sugars. The sugars formed by C_4 plants have higher ^{13}C contents than those synthesized by C_3 plants.

Measured against PDB (Pee Dee Belemnite standard scale) the deviation from the ^{13}C abundance of C_3 products (around 25%) is appreciably different from ^{13}C deviation exhibited by C_4 products (around 12%). This difference has been used to detect addition of C_4 sugars (high fructose corn syrup or cane sugar) to C_3 products (orange juice and wine).

Inter- and intramolecular isotope correlations in organic compounds have been studied as a criterion for authenticity, identification and origin assignment. The gas chromatography GC–IRMS technique has been found to provide the basis for fast and sensitive multicompound on-line isotopic analysis for ^{13}C. This has brought out the potential of this technique for proof of authenticity in food and flavour analysis (Schmidt *et al.*, 1993).

Adulteration of fruit juice and fruit juice based beverages is a serious economic problem in some parts of the world. Simple dilution, addition of sugars or acids, or complex mixtures, to simulate the natural product and avoid detection, are practised on many occasions. The methods for determination of adulteration of citrus juices have been reviewed (Widmev *et al.*). Differentiation between natural and reconstituted juice is possible on the basis of $\delta\ ^{18}O$ or $\delta\ ^{2}H$ values. Undeclared addition of cane or corn sugar to citrus juices can be detected on the basis of $\delta\ ^{13}C$ values. The SNIF–NMR method can be useful to detect and quantify beet sugar added to citrus juices. Pulp wash is richer in most minerals than pure juice. An analysis method using inductively coupled plasma–atomic emission spectrometry (ICP–AES), coupled with a pattern recognition programme is reported to give information on the presence of pulp wash in juices (Nikdel, 1991). Utilizing a gradient high performance liquid chromatography (HPLC) method, juice type and juice blends can be distinguished from pure juices (Kirksey *et al.*, 1992).

The geographical origin of an orange juice may be covered by law in some countries. Each geographical site possesses a unique mineral composition which is reflected in the fruit juice mineral composition. The highly sensitive ICP–AES quantifying minerals at ppb or ppt levels has been used to distinguish juice processed from fruit grown in different geographical regions.

1.13.8 RSK values

On the basis of extensive analytical studies on authentic juices as well as commercial samples sponsored by the Association of the German Fruit Juice Industry in cooperation with experts from research, industry and food control, the Association has formulated and published from time to time 'Richwerte und Schwankungsbreiten bestimmer Kennzahlen' or RSK for fruit juice manufactured and marketed in Germany. The meaning is guide value, range and reference number. The guide value denotes the value which seldom falls below and seldom exceeds the specified data. The range shows the variations in the chemical composition of typical fruit juice components, deviations from which may be due to raw materials used, inadmissible additives or technical procedures. The central value is not identical with the mean but according to the experience of all experts it is the value about which the values of individually produced fruit juices are mostly cumulated. For some fruit juices reference numbers for additional chemical analysis are specified. The common values include:

- A. Sensory analysis: colour/appearance, aroma, flavour.
- B. Chemical analysis: relative density at 20 °C, Brix, soluble solids; titratable acids (pH 7.0) expressed as tartaric acid, ethanol and volatile acids expressed as acetic acid; total sulphur dioxide, lactic acid, D-malic acid, citric acid, isocitric acid, tartaric acid, glucose, fructose, glucose/fructose ratio, sucrose, D-sorbitol, reduction-free extract; ash, alkalinity number, potassium, sodium, manganese, calcium, chloride, nitrate,

phosphate, sulphate, formol number (millilitres 0.1 molar NaOH/100 ml), proline.

Other values included are:

- Apricot puree and juice: L-malic acid, D-isocitric acid.
- Blackcurrant juice: L-malic acid, D-isocitric acid, L-ascorbic acid, no sorbitol.
- Grape juice: free tartaric acid, L-malic acid, no sorbitol.
- Grapefruit juice: flavonoid glycosides expressed as naringin, water soluble pectins expressed as galacturonic acid anhydride, the free amino acids, aspartic acid, threonione, serine, asparagine, glutamic acid, glutamine, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ornithine, lysine, histidine, arginine, ammonia, ethanolamine, pectic substances like galacturonic acid anhydride, oxalate soluble pectin, alkali soluble pectin.
- Orange juice: (as for grapefruit)
  Flavonoid glycosides expressed as hesperidin, total carotenoids, β-carotene as percentage total, carotene ester (cryptoxanthin ester), suspended pulp.
- Passionfruit juice: free amino acids, ammonia, carotenoids as in orange juice.

1.13.9 Identification of fish species in seafoods

New food regulations with specific requirements of labelling have come into existence with the opening of international food markets. The new labelling regulations have requirements that nutritional composition, ingredients and species of origin of the raw material be declared on the label of the product. In the case of processed fish products, none of the morphological characteristics like head, fins or internal organs used for classification of fish species are available, making the species unrecognizable. Therefore some techniques for identification are required. The large number of different edible species of fish, molluscs and crustaceans and the variety of products made from them, suggest that a single technique may not be useful in all cases. The work on techniques which can be used for such purposes has been reviewed recently (Sotelo *et al.*, 1993). Most of the methods used for differentiating and identifying fish species in food products rely on methods of protein and DNA analysis. Electrophoretic or HPLC methodology for identification of different seafood products currently in use has been summarized in the review by Sotelo *et al.* (1993). Among application of immunological techniques, a monoclonal antibody has been developed against a protein of rock shrimp (An *et al.*, 1990).

Antibodies against heat denatured proteins have been developed and could be used to identify species in cooked products (Kangethe and Lindquist, 1987; Dincer *et al.*, 1987). In addition, in analogy with meat analysis, the simple dot blot technique has application, provided the possible cross reactions are properly ascertained. Also as for mushrooms, RFLPs would be useful in differentiating species, especially in the raw products, while for the establishment of the origin of the catch of the fish species, DNA probes could have a crucial role.

1.14 Validation and approval of alternative methods for microbial analysis of foods

Many diverse methods based on recent advances in automation, microelectronics and biotechnology are now available for microbiological analysis of foods. Being rapid and sensitive, they can offer great advantages in monitoring the microbial quality of raw materials during processing and also of the final products. Such methods are required for the food inspection agencies and for international trade in food products. Analytical methodologies must be acceptable to all the concerned parties based on the established technical quality of the methodology. The need for such a mechanism for validating new methods of detection, quantification and identification of spoilage and pathogenic microorganisms in the European countries is now being fulfilled by introducing Microval, a newly introduced Eureka project. It started in June 1993 as a Dutch/French collaboration. It is a four year project having three stages, viz. project planning during the period 1st July 1993–1st July 1994, operation during the period 1st July 1994–1st July 1996 and reporting during 1st July 1996–1st July 1997. This project will touch upon aspects of both quality assurance and national health related to the production and consumption of food (Rentenaar and Van der Sande, 1994).

1.15 Quality management systems

The application of management systems like ISO 9000 and HACCP (hazard analysis and critical control points) to food safety and quality has been introduced in the industry in developed countries and was recently reviewed (Mayes, 1993). ISO 9000 is a specification for quality management system, which is accepted as being applicable to all manufacturing and service industries. It requires manufacturers to define their own standards and demonstrate that they conform to them. HACCP was initially intended for identification of microbiological hazards and now is accepted as the most cost effective means of controlling foodborne diseases and intoxications arising from microbiological, physical or chemical hazards. It is a self assessment system, although review and verification could involve an external person. It is targeted solely at issues of food safety; critical control points (CCPs) are being increasingly required by legislation (Council Directive 92/46/EEC, 16 June 1992). On the other hand, the impetus behind ISO 9000 is customer confidence and manufacturer's desire to control and improve quality standards at all levels of operation. Both these are rapidly being established in the food industry in the USA and Europe. With these management systems, consistent manufacture of products of required safety and quality standards is possible. Thus, in the present context, quality control and maintenance in accordance with these standards is slowly being implemented.

1.16 Clean food campaigns

In the fiercely competitive international food trade, the Australian Food Industry is promoting its image as a supplier of clean, wholesome, contaminant-free produce to increase its share in the world trade. 'Clean Food Australia' launched in March 1993, is an organization of Australian Food Producers, agricultural service industries, processors and retailers promoting quality food systems that are in harmony with the environment to ensure an abundant, clean and sustainable food supply. It is supported by the federal government and also by the Australian Food Foundation. Similarly New Zealand is also promoting its 'clean and green' image for its farm produce. 'Food Watch', a national programme developed under the auspices of the Agricultural Council of America, is aimed at allaying the growing concern among consumers about food safety and to counteract misunderstanding and misinformation about the food industry, especially aimed at the home market. On the other hand, the single issue movement 'The Pure Food Campaign' seeks to preserve the purity of what we eat, by opposing the use of new genetically engineered foods. It is supported by several well known figures in the United States and is expected to spread to Europe and other parts of the world in the near future (Reilly, 1993).

1.17 Current issues in food regulations in the EU and USA

Significant changes in food legislation are currently underway in the European Union and USA. The health and safety of the consumer are of continued importance, with labelling and use of additives remaining a high priority. New extensive labelling regulations in the USA have suggested a number of changes in the way in which components are declared in the list of ingredients. Thus, as per these regulations, beverages claiming to contain fruit or vegetable juice will have to declare the percentage of juice on the information panel. Criteria for naming drinks containing juice are to be established. These regulations in the USA have similarities to the Quantitative Ingredient Declarations (QUID) at the EC level which are effective from May 1994. New labelling regulations include mandatory nutritional labelling of most foods. These changes can help consumers in choosing more healthy diets and can offer incentives to manufacturers to improve the nutritional quality of their products. On the other hand, nutritional labelling is voluntary in the EU, unless a nutrition claim is made. The main requirements for making certain claims for foods in the EU and in the USA, with respect to 'low', 'reduced', 'high', 'free from' etc. differ narrowly (Kernon and Skelton, 1993). The EU aims to regulate those areas of food production, where health and safety aspects are involved. Therefore areas like food hygiene, labelling, contamination, use of additives and inspection are the priority considerations.

The FDA has new legislation for permitted levels of lead in foods. The EU also prohibited use of lead based capsules or foil as a covering for closing devices for containers of spirit drinks, certain wines and wine based drinks with effect from

January 1993. Similarly in the USA, the EPA would strictly enforce the Delaney clause of the Federal Food Drug and Cosmetic Act, which provides that no additive including pesticides may be approved in processed food if it has been found to cause cancer in humans or animals. This may result in banning or having restriction on the use of a number of pesticides commonly used by the food industry. In the USA, the environmental aspects of packaging are being seriously considered by adopting laws on environmentally acceptable packaging, 'recycled content' requirements and use of environmental advertising. In the EU, the proposal for the Council Directive on packaging waste provides a framework containing targets for waste management, to be followed by all member states. Although these guidelines are not mandatory laws, manufacturers have to keep abreast of these trends, as product innovation is a continuous process and consumers' demands keep varying.

The food that the consumer receives from the farm or factory may exhibit important compositional changes which may be relevant to health, social mores or the aesthetic beliefs of the consumer and may not be consonant with the claim, label or trade agreement. The enlightened consumer is now more conscious about what he or she wants and the industry is eager to deliver the quality that the consumer prefers. At the same time scientific advances are making available tools and techniques that are enhancing the sensitivity, specificity and reproducibility of analytical methods. This information fall out arising from basic biological sciences has assisted the analytical researcher in identifying new indicators of quality and authenticity of foods. Mandatory provisions in food legislation in many countries are becoming more rigorous especially with regard to safety aspects. The need therefore has arisen to give the consumer and health authorities much more information about the raw materials used in a food product, over and above the assurance that the product is unadulterated. The objective of the food analyst has now to encompass as well as detection of adulteration, characterization of the food with respect to its source, the history of its handling, storage, pre-processing, blending etc. It is with this view in mind that a survey of the changes in composition that a food article is likely to undergo and the methods available for investigating and evaluating these changes has been attempted. In each of the following chapters, a review of these aspects relevant to each food group will be presented.

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Chapter 2

Food Grains

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Chapter 2

Food Grains

2.1 Introduction

The cereals commonly cultivated for food or feed use are wheat, rice, oats, rye, barley, maize, sorghum and several types of millets in different countries. Wheat is the world's largest and oldest crop, grown for bread and a wide variety of other baked and pasta products, some breakfast cereals and couscous. Rye is the second most widely used cereal for breadmaking, although production is less than 10% of wheat. Rice, the second largest world crop, is the staple food of Asia, which produces more than 90% of the world's production. Oats, barley, maize and sorghum are used mostly in animal feeds, although barley malt is of considerable importance for the production of beer and other alcoholic beverages. Maize is used as a staple grain in South America and provides a variety of food ingredients such as corn oil, starch and corn grits, the last named being used for the manufacture of breakfast cereal after flaking and toasting. Cereals provide the main source of energy, proteins, vitamins and minerals for the vast populations in Asia, Africa and South America. Millets and sorghum, traditionally considered as poor man's food, comprise about 14% of the total food grains in India. The composition of some selected cereals and millets is as given in Table 2.1. Besides, many legumes belonging to the genera, *Pisum*, *Vicia*, *Phaseolus*, *Vigna*, *Cajanus*, *Canavalia*, *Lablab*, *Dolichos*, *Lathyrus*, *Lens* etc. commonly called pea, beans, grams, lentil, etc. have also been consumed extensively in various parts of the world. These have been known to be rich in proteins, the protein content varying between about 14% and 45%. A few of these, notably, *Arachis hypogea* or peanut and *Glycine max* or soyabean are also rich in oil, the former containing as much as 40% and the latter about 18% oil. In spite of their importance as protein sources in cereal dietaries, not much work on the quality aspects other than nutritive has been reported. Cereals are consumed either as cooked whole grains after dehusking and polishing as rice or comminuted grits or flour as with wheat, rye, maize and millets after conversion into bread, cakes, cookies, biscuits, pasta products or sweet or savoury snacks.

Grains belonging to the genus *Amaranthus* and *Chenopodium* are emerging as important pseudocereal crops, the industrial potential for which is yet to be fully realized. Lysine-rich protein (13–16%), waxy starch (55–60%) and squalene-rich oil (7–10%) distinguish amaranth from the other grains (Singhal and Kulkarni, 1988).

Cereals share a general similarity in composition and nutritional properties, being composed mainly of starch, crude fibre, proteins ranging from 5–15% and

Table 2.1 Chemical composition of some selected food grains

Grain	Ash (%)	Protein (%)	Oil (%)	Crude fibre (%)	Carbohydrates (%)
Wheat (whole) (*Triticum aestivum*)	2.70	12.1	1.7	1.9	69.4
Rice (raw, milled) (*Oryza sativa*)	0.60	6.8	0.5	0.2	78.2
Buckwheat (*Fagopyrum esculentum*)	2.30	10.3	2.4	8.6	65.1
Maize (*Zea mays*)	1.50	11.1	3.6	2.7	66.2
Oatmeal (*Avena byzantina*)	1.80	13.6	7.6	3.5	62.8
Sorghum (*Sorghum bicolor L.* or *S. vulgare*)	1.65	12.3	3.9	1.6	73.8[a]
Pearl millet (*Pennisetum americanum L.*, *P. typhoides* or *P. glaucum*)	1.46–3.88	11.6	3.03–7.40	1.96–3.88	56-65[a]
Proso millet (*Panicum miliaceum*)	1.9	12.5	1.1	2.2	70.4
Finger millet (*Eleusine coracana*)	3.2	8.8	1.8	3.6	72.0
Foxtail millet (*Setaria italica*)	3.3	10.6–15.2[b]	4.3	8.0	60.9
Varagu millet (*Paspalum scorbiculatum*)	2.6	8.3	1.4	9.0	65.9
Sanwa millet (*Echinochloa frumentacea*)	4.4	6.2	2.2	9.8	65.5

[a]Only starch.
[b]On dry weight basis.
Source: Hoseney *et al.*, 1981; Kurien and Desikachar, 1966; Gopalan *et al.*, 1981.

lesser amounts of fats and non-starch polysaccharides. Although starch is the primary macromolecule conferring rigidity to the structure of baked products, the initial creation of leavened structure in breads is dependent on protein functionality. Hardness is another important quality characteristic of wheat, and significantly influences the milling behaviour of wheat and the suitability of the flour for its end uses. Hard wheat usually yields more flour with suitable colour, has high water absorption, and therefore gives more and better bread. Soft wheat flour is suitable for cakes, biscuits and cookies. Some wheats are difficult to classify as hard or soft from visual observation of kernel size, shape and colour, especially those varieties resulting from crossing of hard and soft parents.

Cereal varieties differ in their physicochemical qualities with respect to the end uses such as the preparation of varieties of bread, cakes, cookies, pasta products, chapatis, rotis or similar products (in the Indian subcontinent and Arab region) and boiled rice preparations. The suitability improves with ageing in most cases. The quality is thus

determined by the species, variety, season and region, harvesting time and conditions, content of admixtures, storage period and conditions, whether wetted/sprouted, contaminated with dust, stones, chaff or other seeds, and at times, toxic residues of pesticides and other chemicals used in farms and warehouses. The quality needs to be evaluated with respect to type, wholesomeness, including toxic components, ageing, suitability for end use in terms of test production or rheological or physicochemical properties. The products of primary processing such as milling and parboiling may have to be analyzed for different characteristics, contaminants and suitability for end uses and admixture possibilities.

2.2 Contaminants in grains

Grains often become contaminated with weed seeds during harvesting. The possibility is aggravated since mechanical harvesting has been employed. Some of the common weed seeds are given in Table 2.2. Some of these are likely to cause toxic symptoms if ingested in sufficient quantity.

Table 2.2 Some common weed seeds encountered in food grains

Weed seed	Grain in which found
Crotalaria spp. Jimsonweed (*Datura stramonium*)	Widespread, especially in wheat
Justicia quinquangularis (Acanthanaceae)	Rice, maize
Amaranthus spinosus, *A. viridis*, *A. polygamous* *Celosia argentea*, *Digera arvensis* (Amaranthaceae)	Maize, rice Millets, maize
Heliotropium eichwaldi, *H. indicum*	Maize
Chenopodium album, *C. murale* (Chenopodiaceae)	Rabi cereals, peas
Carthamus oxyacantha, *Sonchus oleraceus*, *Ageratum conyzoides* (Compositae)	Rabi crops such as wheat, peas
Euphorbia prostata, *E. hirta*, *E. dracunculoides* (Euphorbiaceae)	Wheat, gram, millets, maize
Echinochloa crusgalli, *Cynodon dactylon*, *Saccharum spontaneum* *Avena ludoviciana* (Gramineae)	Rice Almost all crops Millets, maize Wheat, peas
Argemone mexicana (Papavaraceae)	Wheat, mustard
Striga asiatica, *S. lutea*, *S. euphrasioides* (Scrophulariaceae)	Bajra, sorghum, rice

Source: Indian Council of Agricultural Research (1987).

2.3 Interspecies and intervarietal wheat admixtures

Many pasta products are normally prepared from 100% durum wheat and labelled accordingly. Growing, harvesting and handling practices invariably contaminate durum wheat with non-durum (*Triticum aestivum*) cultivars. This generally causes a quality control problem for manufacturers. Tests that can distinguish between durum and non-durum are therefore generally sought.

Wheat proteins have been conventionally classified on the basis of solubility into four groups: water-soluble albumins and salt-soluble globulins, together forming about 15% of the total protein and termed 'non-gluten'; ethanol-soluble prolamines and dilute acid-soluble glutelins, forming about 85% of the total protein and constituting the 'gluten' fraction. Wheat prolamines and glutelins are called gliadin and glutenin, respectively. The gliadin fraction can be differentiated by polyacrylamide gel electrophoresis at pH 3.2, typically into 20 to 40 individual protein bands, depending on the resolution of the gel. Even with low resolution, four broad groups, named α, β, γ and ω- gliadins can be obtained, which are characteristic of the wheat variety, and can be used for identification purposes. These patterns, determined by the genotype, are unaffected by growing conditions and make a readily identifiable 'fingerprint', and can therefore be used to detect blends of wheat varieties (Frazier, 1992).

Gluten added to durum wheat pasta can be determined as soft wheat content which in turn can be analysed by normal or accelerated electrophoretic methods (Wrigley *et al.*, 1991) or by immunological methods. In some countries like Italy, tolerance levels for soft wheat content are laid down in the regulations. The choice of method in checking the levels of soft wheat content is therefore critical. The immunological method is known to give higher values as compared to electrophoretic methods (Cantagalli *et al.*, 1979). The electrophoretic pattern of soluble proteins used for detecting adulteration of hard wheat products with soft wheat is found to be controlled by genetic differences. The presence of more than one band of γ/β-gliadins is indicative of adulteration of durum wheat flour with flour from some common variety, and is sensitive down to levels of 50 g kg^{-1} or less. Its use is, however, limited because it is complicated and requires a great deal of technical input. It is preferred when all other methods of detecting adulteration have failed (McCarthy *et al.*, 1990). It has been confirmed that the proteins characterizing soft wheat are under direct genetic control (Cubadda, 1974).

The electrophoretic pattern of protein extracts obtained from wheat pastes to detect presence of soft wheat have shown no interfering bands in the migrating zone of soft wheat extracts at temperatures <80 °C. The protein is readily extractable at 90 °C only if the product has a moisture content $>14\%$ (Seibel, 1991). However, some changes in the bands of hard wheat extracts were observed at temperatures >70 °C (Cubadda, 1969). The technique therefore works even with heat treated products containing soft wheat, and can be used to determine the proportion of soft wheat in hard wheat

semolina and pasta products. Most of the procedures that give excellent separation of gluten proteins are labour intensive and their results often difficult to quantify. Capillary electrophoresis offers a superior technique. Capillary electrophoresis is a moving-boundary electrophoresis and in most methods, molecules migrate in open, buffer-filled tubes of diameter 20–75 μm, typically made of silica. These capillaries can be efficiently cooled to dissipate heat generated by the high voltages used. Solute mobilities are a function of molecular size, charge and shape and also vary because of differential interactions with capillary walls. Separation of gliadins using capillary electrophoresis with 0.1 mol l^{-1} phosphate buffer of pH 2.5 contained in a linear hydrophilic polymer has enabled differentiation of wheat classes and promises to become a routine tool for wheat varietal identification (Lookhart and Bean, 1995a) and classification, and for prediction of quality (Bietz and Schmalzried, 1995). This technique, when used in a soluble polyacrylamide sieving matrix in a buffer followed by addition of small amounts of organic solvent to the sieving matrix gives excellent resolution of high molecular weight glutenin subunits that correlate with bread-making quality (Werner *et al.*, 1994). The technique is also useful for rapid differentiation of oat and rice cultivars (Lookhart and Bean, 1995b).

A two year experience of quantitative determination of aestivum wheat in durum wheat by isoelectric focusing has shown its accurate detection at 0–20% in durum wheat. However, accuracy decreases at higher contents, and at aestivum contents >70%, results are only rough estimates. The method is reported to be unsuitable for determination of small amounts of durum in aestivum wheat products and also in pasta products dried at temperatures >80 °C (Stroh, 1986). The immunodiffusion reaction of purified goat serum containing specific antibodies against soft wheat albumin with protein extracts of hard wheat pastes on agarose gel detects soft wheat even at 5% levels. The method remains unaffected by the drying process at 60–80 °C that is used in the manufacture of pasta and is therefore quite sensitive (Cantagalli *et al.*, 1969). None of these methods gives exact quantification of soft wheat in dough, particularly with drying temperatures above 100 °C. Electrophoresis of ω-gliadins, reverse phase HPLC of ω-gliadins and hard wheat immunoassay have been recommended for an international interlaboratory trial (Autran and Bonicel, 1992). Reverse phase HPLC of water soluble protein detects 1% of aestivum wheat in durum wheat with good reproducibility and accuracy. This method could be easily employed in customs and industry laboratories which find electrophoretic techniques difficult to apply (Noni *et al.*, 1994).

Gas chromatography techniques have been used for identification and determination of soft wheat additions to breakfast cereals and nutritional pastes labelled hard wheat. In most cases, additions can be detected with certainity at 20% (Vogel and Berner, 1967). Spectrophotometry of the extracts at 8.2–9.5 μm can also detect adulteration of hard wheat pastes with soft wheat, the method being applicable to one year old pastes (Brogioni, 1969). Near infrared (NIR) spectroscopy has also been used to differentiate between hard red winter and hard red spring wheat. Examination of the

principal component factors has indicated that hardness, protein level and the interaction of water with protein and other constituents are responsible for correct classification based on NIR (Delwiche and Norris, 1993).

Another approach which could distinguish between hard and soft red winter wheat is the image analysis of starch granules isolated from the respective cultivar. Microscopical measurement has been used to calculate the equivalent diameter, defined as $2\sqrt{\text{area}}/\pi$, the aspect ratio, defined as the ratio of length to width, and the circularity shape factor, defined as 4π area/perimeter^2 from the morphology of starch granules. A plot of data for equivalent diameter in the range of 5.5–7.0 μm range versus data for the aspect ratio in the range of 1.65–1.95 μm could distinguish hard red wheat from soft red wheat even when NIR hardness values overlap (Zayas *et al.*, 1994).

The Matweef and Brogioni–Franconi methods are based on the characteristic sterol esters sitosterol palmitate in soft wheat and cholesterol palmitate in hard wheat. Amongst these, the Matweef test permits a better differentiation in all types of products (Alliot, 1957; Montefredine and Salvioni, 1967). This is based on the extraction of steroids from the finely ground material and colorimetric estimation at 640 nm by the Liebermann–Burchard reaction. The sterols from soft wheat extract are precipitated at −3 °C to −4 °C. At temperatures, of the order of −7 °C, sterols from durum wheat are also precipitated making the detection of soft wheat flour in durum wheat incorrect (Zoubovsky, 1959). Extraction of sterols followed by separation on thin layer chromatography (TLC) and detection by spraying with 10% alcoholic molybdophosphate can also be used to distinguish soft wheat from hard wheat (Salvioni, 1969). Fractionation of lipids from wheat pastes by column chromatography on silica by successive elutions with 1:1 diethyl ether:petroleum ether and 1:1 acetone:methanol gives a clue to detection of soft wheats, although amounts less than 20–25% are not detected by this technique. The ratio of both fractions is an index of purity of hard wheat with the value 3.0 being indicative of presence of soft wheat (Cavallaro, 1969).

Mineral contents, in particular N, Mg, Ca, Mn, K and Zn can be indicative of the wheat grain or flour type. In both grain and flour, N, Mg, Ca and Mn are higher for hard wheats, K in grain is significantly higher in soft wheats, and Zn content is significantly higher in hard wheat both in grain and flour. Mg/K ratio is significantly higher for hard wheats than for soft wheats in both grain and flour, and can therefore be used to detect blends of wheat varieties, in both the grains and the flour (Fukuoka and Horino, 1989), and hence also the processing quality.

α-Amylase inhibitors are known to be present in bread wheat, rye, triticale and sorghum, and absent in rice, barley, corn and durum wheat. This fact can be used as an index for detecting admixtures such as the presence of bread flours in macaroni (Saunders, 1975). The polyphenol oxidase activity is another test showing promise in distinguishing wheat types. Polyphenoloxidase activity using tyrosine as a substrate is much lower in durum cultivars compared with most other cultivars (Lamkin *et al.*, 1981). A tyrosinase test that has the potential to detect contamination of durum wheat

by bread wheat, and giving results within 30 min differentiates both hard and soft wheat cultivars from durum, and is suitable for routine quality control. The method is simple, and consists of soaking the wheat seeds in tyrosine solution and then exposing them to air. The grains from aestivum cultivars undergo a rapid darkening and confirm the presence of durum (Mahoney and Ramsay, 1992).

A computer controlled laser scanning system capable of acquiring three-dimensional images of the surface of cereal grains has been developed to distinguish between wheat varieties, and also detect and differentiate sprouted and unsprouted grains. A combination of 14 features based on nine topographic images and five intensity images permits discriminant analysis to classify 92–94% of kernels correctly. In particular, features that measure deformation of the germ end of the kernel are crucial to the discrimination process (Thomson and Pomeranz, 1991). Discriminant analysis based on fluorescence intensity, hardness and protein data has also been demonstrated to allow separation of wheat into proper classes for 100% of durum, hard red spring, club, soft red winter and soft white winter wheats and for 94% of hard red winter wheat (Irving *et al.*, 1989).

Sprouted wheat in sound wheat can be estimated by the enhanced α-amylase levels, which can be quantified in terms of the Brabender amylograph viscosity characteristics (British Standard, 1993b).

2.4 Intervarietal rice admixtures

Several cultivars of rice are grown, the grain varying in dimensions, cooking quality and acceptability. Consumer preference is rigid for regioselective varieties all over the world. It is well known that taste preferences and food consumption patterns are different not only in the various countries of the world, but even within a country. A quality grade that receives the highest premium in one region may be the least preferred in another. The market value of rice is determined largely by grain dimensions, appearance and colour. The quality grades, and price structure differ from country to country as there is no uniform scale for grading rice varieties. Earlier three main groups were recognized in the trading, fine (long slender scented), medium (medium slender and long bold) and coarse (short bold).

This system of classification was found to be unsatisfactory, and one based on length and length/breadth ratio of the kernels was suggested. Additional traits like kernel colour, aroma and degree of endosperm opacity were also recommended. However, this system was also found to be too broad and rigid.

Bhattacharya *et al.* (1982) have classified rice varieties tentatively into eight quality types primarily on the basis of total and hot water insoluble amylose contents and viscogram patterns.

Basmati rice enjoys a special price advantage in the international market. It has earned for itself a unique place in the Middle East, parts of Africa and Europe for years. However, little work has been done on distinguishing this variety from others

that are commonly used to adulterate it. Based on a detailed study of basmati rice samples, indices and minimum standards for qualifying a variety as basmati have been evolved. However, many other non-scented, cheaper varieties of rice can also possess these features. A simple scheme of the Indian Agricultural Research Institute stipulates fine grain, mild to strong aroma (as measured by potassium hydroxide treatment), linear kernel elongation on cooking (>1.5 times) and non-slimy and non-splitting nature as the standard of basmati rice. The last two tests mentioned above could be performed by cooking 10–15 kernels in test tubes over a water bath (Siddiq, 1982).

Aroma is the foremost criterion for distinguishing basmati rice from non-basmati types, yet no reliable qualitative or quantitative method for the precise detection of basmati rice has been developed. In all breeding and genetic experiments, kernel chewing has been considered a reliable method for many years (Indian Agricultural Research Institute, 1980). A technique of heating vegetative plant parts in water in closed vials was found to be unsuitable because of the dominance of a strong chlorophyll smell. One method suggested involves addition of 10 ml of 1.7% potassium hydroxide solution to a small petri plate containing about 2 g of finely minced sample of green leaf or stem. The petri plates are covered immediately and left at room temperature for about 10 min. The plates are then opened and the contents sniffed. The scented rice varieties produce a sharp and readily recognizable aroma. This technique was found to be very useful in breeding experiments but it failed to trap the malpractice of basmati rice adulteration.

Alkaline oxidation value, which represents reducing flavour volatiles, has been evaluated as an indicator of adulteration of basmati with scented as well as non-scented rice varieties. Results of a typical experiment are as shown in Table 2.3.

Detection of an admixture of American long grain rice with basmati rice has been attempted by treating 100 head kernels in a petri dish with a 2% solution of sodium bisulphite. When 5% v/v HCl was added, sulphur dioxide was liberated and the basmati kernels became chalky in about 20 min, while 40 min or more were required for all kernels of American rice to become chalky (Agrawal and Sinha, 1965). Chalkiness is said to occur when rice is harvested at too high a moisture level or in varieties of non-uniform maturities where an excessive number of immature kernels, referred to as immature chalk, exist. Adverse weather conditions and cultural practices were also found to influence the incidence of chalkiness in rice.

Basmati rice has low amylose content (20–22%), medium-low gelatinization temperature, lengthwise elongation on cooking, tenderness of cooked rice and pleasant aroma as its key characteristics. Grains are invariably fine but their degree of fineness varies from short slender (length/breadth ratio above 3 mm with length less than 6 mm) to exceptionally long slender (length/breadth ratio above 3 mm with length exceeding 6 mm). The quality features of the typical export quality basmati varieties, viz. Basmati-370 and Type-3 have been reported by IARI (Indian Agricultural Research Institute, 1980). These include length (6.89 mm and 6.76 mm), breadth

Table 2.3 Effect of admixture of adulterants on the alkaline oxidation value of Basmati-370 rice

Rice variety	Alkaline oxidation value
Basmati-370, 100%	2.4
Pusa-169[a], 100%	6.0
PR-106[a], 100%	9.6
Improved sabarmati[b], 100%	1.6
60% Basmati + 40% Pusa-169	4.8
60% Basmati + 40% PR-106	4.0
60% Basmati + 40% improved sabarmati	1.8

[a]Non-scented varieties.
[b]Scented varieties.
Source: Vaingankar and Kulkarni, 1988 (reproduced with permission).

(1.85 mm and 1.93 mm), length/breadth (3.72 and 3.55), volume expansion after cooking (3.7 and 3.5 times), kernel length after cooking (11.0 mm and 12.5 mm), kernel elongation ratio (1.6 and 1.82) for Basmati-370 and Type-3, respectively. Amino acid profiles of these varieties indicate that these scented varieties possess superior nutritional qualities (Sekhar and Reddy, 1982).

There are several varieties of ordinary, non-scented rice which resemble basmati in one or more characteristics. It is a common practice to adulterate the highly priced, flavourful basmati with such cheaper varieties. Some of these adulterants are pusa-169, improved sabarmati, PR-106, kalimuch, saket-4, lakra and parimal. Amongst these, improved sabarmati and kalimuch possess a mild scent, not as strong as basmati. The percentage adulteration in basmati rice could be determined more precisely by applying the kernel elongation test (Siddiq, 1982). The cooking behaviour of basmati grains in a model system, comprising basmati and its adulterants has shown (Vaingankar and Kulkarni, 1986, 1989) that the differential length/breadth ratio is very promising in this regard and is shown from Table 2.4. A minimum value of length/breadth ratio of 3.92 ± 0.09 to 4.09 ± 0.09 is indicative of pure basmati.

2.5 Cereal/cereal and cereal/legume blends

Not much attention has been devoted to the study of the determination of composition of cereal legume combinations, although data from phytochemistry could be of help in such analyses. Some potential approaches are listed here.

Most proteins contain the generally recognized 20 amino acids but some do contain non-protein amino acids, most of which are structural analogues of one or other of the protein amino acids. Thus, two analogues of proline are pipecolic acid, which has one more methylene group than proline, and azetidine 2-carboxylic acid, which has one less. Pipecolic acid is mainly found in certain legume seeds, while azetidine 2-carboxylic acid occurs characteristically in many members of the Liliaceae

Table 2.4 Differential length/breadth ratios of basmati and adulterants and admixtures of the two

Admixture[b]	Differential length/breadth ratio					
Code[a]	I	II	III	IV	V	VI
$B_{100}A_0$	4.09±	3.92±	3.95±	3.92±	4.09±	4.08±
	0.09	0.09	0.09	0.09	0.09	0.10
$B_{75}A_{25}$	3.58±	2.98±	3.40±	3.79±	3.70±	3.65±
	0.05	0.13	0.09	0.10	0.11	0.08
$B_{50}A_{50}$	3.29±	2.36±	3.11±	3.76±	3.55±	3.48±
	0.08	0.15	0.15	0.10	0.09	0.09
$B_{25}A_{75}$	3.03±	1.85±	2.73±	3.72±	3.36±	3.20±
	0.09	0.09	0.10	0.08	0.12	0.11
B_0A_{100}	2.73±	1.47±	2.30±	3.67±	3.16±	2.99±
	0.11	0.07	0.07	0.09	0.07	0.07

[a]Code: B represents percentage of basmati and A represents percentage of adulterant in a model mixture.
[b]Admixtures: I Basmati-370 and pusa-169 rice; II Basmati-370 and PR-106 rice; III, Basmati-370 rice and improved sabarmati; IV, Basmati-370 and parimal; V, Basmati-370 and kalimuch rice; VI, Basmati from Punjab and lakra rice.
Source: Vaingankar and Kulkarni, 1989 (reproduced with permission).

(Harbourne, 1973). Pipecolic acid could be used as an index of legume content in cereal/legume blend and warrants study. While plants in the same family have the same cytochrome c sequence, there are differences at higher levels of classification. The cytochromes of monocotyledonous plants, for example have 12 to 15 amino acids different (out of a total of 112) from those of dicotyledons (Boulter, 1972).

The purine and pyrimidine bases of nucleic acids are common to all living organisms. These bases also occur at least in trace amounts, bound in low molecular weight compounds in plants. There are a number of unusual bases found in plants which are closely related in structure to the nucleic acid bases. One such compound is 5-methylcytosine, existing in the DNA of wheat germ (Harbourne, 1973). The low molecular weight pyrimydine glycosides, vicine and convicine are known to occur in certain legume seeds of the genera *Vicia* and *Pisum* and could serve as indicators. The presence of lathyrine, a non-protein amino acid having a pyrimidyl ring in *Lathyrus* seeds, often used as an adulterant of other legume flours has been used as an index to detect and quantify the content of this legume.

Rye flour in mixtures with wheat flour can be recognized by characteristic grains of rye starch and differences in the rate of settling of suspensions of wheat flour and rye flour in water (Ferrari, 1953). Polyacrylamide gel electrophoresis (PAGE) has been applied to detect adulteration of chickpea flour with pea flour by the presence of extra bands in the unique electrophoregram of the chickpea. Protein extracts prepared by a single extraction in 5 mol dm^{-3} acetic acid produces patterns which can be used to

differentiate pea and chickpea samples. Using the described procedure, 100 varieties of chickpea did not show intervarietal variation in their electrophoregrams, whereas 11 varieties of pea showed differences in their protein composition. Six of the 25–30 protein bands present in the electrophoregrams of most pea varieties can be used as markers for detection of pea flour in chickpea flour (Hussain and Bushuk, 1989).

2.6 Indices for processing quality of wheat and other grains

2.6.1 Baking quality of wheat flour

The breadmaking process relies on the leavening of an elastic extensible, gas retaining dough, which can be obtained by hydration and development of gluten. Before modern breadmaking methods were invented, doughs were developed very slowly by manual kneading and by the gentle action of yeast during lengthy fermentation. Modern breadmaking processes rely on a short period of intensive input of mechanical energy to develop the dough structure. In a process called the 'Chorleywood bread process (CBP)', the level of work corresponds to 40 kJ kg^{-1}, expended in 4 min or less, and the dough must contain an optimum level of oxidizing improvers to assist development and stabilize the developed structure. An understanding of the rheological effects of dough development is important for selection and optimization of new wheat varieties for the CBP. Conventional dough rheological methods such as the farinograph are unsuitable owing to the high work levels required and time dependent nature of the dough properties. An appropriate procedure consists of mixing doughs to a series of increasing work levels up to 350 kJ kg^{-1}, keeping the work input constant at 20 kJ kg^{-1} min^{-1} by computer feedback control. Replicate dough samples are then moulded into spheres, rested for 45 min at 30 °C and compressed between parallel plates at 10 mm min^{-1} on an Instron materials testing machine, to a load of 1.8 N. The time for the force to decay by 1 N is then recorded as the stress relaxation time (RT). The gluten quality index (GQI), defined as ten times the maximum logRT of a fully developed dough has been used to compare the functionality of different wheat varieties. This index ranges from eight for a very weak variety, to 16 for an optimum breadmaking variety, and up to 20 for an over strong variety (Frazier, 1992). The gluten index may predict faster dough proofing, but does not appear to predict baking performance even when shelf stored for 3 months. It can be used as a rapid predictive test of breadmaking quality, if various other factors affecting gluten functionality such as gluten particle size and sodium content, optimal dough mixing or varied gluten use levels are considered concurrently (Ranhotra *et al.*, 1992). A strong positive correlation between the 'gluten index' value and manual gluten quality score as well as to sodium dodecyl sulphate (SDS) sedimentation tests has been reported. This can be used to predict the durum wheat gluten strength in wholemeal or semolina (Cubbada *et al.*, 1992).

Simple correlations have shown that no simple biochemical component can explain

variation in any given quality parameter. However, a multivariate statistical approach to measured biochemical components can explain >90% of the variation in major quality attributes such as dough handling and loaf characteristics. Protein concentration is a primary factor contributing to both the quality attributes. After the protein effects have been established, flour polar lipids show a positive correlation to dough handling, while loaf textural features are largely correlated to glutenin concentration, water soluble pentosans and flour lipids (Graybosch *et al.*, 1993). These approaches are needed to develop effective models for the observed variation in wheat quality for end use.

A multifactorial mathematical model for predicting the quality of the flour system has been derived in the form,

$$Y=a+a_1x_1+a_2x_2+a_2x_2^2+a_3x_2+a_4x_2^2+a_5x_3+a_6x_3^2 \qquad [2.1]$$

where Y = quality index manifested either as loaf volume or loaf height/diameter (H/D) ratio, x_1 = saccharification capacity of the flour, x_2 and x_3 = dough structural and mechanical properties like shear, viscosity and specific rheological characteristics and a_0 to a_6 are constant coefficients. The values for a_0 to a_6 can be derived or calculated (Kuzminskii *et al.*, 1978). Quality evaluation of wheat varieties to assay gluten quality and quantity has shown a correlation of 0.88 for hard red spring wheat between dough volume and loaf volume. A correlation of >0.80 has been observed in commercial wheat samples between protein content and dough volume. Dough volume has also been known to correlate well with sedimentation values, wet gluten content and wet gluten meal fermentation time tests (Greenaway, 1977). The sedimentation value is based on the rate of sedimentation of the solid phase from an acidulated suspension of flour in water and is a very good predictor of loaf volume of bread since it depends on both gluten quality and quantity. Specific sedimentation, defined as sedimentation value per unit protein content is also a measure of gluten quality. Zeleny sedimentation tests developed to measure the quantity and degree of gluten swelling (Zeleny, 1947) work well for hard flours, but not for soft flours. This method however has been modified (by using a hydration time of 160 min and the addition of lactic acid) and can be used for soft flour (McAuley *et al.*, 1953). This has been accepted as the standard method (British Standard, 1993a).

Alveograph measurements and gluten contents are both valuable in predicting baking quality of flour (Andino, 1950). Changes in potentiometric properties offer criteria for evaluation of dough ripeness, which may be used for periodic or continuous automatic monitoring of dough ripeness using simple instruments like pH meters and potentiometers (Chernykh *et al.*, 1978). Analysis of 27 bread varieties differing widely in quality and hardness for 12 quality characteristics has been carried out. These include extensigraph maximum resistance, protein insoluble in 0.05 mol dm^{-3} acetic acid, farinograph dough breakdown, pelshenke time, farinograph water absorption,

particle size, flour protein, loaf volume, loaf volume/unit volume, Zeleny sedimentation value (Bolling and Meyer, 1973), extensigraph extensibility (Graber and Kuhn, 1992) and farinograph development time. These analyses have shown that bread quality may be evaluated on the basis of protein quality, as determined by the proportion of protein insoluble in acetic acid, grain hardness (Ittu *et al.*, 1979), as evaluated on basis of particle size of flour, and protein content (Bolling and Meyer, 1973). The time required to pearl 10% of the grain has been indicated to be an index of hardness of the grain (Ali and Wills, 1985). These tests together with milling yields are known to provide valuable information for use in selection programmes (Orth *et al.*, 1976).

Wheat hardness measurements have been summarized in several reviews (Obuchowski and Bushuk, 1980; Miller *et al.*, 1982a; Wu *et al.*, 1990). Wheat protein content and grain hardness can be rapidly determined by IR (Bard, 1991; Beresh *et al.*, 1990) and NIR spectroscopy (Delwiche and Weaver, 1994). The NIR and Brabender hardness tester results correlate significantly with percentage of dark hard and vitreous grains as shown by commercial red winter wheats which have similar protein contents (Miller *et al.*, 1982b). These techniques can also be used to predict results of quality tests such as the Zeleny and rapid mix tests, Chopin alveograph results, water absorption and damaged starch results. These methods however require calibration and are sensitive to particle size distribution in milling flours (Schorch, 1983; Bolling and Zwingelberg, 1982). Hardness of wheat can be simply and conveniently measured by determining the time required to collect 17 ml of ground wheat from a 20 g sample in a commercial microhammer cutter mill at 3600 rpm. Hard and soft wheats differ in the grinding time and to the extent that the machine's speed is reduced. No correction for grinding time and speed reduction is necessary within moisture levels of 9.3–12.7% (Wu and Nelsen, 1991).

The swelling index is known to give surer results compared with sedimentation methods in picking out poor flours. This is particularly relevant for the bakery and for manufacture of doughs (Rotsch, 1952). The combination, sedimentation value plus crude protein content is believed to be a more useful quality parameter than wet gluten content/gluten quality (Mosonyi and Feher, 1991). However it is recommended that for realistic evaluation, correlation between the quality parameters for wheat and flour should be determined (Frenzel, 1984). An overall index of grain quality based on individual quality indicators such as content of water, gluten and admixtures, volume weight, content of crude protein and viscosity number has also been suggested to enable grain quality to be judged as a whole as well as its technological suitability for special processing requirements such as milling, baking or brewing. This index also enables comparison of grains from different regions, different seasons and different growers (Kozakov and Kazakova, 1983). Quality indicators based on temperature, moisture content and acidity of the bread dough can be controlled by the use of microcomputers and are suitable to form part of an automatic production control. Alarm signals to supervisors could be given when quality indicators are not observed

(Skugarev and Filyakin, 1985). Regression equations for dough quality based on water content, titratable acidity and dough temperature and taking into account the actual conditions and characteristics of the dough rising process have been developed and the applicability has been confirmed by tests with automated dough control (Blagoveshchenskaya and Petrov, 1984).

A study of baking characteristics, flour ash and protein has shown no correlation between baking quality and ash content, although recently pattern recognition techniques have established a positive correlation between some of the baking parameters and magnesium content of the flour (Zagrodski *et al.*, 1995).While loaf volume is known to be dependent on protein content, endosperm ash has no influence upon the baking quality of flour (Wichser and Shallenberger, 1948). The degree of milling of the flour can be evaluated on the basis of decrease in major flour constituents, fibre content being particularly indicative (Kamimura, 1950). Flour and ash yields could also be used to evaluate milling properties in the case of newly developed wheat varieties (Bolling and Meyer, 1973). Holographic interferometry offers an improved method of evaluating the mechanical properties of cereal grain. Its use reportedly eliminates inaccuracies in estimating mechanical damage. This could also be extended to evaluate milling characteristics of the cereal grains. Determination of flavone content is reported to be more reliable than ash for evaluating the degree of extraction of cereal flours and other milled products. It also correlates with ash, protein and fat contents in the milled product (Koracsonyi, 1947). Bran and germ particles in wheat flour can be determined by differential staining of germ/bran and endosperm with 0.05% crystal violet. Bran/germ appear as violet particles and endosperm cell walls, and dispersed starch and protein are colourless (Larkin *et al.*, 1952).

Analysis of experimental doughs on a farinograph for kneading time, dough temperature and dough resting time and on a valorigraph and penetrometer, for dough consistency has shown dependence of dough consistency on dough temperature and requires studies on a practical scale to arrive at an index for manufacture of high quality bakery products (Wintz and Wiede, 1975). The Pelshenke index is known to increase with decreasing dimensions of the flour particles and increasing ash content (Deschreider, 1948) and is therefore not a very sensitive indicator of the baking quality of the flour.

The population of the Middle East region relies heavily on the traditional bread types as a staple component of the diet. These products along with similar types from other parts of the world, are often collectively referred to as flat breads. These have been recently reviewed by Faridi and Rubenthaler (1983). The Arabic bread is a flat circular loaf prepared from flour, yeast, salt and water, which separates into two layers (pocketing). Doughs are scaled off in pieces (normally 100–200 g), fermented, flattened to resemble a pancake with about 20 cm diameter and allowed to ferment again prior to baking. During baking, crust forms in a few seconds and the temperature of the dough rises. This causes internal steam formation that results in the dough puffing to produce the pocketing effect described above. There is a continuous need to

evaluate wheat flour quality in the context of Arabic bread. Scoring patterns based on area index, crust smoothness, shape, crust colour, cracks, blisters, ability to roll and fold, quality of separation, evenness of the layers, grain appearance, grain uniformity, crumb texture, quality of tearing and crumb colour can give an overall index of bread quality (Qarooni *et al.*, 1987). Various workers have described test baking procedures, equipment and loaf evaluation methods (Faridi and Rubenthaler, 1984; El-Samahy and Tsen, 1981; Mousa *et al.*, 1979). Requirements of the test baking procedures include small sample size for testing, rapid throughput and accurate control of conditions at all stages of test procedures and methods of quality evaluation. Although these apply to all baked products, there are problems inherent in such testing for Arabic bread. In particular, sheeting of the dough and the very high oven temperatures complicate the control of testing conditions. On the basis of extensive ranges of experiments, baking absorption was judged to be best predicted by the proportion of water required to produce a consistency of 850 BU (Brabender units) on the farinograph. Similarly the dough mixing time is estimated as dough development time plus one minute. Sheeting thickness was selected at 3 mm after evaluating a range of 2–6 mm.

Baking quality of wheat has also been recently linked to high glutenin/gliadin ratio (Brunori *et al.*, 1989) as well as certain high molecular weight subunits of glutenin, partly due to -SH groups in flour, because they can increase dough strength and improve loaf volume. This effect of high molecular weight glutenin subunits (HMW-GS) has been confirmed by using size exclusion chromatography (Bottomley *et al.*, 1982; Huebner and Wall, 1976). Bread improvers remove -SH groups and prevent the harmful effects of breaking glutenin molecules. Improvement of flour during first few months of storage is believed to be due to aerial oxidation of -SH groups to -S-S-. Each -SH attaches to a glutenin molecule or blocks an -SH on glutenin to form -S-S- by oxidation. Free -SH on glutenin, either in its original form or from reaction with thiols, is therefore related to the number of ends and therefore inversely related to the number average molecular weight which in turn is related to the baking quality (Ewart, 1990). A high content of high molecular weight glutenin favourably affects the sedimentation value and rheological properties of the dough, while acetic acid soluble protein impairs them (Subda, 1992). Low content of acetic acid and SDS soluble protein in wheat flour is also beneficial for good baking results (Subda, 1989; Subda and Biskupski, 1987; Chung and Pomeranz, 1978).

Rapid antibody-based test methods using antibodies specific for quality related components in flour would provide the potential for developing new techniques for testing of wheat quality. Such methods could be suited to the simultaneous testing of hundreds of small (100 mg) flour or wholemeal samples, for example in early generation screening for quality in wheat breeding programmes. It could also be applied as a rapid dough quality prediction of the grain when it is received at the mill, bakery or elevator. Using the enzyme linked immunosorbent assay (ELISA) format and several antibody combinations, most useful antibodies have been found to bind selectively to HMW-GS, which is known to exert maximum effect on dough strength.

Other gluten proteins (e.g. low molecular weight glutenin subunits, LMW-GS) can also influence dough strength (Gupta and Shepherd, 1988). The extent of correlation between antibody binding and dough strength needs to be established for different sets of wheats. This ELISA test does not discriminate quality, unless a reducing agent is used. This could probably be because many subunit-specific regions of amino acid sequence on HMW-GS are located near the cysteine residues (Anderson and Greene, 1989) and are exposed only after reduction (Skerritt, 1991a). Extraction with a detergent, SDS, and a reducing agent, dithiothreitol are best suited for this ELISA assay.

A high performance liquid chromatography (HPLC) method has also been developed for quantifying glutenin aggregates after sonic disruption of the glutenin complex (Singh *et al.*, 1990a), and has also shown good correlation with several aspects of gluten strength (Singh *et al.*, 1990b). Other HPLC methods for predicting dough strength have also been described (Huebner and Bietz, 1985; Krueger *et al.*, 1988; Dachkevitch and Autran, 1989; Sutton *et al.*, 1989). Although the HPLC method provides useful information on gliadin monomer-to-glutenin aggregate ratios, the antibody test offers advantages of lower capital cost, lower per-sample cost, much higher throughput, ease of interpretation and greater differentiation of samples differing only moderately in strength (Skerritt, 1991b). The method has been tested in a collaborative trial in eight laboratories. Each laboratory reported a highly significant correlation between colour developed in the ELISA assay and rheological measurement of dough strength such as farinograph development time and extensigraph maximum resistance. Good estimates within and between laboratory precision obtained, indicated the suitability of the method in quality assessment in wheat breeding (Andrews *et al.*, 1993). Starch constitutes the most abundant component in wheat flour and its significance is seen during milling, mixing, fermentation, baking and storage. A relation between starch gelatinization temperature, as measured by differential scanning calorimetry (DSC), and loaf volume is reported (Soulaka and Morrison, 1985a, 1985b; Eliasson, 1989). Gelatinization parameters determined by DSC have been found to differ in ordinary bread wheat and durum wheat (Lindhal *et al.*, 1993). Screening of large number of wheat varieties for DSC profile and end-use quality of wheat has shown a correlation between DSC parameters and other quality parameters such as the Zeleny value, falling number, kernel hardness and baking quality. Wheat varieties have typical DSC values depending on end use of the wheat. Durum wheats have a high gelatinization temperature (t_m) values and low gelatinization enthalpy (ΔH_{gp}), wheats for feed or biscuit have low t_m and high ΔH_{gp}, whereas wheats for baking were in between. An increased t_m and decreased ΔH_{gp} improves baking quality. A strong relation between t_m and ΔH_{gp}, and kernel hardness is also found (Eliasson *et al.*, 1995).

IR spectroscopy is a rapid and simple method for evaluation of wheat quality immediately prior to silo storage. Protein content as determined by IR measurements is known to correlate with loaf volume in test baking (Beuch, 1983). This technique

can also be used to evaluate grain quality at delivery reception. The protein content as calculated by IR spectroscopy and Kjeldahl nitrogen is known to differ by <0.5%. A good correlation of protein content as calculated by IR with sedimentation value is reported (Hohne, 1983).

2.6.1.1 Maturity indicators of wheat and their relation to bread quality

Immature and heavily frosted kernels (Geddes *et al.*, 1932) give lower flour yields. The stage of maturity was found to have little effect on protein content or baking quality, whereas flour ash and diastase activity showed elevated levels at the early stages (Mangels and Stoa, 1928). The overall quality of red winter wheats in terms of test weight, flour yield and loaf volume improves during maturation until about two weeks before ripeness (Hoseney *et al.*, 1966). Baking absorption decreases during maturation. These changes are accompanied by a decrease in water and salt extractable flour proteins and a gradual increase in the average molecular weight and complexity of the proteins being synthesized. Maturation is also associated with an increase in dough strength properties, decrease in flour starch damage and decreasing farinograph and baking absorption. Loaf volume is only adversely affected at the most immature stages. Crumb colour generally improves with maturity.

Near infrared reflectance (NIR) spectroscopy has found increasing applications in following changes which take place during the processing of agricultural crops and production of foods as well as in continuous quality control. A study by Czuchajowska and Pomeranz (1989) identified the presence of up to four peaks using NIR reflectance. These disappear during wheat maturation. The peak at 2276–2288 nm seems best suited to identify immature wheat in a mixture because it is the most conspicuous and consistent and gradually disappears during maturation. Thus, for instance, when ten mixtures (ranging from 10% to 100% in 10% increments) of immature seven days after flowering (DAF) and mature (42 DAF) Nugaines wheat were prepared, the simple correlation coefficient between the percentage of immature wheat in the mixture and reflectance at 2276 nm was 0.992. Similarly high simple correlation coefficients have been recorded for the wavelengths 1618 nm and 1842 nm (0.991 and 0.992, respectively). A combination of the three wavelengths 2276 nm, 1618 nm and 1842 nm increased the multiple correlation with percentage of immature wheat in a blend to 0.998. The compounds in maturing wheats from various classes as well as in flours milled from these need to be investigated (Czuchajowska and Pomeranz, 1989).

Studies have suggested that quality is affected by both the degree of frost and the maturity of the plant at the time of the frost. A recent study has investigated the effect of frost on the quality characteristics of wheat at various stages of maturity (Preston *et al.*, 1991). The effect has been shown to be dependent on both temperature and maturity. At early maturity, temperatures below −3 °C caused decreased kernel weight and protein content but increased kernel hardness. Effects are less evident at later

maturity where the seed moisture is below about 45%. Low temperatures have little effect on dough strength properties. However, exposure at all but the most mature stage results in a significant increase in starch damage and farinograph absorption. Milling related parameters, i.e. test weight, wheat ash, flour ash, flour colour and yield, have all been shown to be adversely affected by the low exposure treatment except at the most mature stages. Baking quality in terms of loaf volume, baking strength index, and crumb and crust characteristics is also adversely affected during the early maturation phase (Preston *et al.*, 1991).

2.6.2 Flour quality for chemically leavened baked products

A test commonly used to evaluate the suitability of experimental and commercial soft wheat flours for the production of cookies, cakes, crackers and pastry goods is the classic acid viscosity test in its several modifications such as no-time, one-hour digestion, and 2 g protein tests. Although valuable, it is not consistently reliable as an index of soft wheat quality. Since the doughs and batters involved in the production of most baked products from soft wheat have a pH value higher than 7 due to the chemical leavening agents employed, it was perceived that the alkaline viscosity test would more accurately reflect the reactions occurring between the flour components and hence the associated quality attributes. Experiments have shown alkaline viscosity tests to correlate with cookie diameter ($r=-0.93$, significant at the 0.1% level), the regression equation being $X=-0.024X+18.40$, where Y and X are the cookie diameter and alkaline viscosity of a 4% flour suspension, respectively (Finney and Yamazaki, 1953).

2.6.3 Indicators of cooking and eating quality of extruded products

This group of products includes noodles, macaroni, spaghetti and so on. Properties of wheat flour which are preferred for noodle quality are summarized as: colour grader value, below 0.99; protein content, above 8.3%; breakdown, below 209; and amylose content, below 24.9%. Quantitative analysis using these parameters is suitable for predicting eating quality of noodles from the physicochemical properties of wheat flour. The correlation between observed and predicted eating quality scores is given by an equation, $y=0.635x+26.0$, where y and x are the predicted and observed eating scores in noodles (Watanabe and Suzuki, 1992). High molecular weight glutenin subunits, α-gliadins and β-gliadins are statistically correlated with pasta cooking quality (Autran and Galterio, 1989).

Semolina colour is considered to be the most important characteristic related to durum wheat and is a very useful technological parameter for pasta makers. Several chemical, physical and visual comparison techniques for the estimation of semolina colour have been reported (Fifield *et al.*, 1937; Irvine and Anderson, 1953; Matz and Larsen, 1954). Visual methods are unsuitable for routine assay. Alternative methods

include pigment extraction and use of electronic instruments. Reflectance measurements have been used to measure colour, but are known to be affected by variables such as ash and protein content. A good correlation between yellow index and β-carotene ($r=0.83$) and free lutein ($r=0.85$) has been reported (Johnston *et al.*, 1980). Objective measurements of perceived colours of streams can be obtained in terms of CIELAB (Commission Internationale d'Eclairage) colour space parameters. L, A and B are the tristimulus values used to define colour; L is for lightness, A is for redness or greenness and B is for yellowness or blueness. L^* correlates with flour ash content, b^* with flour yellow pigment content, and flour colour index (L^*-b^*) with both ash and pigment content (Oliver *et al.*, 1993). Semolina colour, as measured by the b and L values (yellowness and lightness) has shown a good correlation between the b value and colour, and is not affected by ash or protein content. The L value is however influenced by the extraction rate of semolina because of the presence of ash in flours. This b value also correlates with lutein ($r=0.99$) and carotene pigment content ($r=0.92$) (Acquistucci and Pasqui, 1991). This is therefore a simple and rapid system and can be reliably used in routine quality control. The colour of the wheat flour can also be measured objectively using a Zeiss lactometer for remission value, R, and samples can be routinely examined compared to a standard value (Moor and Szalai, 1974). The pekar test based on comparison of colour against a standard flour has been used, but is not recommended with the higher extraction flours owing to variation in the colour of hulls (Drevon, 1952). Correlations between 'pekar' formula for determining flour extraction and ash content have also been attempted, but results are variable (Drevon, 1952).

The enzyme polyphenol oxidase (PPO) causes undesirable colour changes in numerous wheat-based end products such as chappatis, Middle East flat breads, steamed bread and noodles. A quantitative assay for PPO levels in grains without the requirement for seed grinding has been developed. The seeds are steeped in water for 16 h after which catechol substrate is added. The colour is then measured after 30 min. This correlates well ($r=0.85$) with the rate of decrease in brightness (L^*) or increase in yellowness (b^*) of raw Cantonese noodle sheet after storage for 4 h and 24 h. This simple method could be useful to plant breeders for selecting wheat cultivars for noodle making (Kruger *et al.*, 1994).

Gluten strength has been taken as an indicator of gluten quality, since durum wheats with short, inextensible gluten produce spaghetti with the best cooking characteristics. Mixing characteristics of semolina at low absorption as assessed on a farinograph can give an indication of gluten strength. Protein content, semolina granulation, temperature and absorption can influence farinograph curves. Stretching test and extensibility tests have been useful in screening new varieties for gluten strength, and could possibly be correlated to spaghetti and noodle cooking quality. A strain gauge, which is sensitive, has been devised and is taken as the force required to break a strand of wet gluten (Matsuo, 1978). These data can be linked to eating and cooking quality of spaghetti and noodles, but this requires experimental evidence.

Table 2.5 Simple correlation coefficients between starch and flour properties and components of noodle eating quality

	Noodle eating quality parameters			
	Softness	Elasticity	Smoothness	Total score
Starch peak viscosity	0.77[b]	0.69[b]	0.34	0.77[b]
Starch swelling power	0.70[b]	0.79[b]	0.49	0.84[b]
Starch swelling volume	0.85[b]	0.79[b]	0.49	0.88[b]
Flour swelling volume	0.55[a]	0.68[b]	0.36	0.69[b]

[a] $P<0.05$
[b] $P<0.01$
Source: Crosbie, 1991 (reproduced with permission).

The high quality of Australian Standard White (ASW) from western Australia is highly regarded in Japan for its suitability for noodle making. This reputation is largely attributed to the starch component and its effect on the eating quality and textural characteristics of the boiled noodle (Nagao *et al.*, 1977; Moss, 1980; Toyokawa *et al.*, 1989). A high starch paste peak viscosity as determined on a Brabender amylograph is a commonly observed characteristic of all wheat varieties giving noodles of good eating quality. Use of a Newport Rapid Viscoanalyser (RVA), wherein a 3–4 g sample is suspended in distilled water and heated to 60 °C for 4 min, followed by increasing the temperature to 95 °C holding for 8 min and then finally cooling to 50 °C for 8 min gives peak viscosity for both flours as well as starch, which correlates well with results from the Brabender viscoamylograph as well as with the sensory eating quality of Japanese and Korean-style salted noodles (Panozzo and McCormick, 1993). Table 2.5 gives simple correlation coefficients between starch and flour properties and components of noodle quality. It can be observed that starch swelling properties provide a method of using starch paste peak viscosity to predict noodle eating quality (Crosbie, 1991).

Although the swelling power test is less time consuming than paste viscosity measurements, the sample throughput is limited by the need to separate carefully the sedimented gel and supernatent layers after centrifuging, to weigh the gel and estimate the soluble dry matter in the supernatent by means of evaporative or colorimetric procedures. The swelling power method without the last steps has been described by Toyokawa *et al.* (1989). Assessment of the swelling power test with this modification also correlates with noodle eating quality as well as starch peak viscosity (Crosbie and Lambe, 1990). This test is simple, rapid and inexpensive and can be applied to a wide range of flour types ranging from high purity flours to wholemeal derived from both soft and hard grained wheats. It can also be used to identify hard grained wheats having potential as parental material in a noodle wheat breeding programme. A further simplification involves simply rating the height of the gel related to that of a standard cultivar (Crosbie *et al.*, 1992). It has been also shown that the swelling power of the starch depends on the cultivars, the growing season and the growing site of wheat.

Inclusion of wheat protein and wheat softness with starch swelling in multiple regression equations provides improved relationships for most noodle eating quality parameters. The predictions so obtained are also useful for varying seasons, and this has been tentatively demonstrated (Konik *et al.*, 1993).

Early studies on pasta cooking quality have stressed the importance of resistance to disintegration during cooking, and this remains an established quality parameter. This is manifested as the amount of residue in the cooking water, which is in fact used as an indicator of cooked spaghetti. Cooking losses can be rapidly estimated by measuring the absorbance at 650 nm of a complex between a clarified aliquot of the cooking water and iodine.

Two of the most important factors which contribute to the macaroni-making quality of wheat flour are higher pigment content and lower lipoxidase activity. These factors have been recommended as indicators in selecting breeds favourable to macaroni manufacture and eliminating unfavourable ones at a much earlier stage of development (Irvine and Anderson, 1953). Technically, these indices could also serve to indicate the suitability of a wheat flour for the manufacture of macaroni and related products and need experimental confirmation.

2.6.4 Pancakes

Pancakes, called hotcakes in Japan are a unique food product, designed to be easily made in a frying pan and are eaten hot immediately after baking. They are characterized by low springiness and strong gumminess. Chlorination of flour is known to improve the texture attributes of these pancakes. Hydrophobicity of the chlorinated starch granule is believed to be related to this improvement. This is explained as follows. Air bubbles trapped in the batter contribute to the development of cake structure and therefore factors that increase bubble stability would automatically improve texture. Chlorination levels increase the stability of air bubbles and hence improve the texture (Seguchi, 1993). Hydrophobicity of the starch granule could thus be considered as indicative of good processing and eating quality of pancakes.

2.6.5 Indicators of malting quality of barley

The quality of the malt determines the saccharification of starch as well as the flavour, body and aroma of the brew and the haze given by the proteins. Therefore tests are needed to evaluate the suitability of barley. These tests are needed by plant breeders during the early stages of a barley breeding programme to enable prediction of malting performance of barley lines. The malting quality of barley depends on the structure of the barley and on the enzymes which develop during malting, and it is unlikely that any single test on barley will ever completely predict the malting quality. The Zeleny sedimentation test, developed to predict the baking quality of wheat varieties is

promising for the evaluation of malting potential of unknown varieties (Reeves *et al.*, 1979) and is used worldwide. Other tests suggested for the purpose are the sedimentation test devised by Palmer (1975), a milling energy test (Allison *et al.*, 1976) and the falling number test (Morgan and Gothard, 1977). The Zeleny sedimentation test is empirical and it is likely that several factors contribute to the sediment formation. The association between fibrils of the protein from the flour particles is believed to be primarily responsible (Muller and Bernardin, 1975). In barley, the counterpart of gluten in wheat is the storage protein, hordein, which has an important influence on malting. The starch/protein interaction and the variations between hordeins from different barleys might be of relevance in brewing.

2.6.6 Cooking quality of rice

Quality is the most sought after factor in rice used for consumption. Since the edible part of rice is the starchy endosperm, its cooking, eating, processing and nutritive quality is of prime significance. The cooking quality of rice is mainly judged by certain basic characteristics of cooked grains such as degree of swelling, water uptake and total solids lost in residual cooking water and has been studied by many workers (Singh *et al.*, 1977). In the Indian subcontinent, freshly harvested rice is not considered suitable for culinary use since it cooks to a pasty mass, does not swell completely during cooking and loses more solids in the cooking medium. These undesirable properties are eliminated by storing freshly harvested rice for a period of about 3–4 months under ambient conditions. The rice grain changes progressively in some physicochemical properties after harvest, especially during the first 3–4 months of storage (Barber, 1972; Villareal *et al.*, 1976; Perez and Juliano, 1981).

The amylose/amylopectin ratio is the major factor affecting water absorption and volume expansion during cooking, as well as gloss and texture of cooked milled rice (Juliano *et al.*, 1972; Perez and Juliano, 1979). However varieties with similar amylose content may differ in eating quality due to differences in starch gel consistency. Gel consistency, therefore in conjunction with amylose content gives a good index of eating quality. Varieties with high amylose content show better cooking quality. Cooked rice grains with high amylose content become flaky, whole and free from slimy liquid whereas those with low amylose become moist and sticky during cooking (Chakrabarthy *et al.*, 1972). Hardness and stickiness values of cooked rice, as measured by an Instron food tester (Mossman *et al.*, 1983), correlated (individually either) to the final gelatinization at birefringence end point temperature or to neutral gel consistency or to both of these properties of raw rice (Perez *et al.*, 1979). Textural properties of rice, i.e. stickiness, consistency of cooked rice and viscogram characteristics could not be explained on the basis of total amylose content alone, but correlated with the total insoluble amylose content (Bhattacharya *et al.*, 1978). Amylopectin content is also suggested as important in determining textural properties (Nava, 1964).

Cooking changes the configuration of starch. The Raman spectrum of uncooked

rice was found to be similar to that of amylopectin and of cooked rice to that of dextran (Shih and Koenig, 1972). Simple alkali degradation is of great value as a rapid test for overall quality (Bhattacharya and Sowbhagya, 1980). The alkali degradation of rice and equilibrium moisture content attained by it after soaking in water also have an important relationship with rice quality (Bhattacharya *et al.*, 1978). Rice having good palatable qualities was found to be richer in sulphydryl content (Moritaka and Yasumatsu, 1972). Sulphydryl and disulphide groups have been positively associated with palatability, especially from the viewpoint of both the texture and aroma of cooked rice (Moritaka and Yasumatsu, 1972). Water absorption and protein were negatively correlated while cooking time was positively correlated with the protein content and in general with the gelatinization temperature (Juliano and Onate, 1965). Carbonyl and sulphur compounds have also been shown to be important determinants of the flavour acceptability of rice.

2.7 Indices for microbial quality of cereals and cereal-based products

About 2% of the total world production of grain is damaged by microflora, causing changes in fat, protein, carbohydrates, minerals and vitamins (Boyacioglu and Hettiarachchy, 1995; Doharey, 1989; Christensen, 1957; Christensen and Kaufmann, 1965). The biochemical changes accompanying the invasion of storage fungi are lipolytic (Goodman and Christensen, 1952; Dirks *et al.*, 1955; Negel and Semeniuk, 1947), proteolytic (DeVay, 1952; Ghosh, 1953), saccharolytic (Milner and Geddes, 1946; Ghosh, 1951, 1952a, 1952b), and changes in vitamins (Bayfield and O'Dennel, 1945; Kondo and Okamura, 1933a, 1933b) and minerals (Ghosh, 1952a, 1952b). It is obvious that conditions such as moisture content and relative humidity (RH), which influence mould growth would also influence these changes (Bottomley *et al.*, 1950, 1952).

Infection of cereals such as corn and milo with fungi has been checked for fat acidity (Hummel *et al.*, 1954; Sorger-Domenigg *et al.*, 1955). Most fungi produce a considerable increase in fat acidity (>160 mg KOH/100 g) in less than 15 days (Tsuruta *et al.*, 1978) and could be used as tentative indices of microbial quality of cereals (Baker *et al.*, 1957). The fat acidity is also positively correlated to other types of damage, such as 'heat damage', 'rancid damage' and 'sick damage' in various grains such as wheat, corn, sorghum and soyabean (Baker *et al.*, 1959).

The acidity values of flour, dough and bread are believed to be indicators of raw material quality with regard to baking quality and incidence of germination damage, but this is not reliable. McGee and Christensen (1970) observed that the action of storage fungi became obvious before fatty acids were measurable, and therefore the application of fatty acid content as an index of contamination by storage fungi is debatable.

There is always a potential risk of mycotoxins and allergens forming during fungal growth. Aflatoxins do form in peanuts, as they also do in cereals, and can be estimated by sensitive ELISA techniques (Azimahtol and Tey, 1992). Ochratoxins are produced

in cereals such as wheat, barley and maize under conditions of high moisture (Abramson *et al.*, 1992). Zearalenone is produced in scabby wheat due to contamination by *Fusarium graminearum* (L'vova *et al.*, 1992). Deoxynivalenol is another fusarium metabolite that is toxic and stable at baking temperature (Boyacioglu *et al.*, 1993).

Current methods for the detection of mould contamination and mould growth have a number of drawbacks. Counting of colony forming units (cfu) is time consuming and not related to actual activity. Representative sampling is also difficult. Hence, investigators have been in search of other methods to quantify mould contamination in cereals. Some of these are described below.

2.7.1 Ergosterol content

Ergosterol concentration will give a direct measure of the biomass of organisms that cause grain mould. It has been proposed as an index of fungal contamination in stored cereals such as soft wheat, sorghum, barley, corn and rape (Seitz *et al.*, 1977, 1979; Jambunathan *et al.*, 1991; Cahagnier *et al.*, 1983; Cahagnier, 1984, 1988; Naewbanis *et al.*, 1986). Ergosterol is, in effect, quantitatively the most important mycosterol present in the grains derived by systematic leaching of moist mould cells. It is a principal structural constituent of the cytoplasmic membrane of the fungi. For wheat, in particular, ergosterol concentration has been found to be a function of kernel weight. It is relatively high in small kernels and in impurities, that is material not classified as whole sound grains (Regner *et al.*, 1994). For rice, ergosterol also appears to be an early sensitive indicator of aflatoxin production (Gourama and Bullerman, 1995).

A simple and rapid screening suitable for routine monitoring of ergosterol as an index of fungal contamination has been reported recently. The method makes use of the reaction between iodine and ergosterol forming a highly fluorescent addition product showing a characteristic greenish-blue fluorescence under longwave UV light. The iodination reaction is specific to ergosterol and the fluorescent product is highly stable. The method takes only 2 h and the minimum detection limit is 500 ng/spot. The chemical confirmatory method involves treatment of the iodinated ergosterol with sulphuric or hydrochloric acid which changes the fluorescence from greenish-blue to brilliant green. The screening method has been successfully applied to wheat, refined wheat flour, wholewheat flour, maize and sorghum (Sashidar Rao *et al.*, 1989). Besides cereals, the presence of ergosterol in sunflower seed oil is documented to point towards the infestation of raw material with moulds such as *Sclerotinia sclerotiorum* and *Alternaria tenus* (Grigor'eva *et al.*, 1982). Two techniques, photoacoustic spectroscopy (PAS) and diffuse reflectance spectroscopy (DRS), have been coupled to Fourier transform infrared (FTIR) spectroscopy to provide information about the mid-infrared absorption spectra of solids. Since most biological compounds have distinct patterns in the mid-infrared region, coupling FTIR with PAS or DRS could provide powerful tools for analysing grains. The technique has an added advantage of requiring

less sample preparation and being generally non-destructive. A correlation coefficient of 0.993 has been observed between fungal content calculated from the relative ergosterol content, and FTIR–DRS amide II absorption (the range of wavelength being 1600–1500 cm^{-1}) (Greene *et al.*, 1992). The absorbance in the amide II region therefore proved to be a very reliable indicator of fungal contamination. PAS has been shown to be more sensitive than DRS, but from a practical standpoint PAS can presently analyse only one intact kernel at a time.

2.7.2 Volatile compounds as indicators of microbial growth

The odour of cereals is often used as indicative of fungal infection. This is however subjective and many volatiles of fungal origin may not have a characteristic odour. The search for improved methods is therefore of great importance. One promising new technique is the analysis of volatile compounds in the headspace gas surrounding a sample where fungal infection is suspected. It can be developed into a simple and rapid method with high sensitivity. A combination of gas chromatography and mass spectrometry was used by Kaminski *et al.* (1974) to identify a number of volatile compounds produced by different fungi during growth on wheatmeal. The method, however, is time consuming since the volatiles have to be separated by a distillation procedure. A much faster method, where a sample of the headspace gas is injected directly into a gas chromatograph was employed by Norrman (1977). A further development consisted in the use of a porous polymer to trap the volatiles (Harris *et al.*, 1986) and concentrate prior to analysis (Hyde *et al.*, 1983). Studies on long term monitoring of storage conditions of cereals have identified 3-methyl-1-butanol, 1-octen-3-ol and 3-octanol as the volatiles associated with microflora (Abramson *et al.*, 1983). 3-Methyl-1-butanol can be detected even when the wheat is ventilated (Sinha *et al.*, 1988). It is important to choose compounds specific to fungi that do not appear in sound cereals and are detectable at an early stage of fungal growth. Even closely related fungi can be discriminated by using the pattern of non-volatile metabolite formation (Frisvad and Filtenborg, 1983). A study by Borjesson *et al.* (1989) attempted to recognize fungi by studying the pattern of volatile compounds. Their results are shown in Table 2.6. It is observed that compounds produced in greatest quantities are alcohols, alkanes and terpenes. Some compounds predominate in the early stages of fungal growth, for example, 3-methyl-1-butanol produced by *A. flavus* and *P. cyclopium*. Terpene production is the single major difference between various speciesof fungi (Collins, 1975; Sprecher and Hanssen, 1982), suggesting its utility not only for species recognition, but also for identification of strains. The effect of different factors such as moisture content or the cereal variety needs to be further evaluated.

Table 2.6 Volatiles in headspace gases (in ng l^{-1} of air) produced by different fungi during 6 days of cultivation with continuous air flow

Fungus/ Volatile compound	Days 1	2	3	4	5	6
Control						
2-Methylfuran	11	4	–	–	–	–
2-Methyl-1-propanol	2	1	–	–	–	–
2-Pentanone	7	6	–	–	–	–
3-Methyl-1-butanol	7	2	–	–	–	–
Aspergillus amstelodemi						
2-Methylfuran	22	10	13	19	39	58
Methylbutenol[a]	–	–	–	–	–	–
2-Methyl-1-propanol	4	6	5	6	5	6
2-Pentanone	10	15	28	19	17	10
2-Methyl-1-butanol	41	81	9	2	1	–
3-Octen-2-ol	2	1	6	6	6	5
3-Octen-3-ol	1	3	10	10	10	7
Aspergillus flavus						
2-Methylfuran	20	11	12	42	89	119
2-Methyl-1-propanol	4	24	12	48	84	76
3-Methyl-1-butanol	16	86	29	8	5	2
Fusarium culmorum						
Ethyl acetate	20	51	33	72	132	143
2-Methyl-1-propanol	3	30	89	145	201	202
Monoterpenes	6	2	7	8	15	17
Sesquiterpenes	2	4	24	25	36	34
Fusarium cyclopium[b]						
2-Methylfuran	–	64	37	83	92	100
2-Methyl-1-propanol	–	5	84	200	140	120
3-Methyl-1-butanol	–	35	20	17	9	7
3-Octen-2-ol	–	6	47	48	16	12
1-Octen-3-ol	–	6	36	45	28	21
Sesquiterpenes	–	2	3	6	7	5

[a]Could not be separated from 2-methylfuran. Mixture of 2-methyl-3-buten-2-ol and 3-methyl-2-buten-1-ol.
[b]This concentration of volatiles on day 1 was not measured for this fungus.
Source: Borjesson *et al.*, 1989 (reproduced with permission).

2.7.3 Physical properties of metabolites as indicators of fungal contamination

Mycotoxins are fungal metabolites that are toxic and pose a health hazard to humans and animals. The feed manufacturers' concern about mycotoxins in general and aflatoxins in particular has created a need for a fast, reliable and simple method of evaluating grains before purchase and of monitoring the quality of stored grains. One such method is the examination of corn under longwave (365 nm) ultraviolet radiation (Shotwell *et al.*, 1972), which gives bright greenish yellow fluorescence (BYGF) presumed to be due to a plant peroxidase product of kojic acid. A correlation does exist between the number of BYGF particles and the level of aflatoxin contamination (Kwolek and Shotwell, 1979; Shotwell and Hesseltine, 1981). This test is only presumptive and is liable to some false positive results (Hunt, Semper and Liebe, 1976; Shotwell *et al.*, 1975; Shotwell and Hesseltine, 1981). False negative results are infrequent and are seen only in the case of an extremely low level of contamination.

Density differences between aflatoxin contaminated corn and uncontaminated corn have been noted (Huff, 1980) and density segregation can be used to separate aflatoxin contaminated corn from uncontaminated corn. However, it is not a reliable method of predicting aflatoxin contamination or the level of such contamination. The density would also be influenced by damaged or cracked kernels in the sample (Huff and Hagler, 1982).

2.7.4 Other methods

Mould frequency index, defined as the sum of the maximum recorded number of kernels contaminated by different moulds, has been proposed as a measure of contamination of barley and malt (Flannigan, 1982).

Quantiation of mycotoxins could be achieved by using as little as a 10 g sample to indicate mould contamination in corn (Francis *et al.*, 1988).

2.7.5 Ergotism

Ergot is a plant disease caused by a fungus belonging to the genus *Claviceps* and characterized by the presence of dark, fungal sclerotia or ergot bodies in the fruiting parts of the affected plant. Rye, barley, triticale, oats, wheat and bajra are among the hosts of the fungi responsible for the disease. The sclerotia contain the toxic alkaloids ergocristine, ergometrine or ergonovine, ergosine, ergotamine, ergocarmine and L-ergocryptine and their ingestion has been the cause of severe illness in man and animals, referred to as ergotism (Alexopoulos, 1962). Reproductive problems are among the recognized effects of the alkaloids in animals (Campbell and Burfenig, 1972; Burfenig, 1973). Ergotism in humans has largely been eliminated by current grading and milling procedures for grains meant for human consumption. A

maximum level of 0.05% ergot by weight has been suggested as an acceptable safety level for flour (Lorenz, 1979).

Development of a liquid chromatographic method with fluorescence detection of ergot alkaloids in flour has been reported (Scott and Lawrence, 1980). Ergot particles in grain products can be evaluated microscopically as well as by the presence of the ergot alkaloid, ergocristine. The latter method is simple and inexpensive and enables rapid screening (McClymont Peace and Harwig, 1982). A screening method for ergot particles in grain products consists of suspending 100 mg units of grain in aqueous glycerol and differentiating ergot particles by their microscopic structures and properties. For flour intentionally spiked with ergot particles of 0.1–0.25 mm diameter, recovery rates range from 79–90% at 0.001% contamination and 70–89% at 0.0001% contamination. Rye flour has been shown to contain 70–414 ng g^{-1} alkaloids with one sample showing a value of 3972 ng g^{-1}. Wheat flour contains much lower quantities (15–68 ng g^{-1}). The values for triticale flour have been reported to be 46–283 ng g^{-1} (Scott *et al.*, 1992).

Studies with wheat indicate that most of the ergot bodies are removed during cleaning, while as a result of the milling process, the feed streams (bran or shorts, depending on the mill used) contained the highest ergot levels (Shuey *et al.*, 1973). However results from ergot-containing rye have shown higher concentrations of ergot in the flour fractions relative to bran (Wolff *et al.*, 1983, 1984).

2.8 Indices of insect infestation of grains

Cereals are often attacked by insects during storage (Atwal, 1976). Certain heteropterous insects or wheat bugs occasionally feed on immature wheat grain and leave salivary proteinase in the kernel. In such bug-damaged wheat grains the enzymes destroy the gluten structure to produce slack, sticky doughs and loaves of poor volume and texture. These need to be detected and rejected before milling.

Flour and bakery products are very often contaminated with insects and their eggs, larvae and their fragments and rodent hairs. A filth test can be used to test flour acceptance, particularly in importing countries (Bouchard, 1983). Wheat quality standards address, among others, insect content of wheat. United States Department of Agriculture Standards (USDA/FGIS 1980, 1987) are based solely on the presence of visible insects (two live insects injurious to grain/100 g). These standards take no account of the immature insects that may inhabit grain kernels (hidden insects). USFDA (1980) standards are based on insect fragments found after milling and thus include at least the more mature hidden insects. Further, there is generally a four-fold increase in infestation from hidden insects in a generation (Schatzki and Bryant Fine, 1988; Arteman, 1981; Chambers, 1987). The various methods for detecting insect infestation are described below.

2.8.1 Physicochemical methods

Among the methods that have been proposed for detecting hidden insects are radiography, staining of the egg plug deposited by the female on egg laying (Reed and Harris, 1953; Frankenfeld, 1950; Goossens, 1949; Milner *et al.*, 1950), visual inspection for exit holes left by emerging insects (Nicholson *et al.*, 1953a), flotation of kernels to detect internal voids left by the feeding insect, cracking kernels and concentrating the removed insect parts (Harris *et al.*, 1952), crushing kernels and staining with ninhydrin to detect amino acids representative of the living insect, detection of uric acid present in the excreta of the insects (Galacci, 1983; Roy and Kvenberg, 1981; Wehling and Wetzel, 1983), IR/CO_2 gas analysis to determine the respiration of insects (Bruce *et al.*, 1982; Sinha *et al.*, 1986a, 1986b; Street and Bruce, 1986a, 1976b) and sonic detection of chewing insects (Adams *et al.*, 1953, 1954). Yet another method consists of gelatinization of suspected grains in sodium hydroxide (5 g or 100 kernels in 50 ml of 10% sodium hydroxide) for 10 min to clear the starch so that the kernels become translucent and it is possible to see insects within the kernels under a low power binocular microscope (Keppel, 1953). This method is not as sensitive as the stain test for detecting internal infestation of wheat. It detects only well developed insect forms, indicating its utility in detecting infestation only in the more advanced stages.

With respect to X-ray imaging in particular, Nicholson *et al.* (1953a, 1953b, 1953c) investigated the pertinent exposure parameters and the method has been adopted as official (AACC, 1969). Exposure of the film is used when the development of the immature insect is to be followed in detail (Sharifi and Mills, 1971). While some methods are under active development, the current choice of method is clearly X-ray radiography. About 40% of US millers and processors use this method of quality control for accepting shipments (Arteman, 1981). This method is however subjective. No quality standards exist for any method to determine hidden insects. Schatzki and Bryant Fine (1988) used daily film radiograms on hard winter wheat kernels following one day exposure to each of the four hidden pests of North American wheat (*Sitophilus zeamais*, *Sitophilus oryzae*, *Ryzopertha dominica* and *Sitotroga cerealella*). The films were viewed in transmitted light using microscopic lenses and image enhancement. The infestation could be determined with 80% accuracy 8, 7, 27 and 15 days after oviposition. Insect detection has been found to be a sigmoidal function of insect age (Keagy and Schatzki, 1991). False negatives generally decrease exponentially with insect maturity while false positives amount to 0.08% (Schatzki and Bryant Fine, 1988).

2.8.2 Staining methods based on the cell wall constituents of the insects

Insect fragments can be detected by using a chitin sensitive stain, phloxin B; whole hairs and feathers by using a keratin sensitive stain, Remazol brilliant blue. These tests

can be automated to give quantitation of filth by a manual optical image analysis (Doring, 1978).

Comparative analysis of gluten from normal wheat flour and that made from flour containing 4.2% grain damaged by chinch bug showed an altered proportion of high molecular and low molecular fractions, when examined by Sephadex G-200 filtration. It is believed that depolymerization of gluten by the action of the insect enzyme in chinch bug infected wheat causes a marked reduction in the content of the high molecular fraction (Koz'mina and Tvorogova, 1973). The proteins of damaged grain show a characteristic zone preceding α-gliadins and corresponding to glutenin breakdown products. The gliadins are strongly resistant to the action of chinch bug enzymes (Yakovenko *et al.*, 1973). The increase in α- and β- amylase and lipase is related to an increase in the proportion of affected grain and could serve to indicate chinch bug infestation, but not the extent, as the changes in enzyme activity are determined by cultivar characteristics. Illumination by electromagnetic radiation can also distinguish damaged and undamaged grain (Brizgis *et al.*, 1987).

2.8.3 Methods based on the estimation of non-protein nitrogen especially uric acid

Insect infestation is characterized by the increase in uric acid, an excretory product (White and Sinha, 1980; Wehling *et al.*, 1984). It is also reported to reduce protein digestibility (Hira *et al.*, 1988; Jood and Kapoor, 1992) while causing an increase in total nitrogen (Pingale *et al.*, 1954; Sudhakar and Pandey, 1987), and a decrease in protein content (Pushpamma and Reddy, 1979; Sharma *et al.*, 1979; Nirmala and Kokilavani, 1980) has been reported as a result of insect infestation. Table 2.7 shows the effect of insect infestation on the nitrogen contents of wheat, maize and sorghum.

Storage studies up to three months have shown minor variations in the total nitrogen, protein nitrogen, non-protein nitrogen and uric acid contents of the cereals. Uric acid is considered a significant compound, primarily as an index of soluble insect excreta (Barry, 1951). It can therefore serve as an index of insect infestation (Subrahmanyan *et al.*, 1955). Uric acid can be determined by enzymatic (Sen, 1968; Bhattacharya and Waldbauer, 1969), colorimetric (Laessig *et al.*, 1972) and thin layer chromatography (TLC) methods (Sen Gupta *et al.*, 1972). However there are many inherent disadvantages associated with their application to the trace analysis of uric acid in complex sample matrices (Young *et al.*, 1975). A simple sensitive technique which is not subject to interferences, and based on combination of high pressure liquid chromatography and thin layer amperometric detection has been reported to monitor insect infestation in cereal products (Pachla and Kissinger, 1977).

2.8.4 Enzymic methods to detect insect infestation in grains

Insects infesting wheat are known to secrete an amylolytic enzyme in their salivary

Table 2.7 Effect of insect infestation on nitrogen content (% on dry matter basis)

Insect species	Infestation level (%)	Total nitrogen	Non-protein nitrogen	Protein nitrogen	True protein	Total protein	Uric acid (mg/100g)
Wheat grains							
Control	0	2.11	0.04	2.07	11.8	12.0	0.04
Trogoderna granarium	25–75%	2.64	0.86	1.78	10.1	15.0	20.3
Rhizopertha dominica	25–75%	2.81	0.84	1.98	11.2	16.0	24.9
Trogoderna granarium+ *Rhizopertha dominica*	25–75%	2.75	0.83	1.92	11.0	15.7	23.0
Maize grains							
Control	0	1.83	0.02	1.81	11.3	11.5	0.06
Trogoderna granarium	25–75%	2.29	0.74	1.55	9.68	14.4	17.6
Rhizopertha dominica	25–75%	2.47	0.77	1.70	10.7	15.4	21.6
Trogoderna granarium+ *Rhizopertha dominica*	25–75%	2.33	0.67	1.67	10.4	14.6	20.0
Sorghum grains							
Control	0	1.74	0.01	1.73	10.8	10.9	0.05
Trogoderna granarium	25–75%	2.30	0.67	1.64	10.2	14.4	18.9
Rhizopertha dominica	25–75%	2.37	0.79	1.57	9.82	14.8	22.8
Trogoderna granarium+ *Rhizopertha dominica*	25–75%	2.32	0.69	1.63	10.2	14.5	21.2

Source: Jood and Kapoor, 1993 (reproduced with permission).

juice. However the characteristics of amylase secreted by the insect are similar to the enzyme in the flour from sound wheat and the activity is not significantly greater than that in the sound grain (Dubois and Vaudzic, 1952).

A simple proteinase microassay (Every, 1991) has been developed to detect bug-damaged wheat flour. This test compares with other methods of detecting bug-damaged grains such as visible damage test and a modified SDS-sedimentation test. Relationships between baking scores (*BS*) and proteinase activity (*P*) are given by the equations:

$$BS = 26.93 \times 0.919^{P/100}, \text{ for high quality cultivars} \quad [2.2]$$

$$BS = 17.41 \times 0.848^{P/100}, \text{ for low quality cultivars} \quad [2.3]$$

It is believed that if the alkaline proteinase level is greater than 280 U g^{-1} (U=unit of enzyme activity defined by Every, 1992; p. 185) for high bread quality cultivars and 100 U g^{-1} for low bread quality cultivars, the wheat can be judged as contaminated by insects (Every, 1992).

2.8.5 Detection of insect eggs in stored grains

Detection of insect eggs such as *Tribolium castaneum* in milled grain products could be

achieved with 100% accuracy by flotation as a scum in supersaturated brine having a specific gravity of 1.198 followed by microscopy (Girish *et al.*, 1972).

Luminescence by examination of the sample under UV light has been reported to indicate flour freshness. The fluorescence changes from blue to yellow on spoilage of the flour which has been dried at 105 °C or rapidly at 130 °C (Lesko, 1952).

2.9 Detection of damaged grains in sound grains

Corn kernel defects such as broken, chipped and starch-cracked kernels can be detected with 100% accuracy and split kernels with 80% accuracy by using reflectance differences, when a low power helium–neon laser (632.8 nm, red light) is used as light source, engineered into an optoelectronic instrument (Gunasekaran *et al.*, 1986).

Specific gravity tables fractionate samples on the basis of density differences and are effective in removing light foreign material from seeds. Specific gravity tables have been used to identify and segregate low grade European bread wheat to recover portions with improved test weight and reduced α-amylase activity (Hook *et al.*, 1988; Munck, 1989). The most severely sprouted, broken and damaged kernels are concentrated in the least dense fraction (Dexter *et al.*, 1991). These could be separated and used to get a crude indication of sprouted or damaged wheat in sound grains.

Frost damage of hard red spring (HRS) wheat was shown (Dexter *et al.*, 1985) to reduce the loaf volume and give poor crumb and crust characteristics, as the visual degree of frost damage increased. In addition, physical dough properties weaken, flour starch damage increases and farinograph absorption increases with rise in visual frost damage. Frost resistant and common wheats can be distinguished by examination of the activity and thermal coefficient of activity of the enzymes catalase and saccharase. Frost resistant specimens show a decidedly better quality, manifested as a lower temperature coefficient (Blagoveshchenskii and Gavrilova, 1954).

2.10 Other grains

Information on several cereal grains is given in Table 2.1. Very little attention has been given by food scientists to these and other grains grown, processed and consumed in parts of Asia, Africa and South America. Some of the edible legumes grown in these areas and which form a major source of protein for the population have been attracting attention only recently. Apart from their nutritional significance they contribute to the textural, taste and flavour characteristics of the food products derived from them and their blends with cereals. In several cases even grading on the basis of scientific criteria has not been done as yet. Of late the problem of the 'hard-to-cook' phenomenon in beans and peas has been receiving some attention (Reyes-Moreno and Paredes-Lopez, 1993). Several of these are cultivated in areas where commercial crops are difficult to cultivate so that such coarse cereal grains, legumes, amaranth should have good scope in future feeding programmes. It is surprising that very little attention has been given

even to oat, rye and triticale available in Europe and America as staple food grains.

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Chapter 3

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Chapter 3

Fruit and Vegetable Products

3.1 Introduction

Fruits and vegetables form an essential component of the daily diet and have been mainly responsible for contributing a variety of tastes and flavours, for widening the recipe range and attractiveness, and in the design of a nutritionally well balanced diet especially with respect to vitamins, minerals, nutrient fibre and as-yet unidentified protective and growth factors. Fruit and vegetable products break the monotony of cereal and meat dietaries. A wide range of fruit and vegetable species, both cultivated and wild, have been used by the human race for edible purposes. The wild varieties have been in use traditionally in different countries and have not received much attention from food scientists. During the past couple of centuries there has been a noticeable trend for dietary vegetables to be restricted to some cultivated species.

Intensive horticultural researches in severeal countries have resulted in the developemt of a large number of cultivars of every species of fruit and vegetable. These cultivars when grown in different regions, climates, soil types and seasons, using different horticultural practices, harvesting methods and timings and different storage, packaging and transport practices, offer widely varying patterns of chemical composition, nutritional, textural, taste and flavour characteristics, apart from size, shape, colour, appearance and other morphological features. On the basis of acceptability as well as end use consideration these cultivars differ in market price as well. The need is therefore felt to classify them into grades and in many countries such gradations have mainly been laid down mostly on the basis of characteristics such as cultivar, region, size, uniformity, appearance, colour, aroma and taste. Some fruits and vegetables go from the farm to the consumer directly, whereas some pass through ambient or cold storages and some are processed into products for direct consumption such as canned, bottled or frozen pulps, juices, juice concentrates, drinks, confections, sauces, pickles, wines and cider, or into intermediates for further use in household cuisine such as cut or cleaned vegetables, pulps, dehydrated products, semi-processed frozen forms, etc.

Those items marketed intact for consumers are graded on the basis of external characteristics that are familiar to the consumer. However the presence of agrochemical or pesticide residues, occasionally of radio isotopes, and in the case of fruit whether artificially ripened, are the aspects that do affect the quality of these commodities.

Processed products on the other hand may offer scope for mixing with inferior

cultivars, over- or undermature material, peel, seeds and other inedible portions, other species, additives such as organic acids, sugars, thickeners, artificial colours, flavourants and preservatives. Since the chemical composition of any species varies widely it is difficult to lay down standard specifications with respect to gross and trace components and develop unequivocal methods for establishing authenticity.

The separation of the edible portion may be, depending on the fruit/vegetable type, by comminution, compression, vacuumizing, homogenizing, followed if necessary by screening, filtration or centrifugation. With a view to recovering maximum yield, the pressure and extent of comminution may be varied. In the mechanical processes employed, depending on the severity of the method, varying extracts from the core, skin, rind, seeds or finely comminuted fibrous materials may be found in the pulp or juice. The enzymes pectinase, cellulase and amylase are often used to enhance the yield of the juice, as in case of apples, or for clarification such as in apple, guava and banana juices.

The chemical composition of the pulp or juice from the same variety of fruit may vary significantly depending on maturity, the season and region where it grows and the mode of extraction. Extracts from rinds and seeds of citrus fruit add bitterness and strong aroma. The quality of the juices in terms of acidity pH, content and nature of sugars, starch, pectin, polysaccharides, carotenoids and other pigments, essential oils, phenolic compounds and ascorbic acid can vary widely. Sweetness, sourness and astringency may vary not only in intensity but qualitatively depending on the constituent sugars, acids, the phenolic compounds and taste modifying agents. Consistency depends on the pectin, starch and other polysaccharides. Several cultivars of each species have been developed in different parts of the world and these show wide variation in characteristics. A multivariate relationship between analytical and sensory characteristics has been demonstrated with whole apples (Dever and Cliff, 1995).

In the manufacturing process, some water addition may be necessary to facilitate quantitative extraction and separation of the edible portions. To maintain uniformity of sweetness and sourness, required amounts of sugar and organic acid may have to be added. In the case of pulp, this may have to be diluted with water to some extent to adjust its fluidity/viscosity. The juices for preservation are subject to pasteurization or sterilization and also the addition of chemical preservatives such as benzoate and sulphur dioxide. Vacuum concentration using a suitable design of evaporator may be necessary to concentrate the juice, so as to minimize thermal damage, which may involve browning, loss of native aroma, caramelization, a cooked flavour and altered taste such as bitterness in citrus juice. While concentrating juices the aroma distilled out may be collected and added back. Alternatively the concentrated juice is supplemented with a proportion of the native juice to replenish the fresh aroma.

The major commercial fruit juices include those of orange, grapefruit, sweet and sour lime, apple, pear, prune, peach, apricot, mango, pineapple, grape, berries, tomato, etc. Besides juices, nectars or other fruit drinks are prepared as per the standards laid down in different countries. The quality of the marketed juice or drink is primarily

evaluated by sensory analysis. Of late varieties of fruit drinks such as juice cocktails are coming into vogue consisting of blends of compatible juices and, in the case of citrus juices, comminuted juices wherein some portion of the finely homogenized rind is suspended in the juice forming a composite drink.

It is a challenging task for the analytical scientist to collect information on several parameters of chemical composition, physical and rheological properties, sensory properties, pattern of trace constituents, enzymes, electrophoretic and immunological behaviour of proteins and chemotaxonomic characteristics, among others and attempt to develop authenticity criteria for every commodity, its cultivars, grades and so on.

3.2 Quality indices of fruit and vegetable juices

Food quality is often measured on the basis of weighted estimates of aspects of quality such as physical, chemical, biological and sensory characteristics. The relative importance of these aspects in determining the quality has to be assessed. When applied to fruit juices, suitable quality criteria include viscosity, colour and browning, natural and 'cooked' aroma and flavour, taste and in some cases bitterness (Luu and Westphal, 1981), and the chemical parameters, total and reducing sugars, acidity and pH and ascorbic acid content.

One aspect of composition that is commonly regulated is the fruit content. The original motive was to protect the consumer, and later due to pressure from fruit growers who want to sell more fruit. Constituents that can be followed right up to the final product are used as an index of fruit content, the ideal constituent being stable to processing, amenable to convenient and easy determination, rare so that it is unlikely to be used as an additive, and above all being at a constant concentration in the fruit. Over the years many trace constituents of fruits have been investigated as indices of fruit content. These include inorganic salts, nitrogenous compounds, polyphenolics, vitamins and pigments (Kefford, 1969).

The adulteration of citrus juice is a significant worldwide problem. Fruit juices may have undergone dilution with water (Boland, 1988), addition of sugar (Brause *et al.*, 1987), acidification and aroma intensification (Benk, 1976; Richard, 1978). Addition of substances foreign to the juices or addition of juices from other fruits are the other fraudulent practices (Iranzo, 1977). A Food and Drug Administration (FDA) probe into quality of chilled orange juice has found some packers to make large profits by illegally diluting their products with water, sugar and pulp wash solids (Anon, 1981). Other fillers identified in samples of orange concentrate are polysaccharides, starch hydrolysis products, essential oil supplements and emulsions (Vladimirov, 1972a). Many such practices remain undetectable. Compositional ranges for genuine products are generally used to detect such frauds (Wallrauch, 1975). Multidimensional statistical methods (Richard and Coursin, 1982), which have been used with citrus and pineapple juices are reported to give excellent results in identifying adulteration (Richard and Coursin, 1980). Statistical analyses which can detect citrus juice

adulterations include regression analysis, discriminant analysis and descriptive analysis such as principal component analysis (Nikdel *et al.*, 1988; Roussef and Nagy, 1987) and analysis of correspondence and hierarchical classification (Richard and Coursin, 1978). In the case of orange juice, principal component and discriminant scores based on the measurement of the areas of 34 different volatile components, measured by high performance liquid chromatography (HPLC) and 10 metal ions, measured by inductively coupled plasma spectroscopy (ICPS) provide an easy and informative way of dealing with complex adulteration problems (Albert and Zervos, 1989). Similarly, application of principal component and factorial discriminant analysis of near infrared (NIR) reflectance spectra over a wavelength range of 1100–2498 nm offers a simple practical method to detect 10% pulpwash in orange juice, and sugar–acid mixture with an accuracy of 90% (Twomey *et al.*, 1995). Effects of crop season and variety of the fruit on these analyses is significant, and detection efficiency increases when a suspicious sample is tested against its own population group, that is the same crop, same season and same varietal type (Aristoy *et al.*, 1989). Besides, the quality is also influenced by fertilizer application, growth regulators and also during storage (Testoni and Gorini, 1987).

In recent years, computerized pattern recognition programmes have become available which make it possible to categorize different samples of food by considering many variables that can be measured, often in a single analytical determination (Massart *et al.*, 1988). For instance, either fresh squeezed orange juice, pasteurized juice, single strength juice reconstituted from concentrate or aseptically packaged single strength juice from concentrate can be compared. This is possible on the basis of headspace analysis of 16 volatile compounds, identified by gas chromatography (GC) (Shaw *et al.*, 1993). Multiple regression analysis between rheological parameters and fruit content, °Brix, and total pectin content have been used to select several rheological and chemical indices and equations to determine fruit content, for example in strawberry jams (Carbonell, 1991).

However certain difficulties need to be surmounted before these methods are established as official methods of detection for the benefit of both producers and consumers (Navarro *et al.*, 1984). The importance of minimizing the number of independent variables in regression equations and of informed judgement when selecting variables has to be emphasized (Cohen, 1983). The correlations existing between the various natural constituents of fruits are deemed to be of value in ascertaining the purity of products such as nectars (Eksi, 1981). Ratios between contents of individual components offer another guide in juice evaluation (Fischer, 1973a, 1973b). Mathematical methods allowing qualitative and quantitative evaluation of the nature and amounts of possible additions to fruit juices and hence a full evaluation of the adulteration have been described. These are based on mathematical expressions of conformity to standards, conformity to ratios and classification of products, and some other mathematical aspects, such as constraints and accuracy of determination and of results (Richard and Coursin, 1979a, 1979b). Indices based on

four components, namely organoleptic properties, composition, weight or volume, and packaging and labelling have also been described as methods of food quality evaluation, wherein a mark from 0.0 to 1.0 is allocated to each component (Szilagyi, 1972). In the case of citrus juices, adulteration could be detected on the basis of organoleptic, physical and chemical characteristics by analytical means (Scholey, 1974; Petrus and Vandercook, 1980) from the composition of authentic samples of orange, lemon, grapefruit, mandarin and tangerine juices (Benk, 1974). Paper chromatography and HPLC (Low and Wudrich, 1993) of sugars (sucrose, glucose and fructose) and organic acids (malic and citric), gas liquid chromatography (GLC) and electrophoresis on cellulose acetate membranes (Gils and Bergh, 1974) for the amino acids (proline, arginine and γ-aminobutyric acid), and chemical characteristics such as acidity, formol value, pentose content, inorganic constituents and amino acid content and their various ratios are the various analytical approaches for detecting adulteration in fruit juices such as lemon, grapefruit and orange (Vandercook *et al.*, 1975; Katsouras, 1971, 1974; Richard *et al.*, 1984; Iranzo, 1972, 1975; Schatzki and Vandercook, 1978) as well as in carrots (Otteneder, 1982), raspberry, strawberry, lingonberry and blackcurrant juices and syrups (Fuchs and Wretling, 1991; Otteneder, 1978) and tomato juice (Otteneder, 1975). Some unidentified ninhydrin-positive substances, labelled as 'A', 'C' and 'M', which are in addition to the normal protein amino acids, have also been recommended as indices of adulteration of orange juices, and could be used for rapid semi-quantitative screening (Rossetti *et al.*, 1976). A chemical matrix method which allows identification of several compounds such as L-malic acid, chlorogenic acid and the fructose/glucose ratio, sucrose, proline and sorbitol can detect adulteration in apple and orange juices such as dilution with water, and addition of sugar, high fructose corn syrup, beet sugar and spent process water (Brause *et al.*, 1987). However, these should be used very carefully, since the varietal differences can mar or mask the results, for example Amasya, a Turkish apple variety used predominantly for juice and concentrate production is characterized by a low glucose/fructose ratio (Eksi and Karadeniz, 1991).

Apart from sugar profile analysis, UV ratio and metal analysis, isotope analysis and an extractive procedure which can detect trace organic materials present in beet sugar/invert sugar but not in authentic juice by GC–MS have also been incorporated in chemical matrix method designed to test juice samples for authenticity (Brause *et al.*, 1986). These also include checking for peel preparations in orange juice and concentrates (Benk, 1972). Amino acid concentrations and proportions are particularly sensitive indicators of authenticity (Ooghe and Kastelyn, 1985) with respect to blending as well as adulteration of fruit juices such as apple (Tanner and Sandoz, 1973a), orange, grapefruit and black grape or addition of protein hydrolysates (Bielig and Hofsommer, 1982). These have been suggested as a basis for evaluation of fruit juice quality (Ooghe and Waele, 1982a, 1982b). Visible and UV absorption and fluorescence and emission characteristics of alcoholic solutions of frozen orange concentrates and single strength orange juices can give qualitative detection and

quantitative approximation of orange pulp wash in orange juice (Petrus and Dunham, 1980). The absorbance sum at 443 nm, 325 nm and 280 nm and ratio of absorbance at 443/325 nm can provide an estimate of the percentage total citrus material, orange juice, pulp wash and dilution of the sample. UV/visible absorption and room temperature fluorescence excitation and emission spectra have been adopted as the official first action for detecting adulteration of Florida orange juice with pulp wash (Petrus and Attaway, 1985).

NIR spectroscopy gives a good idea of fruit content, particularly for strawberry jams, for which peaks are obtained at wavelengths of 750 nm, 830 nm, 890 nm and 1070 nm and troughs at 770 nm and 1090 nm (Scotter *et al.*, 1990). Of the many new developments in spectroscopy, perhaps the most important for the food industry is Fourier transform infrared (FTIR) spectroscopy operating in the mid-infrared region (Wilson, 1990) which offers tremendous potential for quantitative and qualitative food analysis. FTIR can distinguish the fruit type in fruit purees (Belton *et al.*, 1995). It can also detect whether fresh or freeze-thawed fruit was used for puree making, the level of ripeness in some cases, for example raspberry (but not strawberry), fruit variety, for example of apples, and any added sulphur dioxide (Defernez *et al.*, 1995). An investigation into the potential of FTIR for the determination of fruit content of jam has been reported recently. A quantitative method which has been developed used dried jam and the potassium bromide pellet technique, in combination with simple linear regression and partial least square (PLS) analysis. PLS analysis in particular offers one of the best methods for the determination of fruit content in strawberry jam. The FTIR method, using diffuse reflectance of jam solids washed on filter papers produces spectra of unusual appearance, but can reliably and reproducibly distinguish between jams of differing fruit content. Furthermore, the spectra obtained are characteristic of fruits, and can therefore act as fingerprints for different fruit types. These methods therefore have good potential for the verification of product authenticity and for detection of adulteration (Wilson *et al.*, 1993).

Quantitative descriptive analysis (QDA) has been developed for sensory evaluation of foods (Stone *et al.*, 1974; Szczesniak *et al.*, 1963; Weiss, 1972, Piggot, 1988) and relies on statistical analysis to determine terms, procedures and panelists to be used for analysis of a specific product. A quality index (QDA score) representing overall quality and based on statistical analysis of chemical, physical and organoleptic properties such as content of soluble solids, added sodium chloride, sugars and ascorbic acid, acidity, viscosity (instrumental and sensory), brightness and acceptability (flavour and colour) has been proposed to be of potential use in regulating quality standards for strained tomatoes (Riva and Pompei, 1986). Amongst the physical characteristics, refractive index, as obtained from hand refractometers and Abbe refractometers, has been considered suitable for quality control of fruit juices (Winkler, 1985). The correlation matrices from QDA are applicable in making comparisons with competitive products, developing new products and assessing the changes in old ones (Porretta *et al.*, 1992).

3.3 Organic acids and other additives

Fruit juices may be adulterated by addition of foreign, cheaper juices or by addition of sugar solutions acidified with organic acids. Crystalline deposit in casks of orange concentrates is a strong indication of adulteration. In one case, the deposits were identified by IR spectroscopy and melting point as tripotassium citrate (Hils, 1973). Lemon juice is sold commercially on the basis of its total acidity. Since synthetic citric acid costs about one-fifth the price of the acid in lemon juice, the temptation exists to add citric acid. Orange oil and β-carotene are often used to mask the adulteration (Anon, 1980). Extracts of carotenoid containing plants such as tagetes or marigold flowers have also been reported to mask these adulterations (Wild and Dobrovolny, 1976a, 1976b; Wild, 1976; Hadarim Hod Hasharon Ltd., 1976). It is believed that although the total carotenoids in orange juices vary with variety, season and location, the percentage composition of individual carotenoids remains within a narrow range. While cryptoxanthin palmitate predominates in orange juices, myristate and laurate esters predominate in tangerine concentrates. Similarly tagetes extracts can be identified by the presence of an increased concentration of xanthophyll esters, and in particular lutein dipalmitate. Quantification of these carotenoid esters enables detection of added carotenoids (Philip *et al.*, 1989). Turmeric or annato colour addition can be estimated by visible spectrophotometry (Petrus *et al.*, 1984) or by HPLC (Ting and Rouseff, 1986)

Analyses of non-volatile acids and anthocyanidin profiles by liquid chromatography, pigment concentration, polymeric colour and percentage polymeric colour by UV/visible spectral measurements have demonstrated their feasibility in detecting adulteration in certain juices and concentrates like cranberry and apples.

3.3.1 Organic acid profile

A non-volatile organic acid profile can be obtained by simple paper chromatography, and the intensity of the identified spots can yield information about quantity of the acids (Fitelson, 1969a). The profile can also be obtained by liquid chromatography (Fuleki *et al.*, 1993) or HPLC (Pilando and Wrolstad, 1992). These workers have shown the acid profile of some juices as follows: apple (malic, also citric); blackberry (citric, malic); black raspberry (citric, also malic); Morello and Montmorency cherry (malic, also citric); elderberry (citric, malic); Concord grape (tartaric, malic, also citric); California red grape (malic, tartaric, citric); strawberry (citric, malic).

A distinct alteration in the acid pattern of a fruit juice, accompanied by a change in acid intensities, will demonstrate the absence of normal amounts of characteristic acid and the presence of foreign acids. It could distinguish batches of imported cherry and blackberry juices that had been adulterated. A multiple regression analysis of the data obtained from analysis of various samples of commercial lemon juice for their total amino acids, L-malic acid and total polyphenolics gives an estimation of citric acid

content. The equation is: citric acid=36.54+12.04×amino acids+2.71× malic acid+30.06×total phenolics, all in mequiv/100 ml juice. If the actual citric acid is more than 20 mequiv/100 ml above the calculated value, the sample falls outside the acceptable limits, and therefore should be considered abnormal (Rolle and Vandercook, 1963). A significant relationship (99% confidence limit) between citric acid and total phenolics (correlation coefficient, $r=0.788$), as measured by UV spectra has also been established, and can serve as a quick method of establishing the purity of lemon juice (Vandercook and Rolle, 1963). The prediction of citric acid content by this multiple regression approach is independent of commercial fruit storage and processing practices (Vandercook *et al.*, 1966), as well as added preservatives such as sulphur dioxide, benzoate and potassium sorbate (Vandercook and Guerrero, 1968). Correlations between citric acid content and carotenoid and sterol content have been attempted, but were found to be low in lemon juice (Vandercook and Yokoyama, 1965). Isocitric acid is not present in commercially produced citric acid and because the ratio of isocitric to citric acid in natural products generally lies within the range 1:50 to 1:300, depending on the type and origin of the fruit, it can be used as an aid to detect added citric acid in natural juice (Rother and Neugebauer, 1976). Isocitric acid can be determined enzymically (Bergner-Lang, 1974). D-isocitric acid has been suggested as an indicator compound for orange juices. The levels are seldom less than 40 mg l^{-1}, and can be used to detect added acids or dilution with water (Calabro *et al.*, 1978). However, this should be used carefully, since its levels and also the ratio of citric acid: D-isocitric acid can vary with the season, as has been shown with Sicilian lemon juices. This ratio varies from 204–272 in summer and decreases to 170–209 in winter (Petronici *et al.*, 1978). Hence guiding values and tolerances for each parameter need to be properly adopted (Benk, 1979). The citric acid:acetic acid ratio is also an important criterion for detection of adulteration of citrus products (Tanner and Zanier, 1976).

The paper chromatographic methods are time consuming, requiring extraction, concentration, and often derivatization. Gas liquid chromatography requires the preparation of suitable volatile derivatives, mainly esters. Heatherbell (1974) separated acids from sugars in the ethanolic extract of the fruit by precipitation as their lead salts. The acids are converted into the trimethylsilyl derivatives for determination by GLC on SE-52 and XE-60 columns. Isolation of the organic acids in fruit products by anion exchange procedures and derivatization followed by GLC determination is also reported (Baker, 1973). Separation can be achieved by use of microcrystalline cellulose powder (Stahl *et al.*, 1974), Aminex A-25 anion exchange resin (Palmer and List, 1973), cation exchange resin (Turkelson and Richards, 1978) and a reversed phase column (Grushka *et al.*, 1975). In a comparison of an HPLC method with enzymatic and Rebelein (1967) methods, the HPLC method showed good recoveries for these acids. While the HPLC and Rebelein method determine the total (D, L)-malic acid, the enzymatic method measures only the L-malic acid. HPLC is versatile and can survey the complete 'organic acid' profile. From these 'fingerprints', conclusions concerning

possible adulteration, or recognition of juices distributed by the same factory can be obtained (Jeuring *et al.*, 1979). The use of a chiral stationary phase like chirasil Val in gas chromatography on fused silica column allows separation of enantiomers of malic acid after formation of suitable volatile derivatives, and can be used to detect synthetic acid in fruit juices (Bricout, 1987). Chiral liquid chromatography is shown to be effective in resolving malic acid enantiomers in apple juice adulterated with synthetic malic acid (Doner and Cavander, 1988).

The presence of D-malic acid is a clear indication of adulteration because this isomer does not occur naturally. D(+)-Malate can be determined by using an enzyme, D(+)-malate NAD oxidoreductase, isolated from a strain of *Pseudomonas fluorescens*, which decarboxylates D(+)-malate to pyruvate and reduces the NAD. The amount of NADH can be determined spectrophotometrically (Knichel and Radler, 1982). L-Malic acid and total malic acid in apple juice are measured to confirm adulteration. Fumaric acid and citric acid levels above trace amounts are inconsistent with pure apple juice. Measurement of these may also be needed for an authenticity check (Evans *et al.*, 1983). Fumaric acid is a minor contaminant in synthetically produced malic acid and is readily detectable by liquid chromatography. It has been suggested that quantities of fumaric acid above 3 mg l^{-1} indicate addition of synthetic malic acid, although this figure may have to be revised upward for juice made from concentrate (Junge and Spadinger, 1982). Relationships between °Brix (°B) and total percentage sugars and acids can also be considered as parameters of composition of the juices, and hence as indices of adulteration (Iranzo and Peres Toran, 1977). In addition the °B/acid ratio also correlates with flavour significantly (Fellers, 1991).

3.3.2 Anthocyanin patterns

The anthocyanin patterns are useful in detecting adulteration of dark coloured juices by other juices or colours. They are a better test than the anthocyanidin patterns (Fitelson, 1969b). For instance, substantial quantities of delphinidin and malvidin are indicative of possible addition of grape skin extract to cranberry juice (Hong and Wrolstad, 1986).

Detection of added citric acid in lemon juice is independent of dilution, and can be done on the basis of expressing constituent parameters as ratios rather than concentrations. The ratios of amino acids to total phenolics (AA/TP) and L-malic acid to total phenolics (MA/TP) are independent of dilution or added citric acid, but the ratio of citric acid to total phenolics (CA/TP) although independent of dilution would reflect added citric acid. A multiple regression approach has been used to predict CA/TP as a function of AA/TP and MA/TP and is reported to show a high correlation. Statistical criteria have been established to determine the number of samples required to detect any level of added citric acid at any probability of rejecting authentic and adulterated samples (Vandercook *et al.*, 1973).

3.3.3 Microbiological methods

Microbiological assay using *Lactobacillus plantarum* has been used to detect orange juice adulteration (Vandercook *et al.*, 1976; Vandercook and Smolensky, 1976, 1979; Vandercook, 1977). The organism requires many nutrients to grow and hence simultaneously assays a number of compounds which are also important in human nutrition. Its growth is independent of common beverage ingredients such as sugar, acids, butylated hydroxyanisole and orange oil. Growth is measured in terms of pH changes which parallel juice content of the basal glucose–buffer medium.

Fortification of orange juice with mixtures of sugar, buffered citric acid, ascorbic acid and vitamin A can be detected. However, sophisticated nutrient mixtures could be added which would promote growth response in bacteria similar to orange juice (Vandercook *et al.*, 1980). Detection of grape juice in apple juice is also possible by these microbiological methods (Smolensky and Vandercook, 1980).

3.3.4 Miscellaneous compounds

Sugarcane molasses are often used as an adulterant in tamarind concentrates. Molasses contain significantly more total ash, phosphorus and calcium than tamarind concentrates and these constituents may therefore be used to detect adulteration with 15–20% molasses (Chaudhuri *et al.*, 1979).

The ratio between sugar content and Brix has been reported to be useful in detecting additions of sugar to citrus juice (Lifshitz, 1983). Analytical detection of an adulterated consignment of apple juice and identification of extraneous materials have been described. Comparison of the suspect juice with the authentic one from the same country showed the former to have an abnormally high sugar-free extract, a high glucose content, smaller amounts of ash, potassium and phosphate, and an exceptionally high chloride content. High glucose content and simultaneous increase in sugar-free extract is indicative of adulteration with an incompletely hydrolysed glucose syrup. Thin layer chromatography (TLC) could confirm the presence of maltose in the suspected sample and the absence of maltose in the authentic sample. A similar confirmation was obtained from a remarkably high calcium content and an absence of fructose in the suspected sample (Niedmann, 1976a). The fructose/glucose ratio was studied by Stepak and Lifshitz (1971) but not found to be useful in detecting adulterations. Indices such as dry matter/ash, acidity/ash and the ratios of individual sugars with one another such as glucose, fructose and sucrose are useful in detecting addition of water, beet sugar or sugar syrup to natural mandarin juice (Fishman *et al.*, 1988). A liquid chromatographic method with pulsed amperometric detection of added sugars was recently shown to be useful in detecting 1% HFCS (high fructose corn syrup) in orange juice within 96 min (Wudrich *et al.*, 1993) and 5% of beet medium invert sugar (White and Cancalon, 1992a). Another promising technique for detecting added sucrose in fruit juice concentrates is isotope ratio mass spectrometry

(IRMS) (Yunianta *et al.*, 1995), the concept of which is explained later in this chapter. Proton NMR spectroscopy in combination with pattern recognition techniques is also being projected for the same purpose (Vogels *et al.*, 1996).

Added acids in peach pulps can be detected with the aid of the ratios of total acid:potassium, total acid:isocitric acid:malic acid, malic acid:potassium and malic acid:total ash. Similarly citric acid in peach pulps can be detected by the ratio of citric acid:isocitric acid and citric acid:total ash and sugar can be detected by the ratios of total sugar:total ash, total sugar:P and total sugar:Mg (Eksi, 1981). Immunoassays for detection of beet sugar adulteration of fruit juices and concentrates employing antibodies to proteinaceous components have been patented (Potter and Mansell, 1992).

Buchu oil has 8-mercapto-*p*-menthan-3-one as one of its constituents. It has a catty odour similar to that of blackcurrants and is therefore used as an additive to improve quality in commercial products such as concentrates, flavours, jams, juices, carbonated lemonades and wines. This is generally not declared on the label. The retention times on the gas liquid chromatography (GLC) column of the two catty odours are different, so that sniffing the effluent of gas chromatographic column is a way to detect the addition of buchu oil (Nijssen and Maarse, 1986).

Organic dyes are sometimes added to tomato concentrates (Safina and Trifiro, 1954a). These colouring matters which are not fixable by wool can be identified as a specific red ring followed by a pink band, when an isoamyl alcohol extract is adsorbed on alumina and then eluted with 15% citric acid solution (Safina and Trifiro, 1954b).

The pulp expelled from citrus juice finishers, called 'finisher pulp' contains about 80% juice. The soluble solids can be recovered by countercurrent washing and refining. Addition of this pulpwash to frozen orange juice and frozen orange concentrates is currently prohibited in certain places such as Florida. UV/vis spectrophotometer analysis in the range 600–200 nm (Petrus and Attaway, 1980), water soluble pectin levels (Wallrauch, 1986; Rouse *et al.*, 1959), mineral profiles especially calcium, silicon and sodium (Nikdel, 1991) and ratio of chemical constituents such as narirutin to hesperidin (Kirksey *et al.*, 1990; Rouseff and Marcy, 1984) provide various ways of detecting such additions.

3.4 Peel homogenates in citrus juices

The addition of peel extracts to concentrated orange juices with high turbidity can be detected via free amino acids. An increased concentration of the free amino acids, valine, methionine, isoleucine, leucine (Giacomo *et al.*, 1979), tyrosine and phenylalanine, from a total of 1.5% in natural juice to 3.7% in pulp and peel extract and 7.3% in commercial concentrates serves to recognize these additions (Gherardi *et al.*, 1976). Unlike simple flavonoid compounds which occur widely distributed in various fruits, several of the permethoxylated flavonoids (PMF) are probably unique to citrus fruits. The compounds originate in the oil sacs of the flavedo and concentrations

approximately 50 times greater in orange peel juice than in orange juice have been reported (Veldhius *et al.*, 1970). The addition of 10% peel juice to orange juice would significantly increase the total PMF content from 5 ppm to 15 ppm, which is not detected by sensory evaluation. Investigations of the possibility of distinguishing peel juice from juice on the basis of carotenoid patterns were successful in isolating a red coloured compound from Shamouti peel which was not found in the juice. This compound on saponification produced β-citraurin and reticulaxanthin. Immunoassay for limonin is also useful in uncovering pulpwash addition and dilution of orange juice (Boland, 1988). Also, an abnormally high pentose equivalent is indicative of peel and pulp (Sawyer, 1963).

Carotenoid-containing coloured extracts from orange peel in orange juices and concentrates can be detected from the content of total carotenoids. Levels of 10.8–12.8 mg % in two times concentrates may be regarded as abnormal. The content of cryptoxanthan esters in natural orange juice varies from 5.6 to 15.1 mg % (the exception being the mandarin group, which has levels of 30.5–51.9 mg %) (Benk, 1971b).

3.5 Dilution of fruit juices with water

A 1990 amendment to the US Food, Drug and Cosmetic Act states that if a food purports to be a beverage containing vegetable or fruit juice, it shall be deemed misbranded unless its label bears a statement of the total percentage of such fruit or vegetable juice content (Lindsay, 1993).

Analytical data for commercial juices have given a strong indication of dilution with water (Bechler, 1972). Discussion on dilution of juices necessarily also relates to fruit content. Aerometric specific gravity measurement has been recommended for calculation of added water in fruit and vegetable juices. Since the changes in specific gravity on dilution of juices are not rectilinear, empirical curves showing water addition for plum, cherry and tomato juices have been worked out (Lovacheva and Eliarova, 1974). A method for detecting dilution of elderberry mother juices based on determinations of sugar, acid and nitrogen-free extract has been developed (Fischer *et al.*, 1972). Electrical conductivity is also useful, but is influenced by various additives and also changes during storage (Moreno *et al.*, 1976). The methods generally used are as discussed below.

3.5.1 Inorganic indicators

Early procedures recommended for fruit content of citrus products involved determining the ash content as a gross measure of inorganic compounds, the alkalinity of ash, expressed as potassium carbonate as a measure of potassium, and the phosphate content (Stern, 1943, 1954; Morgan, 1954). Ash and phosphorus content are fairly independent of regional variation and soil and are a fair criterion of added water in berry and fruit juices (Tikka and Johansson, 1947). Analyses of serum from orange juices for Brix, acidity, maturity index, sulphite, sulphate, chloride and nitrate contents have shown increased levels of nitrate (Iranzo, 1971; Benk *et al.*, 1971) and sulphite (Benk and Cutka, 1972) to indicate likely dilution of the concentrate. The severity of the problem of dilution can be gauged from a report published in 1973 indicating that 53% samples were adulterated, some to the extent of 30–50% with water as found out by constituent and ratio analysis (Fischer, 1973c).

Increased levels of nitrate in orange juice samples do not originate from peel extracts but from nitrate containing water or pulp or peel extracts prepared using such water. The nitrate content in genuine samples is however dependent on its origin (Benk *et al.*, 1971). Further, even normal nitrate contents cannot be used as evidence of absence of adulteration, since nitrate-free or demineralized water could have been used for adulteration (Benk *et al.*, 1972).

Addition of peel in orange juices causes a slight increase in sulphite. Abnormally high sulphite concentration in some commercial products is attributed to dilution with sulphite-containing water or processing of excessively sulphited juices (Benk and Cutka, 1972).

Total nitrogen (T), amino nitrogen (AN) and the T:AN ratio of orange juice serum (Iranzo and Cervello, 1973a) are reported to be good parameters for detecting adulterations (Iranzo and Cervello, 1973b). The T:AN ratio is especially useful in detecting adulteration of commercial lemon juice (Iranzo *et al.*, 1977, 1978). The T:AN ratio together with the content of characteristic mineral elements such as calcium, magnesium and phosphorus are the best parameters for detecting citrus juice adulterations such as that of orange (Iranzo and Cervello, 1973a, 1973b). Albuminoid amino nitrogen is also useful in determining fruit content (altered due to dilution, or addition of sugar and colour) (Khanwalker and Dubash, 1976), and is also independent of pasteurization of the juice. However, it decreases on prolonged storage of bottled or canned juice, and may be affected by locality, stage of maturity and method of preparation. Therefore the effect of these factors would have to be considered before the limits for authentic juices are fixed (Siddappa and Raja Rao, 1955). The presence of nicotinic acid and betaine (Khanwalker and Dubash, 1976) as well as the concentration of free amino acids are other indicators of dilution of orange juice. A comparison of hand pressed and commercial orange juice has shown that the former contained around 30% more betaine (82 vs. 62 mg/100 ml) and about twice as much nicotinic acid (0.29 vs. 0.14 mg/100 ml) than the latter.

Analysis of percentage juice, refractometric extract, acidity and ascorbic acid in the juice of bitter orange (*Citrus auranticum L.*), and total and reducing sugars, proteins, formol index, calcium, magnesium, sodium, potassium, phosphorus and sucrose have indicated ash, mineral elements and protein and formol index to be useful parameters for detection of adulteration. Sodium content and the ratio of Brix/acidity+total sugars are particularly promising owing to the low sodium concentration (0.52–1.37 mg/100 ml) in the juice of bitter orange (Iranzo and Guzman, 1973). Similarly, organic acids, potassium levels and formol index are useful in characterizing pineapple juices and nectars (Camara *et al.*, 1995). Determination of chloramine value, formol number, and ash, phosphorus, boron, bromine, potassium, sodium, calcium, magnesium and hesperidin in commercial orange and lemon beverages, juices and nectars in Spain has shown phosphorus, hesperidin and boron contents to be most suitable for evaluation of fruit content or genuineness. If a single parameter is required, hesperidin is the most sensitive index, but all three should be determined if the product has been prepared from comminuted material (Termes and Torre Boronat, 1979). The chloramine value has been used as an indicator of the presence of true juice content, but requires extraction with petroleum ether to eliminate the interfering essential oils in its determination (Safina and Trifiro, 1957). In fruit juices containing low juice content such as that in lemon juice, reference values based on potassium, phosphate, proline, formol number and isocitric acid can successfully calculate the juice content of even a 6% lemon juice drink (A report, 1981). Using these indices it was possible to identify some samples which consisted solely of acidified,flavoured, coloured and emulsified sugar syrups and some samples which were 70–80% pure orange juices (Vladimirov, 1972b). Potassium content in a juice is known to vary widely according to the horticultural history of the sample. Some varieties have an abnormally low potassium content such as that in Israeli Shamouti oranges. Comminuted fruit beverages prepared from whole citrus fruits of this variety also have a lower potassium content than the corresponding juice, while the general trend is towards higher potassium content in the entire fruit than in the juice. These observations draw attention to the need to determine the potassium and nitrogen content to assess the edible fruit content of comminuted fruit drinks, preferably in each variety of the citrus fruit (Money, 1966). Similar considerations apply when using the phosphorus or nitrogen content as an index. A more reliable measure of fruit content can be obtained by calculations making use of more than one index compound (Steiner, 1949). A combined formula for the authenticity of comminuted orange drinks is given by Hulme *et al.* (1965) as: fruit content=0.05 (7K+10P+3N), where K, P and N are the calculated fruit contents based on analyses for potassium, phosphorus and nitrogen, respectively. An inverse relationship between inorganic phosphorus and ethanol insoluble phosphorus, as percentages of total phosphorus is believed to be more indicative of fruit content. The ratios K:Ca and K:Mg have been proposed as indices of adulteration (Benk, 1980), the values for genuine California oranges being 8.8–27.0 and 9.6–22.7, respectively. Ranges of this nature are broad, and can only detect an

Table 3.1 Linear relationships between selected parameters with potential for detecting sophistication in orange juice

Function	Correlation
Alkaline ash = 17.0 − 0.0168 (Ca)	−0.797
Alkaline ash = 16.2 − 0.172 (Na)	−0.812
Total phenols = 149 + 0.201 (Ca)	0.800
Na = −3.72 + 0.0950 (Ca)	0.950
Mg = 88.0 + 0.0822 (Ca)	0.828
K = 1420 + 2.48 (Ca)	0.925
K = −262 + 20.7 (Mg)	0.767

Source: Vandercook *et al.*, 1983 (reproduced with permission).

extreme level of adulteration. Potentially useful correlations between minerals, in the form of linear regressions, could verify the authenticity of samples where a few of the analytical values are outside the normal range. Some of these correlations are shown in Table 3.1.

A multivariate test which can detect adulteration or dilution at 15% level at 1% significance has shown °Brix, formol value, chloramine T number, total sugars and chlorides to be key parameters in testing the purity of lemon juices. The application of a computer to standardize analytical characteristics of citrus juices throughout the year and to calculate mixing parameters has been described. This versatile 'mixer' programme using 'basic' language with respect to quality standards for acidity, sugars, ash, sodium, potassium, calcium, magnesium and phosphate also enables grading of standard juices according to their commercial value (Ipsale *et al.*, 1985). Authenticity criteria for grape juice, as established by the Netherlands collaborative working committee on fruit juices and related products are as follows: extract (corrected for acidity) >15.9 Brix; sugar-free extract >20 g l^{-1}; ash >2.2 g l^{-1}; K >950 mg l^{-1}; Na <30 mg l^{-1}; Mg >80 mg l^{-1}; Cl− <50 mg l^{-1}; NO_3− <15 mg l^{-1}; P >97 mg l^{-1}; L-malic acid >3.0 g l^{-1}; D-malic acid 0; citric acid <500 mg l^{-1}; free tartaric acid <1g l^{-1}; sucrose 0; glucose/fructose ratio <1.03; formol value >11 ml 0.1 M NaOH/100 ml and proline >150 mg l^{-1} (Dukel, 1983, 1984). Similar reports judging the authenticity of apple juice have also been published (Dukel, 1982). Apart from chemical and sensory analysis, Richtwerte und Schwankungsbreiten bestimmer Kennzahlen (RSK) values have been in use by the Association of the German Fruit Juice Industry for evaluation of apple, grape and orange juices (Bielig *et al.*, 1982). It is however pointed out that RSK values must not be slavishly adhered to, but are valid as an aid to juice evaluation for authenticity and no single value can be used in isolation (Hofsommer and Bielig, 1982). It is believed that the use of RSK values restricts the possibilities of fruit juice adulteration and that further refining of the concept is necessary (Wallrauch, 1985).

There is a great temptation to add water to juices and then the next step is to add

some sugar, acid, potassium, ammonium salts and phosphates to bring the analytical results in line. Use of 'orange ash sugar' or sugar doctored to make its ash conform with that of orange juices needs better methods of detection to outwit the fraud.

Labelling of the percentage of ingredients in prepared foods has been debated for some time and is especially appropriate in baby foods. Of the several categories of baby foods, those based on 'fruits' are the most popular. The presence of sugar and starch in baby foods precludes use of carbohydrate analysis in quantitative estimation of fruit products. Since the non-fruit ingredients are not rich in potassium, and fruits such as bananas and apricots are, and addition of potassium salts is unlikely, potassium could be a reliable index of fruit in baby foods. However, actual confirmation by experiment led to fruit level estimates lower than probable, mainly because experiment is useful only for monofruit products and even with the same fruit, potassium content varies due to soil conditions, seasonality, climate and fruit variety. Apples and pears contain much less potassium than bananas, and therefore would be less sensitive for verifying compositions of products made with low potassium fruits, especially products made with two or more fruits nearly equal in potassium content (Harvey and Theuer, 1991).

3.5.2 Organic components

3.5.2.1 Amino acids

The determination of free amino acids is currently the most accepted method of detecting adulteration in citrus juices. The influence of harvesting date and of climate on the free amino acid composition of orange juice has been studied. The concentration of some amino acids varies with the harvesting date, while that of others remains practically constant. This has generated the possibility of using the amino acid analysis for detection of adulteration and its validity is demonstrated by analysis of adulterated samples (Wallrauch, 1980). Coffin (1968) indicated that the amino acid content of orange juice could be estimated from an equation based on the ash content of the juice and utilized in determining the purity of orange juice.

Orange juice samples which were supposed to be adulterated contained on an average a quarter of the concentration of amino acids found in unadulterated samples (Weits *et al.*, 1971). Examination of 110 samples of orange juices have shown that proline, glycine and hydroxyproline remain constant while the levels of lysine, histidine and arginine fluctuate considerably and decrease to about half their original concentration after storage for two years at 15 °C. Quantitative amino acid analysis also allows detection of added protein hydrolysate and amino acids (Wucherpfennig and Millies, 1972).

The reduction in formol value caused by the adulteration of orange juices can be masked by the addition of ammonium salts (Benk and Seefried, 1975). In such cases detection of ammonia nitrogen as a percentage of total nitrogen or formol value (expressed as millilitres of 0.1M acid for 10 ml or 10 g of the product) are appropriate

indicators of adulteration. In genuine samples, ammonia nitrogen never exceeds 10% of formol value (Rother, 1971b) or 7% of the total nitrogen content. In adulterated samples, ammonia nitrogen can be up to 92% of the formol value and up to 78% of the total nitrogen. However, a corrected formol value can be obtained after liberating ammonia nitrogen and redistilling at low temperatures under vacuum (Rother, 1971a). This can be calculated from the formula $F-(a-b)/100$ where F is formol value, a is 0.1N acid and b is the 0.1N alkali used (Rother, 1971b). These values are, however, different for orange concentrates (40–67 °B) and also vary with the citrus variety, that is different for grapefruit and lemon (Benk and Bergmann, 1971). A regression equation predicting formol number from concentration of proline, arginine, alanine and γ-aminobutyric acid has been developed but is sensitive to production season. Use of a dynamic model approach, that is using a model that can be changed according to circumstances to detect adulteration is also reported (Cohen, 1983). However, the use of formol number alone to confirm authenticity of juices should be done with caution. Ethanolamine has been implicated as an adulterant in orange and grape juice (Wallrauch, 1979), where it increases the formol value and may mask dilution with sugar solution. Unlike ammonia, it is not detected by steam distillation, but can be detected by amino acid analyser or paper chromatography (Benk, 1978).

Apart from protein amino acids, all natural and most commercial grapefruit juices are known to contain the non-protein amino acid, ornithine, the concentration of which is correlated to that of a number of other free amino acids. Levels lower than the minimum concentration found in natural juices can indicate adulteration and could be used as a quick screening test (Menziani *et al.*, 1976). Qualitative and quantitative analysis of amino acids in grapefruit have shown it to be independent of the origin of the juice and can be used as a check for adulteration (Otteneder, 1977). β-Alanine, γ-aminobutyric acid, histidine, methionine sulphoxide and isoleucine are among the 21 amino acids found in grapefruit (Gherardi *et al.*, 1971). γ-Aminobutyric acid and arginine are particularly useful in detecting adulteration in orange juice (Vandercook and Price, 1974). Ratios of γ-aminobutyric acid/arginine, arginine/asparagine and γ-aminobutyric acid/asparagine are more stringent than the individual amino acid concentrations and can be used to detect dilution with water, and also with other fruit juices and protein hydrolysates (Ooghe, 1980). This method, although expensive, has the merit that the high price of these amino acids makes juice adulteration not economically feasible (Lifshitz, 1983). Capillary GLC on a chiral phase can detect adulteration in fruit juices via the determination of D- and L-aspartic acid. Additions of synthetic amino acids in commercial orange juices can be detected (Ooghe *et al.*, 1984). The efficacy of the formol index, nitrogen (total, ammonia and amino), potassium and phosphorus as indicators of dilution of lemon juice has been shown. The differences between calculated and determined concentration of constituents were all less than 5% and the reference standards could be applied to industrial products (Romojaro *et al.*, 1980). Amino acid composition and formol value are particularly recommended for detecting the adulteration in lemon juices (Romojaro *et*

al., 1976). Citrus juice adulteration by dilution with water can be masked by addition of sugars, citric acid, potassium, phosphate, ascorbic acid and amino acids such as glycine thus rendering analytical quality control valueless in terms of total acid, total sugar, ash, alkalinity and ascorbic acid estimation. Glycine additions can be detected by the ratio of glycine:alanine, the values being 1:2.49–11.10, 1:3.86–13.95 and 1:4.84–12.82 for natural orange juice, and that obtained from first and second pressure extraction of the albedo, respectively (Giacomo *et al.*, 1979). Similarly, protein hydrolysates added for the purpose of masking dilution can be detected by the difference in the relative concentration of leucine/isoleucine and γ-aminobutyric acid in the protein hydrolysate and the citrus juice (Vandercook *et al.*, 1963).

Addition of ammonium salts to orange juice does not basically alter the amino acid chromatograms but causes dullness in the otherwise intense amino acid spots (Benk, 1971a). The content of natural amino acids however decreases by as much as 20% of nitrogenous compounds.

According to Lifshitz and Stepak (1971), the amino acids, glutamic acid, alanine, glycine and aspartic acid are especially useful in detecting dilution of lemon juice. In a study on the characterization of California and Arizona lemon juice, Vandercook *et al.* (1963) reported that lemons may differ in amino acid content in various growing areas and at different times during the season. This was confirmed by Baron *et al.* (1977), who questioned the reliability of using formol values to detect illegal dilution. They found that high formol values were related to high nitrogen fertilization of the orchards. Furthermore, addition of protein hydrolysates would mask the results obtained from formol values (Niedmann, 1976b).

The ratio of formol value/proline has been claimed to be a sensitive indicator of adulteration of citrus juices. It is suggested that a ratio of >30 should be regarded as indication of likely adulteration, although it does require some further consideration before it is acceptable as a dependable indicator (Wallrauch, 1974). The formol value, proline content and formol value/proline ratio should all together be considered in testing for adulteration of citrus juices (Benk and Dittrich, 1976).

Apart from citrus fruits, the amino acid composition of apples, pears, grapes, strawberries, blueberries, cherries, plums, peaches, apricots, bananas, pineapples and watermelon could be used to distinguish them and also to detect admixtures and the degree of ripeness of the fruit (Bielig and Askar, 1972). Assessment of the genuineness of blackcurrant and red currant musts and mother juices has shown total acid:ash, total acid:potassium, total acid:phosphate and citric acid:malic acid ratios, and concentrations of isocitric acid and amino acids to be satisfactory quality parameters (Frank, 1975).

Canonical correlation analysis has been used to detect orange juice dilution masked by addition of citric acid and sugars. Application of canonical correlation analysis to two groups of 28 determined characteristics (such as amino acids, minerals, absorbance, etc.) has shown a correlation coefficient of 0.966 in one pair of canonical correlation variables. Test sets to check the efficiency of predicted equations has shown

dilutions of 10, 20 and 30% to be detected in 28, 62 and 91% of juices from test sets (Capilla *et al.*, 1988). This indicates that the canonical correlation analyses are valid only at high dilutions of the juice.

3.5.2.2 Vitamins

In addition to being nutritionally important in citrus juices, vitamins may have value as indices of fruit content. Carotenoids can be used as a quality index for orange juices. However, in order to do so, the proportion of the pulp must be strictly standardized. The amount of carotenoids are also dependent on variety (Iranzo and Gimenez-Garcia, 1974), and hence are of questionable use. Ascorbic acid is suggested to be useful in detecting adulterations in mandarin juices (*Citrus reticulata*) (Iranzo and Pauletti, 1974). It is however of little use since it is a common additive. However, differentiation between ascorbic acid of natural and synthetic origin is possible by IRMS technique. While commercial L-ascorbic acid has in general δ^{13}c values near -11.3%, the mean δ^{13}c value of ascorbic acid from authentic juice is -20.7% (Gensler *et al.*, 1995). Nicotinic acid content of orange juice is also recommended as an index compound (Sawyer, 1963), the levels of <0.15 mg/100 ml juice being regarded with suspicion. Inositol is another compound examined as an index of fruit content (Lisle, 1965). A formula combining nicotinic acid (B mg/100 ml) and inositol (A mg/100 ml) has been used to calculate the concentration of orange juice (C): $C=2.20\,B+0.0025\,A$.

3.5.3 Stable isotope ratio analysis

Apart from liquid chromatography, UV/visible spectrophotometry and atomic absorption spectra for potassium and sodium, and stable isotope ratio analysis of ^{13}C and ^{18}O all verify the authenticity of orange juice. All authentic juices possess positive ^{18}O ($^{18}O=+0.3$–0.5%) values, while those of ground water and water in beet invert syrups are negative. Therefore addition of either of these to orange juices decreases ^{18}O values and becomes a strong foolproof method for detection of non-authentic products (Brause *et al.*, 1984). The technique is based on the fact that rain water or irrigation water, when transported from the roots to the fruit is fractionated, probably by evapotranspiration (Bricout *et al.*, 1972; Bricout *et al.*, 1973), in such a way that the light isotopes (hydrogen and ^{16}O) are lost, preferentially to the heavy isotopes (deuterium and ^{18}O).

Apart from ^{18}O and ^{13}C, genuine citrus juices have been found to be highly enriched in deuterium (D=about+0.5%) compared with fresh water. The deuterium content in fruit juices is, however, found to be too varied to be applicable for this purpose (Nissenbaum *et al.*, 1974). The ratio of $^{18}O/^{16}O$ in the case of grapes does not change significantly during the course of 24 h, nor is it significantly affected by the maturity of the grapes and fermentation. Rain affects the grape juice ratio of $^{18}O/^{16}O$ only when the fruit is ripe, presumably because of the uptake of this water in ripe grapes (Dunbar,

1982). The results of a collaborative study on frozen concentrated orange juice have shown a correlation coefficient of >0.999 for a plot of mean ^{18}O values and the levels of adulteration. It has been accepted as a first action by Association of Official Analytical Chemists (AOAC) International (Doner *et al.*, 1992).

A major drawback to applying isotopic analysis on a routine basis is the need to distil the sample prior to analysis, since low temperature vacuum distillation (Bricout, 1973) is time consuming. A rapid technique for sample preparation that circumvents the need for distillation has been proposed by Nissenbaum and Feld (1980). The method consists of centrifuging down the suspended particles, treatment with activated charcoal for 60 min in a closed flask, centrifuging in closed cuvettes at 15 000 rpm for 10 min, filtration through a Whatman No. 2 filter paper and then carrying out the mass spectrometric measurement. Care has to be taken during centrifugation and filtration, since errors of the magnitude of 20% can occur if open cuvettes are used during centrifugation. The accuracy of this method is superior and is recommended for the detection of adulteration of citrus juice (Cohen and Saguy, 1984).

Plants use either the Calvin (C_3) or Hatch-Slack (C_4) pathway for photosynthetic carbon dioxide fixation. This pathway of metabolism is reflected in differences in leaf anatomy and $^{13}C/^{12}C$ ratio in their organic carbon. Cane (C_4) sucrose can be distinguished from beet sugar (C_3) by this analysis, a distinction not possible chemically. A report establishing the uniformity of $^{13}C/^{12}C$ ratios of apple (C_3 plant) juices prepared from the commercial apple varieties provided baseline data for the addition of inexpensive corn or cane sugar. The results are generally denoted as ^{13}C values, defined as $[(^{13}C/^{12}C)\ \text{sample}/(^{13}C/^{12}C)\ \text{standard} - 1] \times 103$. The ^{13}C values for 40 apple juice samples derived from 26 geographical regions were found to be within the range of 22.5 to 27.9 (Doner *et al.*, 1980).

The uniformity of the data suggests no correlation between ^{13}C value and apple variety. The small variation in isotopic composition of carbon in pure apple juice works well for the detection of added corn or cane sugar in apple juice. The $^{13}C/^{12}C$ ratio in the solid material of citrus juice is very similar to that of beet sugar, but very different from this ratio in cane sugar (Nissenbaum *et al.*, 1974). Deuterium concentration determined by mass spectrometry has also been used to quantify sugar levels, particularly levels of beet sugar added to orange juice (Bricout and Koziet, 1987; Doner *et al.*, 1987; Dunbar and Schmidt, 1984). Orange sugar is much richer in ^{2}H than beet sugar, making the detection of beet medium invert sugar possible. However, the exchangeable oxygen-bound hydrogen atoms have first to be removed through the formation of nitrate esters, before the level of non-exchangeable carbon-bound ^{2}H can be determined. This makes the method more complex and prone to explosions. More recently, a method has been developed that takes into account the deuterium distribution within the molecule. It has been shown that deuterium is not randomly distributed in the molecule. A method based on this fact and called site-specific natural isotope fractionation – nuclear magnetic resonance (SNIF–NMR) has been developed, and can detect beet sugar in citrus juices (Martin *et al.*, 1991; Martin, 1992).

Another approach to the detection of adulterants is monitoring minor contaminants present with the main compound. It was thought possible that beet sugar adulteration can be detected by monitoring four oligosaccharides specific to medium invert sugar, which is in turn obtained from beet sugar (Low and Swallow, 1991; Swallow *et al.*, 1991; White and Cancalon, 1992b; Weissenberger *et al.*, 1992). However, this approach has not as yet yielded fruitful results (Widmer *et al.*, 1992).

3.6 Juice blends

It is in the interest of both producers and consumers to characterize the juices, to differentiate between them on the basis of variables difficult to falsify and to be able to quantify the proportions of each of them in blends. This is generally done to extend juices with less expensive fruit juices and is based on the presence/absence of a characteristic component. For instance grape juice concentrates or deacidified grape juices are often used in blends with apple juice. This problem is important because of the tariff regulations for the import of grape and apple juices in some countries. Apple juice may be imported free of duty, whereas mixtures of apple and grape juices are assessed at the higher rate applicable to grape juice.

3.6.1 Carbohydrate analysis

Fitelson (1970a) examined the sugars and sorbitol patterns in fruit juices by GLC which indicated that all fruit juices exhibit typical sugar patterns and some fruits contain sucrose and sorbitol in significant quantities.

Detection of the presence of raspberry juice in the juice of sour black cherries, cherries, apples and other fruits of the family *Drupaceae* and *Rosaceae* is based on the absence of sorbitol in raspberries, but its presence in detectable amounts in these juices. The method for detection of sorbitol by two-dimensional limited-time TLC is also valid for detecting admixtures in wine, vinegar and fermented juices (Mattioni and Valentinis, 1971). Sorbitol content is highest in pears, cherries, plums, apples and peaches, and very low or absent from grapes, blackberries, raspberries and strawberries. In products like marmalades and conserves, sorbitol analysis is applicable in detecting apple marc only when present in larger amounts. This must therefore be re-examined more thoroughly (Philippi, 1954). In some countries, the practice of adding 'apple gel extracts' to marmalades and conserves is permissible. This introduces sorbitol and therefore is excluded as an analytical index for adulteration with apple marc (Gopfert,1954).

Pears and apples contain more fructose than glucose, peaches contain more glucose than fructose, while other fruits contain essentially invert sugar so that sugar ratios may prove crucial. Detection of apple juice in pear juice can be done from the ratios of sorbitol/sucrose and sorbitol/total sugars. Different varieties of apples exhibit a sorbitol/sucrose ratio in the range 0.136 to 0.385 and sorbitol/total sugars 0.037 to

0.078, whereas pear varieties show the corresponding value ranges of 0.829 to 2.351 and 0.144 to 0.302. These differences can form the basis for differentiating the two juices and for identifying blends of fresh juices. Heat treatment is known to cause an increase in reducing sugars and a corresponding decrease in sucrose content which in turn alters the sorbitol/sucrose ratio. The method is therefore not suitable for commercial samples. Also, the use of 'water core' apples in juice manufacture may result in miscalculation of the juice, provided the juice has low sucrose content (Sharkasi *et al.*, 1981).

Additives to apple juice such as juice concentrates of hard pears, soft pears, figs, prunes, raisins, white grapes and pineapples as well as sweeteners like invert beet, invert cane and high fructose corn syrup which have been previously characterized by sugar profiles, non-volatile acid profiles, UV spectra and mineral content can be clearly distinguished from apple juice by the pattern recognition methods, the database for which was obtained from HPLC analysis of sugar and non-volatile acids and isotope carbon analysis (Pilando, 1987).

3.6.2 Phenolic constituents

As a result of recent advances in chromatographic techniques, especially HPLC, phenolics have been characterized individually, mainly those of the different flavonoid families (Hermann, 1979; Harbourne *et al.*, 1975; Fleuriet and Macheix, 1976; Markham, 1982; Tomas-Lorente *et al.*, 1988; Gonzalez-San-Jose *et al.*, 1990). Thus phloretin and isohamnetin derivatives can distinguish between apple and pear juices (Wald and Galensa, 1989); naringin and neohesperidin can differentiate between orange and grapefruit (Rousseff, 1988). Methoxylated flavones and flavonone glycosides are useful in detecting grapefruit juice in orange juice (Perfetti *et al.*, 1988). Kiwi fruit can also be estimated in products on the basis of the content of the flavonoids quercitin-3-*O*-rhamnoside, luteolin-6-*C*-glucoside and quercitin-3-*O*-rutinoside (Mareck-Engelke *et al.*, 1991).

Low molecular weight phenolics in fruits have been scarcely studied, except cinnamic acids and their esters with quinic and tartaric acids and some of their glycosides (Ribereau-Gayon, 1963; Wardale, 1973; Mosel and Herrmann, 1974a, 1974b; Melin *et al.*, 1979; Moller and Herrmann, 1982; Billot, 1983; Macheix and Fleuriet, 1986; Risch and Herrmann, 1988a, 1988b; Peleg *et al.*, 1991). It is thought that the free acids are released by partial degradation of the combined forms during extraction or processing of fruits (Ramirez-Martinez and Luh, 1973; Fleuriet and Macheix, 1976). Acids other than cinnamic have been identified in the following fruits: grape — gallic,

Table 3.2 Ranges of concentration (mg l^{-1}) of certain non-flavonoid phenolic compounds in commercial juices and nectars

	Peach	Peach + grape	Apricot	Apple	Apple + peach	Pear	Pear + grape	Grape	Orange	Pineapple
p-Hydroxybenzoic acid	0.01–0.16	0.98–3.42	0.06–0.39	t	0.09		t	0.81–2.60		0.17–0.72
Protocatechuic acid		0.32–0.69								
Gallic acid		0.38–0.84					0.01	0.86–2.01		
p-Hydroxybenzoic aldehyde	0.02–0.06			0.02–0.12					t	0.02–0.11
Vanillic aldehyde	0.01–0.08	0.01–0.04	0.01–0.16	t	0.01	0.02–0.12	0.03		t	t
Syringic aldehyde	0.01–0.04	t	0.01–0.07	t		0.03–0.11	0.03		0.10–0.25	0.10–0.38
3, 4-Dihydroxybenzoic aldehyde	0.03–0.18	0.10–0.20	0.04–0.08	0.05–0.26	0.10	0.02–0.06	0.04	0.03–0.18		t
p-Coumaric aldehyde	0.06–0.15	0.11–0.22	0.07–0.11	0.56–0.77	0.06	0.08–0.12	0.39	0.26–1.91	0.10–0.30	0.11–0.44
Ferulic acid	nd–0.05	0.02–0.07	0.05–0.18	t	0.19	t	t	0.10–0.12	0.23–0.54	0.11–1.77
Caffeic acid	0.02–0.31	0.20–0.37	0.09–0.27	0.86–1.05	0.18	0.01–0.07	0.04	0.38–0.81		0.08–0.16
Sinapic acid									0.36–1.11	0.22–0.64
Scopoletin			0.03–0.07						t	
Aesculetin	t		0.02–0.05	0.20–0.45						
Chlorogenic acid	0.22–7.12	1.18–1.48	0.65–3.09	2.49–9.14	3.52	1.14–7.88	11.56		0.13–2.04	
p-Coumaroylquinic acid		0.27–0.48[a]		0.81–2.58	0.52	0.17–0.43	0.46			0.25–0.39
Feruloylglucose				0.02–0.14		nd–0.30	0.03		0.08–0.19	0.09–0.14
Caffeoyl ester	0.11–1.68[a]	0.14–0.37[b]	0.36–1.06[a]		0.99[a]			0.10–1.78[b]		
Feryloyl ester		0.07–0.16[b]						0.74–1.01[b]		
p-Coumaroyl ester		0.05–0.07[b]						0.78–1.61[b]		

nd, not detected; t, traces (only detected by TLC).
[a]Possible 3′-caffeoylquinic acid.
[b]Tartaric ester.
Source: Fernandez de Simon *et al.*, 1992a (reproduced with permission).

Table 3.3 Ranges of concentration (mg l^{-1}) of certain flavonoid phenolic compounds in commercial juices and nectars

	Peach	Peach+ grape	Apricot	Apple	Apple+ peach	Pear	Pear+ grape	Grape	Orange	Pineapple
(+)-Catechin	0.12–4.64	0.28–2.56		0.02–0.83	3.44			0.22–2.03		
(−)-Epicatechin	0.41–3.60	1.09–1.46		0.02–0.74	4.30	nd–0.39		0.06–1.40		
Myricetin	0.92–0.99	2.05–4.14								
Quercetin	0.03–0.11	0.07–0.13	0.04–0.16	0.08–0.43	0.14	0.03–0.14	0.05	0.07–0.50		
Kaempferol	0.03–0.04	0.05–0.08		0.03–0.06				0.06–0.27		
Q 3-*O*-rutinoside	0.03–0.11	0.07–0.22	0.37–1.52	0.06–0.10	0.23	0.01–0.14	0.07	0.05–0.16	0.30–1.22	
Q 3-*O*-galactoside	0.01–0.04	0.12–0.18		0.75–3.78	0.36	0.15–2.46	0.04	0.71–2.44		
Q 3-*O*-glucoside	0.04–0.16	0.25–1.37	0.02–1.28	0.01–0.08	0.11	nd–0.16	0.04	0.06–0.23		
Q 3-*O*-arabinoside	0.01–0.12	0.04–0.22		0.24–0.70	0.07	0.27[a]				
Q 3-*O*-rhamnoside				1.33–2.68	0.36	0.35[a]		0.03–0.20		
Ph 2′-*O*-glucoside				13.81–26.9	3.79	2.54[a]				
K 3-*O*-rutinoside								0.01–2.72		
Kaempferol glycoside[b]			0.08–0.75							
M 3-*O*-rhamnoside								0.01–0.07		
Luteolin 7-*O*-glucoside									0.09–0.97	
Apigenin 7-*O*-glucoside									2.40–7.82	
Isorhamnetin glycoside[b]						0.03–4.00	0.05–0.90			

Q, quercetin; Ph, phloretin; K, kaempferol; M, myricetin; nd, not detected.
[a]Supposed mixture with apple.
[b]Expressed as rutin.
Source: Fernandez de Simon *et al.*, 1992a (reproduced with permission).

protocatechuic, *p*-hydroxybenzoic, vanillic and syringic (Fernandez de Simon *et al.*, 1992); blackcurrant — salicylic, vanillic, 2,5-dihydroxybenzoic and shikimic (Tanchev *et al.*, 1986); apple — protocatechuic and *p*-hydroxybenzoic (Bilyk *et al.*, 1988); bilberry — *p*-hydroxybenzoic, *m*-hydroxybenzoic, gallic, protocatechuic, vanillic and syringic (Azar *et al.*, 1987). Screening of 3-flavanols, flavonols, chalcones, benzoic acids and aldehydes, and cinnamic acids and their derivatives in the form of esters for the purpose of investigating the marker compounds that could be useful in characterization, and hence for the detection of admixtures has been recently reported (Fernandez de Simon *et al.*, 1992b). The results of this study are shown in Table 3.2 and Table 3.3 (Fernandez de Simon *et al.*, 1992b). It was reported that hydroxycinnamic acid esters with tartaric acid are typical of grapes, phloridzin is characteristic of apples, and isorhamnetin glycosides are typical of pears. Myricetin is found only in peaches, and luteolin and apigenin glucosides only in oranges. Apricot could be detected by the presence of two coumarins and pineapple by the presence of sinapic acid and the absence of the other flavonoids. Umbelliferone is another phenolic compound characteristic of orange juices. Thus grape juice can be detected by the presence of caffeotartaric acid, *p*-coumaroyltartaric acid and feruloyltartaric acid, while the presence of hydroxycinnamic acid ester with quinic acid would imply the presence of other fruits. The mixture of apple plus peach could be proved by high concentrations of phloridzin in apple or the presence of myricetin in peach. Another indicator could be quercetin 3-*O*-rhamnoside, typical of apple but not in peach. Fruits of *Sorbus domestica* and apple juice contain 3-caffeoyl-L-(-)-quinic acid or neochlorogenic acid at levels of 1500 mg/kg at the minimum and 2 mg l^{-1}, respectively. This can unambiguously indicate additions of 0.5% *Sorbus domestica L.* juice to apple juice and even apple wine (Ritter *et al.*, 1994). Similarly, kaempferol and quercetin glycosides can be indicators of kiwifruit (Mareck *et al.*, 1990).

The presence of orange juice in grapefruit juice can be detected through the flavone glycosides, naringin and hesperidin by HPLC (Galensa and Herrmann, 1980) or by several isocratic and gradient methods in underivatized or benzoylated extracts. Additions of enzymically debittered grapefruit juice can be detected via the products of hydrolysis, naringenin and prunin (Siewek *et al.*, 1984a). Similarly flavonol glycoside composition can indicate blends of blackcurrant with redcurrants. Fruits, juices and nectars show similar composition, with myricetin glycosides predominating in blackcurrants, followed by quercetin glycosides with only minor amounts of kaempferol glycosides. In redcurrants, quercetin glycosides are predominant and myricetin and kaempferol occur only in traces in fruits and are not detected at all in commercial juices and nectars. It has been suggested that quercetin-3-*O*(2″*O*-α-α-rhamnosyl-6″-*O*-β-β-rhamnosyl)-β-D-glucoside (QdRG) be used as an index of addition of redcurrant juice to blackcurrant juice or nectars. The sensitivity of detection is as low as >3% redcurrant juice in blackcurrant nectars (Siewek *et al.*, 1984b). The occurrence of two isomeric flavone *C*-glycosides that occur in fig juice but not in grapes and which are also not hydrolysed during fermentation have been the

basis of detection of fig juice in grape juice. Scaftoside (apigenin-6-*C*-β-D-glucopyranosyl-8-*C*-β-β-arabinopyranoside) and isoschaftoside (two sugar moeities exchange positions in the isomer) are the two glucosides on the basis of which it may be possible to detect addition of 5% fig juice to grape juice, wine or sparkling wine (Siewek *et al.*, 1985). As a general rule, the phenolic compounds present in fruit jams coincide with those in the corresponding fruits. The content of these in cultivars and at the maturity stage of the fruit may vary. The detection of mixtures of fruits in jams, may be based on the identification of such characteristic compounds in different fruits (Tomas-Lorente *et al.*, 1992).

Dihydrochalcones are mainly confined to the *Rosaceae* and *Ericaceae* families (Williams, 1966). The dihydrochalcone, phloridzin, isolated from apple, is associated with disease resistance in this plant. Phloridzin can logically be used to detect apple juices, or to determine amount of apple in mixed fruit products.The occurrence of the hydrochalcone glycosides, phloretin glucoside and phloretin xyloglucoside in apple juice has been known (Johnson *et al.*, 1968) and characterized as 2′,4′,6′,4-tetrahydroxydihydrochalcone-2′-*O*-β-D-glucopyranoside and 2′,4′,6′,4-tetradihydroxydihydrochalcone-2′-*O*-(6′-β-D-xylopyranosyl)-β-D-glucopyranoside (Tomas-Barberan *et al.*, 1993). In fact, these substances have not been detected in any other fruit as yet (Herrmann, 1990), and therefore their analysis is useful in food authenticity studies. Dihydrochalcones are also important since they are oxidized rather easily (Dziedzic *et al.*, 1985), and their oxidation contributes to apple juice browning (Burda *et al.*, 1990; Spanos *et al.*, 1990; Oszmianski and Lee, 1991). Phloridzin content in apple juices may be 2.66 to 5.43 mg l^{-1} and xylopyranoside in the range of 2.09 to 3.78 mg l^{-1}. In apple jam, their concentration may vary in the range 1.0 to 9.6 and 0.42 to 5.18 mg kg^{-1} respectively (Tomas-Barberan *et al.*, 1993).

3.6.3 Organic acids

Total non-volatile acids like the individual acids vary considerably in fruits and also change during ripening, but the tartaric acid in grapes, lactoisocitric acid in blackberries and benzoic acid in cranberries can sometimes be used as markers. For instance adulteration of pome, stone and berry fruits with grape juice can be detected on the basis of tartaric acid (Wucherpfennig, 1976). HPLC analysis of the juice of billberries (*Vaccinium myrtillus*) has shown 6.5 g l^{-1} of quinic acid, traces of shikimic acid, 5 g l^{-1} citric acid and about 2 g l^{-1} of malic acid. These facts are very useful in detecting its admixture with other juices such as gooseberries, which contain a sufficient quantity of shikimic acid (3 g l^{-1}) (Tanner and Peter, 1977).

3.6.4 Amino acids

Thin layer chromatography for amino acids has been applied to detect the presence of other materials in citrus and non-citrus juices (Alvarez, 1967). Amino acid contents

Table 3.4 Distribution of amino acids in grape, apple and pineapple juices

Amino acid (n=17)	Grape (n=11)		Apple (n=6)		Pineapple	
	Mean (mg l^{-1})	SD	Mean (mg l^{-1})	SD	Mean (mg l^{-1})	SD
Aspartic acid	19.3*	6.1	81.2**	52.6	25.6*	18.1
Glutamic acid	29.9*	10.3	20.1**	5.4	29.3*	11.6
Asparagine	3.9*	2.3	323.2**	116.6	247.1**	131.0
Serine	23.5*	42.7	15.7**	9.0	44.9*	13.4
Glutamine	3.3*	3.9	1.8*	1.0	15.2**	7.2
Histidine	16.0*	6.1	1.1*	1.4	7.5***	2.9
Threonine+glycine	43.8*	17.3	29.4**	10.7	63.4***	19.9
β-Alanine	5.1*	3.1	4.6*	1.2	12.0**	6.1
α-Alanine	27.7*	8.9	14.4**	9.0	25.4*	8.9
γ-Aminobutyric acid	34.8*	14.2	5.2**	3.0	10.7**	4.3
Tyrosine	8.3*	2.8	0.6**	0.8	11.0***	3.6
Arginine	207.8*	69.9	2.5**	1.0	13.1**	5.1
Methionine	1.0*	1.0	0.04*	0.13	17.6**	5.5
Valine	12.7*	5.7	3.7**	3.1	10.5*	3.2
Tryptophan	1.5*	2.0	ND**	–	3.0***	4.6
Phenylalanine	11.3*	4.4	1.7**	0.8	7.9***	2.3
Isoleucine	7.1*	3.2	4.4*	3.2	7.2*	4.1
Leucine	11.2*	4.4	1.8**	0.7	9.2*	2.2
Ornithine	5.7*	6.1	6.3*	3.8	10.8*	8.5
Lysine	7.5*	8.2	3.7**	1.8	12.9***	2.6
Proline	382.2*	116.3	5.2**	1.3	13.7**	4.1

Means within rows with the same number of superscript asterisks are not significantly different ($P<0.05$).
ND – not detectable.
Source: Dizy *et al.*, 1992 (reproduced with permission).

are low in apples and cranberries, but relatively high in grapes, blackberries, plums and peaches, but their usefulness may be altered by processing or Maillard reactions. In particular, proline is very low in apples (Tanner and Sandoz, 1973b), blackcurrant juices (18.4 mg l^{-1}) compared with grape (345 mg l^{-1}), Morello cherry juice (56 mg l^{-1}), Maraska cherry juice (425–1100 mg l^{-1}) and orange (828 mg l^{-1}). Hence blending with blackcurrant juice is easily manifested as increased proline content (Wallrauch, 1977).

Addition of 5% lemon juice to orange juice can be detected by characteristic bands and zones in extracts after chemical treatment and column chromatography through alumina when viewed in ultraviolet light. Pasteurization of the juice or treatment with sulphur dioxide does not interfere with the analytical test (Safina and Trifiro, 1953). Addition of 1–2% orange juice to passion fruit juice and concentrates can be detected by determination of limonene and hesperidin, and by the free amino acid pattern of the sample (Kuhlman,1984). Interspecies blends of citrus juices such as lemon or orange

juices in grapefruit are difficult to judge from amino acid analysis alone (Gherardi *et al.*, 1971). However grape, apple and pineapple juices can be discriminated on the basis of their amino acid composition. Several multivariate statistical methods such as principal component, cluster, stepwise discriminant and multilinear regression analysis have shown differentiation to be possible by the analysis of methionine, proline, asparagine, arginine and glutamic acid. The determination of amino acids is faster than many of the traditional parameters (Ooghe and Kastelyn, 1985) and is therefore preferred. Table 3.4 shows the distribution of amino acids in the three groups of juices.

It can be seen that grape juice is notable for its high content of arginine and proline which are found only at low levels in apple and pineapple juices. Similarly, asparagine content is very high in apple and pineapple juice but very low in grape juice. Methionine levels may be used to differentiate between apple and pineapple juices. The use of multiple linear regression analysis to estimate the proportions of grape and apple from amino acid composition has been determined using only five variables (r^2=95.0% and s=7.82) in the following equation.

$$\text{Percentage of grape} = 53.81 + 22.10\ \text{proline} - 148.92\ \text{glutamic acid} - 7.01\ \text{asparagine} + 31.65\ \text{arginine} - 237.64\ \text{phenylalanine}$$

Proline and asparagine ratios are also valuable for differentiating among varieties, including genetic differences in grapes (Huang and Ough, 1991).

3.6.5 Pigments

Like other fruit juice constituents, pigments have been utilized for the determination of authenticity. Concord grape juice is sometimes blended with the juices or extracts of other grape varieties, such as California wine grapes. Therefore, Fitelson (1967) developed a method based on the separation and identification of the anthocyanidins of Concord grape juice. The polyphenolic portions include five compounds, malvidin, peonidin, petunidin, cyanidin and delphinidin, which are obtained by acid hydrolysis of the anthocyanins. These five compounds were separated as distinct spots, the intensity of which indicates one grape juice variety in another. This method has been applied to several dark coloured fruit juices such as cherry, raspberry, blackberry and strawberry, and has been found successful in detecting adulteration (Fitelson, 1968, 1970b). However, the use of some characteristic anthocyanin pigments is also limited by changes in processing and storage (Wrolstad *et al.*, 1981; Reyes, 1981).

Tomato in pimento products can be detected by TLC of acetone extract of carotenoids on silica gel G with 80:20 of petroleum ether (b.p. 40–60 °C):benzene. Tomato is characterized by a reddish spot corresponding to lycopene (R_f=0.15) and pure pimento by a yellow spot corresponding to its main pigment β-carotene (R_f=0.35) and a red spot with yellow halo corresponding to xanthophylls (Casas and Mallent, 1983). Similarly detection of red pumpkin in tomato ketchup can be based on the differences in their carotenoid composition. Red pumpkin contains a pigment,

tentatively identified as an esterified xanthophyll, which is absent in tomato (Oke and Shrikhande, 1977). TLC studies on mango carotenoids indicate the Alphonso variety to have a much higher esterified xanthophyll:free xanthophyll ratio than the cheaper varieties (Oke and Shrikhande, 1979).

3.6.6 Miscellaneous constituents

3.6.6.1 Proteins

The protein composition of agricultural products is genetically determined and is not affected by the condition of cultivation. Hence it is used to identify varieties (Cooke, 1984), and also to distinguish between fruit juices such as orange and lemon (Askar and Bielig, 1974). Experiments have been carried out on varietal characterization of grapes and grape musts, based on their electrophoretic protein fractions (Wolfe, 1976). Chromatographic (HPLC) and electrophoretic (polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulphate (SDS) and isoelectric focusing (IEF)) techniques have been utilized to study the soluble protein fractions of grape musts. Stepwise discriminant analysis (SDA) and the nearest neighbour method (KNN) have also been applied to data in order to differentiate and classify the musts according to the variety. A 100% correct classification is obtained with SDA (Polo *et al.*, 1989). This may also be of aid in detecting admixtures of one grape variety with another. Starch gel electrophoresis of the enzymes aspartate amino transferase, phosphoglucoseisomerase and phosphoglucomutase can discriminate between kiwifruit cultivars (Messina *et al.*, 1991), while that of esterase and peroxidase can discriminate between potato cultivars (Choi *et al.*, 1991), and could be useful in detecting blends of one cultivar in another.

3.6.6.2 Lipid constituents

Investigations on the lipid profiles of citrus fruits have shown that citrus species may be characterized and differentiated by distinct lipid patterns, the composition of fatty acids over the region C_{12}–C_{26} being particularly important. Differentiation between cultivars is also possible on this basis (Nagy and Nordby, 1971; Nagy, 1977). Comparative examination of the fatty acids of sterol esters, triglycerides and mono-galactosyl diglycerides shows that each citrus species possesses its own characteristic profile of each lipid class. For instance, Temple orange has been shown to contain high levels of linolenic acid, while Valencia has a relatively high concentration of linoleic acid (Mears and Shenton, 1973). Long chain hydrocarbons have also proved useful in discriminating between citrus species. While C_{23} hydrocarbon predominates in orange, it is C_{25} which predominates in grapefruit.

Use of these constituents presents certain problems. Addition of citrus oils to juices is permitted to restore the flavour lost during processing. Various oils such as juice oil, cold pressed and distilled oils are used for this purpose, making the decision

questionable. The oil composition is also dependent on the extraction process used. In spite of these problems, however, oil component analysis may prove to be significant in assessing the authenticity of citrus oil and hence be of indirect value in characterizing juices.

3.6.6.3 Histological features

Turnip root, parsnip root, potato tubers and corn kernels have been used in the preparation of horseradish sauce and horseradish powders. This can be diagnosed from the histological features of horseradish root and the additives by viewing through a polarizing microscope with cross polars and a first order red plate (Galacci, 1989).

3.6.6.4 Carotenoids

This analysis is of value in distinguishing orange juice from tangerine and mandarin juices. The presence of less than 10% of β-carotene in the total carotenoids is characteristic of orange juice (Higby, 1963; Bernath and Swisher, 1969). Some workers (Koch and Hess, 1971) suggest a somewhat more stringent value of less than 5%. Carotenoid esters in orange juice range from 10 to 16%, but this is not very useful since heat processing alters the range to 30% (Mears and Shenton, 1973).

3.6.6.5 Aroma constituents

The ethyl esters of (*E*)-2-octenoic, (*Z*)-decenoic and (*E*,*Z*)-2,4-decadienoic acids have been identified as authentic components of apple aroma at about 10 $\mu g\ kg^{-1}$, 230 $\mu g\ kg^{-1}$ and 280 $\mu g\ kg^{-1}$ respectively. The concentration of these esters increases markedly with further storage (a four-fold increase in 24 h) with additional formation of (*Z*)-4-octenoic acid. These could be used to detect adulteration of apple products by differentiating the above mentioned esters and those of (*E*)-2-decenoates and (*E*,*E*)-2,4-decadienoates found in pears (Berger *et al.*, 1984).

3.6.6.6 Biogenic amines

Following the demonstration of 5-hydroxytryptamine (serotonin), 3,4-dihydroxyphenylethylamine (dopamine) and norepinephrine in bananas (Waalkes *et al.*, 1958), many other fruits and vegetables were screened for such physiologically active biogenic amines (Udenfriend *et al.*, 1959). It appears that fruits and vegetables have characteristic patterns of amines, suggesting the possibility of using these as an indicator of interspecies admixtures. For instance, red plum in blue plum can be identified by using serotonin as a marker compound, it being present in the former but not in the latter. Similarly addition of orange to grape juice can be identified by tyramine, which is

present in orange juice at 10 μg g^{-1} and totally absent in grape juice. The level of these amines is dependent on the stage of ripeness. For instance, the levels of serotonin in banana pulp in hard green, ripe and overripe stages are 24 μg g^{-1}, 36 μg g^{-1} and 35 μg g^{-1}. The effect of processing on these amines needs to be known in order to use these as marker compounds in processed products. Work in this direction should prove rewarding. Synephrine, feruloylputrescine, tyramine and octapamine in citrus juices have been considered as index compounds (Stewart and Wheaton, 1964).

3.7 Maturity and ripeness indices of fruits and vegetables

Maturity and ripeness indices are of utmost importance in fruit processing since the quality characteristics of fruit products are dependent on the fruit quality. Maturity can be determined using various instrumental techniques or various chemical indicators.

3.7.1 Instrumental techniques

Many non-destructive methods such as resonance properties, random oscillation and impulse response methods implemented by passing electrical or sound waves through the raw fruit are recommended to test the quality of the fruit (Yamamoto, 1982). An impulse waveform of acoustic signals induced by the impact of musk melons is transmitted along the equatorial surface at a uniform velocity. This velocity varies with melon maturity and can be used as its indicator (Hayashi *et al.*, 1992). A mathematical relation between the amplitude in a fast oscillograph method and the number of damaged cells is reported. This is important because the amount of juice extracted depends upon the preliminary treatment of the vegetative tissue. The higher the number of damaged cells in the pulp, the higher is the juice yield. This has been recommended as a quick method to determine the preliminary treatment before pressing (Flaumenbaum and Gereev, 1972). Reflectance properties of apple tissue cannot reliably predict bruise depth. But two-wavelength derivative models distinguish between good and bruised tissue better than non-derivative models (Bilanski *et al.*, 1984). A method of determining the degree of damage (mechanical and/or spoilage) to potato tubers has been described (Weber and Fechter, 1979), based on rinsing potatoes in water to separate easily removable starch from injured tissue and determining the starch liberated. Starch content in the rinse water correlates closely with the degree of damage to the tuber (r=0.8–0.9). The main limitation of this technique is the wide variance of multiple random samples from a large batch. IR and NMR methods (Zion *et al.*, 1995) can determine the sugars in fruits which have a thin skin such as apples, peaches, prunes or cherries, which in turn can be correlated with the state of ripeness. Sensors based on this fact have been developed. These are however ineffective for thick skinned fruits (Bellon *et al.*, 1992a, b). An IR image method has also been described for detecting surface bruise on agricultural products

such as Japanese pear (*Pyrus serotina*), sweet potato (*Ipomea batatas*) and potato (*Solanum tuberosum*) (Miyazato *et al.*, 1981). Near infrared spectroscopy (NIR) is a totally non-destructive technique and in combination with a visual system using a black and white camera, can segregate marketable from non-marketable products (Bellon *et al.*, 1992b).

Delayed light emission (DLE) is a low intensity light that is emitted from a chlorophyllous product for several seconds after illumination with a light source (Strehler and Arnold, 1951). The duration and intensity of DLE produced has been shown to be related to the content of chlorophyll and related compounds. The feasibility of applying DLE to measure fruit maturity has been demonstrated for netted musk melon (Forbus and Senter, 1989; Forbus *et al.*, 1991a), papaya (Forbus *et al.*, 1987; Forbus and Chan, 1989), Japanese persimmons (Forbus *et al.*, 1991b), peaches (Forbus and Dull, 1990) and recently to canary melon (Forbus *et al.*, 1992). Table 3.5 summarizes variable maturity ranges for canary melon fruits from immature to ripe for two crop years. The DLE, chlorophyll, yellow pigments and firmness decrease with ripening while the soluble solids content increases. The maturity index MI, is an estimate of the relative maturity of a fruit with respect to all the other fruits in a specified population. DLE correlates well ($r=0.85$) with MI, which is calculated for each melon based on values for chlorophyll, yellow pigments and soluble solids content.

Cross-sectional X-ray computed tomography (X-ray CT) images through the equator of the tomato fruit (*Lycopersicum esculentum* Mill., cv. Sunny) ranging in maturity from immature (M1) to advanced mature (M4) have revealed localized differences in X-ray absorption related to the formation of gel during maturation of the fruit. Although the maturity stage has been found to be poorly correlated with average X-ray absorbance or with average fruit density or water content, a close relationship is known to exist between maturity stage and the number of image pixels with absorbance values >10 (M1 vs. M2 vs. M3) or 20 (M3 vs. M4) Hounsfield units. Using discriminant analysis, a relationship has been developed that correctly identifies the maturity class of 77% of the fruit and places 97% of the tomatoes in the correct or adjacent maturity class (Brecht *et al.*, 1991). It is feasible that mechanical and optical data may be combined to provide an on-line system for the evaluation of ripeness of tomato fruit. Sensory attributes and instrumental parameters significant in the assessment of ripening tomatoes are translucency, green, orange lightness, hue and chroma. The chromatic coordinate significantly correlates with the mechanical modulus of the fruit, which in turn is inversely related to ripening colour changes from green to red. The standards developed on these lines will have to be revised for genetically modified tomatoes, as has been recently demonstrated (Langley *et al.*, 1994).

There is a need for a quick, objective and non-destructive measurement of onion firmness. Firmness is related to the turgidity of the bulbs and is also a reflection of cellular integrity. Sensory measurement must be made by skilled personnel. The

Table 3.5 Variable ranges for canary melon fruits from immature to ripe for two crop years

	Range from immature to ripe by year			
	1988		1989	
Variables[a]	Immature	Ripe	Immature	Ripe
DLE (V)	0.86	0.06	0.72	0.07
Chlorophyll ($\mu g\ g^{-1}$)	40.0	3.0	60.0	16.9
Yellow pigments ($\mu g\ g^{-1}$)	6.5	0.4	5.2	1.0
Soluble solids (%)	5.2	14.0	5.5	12.4
Firmness (N)	116	46	101	41
I[b]	82.9	69.0	80.3	68.7
a[b]	−11.2	−4.6	−12.6	−6.7
b[b]	24.3	16.9	26.7	20.1
θ, degrees[c]	114.8	102.5	115.2	105.4

[a]DLE values are means of measurements at four locations on each fruit; chlorophyll, yellow pigments and soluble solids are means for three locations; firmness values are means of two locations; and colour values are means of two measurements made on internal flesh at one location.
[b]I, a and b are Hunter colorimeter values.
[c]θ=hve angle=$\tan -b/a$.
Source: Forbus *et al.*, 1992 (reproduced with permission).

instrumental methods include the Magness–Taylor fruit tester (Magruder and Knight, 1933), the Kramer shear press (Kramer *et al.*, 1951) which compresses the onion between flat plates using the slope of the force–deformation line up to the point of rupture as an index of firmness (Ang *et al.*, 1960) and similar methods (Bourne, 1973; Brinton and Bourne, 1972). However, these techniques are slow and do not properly simulate the 'squeeze test' used in sensory evaluations. The results may not be indicative of human reaction to firmness (Sherman, 1973). Also, it has been shown that food firmness is best measured by small deformations (Bourne, 1967a, b, 1967c; 1973). An objective instrument test comparing well with sensory evaluation of onion firmness during storage is the time taken for the force applied to change from 400 g to 3200 g when the bulbs are compressed at 15 cm min^{-1}. This is indicated automatically. Since the deformation rate is constant, this time is a direct measure of bulb deformation (Crete *et al.*, 1974).

The sensory and instrumental readings are related, but the relationships are not linear since the instrument provides a consistent measure of firmness, whereas the sensory tests do not have a reference for standardizing the evaluations. The differences between cultivars, the effect of chemical treatments and storage time can all be evaluated by this test (Crete *et al.*,1974).

Preliminary results have indicated that viscoelastic constants obtained from experimental force–time data on a digital computer correlate with flesh firmness of pears and could be used for non-destructive evaluation of maturity and storage. The method is directed towards general Maxwell materials subjected to a simple

deformation history, but can be extended to other linear viscoelastic models by using the appropriate relaxation modulus and deformation history (Chen and Fridley, 1972). Measurements of force and soluble solids concentration (SSC) in pears are also reported to be useful in characterizing and selecting cultivars that would be most suitable for once-over harvest (Horton, 1992). The correlation of fruit density, floating angle in water and bulk compressibility of tomatoes has been studied with a view to possible non-destructive evaluation of the degree of puffiness. Using a scoring system of 0–4, the results of trials with 100 tomatoes picked at different degrees of maturity showed that density was affected much more by puffiness than by maturity. Extremely puffy fruits (puffiness score >3) could be separated by mechanical sorters. The floating angle of fruits increased with puffiness score between 0 and 3, but decreased at score 4. It could be used to separate fruits with puffiness scores of 2, 3 and 4 from less puffy fruits. Bulk compressibility showed good potential for continuous evaluation of puffiness (Chen and Studer, 1976).

The sonic and vibration response method is one of the techniques used for predicting the textural quality of agricultural products non-destructively. Abbott *et al.* (1968) and Finney (1971, 1972) developed the methodology for intact products and reported that f^2m (f=natural frequency; m=mass), which is designated as the stiffness coefficient or index of firmness, is highly correlated with texture. Ripeness and defects in horticultural products such as apples and watermelons are often judged by listening to the sound produced by thumping. An instrument which can measure the sound when the product is hit by impact has also been developed in this regard (Sawaji, 1970; Takeda *et al.*, 1970). The technology for analysing sound is to find a relationship between natural frequency and the maturity of the product. However, the frequency resolution (80 Hz) of the equipment makes precise frequency analysis impossible. The same idea has been used to predict the ripeness of watermelon (Clark, 1975) and skin cracks in tomatoes (Sorkor and Wolfe, 1983). The time of decay of acoustic sound is measured in relation to an optical property of the product, but the possibility of error on the determination of end point on an oscilloscope cannot be ruled out. A signal analyser which has a function to compute the power spectrum of sound waves by means of a fast Fourier transform (FFT) has been reported, in which the frequency resolution is dependent on the sampling interval and data size. The acoustic impulse response method has an advantage from the standpoint of simplicity of instrumentation compared with the sonic vibrating resonance method. In the latter case, an accelerator has to be attached to the surface of the product to detect a resonance vibration. In the case of acoustic impulse response, a microphone can take the place of the accelerometer making non-contact sensing possible, and a ball pendulum takes the place of the vibrating system and the sound can be measured instantaneously. The correlation coefficients for apples and watermelons with various parameters of objective measurement such as f, mf^2 and $m^{2/3}\rho^{1/3}f^2$ ($m^{2/3}f^2$ in the case of watermelon; ρ=density) are reported. In the case of apples, a significant correlation coefficient of 0.65–0.77 (depending on the variety) between sensory rated firmness and overall

reaction has been obtained. In the case of watermelons, the correlation coefficient with hardness is poor for every acoustic index, and could be due to the poor measurement of firmness by the hand operated tester. A precise measurement of flesh firmness instead of a hand operated pressure test is necessary for comments on the validity of the computed indices (mf^2, $m^{2/3}f^2$). The correlation coefficient between ripeness and sensory firmness is significant (Yamamoto *et al.*, 1980). The fact that there are no significant differences in the internal quality of apples and watermelons among the various ripeness classes, as evaluated by external observation, makes this method advantageous (Yamamoto *et al.*, 1981).

3.7.2 Chemical indicators

Analyses of free amino acids in the main cultivars of orange, tangerine, and grapefruit have shown a total of 22 free amino acids with a concentration range of 150–300 mg/100 ml juice. Proline, arginine, asparagine, aspartic acid, γ-aminobutyric acid and serine together account for 91–93% of amino acids found. During ripening, proportions of proline and arginine increase, while those of arginine and aspartic acid undergo an equivalent decrease. Characterization of citrus juices is possible on the basis of the ratio of (arginine+proline) to (aspartic acid+arginine). The ratio reaches a maximum (about 3) in species having a high sugar:acid ratio such as orange, clementine and tangerine, a minimum (0.3) when the sugar:acid ratio is low such as in lemon and intermediate values of about 1.0 in grapefruit (Zamarani *et al.*, 1973). These are useful indices of ripening of citrus fruits.

Maturity of green peas is critical with respect to the quality of processed products. Physical methods based on specific gravity (Lee, 1941a, 1941b), and chemical methods based on alcohol-insoluble solids (Kertesz, 1934, 1935) and starch (Nielsen *et al.*, 1947a, 1947b; Nielsen and Gleason, 1945) have been documented. These methods are quite satisfactory, and in the case of borderline zone samples, a brine flotation test has been recommended (Lee *et al.*, 1954). Determination of the albumin appears to be a useful indicator of pea quality, since it is also an index of modification of pea protein in the growth period of the pea seeds between 4.7 mm and 8.8 mm in size (Ros and Rincon, 1990). In the case of strawberries, concentrations of alanine and ethyl esters, particularly ethyl butanoate and ethyl hexanoate are profoundly influenced during ripening (Perez *et al.*, 1992), and can serve as tentative indices of ripeness. A comparison of ethyl ester concentration and alanine content during ripening has shown that, from 41 to 46 days after blooming, ester biosynthesis increases about three-fold while alanine levels decrease from 16.7 mg/100 g to 1.6 mg/100 g. It appears that the ratio of ethyl esters to alanine may prove to be a useful index of ripeness and maturity and needs to be investigated.

Fruits belonging to *Pirus communis*, *Citrus mobilis*, *C. sinensis*, *C. limonia*, *Musa sapientum* and *Prunus persica* show an oxidation–reduction potential on the reduction side during ripening, while it is more on the oxidation side at the overripe stage.

Acetylmethylcarbinol progressively increases during ripening, whereas 2,3-butylene glycol reaches a maximum at the onset of overripeness and then decreases. The numerical value differs with the fruit variety and therefore the limiting concentration at the peak of ripeness needs to be set for each variety. For instance, in apples (*Pyrus malus*) of the stark delicious variety, 2,3-butylene glycol is >5.0 mg/100 g at the optimum stage of ripeness. A value >5.0 is suggestive of overripeness, while a value of >10 is the first evidence of decay (Claudio and Giuseppe, 1955). These findings fit generally with the postulated acetoin condensation (Giuseppe, 1956). Similarly, the presence of an optimum level of 2,3-butyleneglycol and minimum level of acetylmethylcarbinol is considered to be indicative of commercial maturation in fruits such as *Curcumis melo*, *Vitis vinifera*, *Fragari vesca* and *Eriobotrya japonica* (Giuseppe, 1957). In grapes, the relationships between polysaccharide concentration, phenols, nitrogenous compounds, especially proline and ammonia, varietal aroma compounds particularly terpenic and the linalool/geraniol ratio, minerals especially K^+/malic acid ratio and various enzymes have been proposed as ripening indices. Lipids in grapes, particularly LOP (palmitooleolinolein) and OOO (triolein) molecular species exhibit metabolic activity directly related to metabolism during ripening (Barron and Santa-Maria, 1990), and could serve as ripening indices.

The amount of lycopene in the combined carotenes is taken as the ripeness index for tomatoes. Lycopene is present in nearly constant amounts, averaging 1000 $\mu g\ g^{-1}$ of the dry extract. By relating the values of β-carotene and combined carotenes with the ripeness index, the degree of ripeness of tomatoes used in the preparation of preserves, or the addition of carotene containing colourants can be determined (Sanahuja, 1953).

In avocados, the official standard for assessing maturity for picking was 8% oil. However, this determination is fraught with difficulties, and hence an alternative of 21% dry matter has been recommended as a maturity standard (Lee, 1981). Dry weight can be very conveniently determined using microwave ovens with an output of 500 W at 2450 MHz (Swarts, 1978). A patent from the Former Soviet Union suggests analysing the comminuted representative samples of fruits for total dry matter (DM) by drying and soluble dry matter by refractometry. The difference between these values on the ripeness scale of −4.4 to 12.5 is indicative of the ripeness of fruits (Skorikova *et al.*, 1991).

Banana fruit ripening is accompanied by a decrease in the uronic acid content of the cell wall material of edible pulp, and an increase in low molecular size uronic acid soluble in aqueous ethanol or phenol:acetic acid:water (2:1:1 w/v), consistent with the hypothesis that exopolygalacturonase has acted upon the cell wall polysaccharides (Wade *et al.*, 1993). This increase in low molecular weight uronic acid varies with variety over the entire ripening period and could possibly be used as indicator of ripeness. Principal component analysis based on characteristics like titratable acidity, vitamin C, concentration of phenolics, pH and crushing force is useful in discriminating ripe from overripe cherries (Fils-Lycaon *et al.*, 1988).

3.8 Non-microbial methods for determining microbial quality

The United States Food and Drug Administration (US FDA) considers cranberry juice to be adulterated under the Federal Food, Drug and Cosmetic Act if the average mould count for >6 subsamples exceeds 15% and/or if any one subsample exceeds 50% (US FDA, 1978). Mould contamination in fruits influences the sensory characteristics of the products. For instance, mould contamination of strawberry fruit results in pronounced colour degradation in strawberry wine. Enzymes derived from mould are believed to play an important role in anthocyanin pigment degradation (Huang, 1955) and in polymerization and browning reactions. Anthocyanin degradation may be caused by both polyphenoloxidase (Pifferi and Cultrera, 1974), and glycosidases (Yang and Steele, 1958; Blom, 1983). The changes resulting from mould contamination are more pronounced in stored concentrates (Rwabahizi and Wrolstad, 1988). The contribution of a mycotoxin, patulin, to apple products by moulds such as various *Penicillium* species, *Aspergillus* species and *Byssochlamys nivea* is well known (Scott, 1977). A quality defect in canned apricots, wherein the fruits in some batches soften and break down within months of processing and thereby seriously affect the marketing of this attractive product, has been attributed to fungal contamination by *Byssochlamys fulva* and *Rhizopus nigricans* (Harper and Beattie, 1971).

The use of considerable amounts of rotten fruit in the manufacture of fruit juices, jellies and butters is easily concealed because their presence cannot be detected by either odour or taste. The mould count method and the rot fragment count suitable for fresh fruit products are of little value when applied to clear jellies and juices. The microorganisms that invade the damaged fruit and cause it to rot constitute a rich source of pectic enzymes. Therefore degradation products of pectin would yield information of the quality of fruit used for processing. Alcohol soluble non-dialysable substances and alcohol soluble furfural yielding substances have been investigated on this basis but did not prove to be promising. The enzyme polygalacturonase, a member of the pectinase group, liberates galacturonic acid from polygalacturonides and is not normally found in apples (although present in tomatoes). The increase in galacturonic acid in apples and strawberries (Mils, 1951) on rotting is evidently due to the action of galacturonase of microbial origin. It is reasonably heat stable and non-volatile and can be determined in microgram quantities after removal of interfering substances. A number of procedures have been tried for quantification of galacturonic acid from fruit products. These include (i) chromatographic separation followed by titration or oxidation, (ii) separation of acids by lead precipitation and determination by oxidation or with naphthoresorcinol, and (iii) separation of the acids by ion exchange resins and determination by oxidation or with naphthoresorcinol. There are two main drawbacks to the first method, its low sensitivity and the occurrence of erratic blanks in solutions of citric and malic acids (unexplained observation). The oxidation method is unduly influenced by natural colouring matters and traces of sugars. The lead precipitation method permits loss of considerable amount of acids in the filtrate and the precipitate

is contaminated with large amounts of sugars. The ion exchange method is found to be sufficiently accurate to merit accumulation of authentic data on samples of fruit juices (Winkler, 1951). This method would be of no use in cases where commercial apple juice is clarified by pectic enzyme preparations prior to pasteurization and filtration. However, the method is suitable for products such as apple butter, jelly and juice not clarified by enzymes (Harris, 1948).

Estimation of chitin, a cell wall constituent of fungi which is not found in higher plants, or an analysis of its degradation product chitosan has shown a high correlation with the Howard mould count for non-homogenized juices and purees. The correlation is however low for homogenized products. Previous methods of chemical analysis of chitin utilized acid, with or without subsequent enzymatic analysis. These procedures were lengthy and also subject to interference by plant materials. Yet another method (Ride and Drysdale, 1972) utilized degradation of chitin to chitosan, which when treated with nitrous acid yields an aldehyde which can be measured colorimetrically (Tsuji *et al.*, 1969).

Glucosamine, another constituent of fungal mycelium is recovered to the extent of >95% of the expected level and can serve as a chemical criterion for the estimation of mould in tomato products (Jarvis, 1977). This is evident from the recovery of 93.6–103.5% of the expected level (Bishop *et al.*, 1982).

Information is lacking on whether the chitin content of moulds differs, and to what extent the chitin content is affected by substrate, age and growth conditions. Since the exoskeleton of insects also contains chitin, work needs to be done to assess the effect of insect contamination. The results of a study undertaken by Bishop *et al.* (1982) have indicated that a mean value of 29–30 μg of glucosamine/mg dry weight of a diverse fungal population may be a statistically valid figure. Experiments with added exaggerated insect fragments (600 insect fragments/100g) has shown the glucosamine levels to be increased only slightly. The microbial population in orange juice has also been estimated by bioluminescence (Graumlich, 1985), a measure of ATP (adenosine triphosphate) of microbial origin.

Chlorophyll a fluorescence (Fvar) has been highly correlated to respiration, and is an indicator of post harvest changes in broccoli. It can be used as a non-destructive indicator of early changes in tissue condition (i.e. degree of freshness) of broccoli during storage (Toivonen, 1992).

Succinic acid has been found among the products of mould metabolism and is present in decomposed eggs and fish. Preliminary studies using succinic acid as an index of decomposition of tomato products have shown considerable promise (Van Dame, 1952). Besides succinic acid, acetic, formic and lactic acids are also produced in tomatoes by moulds and bacteria (Hillig, 1945). It has also been shown that lactic acid is formed and that citric acid disappears with decomposition due to moulds and bacteria. The work of Van Dame (1951) showed the correlation between mould count and succinic acid, and recovery of succinic acid from the products of about 85%. The acid content in tomato juice has been correlated to conductance by an equation,

$y=0.4148x-0.2344$, where y and x denote the acidity and conductance, respectively (Ohta *et al.*, 1980). Hence the degree of decomposition may also be linked to conductance values. This needs to be confirmed. Chromatographic separation of acetic, formic, lactic, malic, succinic and citric acids and individual determinations of each acid are reported by Van Dame (1953). Perhaps the ratios between some of these acids may have a better correlation with the microbial growth in tomato products. Appropriate preparation of the sample is recommended to validate the use of succinic acid as an index of decomposition in spinach and other oxalic acid-rich vegetables because of interference by oxalic acid. Separation of the two acids can be achieved on a silicic acid column using n-butanol–chloroform (15:85) as the mobile solvent with good recoveries (Silverberg, 1955). Analysis of succinic acid by HPLC or other analytical instruments could make it a more sensitive method for assessing microbial decomposition in vegetables.

Quality indices based on the first derivative of spectral reflectance of tomatoes at 590 nm and 710 nm can be used to separate good tomatoes from those with black mould, grey mould and sunscald (Ruiz and Chen, 1982).

The pattern of volatile amines in apple fruits is a good indication of contamination by different moulds. In healthy fruits, 11 amines were found, of which 10 could be identified. *N*,*N*-di-1,4-diaminobutane and *N*-di-1,4 diaminobutane were present in highest concentration. Contamination with *Paecilomyces* sp. or *Aspergillus flavus* causes significant changes, while the changes are small with *Fusarium* contamination. Most of the volatile amines decrease in concentration, the only increase being recorded for ethylamine. Butylamine and isobutylamine which are not present in healthy fruits occur in some mould contaminated ones and could therefore be used as an index of mould contamination (Hrdlicka and Curda, 1971). Growth of *Penicillium expansum*, a causative agent of rot in fruits, secretes extracellular protopectinase (Gupta, 1960), which can be considered as an index of penicillium contamination.

A rare carotenoid, 3,3′-dihydroxyisorenieratene reported in a *Streptomyces* species (Harbourne, 1973) could serve as a potential index of *Streptomyces* contamination in fruit and vegetables and their products. However, documentary evidence needs to be furnished. Polyacetylenes or acetylinic compounds are an unusual group of naturally occurring hydrocarbons, and are also found in higher fungi, in the two families of the *Basidiomycetes*, the *Agaricaceae* and *Polyporaceae* (Bohlmann *et al.*, 1973). The chain length of the fungal acetylinics is mainly between C_8 and C_{14}. The analysis of polyacetylines could be of help in investigating spoilage of fruits and vegetables by the *Basidiomycetes* group of fungi, and levels of polyacetylines could probably be correlated to fungal load. This again needs to be thoroughly investigated.

One of the responses of plant tissue to infection by microorganisms is a large increase in the synthesis of characteristic phenolic compounds. It is presumably a protective response to invasion, although it does not necessarily prevent the organism establishing itself in the tissue. As an example of this, the coumarin scopolin is formed in high concentrations in infected plants of the *Solanaceae* and is particularly easily

observed in blighted tubers (Hughes and Swain, 1960). Scopolin could therefore be used as an index of potatoes being infected by microbes and deserves merit from investigators.

Acetylmethyl carbinol and diacetyl in orange concentrates are suggested to be indicators of the growth of certain bacteria which produce off-flavours (Hill *et al.*, 1954) such as various fungi and lactic acid bacteria (Gierschner and Herbst, 1981). The individual concentrations of these two compounds are useful in determining not only the degree of buttermilk off-flavour due to diacetyl, but also the potential off-flavour spoilage due to acetylmethyl carbinol, since the latter can be oxidized to diacetyl or reduced to 2,3-butylene glycol depending on the conditions present. There seems to be no correlation between individual concentrations of diacetyl and acetylmethyl carbinol in off-flavoured juices. Various amounts of acetylmethyl carbinol can be found in normal juice samples, but only the slightest trace of diacetyl has been detected. The data available do not permit establishment of limiting values for differentiation of perfect from spoiled products, but can be used for in-process quality control (Gierschner and Herbst, 1981). The chemical test depends on the formation of a red coloured compound with diacetyl, creatine and α-naphthol (Byer, 1954). For a general control measure the relative changes in the concentration of these two compounds in juice at various processing points is a good indication of plant sanitation. The points usually selected are the cut-back juice, evaporator feed, 20 °Brix stage and the final product. The 20 °Brix stage of the evaporation system is known to have the greatest growth of gum forming organisms which may produce diacetyl (Hays and Riester, 1952; Murdock *et al.*, 1952). In the case of tomatoes, however, the presence of diacetyl and acetoin in the fruits, puree and the juice does not affect the organoleptic rating. It is therefore inappropriate to use acetoin and diacetyl concentration as a quality indicator for tomato products without additional information on the bacterial load (Jacorzynski *et al.*, 1990).

The moulds, *Alternaria alternata*, *Rhizopus stolonifer*, *Botrytis cinerea*, *Aspergillus niger*, *Cladosporium herbarum* and *Byssochlamys fulva* are collectively identified to represent approximately 70–80% of fungal contamination in common high acid soft fruits. Cross-reactivity of freeze dried mould mycelia has been assessed with the mixed mould I_gG (immunoglobulin G) for antibody conjugation. It is perceived that alkaline phosphatase is a suitable conjugate enzyme compared with horseradish peroxidase for use in such immunoassays (Patel and Curtis, 1989).

Microbial contamination and subsequent spoilage of carbonated beverages are most often caused by osmotolerant yeasts (Speck, 1976; Woodruff and Phillips, 1981). Current quality control methods used to detect yeast contamination in these products normally take 3–5 days (Speck, 1976). This is often too long for quality control management to initiate prompt corrective action if product contamination is detected. As a result, recent research efforts have focused on developing more rapid methods for detecting yeast contamination in beverages. These efforts have led to the development of the radiometric assay (Hatcher *et al.*, 1977), the impedimetric method (Weihe *et al.*,

1984) and the direct epifluorescent filter technique (Pettipher, 1983). The firefly bioluminescent assay for ATP has been used to estimate yeast levels in beer (Miller *et al.*, 1978) and fruit juices (Stannard and Wood, 1983). Figure 3.1 shows the linear relationship of three individually spiked yeasts in a cola beverage between predicted and conventional CFU levels. A close correlation ($r>0.9$) between the bioluminescent predicted colony forming units (CFU) and conventionally obtained CFU has been demonstrated with good reproducibility. The time requirement is in minutes instead of days (LaRocco *et al.*, 1985).

The Malthus 128H system, a rapid automated conductance method, has been studied to find conditions for the rapid detection of yeasts in orange juice. The detection time is influenced by the incubation temperature, with better results being obtained at 30 °C than at 25 °C. This tendency is apparent particularly when the cell number is small. The type of medium influences the measurements over a 30 h period.

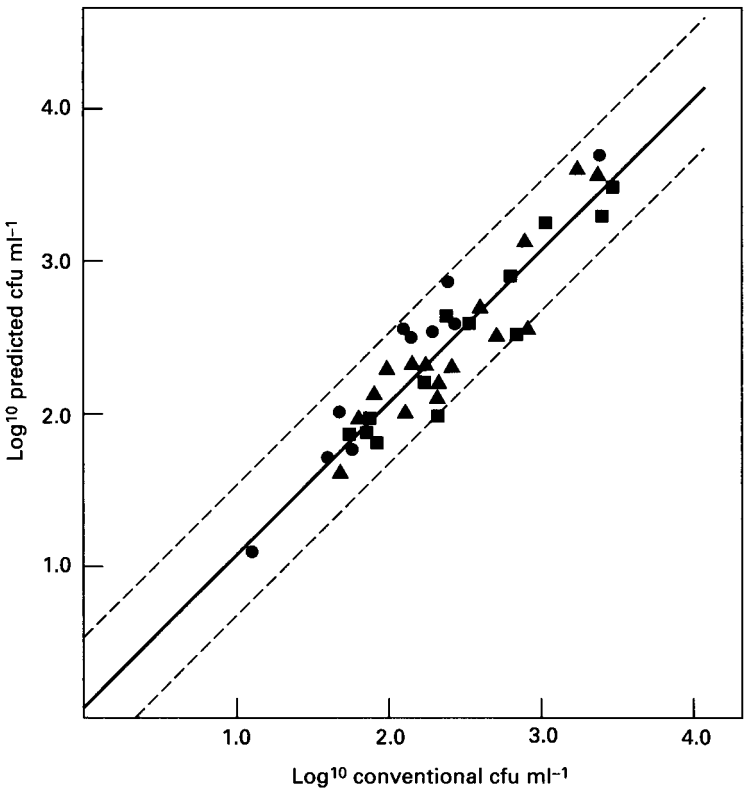


Figure 3.1 Three yeasts individually spiked in cola beverage: ●, *S. cerevisiae*; ▲, *Candida albicans*; ■, *T. kefyr*. Linear relationship between predicted and conventional CFU (colony forming unit) levels: ---, 95% confidence limits; $r=0.946$, $n=42$. (Source: LaRocco *et al.*, 1985, reproduced with permission)

The detection time can be shortened in a shaking incubation system (Miyake *et al.*, 1990a, 1990b).

Uric acid occurs in fruit products as a result of the use of insect contaminated raw stock. A simple paper chromatographic method using phenol–acetic acid as solvent has been described (Tilden, 1951), for identifying uric acid in minute amounts from syrups to which it is added. Development of a mercury complex of uric acid, after chromatographing, helps in locating uric acid spot. It seems possible to develop the procedure into a fairly satisfactory method for determining uric acid in fruit syrups made from insect infested raw stock.

Besides microbial contamination, pesticide residues in postharvest treated fruits need to be monitored in fruit products. Pesticide residues can be retained up to 95% of initial content after several weeks of storage (Tsumara-Hasegawa *et al.*, 1992).

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Milk and Milk Products

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Chapter 4

Milk and Milk Products

4.1 Introduction

The term quality, as applied to milk and products made from milk, embraces a variety of features. These include such diverse properties as absence of dirt, antibiotics, off-flavours, pathogenic organisms and abnormal numbers of body cells; evidence of cleanliness and care in production and handling as indicated by microbiological analysis; chemical analysis to check for dilution with water, removal of fat, and any added adulterants; possession of desirable aroma and flavour; and adequate amounts of those constituents which are of nutritional importance. Yet with milk, the bacteriological aspect has received the greatest attention. With butter and cheese, flavour is of far greater relevance. With ice cream, both the aspects are of interest (Johns, 1959).

Adulteration in market milk implies addition of any substance to normal milk or removal of any of its constituents or both to deceive the consuming public and derive an extra profit from a given volume of milk. The forms of adulteration like addition of water, skimming or removal of fat and addition of fluid skim milk can be detected from specific gravity and fat content. Accidental adulterations in milk or unhygienic and insanitary practices result in the entrance of dirty water, alkalis from detergents, vegetable cells, hair, household dust and dirt, animal urine, dung feed etc. These are usually detected visually or on the basis of smell and taste. A study of various forms of adulteration in a locality is of great help in safeguarding and improving the quality of market milk in the area concerned. In the Netherlands, the raw milk quality is assessed on the basis of six characteristics associated with milk production hygiene and mechanical treatment of the milk, and two associated with the prevention of residues and contaminants. Farmers receive penalty points and consequently lower payments for milk that fails to satisfy these quality standards. Attention is generally focused on the control of residues of antibiotics (Moats and Harik-Khan, 1995; Bell *et al.*, 1995; Reeves, 1995), radioactive substances, polychlorinated biphenyls (PCB) and heavy metals in raw milk. The monitoring of radioactivity has been put in as an emergency measure, following the nuclear accident at Chernobyl in 1986 (Berg van den, 1988).

4.2 Milk of different origins

Blends of cow with buffalo milk or cow with goat or ewe's milk are encountered in

Table 4.1 Composition of milk from various animals (wt %)

Animal	Water	Proteins	Lactose	Fat	Ash
Cow	87.3	3.4	4.8	3.8	0.7
Goat	87.0	3.5	4.3	4.2	0.9
Sheep	80.7	5.2	4.8	7.9	0.9
Buffalo (Egyptian)	82.1	4.2	4.9	8.0	0.8
Buffalo (Indian)	82.8	3.6	5.5	7.4	0.8
Camel	87.6	3.0	3.3	5.4	0.7
Horse	89.0	2.7	6.1	1.6	0.5
Donkey	89.0	2.0	6.1	2.5	0.4
Reindeer	63.3	10.3	2.5	22.5	1.4
Lama	86.6	3.9	5.6	3.2	0.8
Human milk (for comparison)	87.4	1.6	7.0	3.8	0.2

Source: Stein and Imhof, 1990.

many parts of the world. Table 4.1 summarizes the composition of milk from different animal species available commercially.

4.2.1 Ewe's, goat and cow milk

Ewe's milk is abundantly available in some parts of the world and has been used for extending cow's milk. Also, commercial production of caprine milk as a speciality and high priced product brings the possibility of adulteration with added water or bovine milk. When goat milk is blended with cow milk the resultant mixture is not obviously different from pure goat milk, especially if the level of addition is below 15%. This substitution can become a serious problem in cheese manufacture as the composition of milk influences the organoleptic characteristics of the final product. Consumption of products containing undeclared milk may cause allergies in sensitized individuals (Taylor, 1986; Miller, 1987). Detection of species origin of milk used in cheese manufacture is of particular relevance in cheeses made from ewe or goat milk, which are to be exported to European Union (EU) countries where product authenticity must be assured. A number of tests using a variety of methods for detecting this level of adulteration have been reported.

Immunological methods (Aranda *et al.*, 1993) as well as non-immunological methods based on the identification of one specific component of milk include gel electrophoresis (Hillier, 1976; Ng-Kwai-Hang and Kroeker, 1984) and isoelectric focusing (Addeo *et al.*, 1984; Ruiz and Santillana, 1986) for proteins, gas liquid chromatography (GLC) (Sadini, 1963; Iverson and Sheppard, 1985; Prager, 1989) and high performance liquid chromatography (HPLC) (Cerbulis *et al.*, 1982; Kaiser and Krause, 1985), especially of caseinomacropeptides (Lopez-Fandino *et al.*, 1993).

The chemical and UV spectrophotometric characteristics and fatty acid

Table 4.2 Chemical indices to distinguish between cow milk and goat milk

Fatty acid ratio	Cow milk fat	Goat milk fat
C_4/C_6+C_8	1.0–1.8	0.5–1.0
C_8/C_6	0.4–0.6	0.7–1.0
C_{10}/C_6	0.5–1.3	1.5–3.0
C_{10}/C_8	1.5–2.5	2.5–3.5
C_{16}/C_8	15.0–30.0	5.0–15.0
C_{12}/C_{10}	1.0–1.5	0.5–0.8
C_{14}/C_{10}	2.5–5.0	1.0–2.0
C_{16}/C_{10}	10.0–15.0	2.0–5.0

Source: Gattuso and Fazio, 1980.

composition can distinguish between the two milk types. The tetraenezone, particularly the K^{300} is a useful index of adulteration of cow or goat milk. Other useful indices are ratios of fatty acids, especially to short chain fatty acids (Gattuso and Fazio, 1980). GLC analysis of fatty acid butyl esters has shown goat milk cheese and ewe milk cheese to have characteristically different lower chain length fatty acid patterns as compared to cow milk cheese. For instance, the lauric:capric ratio for cow milk cheese averages around 1.16 versus 0.46 for goat milk cheese and 0.58 for ewe milk cheese. This ratio could thus be used to indicate the level of cow milk in cheeses labelled as goat or ewe milk cheese (Iverson and Sheppard, 1989). The fatty acid ratios are tabulated in Table 4.2.

Cow milk added to goat milk can be detected by the presence of β-carotene, which is completely absent in goat milk. Dilution of cow milk however cannot be detected because of wide variations in β-carotene levels (Kuzdzal and Kuzdzal, 1959). The admixture of 20% goat milk in cow milk can be detected by ultraviolet light (Toman, 1935). This detection is also possible by Bovitest reagent which contains dibasic sodium phosphate, tribasic potassium phosphate, sodium azide, formaldehyde and triphenyl tetrazolium chloride (TTC). The test is based on the fact that addition of cow milk introduces riboflavin-derived coenzyme of xanthine oxidase into ewe milk, and the xanthine oxidase converts formaldehyde to formic acid, providing a red complex with TTC (Wagner *et al.*, 1984). An enzymic method for the detection of elevated levels of xanthine oxidase from cow milk has also been described. It has a sensitivity for detecting 2% adulteration levels. However, this method would not be effective in the case of pasteurized cow milk.

The ash content in the milks of ewe, goat and cow is fairly constant, but the relative proportion of different mineral salts is variable. The proportions of the minerals vary during processing, for example in cheese manufacture, due to the association of some minerals such as Ca, P and Mg interacting with casein. Nevertheless they are quite useful in differentiating between milks and cheeses of different species origin (Fresno *et al.*, 1995). The average Ca/Mg ratios in cheeses derived from cow milk and ewe milk

are reported to be 23.3 and 17.2, respectively. These can serve as indices for detecting admixtures of these milks, and along with lactose data, can also evaluate adulteration of grated cheese with processed cheese or whey solids (Pollman, 1984). Stepwise discriminant analysis on ewe, goat and cow milk has yielded the variables K/Mg, Na/Ca, Zn, Cu/Zn and Cu/Na content as the most useful in differentiating between them, achieving a correct classification of 98.2% of cases. In the case of cheeses, the most useful variables have been found to be Fe/K, Na/Ca, Zn/Cu, Na/Mg and Zn content which give a correct classification in 97.1% of cases (Martin-Hernandez *et al.*, 1992). Multivariate analysis using nine trace metals such as Cr, Mn, Fe, Ni, Cu, Zn, Mo, Cd and Pb can also successfully differentiate between the milk species (Favretto *et al.*, 1992).

Other methods of detecting adulteration of ewe milk with cow milk, particularly in processed products like cheeses are based on the differences in the electrophoretic mobility (Furtado, 1983; Aschaffenburg and Dance, 1968) between bovine and ovine *para*-K-caseins (Anufantakes *et al.*, 1985) and/or whey (Solberg and Hadland, 1953) using polyacrylamide gel column (Pierre and Portman, 1970). The high mobilities of α-casein and β-lactoglobulin fractions from cow milk relative to goat milk make them better indices of adulteration. In a densitometric scan of caprine milk adulterated with 10% bovine milk, the bovine α-s_1 casein is clearly visible and can be detected down to 5% (Szijarto and Van de Voort, 1983). The β-lactoglobulin fraction in polyacrylamide gel electrophoresis (PAGE) serves as an indicator for detecting >5% goat milk in ewe milk (Cattaneo *et al.*, 1989). In suspected samples lower percentages can be detected by increasing the sample concentration applied to the gel.

The sensitivity is lower when cheese is prepared from mixed milks. In such cases, α-casein provides a more superior index of quality than does β-globulin, as the latter is present in relatively small amounts and is readily denatured (Foissy, 1976). This method is based on the assumption that the proportion of α-casein is constant in cow milk. However, regional or individual variations and effects of renneting decrease the precision of this method, when <5% cow milk is present in goat milk and cheese (Pierre, 1977). Gel isoelectric focusing of the urea extract of cheeses in an appropriate pH gradient allows accurate identification of bovine, caprine and ovine *para*-K-casein and can detect adulteration of pure ewe or goat milk cheeses (Addeo *et al.*, 1986). Isoelectric focusing followed by densitometric evaluation of pherograms using α-casein as indicator protein can distinguish 1–2% cow milk in ewe milk and in cheeses prepared therefrom (Krause *et al.*, 1982). Densitometric analysis of isoelectric focusing patterns can be used to calculate the colour density ratios of the C/E bands, C and E representing cow and ewe proteins, respectively. An index correlating the percentage of cow milk in the mixture used in cheese manufacture can be derived (Rispoli and Saugues, 1989). A fast performance liquid chromatography (FPLC) technique can also detect bovine milk in cheese made from goat or sheep milk with a sensitivity limit of about 2%. With the fully automated system, the FPLC unit is capable of calculating rapidly the area under the peak indicative of the quantity of the

adulterant. Durand *et al.* (1974) described both an immunodiffusion method and an immunoelectrophoretic study of cow and goat milk. Immunological (Levieux, 1977) and serological detection of cow milk added to goat milk (Solberg and Hadland, 1953), particularly that based on hyperimmune antisera to soluble whey protein from cow milk is noteworthy, since the method can detect >2.5% cow milk in the form of a visible precipitin reaction (Eguaras, 1975). Techniques of immunodiffusion in agar gels using highly active and specific ox- and mutton-precipitating sera can distinguish 5–10% cow milk in mixed cheeses. Pure sheep cheese reacts positively only with antimutton serum, whereas mixed cheeses react positively with both. The results obtained by this technique correlate well with those for serum precipitation (Cantagalli and Piazzi, 1965). Radial immunodiffusion methods have been used to detect cow milk in goat or ewe's milk, but the precision of these methods needs to be improved further (Gombocz *et al.*, 1981; Barbosa and Goncalves, 1986).

A new technique which is simple, accurate and reliable for detecting cow milk added to goat milk is rocket immunoelectrophoresis. The method employs anticow milk serum raised in a goat (Radford *et al.*, 1981). Using this technique, cross reaction between goat milk and antibody is almost totally absent, and tedious purification of the antiserum essential when the antibody is developed in other animals is avoided. It is expected that a goat would not generate antibodies to proteins which are antigenically similar to those in its own milk. This technique provides a quantitative, unambiguous indication of adulteration of goat milk in a form which is easily preserved as a permanent record. Concentrations of 5% cow milk in goat milk are very easily demonstrated, but as little as 1% can also be detected (Radford *et al.*, 1981). The method is applicable to pasteurized or homogenized milk as well as raw milk.

A cow milk identification test (COMIT), capable of detecting >3% cow milk in ewe milk uses polyclonal antibodies raised in goat against bovine whey proteins which have a higher antigenicity than casein fractions (Ramos and Juarez, 1984). COMIT offers the advantage of being accurate, cost-effective and suitable for field use by less experienced operators. Both antisera and positive and negative milk reference discs can be conveniently supplied in a very stable, standardized and ready-to-use form. This is, however, only a detection test and quantification would require other techniques such as enzyme linked immunosorbent assays (ELISA) (Garcia *et al.*, 1989).

The use of affinity purified antibodies against bovine caseins and a sandwich ELISA to detect and quantify the presence of bovine milk in ovine milk and cheese has recently been reported (Garcia *et al.*, 1990, 1991; Sauer *et al.*, 1991; Rodriguez *et al.*, 1990, 1991, 1993). However, mixtures of ovine milk with commercially pasteurized, sterilized and ultra high temperature (UHT) treated bovine milk are known to give lower immunological response than expected, obviously as a result of denaturation.

In collaborative studies, the electrophoresis of the protein fraction soluble at pH 4.6 has been shown to be better than radial immunodiffusion for quantitative determination of the percentage of cow and ewe milk in mixed milk cheeses of known composition (Amigo *et al.*, 1989). Tests on 24 market samples of cheese declared to be

made from ewe milk showed that two contained goat milk and two contained cow milk. Sixteen cheeses were declared to be made from mixed milk, but three were found to contain only cow milk, and five, four and four, respectively contained 50–100%, 25–50% and <25% cow milk (Ramos, 1989). PAGE has been confirmed to be more reliable and accurate than agar radial immunodiffusion for the detection of adulteration of ewe milk with >5% goat milk (Cattaneo *et al.*, 1989).

4.2.2 Cow milk and buffalo milk

Due to its lower price, cow milk is frequently used to adulterate water buffalo milk in the manufacturing of mozzarella cheese, a typical Italian cheese variety. Electrophoretic methods, based on the differences in the electrophoretic mobility of homologous fractions have been proposed for detecting the mixture of cow and water buffalo milk. The differences in the electrophoretic mobilities of casein components from cow and buffalo have been reported by Aschaffenburg and Sen (1963) and Ganguli and Bhalerao (1964). The proportion of α- and β-caseins in whole casein in cow and buffalo milk are known to differ.

The densitometric method for evaluating cow and water buffalo α-s_1 caseins resolved by cellulose acetate electrophoresis (Albonico and Resmini, 1967) and polyacrylamide gel electrophoresis at alkaline pH has been found to give the best results. In aged buffalo cheese, however, an extensive proteolysis of the α-s_1 casein produces a new peptide that has a mobility similar to that of bovine α-s_1 casein. Each α-casein component in cow milk has a counterpart in buffalo milk (Trieu-Cuot and Addeo , 1981), which can be separated by gel isoelectric focusing (Trieu-Cout and Addeo, 1981; Krause and Belitz, 1985). A report by Ganguli *et al.* (1964) has revealed that cow milk casein is hydrolysed at a faster rate than buffalo milk casein by proteolytic enzymes. However, fractionated caseins like α- and β-caseins from buffalo milk are hydrolysed faster than cow milk casein components. The polypeptides isolated after trypsin action on caseins from cow and buffalo milks are found to have different electrophoretic mobilities, although the *N*-terminal amino acid remains the same in both caseins before as well as after trypsin action (arginine and lysine) (Singhal and Ganguli, 1965). This differential behaviour could be utilized as a tool for detecting cow milk in buffalo milk and vice versa.

A method based on the *in vitro* formation of γ_2 and γ_3 caseins by the addition of plasmin to cheese and/or casein solution, their subsequent detection by polyacrylamide gel isoelectric focusing in the pH range of 5–8 followed by a densitometric evaluation of the electrophoretic bands is reported. The sensitivity of the method is believed to be to 1% addition of one milk in another. The detection can be sharpened to 0.5% by using a silver staining procedure (Moio *et al.*, 1989). The level of adulteration is determined by either using a calibration plot established by analysing cheese samples containing known amounts of milk from the two species or by calculating the ratio of bovine γ_2 casein to the total γ_2 casein. Reliable results are

Table 4.3 Bovine milk determination in eight samples of mozzarella cheese using different methods

No.	Theoretical values	Bovine milk (%)			
		Ratio R_1	Ratio R_2	Eqn 4.1	Eqn 4.2
1	25	26+1	21+3	25+0.5	26+0.4
2	50	52+4	41+3	50+0.3	51+0.1
3	20	22+3	14+1	21+0.7	17+0.3
4	0	0	0	0	0
5	60	61+4	49+5	59+0.1	61+0.2
6	15	17+2	10+1	16+0.2	12+0.5
7	35	35+1	28+2	34+0.1	35+1.0
8	30	31+4	24+3	30+0.3	30+0.2

Source: Addeo *et al.*, 1989 (reproduced with permission).

obtained even when the cheese samples are stored for several days at room temperature (Addeo *et al.*, 1989). Table 4.3 reports the percentages of bovine milk determined in eight cheese samples of known composition obtained from the following data:

$$\text{Ratio, } R_1 = \frac{\text{bovine } \gamma_2 \text{ casein}}{\text{bovine } \gamma_2 + \text{buffalo } \gamma_2 \text{ caseins}} \times 100 \quad [4.1]$$

$$\text{Ratio, } R_2 = \frac{\text{bovine } \gamma_3 \text{ casein}}{\text{bovine } \gamma_3 + \text{buffalo } \gamma_3 \text{ caseins}} \times 100 \quad [4.2]$$

In equation 4.1, $Y_1 = 1.03X + 0.17$ and in equation 4.2, $Y_2 = 0.8X + 0.11$, where Y_1 is the percentage of γ_2 casein, Y_2 is the percentage of γ_3 casein and X is the percentage of bovine milk in the mixture.

Electrical conductivity has been used as a tool to detect mixtures of cow milk and buffalo milk. Conductivity of buffalo milk is known to increase progressively with the addition of cow milk. Even with skim milk addition to buffalo whole milk the electrical conductivity increases, the values ranging from 2.9% with 10% skim milk to 23.5% with 90% skim milk (El-Shabrawy and Haggag, 1980). GLC can also be used for detection and estimation of cow milk admixtures in buffalo milk. The analysis is based on initially separating milk fat by fractional crystallization at −20 °C in two distinct fractions, semi-solid and liquid, followed by the fatty acid profiles in the two fractions. The liquid fraction fatty acids are characterized by significant changes in concentration of $C_{16:0}$ and $C_{18:1}$, as the adulteration of cow milk with buffalo milk increased. Simple regression equations for these acids can detect 5% cow milk. Equations for the semi-solid fraction fatty acids are more sensitive indicators of cow milk than those for

Table 4. 4 Composition (percentage) of the semi-solid and liquid fraction fatty acids in buffalo and cow milk

	Semi-solid fraction		Liquid fraction	
Fatty acid	Buffalo milk	Cow milk	Buffalo milk	Cow milk
8:0	0.3	0.9	0.7	0.9
10:0	0.5	1.0	1.8	3.5
10:1			0.1	0.5
11:0			0.1	0.4
12:0	1.4	2.8	2.0	2.6
12:1			0.1	0.3
13:0			0.1	0.3
14:0	12.8	12.8	7.5	12.4
14:1	0.4	1.0	2.0	2.2
15:0	0.2	0.7	0.3	0.5
15:1			0.2	0.2
16:0	51.0	38.9	17.8	25.2
16:1	0.6	0.5	3.8	1.8
17:0	0.1	0.6	0.2	0.3
17:1			0.2	0.3
18:0	14.0	17.3	3.2	3.5
18:1	18.3	23.0	57.5	42.0
18:2	0.3	0.5	2.7	3.0
DU[a]	0.2	0.26	0.69	0.53

[a]The degree of unsaturation [1 (% monoenes/100)+2 (% dienes/100)].
Source: Farag *et al.*, 1984b (reproduced with permission).

liquid fraction fatty acids (Farag *et al.*, 1984a, b). Table 4.4 shows the percentage composition of the semi-solid fatty acids and mother liquor fatty acids obtained from buffalo milk and cow milk. Since the composition of milk fatty acids varies with the season, region and the diet of the animal, it is recommended that every region should have its own regression equations for certain fatty acids to characterize milk adulteration.

An on-the-spot testing for detecting cow milk and buffalo milk in a mixture is based on the Hansa test (Jairam *et al.*, 1984). Addition of buffalo milk to cow milk plus water and addition of water to buffalo milk to resemble cow milk can also be detected by a simple and rapid Hansa test. The test is based on reactions of antiserum to micellar casein from buffalo milk produced in rabbits. For detection of buffalo milk, there are other tests too. Ahmed (1958) reported a rapid platform test for raw milk. Tests based on carotene estimation and starch gel electrophoresis have also been reported (Majumdar and Ganguli, 1972).

Certain bacteria are known to be agglutinated in the milk serum (Chambers, 1920). Two inhibitory substances in milk, that is, lactenin I and lactenin II have been

demonstrated (Auclair, 1954; Auclair and Hirsch, 1953). Lactenin I has been identified to be a glutenin and lactenin II to be a peroxidase. Also, slow acid producing cultures are known to be preferentially agglutinated over the fast acid producers (McPhillips, 1958). Buffalo milk contains more lactenins but less agglutinin than cow milk (Natarajan and Dudani, 1961; Natarajan *et al.*, 1964). Lactenin has been associated with the β-lactoglobulin fraction of milk protein (Sasaki and Aibara, 1959). The lactenin content of cow and buffalo milk was shown to be useful in detection of cow and buffalo milk by means of a rapid ring test (Vedanayakam *et al.*, 1972). The ring test is based on the principle of Wood (1950) for Brucella in that, when stained bacteria are added to milk and if acted upon by agglutinins are carried to the surface along with the cream forming a coloured ring. The bacterial ring test with *Streptococcus lactis* 57 as antigen in cow and buffalo milk has shown all 323 samples of buffalo milk to give negative results and all 487 samples of cow milk tested to give positive results. It has also been seen that admixtures of 10% or more of cow milk to buffalo milk give a positive result, and can therefore be used as a platform test for this differentiation between the two milk species (Vedanayakam *et al.*, 1972).

Studies on the volatile constituents from milks of four different species, that is, buffalo, ewe, cow and goat have shown some interesting results. While the volatiles are similar in all the four species, several quantitative differences exist, which might offer a new approach to detecting admixtures of one milk type in another. For instance dimethylsulphone comprises 25% volatile components in bovine, caprine and ovine milks, and only 4% in buffalo milk, enabling it to be an indicator. 3-Methylbutanal is found only in buffalo milk, phenylacetaldehyde and benzaldehyde are in large amounts in caprine milk, 2-methylketones and 1-octen-3-ol are in larger amounts in buffalo milk and phenylethanol, which is absent in ewe and goat milk, is present at level 100 times more in buffalo milk than in cow milk (Moio *et al.*, 1993). These facts need to be considered in a new light using appropriate analytical methodologies.

4.2.3 Human milk

Simple flocculation tests as analytical methods are reported in the literature to detect added cow milk in mother's milk (Ivady and Kottay, 1953). Freudenberg's reagent (based on calcium acatate) has been used to detect human and cow milk. This reagent precipitates only the caseinogen in breast milk at 37 °C, but all whey proteins at 60 °C. Human colostrum is not precipitated at either 37 °C or 60 °C (Flies, 1952). Detection of adulteration of human milk with cow milk is also possible by observation of granulation of proteins on the sides of test tubes, when two drops of saturated copper sulphate solution to ten drops of milk or 4 ml of 0.4% cadmium sulphate to 2 ml milk are added (Alison, 1952). These tests are applicable whether raw, pasteurized, boiled, sweetened or unsweetened condensed milks are used as adulterants. Human milk has higher amounts of phosphate than cow milk (Romeyer, 1949). Standards prepared with known mixtures of mother's and cow milk based on phosphate content can be

used to furnish a means of determining the content of mother's milk in a mixture (Romeyer, 1950). Dilution of human milk with water can be detected by freezing point depression when >10% of water is added. However, the milk used must be fresh. The results must be used cautiously since freezing point varies for milk from the same individual at different times, and even between the two breasts of the same woman (Miller and Ellis, 1953).

Reversed phase HPLC enables detection of 1% cow milk in human milk on the basis of bovine β-lactoglobulin, bovine α-lactalbumin in the whey fraction and K-casein in the casein fraction (Urbanke *et al.*, 1992). The amino acids, taurine and glutamic acid have been found to be 1.9 and 33.5 μmol/100 ml, and 28.8 and 262.7 μmol/100 ml in cow and human milk, respectively (Mehaia and Al-Kanhal, 1992), and could be used as indices of blending.

4.2.4 Soy milk in cow milk

In recent years soy milk and soy protein have received considerable attention from food manufacturers as alternative sources of economical and nutritive protein. This is especially true in developing countries where shortages of animal proteins exist and soy proteins may be particularly useful in combating malnutrition. Moreover, soy milk and dairy-like products containing soy protein are now being marketed as an ideal alternative for both vegetarians and patients with bovine milk allergies. However, despite the good nutritional and functional properties of soy proteins, the authorities in many countries are reluctant to give legal clearance to the use of non-milk proteins as supplements in bovine milk and its products.

Regardless of this lack of legal acceptance, developments in food processing methods have made it difficult to detect the presence of non-milk proteins in dairy products. It is found that cheese and yoghurt made from cow milk containing 10–20% soy milk does not differ organoleptically from controls. While addition of soy levels up to 20% require no modification in the production parameters, at higher concentration clotting times are known to be extended (El-Safty and Mehanna, 1977; Metwalli *et al.*, 1982a, 1982b; Abo El-Ella *et al.*, 1978). The detection of this type of adulteration presents a peculiar problem to the food analyst who needs to identify specific distinguishing characteristics of the adulterant. Many techniques have been used to identify and quantify soy protein in milk products, including sodium dodecyl sulphate (SDS) – gel electrophoresis (Guy *et al.*, 1973), a serological procedure (Flint and Meech, 1978) and peptide analysis (Bailey, 1976).

This detection is mainly based on the electrophoretic differences in soy and bovine milk. PAGE using tris buffer at pH 8.6 of cow milk and soy milk shows six and nine bands, respectively. Electrophoretic mobility of the prominent globulin of soybean milk is higher than the mobility of x-casein, but lower than of γ-casein. In the mixed milk, this protein band can be seen, even with 2% soybean milk in cow milk (Kim and Park, 1971, 1973). Besides, urea alkaline PAGE, SDS–PAGE and FPLC can be used to

detect quantitatively soy milk in pasteurized bovine milk. While urea gel electrophoresis is not promising, SDS–PAGE can detect 5% soy milk in bovine milk and FPLC is even more sensitive (Hewedy and Smith, 1989). Comparison of the FPLC chromatograms shows distinct differences between bovine milk elution and other profiles. Peaks corresponding to soy milk appear in the mixtures. Areas under these peaks are indicative of the quantity of the adulterant. Using these peaks as the reference, it is possible readily to distinguish mixtures containing >1% soy milk from pure bovine milk. Unfortunately such techniques are time consuming, expensive and need highly trained personnel. In contrast, immunoassays can be simplified to eliminate these limitations. A modified ELISA technique has given good, reproducible and sensitive quantitation of soy milk in bovine milk; detection is possible at 1% (Hewedy and Smith, 1990).

Besides soy milk, other vegetable milks such as coconut milk along with water are also known as adulterants of milk. Simple rapid tests to detect these are also reported.

4.3 Whey or buttermilk in milk

Increasing cheese production gives rise to larger volumes of whey, the disposal of which is posing a problem. Whey protein concentrates are now finding use as ingredients in a variety of processed foods being less expensive than non-fat dry milk (Greenberg and Dower, 1986).

Skim milk powder offered for nutritional intervention programmes shall, according to Regulation 2188/81 of EEC, be prepared exclusively from skim milk and shall not contain solids from whey and buttermilk. The absence of rennet whey solids from skim milk powder is also required according to Regulation 1725/79. In some parts of the world, buttermilk powder made from sweet cream has been used as an additive to skim milk powder. The primary tests which can indicate rennet whey solids are: (a) whey peptide test, which is positive, if the mass fraction determined is higher than 3%; false positive results are obtained with high heat, medium heat and roller dried milk powder; (b) lactic acid determination, which is positive if higher than 150 mg/100g; (c) ash content, which is positive if higher than 8%.

This detection can also be done by electron microscopy at as low a level as 5%. Scanning electron microscopy has shown that particles of pure spray dried buttermilk have shallow wrinkles on their surfaces as opposed to deep wrinkles on skim milk particles. Acid coagulated precipitates of reconstituted buttermilk and skim milk can be used to distinguish between them. When observed under thin section electron microscopy, skim milk precipitates consist exclusively of casein micelles, while those of buttermilk contain additional fat globule membrane fragments and cellular debris. This becomes even more evident on examination of pellets obtained by ultracentrifugation of reconstituted skim milk and buttermilk. Electron microscopy may thus contribute to detection of buttermilk made from sweet cream added to skim milk (Kalab, 1980).

Adulteration of pasteurized milk with whey is an increasingly severe problem in

many countries. The availability of large amounts of whey, its low price and organoleptic characteristics not too dissimilar from those of milk make intentional adulteration economically attractive. Detection is possible by determination of the ratio of casein to whey protein in milk. Casein nitrogen (N_{cas}) can be obtained by nitrogen determination after precipitation at pH 4.6. From the values of casein bound phosphorus (P_{cas}), N_{cas} can also be calculated by the formula, $N_{cas}=(P_{cas}/0.85/6.34)\times 100$. A linear relationship exists between P_{cas} and the casein content in milk. Only caseins have ester-bound phosphorus as esters of serine and occasionally of threonine (West, 1986); the whey proteins have none. The difference between the values of casein content calculated from nitrogen determination after precipitation at pH 4.6 and values calculated from P_{cas} is an indirect measure of the amount of whey protein that reacts with casein during pasteurization and could be used to detect this adulteration (Wolfschoon-Pombo and Furtado, 1989). Analysis of blends of pasteurized milk and cheese whey have shown that the former contains P_{cas} 21.8 mg/100 ml, protein N 500.3 mg/100 ml and casein N 404.5 mg/100 ml, compared with 0 mg/100 ml, 109.7 mg/100 ml and 0 mg/100 ml, respectively for whey. A linearity in reduction in these values has been demonstrated.

4.3.1 Whey proteins in milk products

Quantitative determination of various protein constituents in processed dairy products such as ice creams is a problem for manufacturers and regulatory agencies. The frozen dessert standard of identity requires a minimum fat content of 10% and milk solids content of 20% (Federal Register, 1978). A method that can distinguish between casein and whey protein contribution to solids in a given formulation of ice cream is needed. Furthermore, extension of this method to other dairy products would be desirable. Dye binding methods are suitable for protein determinations in ice cream, but results are slightly different for total milk proteins, caseins and whey proteins depending on which protein has been used as the calibration standard (Kroger *et al.*, 1978). Total nitrogen determinations of casein or non-casein protein fraction would not be reliable, because in processed products whey protein complexes with casein when subjected to heat (Douglas *et al.*, 1981; Elfagm and Wheelock, 1978; Kannon and Jenness, 1961). To determine the amount of whey protein or casein in a processed dairy product, this complex must be broken down, or some other unique property of the proteins must be utilized. A method of determining casein by its ratio of phosphorus to nitrogen has been described (Douglas *et al.*, 1982). This ratio can determine the ratio of casein to whey, assuming 0.85% phosphorus in casein. Further flexibility can be built into the system by using radial immunodiffusion so that the products can be assayed specifically for casein, whey protein or any suspected protein adulterant. Table 4.5 shows a comparison of milk solids-not-fat content calculated from the phosphorus test with respect to formulation contents and Table 4.6 shows a comparison of casein by phosphorus and radial immunodiffusion (RID) determination.

Table 4.5 Comparison of milk solids-not-fat content calculated from the phosphorus test with respect to formulation contents

Sample	% Milk solids-not-fat	
	Estimated from experimental[a] μg P	Intended
Vanilla ice milk	10.06	12.25
Vanilla ice cream	7.88	7.88
Chocolate ice cream	7.27	6.92
Premium ice cream	11.85	11.50

[a]The complete test was run on the samples and the μg P/g of the product determined.

Assuming 0.85% P(μg P/g casein) in casein, and casein is 80% of total protein, and protein comprises 36% of the milk solids-not-fat, the entry given in the table can be calculated.

Source: Douglas *et al.*, 1982 (reproduced with permission).

Table 4.6 Comparison of casein by phosphorus and radial immunodiffusion (RID) determination

Product	% Casein	
	RID	Phosphorus
Coffee creamer	2.63	2.98
Non-fat dry milk	25.85	26.56
Vanilla ice cream 1	2.50	2.33
Vanilla ice cream 2	3.27	3.51
Vanilla ice milk	2.41	2.98
Chocolate ice cream	3.46	2.15
Strawberry ice cream	1.94	2.07

Source: Douglas *et al.*, 1982 (reproduced with permission).

The determination of casein by the phosphorus method can also be applied successfully to sodium caseinate and processed whole milk. Chocolate ice cream, as tested by the RID method is known to give high values because of an interfering ingredient in cocoa.

Addition of dried whey, buttermilk or caseinate to dried skim milk can also be detected by determination of cysteine–cystine (–S–S– complex) and sialic acid (Wolfschoon-Pombo and Pinto, 1985; Rampilli *et al.*, 1985). The cysteine plus cystine content can be estimated by means of modified ninhydrin reaction (De Koning and Van Rooijen, 1971). An upper limit of 86.4 μg SH group/g protein has been established for dried skim milk. Addition of whey or whey protein concentrate causes a linear increase in –SH grp concentration. Calculations determined by regression analysis indicate that 10% whey powder added to dried skim milk would cause the SH

grp/g protein to exceed the upper tolerance limit established for dried skim milk (Hill *et al.*, 1988). Determination of –S–S– content or the ratio cysteine/cystine can be done polarographically (Mrowetz and Thomasow, 1980; Lechner and Klostermeyer, 1981). Both the determinations are recorded in the EC ordinance 625/78 of 30th March 1978. An –S–S– content of >62.5 μg/mg N indicates adulteration of dried skim milk with whey, but the results are masked when other adulterants like caseinate are present simultaneously. This is overcome by using the –S–S– content along with the determination of sialic acid and then making a judgement (Lechner, 1981). A cysteine/cystine ratio higher than three and a sialic acid content higher than 3% are indicative of positive results. A report indicates that any milk sample containing >80 ppm sialic acid should be suspect (Zalazar *et al.*, 1992). Microscopic methods (Loko, 1982), HPLC of indoxyl sulphate (Wolfschoon-Pombo and Klostermeyer, 1986) and gel electrophoresis (Basch *et al.*, 1985) are other approaches.

A turbidimetric method developed by Harland and Ashworth (1947), and further modified by Leighton (1962) claimed to estimate the amount of non-denatured whey protein in non-fat-dry milk (NDM), but has been proved to be inadequate (Basch *et al.*, 1985). An amino acid based method that can detect whey protein concentrate (WPC) at levels greater than 10%, works well with acid or sweet (rennet) whey and is not affected by heat treatment of the skim milk (Greenberg and Dower, 1986). Estimation of whey protein nitrogen (Best, 1988) can give an indication of whey adulteration of skim milk powder (Lechner and Klostermeyer, 1981). Another term used is whey protein index, defined as milligrams of undenatured whey protein nitrogen per gram of dried skim milk powder, which can indicate whey adulteration (Voss and Moltzen, 1973). Qualitative detection of whey solids can be done by measuring ammonia in milk powder by means of a specific electrode (Montana Lampo *et al.*, 1982).

Determination of glycomacropeptide (GMP) by HPLC or spectrophotometry (De Koning *et al.*, 1966) is suggested as an index of rennet whey solids in dried skim milk, however false positive results could arise through the action of bacterial proteinases in the milk before drying. Good results are obtained for detection of rennet whey solids in sweet buttermilk powder, but proved to be less satisfactory when applied to acid buttermilk powder. It did not appear suitable to detect dried acid whey added to dried skim milk (Olieman and Bedem, 1983). Table 4.7 shows the recoveries of weighed amounts of rennet whey powder added to skim milk powder, and analysed by different methods.

A comparison of the results of the HPLC method with the current EU methods shows HPLC to be more sensitive and accurate. Even 0.5% rennet whey powder could be detected and a much better linear relation between the response and the concentration of whey total solids has been obtained.

Dried rennet whey derived from cheesemaking has more water soluble molecules per unit mass than does milk, since the former contains more lactose, sodium, potassium and chloride. Therefore, it seems quite plausible that the freezing point depression of an appropriately diluted dried milk will be greater if it contains added

Table 4.7 Recoveries of weighed amounts of rennet whey powder added to skim milk powder

Sample no.	Added amount of rennet whey (powder) (% mass/mass)	Method of addition	HPLC method		EU method			
			Rennet whey total solids found (% mass/mass)	Recovery (% mass/mass)	Protein (N×6.38) (% mass/mass)	Sialic acid (μg g^{-1})	Rennet whey total solids calculated (% mass/mass)	Recovery (% mass/mass)
A1	0.5	dry	0.57	104	35.9	48	−0.28	
A2	1.0	dry	1.02	102	35.8	47	−0.27	
A3	1.5	dry	1.42	95	35.8	52	−0.23	
A4	2.0	dry	1.98	99	35.35	56	1.05	
A5	3.0	dry	2.86	95	35.5	58	1.13	
A6	5.0	dry	4.92	98	35.1	68	2.44	
B1	2.7	wet	2.60	96	35.5	61	1.40	85
B2	5.1	wet	5.19	102	34.9	80	3.80	94
B3	10.2	wet	10.39	102	33.8	117	7.70	84

Source: Olieman and Van den Bedem, 1983 (reproduced with permission).

Table 4.8 Fat and moisture content (*F* and *M*, respectively, in %), FPD (θ, in m °C) and corrected FPD (θ*, in m °C) from whole and skim milk powders with increasing amounts of added whey powder

Milk powder		% Added whey powder					
no.		0	5	10	20	30	50
1	θ	534	550	564	594	626	686
	F	1.80	1.76	1.72	1.64	1.56	1.40
	M	5.40	5.31	5.22	5.04	4.86	4.50
	θ*	584	599	613	640	671	727
2	θ	542	558	571	604	627	689
	F	1.00	1.00	1.00	1.00	1.00	1.00
	M	3.70	3.69	3.69	3.68	3.67	3.65
	θ*	575	591	604	637	660	722
3	θ	542	558	–	599	630	688
	F	1.40	1.38	–	1.32	1.28	1.20
	M	3.70	3.69	–	3.68	3.67	3.65
	θ*	577	593	–	633	664	721
4	θ	410	430	450	495	543	624
	F	25.00	23.80	22.60	20.20	17.80	13.00
	M	4.30	4.27	4.23	4.16	4.09	3.95
	θ*	575	589	602	633	668	722
5	θ	393	419	440	484	527	618
	F	27.10	25.80	24.49	21.88	19.27	14.05
	M	5.26	5.15	5.04	4.83	4.61	4.18
	θ*	576	594	608	636	664	724

Source: Castaneda *et al.*, 1987 (reproduced with permission).

rennet whey solids. A cryoscope method based on this principle can detect and estimate rennet total solids in whole and skim milk powders. A regression equation giving the percentage of added whey (% AW) with three other independent variables is given as:

$$\%\ \mathrm{AW} = a + b\theta + cF + dM \qquad [4.3]$$

where θ is the freezing point depression, *F* and *M* are the fat and moisture contents respectively and *a*, *b*, *c* and *d* are constants. The value of *a* has been found to be −199.2335, *b* to be 0.3444, *c* to be 1.8279 and *d* to be 2.5694. The equation has been simplified for plotting purposes as:

$$\%\ \mathrm{AW} = a + b(\theta + f_{\mathrm{FM}}) \qquad [4.4]$$

where f_{FM} is the correction factor for fat and moisture to be added to θ and equal to $f_{FM}=cF/b+dM/b=5.3075F+7.4605M$.

The corrected freezing point depression, FPD, $\theta^*=\theta+f_{FM}$ can convert two groups of data into one. Table 4.8 shows fat and moisture content, FPD and corrected FPD from whole and skim milk powders with increasing amounts of added whey powder. Analysis of the data in Table 4.8 showed an error of 2.5% between the calculated and actual amounts of whey solids. This error is ascribed to the natural variation in the composition of the milk powders. It can be concluded that cryoscopy can detect and quantify >2.5% added total whey solids. This method is easy, quick and cheap since it is based on three simple determinations. However, the method is not specific and any water soluble foreign material added to milk powder can give false positive results. Also, mixtures of milk powder with demineralized or delactolized whey powder or milk powder from neutralized acid whey cannot be detected (Castaneda *et al.*, 1987).

All of these methods require substantial time for either preparation or analysis. Infrared spectroscopy has been widely used to measure components in mixtures quantitatively. It is rapid, non-destructive and does not require components to be separated before measurement. The increased signal-to-noise ratio and computing ability achievable with commercially available Fourier transform infrared (FTIR) instruments have overcome the major limitations once associated with infrared methods. Proteins have three characteristic absorbances in the mid-infrared spectrum. Two of these, the amide I (about 1600 cm^{-1} to 1700 cm^{-1}) and the amide III (about 1200 cm^{-1} to 1400 cm^{-1}) absorbance bands are sensitive to polypeptide backbone conformation and might be able to distinguish between proteins (Nyden *et al.*, 1988). The amide I band is more intense, but it overlaps with an intense water deformation band at 1645 cm^{-1}. The amide III band, although less intense is not overlapped by water absorptions. This band has been used as a tool to detect adulteration of NDM with WPC. Figure 4.1 shows a typical spectrum of NDM and WPC in the region of 1400 cm^{-1} to 1200 cm^{-1}. After various computations, predicted versus true concentrations for the 135 blends samples have showed a correlation coefficient, $r>0.99$ (Mendenhall and Brown, 1991).

4.4 Reconstituted milk

Different methods for detecting added dried milk in liquid milk by identification of heat-denatured proteins have been attempted. Methods based on dye binding (amido black and orange G) were found to be unsuitable owing to the difference in dye binding by native and denatured proteins. Gel electrophoresis, which can be used to identify complexes formed by interaction of K-casein, α-lactalbumin and β-lactoglobulin during heating also did not reveal any differences from raw milk and were of no help (Potgieter, 1982). However the ratio of β-casein to α-lactalbumin can detect adulteration of fresh milk with 25% dry milk (Chen and Ji-Hong, 1992).

Table 4.9 Variation in PRS values in fluid milks among 22 collaborating laboratories

Sample no.	1	2	3	4	5	6
min	1.84	2.08	2.68	2.72	5.76	2.72
max	4.40	4.40	6.08	4.92	10.40	6.00
avg	2.48	2.99	4.03	3.42	7.73	3.64

Sample 1: mixture of market milks.
Sample 2: 5% reconstituted milk—high heat milk powder with market milk.
Sample 3: 10% reconstituted milk—high heat milk powder with market milk.
Sample 4: 10% reconstituted milk—high heat milk powder with market milk.
Sample 5: 20% reconstituted milk—low heat milk powder with market milk.
Sample 6: 10% reconstituted milk—low heat milk powder with market milk.
Source: Junker, 1960.

From freeze-fracturing and electron microscopic techniques, reconstituted and heat treated dried milk has been seen to contain typical aggregates of >500 nm diameter, which are not seen in pure liquid milk. This method has been suggested for routine detection of the presence of >20% dried milk (Resmini and Volonterio, 1974). Fractionation of whey proteins can grade milk powder according to the severity of heat treatment and can also detect adulteration of fresh milk with milk powder. This method is based on the fact that in milk previously heated, before precipitation of casein by acidification to pH 4.6, a part of the whey protein is carried down with casein (Babad *et al.*, 1951). Addition of reconstituted dry milk to raw or pasteurized milk can be detected by colour changes with resazurin (Belle and Caspar, 1959; Toubol, 1960). The value of protein reducing substances (PRS) in cow's milk as an indicator of adulteration of whole milk with reconstituted dried skim milk has been assessed (Merritt *et al.*, 1956), but the previous heat treatment given to whole milk (UHT or aseptic processing) eclipses the true values and PRS cannot therefore be directly applied as an indicator of the presence of reconstituted dried milk (Matsumoto *et al.*, 1974), however, it could be a good first action or screening test. It is suggested that the average PRS value of a raw, pasteurized or homogenized milk is well below three, with possibly a few samples running over four. A sample showing a PRS value of 4.5 to 5.0 would therefore be suspicious and a sample having a value of 5.0 or above would definitely be considered to contain added milk powder (Junker, 1960). A collaborative study of six samples of fluid milks having varying amounts of reconstituted milk powder and subjected to varying heat treatments was carried out in 22 different laboratories where the PRS were determined. A summary of the findings is presented in Table 4.9.

It is thought that if freezing point and density are normal, a high content of nitrate and of neutralizing substances such as sodium bicarbonate in milk is indicative of the presence of reconstituted milk (Alosi *et al.*, 1982; Niola *et al.*, 1982). The Italian legal limit of nitrate in milk is 2 mg kg^{-1}. It is believed that this value is too high and that it should be brought down to 1 mg kg^{-1}. Nitrate could be converted to nitrite and analysed by chemiluminescence (Doerr *et al.*, 1982). This is a sensitive method which

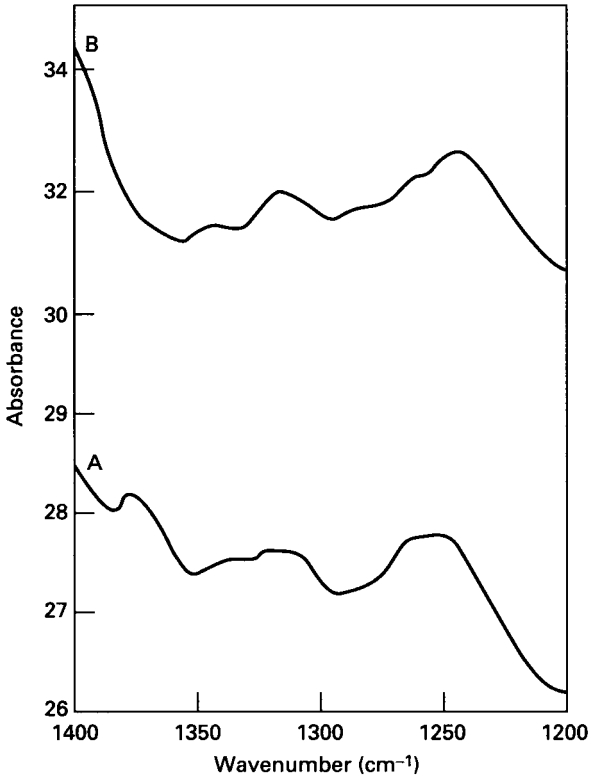


Figure 4.1 A typical spectrum of: (A) whey protein concentrate, (B) non-fat dry milk in the wavenumber region 1400 cm $^{-1}$ to 1200 cm^{-1}. (Source: Mendenhall and Brown, 1991, reproduced with permission)

requires no sample preparation and is also free from other interferences. The cryoscopic index is not suitable for detecting addition of reconstituted milk; the nitrate concentration is said to be of use in such detections (Sipio and Trulli, 1989).

4.5 Adulteration in milk and other dairy products

The high manganese (Mn) content of non-milk components used in calf milk replacer serves as an index to detect the adulteration of milk with milk-based calf replacer. Analysis of 55 milk samples averaged 0.0211 mg Mn l^{-1} while that of calf milk replacer was around 13 mg kg^{-1}. The method could reliably detect 2% of reconstituted calf milk replacer in milk (Vannini, 1984).

Extension of pure milk with filled milk (milk in which vegetable fat is substituted for milk fat) can be determined by nitrogen content of soluble whey proteins after coagulation of casein with 10% acetic acid. A steady decrease is noted after addition of increasing levels of filled milk to pure milk. Adulteration at 30% can be detected with

certainty and sometimes as little as 10% is possible (Cordova and Martinez, 1955).

Raw milk in pasteurized milk can be detected using phosphatase activity as a screening method, even at 0.2% levels (International Dairy Federation, 1987). Added whey to cream can be determined by decreased casein content, which can be estimated by formol titration after separation with 50% magnesium sulphate and subsequent warming (Effern, 1943).

Distinction of mozzarella cheese from imitation mozzarella cheese made from calcium caseinate can be made by differential scanning calorimetry. The enthalpy of the milk fat melting transition at 18 °C decreases with increasing caseinate concentration. Scanning electron microscope (SEM) studies have shown an agglomeration of lipids in imitation samples, whereas natural cheese has a uniform dispersion of fat globules. Addition of caseinate apparently affects fat crystallization, leading to an enthalpy reduction. This differentiation is not possible by techniques such as electrophoresis or atomic absorption (Tunick *et al.*, 1989). In one particular instance, a white powder and white viscous milky liquid suspected of being mixed with water and used to adulterate raw milk as substitutes for milk SNF (solids-not-fat) and fat were found to be 94.96% lactose monohydrate and an oil-in-water (O/W) type of emulsified fat used commercially as an ingredient for breadmaking, respectively. Physical and chemical characteristics of the emulsion were found to be: water, 38.6%; melting point of the fat, 36.1 °C; and butyric acid value, 0 (Iwaida *et al.*, 1971).

Glucose, cane sugar, urea, ammonium sulphate and other substances have been encountered as additives (Mittal and Roy, 1976a, 1976b) for the purpose of masking the effects of dilution with water. Even a sensitive test like freezing point of milk fails to unmask this adulteration (Dharmarajan *et al.*, 1953). These substances can be detected by using glucose oxidase and redox indicators, or by using various instrumental techniques (Madrid Vicente, 1972; Reineccius *et al.*, 1970; Ramachandra *et al.*, 1955). Simple methods arising out of changes in various physicochemical properties can also form the basis of such detections. Electrical conductance has been evaluated, but the increase observed on addition of glucose to milk was not proportional to the concentration. Furthermore acidity developments during subsequent storage masked the results. Density differences have been noted on addition of glucose, urea and ammonium sulphate, but again the increase in density was not exactly proportional to the molar concentration of the added solute. Also, rise in milk density due to glucose was greater than that due to urea and lower than that due to ammonium sulphate (Mittal and Roy, 1976a, 1976b), and was not in molar proportions. Viscosity monitoring also proved to be futile because of changes associated with bacterial degradation and acidity development during storage (Roy and Mittal, 1977). A platform test based on a rapid colorimetric method to estimate extraneous glucose in milk has been developed (Roy and Mittal, 1977). Quantitative determination of sugar is rather cumbersome. After taking a direct polarimetric reading, the sugar has to be hydrolysed to obtain the invert sugar reading and the amount of sucrose is calculated from the two readings. The differences in the R_f values

of the sugars makes paper chromatography a simple and sensitive technique for detecting added sugars. Sugar levels as low as 0.75% can be indicated on the chromatogram.

Addition of sugar to milk is a common problem in the dairy industry. Addition of 0.2% sugar to milk increases the lactometer reading by one degree at 60 °F. A rapid, simple and accurate method, which can be used as a platform test to detect up to 0.05% added sugar has been reported. Sugar is hydrolysed to glucose and fructose by the enzyme invertase and the resultant glucose is estimated enzymically using glucose oxidase– peroxidase strip (Mal *et al.*, 1988). The strip shows changes in colour from sky blue to green to brown, and indicates the presence of added sugar in milk (Pal *et al.*, 1989). If the milk is preserved by formalin, the common resorcinol test needs some modifications, but in this method there is no interference from formalin.

Addition of colouring matter like annato along with water may be suspected when specific gravity, fat content, SNF and total solids of a milk sample would decrease without any change in the appearance of milk. Addition of common salt and water can be detected by a simple test. Sodium chloride can be added to milk up to 0.4% without affecting its normal flavour, odour and taste, while at the same time 13.06% water can be added to milk and yet its specific gravity can be maintained at almost normal.

Milk controls for detecting the presence of ammonia were sought some years ago (Shidlovskaya *et al.*, 1974), since it is often used to conceal the development of acidity. Preservatives like sodium bicarbonate or penicillin are added to milk to prolong its keeping quality. Non-acceptance of milks positive to clot-on-boiling (COB) prompts widespread addition of sodium bicarbonate to milk. Further, addition of sodium bicarbonate to the extent of 0.3% in milk may be of advantage to the vendor by increasing the lactometer reading by 3 degrees, or 9.9% water can be added without affecting the specific gravity (Mishra and Dehury, 1974).

4.6 Other fats in milk fat, butter or ghee

Milk fat in the form of butter or ghee is often extended by other fats and this is extensively discussed in the scientific literature (Jorge and Osvaldo, 1955). Large price difference between butter fat and substitutes prompts this adulteration. Vegetable oils, mainly cottonseed, and beef tallow are common substitutes for butter fat in Egypt. Amongst a total of 27 052 samples of butter and ghee analysed over a period of 13 years, 4472 contained a cheaper adulterant fat at an estimated average level of 16% (Singh *et al.*, 1975). Many importing countries have set their own quality standards to judge the authenticity of butter. The detections are generally based on composition and structure of triglycerides (Kuksis and McCarthy, 1964), fatty acids (Luf, 1988; Ulberth, 1994), composition of unsaponifiable matter, that is, sterols, sterol esters, hydrocarbons, tocopherols (Windham, 1957) and carbonyl compounds (Farag *et al.*, 1982, 1983; Hallabo and El-Nikeety, 1987) or physical properties measured by molecular refraction (Chatterji and Chandra, 1957), differential thermal analysis (Lambelet

et al., 1980; Amelotti *et al.*, 1983), differential scanning calorimetry or IR spectroscopy (Juorez, 1980). A statistical linear model has been developed to characterize pure milk fat and its sensitivity to adulterants. The model is based on triglyceride analysis by GLC. A discriminant function, F_1, based on triglycerides with C numbers 34, 36, 38, 40, 42 and 44 could successfully allow correct classification of 100% and 96.9% of samples adulterated with 10% and 5% non-milk fats (Villanueva *et al.*, 1988). Multiple linear regression analysis in which the percentages of triacylglycerols, C_{40}, C_{42} and C_{44} are inserted can also certify the milk fat purity. For instance, if $11.1747 \times C_{40} - 14.3604 \times C_{42} + 13.6548 \times C_{44} = R = 93.15$ to 108.60, then the butter fat could be taken as pure. This formula sometimes is unable to detect a 15% admixture of a foreign fat, and hence to overcome this, a formula of the type, $R = a_1C_1 + a_2C_2 + a_3C_3 + \ldots$ has been recommended for detection of different fats. For instance, palm oil can be detected by using a formula, $9.8271 \times C_{32} + 0.9229 \times C_{36} + 2.4431 \times C_{44} + 1.5861 \times C_{46} - 4.8307 \times C_{48} + 7.2032 \times C_{50} = 95.83$ to 112.05. Similar equations are available for other fats (Precht, 1992a). These methods also allow quantitative detection of foreign fats in milk fat (Precht, 1992b). However, these need to be used cautiously, since the triglyceride composition varies with season and region (Bornaz *et al.*, 1992). Winter milk fat is reported to contain more short-to-middle chain saturated triglycerides than summer milk fat (Hinrichs *et al.*, 1992). An excellent review for the analysis of triglycerides in milk fat has been published recently (Lipp, 1995).

Attempts have been made to detect <10% of vegetable or animal fat in butter fat by measuring intensity of reflected ultraviolet light, but were unsuccessful (Clercq, 1937). The mole percentage of butyric acid (Keeney, 1956; Anglin and Mahon, 1956) and fatty acid composition of Reichert-Meissel and Polenske fractions (Klayder and Fine, 1956, 1957) can be used to detect adulteration of butter with other fats (Laruelle *et al.*, 1976). Various chromatographic techniques (Smith, 1961; Farag *et al.*, 1980) such as HPLC (Pretel, 1984), GC (Guyot and Piraux, 1965), and GLC in conjunction with IR spectroscopy (percentage transmission at 967 cm^{-1} and 948 cm^{-1}, denoted as T_{967} and T_{948} and assigned to isolated *trans* and conjugated *cis–trans* isomers) can reliably distinguish butter and its adulterant substitute fats (Valencia Diaz *et al.*, 1975). For instance, *trans*-unsaturated fatty acids, as determined by IR spectroscopy at 967 nm increase from 5.3% to 14.3%, as the level of adulteration of butter with cottonseed oil increases from 0 to 30%. A correlation between the mean percentage content of *trans* isomers and absorption at 232 μm and iodine number has been established (Galoppini and Lotti, 1965) and could be used to detect adulteration of butter fat.

The *trans*-unsaturated fatty acids occur naturally in milk fat (Parodi and Dunstan, 1971), but not in other fats unless they are exposed to catalytic hydrogenation (Gurr, 1983). This difference in fat composition could be used to an advantage to measure the level of *trans*-unsaturated fatty acids as an index of milk fat adulteration. These results must be cautiously used, since *trans*-unsaturated fatty acid content in butter fat is influenced by the type of the feed (Parodi, 1970; Gray, 1973) such as unsaturation in the diet of the cow (Younes and Soliman, 1988). The differences in *trans*-unsaturation

levels in milk fat are also related to the extent to which the unsaturated fatty acids are hydrogenated in the rumen. Generally it is possible that the process of hydrogenation and the production of *trans* acids by the rumen microflora could contain a reversible hydrogenation–dehydrogenation system that could produce a *trans* acid from the hydrogenated or partially hydrogenated derivative of a *cis* acid (Hartman *et al.*, 1954; Shorland *et al.*, 1957; Tove, 1960; De Man, 1961; Hawke, 1963; Munford *et al.*, 1964).

The official Italian methods of testing butter for adulteration are based on the ratio of fatty acids. The indices for unadulterated butter, along with their limits are (Gorgano, 1979):

- C_4/C_6+C_8 : 0.7–1.7
- C_{12}/C_{10} : 0.9–1.3
- C_{14}/C_{12} : >2.70
- C_{18} unsaturated/ C_{18} saturated : 2–3

The Japanese official methods for milk fat are based on GLC of sterols, butyrate and caproate. Their study concludes that C_4/C_6 ratio can detect addition of prepared fat containing beef tallow and coconut oil with butyrate incorporated by transesterification (Iwaida *et al.*, 1979). However, seasonal changes, partly related to feeding differences (stall feeding vs. grazing) alter the ratios between fatty acids. The changes are more marked in the higher and medium chain acids than in the shorter chain acids. Contents of C_{10}, C_{12}, C_{14} and C_{16} acids become higher in the stall feeding season than in the grazing season; those of C_{18}, $C_{18:1}$ and $C_{18:3}$ become lower (Huyghebaert and Hendrickx, 1971). A recent analysis has shown that seasonal variations and regional differences are much smaller than the differences between triglyceride composition of milk fat and its adulterant fats and oils (Luf *et al.*, 1987a). The ratios of lauric:capric, myristic:caproic, myristic:lauric, and contents of $C_{10:1}$ and $C_{14:1}+C_{15:1}$, RM (Reichert-Meissel), Polenske, xylol and Crismer values are compared with a reference standard to detect adulterated butter samples (Garcia Olmedo and Gastanaduy, 1971).

The following adulterants of milk are briefly considered:

1 Vegetable fats
2 Fats of animal/marine origin
3 Other adulterants

Table 4.10 Values of six selected fatty acid ratios and two individual fatty acids for evaluating the purity of a milk fat sample

Ratio	Mean	Coefficient of variance (%)	r^2[a]	Relative maximum sensitivity (%)
$C_4/C_{18:1}$	0.1593	8.16	0.748	6.1
$C_4+C_6/C_{18:1}$	0.2617	9.25	0.681	7.8
$C_4+C_8/C_{18:1}$	0.2154	8.96	0.677	7.6
$C_4+C_{10}/C_{18:1}$	0.2854	11.04	0.570	9.3
$C_{10}/C_{12}+C_{16}+C_{18:1}$	5.34	9.59	0.237	14.6
$C_{18:1}/C_{14}$	2.16	13.94	0.418	9.2
C_4 % of total	4.09	6.06	0.551	7.5
$C_{18:1}$ % of total	25.94	7.75	0.499	10.1

[a] r^2 is the degree of correlation between the various ratios and percentage vegetable fat.

Source: Fox *et al.*, 1988 (reproduced with permission).

4.6.1 Vegetable fats

The fatty acid composition of butter fat (Iverson and Sheppard, 1977; Iwaida *et al.*, 1979; Jensen *et al.*, 1967; Toppino *et al.*, 1982), triglycerides (Guyot, 1978; Kato, 1971; Timms, 1980) and monoglycerides assists in the detection not only of vegetable fat, but also animal fats (Younes and Soliman, 1986; Soliman and Younes, 1986). Accommodating the differences between breeds, climatic conditions and seasonal variations, a ratio of 0.82–0.96:1.0 for lauric:capric acid has been considered suitable for detection of adulteration with vegetable fats (Chernev *et al.*, 1979). Addition of 10% cocoa butter, palm oil or rapeseed oil and 5% soybean oil in butter can be detected from the ratio of long chain (C_{50}–C_{54}) to medium chain triglycerides (Luf *et al.*, 1987a, b). In products like cheese, adulteration of milk fat with pure and partially hydrogenated soybean oils, and other similar vegetable oils such as cottonseed oil and corn oil can be detected by GLC analysis of potassium salts of fatty acids, obtained after saponification of the fat. A method that converts fatty acids to potassium salts and then analyses them on a gas chromatograph eliminates a major problem, that is, sample losses due to vaporization. Table 4.10 gives the values of six selected fatty acid ratios and two individual fatty acids used to evaluate the purity of a milk fat sample. Each ratio has been calculated from the collected data and subjected to regression analysis to determine the r^2 value for increasing vegetable fat concentration in milk fat using that ratio.

The $C_4/C_{18:1}$ ratio was the most sensitive and could detect the said adulterants at the $<10\%$ level. Figure 4.2 shows the effect of addition of mildly hydrogenated fat on the ratio of $C_4/C_{18:1}$. This test is not suitable for detecting coconut oil (Fox *et al.*, 1988, 1989). This method is believed to be appropriate for use as a screening method

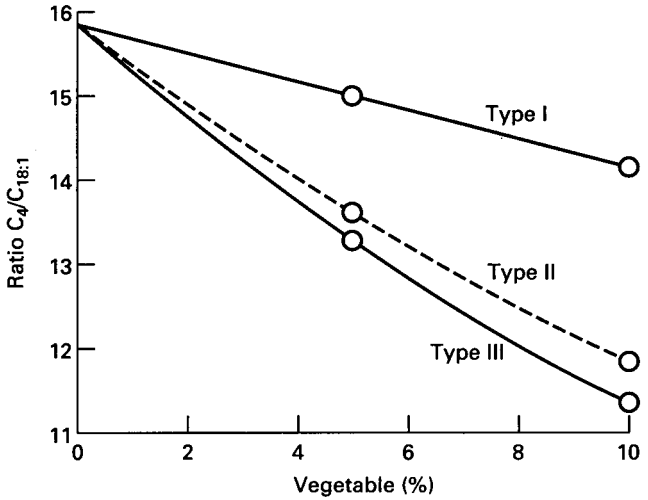


Figure 4.2 Response of the ratio C_4: $C_{18:1}$ in fat from Mozzarella cheese of the addition of vegetable fat of increasing hydrogenation. Type 1 is pure soybean oil, iodine value=130.6. Type 2 is partially hydrogenated soybean oil, iodine value=94.3. Type 3 is partially hydrogenated soybean oil, iodine value=68.6. (Source: Fox *et al.*, 1988, reproduced with permission)

where multiple samples are routinely tested. The sensitivity of this test is limited by the normal variability in the value of the ratio in milk fat due to factors such as season, stage of lactation, type of feed and the breed of the cow (Boatman *et al.*, 1965; Gray, 1973; Hutton *et al.*, 1969; Jensen *et al.*, 1962). Fat is generally extracted from the dairy sample quantitatively by Mojonner's method. Fatty acids in the lipids recovered from only the first extraction could be used to establish the authenticity of a suspected sample. This makes the method more rapid and less expensive than the classical Mojonner's method (Duthie *et al.*, 1988).

Phulwara butter obtained from the north Indian tree *Madhuca butyracea* is a known adulterant of ghee. The fat is usually extracted as a cream-like paste by crushing whole seeds or kernels, the yield of fat being 42–45%. The yellow coloured fat is hard, similar in appearance to ghee and is cheaper. The Reichert-Meissel value (Tamraekar, 1977) and spectrometric methods have been reported to track this adulteration. It can also be identified at >5% levels by TLC analysis of an unsaponifiable fraction on silica gel using chloroform/acetone (95:5) for development (SenGupta and Sil, 1983). With direct spotting, ghee is known to give two pink spots, whereas phulwara butter shows an additional spot. The differences in the sterol composition of butter fat and adulterant vegetable fats (Sebastian and Rao, 1974) have formed the basis for detecting adulterants, even at low levels (<5%), either as such, or as derivatives such as digitonide (Chinese National Standards, 1986) or as silicate ether by paper chromatography (Sulser and Hogl, 1957; Peereboom and Roos, 1960), TLC (Peereboom and

Beekes, 1965; Ramamurthy *et al.*, 1967) or GLC (Panetsos *et al.*, 1974). Butter fat sterols are >99% cholesterol (Torre Boronat *et al.*, 1977), 0.4–1.2% campesterol and ergosterol (Guyot and Sadini, 1974), whereas a vegetable oil like cottonseed contains basically β-sitosterol along with γ-sitosterol and stigmasterol. β-Sitosterol is not present in butter fat (Homberg and Bielefeld, 1980). Adulteration of butter oils with vegetable fats is therefore indicated by a decrease in cholesterol and an increase in stigmasterol and β-sitosterol concentrations (Hallabo and El-Nikeety, 1987). β-Sitosterol content in butter fat increases from 0% to 4.54% for addition of 0–6% cottonseed oil. Processing operations such as bleaching with bleaching earths or activated carbon, deodorization by steam injection or hydrogenation decrease the content of sterols, primarily due to degradation of cholesterol. However, refining does not interfere with the phytosterol test for the detection of adulteration of butter oil (Huyghebaert and Moor, 1974). An animal fat with a similar sterol composition to butter fat, for example, tallow, cannot be differentiated on this basis (Younes and Soliman, 1987).

Vegetable oils such as >2% corn oil or rice oil, >5% cacao butter, rapeseed, sesame, soybean, linseed or peanut oils, >20% coconut or palm oil and >35% palm kernel oil can all be determined from phytosterol contents and their melting points (Tsugo *et al.*, 1965; Cannon, 1955, 1956; Vitagliano and D'Ambrosio, 1957). Cholesterol acetate has a melting point (m.p.) of 125 °C, whereas on mixing with phytosterol acetate at 6.25%, 12.5%, 25% and 50% level the m.p. rises respectively by 2 °C, 5 °C, 14 °C and 18 °C (Cannon, 1957). The sensitivity of the method depends on the amount of phytosterol present in proportion to cholesterol. The kind of phytosterol present determines the slope of the melting curve as a function of cholesterol:phytosterol ratio. Sunflower seed oil shows steep slopes for total and free sterols but the curve is relatively flat for bound sterols. The curve for total sterols in sunflower seed oil reaches a maximum at certain mixtures of sterol acetates showing higher melting points than either of the pure substances. It appears that the bound sterol or sterol ester method appears unsuitable for sunflower oil, but works very well for coconut oil and is therefore recommended.

The ratios of total hydrocarbons to total sterols (TH/TS) in the unsaponifiable matter are very different for lard, margarine and ghee. Lard and margarine contain 19.9 and 33.7 times as much hydrocarbon as pure cow ghee, and 10.4 and 17.5 times as much hydrocarbon as pure buffalo ghee. Table 4.11 shows the effect of adulteration of cow ghee and buffalo ghee by lard or margarine on the ratio of TH/TS. A simple correlation coefficient between percentage adulterant and concentration of various hydrocarbons is found to be highly significant at the 0.1% level. It is possible to determine the extent of admixture of lard or margarine with either cow or buffalo ghee by applying a simple regression equation for each unsaponifiable component. By analysing the unsaponifiable component of the lipid samples the adulteration levels can be easily determined by the equation

Table 4.11 Effect of adulteration of cow ghee and buffalo ghee by lard and margarine on the ratio of total hydrocarbons to total sterols (TH/TS)

Extent of admixture	Lard		Margarine	
	Lard	TH/TS	Margarine	TH/TS
Cow ghee				
100	0	0.13:1	0	0.13:1
95	5	0.17:1	5	0.18:1
90	10	0.21:1	10	0.22:1
85	15	0.28:1	15	0.30:1
80	20	0.31:1	20	0.38:1
75	25	0.37:1	25	0.40:1
70	30	0.41:1	30	0.48:1
0	100	2.59:1	100	4.38:1
Buffalo ghee				
100	0	0.25:1	0	0.25:1
95	5	0.29:1	5	0.30:1
90	10	0.34:1	10	0.37:1
85	15	0.39:1	15	0.42:1
80	20	0.44:1	20	0.52:1
75	25	0.50:1	25	0.56:1
70	30	0.50:1	30	0.67:1
0	100	2.60:1	100	4.38:1

Source: Farag *et al.*, 1982 (reproduced with permission).

$$Y=A+BX, \qquad [4.5]$$

where Y=adulteration ratio; A=a constant value, the intercept of the regression line; B=regression coefficient and X=the concentration of the individual samples (Table 4.12). The compounds intended to be used as indicator of adulteration should only be confined to the adulterants, and not to the pure lipids or to substances occurring in the adulterants in amounts higher than in pure lipids. Compounds present in higher amounts in pure lipids than in the adulterants should not be used as indicators of adulteration.

Compounds characteristic of the adulterants such as sesamin and sesamol in sesame oil can also serve as indicators of adulteration, and can be judged by various colour reactions characteristic of the compound present in the adulterant (Isidoro and Pavolini, 1950). The tocopherol content of butter fat has also been implicated as an index of adulteration with vegetable oils (Keeney *et al.*, 1971; Mahon *et al.*, 1955; Markuze, 1962; Anglin *et al.*, 1955). An exception amongst vegetable oils is coconut oil (Mahon *et al.*, 1955; Windham, 1957).

A presumptive crystallization of the fat and properties of these crystals serves to

Table 4.12 Linear regression equations for the adulteration ratios (Y) of lard and margarine mixed with pure ghee and individual unsaponifiables (X)

Unsaponifiable component	Mixing with lard	Mixing with margarine
	Cow	
C_{28}	$Y=100.41-96.33\,X$	$Y=732.16+718.28\,X$
C_{29}	$Y=-17.52+1.72\,X$	$Y=166.51-16.53\,X$
C_{30}	—	$Y=-4.44+56.07\,X$
C_{31}	$Y=-12.24+27.87\,X$	$Y=-6.59+14.66\,X$
C_{32}	—	$Y=-0.72+1.56\,X$
Cholesterol	$Y=145.64-1.67$ X	$Y=101.28-1.16\,X$
β-Sitosterol	$Y=111.59-116.96\,X$	$Y=-6.20+6.1\,X$
TH/TS	$Y=4.66+37.52\,X$	$Y=8.44+21.25\,X$
	Buffalo	
C_{28}	$Y=99.73-30\,X$	$Y=147.29-45.08\,X$
C_{29}	$Y=-30.69+1.92\,X$	$Y=133.60-9.05\,X$
C_{30}	$Y=-18.93+29.61\,X$	$Y=2.38+57.59\,X$
C_{31}	—	$Y=-4.63+14.38\,X$
C_{32}	—	$Y=-0.82+1.56\,X$
Cholesterol	$Y=155.82-1.87\,X$	$Y=101.26-1.29\,X$
β-Sitosterol	$Y=105.38-16.19\,X$	$Y=-3.75+6.02\,X$
TH/TS	$Y=-0.57+39.54\,X$	$Y=4.89+22.08\,X$

Source: Farag *et al.*, 1982 (reproduced with permission).

identify about 5% adulteration of milk fat with vegetable oils like corn, peanut, cottonseed, coconut, refined coconut as well as beef fat (Keeney, 1953). Differential scanning calorimeter can be used to determine 5% coconut fat or 5% hydrogenated vegetable fat in milk fat but not 5% tallow and lard in butter fat (Antila *et al.*, 1965). Differential thermal analysis can detect coconut oil and palm kernel oil in butter at >5%, but it is not possible to differentiate between coconut oil and palm kernel oil. Palm oil and beef tallow can be differentiated at 5% and with clear distinction at 10% levels, but discrimination between these is not possible (Niiya *et al.*, 1970). A method based on the separation of suspected butter fat into alcohol soluble and alcohol insoluble triglycerides at 20 °C is reported in literature (Bhalerao and Kummerow, 1956). This method causes the concentration of the adulterant to increase in one of the fractions. Measurement of refractive index of the two fractions can give some indication of adulteration by vegetable fats with a fair degree of accuracy. Valenta and Crismer values also detect adulteration of butter fat by vegetable fats (Antonini and Creach, 1948). The Wollny number (Vitagliano and D'Ambrosio, 1956/1957) has been shown to be relevant in many cases.

4.6.2 Fats of animal or marine origin

The detection of animal body fat in butter fat is difficult as the resulting mass has physical and chemical characteristics which fall within the normal range for pure ghee. Further it is found that ghee obtained from the milk of buffaloes fed cotton seeds acquires characteristics similar to those of samples adulterated with animal body fats. The usual physicochemical characteristics such as the Reichert–Meissel value, Polenske value and BR index (butyro refractometer) are of limited use in the detection of animal body fats in butter fat or ghee because of the wide variation. Dastur (1955) even proposed a special standard for cotton tract ghee. While phytosterols can detect vegetable fats, animal fats in small amounts cannot be easily detected. Several methods have been proposed from time to time, but each one had its limitations. Some of these methods along with their merits and demerits are briefly reviewed in the following pages.

4.6.2.1 Method based on the solubility of ghee

This method depends on the solubility of ghee in a mixture of acetic acid and ethanol in a 3:4 ratio, kept at 30 °C for 30 min. The formation of a precipitate indicates adulteration with animal fat (Venkatachalam, 1937). The main drawback of this test is that the ratio of the two solvents has to be varied depending on the type of ghee. Also the solubility test is not dependable for testing the purity of ghee obtained from animals fed on cottonseed products.

4.6.2.2 Grossfield number

Grossfield number or butyric acid number is a measure of butyric acid and has a very narrow range of 21–25. It can be used in place of RM values. This method cannot detect animal body fats at levels of 10% or less.

4.6.2.3 Critical temperature of dissolution

The critical temperature of dissolution has been proposed as a means of detecting animal fat in ghee. The range for pure ghee is 49.5–53.5 °C, and that for mutton tallow between 70 °C and 73 °C. However, in some cases the critical dissolution temperature has been found to be lower than the minimum limit of 49.5 and that for the samples of ghee from cotton tract between 56 °C and 58 °C.

4.6.2.4 Urea fractionation

The property of urea which enables it to form crystalline complexes with straight chain compounds like fatty acids on the basis of both the chain length and unsaturation

has been successfully used to detect and estimate adulteration of oils, particularly of butter fat (Tawde and Magar, 1957). The ratio of fatty acids precipitated by urea to those not precipitated is appreciably lower for butter fat than for other fats (Holasek and Ibrahim, 1953). Methods based on urea fractionation followed by determining the refractive indices of the fractions have been described (Shipe, 1955). A linear relationship generally exists between the percentage adulterant and refractive index. Arachidonic acid can be concentrated by urea fractionation, which can subsequently be determined by ultraviolet spectrophotometry (Gallardo and Sameth, 1962).

4.6.2.5 Fluorescence in ghee

The observation that almost all the adulterants of ghee produce a blue fluorescence while pure ghee shows a pale green fluorescence serves to distinguish ghee and its adulterants. However, pure ghee from cotton tract also shows blue fluorescence and hence it has a limitation (Achaya and Banerjee, 1945). A spectral method is based on locating three peaks in the triene absorption range of animal fat compared with two peaks seen for butter fat. A similar differential spectroscopy procedure comprises dissolving fats (one pure and other suspected) in carbon tetrachloride, and placing them in matched cells. When exposed to beams of infrared in a spectrophotometer, slight differences in the spectra are evident as deviations (Anon, 1959).

4.6.2.6 Methods based on hydroxamic acid index

This method, suggested by Nelson (1954), is based on the fact that fats from animal milk form water soluble hydroxamic acid–iron complexes of short chain fatty esters and impart colour to the aqueous layer, while animal fats form water-insoluble complexes and give no such colour reaction. The hydroxamic acid index (HAI) is obtained by measuring the colour with a photoelectric colorimeter. For pure butter fat, HAI is generally between 9.9 and 12.57 with decreasing values being obtained on adulteration (Bassette and Keeny, 1956).This method can however detect animal body fat in ghee only at or above the 15% level (Pruthi and Sachday, 1968).

4.6.2.7 Chromatographic techniques

Paper chromatographic techniques to separate and identify mixtures of glycerides using various solvent systems have been reported (Kaufmann, 1950; Kauffmann and Nitsch, 1954). Separation of fatty acids of the same length but with different degrees of saturation is also possible by these methods. Chromatography on the unsaponifiable portion of fat mixture has also been reported (Ramchandra and Dastur, 1959) to detect 10% hydrogenated vegetable oil and 5% animal body fats. The method, however, does not give encouraging results for the detection of animal body fats in ghee other than

the fat from cotton tract. Direct spotting of concentrated solutions of fats (50% in CCl_4) using a solvent mixture of ethyl alcohol, isoamyl alcohol and carbon tetrachloride, 35:55:10, as the mobile phase has been reported (Ramachandra and Dastur, 1960). GC examination of sterols can distinguish the presence of 1% margarine in butter (Eisner *et al.*, 1962). Thin layer chromatography (TLC) techniques are also promising in distinguishing between pure butter and that adulterated with animal fat.

GLC values for methyl esters of genuine butter and that adulterated with 5–30% beef suet were analysed statistically and it was concluded that for a butter to be considered adulterated, it must be above the following limits for all of the following four fatty acid ratios: C_{18}/C_8, 7.63; C_{14}/C_{18}, 1.02; $(C_6–C_{12})/C_{18}$, 0.95; $C_{18:1}/C_{18:0}$, 2.34.

Addition of 10% suet could be detected in 83% of cases using the above criteria, with a detection error of 6% (Toppino *et al.*, 1982). However, neither the fatty acid composition nor the ratios of C_4/C_6+C_8, C_{12}/C_{10}, and C_{14}/C_{12} allow reliable detection of beef suet or lard even at >20% admixture (Vanoni *et al.*, 1978). Triglyceride formulae using GC triglyceride analysis and statistical methods have been set up to allow sensitive qualitative detection of 1–5% of animal fats such as beef tallow and lard. The detection in milk fat is independent of feeding conditions or lactation (Precht, 1989). Beef tallow and pork at 10% and 5%, respectively in butter fat can be detected using the ratio of C_{52} to C_{40} triglycerides (Luf *et al.*, 1987b). When the ratio of C_{52}/C_{40} >1, adulteration of milk fat by animal body fats like lard and beef fat is almost certain (Guyot, 1978).

The Bomer value is defined as the sum of the melting point of triglycerides (isolated by the diethyl ether method) and twice the difference between this melting point and that of the fatty acids obtained after saponification of these triglycerides. Adulteration of cow and buffalo ghee by lard and shortenings could be detected by increased Bomer values. The only exception is 5% lard in cow ghee. Cow and buffalo ghee have a Bomer value of 63–64 while buffalo milk from cotton tract has a Bomer value of 66–68. The Bomer value for body fats of buffalo, goat and sheep is 68–69 and of pig is higher at 75–76. Addition of the former to buffalo or cow ghee at a level of 5% raises the value to 66–67 and at a level of 10% to 67–68. Addition of pork fat to ghee samples at a level of 5% raises the Bomer value to 68–69 and at a level of 10% to 70–71 (Singhal, 1986).

Differentiation between cotton tract ghee from normal ghee is possible by the methylene blue test and Halphen's test. In the methylene blue test, 0.1 ml of 0.1% methylene blue dye solution in methanol:chloroform (1:1) is reduced instantaneously by cotton tract/cottonseed fed animal ghee, whereas normal ghee and adulterated ghee samples do not reduce methylene blue. In the Halphen test, 5 ml of clear liquefied fat sample is mixed with 5 ml of Halphen reagent (1% sulphur solution in carbon disulphide+an equal volume of isoamyl alcohol) and heated in boiling saturated NaCl solution for 1 h. A characteristic colour is produced in the presence of cottonseed tract ghee. It is recommended that cotton tract/cottonseed fed animal ghee be initially differentiated from normal ghee by the methylene blue test or Halphen test, and then doubtful samples be subjected to determination of the Bomer value.

The fatty acid ratios $C_{18:0}/C_{18:2}$ and the total saturated:total unsaturated ratio (Youssef and Rashwan, 1987) are found to be effective in detecting adulteration (Farag *et al.*, 1980). The total C_{16}/C_{18} ratio and USU/SUS ratio (i.e. position of saturated/unsaturated fatty acids in triglycerides) are useful for detecting butter adulteration by lard (Youssef and Rashwan, 1987).

Studies on enzymic lipolysis followed by analysis of the distribution of unsaturation in triglycerides and 2-monoglycerides, and determination of the percent fatty acid composition at the C2 position in butter, beef suet and lard, and percent molar composition of the 2-monoglycerides have been attempted from the point of view of detecting adulteration of butter by these animal fats (Colombini and Amelotti, 1979). Separation of butter fat and beef suet into liquid and solid fractions by fractional crystallization from acetone at 0 °C and analysis of the liquid fraction, in particular the ratio of $C_{16:0}/C_{18:2}$ in the 2-monoglycerides has been shown to be the best parameter for detecting 10% added beef suet to butter (Vanoni *et al.*, 1979). TLC separation of a carbon tetrachloride solution of butter fat on silica gel into long and short chain triglycerides, selective lipolysis of each fraction with pancreatic lipase and determination of the C_{18}/C_{16} ratio in position C2 of the triglyceride in each band gives differences which are indicative of adulteration. The differences are <5 units in genuine butter, 10–15 units in butter adulterated with 5% tallow and 20–27 units with 10% tallow. It also increases from 22 to 340, as the degree of adulteration increases from 5% to 80% with a mixture of hydrogenated coconut (7%), palm (63%) and groundnut oil (30%), although the C_{12}/C_{10} ratio does not exceed 1.40 up to 20% adulteration (Carisano and Riva, 1976). The ratio of fatty acids present in 2-monoglyceride is also useful in detecting adulteration of butter animal fats such as lard (Movia and Remoli, 1977). Isolation of the unsaponifiable fraction followed by separation of hydrocarbons by TLC and analysis by GLC can check the adulteration of butter by hydrogenated animal fat or hydrogenated butter. The latter is used to increase the melting point of normal butter. A marked squalane peak is characteristic of this admixture (Kuzdzal *et al.*, 1975).

Scanning the UV spectrum between 220 nm and 420 nm has revealed successful detection of 10% lard in butter, but not of suet (Colombini *et al.*, 1978). Differential scanning calorimetry has been applied to the detection of beef suet in butter. Thermograms obtained by melting or crystallization do not show any significant differences between the two fats and their admixtures. However, crystallization thermograms show two exothermic transition peaks and characteristic temperature differences between the two peaks (about 6.7 °C for pure butter and 19.4–22.1 °C for pure suet with intermediate values for admixtures). It is possible to detect adulteration at 5% levels, but the extra exothermic peak which appears on adulteration is sharper at 10% levels of animal body fats. Quantitation by this method is possible on the basis of the peak area. Results may, however, be affected by other factors such as oxidation, acidity and possibly the conditions prevailing during butter production. The method is therefore applicable to freshly produced butter or that after frozen storage (Amelotti *et al.*, 1983).

The iodine value of the suspected fat (I) and refractive indices of untreated (D_0) and iodinated fat (D_1) can be used to calculate the unsaturation index (B) from the equation

$$B=(D_1-D_0)/I. \quad [4.6]$$

Regression lines linking B with percent bone fat or pig fat in milk fat have been obtained and can be used to detect adulteration with a relative error of 2.5% (Merzametov and Antoshchenko, 1983).

Fish oils in butter can be easily detected even at 5% levels by various coloured fluorescent spots after chromatographic separation (Cerutti, 1955a). R_b values (i.e. the ratio of the R_f value to that of butyric acid) of volatile acids obtained by distillation of 5 g butter can be satisfactorily applied to butter samples containing 5–20% dolphin oil (Canuti, 1958). Adulteration of butter with triacetin or hydrogenated dolphin fat can be detected by conductivity of distilled fatty acids, the acetic and isovaleric acids in hydrogenated dolphin fat having higher conductivities than the mixed volatile fatty acids of pure butter (Chioffi, 1955). Hydrogenated oils of fish and other marine animals can also be detected by the Bellier method (De Francesco, 1952). Paper adsorption of the fat or solutions according to the Tortelli–Jaffe reaction and successive examination in ultraviolet light shows a fluorescence if dolphin or other fish oils are present. This could be a useful index for detecting adulteration (Cerutti, 1955b). Iso-oleic acid (Cerutti, 1953) is present in porpoise oil (7–12%), and can be used as an index for detecting this adulteration (Ambrosetti, 1951).

4.6.3 Other adulterants

Other adulterants include butter obtained from milk of two different animal species, modified butter fats and hydrogenated vegetable oils.

Modified butter fats which cannot be detected by any other method can be detected at >5–10% levels by differential thermal analysis (Niiya *et al.*, 1970), which is a rapid, simple and reliable analytical method (Sadini, 1964). Adulteration of butter with an interesterified fat having the same analytical constants as butter can be detected to the extent of 20% using gas chromatography, spectroscopy and dielectric constants (Lueck and Kohn, 1963).

Detection of sheep butter in cow butter has been attempted. Gas chromatographic techniques are not sufficiently sensitive and do not allow the determination of small amounts (5–10%) of sheep butter (Sadini, 1964). Interpretations have been based on RM, Polenske, iodine value and refractive indices (Isidoro and Bonarelli, 1950), but successful detection is possible by Polenske number if sheep butter is present at >25% levels in cow butter. However, the Tortelli–Jaffe reaction can always distinguish between the two butters (Cerutti, 1955c). Studies on the physical and chemical characteristics of ghee prepared from cow and sheep milk have shown a lower iodine number and a higher saponification number for sheep ghee. 1,2-Diacylglycerides are absent in sheep ghee. The P/S ratio, obtained by dividing the total polyunsaturated

fatty acids (*P*) (PUFA) by the total saturated fatty acids (*S*), regardless of the chain length is very different for cow and sheep ghee, the values being 0.7 for cow ghee and 0.34 for sheep ghee. These variables could tentatively identify one ghee type admixed in another (Al-Khalifah and Al-Kahtani, 1993). Differences in the melting diagrams and crystallization patterns of various lipids as determined by differential thermal analysis provide a basis for the determination of adulteration in cow ghee by buffalo ghee (Lambelet *et al.*, 1980). Ghee samples prepared from cow milk and buffalo milk have similar flavour components, particularly the carbonyls which include ethanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, undecanal and dodecanal. However, the total carbonyls of buffalo ghee are higher than those of cow ghee irrespective of the method of preparation and temperature of clarification (Ganguli and Jain, 1973), indicating the possibility of identifying the origin of ghee, although it may not identify an admixture of cow butter fat with buffalo butter fat.

Hydrogenated vegetable oil (HVO) is used extensively in India as a substitute for ghee. It is also used as an adulterant in ghee. Detection is based on the TLC analysis of nickel ion, which is used as a catalyst in the hydrogenation of vegetable oils. If the percentage of HVO in ghee is low or the adulterant has a low nickel content, detection is possible with a slight alteration in the recommended procedure (Baruah and Chakravorty, 1980). Methods based on high tocopherol content in most vegetable oils have been developed (Mahon and Chapman, 1954), but this method fails to detect marine oils or vegetable oils that have been refined so as to reduce the tocopherol content. Similarly an infrared method for the detection of hydrogenated fat has been proved to be unreliable (Kummerow, 1953).

Many of the methods used for the detection of butter adulteration are ineffective in detecting low levels of adulteration. Infrared spectroscopy, which can measure *trans* fatty acids can also distinguish between authentic butter fat and its adulterant hydrogenated oil (Parodi, 1973; Parodi and Dunstan, 1971). The difference due to *trans* unsaturation is seen at 967 cm^{-1} and a smaller peak at 948 cm^{-1}. A plot of absorbance at 948 cm^{-1} vs. 967 cm^{-1} could detect 10% added HVO with 99% confidence limits. The results obtained by this method for the detection of various samples of hydrogenated fat are shown in Table 4.13.

Turbidity temperatures of ghee and HVO differ by about 20–25 °C in different pairs of solvents. Suggestions based on using turbidity temperature as an indication of adulteration of HVO in ghee have been confirmed by experimental observation. A detection level of 20% is possible by this method. Earlier work had demonstrated turbidity temperature to be of value in detecting mineral oil in edible oil (Kane and Ranadive, 1951). A similar concept, critical dissolution temperature (CTD) of different fats in a mixture of ethyl alcohol and isoamyl alcohol has shown a value of 39–45 °C for ghee and 61–72 °C for HVO, indicating its suitability for detecting and estimating the latter in ghee (Bhide and Kane, 1952). However, it is difficult to measure the turbidity temperature in aniline, called the aniline point, because moisture, free fatty acids, extent of rancidity and natural variation in glyceride composition are disturbing factors. An

Table 4.13 Detection limits of various samples of hydrogenated fat

Fat	Iodine number	Approx. limit of detection (%)
Margarine		
1	85	3
2	76	4
3	66	4
4	75	5
5	77	5
6	73	5
7	68	5
8	80	5
9	58	5
10	77	5
11	80	5
12	75	5
13	66	5
14	75	5
15	75	6
16	69	7
17	61	9
Hydrogenated soybean		
1	95	2
2	83	2
3	84	2
4	78	5
Hydrogenated herring		
1	64	3
2	76	3
Hydrogenated seal	45	4
Hydrogenated palm	54	9
Hydrogenated peanut	64	6
Hydrogenated cottonseed	75	6

Source: Bartlet and Chapman, 1961 (reproduced with permission).

approach based on the solubility of fats in ghee has already been projected as a superior method for detecting butter fat adulterations by beef fat (Keeney, 1954). Table 4.14 shows the turbidity temperatures of genuine samples of ghee and those adulterated with 20% HVO.

Table 4.14 Turbidity temperatures of ghee with and without 20% HVO

No.	Turbidity temperature			
	Solvent: toluene–ethanol[a]		Solvent: benzyl alcohol–glycerine[b]	
	Ghee	Ghee+20% HVO	Ghee	Ghee+20% HVO
1	23	32	78	89
2	24	32	96	104
3	26	35	77	88
4	26	35	93	100
5	27	35	86	98
6	30	38	92	100
7	31	35	87	95
8	31	38	89	97
9	32	37	95	100
10	32	38	94	101
11	34	39	92	101

[a]Toluene:ethanol = 1:5.
[b]17.1 g glycerine in 100 ml benzyl alcohol.
Source: Desikachar *et al.*, 1957 (reproduced with permission).

The turbidity temperature as measured in a benzyl alcohol–glycerine solvent system is lowered by the presence of free fatty acids and raised by moisture. Elimination of one of these by extraction of the test sample with alkaline 70% alcohol and of the other by drying are suggested to overcome the drawbacks. The turbidity temperature of arachis oil is 107 °C, of coconut oil 58 °C and of sesame oil is 98 °C in the benzyl alcohol–glycerine system. The presence of coconut oil in ghee reduces the turbidity temperature. The oil can be extracted from the fat by alcohol at 30 °C and identified in the extract by evaporating off the solvent. However, when present along with HVO, the detection of coconut oil in ghee becomes difficult (Desikachar *et al.*, 1957).

4.7 Dilution of milk with water

Amongst the commonly adulterated foods, dilution of milk with water is probably the most common. Freezing point determination (Edwards, 1958; Henningson, 1969) with the advanced milk cryoscope (Antila and Kyla-Siurola, 1978; De Man, 1962; Ruegg *et al.*, 1984; Bryant and Biggs, 1956; Shipe, 1959; Shipe *et al.*, 1953; Dillier-Zulauf, 1971) is believed to be a rapid and accurate method of determining the amount of added water in milk (Bauch *et al.*, 1993) and the stage at which it was added (Mikkelsen, 1979). A thermistor cryoscope in which the sample is supercooled to a specified temperature followed by induction of crystallization by mechanical vibration is reported in the French Standard NF V 04-205, 1990. The process involves a rapid rise in temperature to a plateau value corresponding to the freezing point of the milk. The test is sensitive enough to detect 3% of added water (Dastur, 1949). The unit of

measuring freezing point, as given by the standard AOAC method is the degree Hortvet (°H) rather than degree centigrade. Belgian standards lay down a maximum limit of 0.002 °H on two successive analyses by the same analyst and a maximum of 0.010 °H between average results of two determinations in two different laboratories (Belgian Standard, 1977). A great deal of confusion results from the use of °H for measuring the freezing point depression.

After analysis of 10 582 raw milk samples during summer, autumn and early winter of 1977, Packard and Ginn (1979) recommended that adopting a working standard of −0.540 °H would result in a far greater number of farmers being investigated in an effort to control water additions. A change to the use of degrees centigrade for measurement is considered to be more suitable (Richardson, 1979; Packard and Ginn, 1979). The formulae for converting degree centigrade to degree Hortvet and vice versa are:

$$°C = \frac{(0.1915 \times °H) - 0.0004785}{0.199} \quad [4.7]$$

$$°H = \frac{(0.199 \times °C) + 0.0004785}{0.1915} \quad [4.8]$$

For every 1% of water added to fresh milk, the freezing point of raw fresh milk is reported to increase by 0.006 °C. Some authors suggest that results be simplified by using millidegrees and omitting the negative sign, so that −0.525 becomes 525. All values less than 525 would be suspected of dilution with water (Jamotte and Duchateau, 1973). The value of freezing point needs to be corrected when the acidity is 7–8 °SH (Soxlet-Henkel degrees), and are disregarded when acidity is less than 8 °SH. Low acidity values (<5.3 °SH) are in themselves an indicator of possible water addition. (SH is the number of ml of 0.25 N NaOH required to neutralize 100 ml milk. It can be converted to percent lactic acid by dividing by 44.4.)

The Pennsylvania Department of Agriculture had even adopted a regulation which states that milk with a freezing point above −0.525 °C be considered adulterated, unless proved to be free from added water (Barnard, 1977). This method had been in operation at farm level since 1965 in Colorado, where a 'monetary penalty scheme' was practised. The percentage of added water was deduced from the freezing point and the farmer was paid accordingly. This was quite successful in bringing down adulteration (Barnum, 1977; Binder, 1974). This method has also been considered accurate in Switzerland and is included in official food quality control (Zeder, 1984). Internationally accepted interpretations of freezing point depression are: where the freezing point depression is greater than −0.535 °C, the supply is assumed to be free from added water; where the depression is between −0.530 and −0.534, the producer is asked to check his plant; where the freezing point depression is −0.525 to −0.529, there is a strong probability of extraneous water being present; and where the freezing

point depression is −0.525 or less, the onus of the proof of innocence is put on the farmer (Harding, 1990).

On the basis of freezing point depression of original milk (I_1) and diluted milk (I_2), expressed in degrees centigrade, two formulae which express the amount of water in g/100g milk (Y) are: (Brouwer, 1980)

Winter's formula:

$$Y = \frac{100\,(\delta I_1 - \delta I_2)}{\delta I_1} \qquad [4.9]$$

Elsdon and Stubb's formula:

$$Y = \frac{(100 - D_2)\,(\delta I_1 - \delta I_2)}{\delta I_1} \qquad [4.10]$$

where D_2 is the total solids of the diluted milk. A third formula, given in the Netherlands Standard NEN 3460 (Brouwer, 1980) expresses the amount of water in g/100 g original milk (X), taking into account the total solid content of the milk before adding water. This is given as:

$$X = \frac{(100 - D_1)\,(\delta I_1 - \delta I_2)}{\delta I_2} \qquad [4.11]$$

Milk as a physiological secretion has a constant osmotic pressure. The osmotic pressure of cow milk averages 6.78 atm, with 3.03 atm due to 4.7% lactose, 1.33 atm due to 0.1% alkali chlorides (Na^+, K^+) and 2.42 atm due to other salts and ions, making a total freezing point depression of 0.560 °C (Coste and Shelbourn, 1919). Since the osmotic pressure of milk is constant, any variation in the proportion of lactose is accompanied by variation in salts. Thus, an increase in the amount of lactose will lead to a decrease in the total number of molecules and ions dissolved in milk. There is no correlation between osmotic pressure of milk and its freezing point. The osmotic pressure due to non-electrolytes such as lactose and non-ionized salts correlates with degrees of freezing point depression. Similarly osmotic pressure due to strong electrolytes such as sodium chloride follows a predictable pattern of freezing point depression. However organic acids and their salts which contribute 2.42 atm osmotic pressure are rather unpredictable in their effect. Attempts to predict the freezing point on the basis of any one of the constituents of milk have therefore failed (Peters *et al.*, 1959). It may be of interest to know that lactose and chlorides contribute about 80% of freezing point depression in Jersey milk and about 75% in Holstein milk (Cole *et al.*, 1957).

Correlation between freezing point depression and content of fat (*F*), density (*d*) and conductivity (*c*) has been observed after analysis of more than 250 samples (Welboren and Velden, 1974). The equation proposed is:

$$\text{Freezing point depression} = 0.0101F + 10.815d + 0.0312c - 10.7694 \quad [4.12]$$

Freezing point of milk can be affected by various factors (Macdonald, 1950; Unger *et al.*, 1984; Rohm *et al.*, 1992; Buchberger, 1992) such as breed (Zee, 1977), lactation stage and mastitis; geographical area (Henningson, 1959; Dahlberg *et al.*, 1953), season, feeding and management regimes (Schroppel, 1992; Mohammedi *et al.*, 1992); water intake, spontaneous change in milk souring and natural deaeration; treatment of milk like cooling, freezing and heating and addition of preservatives (Belgian Standard, 1977). The effect of vacuum pasteurization on the freezing point value (Henningson and Lazar, 1959) must be considered in the case of applicable retail milks. Freezing point increases by 0.006–0.009 °C during pasteurization (Staub and Krahenbuhl, 1954; Buchberger, 1986) and by 0.023 °C for UHT milk. Therefore although freezing point may detect added water in raw milk, it is not always a reliable indicator in heat treated milk (Buchberger, 1986). Also, the results for samples having acidity >0.18 g lactic acid/100 ml are not representative of the original milk (French Standard N F V 04-205, 1990). Freezing point differences seem to be connected to the temperature of the environment, a lower freezing point being recorded at the milking following exposure of the animal to high temperatures. No significant correlation between milk yield and freezing point has been observed, but a low positive correlation between solids-not-fat and freezing point seems to exist. Small seasonal changes in freezing point, particularly when the cows have access to lush spring pasture have been reported. Age and state of lactation, as well as the time of milking, that is morning or evening on any particular day are not known significantly to influence the freezing point (Aschaffenburg and Veinoglou, 1944). The chlorine/lactose ratio, an indicator of mastitis milk, has not shown any strict relation to the freezing point (Jasinska, 1953). Potassium dichromate is sometimes added as a preservative to samples of milk held as evidence in judicial proceedings. This is known to lower the freezing point. Addition of just 1% soybean milk can increase the freezing point by as much as 0.003 °C (Huh, 1971), and 2% added brine can reduce the freezing point by 0.054 °C on an average (Huh, 1971). A minimum freezing point depression standard, based on area data and administered in a manner similar to a minimum butter fat standard appears to be the most feasible way of utilizing the cryoscopic method for the determination of added water in milk (Henningson, 1961).

Freezing point determination is not sensitive enough to detect dilution of buttermilk with water because of great variation in the freezing point of the serum. Refractive index and specific gravity are suggested for routine control work and also require less preparation. If the buttermilk is not fresh, ash determination is the only

Table 4.15 Use of goat milk freezing point depression (FPD) to estimate added water

FPD		% Added water	FPD		% Added water	FPD		% Added water	FPD		% Added water
°C	°H		°C	°H		°C	°H		°C	°H	
0.520	0.543	5	0.383	0.400	30	0.245	0.257	55	0.107	0.114	80
0.493	0.515	10	0.356	0.372	35	0.218	0.229	60	0.080	0.086	85
0.465	0.486	15	0.328	0.343	40	0.190	0.200	65	0.052	0.057	90
0.438	0.458	20	0.301	0.315	45	0.163	0.172	70	0.026	0.029	95
0.410	0.429	25	0.272	0.286	50	0.135	0.143	75	0.000	0.000	100

Source: Szijarto and Van de Voort, 1983 (reproduced with permission).

suitable method for detecting added water, even though there is a large possible error involved (Kiermeier and Pirner, 1956). The normal range of freezing point accepted is −0.528 °C to −0.561 °C (Atherton and Newlander, 1977).

There is scanty literature on the freezing point of caprine milk. A mean of −0.582 °C has been reported by some Italian workers (Princivalle, 1948). An overall mean freezing point of −0.5527°C has been considered to be representative of caprine milk in Ontario. In the case of caprine milk, relationships for freezing point depression (FPD) in terms of °H or °C and added water are reported. Table 4.15 shows the use of goat milk freezing point depression (FPD) to estimate added water.

Equations [4.13] and [4.14] giving the percentage of added water in goat milk from the freezing point depression are as follows:

$$\% \text{ Added water} = 100(-0.552\ ^\circ\text{C} - \text{FPD})/ - 0.552 \quad [4.13]$$

$$\% \text{ Added water} = 100\ (-0.572^\circ\text{H} - \text{FPD})/ - 0.572 \quad [4.14]$$

It has been reported that the degree of hydrolysis of lactose in milk is directly related to the depression of the freezing point (Nijpels *et al.*, 1980). When all the lactose in milk is hydrolysed to monosaccharides, the freezing point is known to decrease by −0.274 °C. Figure 4.3 (Jeon and Bassette, 1982) shows the regression line that relates percentage lactose hydrolysis in milk to the depression of freezing point. Superimposed upon that line are the freezing points of standard sugar solutions representing

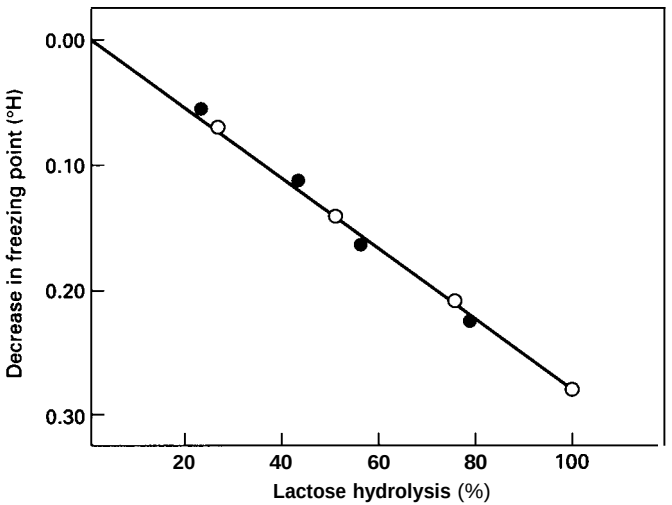


Figure 4.3 Linear relationship between the depression of freezing point and percentage hydrolysis of lactose. ●, Percentage lactose hydrolysis determined by chemical analysis of milk. O Standard sugar solutions containing glucose, galactose and lactose prepared at molar concentrations equivalent to a 5% solution of lactose hydrolysed at 0%. 25%. 50%. 70% and 100%. (Source: Jeon and Bassette, 1982, reproduced with permission)

Table 4.16 Effect of adding water to lactose-hydrolysed milk on freezing point and lactometer readings

Lactose-hydrolysed milk			Water added to lactose-hydrolysed milk			
Lactose hydrolysis (%)	Freezing point (°H)		Water added to milk		Resultant freezing point (°H)	Quevenne reading
	Calculated[a]	Achieved[b]	By ratio	By percent		
0	−0.543	−0.543	0	0	−0.543	32.5
22	−0.603	−0.604	0.110	9.9	−0.540	29.2
35	−0.639	−0.641	0.175	14.9	−0.538	27.5
50	−0.680	−0.685	0.250	20.0	−0.537	26.3
67	−0.726	−0.726	0.335	25.1	−0.536	24.5
85	−0.775	−0.775	0.425	29.8	−0.535	22.3

[a]Calculated from the freezing point depression curve (Fig. 4.3).
[b]Achieved by mixing the lactose-hydrolysed milk with control (unheated milk).
Source: Jeon and Bassette, 1982 (reproduced with permission).

0, 25, 50, 75 and 100% lactose hydrolysis. Table 4.16 shows the effect of adding water to lactose hydrolysed milk on the freezing point and lactometer readings.

By careful manipulation, a considerable amount of water can be added to lactose-hydrolysed milk and still maintain the freezing point within the normal range for milk. In a study, a taste panel also failed to distinguish between control undiluted milk and the hydrolysed milk diluted with water to near its original freezing point.

4.7.1 Other indices for detecting added water in milk

Adulteration of pasteurized buffalo milk with 5-20% water decreases the electrical conductivity progressively (Montefredine, 1942; Grasshoff, 1988). This can be used as an analytical parameter to check fraud (El-Shabrawy and Haggag, 1980).

Correlations between the freezing point of milk and lactose content, and the electrical conductivity at 37 °C and 0 °C to detect added water have been attempted, but are not found to be statistically significant (Peters *et al.*, 1959). This could be because of the great variation in lactose which is dependent on the state of pregnancy of the cow (decreasing with progressing pregnancy); on the feed, which is low with grass; on the acidity, diseases etc. Lactose content however does not vary with the age of the animals, the stage of lactation or the hour of milking (Giuseppe, 1952). Two formulae for checking that milk is not adulterated with water are:

$$(2 \times \text{SNF}) - (\text{protein} + \text{lactose}) > 9.1 \qquad [4.15]$$

and

$$\text{SNF} > \text{lactose} + \text{protein} + 0.7 \quad \text{(Panero, 1975)} \qquad [4.16]$$

Analysis of 2624 milk samples in Spain has shown surface tension and viscosity as

suitable indices for detecting >10% added water to milk (Goded, 1951). Trypsin digestion of the protein in buffalo milk samples followed by precipitation of the undigested protein with trichloroacetic acid and further measuring the decrease of absorbance of the supernatent at 280 nm has been correlated with the degree of dilution. This method gives simple reproducible results compared with those obtained using a cryoscope for freezing point determination (Ali and Hasnain, 1987). Another practical method recommended for testing diluted milk in factories is based on the comparison of percent fat in milk and in the dry substance (Madsen, 1948). The Olivari constant, CSD=Q+3.85C, where Q is the specific gravity of acetic acid milk serum at 15 °C in °Quevenne, and C is the chloride in acetic acid milk serum in g sodium chloride/l can also indicate water in milk. The values of CSD for normal milk range between 34 and 36. Addition of sodium chloride tends to raise the CSD to >36. Dilution with 1% sodium bicarbonate is not detected by the freezing point but can be detected from the Olivari constant, CSD (Chioffi, 1977). Analysis of nitrate in milk could also be used as a clue to detect water addition (Maksimets *et al.*, 1988; Tomeo Ibarra and Bergeret, 1959).

Tests based on specific gravity, using especially designed lactometers (Hostettler, 1956) are considered by some authors to be more suitable for detecting added water in milk in developing countries (Dahlberg, 1955). Some formulae for calculating water addition and/or removal of butter fat are also reported (Siegenthaler and Schultess, 1977). Addition of 1% water is known to decrease the specific gravity by 0.0034 (Huh, 1971). Slide rules, based on the differences between the density of milk and a national or regional standard have been developed to determine the amount of water added to milk (Kapianidze, 1984).

The refractive index of the serum obtained from normal milk (6.3-6.7 °SH acidity) by acidification with acetic acid (Anselmi, 1941), or by boiling milk with copper sulphate (Anas and Noya, 1949; Hoffmann, 1951), potassium ferrocyanide or calcium chloride can detect only above 10% water additions (Slanovec and Arsov, 1977). It is advised that samples in the 37–38 refractometer number range should be regarded as very suspect, those in 33–37 range as diluted and those in 30-35 range as heavily watered (Taborsak and Abramovic, 1978).

Ultracentrifugation of human milk followed by refractometry on ultrafiltrate can be used to detect added water in human milk. Deviations below normal readings of 44-46 indicate dilution with water (Sager, 1952). A refractive constant given by $K=[(n^2-1)]/(n^2+2)d]$ is known to be an effective indicator of watering in milk. The terms n and d denote refractive index of the serum and density as determined with Quevenne's lactometer (Venkatasubramanian and Ramakrishnan, 1951).

A high degree of correlation (>0.99) (Rohm, 1986) has been reported between freezing point determination using a thermistor cryoscope and a vapour pressure osmometer under optimum conditions (temperature raised to 20 °C before testing) in milk samples with known amounts of water added. Added water can therefore be calibrated in terms of the osmometer, which has the advantages of small sample size,

Table 4.17 Percent added water in adulterated milk samples as calculated from the vapour pressure measurement (correlation coefficient. 0.997)

Actual water added (%)	Osmometer reading (mosmol)	Calculated added water (%)[a]
0	265.0	0
1	261.3	1.2
2	259.3	1.9
3	256.7	2.8
4	254.3	3.6
5	250.7	4.7
6	246.7	6.1
7	243.3	7.2
8	241.0	8.0
9	237.3	9.2
10	235.7	9.7
11	232.3	10.9
12	230.7	11.4
13	226.7	12.7
14	224.3	13.5
15	220.7	14.7
20	202.3	20.8
25	189.3	25.1
30	173.3	30.5
40	144.3	40.1
50	129.0	45.2

[a] Percent added water was calculated from milliosmolal reading using the formula:

$$\% \text{ Added water} = \frac{(R-S)\text{X}100}{\text{R}}$$

where R= osmometer reading for milk known to be free of added water (in mosmol) and S= reading of the sample (in mosmol).
Source: Mitchell, 1977 (reproduced with permission).

ease of calibration and the fact that the instrument requires no operator attention once the sample has been inserted (Mitchell, 1977). Table 4.17 shows percent added water in adulterated milk samples as calculated from the vapour pressure measurement.

The major sources of error in the use of the osmometer are variation in sample size and contamination of the thermocouple with milk residue. The vapour pressure osmometer method for quantitating added water in milk has been adopted as official first action (Richardson *et al.*, 1978).

4.8 Indices of microbial quality of dairy products

Interest in bacteriological testing of milk stems from the fact that bacteria in milk can cause spoilage as well as disease. From a public health standpoint the importance of bacteriological examination was quickly recognized and has gradually become a regular practice. In Europe, the International Dairy Federation has been active in standardizing methods for the examination of dairy products. With marked advances in the eradication of bovine tuberculosis and brucellosis together with widespread pasteurization of milk, interest in the bacteriological testing of raw milk has largely shifted from the disease aspect. In present times, examinations are more concerned with obtaining an estimate of the degree of care taken in the production and handling of milk on the farm.

Quality assurance of dairy products entails applications of hazard analysis and critical control points (HACCP) backed up by laboratory analysis of in-line samples and finished products. Two main questions arise in monitoring the microbiological quality of foods: 'what kinds of microorganisms are present?' and 'how many micro-organisms are present?' The number and variety of tests required depend on factors such as the final use of the product, consumer specification and the nature of the process. The standard plate count (SPC) is important as an indication of sanitary conditions of production and handling (American Public Health Association, 1953). Although generally conceded to be most precise for assessing the bacterial population, SPC is not without limitations. No one medium incubated for a short time at a given temperature will bring out all the bacterial types present. Furthermore, colonies may represent single organisms or clumps of several, indicating a considerable inherent error (Wilson, 1935). It should also be emphasized that low SPC in a fresh sample is no guarantee of adequate keeping quality (Johns, 1959; Olson *et al.*, 1953). Acidity monitoring is sometimes done to get a check on quality (Guillermo, 1951). Direct microscopic count (DMC) furnishes a bacteriological estimate within a few minutes. It also enables counts of body cells such as leucocytes, lymphocytes, etc. to be made, a feature especially valuable in indicating mastitis. Somatic cell counts have been correlated best to the percentage of lactose (correlation coefficient, $r = -0.398$) and a slight positive correlation to the protein content ($r = 0.101$) (Packard and Ginn, 1991). While DMC has been recommended in place of SPC for the control of pasteurized milk (Mickle and Bolman, 1943), it has not been generally adopted for this purpose. However, it has been used in the control of skim milk powder (Forest and Small, 1959), where it gives valuable evidence of past history of the product not obtainable by the viable count. A modification of the DMC for making counts of thermoduric bacteria has been proposed (Mallmann *et al.*,1941), but has not been adopted, possibly due to poor agreement with the plate count method (Fischer and Johns, 1942). Thermoduric bacteria are sufficiently heat resistant to survive pasteurizing temperature and thus may be responsible for counts in excess of the legal limits of the pasteurized products. They enter milk chiefly from the surfaces of inadequately cleaned milking and

handling equipment and thus are indication of unsanitary conditions. Thermoduric count is believed by some sanitarians to be more useful than SPC (Barnum, 1959). Thermophilic bacteria capable of growing at holder pasteurization temperature were a serious problem when batch pasteurization was common. With the trend towards higher temperatures with high temperature short time (HTST) and UHT pasteurization, the interest has diminished. These organisms can be detected by incubating plates at 55 °C for 48 h, by direct microscopic examination of the smears or by the dye reduction test (methylene blue or resazurin) incubated at 62–63 °C (Kay *et al.*, 1953).

Positive clearance of product before dispatch is often necessary and may result in lengthy 'holding times'. Contamination of pasteurized milk with unpasteurized milk or unpasteurized cow milk is often implicated in outbreaks of salmonellosis (Ryan et al., 1987; Rowe *et al.*, 1987) and campylobacter enteritis (Jones *et al.*, 1981; Barrett, 1986). Enumeration of *Escherichia coli* seems to have value as an indicator of faecal contamination and thus potential hazard in raw milk (Humphery and Hart, 1986; 1988). The presence of *E.coli* is also indicative of a likely contamination by *Campylobacter jejuni*. For instance, it is reported that the mean number of *E. coli*/ml in campylobacter positive milk is 212.7±105.6, while that of the negative sample is 39.17±20.2. Experience suggests that human infections with campylobacter are more common than salmonellosis (Potter *et al.*, 1984), a fact substantiated from results from various parts of the world (Humphery and Beckett, 1987; Doyle and Roman, 1982; Oosterom *et al.*, 1982; Lovett *et al.*, 1983; De Boer *et al.*,1984; Waterman *et al.*, 1984).

Psychrophilic bacteria growing actively at 7.2 °C (45 °F) should not be overlooked. With milk being held longer at refrigeration temperatures from cow to the consumer, opportunities for the growth of psychrophiles have increased greatly. Many of them are lipolytic and proteolytic, and are capable of inducing flavour changes and other defects in milk and milk products on refrigerated storage. The degree of lipolysis is in fact a quality index of cream and butter (Vyshemirskii *et al.*, 1982). Spoilage in pasteurized milk, cream and cottage cheese is generally due to psychrophilic growth. Pasteurization destroys these organisms, and therefore their presence indicates recontamination. The enumeration of psychrotropic bacteria count (PBC) obtained on the dry petrifilm medium culture plates with triphenyltetrazolium chloride also serves as an indicator of the quality of milk (Bishop and Juan, 1988).

In pasteurized products, the use of the coliform test to detect recontamination has been more generally acoepted. Stemming largely from the work of McCrady and his coworkers (McCrady and Langevin, 1932) the value of this test, to both sanitarians and management has steadily received wider recognition. When applied to products containing other sugars in addition to lactose, for example, ice cream, positive results must be confirmed to avoid misleading conclusions. False positive results have been reported where sweetened and unsweetened fresh fruits are added to the mix (Barber and Fram, 1955). Enterococci are also more useful in detecting faecal contamination of cheeses (Brooks, 1974).

Proper pasteurization of milk or cream destroys yeasts and moulds and therefore

their presence in a product indicates recontamination. Mould and yeast counts are employed by cheese manufacturers and sanitarians as indices of plant sanitation. When cream is stored on the farm at unsuitable temperatures for too long periods, growth of *Geotrichum candidum* takes place and on pasteurization, dead mycelia pass into the butter in appreciable numbers. Their detection is frequently employed by food and drug officials as the basis for seizure and confiscation of butter.

Organisms capable of proteolysing casein are frequently responsible for undesirable flavours in dairy products. Surface taint of butter caused by *Pseudomonas putrefaciens* (Derby and Hammer, 1931) is an example. Organisms attacking fats are often also proteolytic and psychrophiles. This makes them particularly important in butter, cream and cottage cheese, which are frequently held refrigerated for extended periods.

Interest has been aroused by food poisoning outbreaks attributed to the presence of Staphylococcus eneterotoxin in non-fat milk solids and in cheddar cheese and its modifications. While toxin production in raw milk itself rarely presents a hazard to health due to repression of the growth of staphylococci by other types, some growth may take place both before and during the cheese making process (Takahashi and Johns, 1959). Cheese with excessive numbers of coagulase-positive staphylococci must therefore be regarded with suspicion.

The presence of antibiotics in milk, either residual from therapy or by deliberate addition, can influence the results of bacteriological examination (Foley and Byrne, 1950; Johns and Katznelson, 1949; Wilkowske and Krienke, 1951), in addition to antibiotics that cause problems in the manufacture of products dependent upon lactic fermentation and the possible hazard to those individuals acutely sensitive to penicillin. Tests have been reported to detect antibiotics in milk, based upon interference with bacterial growth and activity. One of the simplest is the starter activity test (Silverman and Kosikowski, 1952), patterned after that introduced by Horrall and Elliker (1947). Here, the extent of acid development when inoculated with a lactic starter and incubated for several hours is compared with that of a control. Care must, however, be taken to exclude the action of naturally occurring inhibitory substances. Greater sensitivity can be obtained by using *Streptococcus thermophilus* in place of the common starter streptococci. Another form of test utilizes a redox indicator to reflect interference with bacterial growth when incubated at a suitable temperature; triphenyl-tetrazolium chloride is the indicator commonly recommended (Neal and Calbert, 1956), although methylene blue (Galesloot, 1955; Schipper and Petersen, 1951) and resazurin have been used. The disk assay method has also been studied extensively. In its standard form, it is most useful for detecting the presence of penicillin; the test organism, *Bacillus subtilis* is less sensitive to other antibiotics (Johns and Berzins, 1955). An interesting modification of this method has been described (Shahani and Badami, 1958), wherein the agar layer is flushed with resazurin; the completed test takes considerably less time than the standard disk assay method.

Apart from antibiotics, sulphonamides are frequently used in combination with certifiable antibiotics for the treatment of mastitis in dairy cows. The sulphonamides

appear in milk immediately after the drug is infused into the udder and may persist in subsequent milkings over a period of several days. Such milk is not considered suitable for food use and must be withheld from the channels of commerce. Methods to detect these sulphonamides have been proposed (Selzer and Banes, 1963).

The rapid, accurate and reliable evaluation of total viable cell counts is very important in the efficient monitoring of microbiological quality, especially in raw and ready-to-eat foods. Indirect or non-microbiological methods offer the potential for rapid monitoring of microbial load in terms of metabolic intermediates or end products. Methods such as DEFT (direct epifluorescent filter technique) (Pettipher *et al.*, 1980), polymerase chain reaction, especially for *Listeria monocytogenes* (Starbuck *et al.*, 1992) and Bactosan are in vogue today.

4.8.1 Methods based on the measurement of metabolic activity

4.8.1.1 Dye reduction tests

The dye reduction test (Smith and Zall, 1977) is based on the observation of changes brought about in the medium by the metabolic activities of viable microorganisms. Bacterial dehydrogenases transfer hydrogen from a substrate to a redox dye, which undergoes a colour change. The number of organisms present in the sample is correlated with the rate of colour change reaction. A number of dyes, including methylene blue, resazurin and tetrazolium have been used. The methylene blue reduction test, introduced in Denmark and Sweden around 1912 is probably the most extensively used bacterial test. The SPC at 32 °C is less likely to disagree with the dye reduction test (Harris *et al.*, 1956). Although as a measure of mean keeping quality methylene blue was slightly better than resazurin, standard deviations have shown a wide scatter of keeping quality for specified standards by either dye test (Anderson and Wilson, 1945).

Several factors have tended to distort the relationship between counts and dye reduction times. More productive media and lower incubation temperatures have increased the levels of plate counts. The proportion of thermoduric bacteria influence the reduction, since they are slow reducers. Antibiotics in milk tend to slow down the reduction. Psychrophiles, which sometimes comprise a high percentage of the flora (Johns and Berzins, 1959), fail to grow at 35–37 °C. Finally, with more efficient cooling, some bacteria are extremely dormant at the start of the test and substances inhibiting bacterial growth are conserved. The resulting prolonged lag phase delays reduction in such a manner that some high count milks escape detection. The creaming error, caused by sweeping varying proportions of the bacterial population on to the surface with rising fat globule (Wilson, 1935) has also been referred to. This is, however, overcome by inversion of the tube every half an hour. The methylene blue reduction test is also sensitive to levels of Cu, added sodium sulphate, and is affected by pH (being least at pH 8.0-8.5) and agitation (Maeno and Asahida, 1954). Several

workers have reported that the grading based on dye reduction tests is too lenient. In the winter months, a high percentage of samples with high bacterial counts are not detected (Malcolm and Leitch, 1936; Thomas and Tudor, 1937). The methylene blue reduction time in milk is an index of bacteriophagic activity against true lactic acid bacteria. Compared to other methods, the reduction in milk is more sensitive and shorter. The test also allows the interaction between phage and bacteria to be followed colorimetrically (Miklik, 1951). This technique has been primarily used for the examination of milk, although it has also been adapted for the examination of other foods. In Europe, dairies use the methylene blue reduction test as an index of keeping quality of pasteurized products (Olsen, 1956). A rapid dye reduction test can sometimes be due to aerobic spore formers which survive pasteurization (Olsen, 1956). This test is, however, considered to be inadequate in assessing the suitability of milk for cheesemaking from the viewpoint of bacterial contamination (Gudkov *et al.*, 1979).

Use of the dye resazurin as a redox indicator offered the advantage of earlier indication of change. This is affected by the presence of excessive numbers of leucocytes, etc. and thus could indicate the presence of abnormal milk (mastitis or late lactation) (Ramsdell *et al.*, 1935). Well-cooled milks containing excessive numbers of dormant bacteria often escape detection (Hempler, 1953). In the farm bulk tanks the organisms are so dormant that reduction is delayed appreciably. A preliminary incubation is most useful in overcoming this dormancy and also in encouraging the growth of saprophytic organisms (Johns and Berzins, 1959).

Another oxidation–reduction indicator, triphenyltetrazolium chloride (Mustakallio *et al.*, 1955) is unfortunately extremely sensitive to light. Its usefulness appears to be confined largely to heavily contaminated milks, although it has been advocated for use in the detection of antibiotics and other inhibitory agents in milk (Neal and Calbert, 1956), and in a keeping quality test for pasteurized milk (Day and Doan, 1946; Broitman *et al.*, 1958) and condensed milk (Luk'yantseva *et al.*, 1978).

4.8.1.2 Electrical methods

Impedance is the resistance to the flow of an alternating current through a conducting medium. Impedimetry can be used to monitor the changes in the electrical properties of a culture medium that are brought about by the growth of microorganisms in the medium, as nutrients are converted into metabolic products. Complex uncharged molecules such as carbohydrates are catabolized to smaller charged molecules such as bicarbonate and organic acids. As the microorganisms grow, this process leads to a decrease in the overall impedance of the medium. Thus, measurement of the changes in the electrical impedance of microbial cultures provides a means of detecting microbial proliferation (Gnan and Luedecke, 1982; O'Connor, 1979). The technique can detect as few as 10^2-10^3 cells ml^{-1} within 2 h, depending upon the sensitivity of the instrument used. It therefore reduces the holding time needed for microbiological screening (Wood *et al.*, 1978; Firstenberg-Eden, 1983). In marginal samples, where the

Table 4.18 Comparison of shelf life, impedance response detection time, standard plate count and psychrotropic count for 10 milk samples

Shelf life (days)	Detection time[a] (hours)	Mesophilic plate count[b] (cfu ml^{-1})	Psychrotropic count[c] (cfu ml^{-1})
9	9.4	400	20
9	12.2	7000	30
10	9.6	400	10
10	9.4	200	100
10	10.4	200	—
14	11.1	300	10
14	10.9	400	100
14	11.5	100	30
15	11.4	100	10
15	10.3	200	100

[a]Earliest detection of duplicate vials at 32 °C.
[b]Incubation at 32 °C for 48 h.
[c]Incubation at 7 °C for 7 days.
Source: Cady *et al.*, 1978 (reproduced with permission).

total number of coliforms are low (e.g. positive in 1 g, but negative in 0.1 g), sample variation might be expected to cause variable results, even with duplicate samples tested by the same method. Overall, the impedance method gives more positives in themarginal samples than the standard method, suggesting that low coliforms are more likely to be detected by the impedance method (Fryer and Forde, 1989). Large numbers of bacteria generally require less time to reach the threshold level and produce an impedance change. In Table 4.18 are shown the mesophilic plate count, the psychrotropic count and the impedance response detection times for 10 samples with varied shelf lives. In general, the detection times appear to reflect the values of the shelf life. The detection time, in fact, seems to correlate better with the shelf life than do the standard plate count and psychrotropic count, and shows promise of being useful in predicting keeping quality (Cady *et al.*, 1978). Analysis of 243 samples with counts varying from 3×10^3- 6×10^6 cfu ml^{-1} has shown a coefficient of correlation of −0.657 and a standard deviation of 0.441 between SPC and impedance detection time. The method of sample agitation, that is, standard or violent does not affect the results (Piton and Dasen, 1988). Impedance methods for other groups of organisms such as Salmonella are also available (Easter and Gibson, 1985).

A three-way classification scheme has been explored whereby samples producing impedance changes prior to 7 h were classified as having $>10^4$ organisms ml^{-1}, those with impedance changes between 7 and 9 h as having between 2000 and 10^4 organisms ml^{-1} and those with impedance changes >9 h as having less than 2000 organisms ml^{-1}. This scheme could correctly classify 85% of samples tested. These results can be

Table 4.19 Comparison of the results obtained by the Malthus system and by the standard plate count method

Sample no.	Standard plate count (cfu ml^{-1})	Malthus system		Bacterial flora[a]
		Detection time (h)	Total detection time (h)	
1	190 000	0.6	16.6	-R(-), + S(-)
2	94 000	3.8	19.8	+S(-)
3	220 000	0.6	16.6	-R(+), +S(-)
4	190 000	1.7	17.7	-R(+), +S(-)
5	8 2000	—	—	+S(-)
6	1000 000<	0.6	16.6	-R(-), +R(-), +S(-)
7	1000 000<	3.2	19.2	+S(-)
8	1000 000<	0.6	16.6	-R(-), +S(-)
9	1000 000<	0.6	16.6	-R(-)
10	5 200	18.4	34.4	-R(+)
11	1000 000<	0.6	16.6	+S(-)
12	1000 000<	0.6	16.6	-R(-), +S(-)
13	1000 000<	0.6	16.6	-R(-), +S(-)
14	1000 000<	3.7	19.7	+S(-)
15	1000 000<	0.6	16.6	-R(-), +S(-)
16	< 10	1.1	17.1	-R(-), +S(-)
17	1000 000<	0.6	16.6	-R(-), +S(-)
18	1000 000<	0.6	16.6	-R(-), +S(-)
19	1000 000<	0.6	16.6	-R(-), +S(-)
20	14 000	12.5	28.5	-R(+)
21	240 000	5.9	21.9	-R(-), +S(-)
22	1000 000<	1.1	17.1	-R(-)
23	24 000	—	—	+S(-)
24	100	5.0	21.0	-R(-), +R(-)
25	1000 000<	0.7	16.7	-R(-), +R(-)
28	1000 000<	0.7	16.7	-R(-)
29	1000 000<	0.7	16.7	-R(-), +S(-)
30	<10	9.7	25.7	-R(-)
31	1000 000<	—	—	-R(-), +S(-)

[a] +S: gram positive cocci, +R: gram positive rod, –R. gram negative rod, ( ): oxidase test.
The total detection time=detection time+preincubation time (16 h).
The samples, UHT treated milk, were incubated at 30 °C for 16 h, then examined for viable cell counts by the standard plate count and by the Malthus system.
Source: Kamei *el al.*, 1988 (reproduced with permission).

made available within 10 h as compared to 48 h for standard methods. The samples tested included various types of milk products, including homogenized, low fat and skim milk from many dairies (Dufour *et al.*, 1977). Impedance measurements detect

activity not only from organisms present in the milk, but also from enzymes remaining from bacteria killed by pasteurization. Impedance monitoring may therefore provide a new means of predicting keeping quality. A poor correlation between plate count methods for enumerating postpasteurization contamination and keeping quality with impedimetric measurements on cream alone has been reported. It is possible, with a reasonable degree of certainty, to determine if cream has suffered postpasteurization contamination within 20 h of production (Griffiths and Phillips, 1984).

A conductance method (the Malthus system) has been used to detect postpasteurization contamination of milk (Lanzanova *et al.,* 1993). Detection time is known to depend on type of organism. While *Enterobacter cloacae* has the shortest detection time of about 4 h, Pseudomonas spp. require as long as 13 h. A good correlation between this system and plate count (97% of positive samples could be detected within 1 h, 98% within 4 h and 99% within 9 h, after 16 h preincubation) coupled with short detection times of within 25 h makes this method advantageous for detecting postpasteurization contamination of milk (Kamei *et al.*, 1988). Table 4.19 shows the comparison of the results obtained by the Malthus system and by the standard plate count method.

A study of milk samples from single animals disclosed a disturbance in the secretion when the conductance exceeded 60×10^{-4} mho, except for milk from cows at the beginning or the end of the lactation period; the disturbance in most cases is caused by a Streptococcal mastitis (Miller, 1943). However the conductance of mixed milk samples varies much less.

4.8.1.3 Microcalorimetry

The use of microcalorimetry to detect microorganisms is based on the principle that microbial growth is accompanied by the evolution of heat. Specialized adiabatic calorimeters and thermal fluxmeters are required for the calibration of microcalorimetric data. One of the most widely used microcalorimeters is the Calvet instrument, which is sensitive to a heat flow of 0.01 cal h^{-1} from a 10 ml sample. This has been used to study the bacterial levels in milk (Berridge *et al.*, 1974).

4.8.1.4 Flow cytometry

There seems to be considerable potential for the use of flow cytometry, in which cells are introduced into the centre of a rapidly moving fluid stream and are forced to flow in a single file and at a uniform speed out of a small orifice. The cells pass a measurement station, where they are illuminated by a light source; measurements can be made at a rate of millions of cells per minute. When a particle in the flow stream passes through the light beam, the illuminating light is scattered by the cells, and the intensity of light scattered at different angles can yield information about cell size, shape, mobility, density and surface structure. In most applications of flow cytometry,

fluorochromes are used to label the cellular components of interest. The fluorescence emitted by these molecules, when excited by an illuminating laser beam, can yield information on the expression of the target molecules within the single cells. Flow sorters are then physically able to isolate the cells of interest. Flow cytometry has been proposed for the detection of *Listeria monocytogenes* in raw milk.

4.8.1.5 Fluorescence

Milk from mastitis udders shows variable fluorescence in more or less dull colours. The yellow fluorescence of normal milk disappears upon acidifying to pH >4 or alkalizing to pH>8. The examination of milk in filtered ultraviolet light is a suitable preliminary test for diseased udders. When a deviation from normal yellow fluorescence is found, it is necessary to make a more detailed microscopic and bacteriological investigation (Schonberg, 1943).

4.8.1.6 Enzymic methods

Enzymes such as catalase serve as markers for detecting the postpasteurization contamination of milk with gram negative bacteria. Preincubation of pasteurized milk for 24 h at 30 °C, after adding penicillin and bile salts at 5 IU (1 **IU**=0.6 µg benzylpenicillin sodium) and 1 mg ml^{-1}, respectively raises the numbers and catalytic activity of gram negative recontaminant bacteria to a point where they can be easily detected by oxygen release from hydrogen peroxide (>10 000 cfu ml^{-1}), while suppressing growthof thermoduric gram-positive bacteria. The limit of detection is reported to be approximately 3 - 4 bacteria /100 ml (Spillmann *et al.,* 1988). Milk characterized by an abnormally high chloride and catalase content, by appreciable sediment and by the presence of many leucocytes and streptococci is an almost certain diagnosis of mastitis (Zollikofer, 1941). In raw milk also, bovine catalase can be separated from microbial catalase using the fact that at pH>9, bovine catalase activity decreases, but that of bacterial origin peaks at pH 11. Under these conditions, counts as low as 10^3 ml^{-1} can be easily measured (Doi *et al.*, 1992).

Oxidase activity is not related to total bacterial count of raw milk, but is related to psychrotropic count except in samples with very high total counts. Oxidase negative milk is known to show a better keeping quality after heat treatment (65 °C for 30 min) compared with oxidase positive milks (Kyla-Siurela and Antila, 1974). In a study on milk samples from various farms, 75% of samples with less than 200 psychrotrophs ml^{-1} were found to be oxidase negative, while all samples with more than 200 000 psychrotrophs ml^{-1} were found to be oxidase positive. The cytochrome oxidase test is known to give the same overall distribution of samples among grades as the methylene blue test, although results are sometimes known to differ among individual samples. A linear relationship between cytochrome oxidase activity and bacterial concentration in the range of 10^3 -10^8 has been reported (Rongvaux-Gaida and Piton-Malleret, 1992).

In organoleptic evaluation, the keeping quality of oxidase positive milks after heat treatment is known to be generally poorer than that of oxidase negative milks (Kyla-Siurela and Antila, 1972).

Diacetyl reductase has been shown to be present in many bacterial cultures such as coliforms, Pseudomonas, Alcaligenes, lactic streptococci and *Aerobacter aerogenes* (Seitz *et al.*, 1963). This enzyme is a potential marker of bacterial contamination in certain dairy products.

Milks with high total or high non-acid former counts can be best recognized by the nitrate reductase test. This test is superior to the dye reduction methods for the determination of quality in milks which under modern conditions of milking and refrigeration usually possess a predominantly non-acid former flora (Rapp and Munch, 1973).

4.8.2 Methods based on the measurement of metabolic intermediates and by-products

4.8.2.1 Pyruvate

The biochemical activity of microorganisms can be detected by measuring increases in the levels of certain metabolites, such as pyruvate, lactate, ammonia and free fatty acids (Grappin and Dromard, 1982). The recovery of these metabolites is almost 100% except for free fatty acids, where recovery is a function of chain length. It increased from 67% for C_4 to 99% for C_{16} fatty acids. The pyruvate values and number of cfus run almost in parallel, as has been shown in an analysis of several milk samples from 33 dairies in the Federal Republic of Germany (Suhren, 1982). Pyruvate in excess of 0.5 ppm is attributable to bacterial activity and is therefore an indication of bacterial contamination incurred since milk production (Heeschen *et al.*, 1974). The estimation of pyruvate is rapid, inexpensive, accurate and can be carried out automatically. Pyruvate contents and bacterial counts of raw milk are known to respond similarly to such conditions as transport, storage and cooling, the correlations being comparable to those with counts made by various methods (Suhren *et al.*, 1978). Both the initial pyruvate level and the increase in pyruvate after storage correlate with the Wisconsin mastitis score, which suggests that somatic cells contribute to the pyruvate content of milk. Determination of pyruvate content may be used to identify milks containing more than 10^6 bacteria ml^{-1}, but it does not give an accurate estimation of bacterial numbers. From an overall relationship, it has been predicted that 4 μg pyruvate ml^{-1} would correspond to a bacterial content of 3.7×10^6 cfu ml^{-1} with 95% confidence limits (Cousins *et al.*, 1981). The pyruvate method however exhibits certain limitations, for instance, the failure of preservatives or freezing techniques to stabilize pyruvate content in milk, the variation of pyruvate levels during growth of different bacterial species, the discrepant results obtained by manual and automatic procedures, low reproducibility (Elbertzhagen, 1977) and the poor correlation between pyruvate

values and bacterial counts. Interference by hydrogen peroxide residues in milk or from the hydrogen peroxide treated packaging material in the pyruvate test is also reported (Skrinjar, 1980). Rates of pyruvate production by pure cultures in steamed milk and by mixed natural flora in pasteurized milk were consistent with rates of growth. Cultures of gram positive organisms generally show an increase in pyruvate concentration with time, with the levels levelling off near 11.0 mg l^{-1}. In the case of gram negative bacteria, there are two basic trends observed. With *E.coli* and *Enterobacter aerogenes*, pyruvate content sharply increases, then decreases and then increases again slowly. In cultures of *Pseudomonas fluorescens* and *P. fragi*, pyruvate content increases and then declines. Psychrotropic bacteria reduce pyruvate concentration to undetectable levels after initially producing >10 mg l^{-1} (Marshall and Harmon, 1978). The decrease in pyruvate concentration is associated with the stationary phase of growth. It appears that when the primary sources of energy are exhausted, pyruvate is utilized by the bacteria. However, this method has justified itself in practice by the improvements its use has brought about in milk quality. There is no better test available for judging the probable storage life of milk for consumption (Heeschen, 1977). Pyruvate measurement is also considered to be a suitable test for quality payment. For grading in connection with quality payment, the following three grades have been suggested in descending order of quality: <1.5 ppm, 1.6–2.5 ppm and >2.6 ppm pyruvate (Hackenschmied, 1978). Equipment for this test is expensive and operational expenses add to the cost. The Nixdorf 8850 data communication system, which is capable of handling about 150 000 individual monthly samples of milk, is being used by the milk control association in Münster, Federal Republic of Germany, since it simultaneously assays for fat, protein, lactose, cell counts and pyruvate concentration. This computerized system provides easy access to information about individual cows (Hildebrandt, 1983).

4.8.2.2 Endotoxins by the Limulus amoebocyte lysate test

The use of the Limulus amoebocyte lysate (LAL) test to detect bacterial endotoxins was first suggested by Levin and Bang who observed that infection of the horseshoe crab (*Limulus polyphemus*) by gram negative bacteria of the genus *Vibrio* resulted in fatal thrombosis, caused by the interaction of an endotoxin produced by the bacteria with a protein on the surface of the blood cells (amoebocytes) of the horseshoe crab. The use of this assay as an assay for microorganisms is based on the observation by Jorgensen *et al.* (1973) and Coates (1977) that minute quantities of endotoxin from the outer membrane of many gram negative bacteria will coagulate an aqueous extract (lysate) of Limulus amoebocyte *in vitro*. The LAL test can be employed in a semi-quantitative manner. Serial dilutions of endotoxins are reacted with a standard LAL reagent, and the samples are assessed after incubation at 37 °C for 1 h. The 'titre' is recorded as the reciprocal of the highest dilution that gels (clots) the reagent or, if greater accuracy is required, the samples can be read in a spectrophotometer at 360

nm. Gram negative bacteria can be detected by the presence of a gel in plates that contain a threshold concentration of lipopolysaccharide (LPS). Plates with a sensitivity to LPS of 0.032 ng ml^{-1}, corresponding to about 300 cfu ml^{-1} are available (Sudi and Heeschen, 1983). The Abott MS-2 microbiology system can automatically determine sequential changes in the optical density of up to 176 samples at 1 or 5 min intervals during one hour; a graphic representation of the optical density changes can be viewed on the cathode ray tube or reproduced on a hard-copy printer (Jorgensen, 1981).

Jay *et al.* (1977) have correlated the half-log cycle mean aerobic plate count with endotoxin content, indicating that the LAL test can be used to make a rapid approximation of microbial numbers. The LAL test has been used to assess the bacteriological quality of raw milk (Mikolajoik, 1983), pasteurized milk (Hansen, 1988; Haska, 1979) and dairy products (Sullivan, 1983).

4.8.2.3 Carbon dioxide by radiometry

Radiometric techniques are based on the microbial uptake and respiration of radiolabelled growth substrates, such as measurement of $^{14}CO_2$ produced by the microbial metabolism of a ^{14}C-labelled substrate incorporated into the culture medium (Levin *et al.*, 1956). The time lag between addition of the labelled substrate and the detection of the $^{14}CO_2$ has an inverse linear relationship to the initial number of organisms present in the sample. The usefulness of $^{14}CO_2$ production from [U-^{14}C]glucose, [U-^{14}C]glutamate and [^{14}C]formate as an index of raw milk quality has been investigated to reveal further modifications in the analytical methodology before radiometry can be applied to determine the bacteriological quality of milk (Cogan and O'Connor, 1977). A step in this direction is measuring carbon dioxide by indirect conductance measurements. This technique monitors the electrical changes in an alkaline solution that are due to ionization of carbon dioxide to carbonate (Ascon-Reyes *et al.*, 1995).

4.8.2.4 ATP determination by bioluminescence

The adenosine 5′triphosphate (ATP) bioluminescence assay has great potential, providing rapid results (Kennedy and Oblinger, 1985). All living cells contain ATP. When a microbial cell dies, ATP production ceases, and any ATP already present is rapidly destroyed by ATPases and phosphatases. In the ATP bioluminescence assay, a chemical agent is used to increase membrane permeability, which allows release of ATP in the extracellular environment. The ATP concentration in the sample can then be measured by the enzyme-controlled bioluminescence reaction of purified firefly luciferase (EC 1.13.12.7), which reacts with luciferin in presence of ATP to produce light. The light intensity is detected by a luminometer that measures very low levels of light. The technique can be used to detect as little as 10^{12} pg ATP in 75 min. The test,

called the Lumac Raw Milk ATP-F test, can be carried out using a kit form, facilitating its utility (Anon, 1990). Another instrument called the Bacto Foss is specially designed for rapid screening of milk from milk tankers. It has a repeatability of <0.3 log units and can analyse samples with total bacterial counts in the range of 10 000–300 000 cfu ml^{-1}. Bactoscan 8000 is yet another instrument for enumeration of microorganisms in milk by automated fluorescence microscopy and can analyse 80 samples per hour and can give a reading for bacterial count in raw milk in 5 min (Andersen, 1990). These tests have been improved to make them user friendly (Reybroeck and Schram, 1995). Payment to farmers based on the bacteriological quality of milk as determined by Bactoscan 8000 has been recommended (Rodriguez-Otero *et al.*, 1993).

This method has been found to be suitable for enumerating microorganisms in fermentation and milk samples (Casaleggro *et al.*, 1982). However, studies on ATP of three bacterial species with respect to temperature, pH and medium have shown that the ATP content of bacteria differs according to species, stage of development and growth conditions; the variation is the greatest in the early stages of development (Corlier *et al.*, 1983).

In determining the bacterial ATP content care must be taken to eliminate ATP from the somatic cells. The time necessary to extract the somatic ATP was reduced by increasing the quantity of apyrase added to milk sample to evolve a 5 min ATP platform test to determine the bacteriological quality of milk. A correlation between ATP (Y) and impedance measurements (x) obtained after analysis of 154 samples is given by the regression equation:

$$Y=4.43-0.16x \qquad [4.17]$$

the correlation coefficient being −0.83 and the standard deviation, 0.22. Correlation with bacterial loads have shown ATP values to distinguish between 'bacteriologically highly loaded' milk and 'normal' milk. A classification according to a limit value of one million bacteria per millilitre on the one hand, and the corresponding ATP value of 4000 RLU (relative light units) on the other, showed about 90% agreement for the samples (Bossuyt, 1982). The test appears to be suitable for checking the bacteriological quality of farm milk on arrival at the dairy factory. Antibiotics in raw milk can also be detected by ATP bioluminescence. In this process, raw milk containing various levels of antibiotics is mixed with sterile broths and inoculated with *Streptococcus thermophilus*. Bacterial ATP is then measured and correlated to the antibiotic levels. It has been found to be sensitive to low levels of penicillin G, streptomycin, chloramphenicol and neomycin, and is suitable for low cost manual instrumentation (Hawronski *et al.*, 1993).

4.8.2.5 D-Amino acids

D-Amino acids are quite common in nature as constituents of bacterial cell walls

(Davies, 1977) and have been reported (Palla *et al.*, 1989) in fermented dairy products. A correlation of D-alanine content with psychrotrophic microbial growth and ammonia (Figure 4.4) was observed in fermented milk products. D-Alanine increases only after microbial growth reaches the stationary phase, and is therefore probably related to bacterial cell wall lysis. The D-amino acids are present in trace amounts and should not be of concern from the viewpoint of nutritional and toxicological problems (Gandolfi *et al.*, 1992).

Enumeration of the microbial count or of the metabolites has also been correlated to the remaining shelf life of a pasteurized or UHT treated milk (Bishop and White, 1985, 1986). For instance, bacterial count related to shelf life can be described by the equation:

$$\text{Shelf life (h)} = \frac{1}{\{0.00621[T-(269.55-0.74CFC_{15}-0.11CFC_{15}^2)]\}} \quad [4.18]$$

where $CFC_{15}=\log_{10}$ count after preincubation at 15 °C for 25 h following counting on milk agar containing cetrimide fucidin cephaloridine (CFC) which promotes growth of pseudomonads and other related bacteria and T=storage temperature in K. This model is shown to be quite accurate. Similarly shelf life of pasteurized milk has been correlated to impedance detection time by equation [4.19] (Bishop *et al.*, 1984):

$$\text{Shelf life (days)} = 0.56 + 1.4\,(\text{IDT}) - 0.032\,(\text{IDT})^2 \quad [4.19]$$

where IDT is impedance detection time of preincubated samples. The correlation of shelf life with microbial conditions under various conditions is summarized in Table 4.20. The tests listed in this table have yet to be studied collaboratively. It is, however,

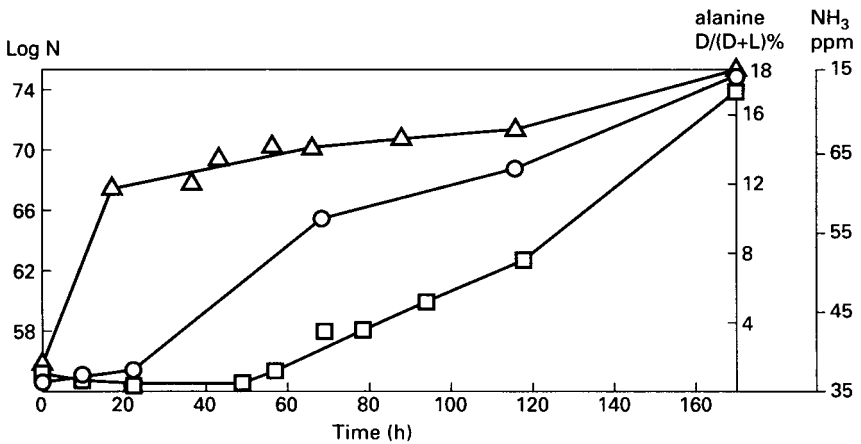


Figure 4.4 D-Alanine ○, ammonia □ and psychrotrophic count Δ in raw cow milk stored at 4 °C. (Source: Gandolfi *et al.*, 1992, reproduced with permission)

Table 4.20 Some methods and tecnniques used for assessing shelf life of pasteurized milk and cream

Method	Correlated to	Correlation coefficient	Principle/performances	Time to complete (days)
Microbial counting				
Without preincubation				
(a)	Flavour related shelf life (FRSL) at 7 °C		Initial psychrotrophie count	2
	skim milk	-0.61		
	whole milk	-0.70		
(b)	Bacterial count related shelf life (BCRSL)=time to reach count of >=log 7.5 ml^{-1}		Initial psychrotrophic bacteria count	2
	at 6 °C	-0.72		
	10°C	-0.62		
With preincubation				
(c) Moseley count	FRSL	-0.77	Incubation of milk at 7 °C/5-7 days then plate count after 48 h at 32 °C	9
(d) Moseley count	FRSL	-0.84		7
(e) Moseley count	Impedance detection time of preincubated samples	0.71		
(f) Preincubation with selective agents at one temperature	FRSL at 7 °C		Preincubation in selective media 18 h/21 °C, plate count after 25 h/21 °C	2
	medium with			
	benalkonium chloride	-0.89		
	or with crystal violet	-0.88		
(g) Preincubation with selective agents at one temperature	BCRSL at 6 °C, shelf life= time to reach count log 7 ml^{-1}		Preincubation 21 °C/25 h in milk containing crystal violet, nisin and penicillin plate count after 25 h/21 °C	2
	milk	-0.82		
	cream	-0.76		
(h) Preincubation in milk, count on milk agar with selective agents to predict shelf life at any temperature up to 1 day accuracy	Correlation at 2-14 °C between BCRSL and T_0	-0.6-0.8	Preincubation at 12 °C, 15°C, 18°C and 21°C in milk, count on 3 different selective milk agar media. Depends on temperature, for 37-89% of samples stored at 2-14 °C shelf life predicted with 15 °C	2.2
Non-enumerative methods				
Related to microbial growth and/or microbial activity				

Table 4.20 (cont.)

Without preincubation				
(i) Impedance detection time	BCRSL at 6 °C see (g) cream	 0.62–0.82	Impedance testing in milk containing selective inhibitors during incubation on milk agar	within 0.5–2 days
(j) Lipopolysaccharide (endotoxin) content in centrifuged down cells	FRSL at 7 °C combined whole/skim milk	 −0.89		
(k) Proteinase activity	Flavour scores skim milk at 4.5 °C skim milk at 7 °C whole milk at 4.5 °C whole milk at 7 °C	 −0.93 −0.92 −0.76 −0.94	Determination of proteinase activity with fluorescamine method after incubation 72 h at 37 °C with microbial growth inhibitor	3.5
(l) Proteinase activity	FRSL at 7 °C whole milk combined whole/skim milk	 −0.51 −0.47	Determination of proteinase activity with *o*-phtaldialdehyde method	1
With preincubation				
(m) ATP content	BCRSL at 6 °C see (g) milk double cream	 −0.79	 Preincubation, see (g) −0.81+measurement of ATP by bioluminescence	1.2
(n) Cytochrome C oxidase activity	BCRSL, shelf life= time to reach count log 6.3 at 5 °C at 10 °C	 −0.89 −0.84	Preincubation 20 °C/18 h in milk with benzalkonium chloride	1.2
(o) Catalase activity	FRSL at 7 °C	−0.79	Preincubation, see (f) +catalase detection with catalasemeter	1.2
Impedance detection				
(p) Without selective agents	last day of use=time at which preincubated samples have impedance detection time <or=2 h	0.88	Preincubation 24 h/18 °C of milk and plate count broth (1:1) impedance testing of 21 °C on milk or plate count agar	2
(r) Without selective agents	FRSL at 7 °C combined skim/whole milk	 0.91	Preincubation 18 h/18 °C +impedance testing, see (p)	within 2

Table 4.20 (cont.)

(s) With selective agents	FRSL at 7 °C medium with benzalkonium chloride medium with crystal violet	0.89 0.91	Preincubation, see (i) +impedance testing, see (p)	within 2

Source: Stepaniak, 1991 (reproduced with permission).

unlikely that a single procedure could be designed to encompass the contribution of all factors influencing keeping quality of pasteurized or UHT milk, the rational design relying on most critical parameters (Stepaniak, 1991).

4.9 Indices of aesthetic quality of dairy products

4.9.1 Sediment

In addition to the microbiological tests, certain tests are commonly conducted which are concerned with aesthetic considerations. Tests for sediment in milk are usually applied to incoming raw milks. Dirt has no place in milk, hence, its presence indicates carelessness in milking or handling.

Sediment tests are occasionally run on bottled milks. Sediment tests for churning cream are also made by enforcement agencies. It is a useful additional criterion of churning cream quality (Nelson *et al.*, 1943). Milk for cheese making may contain appreciable amounts of sediment, which is concentrated approximately eleven fold during the process.

4.9.2 Decomposition

Tests for decomposition may also be regarded with aesthetic considerations. Tests for water insoluble acids and butyric acid in butter are relevant (Hillig, 1947). Lipolysis in churning cream caused by growth of mould or lipolytic bacteria is reflected in higher values for these compounds in butter, and such evidence may be used by enforcement agencies to take legal action.

The souring of milk and cream may also be regarded as a form of decomposition. Cases are known where even certified milk with counts under 10 000 ml^{-1} has been threatened because the titratable acidity was high. The alcohol test, widely used to determine the suitability of milk for condensing, is primarily a reflection of acid development.

4.9.3 Mastitis

While mastitis is primarily a problem of animal health and occasionally is a factor in

the transmission of human pathogenic organisms, the inclusion in the milk supply of the secretion from infected udders may also be regarded as an aesthetic problem. No milk can be considered to be of satisfactory quality if drawn from diseased udders. When the leucocyte count exceeds 100 000 ml^{-1}, the solids-not-fat content is correspondingly reduced (McKenzie *et al.*, 1958). A count in herd milk in excess of 500 000 ml^{-1} strongly suggests an appreciable number of infected udders in the herd. The ability of resazurin to reflect the weak reducing activity of leucocytes can be used to indicate those herds in need of examination. The efficacies of four indirect tests for subclinical mastitis, that is the modified California mastitis test (MCMT), the bromothymol blue mastitis indicator card test (BTB), the modified Whiteside test (MWT) and the modified Aulendorfer mastitis probe (MAMP), have been studied. Samples giving positive results were 84.9%, 75.6%, 73.9% and 72.3% for MAMP, MCMT, BTB and MWT, respectively. MAMP is thus superior for the detection of subclinical mastitis (Buragohain and Dutta, 1991).

In yet another test proposed to detect mastitis, 1 ml sample from individual cows should be mixed with 2% aqueous mastoprim preparation in a plastic plate, with stirring for 10–15 s. Formation of a precipitate is considered as a positive test. If the test is positive, it is repeated with 1% mastoprim solution. The use of this method prevents admixture of mastitis milk to bulk milk, whilst concurrently permitting the identification of diseased quarters (Butkus and Butkene, 1977). Higher alkalinity of ash is also indicative of mastitis (Hamazawi and Hafez, 1992).

4.10 Quality of cheese

Cheese maturity and quality are traditionally evaluated by sensory analysis, according to standard specifications. A series of complex physical, chemical and microbiological changes are known to affect the principal components of cheese. Quality control in different dairies is generally performed by different judges. This traditional judgement of cheese is not sufficient to describe cheese maturation and quality. Cheeses which are not in accordance with the standard are not given any further qualitative description. Cheeses which are unsatisfactory, however, are given a description of defects by a given nomenclature. It is necessary to understand the technological, chemical and microbial information about cheese production to correlate it with precise sensoric description.

Multivariate data analysis is helpful in systematizing information on problems of such complex nature. Principal component analysis (PCA) using flavour and texture variables is reported to be a good tool for manifesting differences in the properties of cheese produced in different dairies. From multivariate data, easily interpreted models can be made which, in combination with knowledge about chemical, microbial and technological conditions, can be easily used in quality assurance (Duus *et al.*,1987).

Organic acids could appear directly as a result of hydrolysis of fats, normal bovine metabolic processes, bacterial growth or direct addition of acidulants. Each of the

organic acids presents a characteristic pattern of change during ripening. Establishing the free fatty acid profile is of great interest in determining relationships with sensory characteristics of cheeses, particularly in those that undergo a high degree of lipolysis (Angel de la Fuente *et al.*, 1993). Unknown samples can be classified according to their age by stepwise discriminant analysis, based on the organic acids content. Regression analysis can estimate the ripening time of the samples according to their levels of the acids – formic, orotic, lactic, uric and butyric (Bevilacqua and Califano, 1992).

Formol ripening indices have been developed for testing the ripening degree of cheeses, and appear to be a useful indicator, the formol titer increasing steadily with increasing age of the cheese (Balatoni and Bakos, 1958; Szonntag, 1958; Tawab and Hofi, 1966). Free amino acids, as measured by Cd-ninhydrin reagent is a good indicator of the degree of ripening (Folkertsma and Fox, 1992). Sensors responding to pH, temperature, conductivity or pressure and which can give signals through a cable or ultra high frequency (UHF) transmitter have also been developed to monitor the extent of cheese ripening (Blanc, 1978).

It should be emphasized that conclusions about the quality of the milk supply should be drawn only after examining a series of samples, not on the basis of a single sample. Because of appreciable differences in bacteriological content from day to day, it is desirable that testing be carried out as frequently as possible. The least expensive dye reduction tests have an advantage. No one method can furnish all the information required in quality control. In addition to the total bacterial populations, tests for thermoduric organisms and for excessive leucocyte counts are essential to the control of raw milk samples. For pasteurized milk, the coliform test is more valuable than SPC in indicating the care taken to minimize recontamination.

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Chapter 5

Meat, Fish and Poultry

5.1 Introduction

The major meats of commerce are beef derived from beef-cattle, pork from pigs, poultry meat from chicken and mutton from sheep and goats. In the developed countries, beef-cattle varieties have been systematically developed over decades, grown specifically for this purpose and slaughtered at the stage when the feed efficiency is at the maximum. Beef derived from culled dairy cattle or farm cattle used for ploughing or for pulling carts is marketed in Asian countries. Of late, beef from water buffaloes is finding increasing use especially in the West Asian region. Poultry meat may include turkey and duck meat. There are serveral varieties of animals and birds in these categories that differ in growth rates, eating qualities and regional and ethnic taste preferences although the trend is towards narrwoing these gaps by using a few stocks derived in the West for scientific cultivation the world over. The only exception is the abstinence from beef or pork on religious grounds or vegetarianism of various shades. Apart from these major sources, exotic animals and birds are finding increasing consumers the world over. Thus, rabbit, deer, bison, wild boar, partridge, crane, frogs and snails have emerged as occasional, choice meat sources.

With the increasing price of commercial meats, the prevalence of incidents of adulteration with cheaper flesh have been on the rise. This is easy in the case of meat products based on comminuted meats such as sausage goods, burgers, patties, etc. This has necessitated development of methods for the detection and quantitation of such admixtures. In the meat industry, the inclusion of soy flour or protein concentrates or isolates is now permissible. Methods of detecting their presence and possibly their content are being actively developed.

The eating quality of beef, pork, mutton and poultry meat varies widely depending on variety, age, sex, region where grown, feed composition, additives to feed, farm and veterinary practices, mode of slaughter, post-mortem handling, storage and such other factors. Organoleptic acceptability is, ultimately, the sole criterion for the price. This therefore prompts the admixture of expensive meats with cheaper varieties. It is a challenge to the analytical chemist to develop methods for detection of such admixtures.

Animal husbandry practices have undergone radical changes during recent years with intensive knowledge of animal physiology, nutrition and pathology. It is known that feed efficiency can be controlled with the use of antibiotics, hormones and

analogous chemicals. In a highly competitive era of marketing and export of meat and meat products, application of the latest scientific knowledge assumes importance. With the discovery of the possibilities of some of these feed additives proving deleterious to consumers, their use has been controlled by public health authorities as these may find entry into meat, eggs or milk. Tolerances for these chemicals have been fixed. This necessitates methods for detecting and estimating such feed additives in meat and meat products.

Many instances could be cited from the literature on meat adulteration from different parts of the world (Patterson, 1985). In Kenya, poaching and substitution of domestic animal meat with game meat has been of considerable concern. In Australia, there have been cases where kangaroo and horse meat have been used in small proportions in exported beef. In Germany, about 50% of the venison supply is imported. Within this supply, meat from less well known non-European species of ground game, for instance African antelope species and kangaroo, is rising. A number of frauds have been observed in which this cheaper venison has been offered as native deer venison. There have also been cases where meats from other animals have been offered for sale as goat meat. Chicken and pork are frequently substituted in beef sausages. Some meat product manufacturers have been accused of adding non-meat nitrogenous ingredients in order to deceive analysts who use nitrogen content as a measure of protein content (Woollen, 1981).

Identification of species origin of meat is of considerable importance in forensic medicine and in the quality control of animal products. Beginning in January 1990 the United States Department of Agriculture (USDA) specifications require the 'species identification' test to be carried out in all animal products manufactured for export (Mezel-Dudonis and Gyorei, 1991), and species of origin has been the subject of many international conferences (Krol *et al.*, 1988) and discussions (Kotter, 1974). The various meat adulterants, analytical methods to detect these and the merits and limitations of one analytical method over the other are reviewed in this chapter.

5.2 Identification of meat species

The various techniques for distinguishing meats belonging to different animal species are based on the examination of muscle extracts. Raw meat or meat product is homogenized and extracted with water or dilute salt solution, filtered and/or centrifuged to obtain a complex solution of soluble components comprising mainly proteins from cell sarcoplasm and residual blood. These are broadly representative of the meat species. Methods for identification (Billington, 1988; Patterson, 1985) of animal species of meats or meat products are reviewed here.

5.2.1 Electrophoretic techniques

Electrophoretic techniques are the most frequently used as a specific spectrum of soluble protein bands is produced for each animal species. The band patterns in a

supporting gel are visualized by simple non-specific staining or by enzymological or immunological methods. Separation can be governed by the use of homogenous gels, concentration gradient gels, pH gradient gels or denaturants such as urea or detergents that dissociate the tertiary protein structure (Patterson, 1985). Occasionally, in regulatory or standards work, it is necessary to have analytical methods to identify fish species. This has assumed importance in international as well as in internal trade due to labelling requirements. Electrophoresis of water soluble flesh proteins in polyacrylamide gels yields useful data. The extraction of proteins from heat processed samples normally requires the aid of solubilizing agents. For instance, cooked horse meat and beef can be distinguished electrophoretically after extraction into 8 M urea, 'renaturation' by dialysis, separation in concentration gradient gels and visualization by specific determination of renatured enzyme activity, for example, adenylate or creatine kinase. Similarly soy proteins can be distinguished from meat proteins by extracting with 8 M urea or sodium dodecylsulphate (Hofmann and Penny, 1971) and separating the soy proteins from the soluble meat proteins by starch or polyacrylamide gel electrophoresis (Fischer and Belitz, 1971; Freimuth and Krause, 1970a; Parsons and Lawrie, 1972). Quantitative results can be obtained for meat pie fillings and canned meat loaf which have been autoclaved at 115 °C for 40 min. This is possible by extraction in 8 M urea and 1% 2-mercaptoethanol at 18–20 °C for 16 h, followed by separation on gels containing 6% polyacrylamide (Guy *et al.*, 1973). The action of urea is to split the hydrogen and other secondary bonds that are formed between the polypeptide chains during heat coagulation. During autoclaving or canning of fish, additional bonds form that are probably covalent in character and in such cases urea is no longer capable of extracting sufficient amounts of protein. In such a situation, splitting at the methionine residue can be achieved by using cyanogen bromide, which liberates a sufficient number of large polypeptides which can be successfully identified by electrophoresis (Connell, 1973; Anon, 1988b; Hofmann, 1988).

Soy ingredients in meat products (Lee *et al.*, 1975; Parsons and Lawrie, 1977) as well as milk protein concentrates (Zhuravskaya and Tropina, 1988) and egg white (Haave, 1977) can also be determined by this method. The various types of electrophoretic techniques are explained here.

5.2.1.1 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) (Malmheden, 1986; Anon, 1988c; Patterson, 1985; Freimuth *et al.*, 1970) is carried out on 'cellogel' strips. The procedure consists of three main steps: extraction, electrophoretic migration and staining–destaining. The stain applied is amido black. Several bands in blue will be observed on the cellogel strip and will differ in width and intensity of staining. They are located transversely on the cellogel and represent the migration of different protein fractions. For a given animal species, the distribution of the bands and their intensity

of staining is stable and characteristic, as examined by the naked eye. By reading the destained strips with a photometric integrator, a pherogram is obtained showing different peaks in which the base width of the bands on the gel and the length are proportional to the intensity of staining and hence enables quantitation of the admixture (Freimuth and Krause, 1970b). These curves are species specific. This method can be applied safely to fresh or frozen meat (Lemos and Moraes, 1992).

5.2.1.2 Polyacrylamide gel isoelectric focusing

Polyacrylamide gel isoelectric focussing (PAGIF) (Patterson, 1985; Malmheden, 1986; Wreede *et al.*, 1982; Collins, 1986) is based on the electrophoretic migration of proteins in a pH gradient; migration stops when the proteins reach a pH corresponding to their isoelectric points. This technique has given good results with beef, pork, mutton, lamb, horsemeat and venison (Anon, 1988b), and has also successfully indicated the species of origin of raw and cooked shrimp (An *et al.*, 1988), fish (Plowman and Herbert, 1992) and fillets of shark meat after cooking in oil (Yeow *et al.*, 1986). Heat treatment at varying intensities and sodium chloride or nitrite curing do not interfere with the results, although the sharpness and intensity of the bands do decrease with severity of heat treatment. The bands characterizing the animal species appear mainly in the alkaline region of the isoelectric focusing pattern. Bands in the acidic region are of value for the assessment of the intensity of the heat treatment (Hofmann and Bluchel, 1992). Myosin light chains, MLC1 and MLC3, found in the alkaline regions can reliably distinguish 5% added beef to pork. A linear relationship, $y=1.2804+0.9046\ x$, between the percentage beef in the mixture, x, and the percentage of beef MLC3, y, has been calculated in its admixture with pork (Luccia *et al.*, 1992). Accurate identification requires comparison of the unknown sample with a known reference sample on the same gel (Hofmann, 1989). Fish species of surimi prepared from a variety of different fish can be identified by peptide mapping of the myosin heavy chain (Rehbien, 1992).

Egg white, used as a binder can be detected in meat products by extraction of the ovomucoid and subjecting the sample to PAGE (Freimuth *et al.*, 1970). Soy protein in meal or isolate can also be determined by PAGIF (Llewellyn and Flaherty, 1976), since the band of soy glycinine is distinctly separated from muscle protein bands.

With this technique, it has been possible to differentiate between meat from the South African antelope and impala from the meat of the roebuck and national deer, and heated beef from the meat of roe deer, red deer, chamois and springbok (Jemmi and Schlosser, 1991). PAGIF can also be applied to game, poultry, other domestic animals and fish. By staining isoelectric focusing gels for particular enzymes (Collins, 1986) like adenylate kinase, it is possible to distinguish between kangaroo and horse meat in beef and peroxidase staining of the myoglobin bands can confirm adulteration of beef goulash with pork (Hofmann *et al.*, 1991). Enzyme stains such as esterase and pseudoperoxidase (Anon, 1988a; Federal Republic of Germany, 1988) distinguish

between some species not differentiated by general proteins; phosphogluconate dehydrogenase distinguishes between sheep (King and Kurth, 1982) and goats and lactate dehydrogenase isozyme is typical for buffalo meat (Gleeson *et al.*, 1983).

Ultrathin layer isoelectric focusing in mini gels is a modification of the above method (Patterson, 1985). This method requires less time, is less expensive and is a good alternative to immunochemical methods. In 90% of cases, the test is sufficient to establish the species.

Since the International Whaling Commission decided in 1982 to prohibit commercial whaling, a conventional identification of seafood meat is required to check for the use of whale meat. Electrophoretic analysis of enzymes in meat and other tissues is a reliable method of identifying whale species (Wada, 1988). Isoelectric focusing has been shown to be a useful and reliable method for taxonomic identification of seafood products (Lundstrom, 1980, 1981, 1983; Taniguchi *et al.*, 1982; Yamada and Suzuki, 1982; Ukishima *et al.*, 1984) and thin layer isoelectric focusing for the identification of whale species. The values are applicable for identification without the need for an authentic standard sample.

5.2.1.3 Polyacrylamide gel electrophoresis – sodium dodecyl sulphate

When using sodium dodecyl sulphate (SDS) with PAGE (Thoren, 1978; Zerifi *et al.*, 1991a, 1991b) in the presence of a reducing agent, 2-mercaptoethanol, proteins migrate in relation to their molecular weight. Protein patterns are therefore interpreted from mobilities relative to molecular weight. PAGE–SDS (pH 3–10) (Kim, 1989) can be used for differentiation of meat from cattle, sheep, lambs, deer and rabbits. It is also applicable to the determination of soybean protein in fresh and cooked soymeat blends, and for identifying meats from raw and cooked crustacean species such as shrimp, prawns and crab. Regression equations quantifying non-meat proteins such as soybean protein or wheat gluten in cooked beef or pork are available (Carrion and Valencia, 1990a, 1990b). The method is applicable to samples heated up to 100 °C, beyond which most of the protein bands disappear. Protein bands with molecular weights lower than 30 kDa are the distinguishing features (Civera and Parisi, 1991). This method also gives a band characterizing milk protein in sausages, the detection limit being 0.5% (Feigl, 1991).

5.2.2 Immunological techniques

Immunological techniques are discussed by Paraf *et al.* (1990), Oswald (1953), Brandly (1954), Anon (1988b), Hofmann (1988) and Billett *et al.* (1996). The ideal immunochemical reaction for the determination of a specific adulterant in extracts of meat products is one which employs an antiserum with antibodies to a specific protein of that species. For instance, antisera to myoglobins (Mb) isolated from ovine, porcine and equine muscles have been used in agar gel diffusion experiments to determine the

presence of flesh from these species in beef products. Rabbit antiovine Mb serum, goat antiporcine Mb serum and rabbit antiequine Mb serum made monospecific by immunoadsorption (Hayden, 1980) can detect lamb, pork and horse meat in fresh ground beef at 3% levels of admixture. Goat antiporcine Mb serum can also detect pork at 10% levels in beef heated at 70 °C (Hayden, 1979). The method is applicable to both fresh and cooked meats (Hashimoto and Yasui, 1957). Soy protein (Baudner, 1977) in heated meat products can also be determined by this technique (Krueger and Grossklaus, 1970). The analysis is simple and the interpretation of results is easier for such systems (Hayden, 1977). The immunological methods also include various enzyme linked immunosorbent assays and precipitating tests in tubes or in double diffusion agar gels.

In most single immunodiffusion methods, the antigen is allowed to diffuse into a gel in which antibodies have been uniformly dispersed. As the antigen encounters the antibody in the gel, it will form a precipitin band (Pike and Sulkin, 1957). This has been found to be useful in detecting lower value white fish in high value crustacean tailmeat (Taylor and Leighton Jones, 1992a) as well as soy protein, hydrolysed milk proteins and ovalbumin, and is suggested to be suitable for routine testing (Kraack, 1973).

In double immunodiffusion methods (Curtis and Chase, 1971), antigens and antibodies are placed in different regions of the gel and allowed to diffuse towards one another. A precipitin band will occur in that region of the gel where the appropriate antigen–antibody complexes are formed. Since the rates of diffusion of antigens and antibodies are dependent on their diffusion coefficient and on their concentrations, precipitin bands of different antigen–antibody complexes can occur independently of one another at different regions in the gel. Horse, pork and chicken meats can be detected by double immunodiffusion as adulterants of bovine meat. Detection limits for this method were 5% for horse meat and 20% for pork and chicken (Beirao Catarino and Cardos Pereira, 1988). Both single and double immunodiffusion methods therefore have the ability to resolve mixtures of antigens. Since some precipitin bands may overlap, the number of precipitin bands formed in immunodiffusion represents the minimum number of different antigens in the solution that can be detected by the antiserum. Various modifications of the techniques are described here.

5.2.2.1 Precipitin reaction

The precipitin reaction for species identification has been discussed by Etzler (1985) and Victor *et al.* (1970). A typical method involves the preparation of a precipitin curve, usually by adding increasing amounts of antigen to a series of tubes containing a constant quantity of antiserum (Kaplan and Buck, 1951). Ethanol precipitated fractions from sera of ox, buffalo, goat and deer have been used to prepare antisera for the detection of flesh from these species in meat products by immunological methods. The amount of precipitate formed is quantitatively analysed for nitrogen or

protein. The precipitin curve then serves as a standard curve for the determination of amounts of the unknown antigen in the solution (Tammemagi, 1954b). Investigators have also reported immunological and serological methods for the detection of 'added' serum in fresh and mildly heated meat products (Herrmann *et al.*, 1973; Kraack, 1973). Serum albumin is the major protein detected in each case (Hayden, 1978). Gamma globulin isolated from species-specific rabbit antiserum is known to give a positive precipitin reaction when mixed with heated extract of skeletal muscle of beef, horse and pork. It does not give any cross reactions between meat samples from different species, is rapid, requiring only 1–2 h compared with 3 days for double gel diffusion and is also much more sensitive than double gel diffusion (Helm *et al.*, 1971). Knowledge of the history of the processed meat product is important because long heat treatments may change the precipitin reaction from strongly positive to negative (Weinstock, 1953; Tammemagi, 1954a). Chicken egg white in meat products has been successfully detected by this method (Victor *et al.*, 1970).

5.2.2.2 Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) (Bjorkroth, 1992; Sauer *et al.*, 1985) encompasses all solid-phase immunoassays using enzyme labelled reagents, but is most commonly used to describe non-competitive solid-phase sandwich assays. In contrast to enzyme immunoassay, there is no element of competition and the amount of enzyme label bound is directly proportional to the concentration of the analytical solid phase reagents used for separation of free label from bound label which also facilitates the removal of excess reagent after each stage. There are many variants of the ELISA procedure, the majority of which use enzyme-labelled antibody as tracer. Peroxidase (Martin *et al.*, 1988), alkaline phosphatase or glucose oxidase are used to label the antigen or antibody. ELISA kits developed by Commonwealth serum laboratories in Australia can identify uncooked beef, horse, kangaroo, sheep, goat, pig, camel, buffalo and donkey meats and also detect 1% contamination of one meat species in another. The test uses urease and substrate solution consisting of urea and bromocresol purple pH indicator, giving advantages for field-type applications (Pelly and Tindle, 1987). In surimi, arginine kinase serves as a good marker for crustaceans and limpet paramyosin for molluscs (Verrez *et al.*, 1992). The detection efficiency is 10–25 g of crab flesh per kilogram of surimi-based product (Verrez-Bagnis and Escriche-Roberto, 1993). Wells in polyvinyl chloride or polystyrene microtitre plates are mostly used for immobilization. ELISA response can also be amplified by an avidin–biotin system to detect whey in meat products (Demeulemester *et al.*, 1990, 1991), with sensitivity levels as low as 0.7 g kg^{-1} whey. Besides ELISA, a dot-blot technique could also detect whey protein in liver pâté at >6.5% levels, or crab flesh in heated and sterilized surimi-based products (Verrez-Bagnis and Escriche-Roberto, 1993). Its sensitivity can be further improved by use of nitrocellulose membranes activated with cyanogen bromide. Even though in heat treated samples whey protein can also be detected,

sensitivity is probably greater for raw meat samples (Abramowski *et al.*, 1990). Production of monoclonal antibodies against red snapper protein using a hybridoma technique has been used in ELISA to identify correctly red snapper (*Lutjanus campechanus*) from amongst 24 various seafoods and meat extracts (Huang *et al.*, 1995).

Techniques are direct or indirect. The direct techniques involve the use of one set of antibodies, usually raised in rabbits for the antigen to be studied. The indirect techniques involve the use of a second set of antibodies raised in goats and specific for rabbit IgG immunoglobins. An indirect ELISA procedure has been applied to detect heated pig meats in a wide variety of meat materials and products, however, it did not prove to be totally satisfactory, since a significant variation in the response of individual pig muscles was observed (Patterson and Jones, 1989). A combination of antiporcine sera raised in sheep and the ELISA method can be used to detect a low percentage of pork even in heat processed meats (120 °C for 30 min) (Sawaya *et al.*, 1990a). Indirect ELISA has also been shown to be useful in identifying the species of canned sardines (Taylor and Leighton Jones, 1992b) and horse meat in raw meat mixtures (Garcia *et al.*, 1994). ELISA is the preferred analytical method since first, the test can be performed in 3 h, second, it can detect at least 10% contamination of one species by another and it is extremely sensitive (<1 mg of meat can be technically identified) and third, less species-specific antisera and even low purity antisera could be utilized, resulting in significant reduction in the cost per test (Whittaker *et al.*, 1983; Slattery and Sinclair, 1983).

Indirect ELISA is the simplest and most versatile method because meat extract components are directly bound on the solid phase. ELISA as well as PAP (peroxidase–antiperoxidase) tests can be employed for identification of raw, partially cooked and boiled meat extracts using species-specific antisera to thermostable adrenal preparations. PAP is more sensitive and gives more consistent results compared with ELISA. A double antibody sandwich technique which has an unlabelled antispecies antibody coated on a microtitre plate has also been used to detect pork and poultry in cooked meat products. After incubation with the sample to be analysed, a second antibody labelled with biotin is added and the reaction is amplified with a streptavidin horseradish peroxidase conjugate and then developed with 2,2′-azinobis-3-ethylbenzthiazoline-6-sulphonic acid. The technique recognizes heat-resistant antigens in simple aqueous extract meat products, and can accurately differentiate between different meat components. Furthermore, there is no interference from product ingredients (Andrews *et al.*, 1992).

A peptide fragment purified from the tryptic digest of autoclaved glycinin has antigenicity against antiglycinin (soybean 11 S globulin), and can be used as an indicator antigen for soy proteins (Yasumoto *et al.*, 1990). An ELISA test kit, the Biokis Soya Protein Assay, has been made available to detect soy protein foods in soy flour (raw and heated at 100 °C), and samples of sausage emulsion containing 2% soy flour and 1% soy protein concentrate or isolate.

5.2.2.3 Enzyme immunoassay

Enzyme immunoassay (EIA) is based on the 'saturation assay' principle in which the analyte is enzyme labelled. The assay comprises three components:

1 a limited and constant quantity of antiserum specific to the analyte,
2 a limited and constant quantity of the enzyme labelled analyte, and
3 standard quantities of analyte for calibration purposes (or unknown quantities of analyte in the test sample).

When the three components of the system are mixed, labelled and unlabelled analytes compete for the limited number of antibody binding sites. Separation of free labelled analyte from that bound to antibody and subsequent addition of substrate to the bound fraction allows enzyme activity to be measured and the degree of competition to be ascertained. The greater the quantity of analyte present, the fainter will be the colour produced. The use of a standard amount of analyte enables calibration curves to be constructed and quantification of analyte. Monoclonal antibodies to meat speciation have potential applications in solving problems of meat adulteration, but the prohibitive costs of producing them are not justifiable until muscle-specific thermally stable antigens of good quality are available (Jones and Patterson, 1988).

5.2.2.4 Counter immunoelectrophoresis

Counter immunoelectrophoresis (CIE) is based on the principle of crossover electrophoresis. If two wells are cut a short distance apart in an alkaline gel (pH 8.6), with meat extracts in the well nearest the cathode and specific-species antiserum in the other well, during electrophoresis, proteins from the extract move towards the cathode and the antibodies in the antisera move towards the anode. If meat and antiserum are homologous, a precipitin line is formed at their junction. The sensitivity of the method ranges from 1/20 000 for the whole lysed blood to 1/300 for samples such as crushed bone, intestines or extremely fatty meat.

5.2.3 Other techniques

Simple correlations between gross composition of meat samples and adulterant vegetable sources such as soybeans can be used with a detection sensitivity of 1%. One such approach is shown in Table 5.1.

Other approaches to detect admixtures in meat are discussed here.

5.2.3.1 Acid phosphatase test as a probe

Acid phosphatase is an enzyme and is present in all forms of life. Attempts have been made to determine the genuineness of meat samples, based on appreciable difference

Table 5.1 Linear regression equations for the mixing ratio (X) of whole soybeans mixed with meat and gross composition (Y)

Item	Regression equation	Correlation coefficient[a]
Proteins	$Y=189.1-3.43X$	−0.95
Lipids	–	0.67
Total sugars	$Y=-54.29+4.92X$	0.96
Reducing sugars	$Y=-0.08+58.02X$	0.99
Non-reducing sugars	$Y=-70.87+103.15X$	0.99
Fibre	$Y=0.19+14.27X$	0.99

[a]Significance at 1% level.
Source: Farag *et al.*, 1986 (reproduced with permission).

in the activity of the enzyme in different meat species (Sarkar and Chaudhuri, 1983), for example the significant difference in enzyme activity between beef and goat meat shows that on admixture, the activity of acid phosphatase should alter accordingly and this can provide an important clue in elucidating adulteration of goat meat with beef. However, it has been noticed that the enzyme activity falls slowly and steadily with age and therefore would not hold good for stored meats.

5.2.3.2 Pentoses and pentosans

Selective analysis of carbohydrates is particularly suitable for detecting soy proteins in meat (Flint and Lewin, 1976). The amount of pentoses in meat is very small (up to 0.1%). As soon as cereals, vegetables, spices and the like are added to meat or meat products, carbohydrates (Farag *et al.*, 1986) and in particular the content of pentoses, mainly as pentosans rises markedly (Bianchi *et al.*, 1981; Fredholm, 1967). This is especially the case when soy meal is added.

5.2.3.3 Specific peptide analysis (Agater et al., *1986)*

Although amino acids in proteins are drawn from amongst the same 18 amino acids normally, two methylated derivatives do occur in some products, such as lean meat, in minute quantities. These are 3-methylhistidine and *N*-methyllysine and they may be used as an index of lean meat content in meat products (Lawrie, 1988; White, 1988; Fuchs and Kuivinen, 1989). While prime cuts in a selection of beef, pork and chicken are reported to yield a consistent content of *N*-methylhistidine (111–130 $\mu g\ g^{-1}$), manufacturing cuts and offals exhibit considerable variations. *N*-methylhistidine is therefore of limited value in detection of illegal addition of foreign proteins in meat products (Ronnestad, 1988; Jones *et al.*, 1988).

Skeletal muscle is known to contain the histidine dipeptides anserine (β-alanyl-L-1-

methylhistidine), carnosine (β-alanyl-L-histidine) and balenine (β-alanyl-L-3-methylhistidine). As the three peptides are present in amounts and ratios that seem to be characteristic for each species (Crush, 1970; Carnegie *et al.*, 1983) and appear to be unaffected by cooking, they are potentially useful for determining the species of origin of meat in cooked products. The proportion of carnosine (*C*) to anserine (*A*) or of balenine (*B*) to anserine can be valuable for detection of pork in processed meat, or for differentiation of red deer from other species (Plowman and Close, 1988). The *C*/*A* ratio can indicate the presence of meats for which this value is very low, for example sheep, chicken, kangaroo and rabbit (Carnegie *et al.*, 1986). This ratio for products made only from beef should be within 5.2 to 7.2. Lower values indicate higher anserine content in meat species such as kangaroo, rabbit and mutton, whereas higher values indicate the presence of horse meat. Balenine is found in significant amounts in red deer. Other species of deer such as blackbuck, chinese water deer and white tailed deer do not contain balenine in this amount and hence enable its identification. Dipeptides and amino acids have been singled out as characteristic components indicative of yeast, meat and connective tissue extracts used in soup manufacture with creatinine, hydroxyproline and nucleotides and purines serving as complementary evidence (Sulser *et al.*, 1973).

A simple quantitative and sensitive method for the determination of chicken or turkey in a blend uses liquid chromatography. The method involves extraction of water soluble proteins followed by injection into the LC system. This method also applies to frozen chicken and turkey, but not to heat treated samples. Chicken and turkey chromatograms have major specific peaks which can be used for species identification (Ashoor *et al.*, 1988). Thus a standard curve for chicken in turkey mixture can be constructed using a specific peak for chicken with a relative retention time of 1.16 and one for turkey with a relative retention time of 1.10. The method is reproducible, can be applied over a range of 5–100% chicken in turkey in the fresh or frozen state and does not suffer serious interference from the presence of other common meats such as beef or pork (Ashoor and Osman, 1988).

Species-specific protein profiles of raw fish have been documented by Ashoor and Knox (1985) and further evaluated by Armstrong *et al.* (1992). Inter- as well as intraspecies variations can be identified by this method. The method relies on visual comparison of a star-symbol plot constructed from the sample's HPLC profile with that obtained from the mean profile data of a morphologically identified species. The profile is unaffected by season and location, and irrespective of whether they are raw, gamma-irradiated or dried with infrared radiation. The analysis involves a 10 min extraction followed by a 60 min analysis. A library of plots, so obtained eliminates the use of an internal standard. HPLC profiles have also been used to detect whey mixtures in animal meats (Frutos *et al.*, 1991).

Table 5.2 Fatty acid composition of the principal lipid fractions of horse and kangaroo meat (muscle)

		Horse					Kangaroo				
Fatty acid	Carbon no.	CE	TG	FA	DG	PL	CE	TG	FA	DG	PL
12:0	12.1		0.1	0.1				trace	0.1		
	13.1			trace		trace		trace			
14:0	14.0	12.3	2.0	2.2	1.6	0.1	6.7	1.9	1.3	4.4	0.3
	14.4					trace					trace
15:0	15.0		0.2	0.5			3.5	2.9	0.9	1.1	0.1
16DMA	15.3					8.5					3.1
16:0	16.0	48.3	26.5	19.6	32.0	14.3	23.0	17.3	23.8	18.1	13.6
16:1		2.9	2.8	2.1							
17:0	17.1					8.1	trace	1.0		0.1	
	17.4	11.4	trace		0.3	1.7		0.5	1.5	3.6	2.9
18:0	18.0		6.2	8.3	7.4	13.1	15.6	13.9	7.2	16.5	8.5
18:1	18.6	13.1	32.7	28.1	25.2	9.3	23.9	45.4	31.5	25.8	28.7
18:2	19.4	3.4	12.8	17.6	12.3	14.0	12.3	8.2	19.9	14.4	22.1
18:3	20.6	8.6	16.6	21.5	18.5	2.0		4.2	5.4	2.8	5.1
	21.4					1.1			5.5		
	21.9										1.3
20:4	22.6				2.7	8.9			7.2	4.9	8.6
	23.4										5.6

CE: cholesterol ester; TG: triglyceride; FA: fatty acid; DG: diglyceride; PL: phospholipid; DMA: dimethylacetyl.
Source: Payne, 1971 (reproduced with permission).

5.2.3.4 Fat analysis

Under the slaughtering laws in Australia, horse and kangaroo meat products cannot be marketed for human consumption. This necessitates the identification of meat and detection of adulteration of permitted meats with others. Serological methods have limitations and need other methods to support their verdict. Fatty acid composition has been used in the identification of meat of bovine, ovine and porcine species, and particularly adulteration of pork with ruminant fat (Hubbard and Pocklington, 1968).

Simple tests like iodine value of the fat can distinguish horse meat in pork, albeit only above 20% (Bellini, 1941). Fatty acid composition of a species can be an aid to its identification in meat mixtures. For example horse meat can be differentiated easily from kangaroo meat by the linolenic acid content (Sinell and Langner, 1966) of the fat and the muscle (Wurziger, 1967; Plowman and Close, 1988). The fatty acid composition of horse and kangaroo meat (Hartman *et al.*, 1955) can be used for both the identification of these meats and for the determination of the degree of adulteration of other meats (Payne, 1971). Table 5.2 shows the fatty acid composition of the principal

lipid fractions of horse and kangaroo meat. It is known that bovine subcutaneous or muscle fat under no circumstances will have a linolenic acid content greater than 4%, and most generally lower than 1.5%. Similarly, pig fat has a maximum linolenic acid of 1%, and therefore becomes an indicator of adulteration with horse fat.

The higher concentration of linolenic acid and its oxides in horse meat is responsible for higher colour development with 2-thiobarbituric acid reagent (used commonly to determine the oxidation of fats) as compared to beef tallow and lard. This test has applications in detecting horse meat admixed in beef products (Masao and Satoshi, 1954). Yet another method is based on the fact that linolenic acid can be precipitated as the hexabromide (Duggan and Petheram, 1952) in cold ether solution. Values of 86.1–127.4 mg hexabromide g^{-1} horse fat and 2.8–9.2 mg hexabromide for other fats are reported (Hynds, 1950). Ultraviolet spectrophotometry has also been used to determine the linolenic acid in the fat extracted from meats and sausages (Cook, 1962).

Rabbit and horse meat have a similar linolenic acid content. They can however be distinguished by their respective cholesterol and arachidonic acid levels (Wurziger, 1967).

Fatty acids are incorporated into the triglyceride according to a species-specific pattern. The relationships between fatty acid distribution in the triglyceride molecule can be used as a reliable criterion for detecting meat adulteration (Federal Republic of Germany, 1991; Verbeke and Brabander, 1980). Pig meats are effectively distinguished from other fats by the positional distribution of palmitic acid and the proportion of oleic acid within the triglyceride. Similarly, beef fat can be differentiated from horse and hen fat using stearic acid as the parameter. Ratios of $C_{16:0}/C_{18:1}$ and $C_{16:0}/C_{18:2}$ could be used as a guide to adulteration levels of beef with pork (El-Khalafy *et al.*, 1987). Hen fat is distinguished from horse fat in using parameters calculated on the basis of distribution of palmitic and palmitoleic acid. The absence of ruminant fat can be deduced with certainty from the lack of *trans* unsaturated fatty acids which originate from the metabolic activity of the rumen microflora (Sinell and Langner, 1966). By combination of the fatty acid composition in the 2-position and total fatty acid composition, species-specific parameters may be calculated, which are then plotted on a cluster graph. This is known to give good separation between pork fat and other fats such as horse, beef and chicken (Brabander and Hoof, 1991). The palmitic acid enrichment factor (Youssef *et al.*, 1988) can be used for detecting lard in meat products and is defined as the ratio of percentage palmitic acid in the β-monoglyceride to that in the triglyceride. Another parameter, unsaturation ratio (Hussein, 1979), defined as percentage of unsaturated fatty acids in β-monoglycerides to percentage in the triglyceride, total C_{16}/ total C_{18} fatty acids ratio, and saturated/unsaturated fatty acids ratio in monoglycerides can also detect and evaluate lard in pure goat and mutton tallows (Rashwan and Youssef, 1989). Gas chromatographic analysis of the triglyceride fatty acids, and analysis of the fatty acid contents in the monoglycerides obtained after lipase treatment of the fat can indicate accurate determination of 5% beef fat in porcine or 10% porcine fat in beef fat. It is believed to be superior to the existing methods in

Table 5.3 Linear regression equations for the mixing ratio (X) of whole soybeans with minced meat and fatty acids (Y) and unsaponifiables (Y)

Component	Regression equation	Correlation coefficient[a]
Fatty acids:		
$C_{14:0}$	$Y=52.36-22.3X$	0.92
$C_{16:0}$	$Y=95.76-3.54X$	0.91
$C_{18:0}$	$Y=93.38-11.07X$	0.87
$C_{18:1}$	$Y=118.69-2.19X$	0.99
$C_{18:2}$	$Y=-6.95+1.55X$	0.99
Unsaponifiables:		
C_{20}	$Y=45.78-1.64X$	−0.96
C_{21}	$Y=37.16+13.48X$	0.85
C_{22}	$Y=192.31-3.79X$	0.96
C_{23}	$Y=-6.73-1.53X$	0.99
Total sterols	$Y=-141.94+2.23X$	0.98

[a]Significance at the 1% level.
Source: Farag *et al.*, 1986 (reproduced with permission).

detecting adulteration of pork fat with beef tallow (Verbeke and Brabander, 1979). The consumption of pork is prohibited in Islamic countries on religious grounds. An increase of the palmitic acid enrichment factor to 0.8 as well as a decrease in the unsaturation ratio to 1.3 or less indicates the presence of 5% or more pork.

Whole soybeans in minced meat can be analysed on the basis of the composition of unsaponifiable hydrocarbons and the concentration of sterols. C_{20} and C_{22} compounds constitute >70% of minced meat unsaponifiable hydrocarbons, while C_{22} and C_{23} compounds constitute >80% of unsaponifiable hydrocarbons in the soybean, while soybean also has about 1.5 times higher concentration of sterols compared with minced meat (Farag *et al.*, 1986). The admixture levels of soybean in meat can be determined by an equation of the type: $Y=A+BX$, where Y=concentration of a particular compound, A=a constant value, the intercept of the regression line, B=the regression coefficient and X=the admixture ratio.

The linear regression equations for the mixing ratio (X) of whole soybeans with minced meat and fatty acids (Y) and unsaponifiables (Y) are given in Table 5.3.

Pork fat is unique in its peculiar fatty acid distribution and triglyceride composition. Since the fatty acid, 11,14-eicosadienoic acid ($C_{20:2}$) (Saeed *et al.*, 1986; Sawaya *et al.*, 1990b) was reported to be present in pork fat and absent in other commonly consumed meats and fats, its presence in meat products can be considered as a positive indicator of 1% pork fat in the sample. $C_{20:2}$ acid has, however, been claimed to be present in some beef and mutton samples (Firestone, 1988).

Depending on the number of saturated (S) or unsaturated (U) fatty acids in the triglyceride molecule, triglycerides are classified into four types: S_3, S_2U, SU_2 and U_3.

Two types, S_2U and SU_2 can exist in two isomeric forms SUS and SSU, and UUS and USU, respectively. Pork fat contains 38% SSU, 41% USU, 1% SUS and 7% UUS. In other animal fats, the triglyceride composition is distinctly different: 9–14% SSU, 13–38% SUS and 28–38% UUS (Chacko and Perkins, 1965). The characteristic composition of fat triglycerides and the application of high performance liquid chromatography (HPLC) have been useful in detecting pork in meat products. Since pork contains mostly the SSU isomer, but no significant amounts of SUS, any addition of pork to pure beef would result in an increased SSU/SUS ratio. Since fat is not significantly affected during processing, the method applies to both fresh and processed meats. The method permits the detection of 2% pork in beef and 3% pork in mutton (Saeed *et al.*, 1989).

There are two main limitations to this method. Sample preparation is tedious and lengthy. The method is not applicable to fats that have been chemically modified, for example, hydrogenated fats.

Fogerty *et al.* (1991) have shown differences in the composition of fatty acids and aldehydes of the ethanolamine and choline phospholipids of various meat species, as seen from Tables 5.4, 5.5 and 5.6. It can be seen that ethanolamine phospholipids of beef, lamb, pork and chicken contain over 40% ethanolamine plasmalogen, whereas fish contain only 13%. The level of choline plasmalogen in choline phospholipids is found to be less than 1% in fish and ranges from 10–30% in various meats. The fatty aldehydes as a percentage of total aldehydes in ethanolamine and choline

Table 5.4 Plasmalogen content and fatty aldehyde composition of meat phospholipids[a]

% Plasmalogen	Fatty aldehyde as percent of total aldehydes of phospholipid[b]					
Phospholipid	A	B	16:0	18:0	18:1$n-9$	18:1$n-7$
Ethanolamine phospholipids						
Beef ($n=4$)	42.1±3.2	42.9±2.0	39.0±6.7	47.5±9.7	9.3±3.2	4.2±0.7
Lamb ($n=2$)	46.4±3.2	46.2±1.8	30.9±1.4	53.3±1.7	10.1±1.1	5.7±1.4
Pork ($n=2$)	46.6±1.6	49.2±0.1	44.5±4.8	33.7±3.0	19.2±1.7	2.6±0.1
Chicken ($n=4$)	39.6±7.3	41.3±2.2	53.6±3.9	27.8±3.8	16.4±1.6	2.2±0.2
Fish ($n=2$)	12.1±0.1	13.5±0.1	18.7±0.1	49.2±0.2	17.8±0.4	14.3±0.2
Choline phospholipids						
Beef ($n=4$)	30.1±5.3	28.1±4.9	80.2±4.1	12.0±4.5	6.4±2.1	1.4±0.1
Lamb ($n=2$)	25.0±0.2	24.5±3.0	68.4±1.4	19.7±1.5	8.4±0.1	3.5±0.1
Pork ($n=2$)	11.1±0.9	12.4±0.7	71.8±1.4	12.6±0.8	14.2±0.4	1.4±0.2
Chicken ($n=4$)	10.6±3.0	9.5±0.6	77.9±4.3	9.2±2.0	11.7±3.5	1.2±0.9
Fish ($n=2$)	0.7±0.1	0.9±0.1	43.1±1.5	38.4±0.4	10.5±1.1	8.0±0.1

[a]Values are mean±range/2.
[b]Calculated before and after acid hydrolysis.
Source: Fogerty *et al.*, 1991 (reproduced with permission).

Table 5.5 Major fatty acids of phosphatidylethanolamine (PE) and phosphatidyl choline (PC) of various meats[a]

Phospholipid	PE					PC				
	Beef (n=4)	Lamb (n=2)	Pork (n=2)	Chicken (n=2)	Fish (n=2)	Beef (n=4)	Lamb (n=2)	Pork (n=2)	Chicken (n=2)	Fish (n=2)
16:0	0.7±0.2	0.9±0.1	1.1±0.4	1.7±0.9	5.5±0.8	38.5±1.3	33.8±0.5	36.1±2.0	38.9±3.1	40.6±1.8
16:1	0.4±0.1	0.3±0.1	0.6±0.2	0.5±0.2	0.6±0.1	3.7±1.2	2.6±0.1	3.0±0.1	4.1±0.3	2.8±0.6
18:0	15.2±4.9	17.3±3.3	13.3±1.3	10.0±5.1	24.7±4.6	4.2±1.0	6.0±0.3	7.3±0.7	5.6±0.8	2.8±0.1
Total 18:1[b]	11.4±1.4	17.9±0.4	11.8±2.5	21.6±4.2	6.7±0.5	32.1±4.5	37.3±1.4	27.0±1.7	26.5±1.0	14.1±1.3
18:2n−6	12.3±3.6	8.6±0.3	23.6±3.1	6.4±0.2	0.3±0.1	10.0±3.2	8.0±1.2	22.2±0.3	20.8±2.7	0.8±0.1
18:3n−3	4.4±1.8	4.4±0.5	1.0±0.1	0.2±0.1	–	3.5±1.3	4.3±0.3	0.7±0.1	–	–
20:4n−6	18.7±1.5	14.9±0.1	20.7±1.5	17.7±1.3	8.4±0.7	1.4±0.7	1.3±0.1	1.2±0.1	1.4±0.6	4.3±0.1
20:5n−3	12.8±1.3	14.9±1.7	6.3±1.6	3.6±0.7	5.7±0.3	0.9±0.6	1.5±0.1	0.2±0.1	–	7.8±0.1
22:5n−3	11.2±1.7	9.7±1.2	6.6±1.9	6.2±0.1	3.6±0.1	0.2±0.2	0.7±0.1	–	–	1.6±0.1
22:6n−3	2.0±0.4	3.0±0.4	5.4±1.9	17.2±2.4	41.2±3.9	–	–	–	–	20.5±0.1
Total PUFA[c]	69.9±6.8	57.8±3.8	69.9±4.7	62.0±2.1	59.9±5.2	16.6±4.1	15.8±1.7	24.5±0.8	23.5±4.0	34.88±0.1

[a]Expressed as a percentage of the total fatty acids: mean±range/2.
[b]±Largely 18:1n−9, plus minor amounts of 18:1n−7 and other positional isomers.
[c]PUFA, polyunsaturated fatty acids.
Source: Fogerty *et al.*, 1991 (reproduced with permission).

Table 5.6 Major fatty acids of ethanolamine plasmalogen (EP) and choline plasmalogen (CP) of various meats[a]

Phospholipid	EP					CP				
	Beef (n=4)	Lamb (n=2)	Pork (n=2)	Chicken (n=2)	Fish (n=2)	Beef (n=4)	Lamb (n=2)	Pork (n=2)	Chicken (n=2)	Fish (n=2)[b]
16:0	2.7±1.1	2.0±0.4	2.3±0.7	1.4±0.2	7.8±1.1	16.4±8.0	11.8±0.1	18.7±4.4	24.6±5.1	
16:1	0.9±0.2	0.7±0.1	1.2±0.4	0.9±0.1	1.7±0.2	2.4±0.4	1.4±0.1	1.9±0.6	2.1±0.4	
18:0	7.0±5.8	9.9±1.6	4.3±0.4	7.2±1.0	10.4±2.4	2.6±1.4	3.0±0.8	4.2±0.2	3.5±0.6	
Total 18:1	14.4±0.6	25.6±2.0	15.1±3.1	30.9±1.3	12.5±0.3	32.0±1.0	37.1±3.5	25.3±2.5	29.5±5.2	
18:2n−6	19.3±7.5	16.5±0.9	41.2±4.6	6.9±0.2	0.6±0.1	17.3±2.7	14.0±1.7	34.5±3.3	20.5±0.8	
18:3n−3	5.8±1.7	8.1±0.5	1.7±0.1	0.3±0.1	–	7.0±1.1	9.8±0.8	1.3±0.2	–	
20:3n−6	3.7±0.2	1.2±0.1	2.6±0.3	2.2±0.4	–	2.1±0.5	0.8±0.1	1.4±0.3	2.2±0.5	
20:4n−6	17.1±3.5	11.9±0.6	15.2±3.0	16.9±0.6	7.5±0.3	6.3±2.5	5.5±0.5	5.1±0.8	6.4±0.5	
20:5n−3	11.8±2.5	11.9±0.1	5.3±1.9	3.5±0.5	7.0±0.3	4.9±0.2	7.1±0.5	2.2+0.7	1.7+0.3	
22:5n−3	8.2±1.7	5.5±0.5	3.9±1.9	5.1±0.1	3.3±0.2	3.5±1.0	4.2±0.2	2.0±0.9	2.2±0.1	
22:6n−3	1.4±0.5	1.4±0.1	2.7±2.0	14.4±1.0	43.5±3.4	–	0.8±0.2	1.3±0.8	3.6±0.3	
Total PUFA	71.6±5.6	56.8±0.1	74.0±5.2	56.0±0.5	62.6±4.3	42.5±9.0	42.6±4.0	48.0±7.6	38.3±1.0	

[a]Expressed as a percentage of the total fatty acids: mean±range/2.
[b]Insufficient for accurate analysis.
Source: Fogerty *et al.*, 1991 (reproduced with permission).

phospholipids show striking differences and could probably be used to detect admixtures in raw meats. Similar differences have been observed for fatty acids of phosphatidylethanolamine and phosphatidylcholine of various meats, and of ethanolamine plasmalogen and choline plasmalogen (Fogerty *et al.*, 1991). These could serve as sensitive indices to detect blends and need to be investigated.

However, hydrolysis of plasmalogens in the phospholipids occurs during heating of meat at 132 °C (Fogerty *et al.*, 1989, 1990), when fatty aldehydes are liberated and recovered in the neutral lipid. Heating also causes losses of polyunsaturated fatty acids (PUFA) from the ethanolamine phospholipids. Therefore this approach may not work with heat processed meat admixtures.

Detection of lard in canned meats as well as smoked sausages can also be done from infrared analysis. With increasing lard percentage, there is a gradual increase in the absorption ratios and these are correlated by using a regression equation: $Y=A+BX$, where Y=lard percentage, X=absorption ratio and A, B=constants. Visual differences between the near infrared (NIR) spectra of meat with respect to lamb, chicken, beef and turkey have been found, which have also been supported by the use of spectral match algorithm and principal component analysis. This discrimination shows promise for providing a reliable method for meat speciation in raw and cooked meat products (Spray *et al.*, 1990).

5.2.3.5 Mineral analysis

Methods based on analysis of mineral constituents can distinguish whole soybeans in minced meat. Soybeans contain high levels of phosphorus, potassium, magnesium (Formo *et al.*, 1974) and calcium, while minced meat has more sodium and zinc which could serve as useful analytical indices to detect this addition (Farag *et al.*, 1986). Table 5.7 shows the linear regression equations for the mixing ratio (X) of soybeans with minced meat and mineral content (Y).

Fraudulent addition of bone powder to sausages can be confirmed by analysis of the Ca content, each gram of powdered bone contains 160 mg of Ca (Anon, 1971).

5.2.3.6 Histological examination

Histological methods such as microscopic examination of sections after Bauer–Calleja staining have been used successfully to detect soy protein in Italian raw sausages (Cortesi *et al.*, 1977). A typical method consists of fixing the sample of the sausage in picric acid, sectioning followed by staining (using chromic acid, schiff's reagent and picroindigocarmine) and then a microscopic examination. Starch and glycoproteins are stained violet/red/blue, while other meat constituents are stained green. Particles of spice can be easily distinguished on the basis of histological characteristics (Feigl, 1992). This method is particularly useful when antisera to the protein are not available. It can also detect gluten and other proteins, primarily because they retain

Table 5.7 Linear regression equations for the mixing ratio (X) of soybeans with minced meat and mineral content (Y)

Element	Regression equation	Correlation coefficient[a]
Ca	$Y=-12.316+31.717X$	0.97
Mg	$Y=-27.803+13.402X$	0.95
Cu	$Y=-10+3999.99X$	0.93
Mn	$Y=1.667+2222.22X$	0.87
Na	$Y=62.65-35.69X$	−0.99
Zn	$Y=134.73-1473.65X$	−0.86
K	$Y=-129.82+13.69X$	0.94
P	$Y=-16.41+25.37X$	0.87

[a]Significance at the 1% level.
Source: Farag *et al.*, 1986 (reproduced with permission).

characteristic structures in the cooked product. Histological methods are inapplicable to caseinate, whey proteins, blood plasma or ovalbumin. Histological examination is also useful in detecting the occurrence of tissues other than meat in sausages (Julini and Parisi, 1978; Julini *et al.*, 1982). The difference between various quality grades of liver sausage is based mainly on the liver content, and can be distinguished by histological parameters as well as by chemical parameters such as percentage water, fat, total protein, meat protein, connective tissue protein-free meat protein, water:protein and fat:protein ratios (Gerigh *et al.*, 1986a, 1986b). Although connective tissue protein-free meat protein increases with the liver content this parameter allows quality evaluation when low quality meat differing essentially in composition from liver tissues is used. It has been recommended that histological examination must always be included until a suitable method for determining liver content is developed (Gerigh *et al.*, 1986c).

5.2.3.7 Differential scanning calorimetry

This technique has been used to analyse the proportions of pork and beef in blends by studying the heat denaturation properties, such as transition temperature and enthalpy, of stroma proteins (Kim, 1989).

5.2.3.8 Biochemical indices

Biochemical indices such as total creatinine, creatine, total nitrogen, net muscle protein, hydroxyproline, tryptophan, creatine phosphokinase and phosphohexose isomerase have been deemed suitable for use in differentiation and identification of different kinds of meats, detection of adulteration/blending of meats and evaluation of the edible quality of meat (Zeng, 1989). Fresh meat from the caprine group can be

distinguished from that of the bovine group by assaying Mg^{2+}–ATPase and peroxidase, the activities being significantly higher in the latter group. Individual species of the bovine group, for example cattle or buffalo, can be identified by phosphatase and catalase activities, while those of the caprine group, for example goat or sheep, can be identified by succinic dehydrogenase and alkaline phosphatase activities (Bhattacharyya *et al.*, 1988). Total pigment and myoglobin contents have been shown to be reliable in calculating meat content of beefburgers and similar products containing admixed soy protein concentrate or wheat flour (Babji *et al.*, 1989).

In Australia, barramundi (*Lates calcarifer*) is considered to be a premium fish species, and lower priced king salmon, threadfin salmon and orange have been substituted for it (Anon, 1982; Bremner and Vail, 1983). Differences in the ratio of inosine to hypoxanthine between these have been shown successfully to differentiate barramundi from its substitutes. Barramundi is known to contain hypoxanthine exclusively, while the other fish are known to have inosine along with hypoxanthine (Williams *et al.*, 1991).

Heating of lipids results in release of characteristic aroma compounds, contributing significantly to the species-specific flavour of meat (Hsieh *et al.*, 1980; Brennand and Lindsay, 1982). For instance, 1-heptadecene and 1-octadecene are specific to beef (Mottram *et al.*, 1982) and 12-methyltridecanal is specific only to stewed beef (Guth and Grosch, 1993). It is believed that 12-methyltridecanal is preferentially a constituent of ruminants, possibly being synthesized by the bacteria in the rumen, and incorporated in the plasmalogens. A search for such a species-specific aroma could be an interesting and a novel approach to identifying the species of cooked flesh.

5.2.3.9 DNA hybridization

For differentiation of the species of origin of meat, serological and electrophoretic methods are valid only for raw meat (Kurth and Shaw, 1983; Patterson *et al.*, 1984). Heat treated meat samples present difficulties due to denaturation of the proteins. Recently a method based on DNA reassociation has been reported (Bauer *et al.*, 1987) which is applicable to cooked samples of meat.

DNA was extracted with NaCl–EDTA–Tris–HCl buffer containing SDS and dithiothreitol, precipitated and washed with phenol–chloroform–isoamyl alcohol, extracted with phenol and precipitated with ethanol. The ethanol precipitate was dissolved in EDTA–Tris–HCl buffer and treated with RNase for 1 h at 37 °C. The DNA solution was sheared by ultrasonic treatment to 0.2–2.0 Kbase pair fragments (Maniatis *et al.*, 1982). The purified DNA fragments were biotinylated with Biotin-11 dVTP by a Nick Translation Reagent kit from Bethesda Research Laboratories Life Technologies Inc. (Gaithersburg, USA) or ^{32}P–dCTP.

The meat sample DNA is heated at 100 °C for 5 min for strand separation and chilled immediately on ice. This is spotted on a nylon membrane Hybond-N, Amersha International (UK) or Hybri-slot manifold with Hybond-N-Silders

(Amusham International and Bethesda Research Laboratories). The DNA solution is applied to the slot-blot filters and exposed to UV for crosslinking to the film. The standard labelled DNA solution is added on to this filter membrane and allowed to hybridize and the excess is washed. The hybridized material is quantitated by laser densitometry of autoradiographic signals or with a BLUGENE nucleic acid detection kit based on colour with biotin, streptavidin and alkaline phosphatase conjugate (Leary *et al.*, 1983).

The technique has been used successfully with canned chicken, pig and beef although some cross reactivity was seen amongst recombinant DNA (Chikuni *et al.*, 1990; Wintero *et al.*, 1990; Tsumara *et al.*, 1992).

5.3 Freshness indicators

Although the biochemical changes taking place in meat are all well documented, attempts to suggest tentative limits of acceptability are of relatively recent origin. Earlier workers such as Jensen (1954) and Turner (1960) had inferred that chemical methods are unlikely to be applicable for specification purposes. However, chemical methods of assessment relating to protein breakdown, fat spoilage, some techniques measuring physical changes, microbiological methods and other miscellaneous methods are suitable as spoilage indicators (Ng and Nobuo, 1989; Pearson, 1968; Babakhanov, 1959).

5.3.1 Protein breakdown products

Owing to a wide variation in fat content encountered between different cuts, the protein contents of meats may vary between 1% and 20%. Protein breakdown may be autolytic or by the bacterial proteolytic enzymes resulting in the formation of soluble peptones and polypeptides followed by amino acids. It has been shown that at death, squid enters a state of uncontrolled enzymatic protein degradation (Tanikawa *et al.*, 1970). The subsequent increase in ammonia, trimethylamine, and amines is by the action of bacterial enzymes and can be very rapid (Takagi *et al.*, 1971). The actual reactions may vary, according to the bacterium, the temperature and whether the conditions are aerobic or anaerobic. For instance, the psychrophilic bacteria growing on beef are mostly Pseudomonas, which are likely to produce ammonia by deamination of amino acids under aerobic conditions (Soudan, 1965; Ayres, 1960). In the case of broilers, onset of spoilage at about 10^7–10^{10} cfu cm^{-2} is generally accompanied by a rapid increase in ammonia (Schmitt and Schmidt-Lorenz, 1992a). Ammonia can also be produced from enzymatic degradation of nucleotides (Tarr, 1966) and of amines (Richter, 1937). Linear relationships between texture and salt-soluble protein content, texture–centrifuge drip content, texture–dimethylamine content and dimethylamine–formaldehyde content have been reported, indicating linear relationships between

texture–formaldehyde (Giannini *et al.*, 1993). Similarly, the concentration of alcohol needed to coagulate the protein in the fish extract can be taken as an index of freshness, 70–80% alcohol being necessary to coagulate fresh meat and 30–40% alcohol being sufficient in the case of decaying meat (Amano and Tomiya, 1950). These methods can be conveniently classified as follows.

5.3.1.1 Total volatile bases

The total volatile bases (TVB) index is by far the most commonly used index of squid (*Illex illecebrosus*) quality (Shimizu *et al.*, 1953; Shimizu and Hibiki, 1953c), particularly in Japan, where most of the world's squid catch is consumed.

Total volatile basic nitrogen (TVBN) has been used as an index of decomposition in meats and fish since 1952 (Motohiro and Tanikawa, 1952; Pearson and Muslemuddin, 1968; Tomiyama *et al.*, 1952; Tomiyama, 1952) and is still widely used (Malle and Poumeyrol, 1989). It is suggested that marine fish may be classified into three freshness groups on the basis of TVBN concentration: class I <30 mg TVBN/100 g, class II 30–40 mg TVBN/100 g and class III >40 mg/100g (Dillon *et al.*, 1979). Earlier reports had set the cut-off point at a maximum of 30 mg% volatile nitrogen bases (Todorov, 1969) for human consumption. Simple, rapid tests for determining volatile amines based on turbidity after reaction of fish extract with 0.1% mercuric chloride are reported (Wierzchowski, 1956). The presence of the substituted amines can also be ascertained by colour reactions with phenol red, hematoxilyn or curcumin impregnated on a wooden stick which can be inserted and withdrawn without disturbing the shape of the seafood. Hematoxilyn changes from yellow to reddish purple, curcumin from pale yellow to deep brownish red and phenol red from pale yellow to bright red, all in proportion to the degree of spoilage as indicated by the substituted amines (Hand, 1953).

Wide dispersal of TVBN levels have been observed for a given decomposition index and are therefore subject to valid criticisms (Oehlenschlager, 1989). The TVB value depends largely upon the analytical variation used (Botta *et al.*, 1982); it is tedious, time consuming and consists of contributions from several volatile amines and ammonia (Rehbien and Oehlenschlager, 1982).

In meat, the volatile nitrogen (TVN) consists entirely of ammonia, with only traces of trimethylamine (Burks *et al.*, 1959). Changes in trimethylamine and total volatile base content correlate well with the sensorily perceptible quality factors in iced white fish. An equation, flavour+6.2 log (1+TVB)=15.0, has been established as a correlation between sensory flavour and total volatile bases (Ehrenberg and Shewan, 1955; Shewan and Ehrenberg, 1957). Elasmobranch fish decomposition is also characterized by large amounts of ammonia formed by the breakdown of urea which is present in considerable quantities (Pearson, 1976) or by bacterial attack, as in shark muscle (Shimizu and Hibiki, 1953b), or by the consumption of urea by the cattle used for meat (Gogoasa *et al.*, 1969). In the latter case, urea passes through the rumen and after decomposition releases ammonia into the blood causing cattle poisoning; the meat

from such a cattle has organoleptic and physicochemical characteristics similar to that obtained from meat with a high ammonia content. Parallels between microbial growth and urease activity in shark muscle have been demonstrated (Shimizu and Oishi, 1951a, 1951b). Ammonia formation occurs even in dried or preserved urea-containing meat such as in shark meat (Murata and Oishi, 1952). In putrefaction of shark, ammonia evolution temporarily ceases due to the coagulation of the muscle protein by which urea is occulted in the protein molecule and is less available to bacteria. This cessation however disappears at higher temperatures (Shimizu and Oishi, 1950). It has been suggested that the resting step in urea decomposition is due to the bacterial consumption of urea present outside the cells (Shimizu and Hibiki, 1953a). This has been considered as an objective index of fish freshness (Ota and Nakamura, 1952), and can be easily analysed by various colorimetric procedures (Stach, 1961; Totescu, 1961). A cut-off point for human consumption has been placed at 30 mg% ammonia (Borowik and Zaleski, 1953). Trimethylamine (TMA) however, provides an accurate indication of bacterial spoilage in some marine and brackish water fish species such as herrings (*Clupea harengus*) and fresh pike (*Esox lucius*) (Folke, 1951). It is formed from an osmoregulatory compound, trimethylamine oxide (TMAO) by bacterial reduction and has traditionally been used for measuring the eating quality or freshness (Dyer, 1945; Laycock and Regier, 1971; Elias and Krzymien, 1990). The fishy, pungent odour of spoiled fish and crustacea is largely attributed to trimethylamine (Castell, 1949). Colorimetry (Obata and Zama, 1950a, 1950b; Dyer, 1945; Aaltonen *et al.*, 1992; Moral *et al.*, 1979), turbidimetry (Wierzchowski, 1956), gas chromatography (Keay and Hardy, 1972; Ritskes, 1975), enzymic determination (using trimethylamine dehydrogenase) (Wong and Gill, 1987), semiconductive sensors (Ohashi *et al.*, 1991) and ion specific electrodes (Chang *et al.*, 1976; Lee *et al.*, 1992) for measuring trimethylamine have been reported (Karl, 1992). In colorimetric procedures using methylene blue or resazurin as indicators, the presence of bacteria capable of reducing them makes them unsuitable for estimating the freshness of marine fish (Castell, 1950). This test is of no value in determining the fitness of cod and mackerel (Gheorghe *et al.*, 1970) for human acceptance (Wierzchowski *et al.*, 1953) and is also the case for freshwater fish since they do not contain or contain very little trimethylamine oxide (Lintzel *et al.*, 1939; Somaatmadja *et al.*, 1961). Similarly in frozen hake (*Merluccius merluccius* L.) stored at $-12\,^{\circ}C$ and $-20\,^{\circ}C$, TMA production does not correlate with decrease in TMAO. It is believed that TMAO may be degraded by an alternate unknown pathway (Sotelo *et al.*, 1995).

In vertebrate fish, quality is judged as unacceptable, when TMA level exceeds 15 mg% (Shaw *et al.*, 1983) for iced fish and 13 mg% for unrefrigerated fish (Fernandez del Riego and Rodriguez de las Heras, 1954). A recent chemical criterion taking both TMA and TVBN into account is the *P* value defined as:

$$P = \frac{\text{TMA}}{\text{TVBN}} \times 100 \qquad [5.1]$$

For cod and mackerel, it has been shown that TVBN and TMA at a given stage of decomposition are associated with species-related factors such as microbial flora, conditions of capture and storage. The *P* values are superimposable and only slightly dependent on species, arguing in favour of use of *P* rather than the associated parameters as a criterion of freshness. The two major components of TVBN are ammonia and TMA, and it is ammonia that predominates in fresh fish, where it may be reutilized in some synthetic reaction and modify TVBN levels. *P* takes into account both these aspects (Malle and Poumeyrol, 1989). TMA can generate large amounts of dimethylamine (DMA) and formaldehyde (FA) in equimolar amounts (Boeri *et al.*, 1993), especially at cooking temperature in squid (Kolodziejska *et al.*, 1994). FA could induce toughening in frozen stored fish by direct crosslinking of the proteins and denaturation of proteins attributed to binding to their side chain groups. This is responsible for toughening and hence texture loss.

As the ammonia production due to deamination of amino acids increases during spoilage, its determination represents a simple method following the course of deterioration of lean meat (Abramayan, 1957) or fish (Ota and Nakamura, 1952). The method consists of distillation of the volatile bases into a suitable system such as boric acid or standard weak acid and then measuring the TVN. A maximum acceptability limit of 20 mg volatile N for fat free meat (TVN/FF) has been recommended for beef (Pearson, 1967). This method is unsuitable for detecting incipient spoilage. Ammonia contents of 3–10 mg nitrogen/100 g fresh beef have been reported. On storage, the meat is not necessarily unpalatable until the value reaches 30 mg.

The ammonia content of squid correlates significantly with the TVB, an accepted indicator of squid quality ($P<0.01$). An enzymatic method of determining ammonia as an index of squid quality has been reported by LeBlanch and Gill (1984). This rapid (20 min) and reproducible (coefficient of variance, CV=2%, $n=12$) method is based on the amination of the α-ketoglutarate to glutamate in the presence of both glutamate dehydrogenase and NADH. The ammonia test is then coupled either to *p*-iodonitrotetrazolium violet or to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, resulting in the formation of coloured formazans. This colour test permits visual assessment of ammonia. It is found to be semi-quantitative and accurate to within ±1 mg% ammonia nitrogen. It can also be performed in the field without the use of elaborate equipment (LeBlanch and Gill, 1984).

5.3.1.2 Amino nitrogen

The determination of amino nitrogen has been used by some workers in the meat field. Determination by the Van Slyke method and the formol titration methods (Kolobolotskii, 1952) are described officially in the USA. Using the formol titration, Broumand *et al.* (1958) observed that the increase in free amino nitrogen in lean samples was not always pronounced or consistent. The 'ninhydrin-positive substances' (α-amino acid) (Jay, 1964; Saffle *et al.*, 1961) tend to show only slight changes as spoilage progresses.

Recent reports have indicated a high correlation between the total volatile nitrogen/amino acid nitrogen (AAN) and shrimp quality. In tests on frozen shrimp the TVN/AAN ratio was >1.5 and had bacterial counts ranging from 11 000–75 000 g^{-1}. Factors which affect this ratio, but not its usefulness as a quality indicator are temperature and salinity of the water (Cobh and Venderzant, 1975).

5.3.1.3 Amino acids

Free amino acids (Jacober and Rand, 1982; Wierzchowski and Fuks, 1967) are good indicators for monitoring the microbial spoilage of protein rich foodstuffs. The degree of autolysis and bacterial proteolysis have been assessed in fish by means of tyrosine value (Sigurdsson, 1947). Suspicious and spoiled wild boar meat is known to show a decrease in aspartic acid, and an increase in the amount of histidine, serine, glycine, glutamic acid, threonine, proline, valine and leucine over that in the fresh meat (Belonosov, 1967; Tserenpuntsag, 1971). The ratio of taurine:hypotaurine has been shown to increase during storage regardless of the storage temperature in the ascidian *Halocynthia roretzi* (Nontratip *et al.*, 1992) and could be considered as an indicator of freshness. Citrulline is a suitable indicator of microbial spoilage of chicken carcasses for three main reasons, it is not found in the skins of fresh carcasses, it can arise only from bacterial action, especially by Pseudomonads and there is a good correlation between the increase in colony counts and citrulline content. The other amino acids are already present in the skin of the fresh carcass and their concentrations merely increase or decrease in the course of spoilage (Schmitt and Schmidt-Lorenz, 1992b). Piperidine is a bacterial degradation product of lysine and can be colorimetrically estimated as an indication of the degree of fish freshness (Obata and Zama, 1950a, 1950b). Similarly, β-alanine, obtained by the decarboxylation of aspartic acid and not present in the free state in living animal tissue, increases during storage even at 2 °C and can be used as a freshness indicator of fish products (Bramstedt, 1955).

A measure of tenderness is the sulphydryl group content which can also serve as an index of freshness. Tenderness changes become apparent when the sulphydryl content of the muscle tissue decreases to about 50% of its value in fresh cooked meat. The amount of buffer extractable nitrogen decreases during frozen storage, whereas the products of proteolysis increase. Above freezing, however, whilst protein breakdown is considerable, changes in the extractability of the nitrogen fraction or -SH groups are negligible. Khan (1965) therefore proposed the use of the 'quality index' to assess deterioration in poultry, which represents the ratio of the -SH groups to the products of protein breakdown represented by tyrosine value. The application of the method to other meats has been considered to be worthy of investigation.

5.3.1.4 Amines

Amines are discussed by Meitz (1977), Yamanaka *et al.* (1987), Yamanaka (1989) and Karube *et al.* (1980). The metabolic pathway for the formation of di- and polyamines is reviewed by Hayashi (1970). The amines include histamine, tyramine, agmatine (specific for squid), cadaverine, putrescine, spermidine, spermine and tryptamine. These biogenic amines can be useful indices of poor quality raw material in processed meat products such as beef (Osamu and Susumu, 1950), the increase being observed earlier than pH changes during storage (Vidal-Carou *et al.*, 1990). One report however shows poor correlation with spoilage in beef, pork and chicken (Tsugo and Saito, 1961). Histamine has been advocated as an index of relative freshness of certain fishery products (Williams, 1956, 1957, 1960; Shimizu and Hibiki, 1955a, 1955b), including the more commonly encountered varieties of tuna (Geiger *et al.*, 1944; Geiger, 1944; Masao and Akira, 1952, 1953; Williams, 1954; Hillig, 1954), seafoods in general (Karmas, 1981) and fish of the family Scrombridae (Struaskiewicz *et al.*, 1977). Its levels in canned fish are also known to correlate with the trimethylamine and volatile nitrogen levels (Yamanishi *et al.*, 1954). Histidine decarboxylase is extensively produced by all putrefactive bacteria (Kimata and Kawai, 1951a) in fish, in particular, a few Proteus strains (Katae and Kawaguchi, 1959). Histamine appears to be correlated to content of free histidine; in fish with no free histidine, it may be derived from autolysis (Kimata and Kawai, 1951b; Masao *et al.*, 1954). Octopus and shark are exceptional and do not produce histidine (Kimata and Kawai, 1953). Histamine does not increase linearly during storage in herring, mackerel, cod and saithe, and therefore the increase observed can only be considered as an index of proteolysis in fish (Vorbeck, 1979). In white-meat fish, it is produced at a much later stage than visual spoilage, and in such cases ammonia appears to be a more reliable indicator (Kimata *et al.*, 1953). Histamine has received attention because of its toxicity at concentrations found in foods (Motil and Scrimshaw, 1979; Miyaki and Hayashi, 1954), especially fish and fermented food products (Doeglas *et al.*, 1967; Ferencik, 1970; Zee *et al.*, 1983; Edwards and Sandine, 1981). Tyramine has also been implicated as a potential health hazard (Blackwell and Mabbitt, 1965; Rice *et al.*, 1975), especially in meat (Santos-Buelga *et al.*, 1981). These biogenic amines are also formed by the contaminant lactic acid bacteria during ripening of dry sausages (Maijala and Eerola, 1993) or by organisms such as *Achromobacter histamineum* (Masao and Mikio, 1955) and *Proteus morgani* (Ganowiak *et al.*, 1979).

Increases in histamine in boiled and hot-air dried sardines have been reported. Substances such as trimethylamine oxide, urea and glycine, in decreasing order of effectiveness inhibit the decomposition of histidine to histamine (Shimizu and Hibiki, 1955a, 1955b). Histamine in tuna is known to be partially destroyed at 102 °C and almost totally at 116 °C, as in cans of tuna (Ienistea, 1971). Amines have been reported to be reliable indices of pork (Lakritz *et al.*, 1975; Nakamura *et al.*, 1979) and ground beef quality (Sayem-El-Daher *et al.*, 1984a). Histamine can be estimated by simple

paper chromatography (Kadota and Hayashi, 1951), thin layer or high performance liquid chromatography. Recently monoclonal antibodies to histamine prepared by immunizing mice with histamine–protein conjugates and exhibiting high affinity for histamine with no cross reaction with other biogenic amines have been used in a competitive inhibition ELISA to quantify histamine (Serrar *et al.*, 1995). This method could be used routinely for large numbers of samples. Oxygen sensor-based simple assay of histamine using purified amine oxidase in scromboid fish has also been developed. The analysis is based on stoichiometric oxidation of histamine to imidazoleacetaldehyde. Based on the equimolar relationship between histamine and oxygen consumption, histamine can be determined selectively by the oxygen sensor (Ohashi *et al.*, 1994). Putrescine and cadaverine in pork, and to some extent in beef, can be correlated with surface microbial count and organoleptic assessment such as odour, surface appearance, tenderness and juiciness. It has been shown that while putrescine could be a good indicator of bacterial count, cadaverine is a good indicator of temperature of storage and not an indicator of freshness since it is undetectable in spoiled beef stored at 4 °C and this is mainly attributed to the inhibition of lysine decarboxylase. Similarly in beef, spermine and spermidine are not good indicators of freshness because of their fluctuation during storage, and so is histidine which is also not a good indicator since it does not increase even in spoiled beef (Sayem-El-Daher *et al.*, 1984b). A high organoleptic correlation of beef with putrescine and 1,3 diamino-propane suggests them to be useful indices of freshness. In the case of broilers also, putrescine and cadaverine are detectable from colony counts of 10^5 cfu cm^{-2}, and can indicate onset of spoilage (Schmitt and Schmidt-Lorenz, 1992a).

An index formulated for tuna using these amines is:

$$\text{Index} = \frac{\text{ppm histamine} + \text{ppm putrescine} + \text{ppm cadaverine}}{1 + \text{ppm spermidine} + \text{ppm spermine}} \quad [5.2]$$

The selection of the formula was due to the simple general observation that histamine, putrescine and cadaverine rose in value while spermidine and spermine fell as decomposition progressed. In very badly decomposed fish, the spermidine and spermine levels were often zero, necessitating a factor of one in the denominator. This index compares favourably with the organoleptic methods. Since it measures several different compounds resulting from several different decomposition reactions, it is a better general index for decomposition of tuna fish (Meitz, 1977).

Agmatine appears to be most useful as a potential index for freshness of common squid (Yamanaka *et al.*, 1987). It has been detected in small amounts in fresh muscle and its concentration increases with storage time, exceeding 30 mg/100 g at the stage of initial decomposition and 40 mg/100 g at a stage of advanced spoilage. This is obviously formed by the action of bacterial enzymes from arginine which is abundant in the free state in squid muscle. Putrescine concentration also increases with the extent of decomposition. The pH value increased at the stage of initial decomposition.

VBN levels were not consistent during storage at different temperatures, but varied from 17 to 30 mg/100 g at the initial stage of decomposition and therefore did not make a good index. It has been rightly concluded that agmatine could be used as a true index of freshness of common squid and that putrescine and pH would be of value as supplementary indices.

Among the amines detected in scallop adductor muscle, putrescine and ornithine produced from arginine appeared to be more useful as potential indices of freshness (Yamanaka, 1989). Amine formation and quality in the shellfish group are being investigated, since this group (molluscs and crustaceans) are known to contain large amounts of arginine (Yamanaka, 1989). Cadaverine is the most suitable index for decomposition of salmon (*Oncorhynchus keta*) and rainbow trout (*Salmo gairdneri*), the levels being<1, 1–10, and >10 ppm at the acceptable, initial decomposition and advanced decomposition stages, respectively (Yamanaka *et al.*, 1989). Tyramine, as measured by tyramine sensors based on immobilized tyramine oxidase serves as an index of microbial count of beef products and is useful for monitoring early putrefaction and evaluation of fermented meat products (Yano *et al.*, 1992, 1995a, b).

Biogenic amines are thus good indices of freshness and degree of staling in fish and seafood varieties. However in different seafood species, different amines seem to be better indicators depending on the free amino acid concentration in the fresh tissues, the storage conditions and the predominant microbial contaminant. Some of the amines may be further metabolized by bacteria so that their concentration may drop as the bacterial load increases.

5.3.1.5 Indole

Among the chemical quality indices suggested for fish (Duggan and Strasburger, 1946), shellfish and marine products such as Dungeness crabs, indole content has recently been accepted (Quaranta and Cuzio, 1984; Quaranta *et al.*, 1985). In fish and shellfish, indole appears to be a product of bacterial metabolism of tryptophan even at refrigeration temperatures (Staruszkiewicz, 1974). In frozen products indole production begins during storage after thawing. It can be determined by spectrophotometric and colorimetric methods, HPLC (Staruszkiewicz, 1979) or by fluorimetric analysis (Ponder, 1978). The indole content of fish on the borderline of fitness for human consumption is reported to be between 3 and 6μg/100g (Wierzchowski and Severin, 1953). Fish can be considered deteriorated when the indole reaches 6.50 μg/100 g.

Indole is a good indicator of organoleptic spoilage in shrimps (Chambers and Staruszkiewicz, 1981). It however fails to detect decomposition, known as 'ammoniacal' (McClellan, 1952). In the case of oysters, however, neither indole nor TMA is a useful index (Duggan, 1948).

The correlation of indole and skatole measurements and organoleptic evaluation of boar taint has aroused considerable interest (Hansson *et al.*, 1980; Lundstrom *et al.*,

1984; Mortensen and Sorensen, 1984; Nonboe, 1992; Garcia-Regueiro *et al.*, 1986; Garcia-Regueiro and Diaz, 1989), with skatole giving a more intense odour than indole in similar concentrations (Empey and Montgomery, 1959). Indoles are not detected in the stomach or small intestine, but found in increasing concentration from the beginning to the end of the colon. A considerable variation in indole concentration has been observed, but not in the skatole content (Wilkins, 1990).

5.3.2 Fat breakdown products

Fat breakdown products are discussed by Pfeifer and Gacesa (1971) and Jangaard and Ackman (1965). The fat content of meat varies from 5% in lean meat to over 90%. The fat of the adipose tissue consists entirely of true fat or the triglyceride. Besides triglyceride (which could have varying degrees of unsaturation), animal fats contain small proportions of phospholipids, sterols, carotenoid pigments and fat soluble vitamins. Many of these components are altered during storage and may affect the odour and taste and thereby the storage life of the meat. These changes are measured in terms of the free fatty acids liberated due to action of lipase on the triglycerides, and oxidative rancidity due to the action of air or ketonic rancidity due to microorganisms. Hematin in various fish exerts a catalytic effect on the lipid oxidation (Brown *et al.*, 1956).

Lipid changes are useful indices in the case of fatty fish (Wood *et al.*, 1969). The formation of free radicals and relatively stable hydrogen peroxide at the early stages of the oxidative deterioration of fat may in turn affect fish protein adversely. These changes may include polymerization of proteins and oxidation of amino acids. At the advanced stages, the hydroperoxides are decomposed to low molecular weight carbonyl compounds responsible for off- and rancid flavour.

5.3.2.1 Free fatty acids

The FFA value has been suggested as a criterion for assessing seafood quality (Woyewoda and Ke, 1980). As fats and free fatty acids are generally insoluble in water, the titration is usually carried out in organic solvents such as ethyl alcohol (British Standards Institution, 1958) and alcohol–diethyl ether (British Pharmacopoeia, 1953).

Lea (1931) reported figures of 1.5% (as oleic acid) in the fat of beef stored for 25 days at 0 °C, after which the acidity increased more rapidly from 5 to 11% after 42 days, which in turn coincides with a change in the flavour from sweet to unpleasant. In the spoilage in bacon fat, excessive FFA is of little importance. In minced beef in retail establishments a maximum value of FFA during storage is suggested to be 1.8% (Pearson, 1967).

5.3.2.2 Peroxide value

Theories relating to the autoxidation of unsaturated fatty acids postulate the primary formation of substances possessing peroxide properties. This applies to chicken as well (Shchennikov *et al.*, 1955). The peroxide value of stored meats, like 'pure oils', shows an induction period followed by a fairly sharp rise (Broumand *et al.*, 1958; Watts, 1962). No threshold peroxide values for the rancid odour of cured and uncured meats and bacon have been designated (Zipser *et al.*, 1964). The peroxide value for extracted fat of fresh beef is reported to be 0–1.0 mEq kg^{-1}, with a value of 5 being taken as a critical acceptability limit. The peroxide value as an index is not always accurate. It increases during the active oxygen absorption period, reaches a maximum and subsequently decreases. The ratio of olefinic protons to aliphatic protons (R_o), measured by NMR, decreases continuously during oxidation (Saito and Nakamura, 1989) as does the ratio of divinylmethylene protons to aliphatic protons (R_m). These ratios have therefore been considered as useful indices of oxidative deterioration (Saito and Udagawa, 1992). The applicability and limitations of this method to various kinds of fish meal needs to be investigated.

5.3.2.3 Thiobarbituric acid value

The thiobarbituric acid value (TBA) has been suggested as an empirical method to measure the oxidative deterioration of fatty foods and measures malonaldehyde as a marker of lipid peroxidation (Botsoglou *et al.*, 1994). Unlike other methods, the TBA test can measure the deterioration in both extractable and non-extractable lipids (Keskinel *et al.*, 1964). A high TBA number is found in lean beef compared with pork. The correlation between odour and TBA number is high. Yet peroxide/TBA ratios appear preferable to single values (Zipser *et al.*, 1964). Spectral changes in beef (Price and Schweigert, 1971) relating to the myoglobin/metmyoglobin ratio have also shown correlation with TBA, peroxide value and other conventional measurements of lipid oxidation (Ukhun and Izi, 1991).

Changes in water activity (a_w) lead to differences in the oxidation status of beef lipid as assessed by TBA values (Greene and Cumuze, 1982). An a_w of 0.33 is the most effective prooxidant in stored beef. Vacuum storage completely retards flavour deterioration as marked by chemical markers such as TBA reactive substances and lipid volatiles (Spanier *et al.*, 1992).

The TBA methodology is of limited value in determination of oxidative rancidity in cured meat products because of interaction of malonaldehyde or sulphanilamide with residual nitrite. It has been suggested that the concentration of hexanal, a major volatile in cooked meats, may be a better index of oxidative rancidity in cured meat products (Shahidi, 1989; Shahidi and Hong, 1991; Lai *et al.*, 1995).

5.3.2.4 Ranco number

Ramsey *et al.* (1964) have developed a method in which rendered pork fat is heated at 70 °C with a solution of potassium hydroxide in isopropyl alcohol under standardized conditions. Rancid fats produce a yellow colour, and the optical density measured at 385 nm is termed as the 'Ranco number'; this has shown a very high correlation with TBA number. This method can be applied to the spoiled rendered fat rather than the meat itself.

5.3.2.5 Kreiss test

This test is based on the reaction between phloroglucinol and the fat under acidic conditions, producing a red colour which appears to be related to the oxygen absorption and is suggested to be due to the degradation products, epihydrin aldehyde or malonaldehyde (Patton *et al.*, 1951). The Kreiss test as applied to meat is believed to be too sensitive in the incipient stages so that non-rancid fats sometimes produce intense colours.

5.3.2.6 Carbonyl compounds

The formation of carbonyl substances (Kim *et al.*, 1974) in sea salmon, red perch and herring fillets is known to increase on storage. These can be measured colorimetrically (Henick *et al.*, 1954) or by the absorption value of the distillate from the fat at 280 nm (Altu'feva *et al.*, 1970). The benzidine test of Holm *et al.* (1957) has shown some correlation with off-flavour development. No acceptable method is as yet available for determining the carbonyls.

5.3.2.7 Hydrocarbons

Pentane has been considered to be an index of rancidity in freeze dried pork. Short chain hydrocarbons like pentane are chemically inert and can therefore be easily separated from rancid fat (Seo and Joel, 1980). Methane has been found to be a major short chain hydrocarbon followed by pentane, propane and butane. Methane may arise from alanine by Streker degradation but the possibility of formation of pentane from a non-lipid source such as amino acid is remote. Pentane however is not responsible for the rancid odour, it is only an indicator of lipid oxidation.

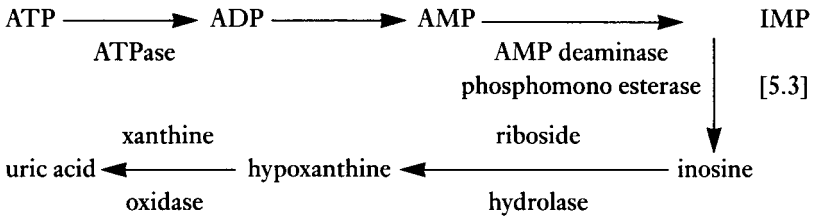
5.3.2.8 Chemiluminescence

Studies on fresh minced meat of the fish species, sardine (*Sardinops melanosticus*), red sea bream (*Pagrus major*), tuna (*Thunnus orientalis*), kichiji (*Sebastolobus macrochir*), mackerel (*Scomber japonicus*) and blue sprat (*Spratelloides gracilis*) have shown that

shelf life, as judged by oxidative deterioration significantly correlates with chemiluminescence intensity of fresh meats. It is shown to be highly accurate for the prediction of fish meat shelf life (Miyazawa *et al.*, 1991).

5.3.3 Nucleic acid breakdown products

The mechanism of breakdown of nucleotides is as shown below (Eklynd and Miyauch, 1964):



Nucleotide degradation products have attracted attention as suitable indicators of quality of fleshy foods (Dingle and Hines, 1971; Fujii *et al.*, 1968; Uchiyama and Ehira, 1970). Evaluation of adenosine-5′-triphosphate (ATP) degradation products in six Finnish fish species indicated different species-dependent degradation rates (Hattula and Kiesvaara, 1992). The nucleotide degradation products arise principally due to autolytic activity whereas trimethyl amine, total volatile bases and volatile reducing substances result from bacterial action, and the latter are of value as spoilage indicators only in the last stages of spoilage (Valencia and Sanahuja, 1969). ATP concentration has been proposed as an index of mesophilic count in minced meat samples. It can be determined by bioluminescence, based on luciferin/luciferase reaction, wherein the light being emitted is proportional to ATP (Jouve *et al.*, 1981). For practical applications, however, the sensitivity of the analysis needs to be improved (Catsaras and Lacheretz, 1982). This is mainly because of its relatively high detection limit ($\sim 5 \times 10^4$ g^{-1}), possibly because of problems with elimination of tissue ATP, and background noise (Bruchon, 1991). Deamination of adenosine-5′-phosphate (AMP) to IMP (inosine monophosphate) generates ammonia. However, ammonia generated by this pathway represents only a small proportion of the total ammonia (Langille, 1983). The use of hypoxanthine has been advocated as an index of quality in some marine fish species (Jones and Murray, 1961, 1962, 1964; Jones *et al.*, 1964; Jones, 1965). However its formation at different rates in different individuals within a species and the variation amongst species precludes its value as a quality index (Dugal, 1967). In the case of albacore, belonging to the Scombridae family, hypoxanthine (HYP) has been correlated with the storage period by the equation, HYP (mg/100 g) $= 1.36 + 0.973 \times$ days (Perez-Villarreal and Pozo, 1990).

Determination of all intermediates in the breakdown of ATP in meat extracts can be done by using reversed phase HPLC; their correlation with the freshness of beef is

under investigation (Watanabe *et al.*, 1989). These purine compounds in meats have a dietary significance for gout patients (Freudenreich and Werner, 1988).

A ratio known as the '*K* value' has been formulated as an index of freshness in fish and meat (Saito *et al.*, 1959).

$$K \text{ value} = \frac{\text{inosine} + \text{hypoxanthine}}{\text{total ATP breakdown products}} \quad [5.4]$$

Studies on freshness of commercial frozen shrimps have shown that it is desirable to keep the *K* value of commercial frozen shrimps to below 40 (Wada *et al.*, 1973). However, *K* values of canned fish meat such as pink salmon and mackerel have been found to be very high at a stage when an abnormal flavour is not detected (Nomoto *et al.*, 1989). The *K* value has been related to the number of days of storage in albacore by the equation, $K = 18.04 + 2.208 \times \text{days}$ (Perez-Villarreal and Pozo, 1990).

A K_1 value, proposed by Karube *et al.* (1984), defined as the ratio of hypoxanthine + inosine to the total amount of inosine-5′-monophosphate, inosine and hypoxanthine, and expressed as a percentage has also shown a good correlation with the *K* value. Several different methods such as simplified column chromatography (Uchiyama *et al.*, 1970), HPLC, enzymatic measurement with an electrode system (Ohashi *et al.*, 1985) and enzyme sensor systems (Karube *et al.*, 1984) have been described for the measurement of the *K* or K_1 value.

A newly discovered enzyme, nucleoside oxidase (Isono and Hoshino, 1988; Isono *et al.*, 1989) catalyses the oxidation of nucleosides, which in presence of *N*-ethyl-*N*-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline and 4-aminoantipyrine forms a colour proportional to the nucleosides oxidized (Isono and Hoshino, 1989). A simple and a rapid colorimetric assay for measuring the K_1 value using the nucleoside oxidase has been reported (Isono, 1990). Besides *K* value and K_1 value, inosine, hypoxanthine and IMP ratios are reported to be adequate indicators of fish freshness (Fujii *et al.*, 1973). The IMP ratio can be calculated as IMP (%) = 100 − K (%) (Nomoto *et al.*, 1989).

These indicators are represented as:

$$\text{Inosine ratio (\%)} = \frac{100 \times \text{inosine}}{\text{inosine} + \text{hypoxanthine} + \text{IMP}} \quad [5.5]$$

$$\text{Hypoxanthine ratio (\%)} = \frac{100 \times \text{hypoxanthine}}{\text{inosine} + \text{hypoxanthine} + \text{IMP}} \quad [5.6]$$

$$\text{IMP ratio (\%)} = \frac{100 \times \text{IMP}}{\text{inosine} + \text{hypoxanthine} + \text{IMP}} \quad [5.7]$$

Figure 5.1 shows the changes in the IMP, inosine and hypoxanthine ratios with

storage time at 18 °C. It can be seen that with the progress of storage, the IMP ratio rapidly decreases, the inosine ratio increases sharply in the early stage and decreases later and the hypoxanthine ratio increases much more rapidly in the later stages than in the early stages (Isono, 1990).

Ribonucleotides exert a major influence on the flavour of flesh foods. Most abundant in chicken muscle is inosinic acid which degrades to inosine and hypoxanthine. Degradation of inosinic acid to hypoxanthine has been associated with bitter off-flavour and its measurement provides a useful index of quality in freshness evaluation of croakers (*Micropogon* spp.) or spot (*Leiostomus xanthurus*) (Guardia and Haas, 1969). Inosinic acid content is more directly related to the flavour of meat than hypoxanthine. In general, hypoxanthine is negatively correlated to quality, while inosine and IMP are positively correlated with overall desirability (Greene and Bernatt-Byrne, 1990).

Hypoxanthine formation has been observed to occur at a fairly uniform rate, reaching a maximum value in 6 days. In contrast, trimethylamine and total volatile bases show practically no changes until after 8 days of storage. Hypoxanthine can thus yield information during early storage, and is particularly suitable for refrigerated fish (Valencia and Sanahuja, 1969). Surimi, an intermediate product in seafood analogue production, is primarily a concentrate of salt soluble muscle protein, and is prepared from deboned, minced and washed fish. Chemical methods recommended for quality evaluation of surimi are hypoxanthine content and free fatty acids, both of which correlate well with sensory grading (Ke and Burns, 1989).

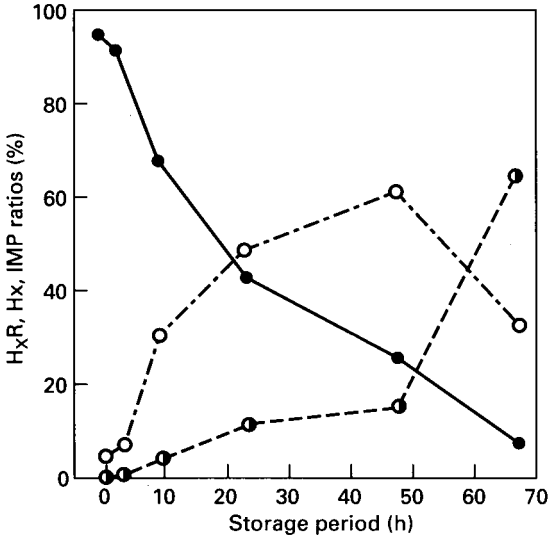


Figure 5.1 Changes in the H_xR (inosine) ratio and IMP ratio in the yellowtail fish (*Seriola quinqueradiata*) during storage at 18 °C. ○, H_xR; ◑ Hx; ● IMP. (Source: Isono, 1990, reproduced with permission)

A biosensor constructed for measurement of hypoxanthine consists of immobilized xanthine oxidase (Shen *et al.*, 1996) and a polarographic electrode on a commercially available preactivated nylon membrane. The polarographic electrode detects hydrogen peroxide and uric acid released during the enzymic reaction (the linear range being 3.6–107 μM). The results obtained using this sensor compare well with the conventional method using the same enzyme. More than 40 assays can be performed with the same membrane and each sample can be assayed in 2–3 min, and hence is a simple, rapid and economical method for the measurement of hypoxanthine (Luong and Male, 1992; Mulchandani *et al.*, 1989). Besides fish, xanthine sensor has also been demonstrated to evaluate ageing in meat (Yano *et al.*, 1995b). Similarly, an enzymic assay for IMP in fish muscle extract using IMP dehydrogenase from *E. coli*, diaphorase, NAD and thiazolyltetrazolium bromide is reported. Test papers based on the assay procedure have been developed, which along with inosine/hypoxanthine test papers can determine the *K* value. A correlation of 0.993 has been observed between this technique and the usual colorimetric method (Negishi and Karube, 1989).

5.3.4 General and miscellaneous techniques

The chemical and physical changes which take place in stored meat may not necessarily directly be associated with the deterioration of protein or fat. Basic as well as acidic substances are produced during such deterioration (Kunisaki, 1967).

5.3.4.1 Colour and pH value

Colour and pH value have been discussed by Kawabata *et al.* (1952), Toyoaki (1951) and Hongmann (1988). Examination of fishes in various stages of freshness has shown pH to correlate with the changes indicated by the soluble and volatile nitrogen (D'Orazio, 1955). It is also a useful indicator to follow the course of putrefaction during meat spoilage (Yamakawa *et al.*, 1956). Tenderness scores of cooked and raw pork are also shown to correlate with pH determined on the freeze-dried muscle (Lewis *et al.*, 1963). The average pH value of 6.99 of five beef carcasses declined to 5.46 and 5.57 in 48 h and 480 h, respectively. Such findings of a decline followed by an increase in the pH of meat from the slaughter stage have been reported by several workers (Jay, 1964a; Rogers and McCleskey, 1961). pH sensors to detect fish freshness have been developed and have been shown to correlate to the *K* value (Li *et al.*, 1992). It has been proposed as an index of freshness in canned red crab (*Chinoecetes opilio, Fabricius*), particularly in the later stages of storage. IMP ratio is suggested for detecting the same spoilage in the earlier stages of storage (Fujii *et al.*, 1972). The muscle pH in fat-rich fish tends to increase with increasing body length; no such correlation exists with low fat fish (Oehlenschlager, 1991a, 1991b).

Indices suitable for assessment of the quality of boiled hams are not suitable for dry hams. An index taking into account the pH of the adductor muscle and the colour of

the vastus muscle has been derived from an observation that consumers prefer dry hams having moderate pH and medium pink colour (Poma, 1991). With deterioration of squid the pH turns alkaline which in turn solubilizes the pigments in the epidermis thereby imparting a reddish colour to meat. Thus, the degree of reddening is an indicator of squid quality. However, colour can be manipulated (Botta *et al.*, 1979; Ohmari *et al.*, 1975; Tanikawa *et al.*, 1970). Quality standards for dried squids include colour as an important parameter. The browning or blackening in dried squid during storage reduces its value drastically. However, in disputed or borderline cases, chemical and/or sensory data are deemed necessary (Ke *et al.*, 1984). The colour could be measured easily by reflectance spectrophotometry at 520 nm (Scharner *et al.*, 1976). An *E*-value is obtained as the optical density at 400 nm of a 5% methanolic KOH solution multiplied by the extract volume and divided by the weight of the sample in grams. This value above 5 is indicative of excessive browning (Hayashi and Takagi, 1980). The colour could be measured using near-infrared spectrometry (Freudenreich, 1992) or by using light sources of different wavelengths which can be amplified and converted into a digital signal to quantify the pigment (Masahiko, 1991).

Objective measurements of colour using colour parameters such as L^*, a^* (L^* and a^* are values used to define colour on the CIELAB colour measuring system; L^* for lightness and a^* for redness) and hue angle can be used to evaluate pork quality on line in an industrial context. This has been confirmed by cluster analysis (Chizzolini *et al.*, 1993a, 1993b). Serum amylase types and levels are also useful indicators for postmortem meat colour in pig meats (Wegner *et al.*, 1970).

5.3.4.2 Volatile acidity

The concentrations and ratios (Suezo, 1953a, 1953b, 1953c, 1953d, 1953e) of individual volatile fatty acids may prove to be a means of evaluating spoilage in beef (Sikorski, 1966), packs of Ocean perch (Hillig *et al.*, 1960b) and fish used for canning (Clark and Hillig, 1938; Hillig and Clark, 1938; Hillig, 1939a; 1939b; Clague, 1942; Sigurdsson, 1947; Hillig *et al.*, 1950a, 1950b). It is an official AOAC method of analysis. It is a measure of shorter chain fatty acids including acetic, formic, propionic, and butyric acids. Volatile acids higher than acetic acid are generally not found in canned fish. Formic and acetic are the acids most frequently detected, with acetic acid predominating (Hillig *et al.*, 1958, 1960).

Spoilage of Atlantic 'little tuna' (*Euthynnus alleteratus*) parallels the levels of formic, acetic, propionic, butyric and succinic acids, and can indicate the quality of the raw material in the canned product (Hillig, 1954). These acids generally impart a disagreeable odour to the meat.

In meats, the lactic acid content appears to stabilize within 1–2 days after slaughter (Bodwell *et al.*, 1965). However, acetic, propionic and butyric acids have been shown to predominate in the volatile acids from the beef carcasses examined (Shank *et al.*, 1962). These acids are suggested to be of importance in the development of a sour non-

microbial off-condition. Succinic acid content has been found to correlate well with the condition of the raw material employed (Hillig *et al.*, 1950a, 1950b). The use of lactic acid has been reviewed as a potential seafood quality index (Jacober and Rand, 1982).

Since acetic acid is the major acid being measured in volatile acids, it is appropriate to measure the acetic acid content of the fish extract by a colorimetric procedure (Suzuki, 1953a, 1953b) or by means of a commercially available enzyme kit, which would be quicker and more specific. High positive correlations of 0.98 and 0.95 have been shown for fresh and canned fish, respectively. Acetic acid can therefore be a reliable indicator of quality of certain seafoods, especially if used in combination with other chemical parameters such as total volatile bases for lean fish and TBA number for fatty fish (McCarthy *et al.*, 1989).

5.3.4.3 Volatile reducing substance

Volatile reducing substance (VRS) closely fits the specifications of an ideal index in that it does not depend on the presence or production of any specific compound or class of compounds, but whatever the spoilage pattern, as long as the deterioration is accompanied by odours and hence of volatile substances, they can be detected as VRS. VRS gives a good correlation with meat (Rubashkina, 1953), raw and canned fish (Farber and Ferro, 1956), as well as that preserved under cold or by salt (Golovkin *et al.*, 1961), as judged organoleptically. Table 5.8 shows values of VRS in chum salmon canned after progressive raw storage at 65 °C.

A similar pattern has been observed for canned tuna of various species, rock cod and Dover sole fillets. The VRS value seems to reflect protein spoilage as well as fat

Table 5.8 Volatile reducing substances in chum salmon canned after progressive raw storage at 65 °C

Hours old	Organoleptic judgement	Volatile reducing substances (avg.)
7	Normal odour and colour	14.6
31	Slightly soft and pink, somewhat stronger odour	28.6
55	Somewhat soft, definite pink colour and strong odour, stale	34.3
77	Soft, pink to red colour, spoiled odour, taint	57.2
99	Honeycomb present, very strong spoiled odour, putrid	120.8

Source: Farber and Cederquist, 1953 (reproduced with permission).

Table 5.9 Volatile catabolites of the amino acids during normal spoilage of refrigerated chicken carcasses and those spoiled by six bacterial species

Amino acid	Volatile metabolites	Normal spoilage (days)	*Pseudomonas fluorescens*	*Pseudomonas putida*	*Pseudomonas fragi*	*Alteromonas putrefaciens*	*Serratia liquefaciens*	*Brochotrix thermosphacta*
Valine	2-Methyl propanoic acid							(+)
	2-Methyl propanoic acid methyl ester	8–12			+			
	2-Methyl propanoic ester ethyl ester	8–16			++			+
	2-Methylpropanal							+
	2-Methylpropanol	8–16	(+)	+	++		+	+
Isoleucine	2-Methylbutanoic acid							+
	2-Methylbutanoic acid methylester	8–16			++			+
	2-Methylbutanoic acid ethylester	6–16			+++			+
	2-Methylbutanoic acid propylester				(+)			
	2-Methylbutanal		(+)		+	+		++
	2-Butanone	6–16		(+)		+	+	
	2-Methylbutanol	6–16		+	++		+	+
Leucine	2-Methylbutanoic acid							++
	3-Methylbutanoic acid ethylester	8–16			+++			
	3-Methylbutanal				(+)	+	(+)	+++
	3-Methylbutanol	6–12		(+)	++	+	++	+++

Source: Schmitt and Schmidt-Lorenz, 1992b (reproduced with permission).

spoilage, particularly the more marked rancidity state (Farber, 1954). Table 5.9 shows the volatile metabolites from the catabolism of amino acids during normal spoilage of refrigerated chicken and carcasses spoiled by six different bacterial species (Viehweg *et al.*, 1989a, b).

Volatile sulphides such as hydrogen sulphide, dimethyl sulphide and methyl mercaptan are produced by bacterial enzymes acting on sulphur amino acids contained within the fish flesh and meat (Herbert, 1970; Martin *et al.*, 1962). Relationships between hydrogen sulphide gas measured from the gills in cod and total viable counts (taken from both the gills and flesh) and between hydrogen sulphide gas and hydrogen sulphide producer counts were of the same linear form and gave similar predictive confidence limits (Strachan and Nicholson, 1992).

The values for the state of incipient spoilage or borderline spoilage are quite distinct and are detectable. The VRS value therefore comes closest to being generally applicable to the determination of the widest encountered spoilage commercially including bacterial breakdown and chemical deterioration of fat. It is not applicable to endogenous enzymic spoilage, such as autolysis which does not produce odoriferous or volatile products (Farber and Lerke, 1958). In the case of shrimps, VRS values are useful in the raw state, but freeze drying lowers the VRS to the extent that fresh and stale could not be differentiated (Moorhouse and Salwin, 1969). Most of the indices put forward in vertebrate fish show random relation when applied to oysters. To overcome this, the total reducing substances (TRS) have been studied and this value nearly approaches the criteria required for a chemical indicator. It also correlates with microbiological tests and organoleptic profile with reproducibility (Liuzzo *et al.*, 1975).

Freeze drying removes additional volatiles and therefore does not permit differentiation between fresh and spoiled beef (Moorhouse and Salwin, 1969).

5.3.4.4 Water holding capacity

The water holding capacity (WHC) is the ability of the meat to hold fast to its own or added water during the application of pressure or mincing. It appears to be influenced by the treatment that the carcass receives prior to storage. It is believed that the change in WHC is a sensitive indicator of alterations in proteins (Hamm, 1960). The WHC of freshly slaughtered meat is high, but drops markedly within a few hours and then increases again during further storage. Hamm (1956) attributed two-thirds of the post-mortem hydration drop to the breakdown of ATP and one-third to the fall in pH, so that there is probably a connection between the hydration decrease and rigor development.

It has been found that during spoilage the free water area on meat decreases linearly with time, and the fall is more closely related to the bacterial numbers. This is confirmed from the observation that meat infused with tetracycline showed a lower bacterial count and higher WHC when compared to control meat.

Extract release volume (ERV) appears to have considerable possibilities for assessing the spoilage of beef (Jay, 1964a, 1964b). It also has a highly significant correlation with WHC. The procedure is based on measuring the volume of the aqueous filtrate released from a slurry of meat in a fixed time. The ERV decreases as spoilage progresses and no filtrate is obtained from putrid meat. An ERV of 25 ml (obtained with 25 g meat with 100 ml buffer solution of pH 5.8 and a filtration time of 15 min) has been recommended as a rejection cut-off figure (Jay and Kontou, 1964). A major drawback is the fairly wide range of values given by fresh meats (21–35 ml).

In view of the simplicity, rapidity of performance and the apparently consistent decrease with spoilage, the ERV has proved useful for routine quality control assessment of meats.

Wierbicki *et al.* (1962) presented a method of measuring the water holding capacity (WHC) of muscle proteins with low water-holding forces which they referred to as meat swelling. Since the tackiness appeared to be related to beef homogenate viscosity, Shelef and Jay (1969) studied viscosity measurements as a possible indicator of meat freshness or spoilage. They found that as incipient spoilage sets in, viscosity values continue to increase beyond the time when extract release volume is zero. Drip losses, used to quantify the WHC of the muscle protein are shown to be the only parameter which can work as a useful texture indicator of frozen fillets, and furthermore it is the only parameter showing a non-temperature dependent relationship with texture (Giannini *et al.*, 1993).

5.3.4.5 Volatile metabolites of microorganisms

In general, the action of bacteria on meat constituents produces a stale, sour or putrid odour, associated with spoilage. Condensation of the volatiles from frozen beef at low temperatures and fractionation by gas liquid chromatography has identified hydrogen sulphide and methyl and ethyl mercaptans, acetaldehyde, acetone, methyl ethyl ketone, methanol and ethanol (Merritt *et al.*, 1959). Ethanol is especially recommended as an indicator for assessment of spoilage, especially in fish containing low or varying amounts of trimethyl oxide and thus producing only small amounts of the widely used spoilage indicator trimethylamine (Rehbien, 1993). It also correlates with endotoxin production by gram negative organisms, as assessed by the limulus lysate test in seafood spoilage (Brown *et al.*, 1979). There is considerable evidence that volatiles from lean meat contribute to the flavour and that flavour differences among species can be traced to the fat. These compounds can serve as tentative indicators of meat freshness. Production of hydrogen sulphide and other volatile sulphides is also illustrative of the different types of the spoilage flora of fish. Aeromonas species, followed by Vibrio, *Alteromonas putrefaciens* and Pseudomonas species are the main organisms producing sulphides (Taampuram and Iyer, 1990).

5.3.4.6 Minerals

Studies on samples of frozen squid (*Ommastrephes saggitatus*) have shown the efficacy of using the sodium:potassium ratio as a quality control indicator. This analysis can be carried out by atomic absorption spectrometric and flame emission techniques (Martinez Para *et al.*, 1985a, 1985b).

Examination of meat or fish samples with a few drops of ammonium vanadate solution after allowing the sample to stand for 5 min gives a good qualitative judgement of state of freshness. If the meat is in a good state, an emerald green colour is formed, with incipient putrification the colour is pale green and when putrification is advanced, the solution becomes white opalescent or milky (Lassandro-Pepe and Maragliano, 1954).

5.3.4.7 Degradation products of creatine

Creatine and creatinine are normal constituents of meat. Creatinine content has been considered as an index of quality of beef (Roulet, 1963). 1-Methylhydantoin is a product of desimidation of creatinine (Szulmajster, 1958a, 1958b). The formation of 1-methylhydantoin in whale meat has been shown during staling (Nakai *et al.*, 1969). Analytical methods to detect these compounds would be paper chromatography or thin layer chromatography, which permit separation of creatine, creatinine, hydantoin and 3-methylhydantoic acid, the last possible intermediate of creatinine decomposition, but whose microbial formation is scarcely reported. A paper chromatography method using solvent systems n-butanol–pyridine–water (20:30:15), n-butanol–pyridine–water (20:30:20) and isopropanol–pyridine–water (20:30:15) followed by heating for 1 h at 110 °C and then spraying with Jaffe's reagent viz 1.3% ethanolic picric acid + 1/5 volume of 10% sodium hydroxide (Williams, 1951; Block *et al.*, 1958) is known to give satisfactory results. Acetoacetic and pyruvic acids, which may occur in the meat also give weakly positive Jaffe's reactions. Acetoacetic acid is however destroyed during heating the chromatograms at 110 °C. Pyruvic acid is absent in whale meats of different degrees of staleness. Therefore the method as proposed above is suitable for the required purpose (Nakai *et al.*, 1970).

5.3.5 Instrumental analysis of meat/fish quality

At a port, typically several hundred tons of fish have to be graded within a period of 0.5–2 h, which most of the time is achieved by sensory analysis, odour being the most predominant basis of quality. Sensory inspection of this kind can be successful, but has some disadvantages. It requires fairly highly trained personnel and obviously has some degree of subjectivity.

Apart from the chemical indices of freshness monitoring discussed above, a promising instrumental technique based on measurement of electrical properties of

the skin and subdermal layers of fish has also been reported. By using a specially designed four-electrode surface contact device, it is possible to measure instantaneously and simultaneously the resistance and capacitance at high frequency of intact fresh or chilled fish. Both resistance and capacitance decrease rapidly as the fish passes through rigor mortis and then change more slowly during subsequent storage. Unfortunately, values of both properties depend on the orientation of the measuring electrodes with regard to muscle. The power factor of intact fish, which is a function of the product of resistance and capacitance, is independent of these factors and decreases uniformly as the fish spoil and can be used as an index of freshness. Earlier work, on the basis of which an instrument called Intelectron Fish Tester had been designed and marketed had used the ratio of the resistances at two different frequencies as an index of freshness. The ratio of resistance to capacitance is largely independent of the fish size and varies regularly with spoilage. This provides a rapid, non-destructive method of grading which is independent of subjective judgement (Connell, 1973). The electrical conductivity of pork has also been suggested as an index of quality (Schwagele and Honikel, 1991).

The four-electrode system was found to be complicated and not convenient for industrial use. An instrument based on identical principles was then developed which had the facility of a meter reading from '0' to '16' on a display system. The efficacy of this system was checked on several fish species. The results with pelagic fish containing higher fat content, however, tend to be more variable because the fat in the flesh has an effect on dielectric properties. This instrument called the 'Torrymeter' measures changes in physical properties of fish muscle and skin in the wet state, and different methods of handling and processing will affect these measurements. Very commonly, fish are held in ice in bulkboard fishing vessels, and the pressure on an individual fish increases with its depth below the surface layer. This can cause damage to the tissues and the meter readings for the fish in the lower layers will usually be lower than if they are stored in shallow boxes with ice. Fillets with skin give identical meter readings as whole fish, but because the dielectric properties of skin and muscle differ, it follows that skinless fillets of the same freshness will necessarily give different meter readings. Freezing changes the original structure of the fish and so it is not possible to determine the original freshness of thawed fish; the meter readings have been found to be in the range 0–3, irrespective of the quality before freezing. This effect can sometimes be used to ascertain whether a fish has been frozen at some time in its history. For example, if the meter readings are low and sensory scores are high, there are grounds for suspecting that the fish has been frozen at some time (Cheyne, 1975).

The use of ultraviolet light as an aid in detecting decomposition in raw shrimp has been of interest to the trade. Preliminary observations demonstrated that putrid shrimp show coloured fluorescent areas under ultraviolet light. However, the distinction between acceptable and spoilt shrimps was not found to be conclusive (Barry, 1957).

Firmness of a variety of foods, in particular muscle foods such as fish fillets, can be evaluated by measurement of the deformation of the sample under a predetermined pressure and the measurement of rebound when the pressure is subsequently released. The firmness, which could indicate freshness can then be determined as a rebound to the deformation ratio.

5.4 Eating quality of fleshy foods

Immediately after exsanguination, complex biochemical reactions collectively regulate postmortem changes which are associated with the transformation of the muscle to meat. The magnitude of these changes has a direct influence on meat quality and appears to be closely related to the rate of anaerobic glycolysis (Briskey and Wismer-Pedersen, 1961) and pH and temperature in the muscle at the onset and completion of rigor mortis. Changes in the glycolytic intermediates and nucleotide levels (Briskey and Lawrie, 1961) also occur postmortem in muscle (Briskey *et al.*, 1966).

The physical manifestation of the biochemical changes is easily visualized. A relatively slow rate of glycolysis resulting in moderately low pH and low temperature is associated with a normal pork muscle colour of greyish pink to red, moderately firm structure, and moderately dry appearance. If the pH remains high, or at least if the rate of decline is retarded, muscles retain dark red colour, firmness and are dry in appearance (DFD). Conversely, a rapid rate of pH decline resulting in acid conditions at a high temperature results in the development of a pale soft and exudative muscle (PSE). Under normal conditions, the combination of low pH and high temperature in muscle immediately postmortem usually is associated with PSE muscle. Individual muscles are known to vary in the extent of PSE (Stamenkovic *et al.*, 1991). It has been postulated that pigs which ultimately have PSE musculature may have some degree of deficiency in adrenocortical hormone production. Studies carried out over a 5 year period have shown that the incidence of PSE and DFD are approximately 6% and 5%, respectively. In the warm periods of the year, the incidence of PSE increases to about 12% (Niewiarowicz and Pikul, 1980).

PSE and DFD are quality defects affecting the manufacturing properties as well as the aesthetic appearance of pork meat. It is generally agreed that the complete elimination of PSE/DFD condition can only be achieved through genetic selection and improvement in the preslaughter and postslaughter handling of the live animal and carcass, respectively (Eikelenboom, 1985). Methods for detecting the PSE and DFD conditions are a pressing need so as to allow proper disposal of these types of products through the food chain and to permit a better quality control by the pork industry. Evaluation of slaughterline instrumentation for quality grouping of pork (Wal, 1987) has been based on light scattering properties of the muscle (Andersen, 1984; Barton-Gade and Olsen, 1984; MacDougall, 1984; Somers *et al.*, 1985; Swatland, 1986) or on the electrical properties of the muscle (Swatland, 1980; Pfutzner *et al.*, 1981; Schmitten *et al.*, 1985; Seidler *et al.*,1985; Seidler *et al.*, 1984; Hald, 1993).

Electrical conductivity and pulse impedance measurements (Schoberlein *et al.*, 1988a, 1988b), 24 h postmortem have been suggested as an indicator for detection of PSE. Dielectric loss factor seems to be especially suited for PSE diagnosis of meat (Chizzolini *et al.*, 1993b). Instruments based on the electrical properties of the muscles such as dielectric constant and electrical conductivity and electrical capacitance of the muscle (Swatland, 1981, 1982) have been developed. These instruments, however, have failed to distinguish reliably among the various Canadian quality standards (based on paleness and structure) used to identify PSE/DFD in pork (Fortin and Raymond, 1988). Subjective evaluation of moistness, colour and texture are believed to give the best results in detecting PSE in intact pork (Wal, 1987). Colour has been shown to correlate significantly with the pH of the intact or chopped muscle, cooked gel strength and cooking loss, and can be used to evaluate the occurrence of PSE in turkey breast meat (Barbut, 1993) and other meats such as pork before and after frozen storage (Irie and Swatland, 1992, 1993). A pH of 6.5–6.6 and 7.0–7.1 in live bird skin is indicative of high probabilities of PSE and DFD defects respectively, in broiler meats (Niewiarowicz and Pikul, 1980). In the German Democratic Republic, the quality of skeletal muscle meat is evaluated on the basis of pH, drip loss and colour. A procedure is given in which deviations from optimum quality are considered as unidimensional vectors. The addition of these for individual quality characteristics gives a sum which in turn gives an estimate of overall quality (Kruger and Schiefer, 1988). Texture, measured with a meat structure tester has also been shown to be applicable in the diagnosis of the PSE meat (Campanini *et al.*, 1991). Rapid cooling is reported to reduce PSE incidence to less than half (Petrovic *et al.*, 1992).

Several related aspects of the eating quality of beef, or more precisely, muscle quality have not been clearly defined. This combination of traits is commonly thought to produce maximum acceptability from the standpoint of the consumer. These traits are economy, a high proportion of lean meat, attractiveness and optimum palatability. No reliable method for evaluating optimum palatability has been found presumably because of the lack of complete understanding of what traits contribute to desired palatability. Such components of beef muscle as lactic acid (Lewis *et al.*, 1963), myoglobin and haemoglobin (Husaini *et al.*, 1950; Craig *et al.*, 1966) and alkaline phosphatase have been studied to determine their association with muscle quality. Many times, conflicting results are reported, but in general, sensory panel evaluations strongly suggest an undesirable effect of lactic acid, ash and pigment concentration on muscle acceptability. Variable but small, negative correlations have been observed between panel traits and the acid and alkaline phosphatase concentrations of the muscle (Dryden *et al.*, 1969).

Since the connective tissue within the muscles plays an important role in determining tenderness (Cover and Smith, 1956; Irvin and Cover, 1959), the effect of age on the amount of connective tissue measured, usually as collagen, has been studied without definitive results. Decreasing tenderness has generally been shown to be associated with advancing maturity in bovine animals (Goll *et al.*, 1963; Herring *et al.*,

1967; Webb *et al.*, 1967) and likewise in chicken (Lowe, 1948; Wells *et al.*, 1962) and goat and sheep meat (Schonfeldt *et al.*, 1993). Contradictory results are reported on the effect of age on collagen in animals (Mitchell *et al.*, 1927; Hiner *et al.*, 1955; Ritchey and Hostettler, 1964). Hill (1966) could not find any difference in collagen associated with beef age, but suggested that the solubility of collagen be considered in relation to tenderness. It appears that the decrease in polysaccharides with advancing age may be a predominant factor affecting the increased insolubility of collagen with ageing. When there are fewer polysaccharides forming a network around collagen fibres, there may be more chances for the formation of intramolecular crosslinkages in collagen. The polysaccharides may play an important part in plasticizing the collagen fibres, and thus contribute to meat tenderness. A significant association between the hexosamine:collagen ratio and the tenderness of muscle has been demonstrated. The hexosamine content is measured as the total connective tissue polysaccharide (Cormier *et al.*, 1971). A study on collagen nitrogen and subjective scores for tenderness in veal and mature beef has shown a highly significant, but somewhat low correlation of −0.54, indicating that collagen in raw meat could be used as an index of tenderness of connective tissue within cooked meat. Panel scores within the biceps femoris are indicative of larger amounts and tougher connective tissue in older animals (Kim *et al.*, 1967). Residual hydroxyproline content as a measure of collagen not converted to gelatin during cooking was shown to increase with age, and showed a good correlation with shear values in case of white leghorn fowl (Wangen and Skala, 1968).

There have been several reports of a positive linear relationship between ultimate muscle pH and tenderness within the pH range normally encountered in postrigor muscle (Dransfield, 1981). Other reports have indicated a curvilinear relationship between tenderness and pH (Bouton *et al.*, 1957; Martin and Fredeen, 1974; Purchas, 1990). Meat quality modelling during beef chilling has shown a first order kinetics between pH and beef quality measured as press juice (Mallikarjunan and Mittal, 1994). Segregation of beef carcasses with ultimate longissimus pH values between 5.8 and 6.19 appears to be an easy, non-destructive, practical means to remove the majority of tough carcasses effectively, regardless of the sex and the breed (Jeremiah *et al.*, 1990, 1991). However, the pH effect can be masked by cold shortening (Purchas *et al.*, 1988).

Fractionation of beef muscle proteins has shown sarcoplasmic protein per total fibrillar protein nitrogen and soluble fibrillar protein nitrogen to be correlated to tenderness (Hegarty *et al.*, 1963). The total amino acid composition of the muscle protein is quite constant regardless of the species or the muscle from which it is obtained (Lyman and Kuiken, 1949; Blum *et al.*, 1966; Schweigert *et al.*, 1945). Free amino acids have also been correlated to tenderness, for example with turkey (William, 1971). Studies on free amino acid composition of various beef muscles have concluded that more tender cuts have higher leucine and isoleucine content (Ma *et al.*, 1961). Several amino acids have been correlated with ham (McClain *et al.*, 1968) and beef flavour (Batzer *et al.*, 1962). Table 5.10 shows partial correlation coefficients of raw muscle protein components vs. quality traits. The amount of total nitrogen in lean

Table 5.10 Selected partial correlation coefficients; raw muscle protein components vs. quality traits

Variables	Partial correlation coefficients
Percent total nitrogen vs. marbling	−0.44*
Percent total nitrogen vs. flavour	−0.51*
Percent total nitrogen vs. juiciness	−0.79**
Percent total nitrogen vs. overall satisfaction	−0.72**
Percent sarcoplasmic protein nitrogen vs. firmness	0.58**
Percent sarcoplasmic protein nitrogen vs. juiciness	0.51*
Percent sarcoplasmic protein nitrogen vs. overall satisfaction	0.45*
Percent soluble fibrillar protein nitrogen vs. marbling	0.47*
Percent residual connective tissue protein nitrogen vs. juiciness	−0.58**

* $P<0.05$.
** $P<0.01$.
Source: Usborne *et al.*, 1968 (reproduced with permission).

meat seems to play an important role in influencing palatability characteristics as exemplified by the high negative partial correlation between percent total nitrogen and palatability characteristics. The percent sarcoplasmic protein nitrogen has also been found to be significantly correlated with firmness, juiciness and overall satisfaction. A significant partial correlation coefficient has also been reported for percent residual connective tissue protein nitrogen and juiciness.

Free amino acids in raw muscle, cooked muscle and in drippings vs. quality traits have been reported. Glutamic acid in raw pork muscle has been found to be significantly correlated to texture, flavour and tenderness. Leucine, serine and phenylalanine are also positively correlated to tenderness. In cooked pork muscle, tyrosine, glutamic acid, aspartic acid and serine have been found to be negatively correlated and glycine to be positively correlated to flavour. Tyrosine and glutamic acid are also negatively correlated to juiciness and overall satisfaction. In the drippings, lysine has been found to be significantly correlated to marbling, glycine to colour of the muscle and aspartic acid to tenderness.

Several of the amino acids have also been correlated to the protein nitrogen components in raw pork, cooked pork and in the drippings. In raw meat, free threonine has been significantly correlated to percent total nitrogen, arginine to non-protein nitrogen, and glycine, valine and leucine to percent collagen nitrogen. Glycine in raw meat has also been correlated to percent residual connective tissue protein nitrogen. In cooked meat, alanine has been found to be negatively correlated to total nitrogen, proline and glycine to be positively correlated to percent collagen nitrogen, and leucine, methionine, valine, isoleucine and phenylalanine to be negatively correlated to percent collagen nitrogen. In the drippings, glycine has been found to be negatively correlated to percent non-protein nitrogen, tyrosine and isoleucine to percent collagen nitrogen, and alanine to percent total nitrogen.

It is difficult to make any definite statements about the relationship of tenderness, collagen and free amino acids since there is generally little difference in tenderness, and percent collagen nitrogen fails to change significantly from the raw to the cooked product. In raw pork, leucine is the only amino acid which has been found to be significantly correlated to the tenderness as well as to collagen content. Concentrations of free leucine could probably be used as an indicator of tenderness, but more experimental evidence is required (Usborne *et al.*, 1968). Proteolytic reactions which occur postmortem are responsible for decreases in myosin and in sarcoplasmic proteins (Lawrie, 1966) and these decreases are reflected by increases in free amino acids (Colombo and Gervasini, 1956). As postmortem ageing of muscle increases, tenderness and flavour of cooked meat improves (Wilson, 1960). The greater increase in the tenderness in the longissimus than in the biceps femoris during ageing has been attributed to greater amounts of connective tissue in the latter rather than to the changes in free amino acids (Field *et al.*, 1971). Differences in free amino acids due to sex and line of cattle have been found, for example steers containing a slightly higher proportion of free amino acids than bulls give a more tender meat (Field and Chang, 1969). Calcium activated neutral proteinases, calpains I and II, and cathepsins B, D and L degrade myofibrillar and cytoskeletal proteins (Dayton *et al.*, 1981; Ouali *et al.*, 1987; Mikami *et al.*, 1987), but their importance to tenderization is only inferred. A recent report has confirmed the link between rate of tenderness and rate of proteolysis by calpain I (Dransfield *et al.*, 1992a) and suggested that first order tenderization begins at a muscle pH of 6.1 (Dransfield *et al.*, 1992b). Calpain I becomes activated when the muscle pH falls to about 6.1. This enzyme is autolysed slowly reducing its concentration and the rate of tenderization. Parameters governing activity of calpain have been derived and can predict 68% of the variation in muscle toughness (Dransfield, 1992).

Collagen content is important to the structure and meat quality and to the functional properties of emulsion products, and is the major protein in skin, bone, tendon and cartilage (Lawrie, 1979). The elastin content of connective tissue is very low and is of little practical importance (Fey, 1977). In Japanese abalone, kuro-awabi (*Haliotis discus*), collagen content has been correlated to muscle toughness; the higher the collagen content, the tougher the muscle (Olaechea *et al.*, 1993). Collagen can be estimated by the Waring blendor method (Hartley and Hall, 1949), in which the tissue is homogenized with water in a Waring blendor, the pH is adjusted to the apparent isoelectric pH 5.0 and the precipitate washed with water by centrifugation. This method generally gives high values for collagen. It gives reproducible results with raw meat, but not with cooked meat. An enzymic method uses proteolytic enzymes, inactive towards collagen, which break open complex connective tissue structures by hydrolysis of the simple proteins so that soluble nitrogenous proteins can be washed off by centrifugation. Collagen, obtained by the enzymic method has a better relationship to shear values and tenderness scores than by the Waring blendor method (Adams *et al.*, 1960). Collagen can also be determined by near infrared spectroscopy (Chevalier *et*

al., 1990). A limiting value of 19 vol% collagen for high quality scalded sausage (Hultsch and Kruger, 1977) and 12% for the collagen to protein ratio has been suggested for commercial meats (Pailer, 1979).

In contrast to other mammalian proteins, collagen contains a high concentration of the imino acid, hydroxyproline. It is therefore accepted as a method of estimating collagen content (Wierbicki and Deatherage, 1954; Etherington and Sims, 1981), and also as a criterion of quality (Joksimovic, 1970). Chromatography, NMR spectroscopy and colorimetric determination (Neuman and Logan, 1950; Stegeman, 1958; Szeredy, 1970a; Kolar, 1990; Pailer, 1979) of hydroxyproline have been reported with the colorimetric procedure being accepted as an official method (ISO 3496-1978 (E), 1978). However, one report does indicate the lack of any correlation between hydroxyproline content and the tenderness of meat (Szeredy, 1970b). The hydrolysate that is used to measure hydroxyproline can also be used to measure glutamic acid enzymatically, so as to calculate the ratio of hydroxyproline:glutamic acid. This seems to be a practicable index for the evaluation of meat products (Moehler and Volley, 1969). The 'tryptophan–peptide' value is also suitable for evaluating collagen content; it is independent of whether the animal protein is raw or processed and can be used to classify meat products (Brieskorn and Berg, 1959). The hydroxyproline:tryptophan ratio has also been used to segregate sausages, the ratio being 4.3 for the lower grade product and 6.0 for first class sausages. The ratio shows less variation than the individual amino acids and is therefore more useful (Gladkov *et al.*, 1968).

Tenderness, juiciness, firmness and appearance of meat are influenced by WHC, press juice and cooking loss (Offer and Knight, 1989) and of course, pH (Dutson, 1983). Press juice has been shown to be negatively correlated to fat thickness and positively to cooking loss and pH; pH also is shown to correlate negatively to fat thickness. The ageing time is known to influence muscle pH and cooking loss significantly (Boakye and Mittal, 1993). Physicochemical properties undergo a change during ageing of carcasses, particularly during the first 11 days, within which tenderness improves and beyond which the palatability is adversely affected. A protein band on SDS–PAGE electrophoresis of 30 kDa has been indicated to be a useful indicator of ageing (Negishi *et al.*, 1991).

Consumer resistance to meat surrounded by large amounts of unused fat (termed 'marbling') coupled with encouragement in the USA by the American Heart Association of the public to consume less animal fat has resulted in a certain amount of meat being offered by some meat processors on a 'trimmed of waste fat' basis. Using odour as a flavour characteristic, the effects of free fatty acids, carbonyls, neutral fats, cephalin, sphingomyelin and lecithin on raw and cooked beef flavour have been examined (Hornstein and Crowe, 1960; Hornstein *et al.*, 1961; Yueh and Strong, 1960), with no clear conclusions. First principal component values (FPC), determined from shear force and palatability attributes of tenderness, connective tissue amount, flavour and juiciness have been correlated to overall maturity (OM), lean colour, marbling score (MS), lean firmness, lean texture, fat colour (FC) and marbling

firmness. Quality grades based on FPC values have corresponded with predetermined acceptability levels for each palatability trait in carcasses (Hodgson *et al.*, 1992). Similar objective tests for pork quality assessment have recently been developed (Garrido *et al.*, 1994). It has also been suggested that marbling score may be receiving more attention than it deserves in relationship to beef palatability (Richardson, 1968). With a few exceptions, low positive correlations have been demonstrated for the serum lipid components to flavour, whereas most of the marbling and subcutaneous fat compounds exhibit low negative correlations to flavour (Thrall and Cramer, 1971). Fat marbling and the water holding capacity can be determined by measuring the intensity of light reflected from the meat surface along a scanning line (Borggaard and Rasmussen, 1991).

Attempts have been in progress to develop objective instrumental methods for predicting quality of meats. Hildrum *et al.* (1994) correlated sensory quality of beef by near IR spectroscopy. Similar studies are being conducted on turkey meat (Swatland and Barbut, 1995) and meat sausages (Ellekjaer *et al.*, 1994). Optical properties, mainly birefringence of myofibrils appear to reflect pH changes, pH-related paleness and associated quality characteristics of meat. Work in this area has been reviewed recently (Swatland, 1994, 1995).

Methods of predicting meat quality in live animals have been suggested and include muscle biopsy, blood group, creatine kinase and halothane tests, with the last two being recommended for practical purposes (Fischer, 1983; Babol and Squires, 1995).

5.5 Evaluation of the age of the animal carcass

The concentration of creatine in semimembranosus has been shown to be closely related to the age of pigs between 69 and 219 days, the amount being very low in the muscles of young pigs (Witkowska and Kortz, 1990). The thermal stability of the bovine intramuscular collagen (IMC), measured as the thermal shrinkage temperature (T_s) has been shown to be greater in older animals than in younger ones (Goll *et al.*, 1964; Judge and Aberle, 1982). Meat tenderness is influenced by the quantity of heat-stable collagen crosslinks, with greater amounts being present in tougher muscle. It has also been suggested that the number of total or heat-stable crosslinks influences the heat stability of collagen, as measured by differential scanning calorimetry (DSC) (Judge *et al.*, 1981; Judge *et al.*, 1984; Judge and Aberle, 1982). One such heat-stable crosslink of collagen is pyridinoline (Fujimoto and Moriguchi, 1978; Eyre and Oguchi, 1980; Fujimoto, 1980). An increase in pyridinoline content in porcine epimysal connective tissue as well as in intramuscular collagen of goat has been demonstrated (Nakano *et al.*, 1985). A positive correlation between pyrolidinone concentration and thermal transition temperature has led to confirmation of the positive correlation between pyrolidinone concentration and maturity of the beef muscles ($r=0.56$; $P<0.001$). The increasing amount of pyrolidinone in intramuscular collagen enhances the thermal stability of maturing beef muscle (Smith and Judge, 1991) (see Table 5.11).

Table 5.11 Effect of maturity on bovine intramuscular collagen thermal characteristics and crosslinking characteristics[a]

	USDA maturity group				
Variable	A	B	C	D	E
No. of carcass	9	9	8	17	6
Pyrolidinone	0.04[b]	0.05[bc]	0.06[bc]	0.09[c]	0.14[d]
conc. mol/mol	0.01	0.01	0.01	0.01	0.02
of collagen					
Thermal	63.2[b]	63.72[b]	63.71[b]	64.92[c]	65.63[c]
shrinkage temp.	0.33	0.33	0.35	0.24	0.40
(°C)					
Solubility (%)	6.66[b]	5.38[bc]	5.28[bc]	3.88[c]	3.24[c]
Enthalpy (cal g^{-1})	5.34	5.26	4.82	5.06	5.19
	0.33	0.33	0.35	0.24	0.40

[a]Least square means±SE.
[bc]Mean values not having a common superscript differ ($P<0.01$).
[d]Heat-labile collagen.
Source: Smith and Judge, 1991 (reproduced with permission).

The haem iron content of the muscles is also a good index for the assessment of maturity of bovine carcass (Dumont and Bousset, 1990).

5.6 Contaminants in flesh foods

5.6.1 Chemical contaminants

5.6.1.1 Hydrocarbons

Some standards for foods, including some EU standards, specify maximum allowable levels of trace constituents or contaminants. A probable contaminant of fish about which not much is known and which does not feature in any standard is hydrocarbon originating from fuel oils released into the marine environment. Concern about these substances stems from their possible toxic effects, primarily in fish eaten as food but also on the whole of the marine ecological system. Kerosene-like hydrocarbons in sea mullet (*Mugil cephalus*) have been reported (Sidhu *et al.*, 1970), causing them to become tainted (Connell, 1974). Fish absorb hydrocarbons from contaminated sediments, food and water. These n-alkanes are readily metabolized when taken up via the digestive tract, but limited metabolism occurs when taken up by the gills (Geiszler Grantham and Blonquist, 1977). This tainting has caused significant economic damage to the sea mullet. Although n-alkanes ranging from n-nonane to n-tridecane and several aromatic hydrocarbons have been identified by gas chromatography coupled with mass spectrometry, the specific compound causing this taint has remained a mystery. This type of contamination has been reported in Brisbane and Queensland in

Australia. The deposition of hydrocarbons in different segments of the muscle tissue of the sea mullet is found to be proportional to the lipid content of the segment. Evidence has also been presented that metabolic processes in the sea mullet result in preferential degradation of the n-alkanes leaving a hydrocarbon mixture in the sea mullet enriched with isoalkanes and related compounds (Connell, 1978). Research efforts are needed to understand the intricacy of the problem in an attempt to find a workable solution.

Pollution of lobsters in holding crates by diesel oil has also been reported in the harbour of Souris, Prince Edward Island, Canada. It can be conveniently analysed by extraction of the hydrocarbons, followed by spectrofluorometry with excitations at 300, 370 or 395 nm (Awad, 1977). A simple method based on steam distillation and isothermal gas chromatography (Ackman and Noble, 1973), previously used to examine tainted fish gave only limited success in identifying the contaminants. Only traces of hydrocarbon were found to appear in steam distillate possibly due to prior removal of shorter chain length hydrocarbons during lobster processing by boiling or steaming. An alternate method involving extraction, column chromatography and temperature programmed gas chromatography which allows concentration of the hydrocarbons, could successfully indicate the specific source of the contaminant (Mackie *et al.*, 1972). Analyses of three samples of commercial marine diesel oils have shown a distribution of straight chain hydrocarbons from C_9 to C_{24}. Pristane (2,4,10, 14-tetramethylpentadecane) and phytane (3,7,11,15-tetramethylhexadecane) are found in large amounts. The ratios of pristane:phytane and n-C_{17}:n-C_{18} can indicate the origin of the diesel contamination (Paradis and Ackman, 1974). On the other hand organoleptically acceptable lobster contains an appreciable background of extractable volatiles upon which a superimposable pattern of pristane and other normal hydrocarbons is observed. Straight chain hydrocarbons and monomethyl hydrocarbons are common in marine algae and plankton (Clark and Blumer, 1967; Blumer *et al.*, 1971; Youngblood and Blumer, 1973). Pristane is found in lipids of marine animals and often in large amounts in planktonic crustaceans (Blumer *et al.*, 1964; Ackman, 1971; Inoue *et al.*, 1973) as well as in the crabs *Chionoecetes opilio*, *Lithodes turritus* and *Paralithodes camtschatica* (Kawada *et al.*, 1973). It is thus uncertain whether the presence of hydrocarbons in marine animals can be definitely linked to oil pollution of the marine environment (Paradis and Ackman, 1974).

Polycyclic aromatic hydrocarbons are often found in sediments in coastal regions. Fish and mussels feed on these sediments which determine their biochemistry. Organochlorines such as benzene hexachloride (BHC) and dichlorodiphenyltrichloroethane (DDT) also find their way into fish (Miskiewicz and Gibbs, 1992), and so do some textile dyes as has been demonstrated in the organs of *Onchorhynchus mykiss* (Riva *et al.*, 1992). A good correlation has been obtained between biochemical measurements in marine organisms and chemical analysis of polycyclic aromatic hydrocarbons in sediments, suggesting the use of biochemical indices for application in chemical contaminant monitoring (Garrigues *et al.*, 1990). These can be estimated

by liquid chromatography with fluorescence detection at levels of 0.01 ppm (Perfetti *et al.*, 1992).

Of particular interest are toxic and potent enzyme-inducing chemicals such as some of the polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxan (TCDD) and some of its congeners which induce the activity of a variety of enzymes including aryl hydrocarbon hydroxylase (AHH) (Poland and Glover, 1973; Bradlaw and Casterline, 1979). PCBs and PCDDs have been screened for their ability to induce AHH, and there is a correlation between the toxicity of certain classes of polyhalogenated organic substances and their ability to induce AHH. The bioassay for AHH does not specifically identify or classify the reactive substances in foods, but is useful as a screen for the presence of substances that induce AHH activity (Casterline *et al.*, 1983).

The question as to how much, if any, of these hydrocarbons is derived from fuel oils released from human activities in modern times cannot yet be answered, since fossil hydrocarbons of the type in question have always been present to some degree in the environment.

5.6.1.2 Heavy metals

Heavy metals are often found as contaminants in carcasses (Kluge-Berge, 1989) and fishery products (Hwa-Jung *et al.*, 1993; Rieder, 1993). Many marine organisms eaten by man are able to accumulate high concentrations of heavy metals from water that may itself be low in these. Screening for metal types and content is therefore undertaken to ensure safety, as in Cuba (Gonsalez *et al.*, 1991). Oysters for instance can contain as much as 10 000 times more zinc w/w than the water they inhabit, 30 000 times more cadmium (which is particularly toxic to humans) and 14 000 times more copper. The metal ions are distributed unevenly in fish organs; brain and flesh accumulate maximum and minimum levels of these minerals, respectively (Gomaa *et al.*, 1995).

Attention was first drawn to the possibility that some Tasmanian oysters contained toxic material when oysters from Ralph Bay, marketed for the first time in 1970, were the cause of gastric disturbances, ranging from generalized uneasiness to acute nausea and vomiting. Subsequent analysis showed them to contain high levels of zinc, a powerful emetic, together with cadmium and copper, which can also cause nausea and vomiting. An added hazard in the case of cadmium is that there is evidence to show that even at very low levels it can produce chronic toxicity (Nilsson, 1970). Mercury is also known to accumulate in seafoods such as river blackfish or *Gadopsis marmoratus* (levels being about 0.64 $\mu g\ g^{-1}$) (Bycroft *et al.*, 1982), and can be easily estimated by cold vapour atomic absorption spectroscopy (Landi *et al.*, 1992).

Cadmium and zinc accumulate preferentially in the liver, while mercury is distributed uniformly between the muscle and liver (Marcovecchio and Moreno, 1993). The proximity of a large electrolytic plant for ore processing could also

contribute to heavy metals in oysters. This has been the case of cadmium contamination in water flowing from the South Esk, a tributary of the Tamar that is known to be polluted by effluents from tin and wolfram mines (Thorp and Lake, 1973; Tyler and Buckney, 1973).

An episode of Minimata disease in Japan was traced to mercury poisoning from fish contaminated by methyl mercury from industrial effluent (Harrison, 1993).

5.6.2 Indicators of microbial quality

From the standpoint of public health and inspection and industrial control, it is desirable for critical limits of acceptability to be applied with reasonable confidence. Microbiological methods, although most desirable theoretically, are usually too lengthy and time consuming for efficient and quick quality control. Numerous relatively rapid chemical methods as well as non-microbial indices of microbial history are suitable as indicators for industrial control.

The quality of the final meat is markedly affected by the care taken during the preprocessing steps such as animal care and feeding, slaughterhouse operations, conditioning, storage of the carcass, cutting, mincing, etc. Generally, the animals are rested prior to slaughter to give maximum lactic acid production. The healthy animals can normally cope with the bacterial invasion, but after slaughter a sequence of catabolic events occurs that favour bacterial proliferation. Also the accumulation of metabolites arising from the breakdown of nucleotides, carbohydrates and proteins presents a good culture medium for bacteria. The keeping quality may be further affected if the meat has been frozen and thawed before mincing. In meat the fall in redox potential encourages the growth of anaerobes (Barnes and Ingram, 1955).

Fish is one of the most perishable foods. It is universally recognized that development of scientific tests that would objectively indicate the freshness of fish and possibly predict keeping quality would be of great value to the fishing industry and trade (Stansby, 1958). Fish decomposition is mainly due to bacterial growth which results in production of various substances, some of which are not normally found in the live muscle tissue, while others which are already present increase logarithmically in parallel with microbial growth. It is difficult to find a consistent pattern of fish spoilage, the complexity of which depends on fish species, catching methods, fishing seasons and bacterial load and types. In the case of broiler chickens, fungal contamination has been associated with a musty taint, attributed to 2,4,6-trichloroanisole (Land *et al.*, 1975).

Besides the rapidity of a potential test indicated to be a quality marker, the test should also comply with several other criteria. These include a reasonably good agreement between replicate determinations, consistency of measures at fresh and spoiled stages, sensitivity in the incipient stages of spoilage, good recovery and a reasonable correlation with organoleptic findings. The tests for quality of fish and meat are generally identical, with only the absolute values varying for each type.

Contamination of fresh seafood with many organisms is quite well known. Apart from public health hazards, contaminations can also cause off-flavours, as has been shown with yeast species *Debaryomyces hansenii* growing on surimi and producing kerosene like off-flavours (Koide *et al.*, 1992). Total microbial load and coliform count are the correct objective indicators of the hygiene and sanitation to which the meat is exposed during the slaughter, or catch, and subsequent handling and storage. These may have to be extended to include specific tests for identification of pathogens Salmonella (Ceol *et al.*, 1992), Shigella, Staphylococcus, *Clostridium perfringens, Klebsiella pneumoniae* (Singh and Kulshrestha, 1992), Brucella, *Mycobacterium tuberculosis* and pathogenic vibrios (Rashid *et al.*, 1992). A recently reported contaminant on which attention has been focused is the Listeria species (Masuda *et al.*, 1992; Dillon and Patel, 1992). The usual methods available for the routine bacteriological testing involve total viable counts by plate counting. Although it gives an approximate estimate of the number of bacteria present, their distribution in the sample cannot be known. In tests on sanitary quality of meat, more samples show positive infection (brucellosis and tuberculosis) by the precipitin method compared with the bacteriological tests (Kheifets, 1952).

The time consuming nature of these tests has motivated the investigators to develop a simple and rapid method which would give an indication of both the number and distribution of the bacteria on the sliced cured meat. The distribution of the organisms can give valuable clues about their origin, and allows sources of contamination along the production line to be rapidly traced and eliminated.

5.6.2.1 Staining procedures

One of the approaches is to press a metal plate first to the surface of the meat and then to a poured agar plate. Incubation of the agar plate and the consequent development of colonies not only gives a guide to the numbers of organisms present but also to their distribution. This becomes cumbersome and also requires a period of 2–3 days. Staining of meat colonies on meat surfaces so as to make them visible is another method. Bacterial growth generally causes the redox potential to fall below −250 mV, and in this range dyes such as methylene blue and resazurin (Uno and Tokunaga, 1954; Wilhelmsen, 1965) undergo a colour change by acting as artificial acceptors of hydrogen liberated during metabolism by dehydrogenases (Straus *et al.*, 1948; Fahmy and Walsh, 1952; Anderson, 1955). Promising results have been obtained by measuring the times required to reduce these dyes. For instance, the resazurin method for beef, pork and mutton carcasses is reported to give a close correlation (r=0.9258–0.9736) with the conventional techniques (Labadie, 1985; Losonczy, 1969). These dyes have been found unsuitable for fish due to the presence of trimethylamine oxide which retards the lowering of the redox potential. Fluorescent staining of bacteria using 0.025% acridine orange is a rapid method for enumeration of bacteria in minced products (the correlation coefficient is 0.97 between this method and standard plate

count) (Sheridan *et al.*, 1990).

Tetrazolium salts are considered suitable for use as stains (Kuhn and Jerchel, 1941; Nineham, 1955; Smith, 1951), since they are more electropositive dyes than methylene blue and resazurin. In particular, triphenyltetrazolium bromide has been found suitable for routine analysis (Kun and Abood, 1949). The colourless water-soluble tetrazolium salt is reduced to the brightly coloured insoluble formazan. This method has been used for vital staining of bacteria (Usdin *et al.*, 1954; Eidus *et al.*, 1959) and for assessing the quality of iced water fish (Shewan and Liston, 1957). The amount of dye reduction in 8 h shows a good relation with the quality of cured fish as revealed by total volatile nitrogen, another indicator of bacteral contamination of fish (Suryanarayana Rao *et al.*, 1956). Test papers impregnated with tetrazolium salts have been used to assess the spoilage of cod and haddock stored in ice. Observations on different types of fish like sardines, mackerel and Lethrinus have shown that the time taken for the initial appearance of red colour is inversely proportional to the extent of fish spoilage. The colour is perceptible within 3 h when the fish passed the organoleptically fresh stage. In the case of spoilt fish, it takes less than 30 min (Kamasastri, 1957).

Amongst the tetrazolium salts studied, 5-phenyl-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)tetrazolium chloride has been found to be the best (Mackay and Liston, 1959). It gives a purple formazan which is formed rapidly and is easily visible. Generally hams with high total counts, due to undercooking, tend to give a fairly uniform development of colour. Postcooking contamination usually gives spots or sharply defined areas of formazan production that can often be related to specific pieces or equipment. The method can be applied to sliced bacon, but the comparatively higher initial counts of bacteria usually obtained on this product, derived mainly from the brine tank, make interpretation difficult. However, distribution of flora between the fat and lean portions of the rashers and between the various muscle areas can be estimated (Bradshaw *et al.*, 1961). The direct epifluorescent filter technique (DEFT) can also be used as a rapid method to enumerate plate count, a correlation of 0.87 between the two methods being reported (Pettipher and Rodrigues, 1982). Observation for fluorescence under UV light in the range 300–400 nm had been reported as a quality control check for fish (Adamova and Spektov, 1947). Fresh fish show deep violet fluorescence with occasional spots of a white, blue or red; sometimes the entire specimen glows with white-blue or deep green colour. The blood of the fresh fish does not fluoresce. Boiling or salting of spoiled fish does not destroy the typical blood fluorescence (Leskov, 1946).

5.6.2.2 Electrical properties

Changes in resistance or conductivity (Bulte and Reuter, 1984) caused by the growth of microorganisms had already been documented at the end of the last century (Stewart, 1899), and are now applicable to the detection of spoilage of fish (Gibson and Clark, 1987). Oker-Blom (1912) assumed a relationship between growth of bacteria and the

ionic concentration of the culture medium. The correlation between the time necessary to detect changes in electrical properties of a bacteriological medium and the original numbers of bacteria in food has already been reported for meat (Strasser, 1979; Bell, 1980) and reviewed by Bulte (1983). Tests on 140 samples of raw meat gave a good correlation, but around the regression line there was a higher distribution of single values. Detection times with a computerized bactometer ranged from 1 h to 11 h, corresponding to a bacterial count of 10^7–10^3 g^{-1}. Martins and Selby (1980) have also determined the coliform count of meat samples within 24 h using a bactometer.

The optimal incubation temperature for impedance measurement may be influenced by the biotype to be investigated. In the case of meat surface microflora, an operating temperature of 30 °C yields good results, namely, fast detection times and low standard deviations may be due to the high content of Enterobacteriaceae (Strasser, 1979). Use of a selective medium for Salmonella followed by measurement of the impedance can quantify Salmonella, but the rate of false-positive results with this technique is high (Donaghy and Madden, 1992). The number of Enterobacteriaceae in meat flora correlates very well with the total viable count, even at different stages of development of the carcass microflora (Strasser, 1979), indicating the justification of impedance measurements for the determination of surface microflora. Distinction between meats with $>10^2$ faecal coliforms g^{-1} and those with $<10^2$ g^{-1} can usually be detected by impedance measurements (Kraeger *et al.*, 1978).

5.6.3 Indicators of hygienic quality

Attempts have been made to devise tests to determine whether dressed carcasses have come from healthy or diseased animals. The catalase activity of fresh extracts of beef from diseased animals has been found to be three-fold that of the normal veal and beef and could be a useful indicator of the hygienic quality of the meat (Nesterov and Stepanova, 1966). Catalase activity is also an indication of degree of spoilage by the growth of microorganisms (Tomiyama and Yone, 1953; Tomiyama *et al.*, 1955). Fish meal has been graded according to the catalase contents as follows: under 3.0 ml, good; 3.0–5.0 ml, still passable or usable; over 5.0 ml, not good (Benno, 1959).

To ensure product safety and quality it is important that the cleaning of meat process plant is effective. The microbiological counts, particularly the Enterobacteriaceae counts (Notermans *et al.*, 1977) are useful and visual methods have been widely used to assess the effectiveness of cleaning routinely, but despite wide usage have a number of limitations, the most important one being the considerable delay between sampling and the result. There may also be little correlation between the amount of soil on the surface and the microbiological count (Abele, 1965). Visual appraisals although rapid have only a limited sensitivity and objectivity. A rapid catalase test has been proposed for hygiene control in slaughterhouses and meat processing plants, based on the splitting of hydrogen peroxide by the enzyme produced by the meat spoilage organisms (Okolov, 1947) and blood residues (Milledge,

1982). The catalase test is very sensitive and can detect blood residues at loading rates as low as 0.05 ml m^{-2} which is 100 times lower than visually detectable. In addition it does not give any false positives and can be applied to a wide variety of surfaces found in the meat processing industry (Milledge, 1982).

Bioluminescence measurements, based on the ATP content of bacterial cells is another rapid method of evaluating the hygienic quality of meat (Danilov and Kondratenko, 1958). Certain factors need however to be considered carefully. ATP determination by luminescence is possible only at cell counts $>10^5$ ml^{-2}. For lower cell counts, cells may be concentrated by absorption on to ion exchangers followed by centrifugation before the determination (Baumgort *et al.*, 1980). The ATP content of the cells varies according to the type and physiological state of the culture. The ATP level during lag and log phase is higher than in the stationary and decline phase. Differences up to 80% can be observed. Rod shaped bacteria yield higher percentage differences than the cocci. In spite of these observations, a good correlation between the ATP values and the total number of viable cells ($r=0.92$) has been reported (Bulte and Reuter, 1985). Exposure of fish, cattle, swine and poultry products to electromagnetic radiation within the range 355–360 nm and a check for any fluorescence also provides a suitable quality control check. Characteristic fluorescence from bones, cartilages, connective tissue and fat can be identified by analysis at 365–490 nm (Jensen *et al.*, 1984). *Escherichia coli* producing a shiga-like-toxin in ground beef (Gannon *et al.*, 1992), Campylobacter in chicken products (Giesendorf *et al.*, 1992) and Salmonella spp. (Way *et al.*, 1993) can be easily and rapidly detected using the polymerase chain reaction. Lactic acid bacteria in meat can be identified by low molecular weight RNA (in particular 5S rRNA and tRna,<150 nucleotides) profiles (Cha, 1992).

5.7 Quality of comminuted meats

An approach to the estimation of the quality of comminuted meat products has been made by determining the content of tryptophan and hydroxyproline (Dahl, 1960, 1963). The high quality animal protein contained in expensive tissues, such as skeletal muscle and red organs, is richer in tryptophan and low in hydroxyproline, while the reverse is true for the protein contained in inferior tissues such as connective tissue, tendons, etc. The protein in smooth muscles occupies an intermediate position. In this group, lungs, pig stomach and intestines of pigs and calves have the highest content of tryptophan and moderate content of hydroxyproline, corresponding to that of such meat in which about 15% of the protein originates from connective tissue. Analyses of various tissues and sausages have shown that the creatine content, calculated as the percentage of crude protein, can be used as an approximate index of the quality of meat products (Ennor and Rosenberg, 1952).

Skeletal and cardiac muscle, as well as tongue, are the tissues which have by far the highest content of creatinine, about 2.5%, 1.5–2.0% and 11.5%, respectively, calculated as the percentage of crude protein. They are followed by pig stomach and

chitterling (about 0.3–0.6% creatine). Blood, skin, connective tissue, liver and lungs are poor in creatine. These findings permit certain conclusions on the quality of ingredients in meat products from the content of creatine. Erroneous results can be obtained in the case of products manufactured from cooked meat without the cooking water as in corned beef.

Amongst important sensory attributes that determine the quality or acceptability for use of high value premium packs, texture is by far the most important. Under commercial conditions, this texture can only be subjectively measured by people touching and visually assessing the product using the 'finger test'. Several attempts however have been made to measure the texture of a raw fish objectively. These include double compression to measure the elasticity of the fillet (Azam *et al.*, 1989); shearing, puncture and compression tests conducted independently of each other (Borderias *et al.*, 1983); shearing using costly non-portable equipment and involving destructive sampling (Gill *et al.*, 1979); double deformation to measure elasticity using a simple mechanical penetrometer which entails destructive sampling; and a hand-held probe and gauge which measures deformation force, the results of which are often dependent on the person using the probe. An electronic fish texture tester, which is quite reliable and gives results comparable to that of experienced fish inspection officers, and that is portable and non-destructive has been recently reported. ATP depletion and other biochemical indices have been related to texture indices of cooked sturgeon meat from fish which have struggled and anaesthetized fish. Penetrometer tests have revealed that cooked sturgeon meat is firmer prior to ATP depletion and onset of rigor than in the postrigor state. It is therefore recommended that tough texture problems sometimes encountered in farmed sturgeon may be minimized by allowing the sturgeon meat to undergo rigor prior to cooking (Izquierdo-Pulido *et al.*, 1992).

5.8 Meat additives and adulterants

5.8.1 Artificial colour in sausages

The rapid colour change which freshly chopped beef and mutton undergoes is arrested by employing powdered saltpetre. The colour remains pink because of the formation of nitroso derivatives of haemoglobin and myoglobin. A larger quantity of saltpetre causes a shrivelled appearance. A variety of other colours like red ochre, coal tar dyes, cochineal, carmine and orchil may also be used (Leach and Winton, 1936). In the Indian subcontinent speciality roast meat is made in a typical open fire device called a 'tandoor'. Meat pretreated with spices often containing artificial colour is used for this purpose.

5.8.2 Fillers in sausages

The generally used 'fillers' are dried breadcrumbs, cornmeal, potato starch, crackers, waste biscuit and boiled rice. Lean meat, when carefully chopped has an enormous combining power and can take large quantities of water. Frankfurters, bologna and pork sausages have been found to be admixed with 0.5–5.0% starch and 5–40% water in addition to that contained in fresh meat (Leach and Winton, 1936). Frozen shrimps as well as broilers (Hildebrandt and Weiss, 1975) have also been reported to contain excess water (Beckman and Mattson, 1980). The addition of water is to obtain a consistency suitable for stuffing into sausage tubings. Such additions are unnecessary with fresh meats, and can be easily determined on the basis of the Feder value. The Feder number is used in Germany and Holland, and is calculated as follows (Pearson, 1976):

$$\% \text{ Organic non-fat} = 100 - (\% \text{ fat} + \% \text{ ash} + \% \text{ moisture}) \quad [5.8]$$

$$\text{The Feder Number} = \frac{\% \text{ water}}{(\% \text{ organic non-fat})} \quad [5.9]$$

Scalded or cooked sausages cannot, however, be assessed by this method (Stadtler, 1942). Starch hastens and increases the absorbing or combining power of lean meat and is the only absorbing agent when inferior cartilaginous tissues are used. Unscrupulous manufacturers use the 'pump cure' method, that is, intravenous injection of water to increase the weight of cured meat. This is, however, easily determined by estimating protein and moisture content. Addition of more than 3% water in uncooked and 10% in smoked or cooked sausage is considered to be an adulteration.

Synthetic creatinine has often been added to meat extracts so as to increase its concentration to the levels prescribed in food legislation. This can be detected by radiocarbon measurement. It is based on the fact that synthetic creatinine has a much lower ^{14}C activity than natural creatinine. This method is however unsuitable for routine testing of meat extracts (Sulser, 1974).

Poultry skin may be used as a low cost filler in processed poultry products. However, in excess it causes a deterioration of product quality because of its high content of lipid and collagen. This can be detected by measuring the fluorescence of chicken skin collagen in a meat slurry through the use of a quartz-glass rod. Fluorescence intensity is strongly correlated to the skin content by r ($r > 0.99$ from 460 nm to 510 nm) and with the processing properties related to the skin content such as gel strength, cooking losses and fluid-holding capacity (Swatland and Barbut, 1991).

5.8.3 Chickpea flour in sausages

Chickpea flour is known to be a potential source of cheap yet good quality protein for use in comminuted meats, the addition of which imparts a softer texture. The effect becomes more marked as the substitution level increases. It also causes discoloration of

raw sausages, owing to metmyoglobin formation, which becomes more pronounced during storage at 0 °C (Verma, 1984).

5.8.4 Gelatin in smoked meat products

Cured meats are sometimes injected with a solution of gelatin, which solidifies during storage and gives additional resistance to knife cutting of meat as well as in retention of water. Its presence is evidenced by the expulsion of a jelly-like substance from the veins themselves by pressure (Leach and Winton, 1936).

5.8.5 Blood added to hamburgers

The deceptive practice of adding blood to ground beef in hamburgers to coat fat particles and make the meat appear lean is a common practice. The skilful addition of a natural product such as blood, is somewhat more difficult to measure quantitatively, since the meat already contains some. Bovine blood is usually added to ground beef since it is more easily available to the processor (Hankin, 1965).

5.8.6 Spleen added to ground beef

Many consumers object to the use of organ tissues in certain meat products for aesthetic reasons and for the fear that they are being fed these materials in a disguised form. Individual tissue types are not readily identified in meat items manufactured by grinding, chopping or emulsifying one or several animal tissues. Spleenic tissue is classified as variety meat and there is no legal provision for spleen addition to ground beef or hamburgers (Bittel and Graham, 1977).

5.8.7 Vegetable proteins and other non-meat proteins in meat products

Since the mid-1980s, the addition of non-meat proteins such as egg (Feigl, 1993) in meat products has become well established (Rangeley and Lawrie, 1976). Although these substitutes do not reduce the nutritive quality or spoil the texture or flavour of the product, they could lead to the replacement of expensive animal protein by cheaper proteins of vegetable or microbial origin. Improvement in functional properties and extension of available animal proteins are other reasons for admixture of vegetable proteins like soybean flour to meat products (Seideman *et al.*, 1979). A high glucosamine level in sausages indicates the presence of various additions such as binders, inner organs, blood plasma, milk proteins or egg white. If the presence of inner organs can be excluded by microscopic examination and if the level of tryptophan is high and that of hydroxyproline low, an increase in glucosamine is suggestive of addition of protein substance which is free of collagen and muscle tissue (Koller, 1961). In sausage

manufacture (Manrique and Thomas, 1976), the relevant functional properties are the emulsifying capacity, emulsion stability and water binding capacity. These attributes are native to the meat proteins, myosin and actin. Other proteins such as soybean protein concentrates and isolates (Baltic and Smiljanic, 1987; Flint and Lewin, 1976), non-fat dried milk, milk coprecipitates and cereal flours have been used as substitutes in sausage manufacture. The chemistry of gel formation has indicated that whey protein concentrates have potential as binders/extenders in meat products (Ikeuchi *et al.*, 1988). Milk constituents confer the beneficial properties of lactose such as masking of salts, phosphates and bitter aftertastes, providing a low sweetness profile (Pizza *et al.*, 1988).

5.8.8 Interspecies meat adulteration

This is a very common practice followed all over the world intended primarily to reduce the cost price by substituting expensive with cheaper meat. Meat of similar pigmentation, for example, beef and horse meat, beef and mutton or poultry and pig meat are virtually impossible to distinguish by appearance once they have been frozen *en masse* in large blocks, or flaked and incorporated into comminuted meat products. It has become a challenging task to identify the species of the meat, especially in processed meat products. Furthermore, the identification of animal species is one of the areas of major concern for the food hygiene laboratories.

5.9 Egg: quality criteria

In the past few years concern has been expressed about the quality of eggs reaching the consumer. The first step was taken by US Department of Agriculture (USDA) in 1925 to ensure quality standards of individual eggs, followed by the issue of commercial grades in 1948. This was followed in 1967, when standards for consumer grades at origin and destination were issued to allow for damage due to handling and transportation (US Department of Agriculture, 1981). External as well as internal characteristics need to be examined. The external characteristics are easily seen by the naked eye, while internal characteristics are observed with the aid of an egg candling light. Objective methods such as gravimetric determination of impurities on the egg shell surface are also reported (Bacikova and Gresa, 1977). Other defects such as cracks in the shell, blood spots (Sherman, 1981; Jong, 1981) and rots can also be viewed with the aid of a candling light. This can be made into a continuous operation enabling many eggs to be checked at the same time. Generally a high quality egg does not show a clear outline of the yolk on candling, since thick egg white obscures it. Candlers used by inspectors vary and can range from a lens and mirror to direct the beam of light to a green or blue filter light to make detection of blood spots easier (Smith, 1991). Automation of egg candling has also been achieved by using a video camera to detect cracked eggs and robot arms for egg manipulation using an inexpensive robot and

computers (Bourely, *et al.*, 1987). Sensors and microprocessors have been described for testing eggs for multiple defects in eggs (Bergemann, 1986). Other methods to replace human eye completely are image analysis, IR transmission spectroscopy and NMR (Baksh, 1987). Spectral analysis has shown that sound eggs have an absorption maximum at 560–563 nm, blood stained eggs at 546–575 nm, and bacterially contaminated eggs at 570–580 nm. In case of Pseudomonas contamination, the luminescent spectrum shifts to the shortwave zone (15–18 nm). The spectral method is therefore recommended to detect inferior eggs (Tsarikov *et al.*, 1975). Some problems associated with egg candling are opacity of brown eggs, fine cracks in the shell not being visible until moisture moves in the crack, scratch marks confused with cracks and speckled brown eggs being identified as dirty eggs. Alternatives to egg candling are automatic sonic, ultrasonic and microwave methods (Anon, 1986).

A legal criterion for the freshness of a hen's egg is the height cell (EEC directive No. 2772/75 of 29 Oct. 1975). This has been examined with regard to its practicality in judging eggs of weight grades 1–4 (>70 to <55 g). During a 5 week storage under various controlled conditions – room temperature; 18 °C, 50% relative humidity – eggs of weight grades 1–4 showed the same relative weight loss. A greater increase in the height of the air cell has been detected in eggs of grade 1 than in those of grade 4. Measurement of the volume of the blunt pole of the eggs showed the same shape for all egg grades. As the increase in air cell volume corresponds to weight loss during storage, the higher absolute loss of larger eggs leads to a greater increase in air cell height under the same storage conditions. The limit of freshness, that is 6 mm air cell height, is reached earlier in larger egg grades than in smaller ones. Hence a new method is proposed for evaluating freshness by the air cell height: 6 mm for eggs >70 g, 5.5 mm for eggs weighing 60–65 g, 5 mm for smaller eggs (equal to or less than 55 g) (Kessler *et al.*, 1990). Albumen quality is considered to be a reliable indicator of an egg's freshness. The Haugh unit (HU) index, a function of egg weight and albumen height, has been frequently used and widely accepted as a measurement of albumen quality in survey work (Hughes, 1972). The albumen quality, and hence the Haugh unit is a function of temperature (Davis and Stephenson, 1991), breed and season (Salahuddin and Howlider, 1991).

Lysozyme activity in eggs shows a close correlation with Haugh unit score and albumen viscosity data, and is considered to be a useful procedure for assessment of egg quality (Trziska and Clostermann, 1993). Acoustic resonance is a technique that offers the potential of a simple, non-invasive and rapid categorization of eggs, particularly with respect to yolk puncture, shell cracks and presence of salmonella, and is therefore practical in routine quality control (Sinha *et al.*, 1992).

5.9.1 Detection of cracks in whole eggs

The physical characteristics of egg shells and current handling procedures during marketing predispose eggs to breakage. Thin shells are causative of breakage and result

when the laying hens are suffering from acidosis (Helbacka and Casterline, 1963).

The latest standards issued by the Agricultural Marketing Service and Food Safety and Inspection Service of the USDA specify the number of checks (an individual egg with broken or cracked shell, but with shell membranes intact and content not leaking) allowed in packaged eggs at both the origin and final destination.

Currently many small 'hairline' checks are not visible to the operators of the candling station, causing the number of checks packaged by automatic equipment to increase. A process to increase the detection of checks by candling personnel consists of applying a special stain to the shells (Moats, 1982) which penetrates the cracks in the shells, staining the shell membrane blue and which is easily visible to the candling personnel. The stain then fades rapidly leaving no residue. The stain is a starch–iodine solution. Iodine is permitted as a sanitizing solution for intact eggs. Based on this concept, an automated system has been developed. Checks not detectable by standard candling methods are visible as blue lines on the shell membrane upon recandling. Commercial use of this system would significantly reduce the number of cracked eggs that would reach the consumer, and also decrease the risk of egg contamination and thereby retain a higher egg quality.

Another approach consists of scanning eggs by a narrowly focused laser beam. The light entering the egg through a crack is scattered, increasing the general brightness level of the egg, which is measured by a photodetector assembly. The photodetector assembly shows a sharp peak when the laser beam scans a crack. Practical problems relate to the selection of the width vs. depth of focus of the laser beam, and effects of the thickness and colour of the egg shell. Thin white shells give a relatively small increase in brightness when a crack is scanned. Field trials have shown that this apparatus can reliably detect cracks with widths >50 μm, and detect narrow cracks with decreasing probability. Cracks <20 μm cannot be detected (Bol, 1981). Inspection by a PC-based computer vision system that can identify $>90\%$ egg cracks has also been developed. The response in this system is dependent on egg size and two software calibration constants (Goodrum and Elster, 1992).

5.9.2 Sensory quality of eggs

Sensory testing of eggs with respect to consistency, colour, aroma, flavour, contaminants and dark particles in dried eggs are all specified in a Polish standard (Polish Standard, 1991). The reliability of a smell test depends on the fundamental property that good egg has no odour and is reliable only when judged by experienced examiners (Callaway, 1937). A detectable odour is associated with one or more egg constituents. For example 'fishy' tainted eggs have been associated with trimethylamine, and believed to come from the diet or from some heritable trait (Hobson-Frohock *et al.*, 1973). Smell test and bacterial counts have long been used in examination of egg and egg products. A simple objective method that could be used to confirm the subjective sensory classification would be highly desirable.

Compounds suggested as chemical indices of egg decomposition are ammoniacal nitrogen, fat acidity, ethyl alcohol, hydrogen sulphide and dimethyl sulphide, and organic acids such as acetic, formic, lactic and succinic acids (Brown *et al.*, 1986).

Spray drying is frequently used to obtain egg powder. During this process, oxidation of carotenoids and Maillard browning are mainly responsible for sensorial defects in powdered egg. Formation of oxysterols in eggs during spray drying has been reported; its content depends on the air heating system used, inlet and outlet temperature, type of atomizer and residence time inside the spray drying chamber. Linear correlations between oxysterol content during the spray drying process and other lipid oxidative processes, and also between oxysterol content and Maillard browning intensity have been found. These results indicate oxysterol content to be a potential indicator of the sensory quality of egg powder (Guardiola *et al.*, 1995).

The development of spoilage odours in liquid, frozen or dried egg products is associated with increased concentration of ethanol, hydrogen sulphide, dimethyl sulphide, formic, acetic, lactic and succinic acids. Hydrogen sulphide is produced when white and/or yolk is heated above pasteurization or contaminated by Pseudomonas, and is an excellent check on the freshness and microbiological quality of egg product (Cantoni *et al.*, 1980). Lactic acid in good eggs never exceeds 50 mg/100 g dried egg, and acetic acid does not exceed 65 mg/100 g dried egg (Pearson, 1976). Succinic acid, a microbial product, is not found in edible quality, but develops on decomposition (Ros, 1988). These are however found to be imperfect criteria since some egg samples rejected on basis of odour did not have abnormal concentration of any of these acids (Morris *et al.*, 1989). The metabolites vary considerably in the microflora and production at the various manufacturing stages. Therefore succinic acid or lipopolysaccharides cannot be used as indicators of quality (Bostel, 1991).

A recent work has focused on using uracil as a potentially useful chemical indicator of spoilage in egg products. Acceptable samples of egg are known to have $<1\ \mu g/g^{-1}$ of uracil. However, on deterioration, uracil content increases accompanied by a decrease in uridine contents. Spoilage odours can be detected when the product contains at least $1.7\ \mu g/g^{-1}$ uracil. The thermal stability and non-volatile nature of uracil makes it more useful for determination of whether dried egg products were prepared from acceptable liquid products (Morris *et al.*, 1989). However more extensive studies are necessary before uracil is accepted as a quality indicator.

5.9.3 Microbial quality of eggs

Microorganisms can either contaminate the shell, penetrate the pores of the shell to the shell membrane, grow through the shell membrane to reach the white or yolk or grow in the egg white despite unfavourable conditions. Bacteria are more often implicated in spoilage than moulds. Raw and undercooked eggs have been implicated in recent outbreaks of salmonellosis (Ament *et al.*, 1993; Pless, 1993) in Italy (Binkin *et al.*, 1993; Scuderi *et al.*, 1992) and in army catering in Bavaria in 1991 (Buchner *et al.*,

1992). Possible routes for these contaminations are faecal contamination through handling personnel, wash water or condensed water on the egg surface. It can also come from the plant, as has been shown with one producing spray-dried egg products (Mikulas and Valik, 1992) or from the environment (Poppe *et al.*, 1992). The outbreaks appear to be regioselective. In the Netherlands, for example, salmonella incidence in eggs is very low (Boer, 1991). Studies on characteristics of 10 *Salmonella enteritidis* strains have shown all of them to survive for 21 days or more at 10–30 °C and can also multiply in egg white during non-refrigerated storage (Reglich and Fehlhaber, 1992; Dolman and Board, 1992). Detection methods include the use of antigens against salmonellae antibodies (Baay and Huisin't Veld, 1993).

In Canada, regulations pertaining to processed egg products consider *Listeria monocytogenes* to be a pathogenic organism of human health significance, and prohibit the marketing of such products (World Health Organization, 1991). Processing conditions such as frying of whole and scrambled eggs are however known to reduce their levels to undetectable limits (Brackett and Beuchat, 1992).

A limulus test enables rapid and inexpensive detection of gram negative bacteria, that is, Enterobacteriaceae from dirty shells by semi-quantitative detection of lipopolysaccharides, from cell walls and endotoxins. Kits for this test are available commercially. Total aerobic cell count may be determined in 1 h by an oxygen consumption method using oxygen-specific electrode and pH meter with a limit of detection corresponding to about 20 000 cells g^{-1}. The presence of antibiotics and disinfectants in egg products can be detected by using a microbial inhibition test based on *Bacillus subtilis* spores (Bostel, 1986).

Bacteria of the genus Pseudomonas are considered to be among the most important of the groups responsible for the spoilage of eggs. They can penetrate rapidly through the intact membrane of the egg, whereas most other spoilage organisms cannot or do so more slowly (Florian and Trussel, 1957). Although odours of spoilage due to pure cultures of Pseudomonas may be mild (Elliott, 1954), accompanying organisms often will cause putridity (Trussell, 1955; Elliott, 1958). Most pseudomonads produce a water soluble pigment called 'pyoverdine' in the albumen of the egg. The fluorescence of pyoverdine under longwave ultraviolet black light is the basis for the black-light egg candler used to detect fluorescent egg shells. However, when the albumen and yolk are mixed, as in the manufacture of frozen whole egg, the fluorescence of pyroverdine is masked by the more intense one of the yolk. A convenient method of empirical nature has been reported for the measurement of pyoverdine and had been suggested as official method. Green rots caused by *Pseudomonas fluorescens*, colourless rots caused by Pseudomonas, Achromobacter, certain coliform bacteria, or other types of bacteria, black rots caused by Proteus species, pink rots by certain strains of Pseudomonas, red rots caused by Serratia are the spoilage modes in eggs. Pin-spot moulding has also been observed, with Penicillium causing yellow, blue or green spots inside the shell, Cladosporium species giving dark-green or black spots, and species of Sporotrichum producing pink spots. Mucor, Thamnidium, Botrytis and Alternaria are the other

fungal spoilages in eggs. These are responsible for offodours such as hay odour caused by *Aerobacter cloacae*, fishy by *Escherichia coli*, and earthy or musty by Streptomyces (Frazier, 1967).

5.9.4 Adulteration in egg products

In the USA and Canada the incorporation of hatchery reject eggs, eggs which have failed to develop into chick, into egg products destined for human consumption is prohibited. Several instances have recently been encountered where infertile incubator rejects were substituted in part or wholly for fresh eggs in frozen egg products on the market (Anon, 1985). Techniques to detect such malpractices are not readily available.

A number of analytical methods based on the proteins such as electrophoresis (Harwalkar, 1968; Csuka *et al.*, 1973) and measuring the increase in reducing proteins (Cattaneo *et al.*) have been reported. Both these methods are based on the denaturation of proteins due to heat in the incubators, and are applicable to infertile as well as fertilized but dead eggs. However, the sensitivity of such methods is low. Methods based on the content of 3-hydroxybutyric acid are also employed (Bethea and Wong, 1968; Robinson *et al.*, 1975; Parry *et al.*, 1980; Heany and Curtis, 1976; Elenbaas *et al.*, 1986; Uijttenboogaart *et al.*, 1986; Salwin *et al.*, 1972). β-Hydroxybutyric acid content in excess of 10–15 mg/kg^{-1} indicates the use of fertilized or incubator reject eggs. Organic acids can be determined by isotachophoresis, which is very rapid (2 h) but very expensive (Bostel, 1986). An observation by Feeney *et al.* (1963) suggested slight differences in the electrophoretic patterns of egg white after incubation at 37 °C for 6 days. Polyacrylamide gel electrophoresis at pH 9.1–9.3 and with 4.5 M urea has shown consistent and reproducible differences in the protein patterns of fresh and incubator-reject eggs. The differences are found in the ovalbumin and conalbumin regions, and are also evident in mixtures of the two types of eggs. This method could be used to detect adulteration of egg products with as little as 15% of incubator rejects (Harwalkar, 1968). As some physicochemical changes are produced by heat pasteurization (Parkinson, 1968, 1970; Garibaldi *et al.*, 1968; Vadehra and Nath, 1973), the presence of hydroxybutyric acid formed as a result of limited embryonic development seems to be the most promising method. It can be extracted by liquid–liquid extraction and analysed on a gas chromatograph. It has also been established that the presence of 15.95 ± 8.54 mg β-hydroxybutyric acid per 100 g of liquid whole egg would strongly indicate that the egg had been prepared from incubator reject eggs (Robinson *et al.*, 1975). Freshly laid eggs contained 0.40–0.56 mg β-hydroxybutyric acid/100 g. The finding that isovaleric acid is also present in cracked infertile eggs, called 'leakers' seems to obviate its claim as an indicator of incubator reject eggs. The number and type of bacteria present generally do not influence β-hydroxybutyric acid.

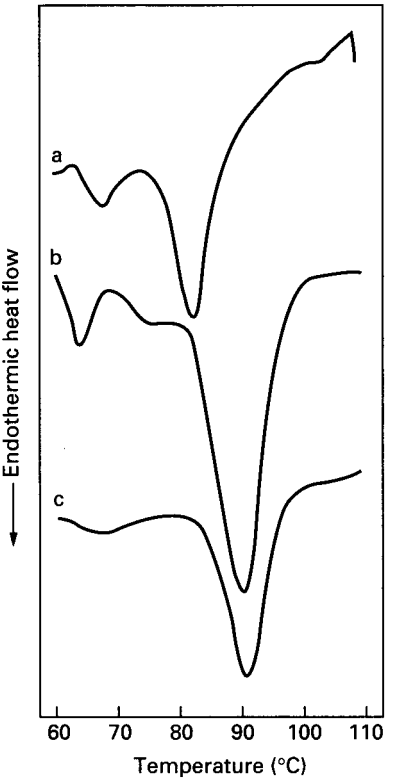


Figure 5.2 Thermograms of egg white from fresh and incubator reject eggs (a) 100% fresh; (b) 100% fertile dead IR; (c) 100% infertile IR. (Source: Raymond *et al.*, 1992, reproduced with permission)

Storage of eggs above 22 °C changes the transition temperature (T_d) of denaturation of ovalbumin (88 °C) to that of a more thermostable S-ovalbumin (91 °C) in DSC. This difference has been used to detect incubator reject eggs in fresh eggs and is evident from Figure 5.2 (Raymond *et al.*, 1992). Detection of as low as 7% incubator reject eggs in fresh eggs is possible by this method.

Liquid whole egg, prepared from infertile eggs, often contains many different types of microorganisms which originate from outside of the shell and the environment in general and are capable of multiplying in the egg when held at low temperarures. Amongst these, strains of Pseudomonas frequently predominate, and there is scanty information as to whether these organisms would metabolize any β-hydroxybutyric acid present in liquid whole egg containing some incubator reject eggs.

Detection of duck egg albumin in hen's egg white is necessary in some cases of irresponsible practices and could prove useful in regulatory work. This can be achieved by immunological techniques, which can detect as little as one part of duck

egg albumin in 12 800 parts of hen egg albumin (Oswald, 1953).

5.9.5 Egg discoloration

Pink discoloration of eggs during cold storage has been encountered and is associated with feeding cottonseed oil or cottonseed meal to laying hens. This discoloration is a result of the combination of conalbumin of the white with ferrous of the yolk to form a pink complex. Increased permeability of the vitellin membrane allows diffusion of proteins and water into the yolk. Reverse diffusion of the complex into white accounts for the pink colour of the white. The yolk enlarges and becomes apricot coloured as a result of the blending of the pink colour with the natural yellow of the yolk. Certain uncommon oils such as kapok seed oil and that from *Sterculia foetida* are also known to cause pink discoloration in eggs. The common feature in all these feeds was the presence of sterculic acid, the causative nature of which was confirmed with studies using pure sterculic acid (Masson *et al.*, 1957).

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Chapter 6

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Chapter 6

Edible Oils and Fats

6.1 Introduction

The major vegetable oils used the world over for edible applications are derived from groundnut, soybean, sunflower, rapeseed, mustard, sesame and safflower seeds grown primarily as annual oil seed crops and oil palm, coconut and olive which are perennial trees. Cottonseed has of late been processed to exploit the oil for edible uses. Similarly corn germ and rice bran are valuable by products of the staple cereals that yield edible oils, the former being a recognized quality oil of commerce. Cocoa butter and avocado fruit fat are speciality fruit fats of plant origin. Of late, some perennial forest tree seeds have been processed in India to yield edible grade fats, *Shorea robusta* or sal seed fat and madhuca or mowra or mahua seed fat are two such fats, the former being used as cocoa butter substitute. Seeds from water melon and pumpkin yield good quality edible oils.

Apart from these, several oils of plant origin have been commercially used for non-food applications in paints, varnishes, surface coatings, etc. Major oils amongst these are nigerseed, linseed and castor oils. At times, these enter into edible oils as contaminants or adulterants.

Fats of animal origin emanate as by products of beef and pork processing in large quantities. Their refined forms are extensively used as edible fats. Milk fat in the form of cream, butter, butter oil and ghee is used worldwide in diverse food products. Marine oils, as such or after controlled hydrogenation find use in canning of fish types.

These oils and fats, whether of animal or plant origin may be used in the mechanically extracted or solvent extracted forms or after further processing like deodorization, decolorization, refining, hydrogenation or fractionation such as winterization.

With the scarcity of edible oils being experienced in many countries, several hitherto unexplored plant materials are being investigated as sources of fats and oils. Seeds from a large number of tropical forest trees have been found to be rich in oils and the oils, after suitable refining treatment, are being used commercially for non-edible applications such as for the manufacture of fatty acids, glycerine and soaps. Mango kernel (*Mangifera indica*), kokum (*Garcinia indica*) seeds, tea (*Camellia sinensis*) seeds, tobacco (*Nicotiana tabacum*) seeds etc. are known to yield substantial quantities of oils.

Geographical classification of some oils such as olive oil is gaining importance, and this may encourage bottling of good quality oils in a way similar to 'appellation

d'origine' wines. Hence there is a need to establish methods of determining the trademarks of such products. The fact that this can be proved by scientific procedures (Boskou, 1990) is under discussion by the European Union. Chemometrics has been made the basis of such classifications (Forina and Tiscornia, 1982; Forina *et al.*, 1983a, 1983b; Derde *et al.*, 1984; Eddib and Nickless, 1987; Leardi and Paganuzzi, 1987; Tsimidou *et al.*, 1987; Forcadell *et al.*, 1988; Alberghina *et al.*, 1991). Graphic, parametric and non-parametric pattern recognition methods have been performed on data sets of fatty acid composition and/or sterol or triglyceride composition to produce visual or numerical estimates of origin (Aparicio *et al.*, 1987, 1991; Derde *et al.*, 1987; Aparicio, 1988). It is believed that non-parametric discriminant analysis after proper transformation of the data seems to be a suitable approach for characterizing the oils according to their geographical origin and may produce a scientific basis for the assignment of an 'appellation d' origine' trademark (Tsimidou and Karakostas, 1993). Tables 6.1 to Table 6.4 summarize the physical and chemical characteristics and fatty acid composition of oils and fats of commercial importance. It may be noted that the oil content has been correlated to specific gravity of the oilseed kernels, as in the case of groundnuts (Misra *et al.*, 1993). The fatty acid composition can be affected by maturation of oilseeds (El-Shami *et al.*, 1994), infection in the plant from which the oil is derived (Conte *et al.*, 1989) and also varies as a function of geographical origin and harvesting time (Parcerisa *et al.*, 1994, 1995).

Table 6.1 Physical properties of some fats and oils of commerce

Oil	Smoke point (°C)	Flash point (°C)	Fire point (°C)	Specific heat (J/g)	Surface tension (80 °C, mN/m)	Interfacial tension (70 °C, mN/m)
Coconut	194–209	288–316	329–341	–	28.4	–
Cottonseed	185–223	290–322	342	2.200 (at 90 °C)	31.3	29.8
Linseed	–	–	–	2.050 (at 70.7 °C)		
Palm	223	314	341	2.400 (at 140 °C)		
Peanut	160–207	290–333	342–363	–	–	29.9
Olive oil	–	–	–	2.300 (at 110 °C)		
Rapeseed	218	317	344	–		
Soybean	213	317	342	2.060 (at 80.4 °C) 2.000 (at 60 °C)	–	30.6
Sunflower	209	316	341	2.500 (at 175 °C)		
Beef tallow	266–316	344				

Source: Thomas, 1987.

Table 6.2 Some constants of oils and fats of commerce

Oil	Saponification value	Iodine value	Unsaponifiable matter (%)	Melting point (°C)	Density (g/L) (t = °C)	Refractive Index (at 20°C)
Coconut	25–262	7–10	0.15–0.60	20–28	932.0–0.745t	1.448–1.450
Corn	187–196	109–133	1.3–2.0			
Cottonseed	190–198	100–117	0.5–1.5	~ 0 °C	931.7–0.755t	1.472–1.477
Olive	185–196	80–88	< 1.4%	− 3 °C	928.5–0.700t	1.467–1.471
Palm	195–205	44–58	0.5	36–40	925.0–0.655t	1.453–1.456
Palm kernel	242–254	16–19	0.2–0.8	23–30	940.0–0.740t	1.449–1.452
Pumpkin seed	185–198	117–130	0.6–1.5	~ − 15 °C		
Safflower	180–194	136–152	0.3–2.0	− 13 to–25 °C		
Sesame	187–193	136–138	0.9–2.3		933.0–0.700t	1.473–1.476
Soybean	188–195	120–136	0.5–1.5	− 15 to − 8 °C	933.4–0.657t	1.470–1.478
Sunflower	188–194	125–144	0.4–1.4		932.7–0.680t	1.474–1.476
Beef tallow	193–200	32–47	0.3– 0.8	40–50 °C	956.8–0.898t	1.454–1.459

Source: Thomas, 1987

Table 6.3 Saturated fatty acid composition of various oils and fats

	Saturated fatty acids[a], g/100 g fatty acids							
	C_{10} and lower	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}
Liquid vegetable oils								
Corn germ		tr–0.5	tr–0.3	9–12	1–3	~ 0.5	tr–0.5	<0.5
Cottonseed		tr	0.5–2.0	21–27	2–3	< 0.5	tr	tr
Linseed		tr	tr	5–6	3-5	<0.5	tr-0.2	tr
Olive				7–16	2-4	~ 0.5	tr	tr
Peanut (Africa)		tr	7–12	1.5–5	~ 1.5	2-4	1-2	
Peanut (South America)		tr	10–13	1.5–4	~ 1.5	3–41.5–2		
Pumpkinseed			7–13	6-7tr				
Rapeseed (high erucic)		tr	tr	2–4	1–2	0.5–1	0.5-2.0	0.5
Rapeseed (low erucic)		tr	tr	3–6	1–2.5	<1	tr–0.5	tr–0.2
Ricebran			~ 0.5	13–18	~ 2	0.5–1	~ 0.5	
Safflower		tr	tr	~ 5	2–3	~ 0.5	~ 1	~ 1
Sesame			tr	8–10	3–6	~ 0.5		
Soybean			<0.5	8–12	3–5	<0.5	tr	
Sunflower		tr	tr-0.1	5.5-8	2.5–6.5	<0.5	0.5–1.0	<0.5
Wheat germ				12–14	ca.1	0.5		tr
Consistent vegetable fats:								
Babassu oil	~ 12	42–44	15–18	8–10	2–3	tr		
Coconut oil	~ 13	41–46	18–21	9–12	2–4	tr	tr	
Cocoa butter		tr	tr	23–30	32–37	<1	~ 0.5	
Palm kernel oil	~ 7	41–45	15–17	7–10	2–3	tr–0.3	tr–0.5	
Palm oil (Africa)		tr	1–2	41–46	4–6.5	~ 0.5		
Palm oil (Indonesia)		tr–0.5	~ 1	41–47	4–6	~ 0.5		
Animal fats:								
Beef tallow		tr	2–4	23–29	20–35	<0.5	tr	
Butter fat	7–9	2–5	8–14	24–32	9–13	2		
Chicken fat			~ 1	20–24	4–7			
Goose fat				20–22	4–11			
Horse fat	tr		~ 0.5	3–6	20–30	6–10	tr	
Lard	tr	<0.5	~ 1.5	24–30	12–18	~ 0.5		
Mutton tallow	tr	~ 0.5	1–4	22–30	15–30	tr	tr	

Table 6.3 (cont)

Marine oils:								
Fish oils								
Japanese	tr	tr	~ 6	~ 16	~ 3	< 0.5	tr	tr–1
Menhaden	tr	tr	~ 9	~ 20	~ 4	tr–0.1	tr	tr–1
Scandinavian	tr	tr	6–8	11–15	1-3	tr–0.5	tr	tr–1
South American	tr	tr	~ 7	17–19	2–4	~ 0.5	tr	tr–1
Whale oil		tr		~ 0.5	4–10	10–18	1–3	tr

atr = traces (< 0.05 %)
Source: Thomas, 1987.

Table 6.4 Unsaturated fatty acids composition of various fats and oils

	Unsaturated fatty acidsb, g/100 g fatty acids								
	$C_{14:1}$	$C_{16:1}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$	$C_{22:1}$	$C_{20:x}$	$C_{22:x}$	$C_{24:1}$
Liquid vegetable oils										
Corn germ		<0.5	25–35	40–60	~ 1	~ 0.5	tr-0.1			
Cottonseed		<1	14–21	45–58	tr–0.2	tr				
Linseed		tr	18-26	14-20	51–56	<0.5				
Olive	1–2	64–86	4-15	0.5–1	0.5					
Peanut (Africa)			<0.5	50–70	14-30	tr	0.5–1.5	tr		
Peanut (South America)		<0.5	35-42	39–44		tr	0.5–1.5	tr		
Pumpkinseed				24–41	46–57					
Rapeseed (high erucic)		~ 0.5	11–24	10-22	7–13	~ 10	41–52			
Rapeseed (low erucic)	0.1–0.5	52–66	17–25	8–11	1.5-3.5	tr–2.5	tr–0.1	tr		
Ricebran				~ 44	30-40					
Safflower		tr	12-20	70-80	tr	tr				
Sesame				tr	35-46	40-48	tr-0.5	<0.5		
Soybean				tr	18-25	49-57	6-11	<0.5		
Sunflower		<0.5	14-34	55-73	tr-0.4	<0.5	tr-0.3			
Wheat germ			~ 30	40-55	~ 7					
Consistent vegetable fats										
Babassu oil			14-16	1-2						
Coconut oil	tr	tr	5–9	0.5–3	tr	tr				
Cocoa butter		~ 0.5	30–37	2–4						
Palm kernel oil			10–18	1–3	tr–0.5	tr–0.5				
Palm oil (Africa)		<0.5	37–42	8–12	tr–0.5	tr				
Palm oil (Indonesia)		~ 0.5	37–41	~ 10	tr–0.5	tr				
Animal fats:										
Beef tallow	~ 0.5	2–4	26–45	2–6	~ 1	<0.5	tr			~ 0.5
Butterfat	~ 2	3	19–33	1–4	2–6					~ 2
Chicken fat		~ 7	38–44	18–33	~ 1					0.5–1.0
Goose fat			41–74	7–19						
Horse fat		3–10	36–40	6–11	4–9	tr–0.5			tr	1–2
Lard	tr	2–3	36–52	10-12	~ 1	0.5–1			<0.5	tr
Mutton tallow	~ 0.5	3–4	31–56	3–7	1–2				~ 0.5	
Marine oils										
Fish oils										
Japanese	tr	~ 7	~ 14	~ 2	~ 1	~ 7	~ 6	~ 15	~ 12	tr–1
Menhaden	tr	~ 11	~ 13	~ 2	~ 1	~ 2	~ 1	~ 14	~ 11	tr–1
Scandinavian	tr	6–11	12-15	1–2	0.5–1	9–16	14–20	6–10	5–11	tr–1
South American	tr	9–11	14–15	1–2	0.5–1	1–2	1–2	7–19	10–14	tr–1
Whale oil	1–3	13–20	24–33	1–2	tr	10–15	4–10	1–6	5–7	tr

[a]tr = traces (< 0.05 %).
Source: Thomas, 1987.

6.2 Indicators of storage changes

The autoxidative deterioration of edible fats and oils is not merely an economic problem of the food industry. As a result of rancidity, the nutritive value of the products also declines due to destruction of essential fatty acids and vitamins. The oxidative stability of an oil or fat is one of the important properties that determines its use in processed foods. Since the most important cause of the oxidative deterioration of lipids is the dissolution of oxygen from air and its subsequent reaction with the unsaturated glycerides, the storability of edible oils is determined by the oxidative stability and the dissolved oxygen content. Oxidative stability of a product is characterized by the length of the induction period of the autoxidation process (Thompson, 1966; Pardun, 1969). A simple, fast and direct method for the determination of dissolved oxygen in fats and oils is provided by an electrochemical procedure using membrane-covered polarographic sensors (Becker and Niederstebruch, 1966). Peroxide value is an effective indicator of the degree of oxidation of lipid and is generally determined iodometrically. This is a long recognized method of oil analysis. Test papers (Asakawa and Matsushita, 1975, 1976) and test solutions based on peroxide values have been developed. However, the difficulties in judging the end point make this method uncertain. A rapid, controlled potential coulometric procedure that also correlates well with iodometry has been developed (Oishi *et al.*, 1992a, 1992b). Many new methods have been developed in the past few years for the detection of lipid peroxidation. The assay for hydroperoxides offers the most direct measure, since these can undergo both enzymatic and non-enzymatic degradation to produce an array of secondary oxidation products. Methods of determining hydroperoxides include spectrophotometric determination of the oxidation of iodine (Hicks and Gebicki, 1979), leuco dyes (Glavind and Hartmann, 1955), thiocyanate (Stine *et al.*, 1954) or diene conjugation (Barthel and Grosch, 1974). Limitations of the iodometric assay include its susceptibility to background reactions, easy oxidation of the iodide to triiodide by molecular oxygen, and oxidation of the unsaturated fatty acids to lipid hydroperoxides. These disadvantages can be overcome by reducing the iodide and acid concentrations through the use of ferrous ions which can accelerate the formation of triiodide chromophore from the lipid chromophore without promoting the oxidation of unsaturated fatty acids (Lovaas, 1992).

The validity of several analytical parameters for quality evaluation of edible oils has been tested in various methods that determine acidity, peroxide number, Stamm's peroxide number, Stamm's qualitative reaction, Kreiss reaction, Watts and Major index, thiobarbituric acid (TBA) index, anisidine index, saturated and unsaturated carbonyls (Berry and McKerrigan, 1958) and optical extinctions at various wavelengths. Test papers based on the TBA test have also been developed (Sinnhuber and Yu, 1958; Ottolenghi, 1959; Asakawa and Matsushita, 1979a, 1979b). The main reactant in the TBA test was identified as malonaldehyde, an indicator which

effectively distinguishes rancid fat-containing products such as nutmeat (Holland, 1971). TBA-reactive substances also correlate linearly with consumed oxygen up to a value of about 500 μmol l^{-1}, indicating it to be suitable only in the early stages of the lipid oxidation (Kishida *et al.*, 1993a, 1993 b). The TBA reaction is now shown to be not specific to malonaldehyde alone and hence methods were developed to estimate malonaldehyde separately. Earlier attempts in this direction were unsuccessful because of the extremely unstable and reactive nature of malonaldehyde. Recently, a high performance liquid chromatography (HPLC) method based on the reaction of malonaldehyde with 2-hydroxypyrimidine (Osawa and Shibamoto, 1992), and a chitosan-based fluorescence sensor which sorps malonaldehyde independent of the temperature (Weist and Karel, 1992), and ion pairing HPLC (Madere and Beherns, 1992; Beherns and Madere, 1991) have been developed. TBA number, anisidine value and Kreiss test all determine secondary oxidation products formed from the hydroperoxides, and are therefore much less sensitive and non-specific. The carbonyl compounds responsible for these tests may further react with one another, resulting in a decrease in these rancidity indices. The measurement of conjugated dienes in terms of UV absorption at 280 nm is partially useful since it is an indication of compounds formed during the early stages of autoxidation, and which may be lost by the formation of secondary products. The ratio of aliphatic to olefinic protons, determined by NMR spectroscopy increases steadily over a length of storage period and can be used to monitor the oxidative stability of oils. This has been successfully demonstrated with canola and soybean oils. A good correlation between peroxide value and NMR results has been found indicating it to be a rapid, non-destructive and simple procedure for evaluation of the oxidative changes during storage of edible oils (Wanasundara *et al.*, 1995).

The sensory changes could probably be monitored by headspace analysis (Solinas and Rossetti, 1977). Pentane, as determined by headspace gas chromatographic method has recently been proposed as an excellent indicator of oil rancidity (Ulberth and Roubicek, 1992). Indications are that the concentrations of polar and non-polar compounds are less dependent on particular oils and may correlate better with sensory assessment (Spark *et al.*, 1981).

6.2.1 Chemical methods

The quality of fats and oils can also be determined from their epoxide content. The method involves dissolving in a solvent, adding a standard solution of picric acid, holding for 45 min in a boiling waterbath to develop the colour, adding an alkali, measuring the optical density at 490 nm, and measuring the amount of oxirane oxygen, which is characteristic of the epoxide content. The precision can be improved and the time can be reduced by using dioxane as solvent (Chumak *et al.*, 1990).

6.2.2 Physical methods

6.2.2.1 Thermal methods

There is a possibility of estimating the oxidative changes in fats and oils and their tendency to further deterioration by studying the thermoanalytical behaviour by thermal analysis. Cross (1970) used the differential scanning calorimeter (DSC) to characterize different oils and shortenings. The exothermic enthalpy change detected under isothermal conditions during rapid oxidation following the induction period shows a good correlation with standard tests. Thermogravimetric and pressure differential calorimetric (Nieschlag *et al.*, 1974; Hassel, 1976) methods for estimating oil stability using static and dynamic conditions have been reported.

Yet another method developed for evaluating oxidative stability and predicting storage life of vegetable oils is by means of a derivatograph under isothermal conditions. Oxidative stability can be calculated from the length of the incubation period (IP), time to maximum weight gain (TG), time of maximum rate of weight increase (differential thermal gravimetry – DTG) and time of maximum enthalpy change (differential thermal analysis – DTA). Results with sunflower and rapeseed oil have shown a good reproducibility of the method. It is rapid, and is suitable for routine use, particularly in conjunction with the determination of the amount of dissolved oxygen (Buzas *et al.*, 1978).

6.2.2.2 Chemiluminescence

Chemiluminescence (CL) is virtually a universal property of oxidizable organic substances (Mendenhall, 1977). CL has been used to study the oxidation of foods (Usuki *et al.*, 1979). Reports of correlation of CL with a wide range of chemical and physical properties of foods and other complex substances are available. The CL method has been used to determine the content and effectiveness of antioxidants in oils and fats used in pharmaceutical preparations (Goldenberg *et al.*, 1978). Usuki *et al.* (1980) studied the chemical characteristics and CL of frying oils collected from food manufacturers. Although no correlation between oxidative deterioration and CL has been observed, the absolute level of CL depends significantly on the type of oil and its thermal history. This makes quantitative comparisons rather difficult. However, a good correlation between CL and oxidized flavour in milk powder and reconstituted milk has been observed (Timms and Roupas, 1982). CL for milk fat has been shown to be closely related to oxidation after an initial slight decrease and closely correlates with the peroxide and anisidine values and flavour. It is suggested that CL is emitted during peroxide breakdown.

The light emitting steps in the oxidation of hydrocarbons have been shown to be:

$$RO_2\cdot + RO_2\cdot \rightarrow \text{excited ketone} + O_2 + \text{alcohol} \quad [6.1]$$

$$\text{excited ketone} \rightarrow \text{ketone} + \text{light} \quad [6.2]$$

Increasing oxygen consumption will lead to an increased concentration of $RO_2\cdot$ and hence excited ketone and increased light emission. It is therefore believed to be a potentially useful method as an indicator of rancidity, but has a low sensitivity. Addition of sodium hypochlorite to an oxidized oil however causes a strong emission of light (Yamamoto *et al.*, 1985), providing a highly sensitive method for the detection of low levels of hydroperoxides. This method is rapid and can be used as a supplement to the standard chemical methods for quality assessment and antioxidant evaluation. Care has to be taken in using this method with fish oils, since the light emission depends both on the composition and rancidity level of the oil (Burkow *et al.*, 1992). Addition of luminol (5-amino-1,2,3,4-tetrahydrophtalazin-1,4-dione) improves the sensitivity of hypochlorite induced chemiluminescence and the method is suitable for evaluating the rancidity of refined, high quality fish oils (Pettersen, 1994).

Fluorescent compounds have also been observed in oxidizing lipids and interest has been shown in the use of fluorescence to follow the progress of oxidation in oils and fats (Gray, 1978). In animal tissue lipids, these fluorescent compounds have been identified as schiff's bases formed from the reaction between *o*-amino groups and malonaldehyde (Dillard and Tappel, 1973; Buttkus and Bose, 1972). These fluorescence methods are 10–100 times more sensitive than the TBA test. The development of fluorescence is linearly related to oxygen absorption. Recently, laser-induced fluorescence at 673 nm has been shown to be indicative of the oxidative quality of crude palm oil; a positive correlation between fluorescence intensity and the carotene and the deterioration of the bleachability index of crude palm oil and the Rancimat induction time of the final refined product makes it a rapid method for determining the quality of crude palm oil (Tan *et al.*, 1995).

6.3 Indicators of quality of heated oils

A large proportion of edible oil is used for frying of foods. Deteriorative changes occurring during frying affect the flavour and nutritive value of foods. These include thermal oxidation, hydrolysis and polymerization (Dobarganes and Perez-Camino, 1988). Tests to determine the suitability of an oil for frying use have been documented. These include physical tests such as viscosity and foaming related to the polymers; colour related to α,β unsaturated carbonyl compounds; ultraviolet absorption related to conjugated dienes and trienes; dielectric constant related to polar compounds; and organoleptic assessment related to the formation of volatile compounds. Deep fat frying of potatoes with fast turnover in sunflower oil has shown more thermoxidative changes than hydrolytic changes. A dramatic rise in polar compounds and those compounds related to thermoxidative alteration of the oil has been reported (Cuesta *et al.*, 1993). Chemical tests include those based on acid value related to free fatty acids, iodine value related to unsaturation, peroxide value and various colorimetric reactions related to oxidized fatty acids. The efficiency of physical tests is conditional on the knowledge of their value for the starting material. Volatile decomposition products

distilling out during frying and fatty acid analysis especially 18:2/16:0 ratio can also form the basis of estimating heat abuse of frying oils (White, 1991). The contribution of the food being fried is more than the effect of frying on the oil with respect to chemical tests such as acid value, iodine value and UV absorption. Rise in peroxide value is surprisingly lower than the rate of decomposition of the oil and UV absorption undergoes a much more marked change in very unsaturated fats independent of total alteration.

Direct measurement of change has a great advantage of eliminating the inadequacies of the classical indices, as, together with the possibility of evaluating compounds specifically related to the degradation, there exists a clear value for initial fat. These include general characteristics such as polar compounds, and specific determinations such as cyclic monomers, dimers, non-polar dimers and polymers of triglycerides. The polar compounds, cyclic monomers and non-urea adduct fraction are the major concerns associated with heated oil toxicity. The formation of cyclic monomers starting from fatty acids with three or more double bonds gives this determination a special importance in the evaluation of oils containing significant amounts of linolenic acid. In France, this has served as the basis for prohibiting use of fats having a content greater than 2% of linolenic acid for frying. These can be conveniently analysed by gas chromatography – mass spectroscopy (GC–MS) using high resolution capillary columns and selected ions abundance (Rojo and Perkins, 1991). Polymeric compounds give a correlation to the changes produced in heated fats, but their separation is still difficult and therefore cannot be quantitated with accuracy. Similarly the method based on non-urea adductable fraction suffers from a disadvantage of ill-defined separations (Gutierrez *et al.*, 1988).

It has been recommended that frying oils containing more than 25–27% polar lipids should be rejected, and the cut-off point be based on the quantitation of compounds impairing the nutritional value of the fat (Dobarganes *et al.*, 1991). A survey of quality of used frying oils from restaurants has shown significant correlation between total polar compounds and Foodoil sensor (Al-Kahtani, 1991). The Foodoil sensor, developed by Northern Instrument Corp, Minnesota, measures the dielectric constant in discarded frying oils relative to fresh oils. A correlation between dielectric constant and thermal degradation of oils has been demonstrated. The Fritol test is a new test based on the ratio of polar substances in oils and can be used for rapid evaluation of thermal degradation of frying oils (Ostry and Ruprich, 1992). Quantification of oligopolymers, diglycerides and oxidized triglycerides by high performance size exclusion chromatography (HPSEC) helps define levels of oxidative degradation and hydrolysis in deep fried fats (Gomes, 1992). The levels for each oil type, and effects of refining on these indicator compounds need to be established. The ratio of 1,3:1,2 diglyceride is also altered on thermal treatment of oils and could be used in addition to the widely used quality parameters such as peroxide and anisidine values (Siew and Ng, 1995). A comparison of various indicators has shown dielectric constant (Fritsch, 1981; Wu and Nawar, 1986) and polymer content to be suitable for monitoring the

quality of heated oils. These are convenient indicators in commercial deep fat frying operations (Smith *et al.*, 1986). Changes in the dielectric constant have also been reported to be useful for determining the effects of treating spent frying shortenings to extend their utilization. It has been shown that dielectric loss (E'') reaches a maximum after 5 h of heating, while the peroxide value reaches a maximum after 15 h. This is believed to be due to the fact that E'' is more sensitive to hydroperoxide formation than to its decomposition. Thus the beginning of deterioration of an oil during deep fat frying can be ascertained from the E'' value (El-Shami *et al.*, 1992). The E'' value also correlates to the polar compounds. A good correlation exists between the potentially toxic non-volatile materials (cyclic monomers, non-urea adduct portion) and the commercial quality indices such as dielectric changes and polar materials, for each oil such as soybean or sunflower. Different oils contain different amounts of toxic substances for the same value of commercial quality indices (Huang *et al.*, 1990).

Near infrared reflectance spectroscopy (NIRS) has been used to determine the sum of dimer and polymer triglycerides and acid value to evaluate frying oils (Boot and Speek, 1994). This is a rapid low-cost technique to assess whether a sample complies with the food legislation. Application of the first and second derivative UV spectrometry over the wavelength range 200–300 nm is also shown to characterize the frying oil (Clemente and Valletrisco, 1992). Worldwide regulations on frying fats and oils have recently been reviewed by Firestone (Firestone, 1993; Firestone *et al.*, 1991).

6.4 Toxic contaminants and adulterants

Oilseeds are subject to potential contamination by weed seeds during harvesting, some of which are toxic. These toxicants find their way in the edible oils during oil extraction and are often manifested by the effect on a section of unsuspecting consumers. These often come to light in cases of outbreaks of food poisoning, which affect a large number of people. Similarly, storage of an otherwise safe oil in unsafe containers can also cause contamination, responsible for toxicity symptoms such as Spanish Toxic Oil Syndrome which occurred in 1981 (see Section 6.4.3).

6.4.1 Contamination by weed seeds

Much of the literature on toxic plants and weed seeds has been summarized by Muenscher (1951), Kingsbury (1964), Bailey and Bailey (1976) and in the Merck index (1976).

The toxic weed seeds identified as contaminants in soybeans, their geographical distribution, and toxic principles are given in Table 6.5.

Table 6.5 Toxic weed species identified in soybeans

Seed	Genus, species United States	Distribution, principle(s)	Toxic
Cocklebur	*Xanthium strumarium*	Widespread	Hydroquinone
Nightshade	*Atropa belladona*	Cultivated	Tropane alkaloids
Cowcockle	*Saponaria vaccaria*	Northwestern states	Githagenin
Corncockle	*Agrostemma githago*	Widespread	Githegenin
Morning glory	*Ipomea* spp.	Widespread	Clavine, Indole alkaloids
Castor	*Ricinus communis*	South central	Ricinine, ricin
Pokeweed	*Phytolacca americana*	Eastern	Saponins, Glycoproteins
Crotalaria	*Crotalaria* spp.	Southwestern	Pyrrolidizine alkaloids
Jimsonweed	*Datura stramonium*	Widespread	Tropane alkaloids

Source: List *et al.*, 1979 (reproduced with permission).

6.4.1.1 Cocklebur

This is a weed widely distributed throughout North America. The toxic principle has been suggested to be a glucoside, xanostrumarium in the germinating seeds (Radeleff, 1964); although Kuzel and Miller (1950) have claimed it to be a hydroquinone. Cocklebur poisoning is known to affect all classes of domestic animals. Hydroquinone is toxic at a level of 0.3% of animal weight. The toxicity survives in the silage and can poison livestock such as pigs and fowl (Kingsbury, 1964). The symptoms of cocklebur poisoning are depression, nausea, vomiting and muscular weakness.

6.4.1.2 Crotolaria spp.

Crotolaria species reported to be toxic are *C. sagitalis*, *C. spectabilus* and *C. retusa*. An alkaloid, monocrotaline has been isolated from the leaves, seeds and stems of *C. spectabilus* (Neal *et al.*, 1935), and *C. retusa* (Adams and Rogers, 1939). It is known to cause severe poisoning in fowl, cattle and horses. Sheep, goats and mules are less susceptible. These contaminant seeds in shelled corn and soybeans can be easily removed by screening. .

6.4.1.3 Cowcockle and corncockle

The toxic principle in these weed seeds is the saponin, githagenin (Merck Index, 1976). Humans are known to be poisoned from eating bread made from wheat contaminated with corncockle seed. However, no reports on poisoning from soybean or any other oil obtained from contaminated oilseeds has been reported.

6.4.1.4 Morning glory

The toxic principles, identified as clavine and indole alkaloids (Taber *et al.*, 1963) are purgative, causing mild distress in hogs, sheep, goat and cattle in Brazil are known to be poisoned by this weed.

6.4.1.5 Castor seed

Castor (*Ricinus communis* L.) plant of the family Euphorbiaceae is distributed in South Central United States. The toxic principle is ricin, a glycoprotein of molecular weight 65 000, and another compound ricinine. Ricin is amongst the most toxic plant substances known. While ricin is not found in the oil, that found in the castor bean press cake can be made non-toxic by heating with alkali. An allergenic compound has been reported in castor seeds (Morton, 1971) which causes bronchial asthma and dermatitis among the castor oil factory workers.

Castor oil is cheaply priced and is used as an adulterant in many common edible oils. Research on detection of castor oil started as early as 1951 (Achaya and Saletore, 1951) by estimating the hydroxyl value and the refractive index of acetylated oils, which is quite low in the presence of castor oil. Colorimetric testing (Anselmi *et al.*, 1959) as well as turbidity tests (Rajnish, 1963; Lakshminarayana and Mani, 1964; Lakshminarayana, 1968) are reported, but are said to be unsatisfactory. A thin layer chromatography (TLC) method is quite effective (Lakshminarayana and Mani, 1964), but is less useful when the oil under investigation is highly oxidized (Nasirullah *et al.*, 1982). However, TLC of the alcohol extract or after methanolysis affords a simple, quick method of detecting adulteration with castor oils (Adhikari and Adhikari Dasgupta, 1982).

6.4.1.6 Nightshade

Its presence as a contaminant in soybeans is difficult to explain since it is a cultivated plant and does not occur in the wild in the American Subcontinent. The toxic principles are atropine and scopolamine.

6.4.1.7 Jimsonweed

This is known by a number of names such as thornapple, apple of Peru, tolguacha and Jamestown weed, which is the most common. The toxic compounds are atropine and scopolamine as in nightshade. Jimsonweed seeds are small compared to soybean, and are generally removed by mechanical screening in the processing line. If the weed does get into cracking rolls for oil extraction, the alkaloids are concentrated in the meal; the alkaloids in the oil are removed during caustic refining wherein they are hydrolysed to water soluble tropic acid and tropine (Holmes, 1950; Polesuk and Ma, 1973) and

subsequently removed during washing. However, since the oilseed meals from soybean are used as live stock feed and to prepare high quality protein products for human food, the effect of processing conditions such as toasting on jimsonweed alkaloids needs to be investigated.

6.4.1.8 Contamination with karanja oil

Karanja (*Pongamia glabra*, Syn. *Pongamia pinnata* of the family leguminosae) oil is one of the most abundantly available non-edible oils in India (KVIC, 1974) and resembles groundnut oil in composition. The presence of a number of toxic flavonoids, identified as karanjin, karanjone, pongapin, pongaglabrone and karanjachrome, and phenolic compounds have prevented edible uses of this oil (Parmar *et al.*, 1976). Techniques for the removal of these substances have been reported (Mandal *et al.*, 1984, 1985), and karanja oil lends itself admirably to admixture with other vegetable oils. This contaminant oil has been detected by the intense yellow colour with antimony trichloride (Thirumala Rao *et al.*, 1969), a bright orange colour with a drop of sulphuric acid (ISI, 1976) and by TLC methods (Srinivasulu *et al.*, 1976; Rao *et al.*, 1977). The colour tests are not specific since a number of compounds such as alkaloids and carotenoids also give the colour test. A rapid colour test has been recently reported which can detect contamination of vegetable oils with karanja oil, either raw or refined and bleached (Mandal *et al.*, 1988). This test is based on the development of the intense orange to bright red colour of karanja oil flavonoids with phosphoric acid, and has a detection limit of 1% . The test can be easily adopted by any laboratory and requires a very small size of the sample. Detection up to 0.01% is also possible by spotting the unsaponifiable matter on a TLC plate using 60:40:1 v/v of petroleum ether:diethyl ether:acetic acid for development and looking for three bluish green spots of R_f 0.34, 0.22 and 0.17, characteristic of karanja alkaloids under UV light (Nasirullah *et al.*, 1992).

6.4.1.9 Contamination with argemone oil

Analysis of 181 samples of rape and mustard collected from wholesale markets in North India has revealed a minimal contamination with argemone oil. However, a report of epidemic dropsy from Andhra Pradesh in India has shown large scale adulteration of edible oils with argemone oil (Krishnamachari and Satyanarayana, 1972), a fact that has been confirmed by the presence of sanguinarine in the urine of the affected patients (Shenolikar *et al.*, 1974). It is also suggested that the permissible level of argemone oil should be fixed at $\leqslant 0.011\%$ (Kaul and Ahuja, 1980).

Adulteration of edible oils with argemone oil is a health hazard, it being implicated in epidemic dropsy (Patwardhan, 1962). An alkaloid, sanguinarine which belongs to the isoquinoline group is present in the oil and is shown to be related etiologically to the toxic manifestations (Sarkar, 1948). Argemone seed is similar in size and colour to

mustard seed. Argemone oil at 0.01% in other edible oils can be detected by separation in ether and extraction in 1:1 HCl followed by treatment with concentrated nitric acid. The orange yellow colour obtained due to the alkaloid is characteristic of argemone and aids its detection (Bose *et al.*, 1969). Microscopic observation of orange red needle shaped crystals which are formed on treatment with ferric chloride can also be used to identify >0.02% argemone oil in other oils (Bose *et al.*, 1970; Prakash *et al.*, 1947). Simple spot tests (Bose, 1974) and micro tests (Chakravarti *et al.*, 1959) based on this reaction are also reported. Extraction of the oil in concentrated hydrochloric acid and further reaction with KBr-$KBrO_3$ solution after letting it stand for 2 h produces an orange red colour, the intensity of which increases with increasing concentration of argemone oil. At concentrations higher than 0.75% of argemone oil, the tint of the colour is too deep for direct matching and the sample should be diluted with pure argemone-free mustard oil (Mitra *et al.*, 1952). The picrate crystals of the alkaloid are practically insoluble in petroleum ether and very slightly soluble in ether. This test has been considered for quantitative determination. The proportionality of the amount of precipitate of picrate crystals with argemone oil content has been demonstrated (Sarkar and Rahman, 1945).

Paper chromatographic (Chakravarti and Chaudhuri, 1955; Nataraja Sarma and Nithyanandan, 1967) or TLC separation of the alkaloid, followed by viewing in UV light (365 nm) enables detection of argemone oil in mustard oil (Sen, 1946; Dhar and Suri, 1974). Solvent systems found best for this chromatographic separation on silica gel G plates are a 4:1 mixture of heptane:acetone and a 2:2:1 mixture of chloroform:benzene:dioxane (Bose, 1970). In another modification, addition of 2 ml oil to 1 ml of concentrated hydrochloric acid and 0.5 ml of ethanol, followed by heating in a boiling water bath for 10 min detects argemone oil at the 0.25% level in mustard oil as a pinkish fluorescence in the lower acid layer when viewed under UV light (Sarkar and Nandi, 1951). Another method for detection in mustard oil is based on the spectrophotometric identification of argemone alkaloids, which show maxima in the UV region at 236, 276 and 326 nm. Quantitative estimation of these alkaloids can be carried out by measuring the absorbance at 326 nm (Pundlik and Meghal, 1977). An HPLC method to determine argemone oil in edible oils is also known (Murthi *et al.*, 1988).

In addition to sanguinarine, various reports have indicated the presence of an unusual fatty acid in argemone oil (Rukmini, 1971; Mani and Lakshminarayana, 1972), identified as (+)6-hydroxy-6-methyl-9-oxooctacosanoic acid and designated as argemonic acid (Rukmini, 1975). This can be used as an indicator of argemone oil in edible oils, although an experimental procedure needs to be developed.

6.4.1.10 Contamination with jatropha oil

Jatropha is a tropical weed of wider occurrence in India. Seeds of *Jatropha curcas* L. resemble castor and contain about 46–58% of a fixed oil. The toxic constituent has

been identified as curcin. This oil gives a characteristic UV absorbance at 259 nm and can be detected even at 0.5% level in other oils (Neelakantan and Manimegalai, 1977).

6.4.1.11 Contamination with kusum oil

Oil obtained from *Schleichera trijuga*, commonly known as kusum oil can be detected in rapeseed or mustard oil by (i) the Cu acetate/benzidine acetate method, (ii) the picric acid method, being sensitive enough to detect 0.1% kusum oil in edible oils and (iii) the ferric chloride method, which fails to detect kusum oil at <0.5%level. The picric acid method is known to give the most reliable results, but it requires more sample than the copper acetate/benzidine acetate method (Mukhopadhyaya and Bhattacharya, 1982). Toxicity of this oil is attributed to the presence of a cyanolipid in the seeds.

6.4.1.12 Contamination with taramira oil

Taramira, Jamba rape (*Eruca sativa Mill.*) grows abundantly in northern states of India, Pakistan, Bulgaria and USA. It belongs to the Eruca genus of the Cruciferae family and is rich in erucic acid (37–46%). The pungency of taramira oil resembles that of mustard/rapeseed oil and is also frequently associated with these oils. The expressed oil yields about 1% volatile oil on steam distillation. It is released by the action of myrosinase on the thioglycosides present in the seeds. It has been observed that taramira oil contains some volatile sulphur compounds other than allyl isothiocyanate and thus differs in its volatile composition from mustard/rapeseed oil. A TLC method, sensitive enough to detect the presence of taramira oils at a level as low as 2% has been developed, and is based on the appearance of greenish brown spots in the chromatogram (Grover *et al.*, 1978). Petroselinic acid, an isomer of the more common palmitoleic acid occurs to the extent of 76% of the total acids in seeds of parsley *Petroselinum sativum* (Harbourne, 1973). It is also present in many other seeds of Umbelliferae, and could be an important indicator of adulteration with parsley seed oil, if it is practised at all.

6.4.1.13 Other contaminants of edible oils

The ambrettolide ketone in ambadi or *Hibiscus cannabinus*, theasin in tea seed or *Camellia sinensis*, linamarin and phaseolunatin in rubber seed or *Hevea brasilensis* are all toxic constituents in the respective seeds which are often found as contaminants in edible vegetable oils. These can be detected by physical, chemical, colorimetric, chromatographic and spectroscopic methods. While the presence of the contaminant oil may not be detected in each sample, a combination of techniques is proved to establish the presence or absence of a particular oil (Nasirullah and Nagaraja, 1987).

Triglycerides of *Caloncoba echinata* and *Hydnocarpus anthelminthica* of the family

Flacourtiaceae, commonly known as chaulmoogric oils contain cyclopentyl fatty acids which aid in their detection by HPLC. Chaulmoogric oils are poisonous when ingested (Shukla and Spener, 1985). The Flacourtiaceae oils also exhibit exceptional dextrorotatory powers owing to their constituent cyclopentyl fatty acids (Spener and Mangold, 1974; Spener and Tober, 1981), and could be indicative of its presence.

6.4.2 Contamination due to faulty storage

In mills producing vegetable oils from oil seeds it is necessary from time to time to examine samples of lubricating oil taken from the expellers for possible contamination by vegetable oil. This provides useful information on the amount of wear sustained by the expeller bearings. Logically, the method used to detect this contamination would also apply to accidental contamination of vegetable oils by mineral oils. Traditionally the estimation of such contamination was effected by determining the saponification values of the lubricating oil samples. This is a time consuming method. A new method has been developed to overcome this drawback. A convenient volume, say 5 ml, of the lubricating oil sample is placed in a stoppered measuring cylinder followed by four volumes of dioxane, containing 30.6% (v/v) furfural. The cylinder is then shaken vigorously and allowed to stand until an upper layer of lubricating oil (sparingly soluble in, and lighter than, the dioxane/furfural) has completely separated, the vegetable oil being freely soluble in the dioxane/furfural. This settling may take an hour or so and in some cases it may be necessary to centrifuge for about 10 min in a graduated centrifuge tube, or better to carry out the whole operation in such a tube. The volume of the undissolved oil is noted against a strong light in case of dark oils. The difference between the volume of the oil sample taken initially and the final volume of the undissolved lubricating oil represents the degree of contamination by vegetable oil. The results can be suitably interpreted to detect mineral oil contamination in vegetable oils (Farmer, 1975).

Mineral oils of various thickness and refractive indices have been reported to be used as adulterants in edible oils and fats, in particular coconut oil. These can be detected by Holde's test, which measures the turbidity of a saponified oil sample after addition of 50% alcohol (Venkatachalam and Sundaram, 1957).

Gas chromatograms of lubricating oils or fractions that have weathered are characterized by the presence of a 'hump' which cannot be resolved by gas chromatography into individual peaks, and is referred to as unresolved complex mixture. Contamination by mineral oil can be easily recognized by the presence of this hump in the gas chromatogram (McGill *et al.*, 1987; Parker *et al.*, 1990; Grob *et al.*, 1991a, 1991b). n-Alkanes are indigenous to the oil and are characteristic of specific plant oils. A markedly different alkane pattern, as has been demonstrated in retail samples of edible oils (McGill *et al.*, 1993) can be indicative of petroleum-based contaminants.

6.4.3 Spanish Toxic Oil Syndrome

Epidemiological data clearly indicate an association between the consumption of industrial rapeseed oil adulterated with aniline and the subsequent development of acute pulmonary and chronic neuromuscular disorders in Spanish Toxic Oil Syndrome (Grandjean and Tarkowski, 1984; World Health Organization, 1985, 1992; Posada *et al.*, 1987; Kyrklund, 1993). It is estimated that about 50 000–100 000 people consumed the poisoned oil which affected over 20 000 people and has caused many deaths (Pestana and Munoz, 1982).

The contaminated cooking oils have been examined for industrial chemicals, pesticides and trace chemicals. Elevated levels of chlorine were found up to 440 ppm by neutron activation analysis (NAA). Fatty acid anilides in several suspected oils were discovered independently by Tabuenca (1981) and Diachenko *et al.* (1982) at levels as high as 2400 ppm. Gas chromatography with an electron capture detector (ECD) is a sensitive technique for analysing these anilides. The procedure involves alkaline hydrolysis of anilides, the isolation of aniline by extraction with isooctane in a Bleidner distillation/extraction head followed by bromination of the aniline to the 2,4,6-tribromo derivative. The ECD detector is very sensitive to bromoaniline compounds and enables the detection of an addition of even small amounts of the contaminated oils to edible oils (Jeuring *et al.*, 1982). While the causative agents remain unknown, it has been shown that most of these oils comprise mainly low-erucic acid rapeseed oil which is severely contaminated with reaction products of aniline, especially fatty acid anilides, and several other compounds in smaller quantities, and which has been blended with other vegetable oils and animal fats (Arribas Jimeno, 1982; Grandjean and Tarkowski, 1984; Vazquez-Roncero *et al.*, 1983a, 1983b; Ventura Diaz, 1982; Vioque and Ventura, 1984). Since the clinical symptoms of the toxic oil syndrome (TOS) are not those of aniline toxicity, it has been proposed that the aetiological agent could be a contaminant in the aniline, introduced during transportation or a reaction between normal oil components or materials used in the refining with either aniline or contaminants (Paz *et al.*, 1991). For instance, fatty acid diester of 1,2-propanediol-3-aminophenyl, is believed to be formed by the reaction of aniline and free diglycerides.

Since fatty acid anilides were detected in very high concentrations in these oils, most analytical and toxicological research has focused on these agents (Aldridge and Connors, 1982; Pestana and Munoz, 1982; Vioque and Vioque, 1982; Grandjean and Tarkowski, 1984; Tucker and Cunningham, 1986; World Health Organization, 1985). However, fatty acid anilides as well as case-related oils have failed to reproduce the complex picture of TOS in experimental animals.

On the basis of their structural resemblances to autoimmunity inducing drugs, it has been hypothesized that phenylthiourea derivatives may have been formed in Spanish toxic oil (Kamuller and Seinen, 1986), and these compounds represent possible aetiological factors in TOS. Plants and seeds of the Cruciferae family, such as rape, turnip, cabbage, brussels sprouts, mustard, etc. are rich in glucosinolates (Fenwick *et*

al., 1983). Since toxic oil samples mainly contain rapeseed oil, naturally occurring glucosinolate-derived isothiocyanates may have reacted with the denaturant aniline to form phenylthiourea compounds. Although this possibility has been mentioned, a causative role for these agents in the pathogenesis of TOS has not been considered (Food and Chemical News, 1981; Viayna Roca, 1982; Koch, 1984). The rapeseed oil glucosinolate, progoitrin is postulated to give a breakdown product, 2-hydroxy-3-butenylisothiocyanate, which in turn reacts with aniline to form *N*-(2-hydroxy-3-butenyl)-*N'*-phenylthiourea (HBPTU), and that cyclization would yield 1-phenyl-5-vinyl-2-imidazolidinethione (PVIZT) (Kammuller *et al.*, 1986; 1987). PVIZT has been chemically synthesized and characterized with the aim of understanding the compounds involved in TOS (Kammuller *et al.*, 1988).

Gardner *et al.* (1983) have reported the isolation and identification of C_{16} and C_{18} fatty acid monesters and diesters of chlorpropanediol in three samples of toxic Spanish cooking oil having the highest levels of cooking oils. Based on the reports that monoesters and diesters of chlorpropanediol can be produced by hydrolysis of the triglycerides with hydrochloric acid at 110 °C (Davidek *et al.*, 1980), the discovery of similar compounds in some samples of Spanish toxic oils suggests that these oils, or portions thereof, were exposed to hydrochloric acid during the refining process. The implication of the presence of these chloropropane mono- and diesters in the Spanish toxic oil syndrome is not known and needs to be investigated.

6.4.4 Contamination due to tricresyl phosphate

Tricresyl phosphate (TCP), also known as tritolyl phosphate, lindol, celluflex or crotinex is a flame resistant oily liquid finding use in several industries. TCP is a mixture of the *ortho* and *para* isomers, the proportion of the toxic *ortho* isomer being usually low. TCP is a lethal poison if taken internally and may cause nausea, vomiting and diarrhoea. Polyneuritis leading to paralysis of extremities has also been reported in severe poisoning cases (Merck Index, 1968). Shah *et al.* (1960) have reported many cases of polyneuritis attributed to contamination of edible oils with TCP. The first dramatic outbreak of 'ginger jake paralysis' was reported in the 1930s in the USA (Morgan and Penovich, 1978). Since then several other epidemics have occurred elsewhere, the outbreaks in Durban (Susser and Stein, 1957), Morocco (Smith and Spalding, 1959) and Bombay (Vora *et al.*, 1962) being some of the worse. An outbreak of acute polyneuropathy, which was restricted to girls attaining menarche and to women after childbirth in Sri Lanka has been traced to tricresyl phosphate found as a contaminant in sesame oil. The contamination was believed to have occurred during transport of the oil in containers previously used for storing mineral oils (Senanayake and Jeyaratnam, 1981).

The methods available for the identification and estimation of TCP are based on chromatographic or distillation techniques using various solvents and spraying agents for phenol (Collins, 1945; Copius-Peereboom, 1960; Chakravarti *et al.*, 1973; Sengupta

et al., 1973) as well as for phosphate after hydrolysis of TCP (Patel *et al.*, 1962). TCP shows an absorption maximum in the ultraviolet region at 262 nm, where it obeys Beer's law, and can be used as a means of quantifying TCP in edible oils (Pundlik and Meghal, 1974).

6.5 Indices of admixtures, blends, contaminants and adulterants—one fat in another

Fraudulent practices common in the international oils and fats trade range from the breach of a signed contract to dishonesty in selling cheaper oils in place of expensive ones (Shave, 1988). A survey of olive oil products available in 1982 in the USA demonstrated that many products contained undeclared esterified olive oil as well as undeclared olive husk oil and seed oils. These findings led to a Food and Drug Administration (FDA) regulatory programme initiated in 1982 to control olive oil adulteration and mislabelling. Surveys conducted by the FDA in 1983–1984 and 1985–1986 showed an overall improvement in labelling of olive oil products. In 1983–1984 17 out of 20 samples were mislabelled, but only seven out of 26 in 1985–1986. Tighter controls by exporting countries and continued surveillance in the USA were believed to be necessary to eliminate such adulteration (Firestone, 1987). Work relating to a vegetable oil authenticity project carried out at the Leatherhead Food Research Association has been reviewed (Rossell, 1991). In particular, stable carbon isotope ratio analysis (SCIRA) is seen as a very promising new technique for establishing the authenticity of oils. Another interesting approach is the use of Fourier transform infrared (FTIR) spectroscopy for authenticity of extra virgin olive oil (Lai *et al.*, 1995). The problems associated with identification of adulterated oils are complicated by the ever changing nature of the adulteration techniques, and the number of procedures that can pass undetected through official quality control (Grob and Romann, 1993). Data presented on chemical analysis of oilseeds and associated impurities support a discussion of the way different impurities find their way into the oils. In particular, it is noted that the oil and free fatty acid contents of shell and fines should be carefully taken into account (Kershaw, 1986).

Edible oils may be blended in order to prepare suitable products, using raw materials which vary in cost and availability. In order to monitor trade in edible oils, it is important that good analytical procedures are available to detect and identify oils used in blends. Blends in proportions other than that indicated on the label amount to adulteration in all parts of the world. The basic concepts in detecting adulteration and determining the composition of a blend are similar and are discussed here.

6.5.1 Admixture of vegetable oils with other vegetable oils

The biosynthesis of fat in each vegetable and animal organism is species specific and detection of adulteration can be based on the basis of various constituents in the fat admixture.

6.5.1.1 Fatty acid composition

There are some oils that due to their fatty acid composition can be added to other oils without being detected by the routine physical and chemical characteristics. In such cases, gas liquid chromatography (GLC) analysis of fatty acids proves useful. For instance, the presence of babasu (*Orbignya speciosa*) and soyabean oils in olive oil can be detected by the presence of caprylic, capric, lauric and myristic acids and the relative composition of other fatty acids (Badoloto *et al.*, 1981). Malva oil as an adulterant in commercial oils can be detected at $\geqslant$10% by the presence of cylic acids (maximum 1.26%) containing the cyclopropane ring and by characteristic presence of Δ-sterols (Lercker *et al.*, 1983). Groundnut oil adulterated with 10% cottonseed or 5% kapok seed oils may be detected by GLC with glass capillary columns on the basis of presence of cyclopropenoic fatty acids (Spanish Standard, 1973a) present in the adulterant fats (Bianchini *et al.*, 1981). The amounts of cyclopropenoic fatty acids are relatively small in cottonseed oils (Badami *et al.*, 1973), but are more abundant in kapok seed oils which are also used as edible oils.

The cyclopropene fatty acids, namely sterculic (9,10-methylene-9-octadecenoic) and malvalic (8,9-methylene-8-heptadecenoic) acids that occur in lipids of cottonseed and other species of the order Malvales are responsible for a number of adverse biological effects consequent to dietary consumption. These are also unstable thermally and this complicates their analysis. Raman spectroscopy, which takes advantage of an isolated strong band at 1870 cm^{-1} associated with the cyclopropene double bond provides a direct method for determination of these cyclopropenoids as components of lipid mixtures at levels down to 0.03%. Potential contamination of vegetable oils intended for consumption can thus be monitored for these deleterious substances without the uncertainty of the chemical methods (Kint *et al.*, 1981). An added advantage is that no derivatization or chemical treatment is required.

Acetylinic acids, for example stearolic acid, are present in certain Malvales, where they occur in close association with cyclopropene fatty acids (Bu'Lock, 1966). However, this compound has not been used as an indicator for detecting adulteration of edible oils with seed oils of Malvales and deserves attention from food analysts.

Mustard oil in groundnut oil can be determined in terms of erucic acid, when methyl esters of fatty acids are prepared and chromatographed on TLC using silica gel G plates (Kaimal *et al.*, 1974). Fatty acids and the melting point of the glycerides in edible fats such as hydrogenated soybean oil are of value in detecting adulteration (Karathanassis, 1973).

The iodine value and butyrorefractometer reading help to indicate some adulterations like linseed oil in edible rapeseed oil (Manandhar *et al.*, 1986a). The degree of unsaturation in fats and oils can also be calculated from the net absorbance at 3007 cm^{-1} and the area under the peak. It shows a good correlation with iodine value ($r = 0.9992$) (Muniategui *et al.*, 1992).

Coconut oil is the highest priced vegetable oil in many countries and adulteration

with other oils is often practised. Coconut oil differs from most other oils in its high content of short chain fatty acids and low unsaturated fatty acids. Determination of saponification and iodine values is therefore usually adequate to detect gross and simple adulteration. However, adroit admixture with various adulterants calls for a number of analytical determinations and specific tests. A rapid one step method, based on silver nitrate–silica gel thin layer chromatography can be used as an alternative or confirmatory test to detect the adulteration of coconut oil. Silver nitrate forms π-complexes with the double bonds of fatty acids in the glycerides. The greater the number of such double bonds, the stronger is the complex formation and the slower the migration. This method can successfully detect adulteration of coconut oil with 5% or more of common commercial vegetable oils. There is no interference from free fatty acids added to the extent of 1% or by the peroxidation products (up to a peroxide value of 5). It is particularly promising in the analysis of a large number of samples (Mani and Lakshinarayana, 1965).

Analysis of methyl esters of fatty acids by GC and evaluation to obtain the ratio of C_{22}:C_{16} acids permits detection of 5% rapeseed oil in peanut oil, the ratios being 0.21:0.35 and 13.2:14.3 in peanut and rapeseed respectively. Addition of coconut or palm kernel oil to butter can also be detected by this technique (Wolff and Wolff, 1960). The presence of linolenic acid is recommended as a criterion for detection of 5% grapeseed oil or <5% sunflower oil in olive oil (Gracian and Martel, 1974; Martel, 1977), which is not possible by other analytical methods (Petruccioli, 1959). Fatty acid methyl ester analysis is shown to be useful for examination of olive oil adulteration with other vegetable oils like sunflower, corn and soybean (Kyriakidis and Dionysopoulos, 1983). GLC determination of linolenic acid can also be used to detect adulteration of other vegetable oils like olive, almond, rice, groundnut (Kaimal *et al.*, 1974), corn, cottonseed (Tsatsaronis and Bostov, 1972) and sunflower (0.00–0.45%) with soybean oil (Vidal *et al.*, 1979). GLC analysis of stearic and linoleic acids is of value in detecting adulterant linseed oil in rapeseed oil (Manandhar *et al.*, 1986a). Adulteration of olive oil with very low levels (1–2%) of linoleic acid-rich oils can be unequivocally detected by reversed phase HPLC (Kapoulas and Andrikopoulos, 1986). High linoleic acid contents in pure olive oil shortenings indicate the presence of other hardened oils. Palm kernel and coconut oils in adulterated cocoa butter are identified by the high lauric and myristic acid content, and cottonseed oil in olive oil is identified by differences in $C_{16:0}$ + $C_{18:2}$ content and the ratio of $C_{18:1}$:$C_{18:2}$ (Tsatsaronis and Bostov, 1972). Adulteration of edible soybean oil by linseed oil is manifested as decreased linoleic and increased linolenic acid, and could be used as indices of this adulteration (Manandhar *et al.*, 1986b). Adulteration of pumpkin seed oil by rapeseed oil can be determined by erucic acid content even at 1%, soybean or linseed oil can be determined by linolenic acid, and sunflower oil by behenic and lignoceric acid (Gorbach and Weber, 1963). Multivariate statistical techniques like principal component analysis, discriminant analysis and hierarchical clustering on a reduced set of variables based on the content of palmitic, stearic, oleic and linolenic acids from

eight different plant oils could serve for assessment of oil samples (Schwaiger and Vojir, 1994).

Semi-drying oils are sometimes used as adulterants of olive and olive foots oils. Fractional crystallization of the oil using methanol:acetone (7:3) solvent at −22 °C followed by purification by TLC and analysis of fatty acid composition by GLC of methyl esters is a useful analytical tool. The ratio of linoleic acid concentration in this fraction to that in the original oil is an index of semi-drying oil content. The sensitivity of detection is 1–5% semi-drying oil in olive oil (Spanish Standard, 1984).

Treatment of the seed oils with pancreatic lipase and separation of the 2-monoglycerides followed by analysis of the methyl ester by GC to give the amount of saturated fatty acids in the 2-position of the glycerol is known to vary within narrow limits and can be used to estimate the quality and purity of an oil (Tiscornia and Bertini, 1970; Rossell *et al.*, 1983) such as sunflower (Prevot, 1987), and adulteration of genuine olive oils with synthetic oil (Taponeco and Ghimenti, 1973).

IR spectra between 2.80 μm and 3.30 μm, and between 8.50 μm and 13.0 μm can distinguish between oils of peanut, sesame, sunflower, first pressed olive oil and refined solvent extracted olive oil and synthetic oil. Triolein, synthetic oil and solvent extracted olive oil have a strong absorption at 2.90 μm, while other oils have a low absorption. Triolein has a strong band at 10.35μm. Peanut oil has a characteristic band at 10.95μm, sunflower at 10.95μm and 11.8μm, and sesame at 10.95μm and 12.32 μm. These differences are of value in detecting oil admixtures and adulterations (Bottini and Sapetti, 1958). IR spectra between 4000 and 850 cm^{-1} have shown differences between olive oil and its adulterant rapeseed oil. Differences have been noted at 3100 cm, 1750 cm^{-1} and 1400–1300 cm^{-1}, the most striking feature of the differential spectrum being the negative peaks at 1130 cm^{-1} and 1080 cm^{-1} with a characteristic contour from 1200–900 cm^{-1}. These characteristics persist in mixtures containing as little as 10% rapeseed oil, enabling its detection in olive oil. These characteristics are attributed to the differences in the unsaturated fatty acids, particularly oleic, linoleic and linolenic acids. Erucic acid in mustard and rapeseed oil having a double bond at C_{13} has only 0.5 times the influence of oleic acid. In contrast, linoleic and linolenic acids have about 1.5 times the influence of oleic acid. A plot of peak heights at 1130 cm^{-1} and 1112 cm^{-1} against % oleic + 1.5% linoleic + 1.5 times % linolenic + 0.5% erucic acids correlates with the degree of unsaturation in the oil. A positive peak at 1050 cm^{-1} is indicative of hydroxy fatty acids, while that at 1065 cm^{-1} is indicative of fats with short chain length fatty acids or a low iodine number. Similarly, the peak at 940 cm^{-1} is attributed to the carboxylic group of the free fatty acids. Refining, but not hydrogenation causes a slight change in the IR spectrum (Bartlet and Mahon, 1958). The metal derivatives, particularly sodium, barium and lead salts of fatty acids have been suggested as a basis for infrared spectrum. It has been shown to be feasible to detect olive oil adulterated with peanut oil. Spectra for lead salts of five saturated fatty acids having even numbers from C_{16} to C_{24} show definite bands with good resolution and offer an elegant method for detecting such

frauds (Gelli and Pallotta, 1959).

The application of urea to the separation of saturated from unsaturated fatty acids has been explored by a number of workers (Narayan and Kulkarni, 1954). The formation of the complex is affected by the temperature of the reaction mixture, the duration of the crystallization period and the concentration of urea and the material being fractionated (Zimmerschied *et al.*, 1950). The tendancy of urea to form crystalline complexes with fatty acids on the basis of chain length and unsaturation has been used to detect adulteration of mustard oil with groundnut oil and linseed oil (Mehta *et al.*, 1956), and of coconut and sesame oils with groundnut oil (Mehta and Gokhale, 1965). To exemplify the efficacy of the method, Table 6.6 shows data obtained by urea fractionation studies on coconut oil adulterated with groundnut oil. As the proportion of coconut oil in the mixture increases, the acid value of the fatty acids increases and their iodine value declines. This is because the proportion of lauric acid in this fraction increases.

Ultraviolet spectroscopy is an important tool in determining polyunsaturated constituents of fats and oils. Unconjugated unsaturated fatty acids can be determined by catalytic isomerization into their conjugated, UV absorbing forms by means of alkali and heat. This technique provides a rapid method for determining soybean oil in cottonseed and other oils which contain little or no linolenic acid. It can also detect adulteration of ground beef, pork or lamb with horse meat, since most animal fats contain tetraenoic acids which are not generally present in vegetable fats. The technique could also be useful for distinguishing between animal and vegetable fat (Firestone, 1954).

6.5.1.2 Triglyceride analysis

Detection of canola oil (low erucic rapeseed oil) as an adulterant in olive oil is difficult due to the similarity in the fatty acid composition. The absence of those of equivalent carbon number (ECN) 40 in olive oil (Damiani and Burini, 1980), and the presence of those with ECN 42 and small amounts with ECN 40 in canola oil has formed the basis of detection of this adulteration. The ECN ratio 46:44 has been found to be useful in detecting canola oil in olive oil, a value less than 3.9 being particularly indicative (Salivaras and McCurdy, 1992). A major difficulty lies in the continuing genetic modifications that canola oil is presently undergoing. For example, 'low-linoleic' spring variety has lower percentages with ECNs of 40 and 42, and increased triacylglycerols with ECNs of 44 and 46 (Prevot *et al.*, 1990). This introduces difficulties in the detection of canola oil in olive oil. Indices obtained on the basis of polyunsaturated triglycerides can detect olive oil products adulterated with hazelnut or esterified oils (Casadei, 1987). Adulteration of edible oils and fats by non-generic fats and oils, for example, olive oil by soybean or sunflower can bedetected using HPLC with a light scattering detector (Palmer and Palmer, 1989) or differential refractometry for determination of polyunsaturatedtriglycerides. Pure olive oil contains no dilinoleyl

Table 6.6 Adulteration of coconut oil with groundnut oil

No.	1	2	3	4	5	6	7
% Groundnut oil in 5 g mixture	0	20	40	50	60	80	100
Saponification value of the blend	256.2	249.0	238.6	232.6	218.1	–	196.0
Wt of adduct (g)	16.3	16.1	15.5	14.2	14.0	12.8	12.7
Iodine value of fatty acids	6.3	17.1	35.0	44.6	65.5	70.1	90.2
Neutralization value of fatty acids	270	268	246.5	240	233	218.1	194.0

Source: Mehta and Gokhale, 1965 (reproduced with permission).

linolenate (vs. 10% in soybean oil) or trilinoleate (vs. 29.6% in soybean oil and 43.6% in sunflower oil). The sensitivity of detection is 1–5% of these oils in Moroccan olive oil (Fellat-Zarrouck *et al.*, 1988). The separation of glycerides by paper chromatography for detecting adulteration in fats has long been known (Priori, 1956). Attempts to detect common adulterants like groundnut, argemone and linseed oils in mustard oil have been made using reverse phase thin layer chromatography. Separation of the glycerides on silica gel plates using plaster of paris as the binder and acetone:methanol (85:15) as the developing solvent gives characteristic spots. The number of spots and their R_f values are given in Table 6.7 (Chakrabarty *et al.*, 1963). Since each pure oil would yield a definite number of discrete spots, any mixtures can easily be detected particularly when more unsaturated glycerides which have higher mobility are present.

A semidrying oil in olive oil can be detected as precipitated brominated polyunsaturated glycerides since olive oil is practically free from polyunsaturated glycerides. This method is generally not applicable to non-refined oils (Spanish Standard, 1973b). Triglycerides with carbon numbers such as 58, 60 and 62 are peculiar to rapeseed oil (Imai *et al.*, 1974).

Another approach consists of fractionating the triglycerides by dissolving in suitable solvents and reprecipitating them, followed by determining the iodine value in the individual fractions. This method has been shown to be suitable for judging the authenticity of various vegetable oils, and in particular olive oil (Mangio, 1960).

6.5.1.3 Unsaponifiable fraction of oil

In certain cases, analysis of fatty acids does not give a clear indication of adulteration. For instance, adulteration of olive oil by olein prepared from the fat of slaughtered animals is rather difficult to detect as the oil characteristics are quite similar

Table 6.7 Thin layer chromatography of pure and adulterated samples of mustard oil[a]

Adulterant	Quantity of the adulterant added (%)	No. of spots	R_f value of the spot[b] (×100)										
			1	2	3	4	5	6	7	8	9	10	11
Nil	–	6	18	22	28	38	47	54	–	–	–	–	–
Groundnut oil	5	7	17	21	28	36	43	52	60	–	–	–	
	10	7	17	21	28	36	43	52	60	–	–	–	
Argemone oil	5	8	18	24	32	41	50	56	65	70	–	–	–
	10	8	18	24	33	42	50	56	63	–	72	–	–
Linseed oil	5	10	19	22	28	38	47	54	71	79	87	96	–
	10	11	19	22	28	38	47	54	68	79	87	96	98

[a]Developing solvent, acetone:methanol (85:15); amount of sample taken, 75 μg; stationary phase, liquid paraffin BP (BDH) and indicator, iodine vapour
[b]Spots serially numbered from the baseline.
Source: Chakarabarty *et al.*, 1963 (reproduced with permission).

(Bortolomeo, 1953). In such cases, the unsaponifiable fraction gives a clue. Unsaponifiable matter varies greatly in olive oils from different geographical sources (Gracian Tous and Martel, 1960) and could probably be used as a criterion to distinguish between them. The hydroxyl number of the unsaponifiable matter of pressed olive oil is consistently lower than that of oils extracted from the residue, and also of most other seed oils. These values can be used for distinguishing the pressed from extracted olive oils and for differentiation of olive oil from other oils (Gracian Tous and Martel, 1960). Simple data processing systems based on the determination of unsaponifiable components and suitable for routine analysis for quantifying blends of vegetable oils have been reported (Abou-Hadeed *et al.*, 1990).

Studies on the unsaponifiable fractions of soybean, sunflower (Prevot, 1987), cottonseed, coconut, olive and avocado have shown differences in the contents of total unsaponifiables, squalene, tocopherols and sterols and also in the composition of tocopherol and sterol fractions (Rossell *et al.*, 1983). The presence or absence of individual unsaponifiable components can help in establishing the identity of each oil and also in detection of admixture with another oil (Gutfinger and Letan, 1974a). Table 6.8 shows unsaponifiable components useful for characterization of several vegetable oils, Table 6.9 shows the content of unsaponifiables, phosphatides and squalene in some seed oils, Table 6.10 shows the sterols in various vegetable oils, and Table 6.11 shows the tocopherols in several vegetable oils, all these tables summarizing the usefulness of these determinations in detecting oil admixtures.

A combination of fatty acid methyl ester analysis, and sterol and tocopherol analyses is adequate for some blends (Van Niekerk and Burger, 1985). These have been used to optimize a simple procedure which can detect blends of sunflower seed oil, groundnut oil, cottonseed oil, maize oil, olive oil and palm oil (Van Niekerk and Hasty, 1989). Methods based on analysis of fatty acid methyl esters, 4-methylsterols, triterpene alcohols, tocopherols and squalene analysis have also been developed (Abou-Hadeed and Kotb, 1988).

Table 6.8 Unsaponifiable components useful for characterization of some vegetable oils

Oil	Type of components		
	Presence[a]	Absence	Traces
Soybean	Tocopherols: $\gamma > \delta > \alpha$ Campesterol ~ 20% Stigmasterol ~ 20%		
Cottonseed	α -tocopherol ~ 50% γ -Tocopherol ~ 50%	δ-Tocopherol Stimasterol	
Coconut	Campesterol ~ 17% Stigmasterol ~ 7%		Squalene Tocopherols
Olive, flesh	High content of squalene[b] α -Tocopherol ~ 90% β -Sitosterol ~ 96%	δ -Tocopherol	γ-tocopherol Stigmasterol
Avocado, flesh	High content of unsaponifiables[c] α -Tocopherol ~ 100%	γ -Tocopherol δ-Tocopherol	Stigmasterol

[a]Percents indicate the component's level in the tocopherol or sterol fraction.
[b]Over 1000 ug/g^{-1} oil.
[c]Over 40 mg/g^{-1} oil.
Source: Gutfinger and Letan, 1974a (reproduced with permission).

Table 6.9 Oil content in some fruits and seeds and content of unsaponifiables, phosphatides and squalene in oils

	Oil content in fruit or seed (%)	Unsaponifiables in oil (%)	Phosphatides in oil (%)	Squalene in oil ($\mu g\ g^{-1}$ oil)
Soybean	19.2–19.6	1.5–1.7	1.1–3.2	123–143
Cottonseed	28.2	1.2	0.7–0.9	91
Coconut	59.0	0.5		16
Olive, flesh	32.4–34.9	0.8–1.5		2500–9250
Olive, pit's, shell	2.3	4.9		2350
Olive, pit's, kernel	43.7	1.5		95
Avocado, flesh	14.1–19.8	4.8–12.2		341–370
Avocado, kernel	1.3	55.5		–
Corn germ oil			1–2	
Palm oil			0.05–0.1	
Peanut			0.3–0.4	
Sesame			0.1	
Rapeseed			2.5	
Sunflower			< 1.5	

Source: Gutfinger and Letan, 1974a (reproduced with permission).

Table 6.10 Sterols in some vegetable oils

Oil	Component sterol (%)			
	Total sterols ($\mu g\ g^{-1}$ oil)	Campesterol	Stigmasterol	β-Sitosterol
Soybean	3430–3870	21.8–23.4	23.3–23.8	53.2–54.4
Cottonseed	3640	7.4	0.0	92.6
Coconut	790[a]	7.1	16.8	76.1
Olive, flesh	1050–2210	1.4–2.8	traces–0.6	96.6–98.6

Table 6.10 (cont)

Olive, pit's shell	6000	2.8	traces	97.2
Olive, pit's kernel	4200	3.6	0.7	95.7
Avocado,[a] flesh	4860	7.7	0.0	92.3
Avocado, flesh	3770	7.3	1.6	91.1
Avocado, kernel	10720	7.9	2.3	89.8

[a]Also contained traces of cholesterol.
Source: Gutfinger and Letan, 1974a (reproduced with permission).

Table 6.11 Tocopherols in some vegetable oils

Oil	Total tocopherols ($\mu g\ g^{-1}$ oil)	Component tocopherol (%)		
		α-Tocopherol	γ-Tocopherol	δ-Tocopherol
Soybean	1,129–1452	7.9–11.0	65.3–69.9	21.3–26.8
Cottonseed	864	45.6	54.4	0
Olive, flesh	121–186	90.0–94.0	6.0–10.0	0
Olive, pit's kernel	291	75.0	25.0	0
Avocado, flesh	140–153	100	0	0
Avocado, kernel	43	100	0	0

Source: Gutfinger and Letan, 1974a (reproduced with permission).

Tailoring of rapeseed towards low erucic acid types is now complete and canola oil is commercially available. Attempts to develop a method for quantitative determination of contamination of rapeseed with wild mustard is now based on the differences in the composition of seed waxes. The method developed can detect contamination with wild mustard at levels as low as 0.5%. It involves capillary GLC analysis of whole, unhydrolysed epicuticular waxes and the use of a simple ratio of two peak areas (Andrew, 1989).

6.5.1.3.1 Sterol analysis

Analysis of sterols from the unsaponifiable fraction without prior separation can detect adulteration of olive oil with soybean oil (Mordret, 1968). The detection of adulteration of olive oil by other fats has been widely reported. A wide variety of officially recognized tests for detection of mixtures is recorded (Goded, 1981). It can be done by separation and determination of the melting points of sterol acetates. Typical values for oil samples are: 117.6–119.7 °C for olive oil, 126.7–128.2 °C for soybean, 122.7–130.0 °C for cottonseed, 123.0–123.6 °C for grapeseed, 123 °C for corn, 132.3 °C for rapeseed, 126.0–126.2 °C for peanut, 125.8 °C for sesame and 113.2 °C for animal fats (Vitagliano and D'Ambrosio, 1960).

The sterol composition obtained by fractionation of the unsaponifiable matter is characteristic of virgin olive oil and can indicate its genuineness. The addition of seed

oils such as peanut, soya, sunflower, grapeseed and sesame alters the sterol balance; the ratio of the content of β-sitosterol to the sum of stigmasterol and campesterol (Wetzler *et al.*, 1977) is 25–30 for virgin olive oil, but reduced to 8–19 with 5–10% addition of seed oils (Amati *et al.*, 1971). An admixture of groundnut oil with safflower oil can also be detected on the basis of some sterol components (Kaimal *et al.*, 1974). GLC analysis of sterol constituents can detect the adulterant linseed oil in rapeseed oil or soybean oil at levels as low as 5% on the basis of gramisterol, a characteristic constituent of linseed oil (Manandhar *et al.*, 1986b). Detection of brassicasterol in sunflower oil can be suitable for routine factory monitoring for contamination by > 5% rapeseed oil (Imai *et al.*, 1974; Desbordes *et al.*, 1983). Addition of sunflower oil to olive oil (virgin, commercial and sansa) can also be detected by the presence of Δ^7-sterols. The method is applicable to all added oils containing Δ^7-sterols (Fedelli and Mariani, 1973). Certain sterols are confined to lower plants, but also appear occasionally in higher plants. One such example is fucosterol, the main steroid of brown algae and also of coconut (Harbourne, 1973). Fucosterol could serve as an indicator of the purity of coconut oil, and needs to be confirmed by experimental evidence.

Characterization of oils on the basis of their trace components presents some problems. Neutralization, bleaching and deodorization reduces the levels of tocopherols in processed soybean oil (Gutfinger and Letan, 1974b), and the sterol content of the oil is also reduced by refining (Johansson and Hoffmann, 1979). The composition of the sterol fraction may undergo change during refining (Jawad *et al.*, 1984). Steryl esters are lost to a much lesser extent during refining (Johansson and Hoffmann, 1979) and could therefore be used as indices in detecting crude or refined oils in blends. Disteryl ethers, formed by dehydration of sterols in low concentration during bleaching and refining of fats and oils have been recently shown to be useful analytical indicators of the bleaching process (Schulte and Weber, 1991). Similarly, refining vegetable oils produces steroidal hydrocarbons which can be used to establish whether an edible oil is crude or refined (Kocchar, 1983). One of these, stigmasta-3,5-diene (STIG), was found as a reaction by product in an experiment on autoxidation of β-sitosterol. Since it is not found in virgin olive oils, it can provide a tool to detect refined olive oils as well as other vegetable oils such as soya, sunflower and rapeseed in virgin olive oils (Cert *et al.*, 1994). STIG appears mainly from the action of bleaching earth, with earth activity and decoloration temperature being the most crucial parameters. The STIG levels under usual refining conditions lie between 2–45 mg/kg^{-1} oil, and its presence can indicate as little as 1% refined vegetable oils. Desterolized edible oils under forced conditions (e.g. 150 °C, 3–5% earth) in order to render them 'undetectable', and mixed with other oils are also reported. Such frauds remain detectable by the olefinic degradation products of the sterols. The degradation products have approximately the composition of the sterols they originate from. The presence of campestatriene derived from brassicasterol reveals the presence of desterolized rapeseed oil. The ratio of the degradation products of sitosterol and campesterol is a sensitive indicator for desterolized sunflower, soybean, palm, or

grapeseed oils in oils of low campesterol content such as olive and walnut. It is believed that desterolised oils are in circulation exclusively for frauds. The main difficulty in detection of desterolised oils by the degradtion products is the lack of information about the amount of oil added and the wide variation in the concentrations of the olefinic degradation products (Grob *et al.*, 1994).

The techniques reported to be promising in this regard are gas chromatography, high performance liquid chromatography (Gordon and Griffith, 1992a) and reversed phase liquid chromatography which have been successfully employed to separate cholesteryl esters (Duncan *et al.*, 1979; Caroll and Rudel, 1981; Chu and Schroepfer, 1988) and phytosterol esters (Billheimer *et al.*, 1983; Evershed and Goad, 1987; Kuksis *et al.*, 1986). The fatty acids esterified with sterols in sunflower and poppy seed oil are significantly different from those of total fatty acids (Johannson, 1979). This is observed for corn oil and peanut oil as well (Worthington and Hitchcock, 1984). Sunflower oil and tomato seed oil contain high levels of saturated long chain fatty acids in the form of steryl esters (Kiosseoglou and Boskou, 1990). The fatty acids are not only esterified to sterols, but also to the long chain aliphatic alcohols. The available methods for the isolation of steryl esters do not separate waxes on account of similar polarity. Recently Kiosseoglou and Boskou (1990) used a combined method of column chromatography and argentation thin layer chromatography to separate steryl ester from wax ester. Their scheme is outlined in Figure 6.1. The results of analysis of the fatty acids in the wax and steryl ester fraction of sunflower, tomato seed oil and soybean oil are given in Table 6.12.

It can be inferred that there is an appreciable difference between the fatty acid patterns of steryl esters and wax esters for the same oil. It is also apparent that significant differences exist between various oils. This information may be useful for chemotaxonomic purposes and for the identification of vegetable oils, considering that these compositions are totally different from the composition of the fatty acids present in the triglycerides. It could further be probed for as a means of detecting admixtures of various oils (Kiosseogolu and Boskou, 1990). Fats with similar fatty acid composition such as coconut oil and palm kernel oil can also be differentiated on the basis of their steryl ester profile (Gordon and Griffith, 1992b). The variability in steryl ester fractions of coconut and palm kernel oils from different origins, and possible changes during refining have to be investigated before the method can be considered for practical application.

6.5.1.3.2 Tocopherol analysis

The possibility of assertaining the purity of oils through tocopherol composition has recently been surveyed (Coors, 1991). This is illustrated with olive – soybean oil mixtures and avocado – cottonseed oil mixtures as manifested in the increase in γ-tocopherols. The concentration of γ-tocopherols in solvent extracted cottonseed oils increased on contamination with soybean oil (Gutfinger and Letan, 1975). Contamination of olive oil with peanut oil can be detected by comparing tocopherols

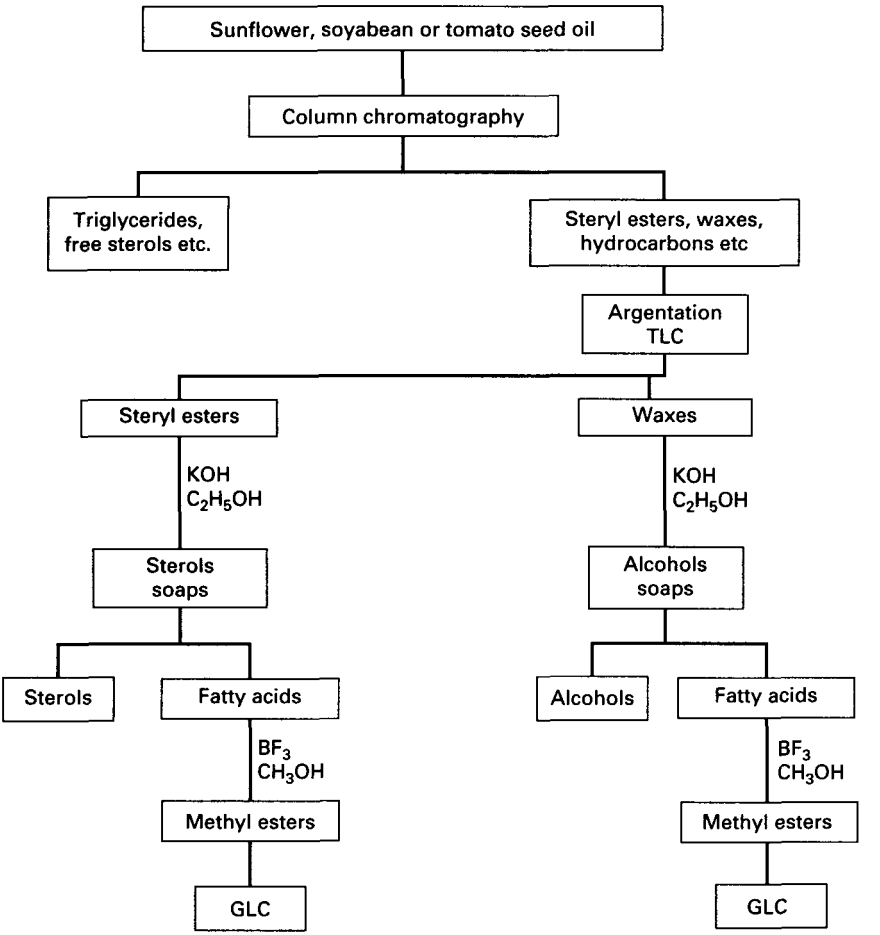


Figure 6.1 Diagram for separation of fatty acids occurring in steryl esters and wax esters. (Source: Kiosseoglou and Boskou, 1990, reproduced with permission)

Table 6.12 Steryl ester and wax fatty acids of sunflower, soybean and tomato seed oil (% of total fatty acids)

Oil		16:0	18:1 + 18:2	18:0	20:0	22:0
Sunflower	A[a]	12.0	39.6	2.5	4.0	41.4
	B[b]	9.7	5.6	2.3	8.5	73.4
Soybean	A	9.8	24.0	3.0	6.7	56.5
	B	7.0	5.0	2.0	6.0	80.1
Tomato seed	A	5.2	79.6	trace	1.4	13.4
	B	8.0	5.8	3.6	6.6	26.1

[a] Steryl ester fatty acids.
[b] Wax fatty acids.
Source: Kiosseogolu and Boskou, 1990 (reproduced with permission).

present in mixtures (Losi and Piretti, 1970). Linseed oil in edible soybean oil can be detected from increased β-tocopherol levels (Manandhar *et al.*, 1986a).

6.5.1.3.3 Phenolics and alcohols

Phenolics, and in particular tyrosol, may be of value in calculation of the proportion of virgin olive oil in blends with refined oil. Tyrosol, 4-hydroxyphenyl acetic acid and hydroxytyrosol comprise about 50% of the phenolic compounds in virgin oils. Unrefined oils contain tyrosol at a concentration of at least 30 mg/kg^{-1}, whereas refined oils are free from this constituent (Sohnos and Cichelli, 1982). Reversed phase HPLC with UV detection and GC–MS evaluation (Angerosa *et al.*, 1995) has been reported as a technique for quantification of these phenols (Tsimidou *et al.*, 1992).

Olive presscake in olive oil can be detected on the basis of the presence of higher fatty alcohols in the former (Spanish Standard 1973c). GLC analysis of tetracosanol and hexacosanol in Greek olive oils has shown them to be useful as indices to detect adulteration of hydrogenated olive oil with B-residue oil (or olive pomace oil) and/or B-residue oil stearins (Dimitrios et al., 1983). Table 6.13 presents the results obtained from the analysis of various olive oils, olive oil stearins, vegetable oils and hydrogenated products. The data show that in olive oil the quantity of tetracosanol plus hexacosanol does not exceed 40 mg/100g oil, while crude or refined B-residue oil gives values ranging from 140–460 mg/100g oil. Equally high contents are found in B-residue oil stearins (above 270 mg/100g). It has been suggested that since the determination of tetracosanol and hexacosanol is easy to carry out, it may be used in routine analysis of cooking fats and margarines before any other sophisticated methods of recognizing B-residue oil are applied.

Admixture of pressed olive oils with dewaxed oils can be detected from the composition of the alcohol soluble unsaponifiable fraction of acetone cleaned oils. Saturated aliphatic alcohols, C_{22} (behenyl), C_{24} (lignoceryl), C_{26} (ceryl) and C_{28} (montanyl), and altered ratios of C_{26} : C_{24} from 1.30 in virgin oil to 1.12 in rectified oil, 0.98 in rectified sansa oil, and 0.30 in acetone dewaxed oil are particularly altered on adulteration. The ratio of C_{26} : C_{24} progressively decreases by 0.20, 0.35 and 0.45 on addition of 10%, 20% and 30% dewaxed oil to virgin and/or rectified oil and can be used as an index of adulteration (Fabrini *et al.*, 1973). Detection of olive presscake oils in olive oils on the basis of the presence of higher fatty alcohols in the former has been described in a Spanish Standard (Spanish Standard, 1973d). Triterpene dialcohols (particularly erythrodiol) can be used as a marker to distinguish between cold-pressed and extraction olive oils. Erythrodiol is separated on TLC along with sterols from unsaponifiable matter. About 5% olive cake oil can be detected in pure and refined vegetable oils (Choukroun *et al.*, 1984). Analysis can be performed by fully automated on-line coupled liquid chromatography–GC using 2% methyl-t-butyl ether/n-hexane as eluent in liquid chromatography (Grob *et al.*, 1989). The Bishop reaction, based on the formation of an oxidation product of hydroxyquinone can detect 5% sesame oil in clear olive oil samples (Pavolini, 1940).

Table 6.13 Tetracosanol and hexacosanol content of Greek olive oils

	Number of samples	Tetracosanol + hexacosanol mg/100 g		
		Min.	Max.	Mean value
Olive oil (virgin or refined)	12	10	32	22
B-Residue oil	8	145	460	254
Olive husk oil (laboratory extracted)	1			792
B-Residue oil stearins	5	275	494	396
Cottonseed oil	5	12	18	15
Sunflower oil	3	11	33	22

Source: Dimitrios *et al.*, 1983 (reproduced with permission).

6.5.2 Blends of vegetable and marine/animal fats

6.5.2.1 Fatty acid composition

GLC analysis of the fatty acid composition can be used to detect admixture of marine oils in vegetable shortenings. These are manifested as increased myristic, palmitoleic, C_{20} and C_{22} acid contents. Both marine oils and animal fats contain odd numbered fatty acids (Tsatsaronis and Bostov, 1972). The purity and contamination of vegetable oils with lard can be determined from the values of methyl esters of C_8–C_{20} fatty acids (Barvir *et al.*, 1968). Animal fats contain tetradecanoic and hexadecanoic acids in greater amounts than does olive oil. For instance, the ox, sheep, and pig fat contain 0.6–4.8% tetradecanoic and 0.1–6.7% hexadecanoic acid, while olive oil contains less than 1% tetradecanoic acid and no hexadecanoic acid. Saponification of the fat, followed by separation of the solid fatty acids as lead salts, methylation of liquid fatty acids and distillation of the first 15% fraction of methyl esters are the various steps involved in separation. The average molecular weight of the distilled fraction from pure olive oil has a saponification number less than 191, while >10% animal fat increases this number to between 194 and 212. These are also valuable indices in detecting such adulterations (Bigoni, 1959). Analysis of four fatty acids viz. erucic, eikosanic, oleic, and linoleic by chromatographic separation, development with $KMnO_4$–benzidine and photometric and planimetric evaluation can also detect animal fat in rapeseed oil (Sulser, 1958).

Spectral analysis is also effective in detecting the presence of marine oils in edible fats. It is based on the fact that hardly any fatty acids with four or more double bonds are encountered in vegetable oils. $E^{1\%}_{1\,cm}$ is the specific absorbance of the oil sample at any wavelength (λ), of an oil sample diluted appropriately in isoctane and is given by: $E^{1\%}_{1\,cm} = A/c \times d$ where c = concentration of the sample solution in g/100 ml; d = cell length in cm and A = absorbance of the sample at the wavelength denoted by λ in the tetraene region (315 μm), and can easily distinguish marine fats in various vegetable

fats such as linseed, palm kernel, cottonseed, olive, peanut, poppy seed, sesame, rapeseed, pumpkin and sunflower (Franzke, 1964a). Linseed oil shows a different light absorption at $E^{1\%}_{1\,cm}$ from fish oils after alkali isomerization (1.3 N potassium hydroxide in ethylene glycol, 180 °C, 45 min), enabling the determination of up to 3% fish oils in linseed oil (Franzke, 1964b).

Adulteration of pork fat can be detected using the Bomer value (British Standard, 1989; Spanish Standard, 1981) as an index. The Bomer value is defined as the sum in °C, of the melting point of saturated triglycerides and twice the difference between this melting point and that of the fatty acids obtained after saponification of these triglycerides (Netherlands Standard, 1982).

6.5.2.2 Unsaponifiable fraction

Either vegetable or animal oil can be detected in the presence of 20 parts of the other on the basis of sterol acetate spots (Hatzopoulos, 1960). The differences in the sterol composition of animal and plant fats offer an efficient method of detecting their admixture. Chromatographic separation of sterols followed by spraying with phosphomolybdic acid and/or detection of stigmasterol by chromatographing bromides of sterol acetates are particularly noteworthy (Riemersma and Stoutjesdijk, 1958). Sterol composition, particularly with respect to cholesterol (Karathanassis, 1973), brassicasterol, campesterol, stigmasterol and β-sitosterol and their appropriate relations derived for various combinations can detect adulteration of lard and vegetable oils like palm oil as well as mixtures of vegetable and marine oils (Karleskind *et al.*, 1966). Adulterant animal fat at 10% in vegetable oils can be detected on the basis of cholesterol. Similarly, kidney fat in rapeseed oil can be detected by chromatographing the unsaponifiable fraction of the fat (Sulser, 1958). The sterols can be separated from the unsaponifiable matter on a TLC plate and analysed by GC, either as such or after preparation of suitable derivatives (British Standard, 1992; International Standard, 1991). This method is however complicated by the presence of cholesterol in some vegetable fats (e.g. up to 8% in palm, palm kernel and coconut oils) and therefore does not necessarily indicate adulteration with animal fats (Homberg, 1991). It is believed that the method is not sufficiently specific to permit establishing a maximum cholesterol value as an index of purity (Woell, 1979).

Characteristic amounts of these sterols can also indicate the presence of fatty binders for painting such as linseed, soybean, corn, grapeseed, chinawood, safflower and castor oil. The presence of sardine fish oil can be indicated by the presence of >5% cholesterol. Even if glycerophthalic resins modified with oils are used as paint binders, the nature of the oil is evident from the sterol composition (Wolff *et al.*, 1966).

6.5.3 Other adulterants in fats and oils

Hydrogenation and interesterification are both means of hardening fats. The presence

of *trans* fatty acids, easily estimated by infrared spectroscopy, can be used to detect straight hydrogenated fats upon admixture with interesterified products. The fact that urea forms a complex with *cis* isomers more readily than *trans* isomers could be the basis for distinguishing between hydrogenated and unhydrogenated fats, since the former contain *trans* isomers not naturally present in the native fats (Schlenk and Holman, 1950). Silroy and Bhattacharyya (1989) showed that hydrogenated fats give cooling curves with a characteristic hump, absent in inter esterified products, which makes it possible to distinguish an interesterified fat from hydrogenated one. The detection of an interesterified fat in hydrogenated fat is more difficult, but is neverthless required. The latter is becoming more relevant because interesterification is permitted in many countries as a means of producing hard fats.

In interesterified fats, fatty acids are distributed randomly, which implies that the proportion of any fatty acid at the 1,3- or 2-position of glycerol is the same and is equal to that of the particular fatty acid in the total triglycerides. In interesterified fats with melting points of 35 °C or more, the proportion of saturated fatty acids, both in the fat as a whole and in the 2-position , is in the range of 30–35% or greater (Adhikari *et al.*, 1981).

Lipase hydrolysis of the triglycerides, followed by gas chromatographic analysis of the fatty acids of the 2-monoglycerides has been used to calculate two indices. These are R_1, the ratio of amounts of palmitic acid present in the 2-position to that in the total glyceride, and R_2, the ratio of saturated acid present in the 2-position to total saturated fatty acids in the fat. In hydrogenated vegeatble oil (HVO), R_1 is always below 10 and R_2 is always below 20. The presence of 5–10 % interesterified fat raised both values and therefore a suitable basis for the detection of interesterified fats in hydrogenated fats (Adhikari and Adhikari, 1992). Table 6.14 shows the fatty acids present in total triglycerides (TG) and in 2-monoglyceride (MG) of HVO, interesterified palmitic-rich fat and of their mixtures in various proportions.

Data from Table 6.14 show that for detection of interesterified fats in HVO, the palmitic acid concentration at the 2-monoglyceride position can be adopted as the primary screening test. If its concentration is greater than 2%, it can be assumed that interesterified fat is present. When it is below 2%, a second confirmation can be made by calculating R_1 or R_2. When R_2 is greater than 16.5, admixture with interesterified fat is indicated (Adhikari and Adhikari, 1992).

Re-esterified oil in oils can also be detected by the percentage of palmitic acid in position 2 of the glycerides. However, certain refining procedures may affect the glyceride structure of the oil and lead to the level of palmitic acid in position 2 of genuine oils that have not been subjected to re-esterification being above the maxima given (Gegiou and Georgouli, 1980). Esterified oil in refined olive oil can be detected by the 'aniline point test', which is the temperature of first appearance of cloudiness when cooling a mixture of 5 ml of dry oil with 5 ml dry aniline in running water. While the genuine oils have an aniline point at $\geq$17 °C, the suspected oils have a value of 9–10 °C (Massarotti, 1975). In this case, the monoglyceride content is also of value (4–5% instead of <1% for suspected oils).

Table 6.14 Fatty acids present in total triglycerides (TG) and in 2-monoglyceride (MG) of HVO interesterified palmitic-rich fat and of their mixtures in various proportions

		Fatty acids					Indices[b]	
Fat[a]	Position	16:0	18:0	18:1	18:2	Others	R_1	R_2
IF(PC)	TG	36.9	3.1	27.4	29.5	3.1	–	–
	MG	36.0	2.8	28.5	28.5	4.2		
VRT	TG	24.6	4.5	50.1	18.9	1.9	2.0	12.0
	MG	0.5	3.0	68.6	25.6	2.8	–	–
VRT + IF(PC)	TG	25.3	4.4	50.5	18.3	1.5	11.5	19.8
(95:5)	MG	2.9	3.0	67.3	25.8	1.0	–	–
VRT + IF(PC)	TG	26.0	4.3	50.3	17.9	1.5	20.0	26.7
(90:10)	MG	5.2	2.9	64.9	26.0	1.0	–	–
VRT + IF(PC)	TG	27.3	4.2	50.3	16.4	1.8	36.2	40.3
(80:20)	MG	9.9	2.8	59.5	26.8	1.0	–	–
IF(RM)	TG	29.2	11.7	37.7	21.2	0.2	–	–
	MG	30.3	11.9	37.8	20.0	–	–	–
VRT + IF(RM)	TG	24.8	4.0	49.5	19.9	1.9	8.0	19.0
(95:5)	MG	2.0	3.5	67.1	26.3	1.1	–	–
VRT + IF(RM)	TG	25.0	5.2	48.9	19.1	1.5	14.0	25.1
(90:10)	MG	3.5	3.9	65.5	25.0	2.1	–	–
VRT + IF(RM)	TG	25.5	5.9	47.6	19.4	1.6	25.1	35.7
(80:20)	MG	6.4	4.8	62.4	24.5	1.9	–	–
IF(RS)	TG	17.2	23.1	40.2	15.4	4.1	–	–
	MG	19.2	23.5	39.5	14.9	2.9	–	–
VRT + IF(RS)	TG	24.2	5.6	49.6	18.7	1.9	5.9	21.5
(95:5)	MG	1.4	4.0	59.2	25.0	0.4	–	–
VRT + IF(RS)	TG	23.8	6.8	49.2	15.8	4.8	14.2	23.8
(90:10)	MG	3.4	4.8	67.7	24.5	0.6	–	–
VRT + IF(RS)	TG	23.1	8.2	48.1	18.4	2.2	18.1	36.1
(80:20)	MG	4.2	7.1	62.9	23.5	2.3	–	–
IF(SuS)	TG	5.5	22.8	36.5	31.5	3.7	–	–
	MG	5.9	22.6	36.4	32.0	3.1	–	–
VRT + IF(SuS)	TG	23.6	5.4	49.4	19.5	3.6	3.9	21.8
(95:5)	MG	0.9	3.9	67.0	25.9	2.5	–	–
VRT + IF(SuS)	TG	22.7	6.4	48.7	20.2	2.5	4.4	20.3
(90:10)	MG	1.0	4.9	65.4	26.2	2.0	–	–
VRT + IF(SuS)	TG	24.2	3.6	49.8	21.4	1.0	6.6	30.5
(80:20)	MG	1.6	6.9	62.2	26.9	2.4	–	–

[a]IF(RS), interesterified fat made from rice bran and sal fat; IF(SuS), interesterified fat made from sal fat and sunflower oil; IF(PC), interestified fat made from palm oil and cottonseed; IF(RM), interestified fat made from rice bran oil and mowrah. VRT coded vanaspati (HVO).

[b]Example: for fat no. 2 (VRT):

$$R_1 = \frac{\text{Conc. of palmitic acid at 2-position} \times 100}{\text{Conc. of palmitic acid in total fat}} = \frac{0.5 \times 100}{24.6} = 2.0$$

$$R_2 = \frac{\text{Conc. of total saturated acid at 2-position} \times 100}{\text{Conc. of saturated acid in total fat}} = \frac{(0.5 + 3.0) \times 100}{(24.6 + 4.5)} = 12.0$$

Source: Adhikari and Adhikari, 1992 (reproduced with permission).

6.5.4 Constituents specific to or characteristic of an oil

Many oils contain a constituent which is characteristic of the plant species. Examples are the presence of sesamin and sesamol in sesame oil, sanguinarine in argemone oil, unusual quantities of squalene in olive oil, etc. These have often been the basis of detecting adulterants. Typical examples are listed below.

6.5.4.1 *Fitelson's reagent*

This gives negative results with mixtures of olive oil and various other seed oils (Fichera and Zappala, 1959). The Fitelson colour test has been modified to distinguish olive oil from seed oils and attempts have been made to make it semi-quantitative by reading the colour spectrophotometrically at 530 μm (Pier and Stella, 1961). The Fitelson reaction can also detect adulteration of olive oil with tea seed oil. The optical density at 540 nm of olive oil samples can also indicate adulteration. While the value for olive oil samples has been shown to be <0.2, results on mixtures of various proportions of olive and tea seed oil gives values of 0.258–0.570, with no direct relation to the concentrations of the two oils (Chindemi *et al.*, 1963).

6.5.4.2 *Linseed oil in mustard oil*

This can be evaluated quantitatively by reacting with bromine in chloroform and then treating with alcohol and ether. A calibration curve prepared by plotting percentage of precipitate (v/v) against percentage of linseed oil in mustard oil is almost linear and can be used as a standard (Chakravorty, 1979).This is evident from Table 6.15, which shows the volume of precipitate per 100 ml of oil containing various admixtures of linseed and mustard oil. This method can detect the adulterant at 2% level, whereas computations based on saponification value, iodine value and butyrorefractometer reading cannot detect even up to 5% adulteration.

6.5.4.3 *Villavachia–Fabris and Pavalini–Isidoro reactions*

A direct identification of sesame oil by the Villavachia–Fabris reaction, based on the reducing action of $SnCl_2$ in samples of edible oils and fats coloured with fat soluble azo dyes is reported (Luigi, 1958). This test can detect adulterant niger seed, groundnut, castor, soybean, liquid paraffin and mustard in sesame oil (Chand *et al.*, 1974). The Pavalini–Isidoro colour reaction for sesamin can identify sesame oil in hydrogenated fats at levels as low as 0.05–0.075% (Daghetta, 1957). An exception to this is detection in butter and lard (Renko, 1952). Colour reactions with furfural and concentrated hydrochloric acid can determine sesame oil in other oil quantitatively (Buhrer, 1950).

Table 6.15 Volume of hexabromide precipitate from linseed – mustard oil mixtures

Linseed oil (% v/v)	100	90	75	60	50	25	15	10	5	2.5
Average volume of the precipitate (percent)	111.7	97.9	81.5	74.5	68.2	35.7	23.1	15.25	9.8	4.75

Source: Chakravorty, 1979 (reproduced with permission).

6.5.4.4 Determination of castor oil

Castor oil can be determined with sufficient precision gravimetrically by the stepwise procedures of extraction of the oil in 90% alcohol, evaporation, dissolution of the residue in petroleum ether and ether (9:1), elimination of fatty acids by adsorption on silicic acid, evaporation of the solvent and condensation of the residue with *p*-dimethylamino benzaldehyde. The orange red precipitate can be weighed to estimate castor oil or determined colorimetrically at 532 μm (Anselmi *et al.*, 1959). A simple method comprising the extraction of the suspected oil in absolute alcohol and identification by TLC can also detect the adulterant castor oil in groundnut and other vegetable oils (Srinavasulu and Mahapatra, 1973; SenGupta *et al.*, 1978).

6.5.4.5 Mustard oil determination

The determination of mustard oil in other edible oils is based on the detection and estimation of allyl-isothiocyanate, a volatile constituent present in mustard oil but not in other edible oils. The Association of Official Analytical Chemists (AOAC) method consists of distilling the sample, and precipitating the allylisothiocyanate as a black precipitate and dark colour with silver nitrate. The intensity of the dark colour and the amount of black precipitate formed are directly related to the amount of mustard oil present (Mitra *et al.*, 1958). Detection sensitivity is about 0.05% mustard oil in other edible oils. Erucic acid is characteristic of mustard and rape, and hence the estimation of erucic acid number by selective oxidation to dihydroxybehenic acid with $KMNO_4$ can be used as an index of the purity of rapeseed and mustard oils (Pathak and Aggarwal, 1954). It can detect a 10% admixture of rapeseed oil in olive or peanut oil (Hadron *et al.*, 1953) and can determine these oils in rice bran oil (Adhikari and Adhikari, 1991).

6.5.4.6 Nigerseed oil

Nigerseed oil (*Guizotia abyssinica*) is widely used to adulterate many edible oils, particularly mustard oil. Not many specific tests are reported for the detection of nigerseed oil in other oils. A TLC method, which can successfully detect nigerseed oil in mustard oil at a 5% level and above has been reported. There is no interference from groundnut oil, sesame oil and argemone oil (Mitra *et al.*, 1971).

6.5.4.7 Determination of tung oil

Tung oil is cheaply available in China and was reportedly used as an adulterant of edible oils. It can roughly be determined by shaking 2 ml oil with 2 ml of 65% nitric acid, letting it stand for 30 min at 0 °C, filtering the voluminous precipitate on asbestos, washing with 50–150 °C naphtha, drying at 100 °C and weighing. Graphs which vary for different oils give percentage tung oil corresponding to the weight of the precipitate per millilitre of oil (Suen and Wang, 1944). Three other colour reactions have been examined for the determination of tung oil, (i) a maleic anhydride reaction in the presence of chloroform, (ii) the Storch and Morawsky reaction with sulphuric acid in acetic anhydride and (iii) an antimony trichloride reaction (reaction with 10% antimony trichloride – chloroform solution). The reactions were applied to many vegetable oils containing tung oil as a mixture and to oils containing conjugated trienoic fatty acids prepared from linseed oil. The maleic anhydride reaction has been observed to be stable with no change in the specific yellow colour following the reaction period. However, this reaction is not suitable for dark coloured oils. The reaction with sulphuric acid in acetic anhydride gave a strong purplish red colour which changes to a different red shade after 20–30 s. The colour resulting from the antimony trichloride reaction is found to be very deep, but the reaction requires nearly 1 h to complete. The reactions could be used to detect tung oil in vegetable oils at 5% concentration. The colour noted during the course of these reactions is due to the presence of conjugated trienoic fatty acids in tung oil. Therefore, conjugated fatty acids prepared from linseed oil show the same colour reactions as tung oil. In the case of mixtures containing tung oil these simple reactions may be used for its detection in preference to complicated methods such as GC or UV spectrophotometry. However care must be taken because other oils containing conjugated trienoic fatty acids will show the same colour reaction (Kitamura *et al.*, 1984).

6.5.4.8 Rice bran oil

Rice bran oil contains oryzanol (methyl ferulate, cycloartenyl ferulate, 24-methylene cycloartenyl ferulate, etc.) in the unsaponifiable matter as a native constituent. This compound has been indicated as a marker of rice-bran oil in other edible vegetable oils (Nasirullah *et al.*, 1992).

6.5.5 Detections based on physical properties

Various physical properties of oils have been exploited by researchers as an aid to detect adulteration. Some of these are summarized here.

6.5.5.1 Atomic absorption spectrophotometry

Atomic absorption spectrophotometry is a rapid and accurate method, and using standards containing virgin olive oil and known amounts of sodium oleate, detection of adulteration of virgin olive oil with refined olive oil or other vegetable oils can be made (Gegiou, 1974). The graph of absorption versus concentration of sodium oleate in oil in the range 3–1000 ppm is linear. Although the method does not distinguish between soap and other forms of sodium present, it is rapid, sensitive and accurate, and can also be useful in the control of refining operations.

6.5.5.2 Detection of stearin in palm oil

Analysis of slip melting point, iodine value, fatty acid and triglyceride composition to detect stearin in palm oil has shown the limits of detection to be governed by the type of stearin and palm oil used in the mixture. Soft stearin with an iodine value of 49, when mixed with palm oil with an iodine value of 55, is difficult to detect. Hard stearins with an iodine value of 21 were more easily detectable in amounts as low as 4% (Tan *et al.*, 1983). The effect of added palm stearin and fatty acids on isothermal crystallization and cooling behaviour has been studied by measurement of the induction and cooling curves, respectively. Whereas palm stearin shortens the induction time, both palmitic and oleic acids prolong it. The cooling behaviour of palm oil is different from that of stearin which shows no plateau region during phase transition under similar experimental conditions. The maximum temperature in the region of supercooling is affected by sample size, stearin and acid contents. The induction period as well as increase in maximum temperature are proportional to the amount of stearin added. Detection of adulteration of palm oil with stearin has therefore been recommended on this basis (Ng *et al.*, 1983). Incorporation of stearin into palm oil can also be analysed by the increased solid fat content over the temperature range 5–55 °C. The effect is larger for hard stearins than for soft stearins and offers a possible means of detecting stearin adulteration in palm oil (Oh and Ng, 1981).

6.5.5.3 Four-temperature test

A four-temperature test based on determination of different glyceride structures of oils can be used for identification and detection of adulteration in edible oils (Heller, 1960). Using graphs based on the four temperatures and isooleic acid as a standard, rapid qualitative and approximate quantitative analysis can be made of variously refined and esterified types of olive oils, virgin olive oil and foreign oils such as grapeseed oil and animal fats (Martinenghi, 1960).

Table 6.16 Bellier figure and butyrorefractometer reading of some common oils

Oil	Bellier figure	Butyrorefractometer reading at 40 °C
Sesame	19–20	59–60
Coconut	13–14	35–36
Safflower	15–16	64–65
Nigerseed	25–26	63–64
Mustard	24.5	60.5
Mustard (Rape)	27.5	59.5
Peanut	39–40	54–55

Source: Narayanaier, 1945 (reproduced with permission).

6.5.5.4 The Bellier test

The 'Bellier figure' or the temperature at which turbidity appears in a saponified and neutralized oil sample under specific conditions was first quoted by Evers (1912), and had been adopted for detecting peanut oil in other vegetable oils (Amato and Wohlers de Almeida, 1952; Kane, 1955) such as sesame oil (Hawley, 1937), olive oil, soybean or cottonseed oils (Lacerda, 1949). Along with refractive index, it appears in most cases to be sufficient to judge the purity of the oil. It has been shown that although the Bellier figure increases with the percentage of peanut oil in the mixture, the increase is not proportional and that there is a steep rise for percentages of peanut oil below 25%, but only a slow rise beyond this level. Table 6.16 gives the Bellier figure and butyrorefractometer reading of some common oil types.

The Bellier test had been used to ascertain the purity of olive oil (Diemair, 1963; Tous, 1968). While soybean oil, cottonseed oil and palm oil give a violet colour, sesame oil and olive residue oil are known to give a green colour. The test seemed not to be influenced by the rancidity level of sesame, linseed or sunflower oils, regardless of the peroxide value of these oils. Its sensitivity decreases as the rancidity level increases in case of soyabean, cottonseed, palm, corn and olive residue oils (Amr and Abu-Al-Rub, 1993).

6.5.5.5 Molecular refraction

Adulteration of vegetable oils can also be detected using molecular refraction, for example adulteration of mustard oil with peanut, sesame, safflower, linseed or argemone oils (Chatterji and Chandra, 1956).

6.5.5.6 Ultrasonic interferometer

The ultrasonic interferometer has been reported to be a suitable tool for the detection of contaminants in pure fats and oils. There is a marked change in the velocity of ultrasound when a pure sample is adulterated. As such, the accurate measurement of

velocity can be used as a tool to detect adulteration and the accuracy of this method is about 1%. This method can detect 1% HVO in ghee, 2–3% coconut oil, <1% of sesame oil, 2% castor oil or 2% mineral oil in groundnut oil and 3% mineral oil in sesame oil. The method, however cannot detect adulteration of sesame oil with groundnut oil because of a very small variation in the velocity of 20 mms^{-1}. Accurate determination of velocity is of prime importance. The method cannot identify the adulterant, but once the adulterant is known, it is easy to estimate the percentage of the adulterant (Raghupati Rao *et al.*, 1980).

6.5.5.7 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is now widely used in food research (Biliaderis, 1983). It can be used for comparison of olive oil with re-esterified oils (Gegiou and Georgouli, 1982), in the identification of edible vegetable oils (Dyszel, 1982) and in the detection of beef suet in butter fat (Amelotti *et al.*, 1983). This technique has been particularly promising for the the detection of beef tallow in lard. The standard procedure to detect this adulteration is separation of the triglycerides fraction, drying and comparison of the melting point with that of the fatty acids prepared from the same triglycerides after KOH treatment (Polish Standard, 1984a). This determination is only qualitative because polymorphism causes complex and uncertain melting behaviour.

The DSC melting curves for lard containing tallow are comparatively more complex than the cooling curve, and hence the latter has been preferred. Figure 6.2 shows the thermograms of lard and beef tallow at a cooling rate of 2 °C min^{-1}. Lard shows two main peaks in the area ratio of about 2:3, with no peaks above 20 °C. The extrapolated onset temperatue (T_{on}) and the peak maximun temperature could be used as indices for purity of lard. Thermograms of beef tallow generally show a sharp peak at T_{on} of 29.3 °C and T_{max} of 27.8 °C. The detection sensitivity is reduced if the cooling rates are very slow; for instance tallow contents of < 1% cannot be detected at cooling rates < 2 °C/min^{-1}. This drawback can be overcome by using faster cooling rates. A cooling rate of 4 or 5 °C min^{-1} gives qualitative as well as quantitative detrmination of tallow in lard. A regression equation obtained on analysis of mixtures of lard and tallow is given by:

$$m_w = 0.999 m_{DSC} + 0.157 \qquad [6.3]$$

Where, m_w and m_{DSC} are percentage contents of tallow and lard by weight and determination by DSC, respectively. The correlation coefficient was 0.998 andstandard deviation ± 0.603 ($P<0.001$). The method has additional advantages of being fast and precise, requires no sample preparation, and needs only milligrams of sample to detect down to 1% tallow in lard. In comparison, the Polish Standard (1984b) can detect tallow in lard above 10%, and estimates of tallow contents are dubious (Kowalski, 1989).

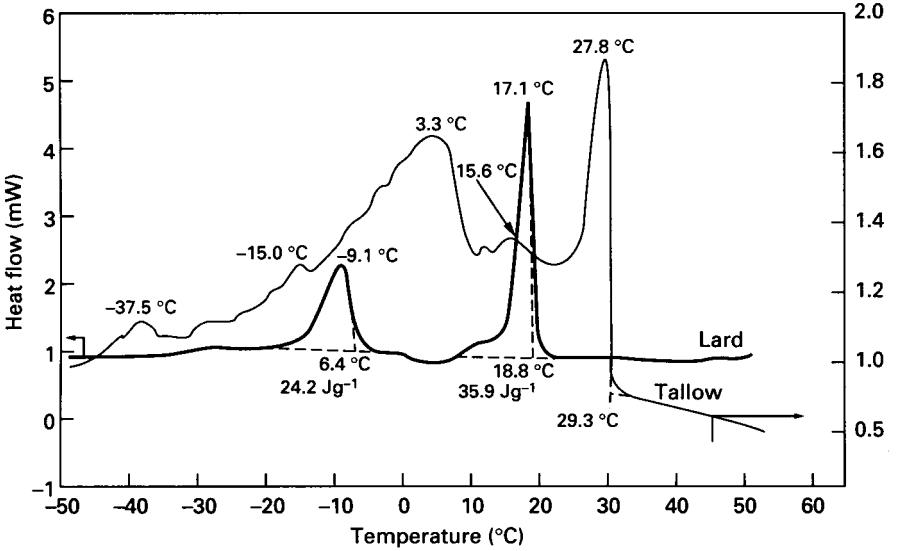


Figure 6.2 Thermograms of lard (8.1 mg) and cow tallow (2.0 mg). Cooling rate $\beta = 2$ °C min^{-1}; scan from right to left. (Source: Kowalski, 1989, reproduced with permission)

6.5.5.8 Refractive index

The importance of refractive index measurements in the analysis of fats and oils is well recognized, but no single property is sufficient to establish the purity of any sample. The Lorentz–Lorenz formula, given by $(n^2-1/n^2+2)\times 1/D$, takes into account two specific properties namely refractive index (n) and density (D), and has been shown to be useful in detecting admixture of mustard oil with groundnut oil (Table 6.17). A full exploitation of this expression awaits much wider data collection (Nayar *et al.*, 1950).

6.5.5.9 UV methods

Analysis of fatty acids or triglycerides are ineffective in detecting adulterations such as virgin olive oil by residue oil or refined oil. Sterol and phenol analysis are also ineffective, since they can detect blends only above 50% and 20%, respectively. UV spectrophotometric methods do not have these disadvantages. Most of the measurements are made at 268 nm, corresponding to the maximum absorption of conjugated trienes derived during refinement. The development of compounds during storage of virgin olive oil also having a maximum absorption at 268 nm, however, precludes this method. Absorption at 315 nm is characteristic of conjugated tetraenes, and has been suggested as another approach to detecting this adulteration. The ranges for $\Delta K^{1\%}_{315}$ (where $K^{1\%}_{315}$ denotes the specific extinction coefficient of a 1% oil solution in a suitable solvent at 315 nm) have been found to be 0.008–0.015 and 0.010–0.030 for

Table 6.17 Observed and calculated specific refraction for various mixtures making use of average 0.0000283 decrease for 1% groundnut oil

Sample	Observed specific refraction	Calculated specific refraction	Difference
Genuine mustard oil	0.30597	..	..
+ 5% Groundnut oil	0.30583	0.30583	nil
+ 10% Groundnut oil	0.30569	0.30569	nil
+ 15% Groundnut oil	0.30553	0.30555	+0.00002
+ 20% Groundnut oil	0.30536	0.30541	+0.00005
+ 30% Groundnut oil	0.30577	0.30573	+0.00004
+ 40% Groundnut oil	0.30485	0.30485	nil

Source: Nayar *et al.*, 1950 (reproduced with permission).

virgin olive oils of the last and older crops, whereas that for refined olive and olive kernel oils exceeded 0.450 and 0.990, respectively. About 5% adulteration of virgin olive oil by refined oils can be detected by this method (Kapoulas and Andrikopoulos, 1987).

In the case of virgin olive oil, absorption at 315 nm is not always visible because of a low tetraene region and/or high absorption at 232 nm. The ratio of the slopes of the absorption curve on either side of 315 nm, that is $(K_{315} \times K_{320})/(K_{310} \times K_{315})$, has been proposed as an index of the above property. Recently, it has been shown that $\Delta K^{4\%}_{315}$ values, obtained directly from the UV spectra are much more reliable estimates of conjugated tetraene absorption at 315 nm, without affecting the sensitivity of the method (Passaloglou-Emmanouilidou, 1990).

6.5.5.10 Pyrolysis mass spectrometry

Recently, a combination of Curie-point pyrolysis mass spectrometry (PyMS) (Irwin, 1982; Meuzelaar *et al.*, 1982; Aries *et al.*, 1986; Goodacre and Berkeley, 1990) with multivariate data analysis using artificial neural networks (ANN) (Rumelhart *et al.*, 1986; Eberhart and Dobbins, 1990; Simpson, 1990; Hertz *et al.*, 1991) has permitted rapid assessment of extra-virgin olive oils with various seed oils (Goodacre *et al.*, 1992, 1993). The detection sensitivity is as low as 50 ml corn, peanut, soya, sunflower or rectified olive oil per litre of virgin olive oil. The running cost of this technique in detecting such frauds is reported to be only £1 per sample.

6.5.6 Detection of mixtures of animal fats

Adulteration of beef fat by pig lard has received attention recently. The detection can be made at not less than the 5% level by determination of *trans* fatty acids (Imamura *et al.*, 1969). Fatty acid composition, triglyceride composition and physical characteristics are the usual approaches used to detect these cases. For instance, comparative

gas chromatographic studies on the fatty acid composition of lard and goose dripping revealed differences in the content of stearic and oleic acids. The ratio of oleic to stearic acid in lard is found to be 0.39–0.60 (average = 0.49), while that of goose dripping is 0.08–0.13 (average = 0.11). Addition of 10% lard to goose dripping changes the ratio to 0.15–0.18 (average = 0.17). Therefore the ratio of stearic acid to oleic acid can be taken as the basis for the detection of about 10% of lard in goose dripping (Brixius and Treiber, 1974). The widely different fatty acid composition between beef and lard (Table 6.18), notably the high percentage of saturated $C_{18:0}$ (Riel, 1963; Stull and Brown, 1964) is useful in distinguishing the two fats and their blends. Furthermore, the ratios of $C_{16:0}/C_{18:1}$ and of $C_{16:0}/C_{18:2}$ are more effective in detecting beef and lard adulteration (Table 6.19). Even a simple test such as iodine value shows remarkable change on admixture and could be used to check this adulteration (Table 6.19)

Detection of suet in lard can be achieved by the Bomer value, fatty acid composition and '*S*' ratio (Wolff, 1963). The myristic:palmitic (C_{14} : C_{16}) acid ratio is considered to be a better indicator than the Bomer index. A value of the ratio of <6 indicates that the sample is pure. At ratios >6, values for myristoleic acid, ($C_{14:1}$), pentadecanoic (C_{15}) and isopentadecanoic (iso-C_{15}) may be used in the calculation of '*S*' ratio, when values greater than 10 indicate a genuine lard (Juarez and Martinez Castro, 1981). The incorporation of palmitic acid, oleic acid and linoleic acid into the two position of the triglycerides of beef and pork fat is shown to be highly correlated to the corresponding acid contents of the triglycerides. On the basis of various regression equations obtained for pork and beef fats, the percent adulteration of pork fat with beef tallow may be determined. The method involves gas chromatographic determination of the triglyceride fatty acids, and analysis of the fatty acid contents in the monoglycerides obtained after lipase treatment of the fat. Results indicate that mixing beef fat with 10% porcine fat may be accurately estimated (Verbeke and Brabander, 1979).

Detection of interspecies meat adulteration is very often based on the differences in fat characteristics, and the reader is referred to the chapter on 'meat quality' for a detailed report. Detection of adulteration of butter fat or ghee is dealt with under dairy products.

6.6 Sensory quality of oils

A new dimension is, of late, being added to the definition of quality of edible oils – the sensory quality. This is based on the voluminous work carried out on the sensory quality of virgin olive oil for which the market has been widening in Europe. A concerted attempt has been made by scientists from the olive oil consuming countries to highlight the acceptability aspects of the virgin olive oil. Pagliarini and Rastelli (1994) and Pagliarini *et al.* (1994) have evaluated the visual characteristics, colour and transparency of virgin oil samples in relation to consumer acceptability. The Commission of European Communities has formulated a standard of quality known as the COI-test developed on the basis of studies by the International Olive Oil Council

Table 6.18 Fatty acid composition of beef fat and lard and their admixtures

Sample %	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Beef I	5.98	30.45	1.00	24.01	36.95	1.61
Lard II	1.95	27.01	2.54	11.87	48.00	8.63
II + I 2%	5.90	30.30	1.20	22.50	37.50	2.60
II + I 5%	5.90	30.00	1.50	21.00	38.01	3.08
II + I 8%	5.85	30.00	1.63	19.52	39.00	4.00
II + I 10%	5.80	30.00	1.80	17.00	39.90	5.50
II + I 12%	5.00	30.00	1.85	16.55	41.00	5.60
II + I 15%	4.80	30.00	1.90	13.00	42.30	7.50
II + I 18%	4.50	28.50	2.05	15.15	42.30	7.50
II + I 20%	4.00	28.50	2.25	14.75	42.60	7.90

[a]Average of ten samples.
II represents lard fat, I represents beef fat, thus for example II + I 2% means concentration of lard in beef is 2%.
Source: El-Khalafy *et al.*, 1987 (reproduced with permission).

Table 6.19 Iodine values and the ratios between the fatty acid which can be used to check the addition of lard to beef fat

Sample	Iodine value	$C_{16:0}/C_{18:1}$	$C_{16:0}/C_{18:2}$
Beef I	37.13	0.824	18.913
Lard II	61.31	0.545	3.130
II + I 2%	39.62	0.810	11.650
II + I 5%	42.19	0.802	8.472
II + I 8%	43.93	0.770	7.500
II + I 10%	47.63	0.752	5.455
II + I 12%	48.85	0.730	5.360
II + I 15%	54.05	0.669	4.000
II + I 18%	53.66	0.670	3.800
II + I 20%	54.85	0.670	3.610

II represents lard fat, I represents beef fat, thus for example II + I 2% means concentration of lard in beef is 2%.
Source: El-Khalafy *et al.*, 1987 (reproduced with permission).

(COI) at the Institute de la Grassa, University of Seville, Spain. The methodology is a Quantitative Descriptive Analysis (QDA) (Stone *et al.*, 1974). The attributes included in the original COI test have of late been found not to represent the widening clientele of virgin olive oil amongst the European Communities. It has been considered necessary to re-evaluate the various parameters that determine consumer acceptability amongst different geographical regions. A detailed study of the consumer acceptability aspects have been carried out, statistical evaluation methodology has been developed and recommendations have been made to the Commission of European Communities on the modification of the COI test (Aparicio and Morales, 1995;

Aparicio *et al.*, 1994a; Aparicio *et al.*, 1994b; Bruggen *et al.*, 1995; Esposito and Peri, 1994; Lyon and Watson, 1994; McEwan, 1994; Pagliarini and Rastelli, 1994; Pallotta, 1994; Peri and Rastelli, 1994; Ranzani, 1994; Servili *et al.*, 1995).

Since every virgin oil has its own characteristic aroma, taste and appearance for which regional preferences have been known, the researches on olive oil may open up new studies on these aspects in the case of other oils, in particular coconut, groundnut, mustard, sesame, and so on.

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Chapter 7

Honey: Quality Criteria

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Honey: Quality Criteria

7.1 Introduction

Honey, the sweet viscous substance elaborated by the honey bee from the nectar of flowers, is perhaps the oldest sweetener discovered by the human race. This definition excludes honeydew honey, which is produced by the bee from honeydew excreted by the various plant sucking insects. Honey has widespread use in foods and pharmaceuticals as a sweetener. It is used as an ingredient in virtually hundreds of manufactured foods, mainly in cereal based products, for sweetness, colour, flavour, caramelization and viscosity. In processed foods, the word 'honey' in advertising and promotion carries a definite connotation of rich quality and 'old fashioned goodness' not conferred by any other sweetener.

7.1.1 Chemical composition and physical properties

The properties and composition of honey are known to vary widely depending on the region, season, variety of bee, plant source of nectar, period for which it is stored in the honeycomb, mode of harvesting and postharvest storage. Normally, honey contains 12.4–20.3% (mean 16.4%) moisture and 60.7–77.8% (mean 69.5%) sugars, of which about 0.2% may be sucrose, 25.2–35.3% (mean 30.3%) glucose and 33.3–43.0% (mean 39.1%) fructose. Up to 1–3% of higher oligosaccharides are reported (Sancho *et al.*, 1991). The ash content is about 0.03–0.04%, is usually alkaline in nature and mainly contains manganese, phosphates and sometimes borates. Honey does not contain any sulphates. Honeydew, however contains up to 73 mg/100 g sulphates expressed as sulphuric acid. Protein is low, usually under 1% (Nicholls, 1952). Other compounds present are organic acids and vitamins (B group and C). The amounts of vitamins are too low to be of any nutritional benefit (Skrokki and Ruottinen, 1994). Pollens, wax and fatty acids are amongst the water insoluable constituents that may occur. Fatty acid composition determined by GC–MS (gas chromatography–mass spectrometry) includes dodecanoic acid, 10-hydroxy-2-decanoic acid, 6,9-undecanoic acid, tetradecanoic acid, 12-(acetyloxy)-9-octadecanoic acid and 14-octadecenoic acid (Lee *et al.*, 1991). The pH of honey varies between 3.95 and 4.10, free acidity is about 30.49 mequiv kg^{-1}, lactone acidity about 10.74 mequiv kg^{-1}, and lactone acidity: free acidity ratio around 0.363. The pH changes linearly during storage (Sancho *et al.*, 1991).

Table 7.1 Standard colour designation of honey and range for each colour[a]

USDA colour standards	Colour range–Pfund scales (mm)	Optical density[b]
Water white	8 or less	0.0945
Extra white	8–17	0.189
White	17–34	0.378
Extra light amber	34–50	0.595
Light amber	50–85	1.389
Amber	85–114	3.008
Dark amber	over 114	–

[a]Based on USDA (1951) United States Standards for Graders of Extracted Honey, 4th issue, Agric. Mark. Serv., Washington D.C.
[b]Optical density (absorbance)=log10(100/percentage transmittance), at 560 nm for 3.15 cm thickness for caramel–glycerin solutions measured versus an equal cell containing glycerin.
Source: White, 1978 (reproduced with permission).

Table 7.2 Composition and quality factors for honey[a]

Compositional criteria[a]	
Apparent reducing sugar content, calculated as invert sugar:	
Blossom honey, when labelled as such	not <65%
Honeydew honey and blends of honeydew and blossom honey	not <60%
Moisture content:	not >21%
Heather honey (calluna)	not >23%
Apparent sucrose content:	not >5%
Honey dew honey, blends of honeydew and blossom honeys	
Robinia, Lavender and *Banksia menziesii* honeys	not >10%
Water insoluble solids content:	not >0.1%
Pressed honey	not >0.5%
Mineral content (ash):	not >0.6%
Honeydew honey and blends of honeydew and blossom honey	not >1.0%
Acidity:	not >40 meq/1000g
Diastase activity and hydroxymethyl-furfural content:	
Determined after processing, blending;	
diastase activity on Gothe scale:	not <8
Provided the hydroxymethylfurfural content is	not >40 $mgkg^{-1}$
Honeys with low natural enzyme content,	
e.g. citrus, diastase content on Gothe scale:	not <3
Provided the hydroxymethylfurfural content is	not >15 mg kg^{-1}

[a]From Codex Alimentarius Commission (1969) Recommended European Standard for Honey, CAC/RS-12-1969, Jt. FAO/WHO Stand. Program, Rome (reprinted in *Bee World* **51**: 79–91 (1970).
Source: White, 1978 (reproduced with permission).

The parameters ordinarily monitored by food manufacturers are moisture, colour, flavour and cleanliness. The standard colour designation of honey and the range for each colour are given in Table 7.1 (White, 1978). The essential composition and quality factors for honey (Molina, 1989) are given by Codex Alimentarius and are shown in Table 7.2 (White, 1978).

The physical properties and chemical composition of honey have been reported by many workers (White *et al.*, 1962; Siddiqui, 1970; Doner, 1977; Mesallam and El-Shaaraway, 1987). Earlier, compositional factors such as sugars, acids and ash were not considered so important for quality control, however of late some investigators believe them to be crucial. 'Ripeness' of honey is a measure of the extent to which the nectar has been processed by the honey bees. It is generally assessed on the basis of the content of the substances secreted into the honey by the bees. These include enzymes such as saccharase, diastase and glucose oxidase, and proline. Proline concentration in association with other constituents can be used for the evaluation of the 'ripeness of honey' (Ohe *et al.*, 1991). Amylase activity has assumed significance as a quality factor as is evident by its inclusion in Codex Alimentarius, although its origin is as yet not clearly understood. Glucose oxidase activity as a measure of honey quality has also been proposed, although a wide variation is observed in authentic honey. Cluster analysis applied to physicochemical parameters has indicated a relationship between free acidity, total acidity, fructose, glucose and diastase activity. Proline content and reducing sugars also form a cluster (Sancho *et al.*, 1991f).

7.1.2 Texture of honey

Honey usually contains a crystal phase and a syrup phase. The amount of the crystal phase is dependent on the ratio of sugars and the water content. The texture is dependent on the ratio of the phases and on the water content. It is essential that any crystallized dextrose should be of as fine a particle size as possible. Poor graining immediately after manufacturing or storage can give rise to clumping of crystals and abnormal appearance of white patches. Grained honey must be warmed and stirred before use for the manufacture of sugar confectionery. Grading of honey dependent on the amount of crystallization has been proposed and is shown in Table 7.3.

The possibility of graining developing in honey is increased as the sucrose and dextrose content rises. Crystallization is less likely to occur when the honey contains more of the higher sugars. A correlation between crystallization and the ratio of dextrose content:water content (D:W) has been observed (Sancho *et al.*,1991h) and is also shown in Table 7.3. Austin and Jamieson (1953) found that the texture and therefore the crystallizability of honey was related to the water content and the ratio of laevulose content:dextrose content (L:D) (Sanz *et al.*, 1994). Optimum conditions for recrystallized honey are an L:D ratio of 1.14 to 1.00 and a moisture content of 16–17%. The crystallization can be inhibited by addition of some fatty acids such as 0.3% isobutyric acid or sorbic acid, or by treatment in a sonic apparatus for 15–20 min

Table 7.3 Grading of crystallized honeys

Crystallization grading (C)	Dextrose/ water	Description
0	1.58	No crystals
1	1.76	A few scattered crystals
2	1.79	Crystal layers 1/16–1/8 inch
3	1.86	A few clumps of crystals
4	1.83	Crystal layer 1/8–1/4 inch
5	1.99	Crystal layer 1/4 inch
6	1.98	Crystal layer 1/2 inch
7	2.06	Crystal layer 3/4 inch
8	2.16	Complete soft granulation
9	2.24	Complete hard granulation

Source: Lees, 1975.

at 9 kc s^{-1} (kilocycles per second) on storage at 15 °C (E.V.A., 1959).

The quality of honey is discussed below in terms of:

1 Honey adulteration
2 Differentiation of honey from that produced from sugar-fed bees
3 Identifying the botanical/geographical origin of authentic honey.

7.2 Adulteration of honey

A natural product of limited supply and relatively high price, honey has always been a target of adulteration. Many adulterants including acid-inverted syrups, corn syrups, sugar, starch and dextrin, syrups of natural origin including maple, sugarcane, sorghum, mahua (*Madhuca butyraca*) flowers, molasses and hydrol are reported in literature. Analysis by GC, high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) has been tried to detect adulteration. TLC is simple and reliable (Allegretti *et al.*, 1987). Tests using a copying pencil (Chudakov, 1973a) and blotting paper (Chudakov, 1973b) are reported, but are considered to be of little diagnostic value. A Russian patent gives a technique which is claimed to be more efficient in detecting honey adulteration (Aganin, 1984). The procedure consists of making a 1:1 aqueous solution of honey, boiling for 1–3 minutes and observing visually for flake formation, sedimentation and illumination of the supernatent. Turbidity values ranging from '−' to '+++' are reported to reveal adulteration with glucose, sucrose and/or invert sugar.

7.2.1 Adulteration with acid inverted syrups

Detection of invert syrup in honey has been a problem for more than a century. Addition of moderate amounts of invert syrup does not cause fructose and glucose levels to fall outside the normal range for honey. Qualitative colour tests depend on the detection of hydroxymethylfurfural (HMF) which is produced by heating in the presence of acid for inversion of sucrose. The presence of 5% invert sugar prepared by acid hydrolysis can be detected by determination of HMF spectrophotometrically (Serra and Gomez, 1986a), by the bisulphite method (White, 1979a, 1979b), by UV spectrometry or HPLC (Serra, 1991). This eliminates the need for complete carbohydrate analysis (White, 1980b, 1980c). Recognition that HMF can arise from heating or storage (Ghoshdastidar and Chakrabarti, 1992; Serra, 1991; Perez-Arquillue *et al.*, 1994) has, however raised doubts about the validity of these tests (Schade *et al.*, 1958; Gautier *et al.*, 1961; Hadorn and Zurcher, 1962; Hadorn and Kovacs, 1960; White *et al.*, 1969; Gonnet, 1963). Honey is processed by heat and straining or pressure filtration to delay granulation and to eliminate yeast spores. The exact procedures differ among packers and would be expected to have variable effects on HMF content. Analysis for HMF in honey before and after processing has shown that fresh samples containing about 410 μg/100 g revealed a mean rise of 850 μg to 1250 μg HMF after melting in a hot oven, settling, bottling and storage for 9 days. The value rose to about 3200 μg after storage for one year (White and Siciliano, 1980). Data from 1728 analyses of HMF in commercial honey samples from four laboratories between 1960 and 1974 showed an average HMF content of 1.24 mg/100 g. European quality standards allow a maximum of 4.0 mg/100g. The HMF content of citric acid catalysed invert syrup is 170–650 mg/100 g. A suggested level of 20 mg/100 g allows selection of honey, possibly adulterated with acid converted invert syrup (White, 1980b). Values for HMF in the range of 50 mg/100 g are considered to be conclusive proof of the presence of acid invert syrup in honey (White and Siciliano, 1980). Honey produced in subtropical climates has high HMF values, exceeding 4 mg/100 g, the maximum standard for HMF in the EU. Similarly, room temperature storage leads to an increase in HMF, whereas cool storage retards it. HMF can be estimated by spectrophotometric (Dhar and Roy, 1972; White, 1979a, 1979b; White *et al.*, 1979; White and Siciliano, 1980; Wootton and Ryall, 1985; Lord *et al.*, 1989) or by chromatographic (Jeuring and Kuppers, 1980; Marini and Righi, 1985; Wootton and Ryall, 1985) techniques.

7.2.2 Adulteration with corn syrup

Corn syrup or dextrose syrup added to honey can be recognized easily from the lowering of the laevulose:dextrose ratio. However the commercial availability of high fructose corn syrup (HFCS) of late has rendered the task of detecting its addition much more difficult. HFCS resembles honey more closely in composition with regard

Table 7.4 ^{13}C values of various nectar honeys (NH1)/syrup mixtures

NH 1/CS[a] 1 (%)[b]	^{13}C (%)	NH 1/CS 2 (%)[b]	^{13}C (%)
00.00	−5.05	1.10	−25.33
1.43	−24.47	5.03	−24.63
6.18	−24.13	9.36	−23.85
9.32	−22.80	18.51	−22.23
17.23	−20.78	34.69	−20.15
313.91	−20.30	50.01	−17.58
50.32	−18.41	100.00	−10.84
100.00	−10.95		
NH 1/HFCS[c] 1 (%)	^{13}C (%)	NH 1/HFCS 1[b] (%)	^{13}C (%)
1.90	−24.86	1.04	−25.19
4.84	−24.84	5.26	−25.17
8.80	−24.29	9.47	−24.90
16.27	−23.39	17.83	−24.84
37.80	−20.57	34.13	−24.31
49.83	−17.98	10.14	−23.67
100.00	−10.11	100.00	−22.22

[a]CS = corn syrup.
[b]Number represents percentage of syrup in the mixture.
[c]HFCS = high fructose corn syrup.
Source: Lipp *et al.*, 1988 (reproduced with permission).

to major components and is also more highly refined. Methods of HFCS manufacture have been evolving and trace constituents unique to syrups may be eliminated by the refining processes. In spite of extensive studies to detect high fructose corn syrup, it has not been possible to identify any unique trace indicator compounds.

A recent approach is based on the difference in the ratio of ^{13}C to ^{12}C in the samples of honey and corn syrup (Doner *et al.*, 1979a; White and Doner, 1978b; Clarke, 1988; Rossmann *et al.*, 1992; White, 1992). Corn syrups are slightly enriched in ^{13}C as compared to honey. This difference is caused by the fractionation of carbon isotopes during photosynthesis (Smith and Epstein, 1971; Bender, 1971). Nectar bearing flowering plants are almost exclusively C_3. The technique of isotope ratio mass spectrometry (IRMS) has been used to analyse genuine honey (White and Doner, 1978a). Table 7.4 shows the ^{13}C values of various nectar honeys (NH 1)/syrup mixtures.

The ^{13}C values of genuine honey are between −23 and −28 parts per thousand (ppt), whereas for HFCS, it is around 10 ppt. Honey with a ^{13}C value less negative than −21 ppt is considered to be adulterated with HFCS. This detection is generally done by stable isotope ratio analysis (SIRA) using MS. Under the United States Department of Agriculture (USDA) loan purchase programme, honey is compulsorily

tested for adulteration by the isotope ratio method (White and Doner, 1978a; AOAC, 1984). It was however realized that a ^{13}C value less than -21.5% was too liberal a limit, allowing up to 15% added corn syrup in honey with a ^{13}C value of -25.4.

A TLC method for high fructose corn syrup in honey has been developed, which is more sensitive than the isotope ratio method (Kushnir, 1979). This is based on the presence of higher molecular weight oligosaccharides and maltodextrins of varying molecular weight in high fructose corn syrup (HFCS) (Mermelstein, 1975). The method is sensitive at HFCS levels of 10% or even less. An added advantage of this procedure is that it detects in honey the presence of all starch derived sugar syrups, regardless of the plant source. A survey of isoglucose adulteration in honey has been made. Suitably modifed TLC methods can detect this sugar (Sangiorgia, 1988). It was subsequently decided to use TLC methods when the ^{13}C value is in the 'grey region', that is, between -21.5 and -23.4 (White, 1980a; AOAC, 1980, 1984).

An additional problem with a ^{13}C limit of -23.4% is that there are certain honey types which normally have a ^{13}C at or lower than this, for example citrus honey, which has shown (White and Robinson, 1983) to average -23.8%, (standard deviation, $s=0.96$) and honey from mesquite (Prosopis spp.) and related plants (Clarke, 1988) that are significantly less negative than the average of all honeys ($^{13}C=-25.4\%$, $s=0.98$) (White and Robinson, 1983). Thus certain genuine samples of citrus honey could be mistakenly branded as 'adulterated'. Identifying the botanical origin of a honey may thus be essential. Using the difference in stable carbon isotope ratio between a honey and its protein fraction permits objective evaluation of possible adulteration of honey with small amounts (7–20%) of corn or cane sugar. The present uncertainity in interpretation of results from pure honeys with ^{13}C values outside the generally accepted limits for pure honey (-27.5 and -21.5 ppt) is eliminated; likewise TLC testing to resolve questionable samples with ^{13}C values between -23.5 and -21.5 ppt is not needed. Tests on analysis of 50 certified samples of pure honey and 38 other samples with ^{13}C values in the 'questionable' range have suggested a difference of ≥ 1.0 ppt between honey and protein fractions as indicative of adulteration (White and Winters, 1989).

^{13}C analysis uses IRMS with a multicollector for simultaneous measurement of $^{13}CO_2$ and $^{12}CO_2$ and a dual capillary inlet to allow frequent comparison with a reference. With careful combustion of samples to CO_2, these instruments can achieve a precision (standard deviation $<0.05\%$) in excess of that required for honey analysis. Manual preparation restricts the testing to around 20 samples per day and introduces a source of error. An alternative technique is the continuous flow IRMS, a technique of dry combustion in a helium carrier that transfers sample derived N_2 or CO_2 directly to the IRMS (Preston and Owens, 1983). Now known as the automated ^{15}N, ^{13}C analysis mass spectrometry (ANCA–MS), it has been recently reviewed (Barrie *et al.*, 1989; Barrie and Lemley, 1989). The ANCA–MS procedure combusts and analyses 66 samples in a batch and takes only 5 min per sample. A comaprison of IRMS and ANCA–MS using honeys containing from 0 to 100% HFCS gives a good agreement within 0.2%. It is

believed that ANCA–MS should be of particular value in quality control and to the investigative laboratory required to monitor foods and flavourings for authenticity. The technique is fast and easy to learn and can be applied to any liquid or solid sample that can be dispensed into a combustion capsule (Brooks *et al.*, 1991).

Most HFCS is produced from maize, but it can also be derived from potato or cassava starch (a C_3 plant product) which would not be detected by SIRA, so that this technique has to be used in conjunction with another test, for example amino acid analysis for proline or gel electrophoresis (Croft, 1987). Apart from honey, adulteration of many products such as alcoholic beverages, eggs, vinegar and flavourants can be detected by measuring the ^{13}C:^{12}C ratio in the samples (Clarke, 1988; Winkler and Schmidt, 1980). Although the identification is rapid, the method is expensive and requires special equipment (Doner *et al.*, 1979a).

Knowledge of the carbohydrate composition of a sample of honey is useful in judging the authenticity. Although a large body of compositional data is available for United States honey (White *et al.*, 1962), its utility is somewhat limited because of the complexity of the analytical procedures needed to obtain it. Since United States honey is an extremely variable and complex mixture of sugars and other components (White, 1975; 1978), the relatively facile GLC and HPLC methods have only limited application in studies of its composition. The presence of at least 22 di- and trisaccharides severely limits attempts at quantitation (Doner, 1977). Table 7.5 shows a general carbohydrate analysis of honey (White, 1980c). The composition of honey depends on two important factors, the floral source and the composition of the nectar. Less important are certain other external factors such as climate and differences in processing. A procedure for detecting adulteration of honey based on isolation of oligo- and polysaccharides by low pressure or low vacuum liquid chromatography, quantification by HPLC and checking the results on TLC is reported to detect adulteration levels at <5% (Lipp and Ziegler, 1989). Concentration of the higher saccharaides characteristic of syrups by medium pressure liquid chromatography (MPLC) using a charcoal/celite mixture, followed by measuring the sugars by a

Table 7.5 Carbohydrate analysis of honey

Sugar	Mean (%)	standard deviation *s* (%)	coefficient of variation (%)	Range (%)
Monosaccharides	69.71	4.13	5.92	58.00–79.95
Disaccharides	8.62	2.08	24.1	3.29–18.16
Glucose	30.31	3.04	10.03	22.89–40.75
Fructose	8.38	1.77	4.61	30.91–44.26
Fructose/glucose	1.229	0.126	10.2	0.76–1.86
Sucrose	1.31	0.87	66.4	0.25–7.57
Higher sugars	1.36	1.11	81.6	0.13–3.85

Source: White, 1980 (reproduced with permission).

refractometer connected to a reversed phase liquid chromatography allows for a sensitive, rapid and quantitative detection of adulteration in honey (Lipp *et al.*, 1988). Since higher oligosaccharides indicative of honey adulteration occur in high fructose syrups (HFS) in very small amounts (average 1.4%, range 0.3–2.3%), they must be concentrated for analysis. Monosaccharides, which occur frequently and cause overloading of columns and plates, have to be separated. Both steps can be acheived in a relatively short time using MPLC. Although they contain considerably more higher oligosaccharides (average 26%, range 16–40%), conventional syrups can still be analysed by MPLC, giving a more sensitive detection. Figure 7.1 shows a TLC chromatogram in which spots are expressed by their R_f values. It has been observed that there are generally distinct blue spots in HFS, which do not appear in authentic honey. Generally honeys are characterized by brownish red spots and syrups by blue spots (Lipp *et al.*, 1988).

A test which was conceived as useful to detect HFCS in honey was based on analysis

				78.5	77.9				
				75	73.2				
65.5	66	66.6	66.6	65.4	64.8				
63.1	63.6	63	63			63			
60.4	60.7			61.3	60.7		61.3	62.5	
	57.1	58.9	58.9	58.3	57.1	59.5	58.9	59.5	
						56.5	55.9	55.9	
54.2	54.7	54.7	54.7	54.7	53.5	53.5	52.9	52.4	
49.7	50	50	50	48.8	48.2	50.6	50	49.4	
						47.6	47		46.4
						44			
							42.2	41	
38.7	38.7			39.8	39.2	40.4			
						36.9	37.5	35.7	
32.7	32.7						32.7	30.9	
				28.5	28.5		28.5	26.8	
25	24.4						24.4		
19.6	19								
13.1	14.2			13	13.6				
	11.3								
						5.9	5.9		
				2.9	2.9				
1	2	3	4	5	6	7	8	9	

Saccharides

Figure 7.1 Thin layer chromatogram of saccharides. Spots expressed by R_f values (×100). 1, Honeydew honey 1; 2, honeydew honey-2; 3, nectar honey-1; 4, nectar honey-2; 5, HFS (corn); 6, HFS (wheat); 7, CS-1 (corn); 8, CS-2 (corn); 9, Maltooligosaccharides (degree of polymerization, DP 2–11). *Source*: Lipp *et al.*, 1988 (reproduced with permission).

of psicose, formed in HFCS at a 1% level due to base catalysed isomerization of fructose (Doner, 1978). This sugar is not present in honey and HPLC methods are available to detect its presence. However, recent improvements in the manufacture and refining of HFCS have made it possible to eliminate this sugar.

The unusually high degree of branching present in polysaccharides of some HFCS samples was made the basis for detecting honey authenticity. The soybean lectin. concanavalin A associates with terminal glucose units in branched polysaccharides through a multivalent interaction and leads to precipitation and quantification by turbidity. This method has also been rendered useless due to the availability of HFCS containing no high molecular weight carbohydrate polymers (Doner *et al.*, 1979a).

In a complex mixture, polarimetric analysis is of limited value, although it has been shown to quantify glucose and fructose in the monosaccharide fraction of honey from the charcoal column. Analysis of oligosaccharides by chromatographic separation on a charcoal/celite column and elution with 50% alcohol, and further analysis on TLC using silica gel G plates with n-butanol/acetic acid/water (2:1:1) as the mobile phase and detection by warming with aniline/diphenylamine hydrochloride/phosphoric acid reagent can also detect adulteration of honey with corn syrup. TLC can be substituted by paper chromatography (Strong and Duarte, 1985). Corn syrup can also be detected by determination of maltodextrin after precipitation of proteins at pH 4.2 (Serra and Gomez, 1986b). HFCS (at >30%) can be determined by gas chromatography for maltose and isomaltose (Serra and Gomez, 1986b). Values of maltose, isomaltose and isomaltose:maltose ratio are reported to be 1.93, 0.64 and 0.34, respectively for domestic honeys (80 samples), 2.17, 0.87 and 0.39 for imported samples (35 in number), and 0.72, 1.50 and 2.09 respectively for HFCS (21 samples) (Doner *et al.*, 1979b)

Indices such as maltose:isomaltose ratio have been proposed for detecting honey adulteration with syrups, but are of little value. A discriminatory equation [$D=2.73-5.35$ (isomaltose:maltose)] has been developed from the data on maltose and isomaltose, which could correctly classify 81% of authentic honey samples and 78% of adulterated honey samples (based on ^{13}C analysis) (Doner *et al.*, 1979b). The sample is classified as adulterated when $D<0$ or >0.51 and as genuine honey when $D>0$ and <0.51. Instead, determinations of sucrose, erlose and melezitose are believed to be more useful (Tunin *et al.*, 1987). The sugar composition of honey has been recently found to be correlated with many compositional properties of honey. Reductions in dry matter, viscosity, sodium, potassium, proline and nitrogen can be taken as good indicators for adulteration of honey with HFCS. It is also reported that increase in moisture, ash, calcium and hydroxymethylfurfural can be considered as simple and rapid tests for adulteration levels ranging between 10% and 50 % (Abdel-Aal *et al.*, 1993).

Tests describing additional steps to eliminate false positives caused by honeydew honey have been based on using a 50% alcohol residue test wherein any value greater than 15 mg and found positive by TLC tests is indicative of honeydew honey. If

Table 7.6 Validity of procedures for detecting mixtures of HFCS in honey

Method	Constituent determined	Found to be applicable
Differential scanning calorimetry	Organic compounds	No
Gas–liquid chromatography ratio	Isomaltose/maltose	Yes
Gel filtration, affinity chromatography	Polysaccharides	No
High pressure liquid chromatography	Monosaccharides (psicose)	No
Immunodiffusion	Polysaccharides, proteins	No
Stable carbon isotope ratio analysis	$^{13}C/^{12}C$ ratio	Yes
Thin layer chromatography	Dextrins, polysaccharides	No
Turbidimetry (with concanavalin A)	Polysaccharides	No
Atomic absorption spectroscopy	Sodium/potassium ratio	No
Colorimetry	Proline	No

Source: Doner *et al.*, 1979a.

suspected, samples could be eluted first by 25% alcohol to eliminate the natural material responsible for the false positive test without affecting the material from the HFCS. It is also documented that since most honeys do not contain honeydew honey (White, 1980a), the requirement of weighing all 50% alcohol residue is unnecessary. Furthermore, it is also possible that some honeydew containing honey may have less than 15 mg residue. Another notable fact is that about 40% honeys contain >1.5% higher sugars, corresponding to a 15 mg residue in the TLC test (White *et al.*, 1962).

A suggestion had been made (Shallenberger *et al.*, 1975) that examination of the sodium:potassium ratio may be useful because HFCS is refined by ion-exchange treatment, and the original cations present in HFCS are replaced by sodium. Honey has long been known to be relatively poor in sodium but rich in potassium. Limited analyses indicate the Na:K ratio in honey to be very different from that in isomerized syrups (K=about 40×Na in honey, Na=10−20×K in syrups) and could be a useful analytical index in detecting adulteration (Fine, 1975). Evaluation of literature data (White, 1977), however, demonstrated that the sodium:potassium ratio is of little use as the sole parameter because of the extreme variability of these values in honey.

Factors such as acidity (free, lactone and total), protein (White and Rudyj, 1979) and amino acid composition, particularly proline (White and Rudyj, 1978) and phenylalanine, could also provide a clue to adulteration of honey with enzymically inverted sugar (Mavrikos *et al.*, 1978). Although a convenient colorimetric method has been available to estimate proline, the wide range of values found in genuine honeys precluded its use as an indicator of honey purity. For instance, proline in Alberta honey has been found to be <200 ppm, which has incorrectly been used as a minimum level for honey authenticity by some honey importers (Sporns *et al.*, 1992). Low proline concentration is also reported to be indicative of heat treatment or poor storage (Lungo *et al.*, 1993). The total acidity in honey indicates the history of the honey

sample and the possibility of fermentation (Rodgers, 1979). The pH of honey is not related to the total acidity because of the buffering action of the various acids and minerals present. An immunochemical approach based on elicitation of antibodies obtained on injection of HFCS materials and protein concentrates from HFCS has also been tried, but has proved to be fruitless. Similar disappointing results have been obtained from differential scanning calorimetry, which gave almost identical profiles for honey and HFCS. The approaches for detection of mixtures of HFCS and honey are summarized in Table 7.6 (Doner *et al.*, 1979a).

7.2.3 Other adulterants

Adulteration of natural bee honey with commercial sugar can be ascertained on the basis of specific rotation. However, this is possible only if the analysis is carried out within 90 days of addition (Nicoletti, 1987). The values of optical rotation for authentic honey are $-12°$ to $+3°$. The method is good and rapid for quality analysis of honey. This adulteration can also be detected by chromatographic separation of bisulphite complexes of fructose and glucose (Chepurnoi and Dmitrenko, 1987). Honeydew samples can also be identified conventionally by polarimetry. The advisory Food and Drug Adminstration (FDA) definition for honey requires that it be levorotatory. Thus a significant (>5%) amount of melezitose is a confirmatory negative test for a dextrorotatory sample that tests negative for conventional corn syrups or added sucrose. Molasses at >5% can be determined by sucrose and ash analysis (Serra and Gomez, 1986a). Isoglucose at >10% indicates adulteration of honey and it can be easily detected by TLC or HPLC, with TLC being preferred for routine analysis (Sangiorgi, 1988). Starch, dextrin and sorbitol have also been identified as major adulterants, which have been confirmed by GLC and TLC (Shimokawa *et al.*, 1970).

7.3 Honey obtained from sugar-fed bees

It is believed that natural floral honey should contain >90% invert sugar in dry matter and sucrose should be <2% by weight, and that honey not satisfying these criteria be considered unripe, made by bees fed sugar syrup or adulterated with syrup (Gensitskii, 1974). However, adulteration of natural honey with that of bee-inverted the sucrose cannot be ascertained on the basis of sucrose content, since during two months of storage, sucrose content of the latter decreases to the level of the former. The bee-inverted sucrose does not have lower amino acids (particularly proline) and ash as well as lower electrical conductivity, which could serve as indicators of adulteration (Rybak and Achremowicz,1988).

Gel filtration, ion exchange chromatography and starch gel electrophoresis of proteins in honey can differentiate floral honey and honey obtained from sugar-fed bees (White and Kushnir, 1967a; Bergner and Diemair, 1975). A larger number of

components in authentic honey differentiate it from honey obtained from sugar feeding.

Starch gel electrophoresis of α-glucosidase preparations can also differentiate between genuine honey and that from stores of sucrose-fed bees (White and Kushnir, 1967b). Acid phosphatase activity in honey from sugar fed bees is one-sixth that of pure honey and can be used as a criterion for differentiating between them (Zalewski, 1965).

7.4 Identifying the botanical/geographical origin of authentic honey

Earlier attempts at distinguishing honey from artificial blends had been based on the volume of precipitate of honey proteins with tannin (Lund, 1909), phosphotungstic acid (Lund, 1910) and/or alcohol (Laxa, 1923). Sediment content has been reported to be a good guide to the floral origin of honey (Sancho *et al.*, 1992b). The value has been found to be between 14 and 99 μl/100 g with a mean of 35.8±1.88 μl/100g. However, this parameter cannot be used in isolation, since dirty and pressed honeys give smaller sediment contents (Sancho *et al.*, 1991b). Pollen analysis has been used to distinguish honey types such as *Eucalyptus*, *Citrus*, *Paspalum*, *Cecropia*, *Rubus*, *Lotus corniculatus*, *Anarrhinum*, etc. (Jato *et al.*, 1991; Ramalho *et al.*, 1991; Sancho *et al.*, 1992c; Bano Breis *et al.*, 1993; Sancho *et al.*, 1991c; Jhansi *et al.*, 1991; Kerkvliet and Beerlink, 1991). For instance, a minimum of 10% *Lavandula stoechas* pollen and maximum of 30% *Echium* sp. pollen are the suggested requirements for characterizing French lavender honey (Bonvehi and Coll, 1993). Immunological tests on honey proteins were attempted as early as 1915 (Langer, 1915; Thoni, 1913) and proteins of hand collected pollen were isolated.

As early as 1927, Tillmans and Kiesgen (1927) proposed formol titration, which gives a measure of total amino acids, to authenticate honey. The relationship between botanical origin of a honey and formol number has been investigated on 319 Dutch honey samples. The values obtained were 0.35–1.25 for white clover honey, 1.5–3.05 for heather honey, 3.0–3.5 for buckwheat honey and only 0.1 for artificial honey. These could serve as tentative indices for identification of the botanical origin of honey (Boer, 1947). Certain ratios of various amino acids such as amides:phenylalanine and aspartic acid:proline could demonstrate their utility in determining the geographical source of honey (Davies, 1975). This ratio has been modified by using a computer aided selection of 60 amino acid ratios (Davies, 1976). However, recent reports indicate that proline content and formol value are not reliable parameters for correct identification of the geographical origin of honeys (Sancho *et al.*, 1991k). Sugar analysis, particularly the glucose:fructose ratio can primarily differentiate between acacia and rape honeys, the ratios being 1.33–1.39 for acacia and 0.90–0.93 for rape. The corresponding ratios for other honey types are 1.03 for linden, 0.99–1.01 for floral , 1.14–1.21 for heather and 1.04 for honeydew. The high disaccharides in acacia (7.62–10.25%) and tri- and oligosaccharides in honeydew honey (2.15–6.22%) also serve to distinguish them from

other botanical types. Therefore a fingerprint of the sugar spectrum could be a probable aid in identifying honey type, and could be acheived by separation on Bio-gel P2 (Krauze, 1991).

Pattern recognition techniques, which have been applied to a wide variety of problems in food chemistry such as geographical classification of olive oils (Derde *et al.*, 1984), concentrated orange juices (Bayer *et al.*, 1980) and wines (Kwan and Kowalski, 1978; 1980a, 1980b; Maarse *et al.*, 1987; Vasconcelos and Chaves das Neves, 1989) have also been shown to distinguish honeys successfully on the basis of their geographical origin (Crecente and Latorre, 1993). Humidity and free acidity have been found to be the most important features for the classification. In such cases, the use of pollen data to acheive a correct geographic classification of the honey samples is not necessary. Apart from this, principal component analysis (PCA), linear discriminant analysis, (LDA) (Sancho *et al.*, 1991a), K nearest neighbour (KNN) and soft independent modelling of class analogy (SIMCA) of the chemical data permits the differentiation between geographic origins of honey samples (Crecente and Latorre, 1993; Lopez *et al.*, 1996). Cluster analysis can indicate the floral origin of honey (Sancho *et al*, 1991g).

Honey phenolics may be divided into three groups (Amiot *et al.*, 1989), benzoic acid and its esters, cinnamic acid and its esters and flavonoid aglycones. The proportion of these three groups varies greatly in honeys with different floral origins.

The distribution pattern of phenolic acids allows differentiation between honeydew, chestnut and blossom honey. The concentration of 2-hydroxybenzoic acid in chestnut honey is 10 times greater than in blossom honeys, while 4-hydroxybenzoic acid is helpful in identifying chestnut honey, the levels being three times higher than in other types and 3,4-dihydroxybenzoic acid identifies honeydew honeys. Similarly 4-hydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid can be used to identify chestnut honey (Joerg and Sontag, 1992). These can all be sensitively estimated by HPLC (Bernwieser and Sontag, 1992). Further investigations need to be carried out to differentiate amongst the various types of blossom honeys.

Phenolic esters have recently been shown to be valuable in characterizing different honey types. The content of various phenolic esters in some honey types, as determined by reversed phase chromatography coupled with a coulometric electrode-array detection system is shown in Table 7.7. It can be seen that rape honey is characterized by a very high level of methyl syringate. Similarly the content of methyl 4-hydroxybenzoate is higher than in other types except for orange blossom honey; the absence of methyl syringate is another unique characteristic of orange blossom honey. Fir honey has a very high level of *trans*-methylferulate, enabling its use as an index for identification of this floral type. Rosemary honey is reported to contain kaempferol-3-sophoriside (93%) and quercetin-3-sophoriside (7%) as the only significant constituents. However, researchers caution that the presence of kaempferol in rosemary honey cannot be considered as proof of its floral origin since this flavonoid can originate from different flower nectars. However, its absence or presence in small amounts

Table 7.7 Content of phenolic esters in honey (μg/kg)

Variety	M4HB	MVAN	MSYR	MCOM	MFER
Robinia	—	—	281.0	—	—
	—	3.5	93.4	—	—
Rape	126.2	—	31.3	—	3865
	—	—	—		
	184.8	237.4	5044		
Chestnut	18.0	10.9	42.2	—	—
Clover	38.3	11.8	1592	—	—
Linden blossom	7.7	20.2	759.5	—	—
Dandelion	—	11.8	551.5	—	1.3
Sunflower	31.8	8.0	322.7	1.5	2.9
Orange blossom	194.2	18.8	—	—	7.9
Fir	—	14.7	539.5	—	1196

M4HB=Methyl 4-hydroxybenzoate; MVAN=methyl vanillate; MSYR=methyl syringate; MCOM=*trans*-*p*-methyl coumarate; MFER=*trans*-methyl ferulate.
Source: Joerg and Sontag, 1993 (reproduced with permission).

(<0.3 μg g^{-1} honey) could be considered as additional evidence of a different floral origin (Gil *et al.*, 1995).

The ratio of flavonoids to total phenolics of 44 sunflower honeys from different regions are reported to be not significantly different (Sabatier *et al.*, 1988), indicating that the flavonoid content is a promising indicator of origin of floral honey. Flavonoids serve as biochemical markers of the plant origin of bee pollen (Garcia-Viguera, 1991; Ferreres *et al.*, 1992; Tomas-Barberan *et al.*, 1989) and could be analysed by GC/MS (Berahia *et al.*, 1993). Amongst the flavonoids identified in honey are pinocembrin (5,7-dihydroxyflavanone), pinobanksin (3,5,7-trihydroxyflavanone), chrysin (5,7-dihydroxyflavone), galangin (3,5,7-trihydroxyflavone) and quercetin (3,5,7,3′,4′-pentahydroxyflavone). Two other minor flavonoids detected are tectochrysin (5-hydroxy-7-methoxyflavone) and kaempferol (3,5,7,4′-tetrahydroxyflavone) (Sabatier *et al.*, 1992). The flavonone, hesperitin (5,7,3′-trihydroxy-4′-methoxyflavanone) has been detected only in citrus honey samples. Its presence in orange anthers has been shown by HPLC analysis. Hesperidin is the major flavonoid detected, suggesting that this could be another source of hesperitin in honey. Studies of honey samples containing from 95 to more than 75 000 pollen grains per 15 g honey have not revealed any significant differences in the relative amounts of hesperitin, indicating that nectar is the main source of honey hesperitin, presumably produced by hydrolysis of hesperidin by the bee enzymes. HPLC analysis of hesperitin could be used along with other techniques in the determination of the floral origin of citrus honey (Ferreres *et al.*, 1994, 1993). It is believed that further studies on flavonoid structures would provide an index of floral origin of honey.

The flavonoid pattern is believed to be more useful in determination of geographical

Table 7.8 Relative amounts of propolis-derived and pollen nectar-derived flavonoids in honey

Honey sample	Propolis flavonoids (%)	Pollen nectar flavonoids (%)
Spain[a]	45–52	45–48
Italy[b]	44–64	25–45
Germany	21	70
China	14	84
Canada	20	78
USA	44	52
Mexico[c]	15–17	75–84
Cuba	15	82
Caribbean	7	87
Costa Rica	0	98
Brazil	0	99
Argentina[c]	14–18	81–80
Chile	29	69
New Zealand	62	32
Australia	0	98

[a]Range for 5 samples.
[b]Range for 8 samples.
[c]Range for 2 samples.
Source: Tomas-Barberan *et al.*, 1993 (reproduced with permission).

origin than in botanical origin studies (Ferreres *et al.*, 1992). A close correlation between the HPLC flavonoid patterns of honey and propolis has been found, supporting this belief. Propolis is a natural constituent of honeycombs and has components distributed in relatively lipophilic beeswax and the more hydrophilic honey. As a general rule, honey samples from the Northern hemisphere show flavonoid patterns characteristic of propolis flavonoids. In contrast samples from most equatorial regions and Australia show only flavonoids from other plant sources. However, several honey samples from Central and South America and from New Zealand do contain flavonoids characteristic of propolis, indicating that imported *Apis mellifera* colonies may locate poplar trees and occasionally an imported specimen may be found in gardens (Tomas-Barberan *et al.*, 1993) (Table 7.8).

The chemical composition of honey flavour is of great interest for tracking its geographical and floral origin. A carbonyl compound, identified as (*R*/*S*)-dehydrovomifoliol [4-cyclohexene-3-one-6-hydroxy-1,1,5-trimethyl-6-(3-oxo-1-butenyl)] is an oxidation product of α-ionone, which in turn is an intermediate in the synthesis of the plant growth and germination inhibitor-abscisic acid (Roberts *et al.*, 1968). (*S*)−(+)-dehydrovomifoliol has been found to be a naturally occurring component in the aerial parts of a sunflower species (Herz and Bruno, 1986), the roots of kidney beans (Takasugi *et al.*, 1973), the seeds of pumpkins (Fukui *et al.*, 1977) and rice husk (Kato *et al.*, 1977). The (*S*)−(+)−dehydrovomifoliol content has been shown to be

Table 7.9 (S)-(+)-Dehydrovomifoliol content in heather honey and honeys of other floral origin

Honey			Content (mg kg^{-1})
Heather honeys[a]			
French	heather,	Calluna	264
French	heather,	Calluna	210
French	heather,	Calluna	208
French	heather,	Calluna	186
Spanish	heather,	Ericaceae	56
Honeys of other varieties[a]			
Australian eucalyptus			6.02
French	chestnut		5.39
	Lime		1.67
German	rape		1.51
Russian	buckwheat		1.37
	acacia		0.40
Spanish	orange blossom		0.19
French	sunflower		0.03

[a]Specified by pollen analysis.
Source: Hausler and Montag, 1989 (reproduced with permission).

56-264 mg kg^{-1} in heather honeys, whereas the levels in honeys of several other floral origins have been shown to be 33 μg kg^{-1} to 6 mg kg^{-1}. The wide difference between the (S)-(+)-dehydrovomifoliol content of heather honeys and that of other floral origin (Table 7.9) offers the possibility of determining adulteration of heather honey (Hausler and Montag, 1989).

Some workers have suggested that methyl anthranilate be used as an indicator compound to distinguish citrus honey from other monofloral or multiflora1non–citrus ones (Deshusses and Gabbai, 1962; Dorrscheidt and Friedrich, 1962; White *et al.*, 1962; Hoopen, 1963; Merz, 1963; Cremer and Reidemann, 1965; White, 1965; Chogovadze *et al.*, 1973; Wootton *et al.*, 1978; Graddon *et al.*, 1979; Bicchi *et al.*, 1983; Serra, 1988). It gives a distinctive and pleasant flavour to citrus honey. While the content of methyl anthranilate ranges from 0.84-4.9 ppm in citrus honey, non–citrus samples averaged 0.07 ppm (Knapp, 1967; White, 1965). A fast and simple reversed phase gradient elution HPLC procedure for simultaneous determination of methyl anthranilate for routine characterization of honey and HMF, as evidence of improper processing and storage or adulteration with invert syrup, was reported recently (Vinas *et al.*, 1992). The recoveries of HMF ranged between 98.7% and 103.5% and of methyl anthranilate between 95.0% and 98.4%. However, methyl anthranilate is a volatile compound and therefore suffers significant changes in concentration with different variables including storage conditions (Serra and Coll, 1995). A novel approach to characterizing citrus honey and detecting adulterations of citrus honey is the

measurement of the $\delta^{13}C$ value of the ethanol produced by alcoholic fermentation. The $\delta^{13}C$ values so obtained exceed the values obtained from other honeys by 5 ppm (Lindner *et al.,* 1996).

Distinguishing between various types of honey such as Brassica, Calluna, and *Trifolium repens* has been investigated and the pollen composition, sugar composition and electrical conductivity are together proposed as a potential screening method (Ravn *et al.,* 1975). The floral source of some unifloral New Zealand honeys was reliably determined from gas chromatographic analysis of the noncarbohydrate organic substances after liquid–liquid extraction with diethyl ether (Tan *et al.,* 1988; 1989a, 1989b; 1990). Manuka *(Leptospermum scoparium)* honeys are characterized by the presence of high levels of 2–hydroxy–3–phenylpropionic acid and syringic acid (Tan *et al.,* 1988), while degraded carotene like substances are known to occur in heather honeys (Tan *et al.,* 1989a). 2–Methoxybutanedioc acid (0-Methylmalic acid) and 4-hydroxy-3-methyl-*trans*-2-pentenedioc acid are proposed as markers of New Zealand rewarewa (*Knzghtea excelsa*) honey (Wilkins *et al.,* 1995). This has been confirmed after examination of more than 200 samples of rewarewa honey. Nodding thistle *(Carduus nutans)* honey has shown the presence of 15–87 $\mu g\ g^{-1}$ (average 43 $\mu g\ g^{-1}$) of linalool derivatives. The 16 compounds isolated in this case have been proposed as suitable marker compounds (Wilkins *et al.,* 1993). The aroma compounds hexanal and heptanal in lavender; acetone in fir; diketones, sulphur compounds and alkanes in eucalyptus and some identified compounds in dandelion and rape have been suggested as indicators. This approach needs to be studied further (Bouseta *et al.,* 1992).

Recent discoveries of certain animal sterols in plant tissues are most intriguing. One of the most remarkable is of the animal estrogen, estrone in pollen. Analysis of estrone may indicate the authenticity of honey, but screening of honeys from different botanical and geographical origins must be conducted for experimental evidence. Cyanogenesis has been detected in at least 750 plant species representing about 60 families and 250 genera. It is known to vary within populations of plant species such as clover or *Trzyolium repens* (Daday, 1954; Conn and Butler, 1969).

Blossom honeys can be differentiated from honeydew honey on the basis of citrate concentration. Honeydew honeys have about six times higher citrate concentration than blossom honey. However, honeys produced by bees partially fed sugar syrup cannot be differentiated from blossom honey on the basis of citrate content. Electrical conductivity can be used to differentiate honeydew honey from blossom honey, but the sensitivity is lower than that obtained by analysing citrate, which is a reliable index for differentiation of the two honeys (Talpay, 1988). Formate values in most honey types are < 1 mequiv kg^{-1}. However, honeys from sweet chestnut, eucalyptus, erica and calluna have high concentrations of formate, up to 11.60 mequiv kg^{-1} and the same of citrate. These data are believed to be useful in assessment of honey authenticity and differentiation of honey types (Talpay, 1989).

Floral honey from clover and buckwheat can be distinguished from honeydew honey or invert sugar on the basis of the greater inhibition of the luminol reaction by

Table 7.10 Some minerals in honey types

Sample number	Scientific name	English name	Minerals (ppm)				
			Ca	Fe	Na	K	P
1	Rhamnus spp.	Buckthorn	5.16	8.39	54.0	254	13150
2	Citrus spp.	Citruses	2.82	4.96	10.0	40.1	5165
3	Calendula spp.	Pot-marigold	13.6	6.97	96.9	793	6475
4	—	Sugar-fed	27.7	1.70	79.6	201	4405
5	Medicago spp.	Alfalfa	3.52	1.80	46.1	75.1	3080
6	Zyziphus spp.	Buckthorn	1.50	5.50	133	979	6700

Source: **Abu-Tarboush *et al.*, 1993 (reproduced with permission).**

the former. Inhibition curves at inhibition concentrations of 10^{-4}-10^{-5} g ml^{-1} are most distinct and steep enough to detect 1–2% adulteration in honey (Ponomarenko *et al.*, 1973).

Palynological and physicochemical characteristics of 36 samples of commercial Spanish honey with respect to acidity, ash, hydroxymethylfurfural, colour, minerals (Ca, Mg, K, Na, Fe, Cu, Cr, Pb) and percentage composition of the sugar fraction (fructose, sucrose, glucose, trehalose, isomaltose, maltose, kojibiose, gentiobiose, melibiose, raffinose, erlose and melezitose) are reported. The samples are characterized by low percentage sucrose and trisaccharides and large quantities of Ca, Na, K and Mg. The mineral composition of six different types of honeys are given in Table 7.10. A significant variation exists in the honey types, which could possibly indicate the floral origin of honey. Generally cotton honey has higher acidity and mineral concentrations than clover honey (El-Sherbiny and Rizk, 1979). While honey from sugar-fed bees is generally high in calcium content, buckthorn honey from Zizyphus spp. has high sodium and potassium content and buckthorn honey from Rhamnus spp. is high in content of phosphorus and iron (Abu-Tarboush *et al.*, 1993). The minerals are generally analysed by atomic absorption spectrometry (Rodriguez-Otero *et al.*, 1992). Neutron activation analysis has recently been used to determine trace elements such as As, Cr, Sb, K, Br, Zn, Fe and Co in honey types (Sevimli *et al.*, 1992). Using a multifactorial discriminant analysis of the minerals encountered, a 100% classification of Mexican honeys has been achieved (Duch and Hernandez-Chavez, 1994).

Colour values of various Spanish honeys, expressed as chromatic coordinates in the CIE-1931 from the Commission Internationale d'Eclairage system (x, y, L, the tristimulus values) and CIE-1976 (L^*, a^*, b^*, the chromaticity coordinates) have shown stepwise discriminant analysis of CIE-1976 (L^*, a^*, b^*) to yield an overall proportion of accurately classified samples. Although 100% classification is not acheived, it can, along with other variables such as pH, electrical conductivity, chromatographic sugar spectrum and palynological analysis, be regarded as a useful complementary tool for determining the botanical origin of honey (Castro *et al.*, 1992).

In a recent study, honey has been considered to be unifloral, when the dominant pollen was found at over 45% of the total pollen. Exceptions were made for lavender and thyme honey, where a finding above 15% was considered enough to typify them (Perez–Arquillue *et al.,* 1995). Multivariate statistical analysis of chemical and physical data such as acidity, mineral content and factors related to degree of freshness could classify geographical origin of Spanish honey with 83% accuracy. Pollen analysis was not necessary to achieve this objective (Sanz *et al.,* 1995).

7.5 Contaminants of honey

In some countries such as Spain, honey must meet a series of microbiological and physicochemical standards which are defined in the Government regulations. Aerobic colony counts are among these requirements. A recent analysis has demonstrated the presence of motile colonies, identified as *Bacillus alvei*. These colonies are less frequent in monofloral than in multiflora1 honeys. This is because monofloral is extracted as soon as the particular species is in bloom, and with greater care which results in better microbiological quality (Bonvehi and Jorda, 1993). *Bacillus alvei* and some other organisms further stimulate toxin production by *Clostridium botulinum* type F in honey, at some stage in the honey ripening (Nakano and Sakaguchi, 1991). *Bacillus larvae* spores in honey have been implicated in American foulbrood outbreaks (Hornitzky and Clark, 1991). Contaminants observed as microscopic impurities are oxalate crystals, vegetal tissues and hairs, bee hairs, soot, germinating yeasts, butterfly wings, bee trachea, mites and spores of *Pericystis apis* (Sancho *et al.,* 1991e). A new mite, *Acarapsis woodi,* widely known as tracheal mite appeared in American beehives in 1984 (Li *et al.,* 1993). Fungal spores and mycelium indicate the presence of honeydew in honey, and depending on the quantity of spores and mycelium, could be classified as abundant, important, common, isolated, insignificant and not observed (Sancho *et al.,* 1991d, 1992a).

Yeasts belonging to the genera *Saccharomyces, Schizosaccharomyces* and *Zygosaccharomyces,* and filamentous moulds of the genera *Aspergillus, Penicillium, Fusarium* and *Alternaria* have been reported in honey Uimenez*et al.*, 1994). Many researchers have observed that some osmophilic yeasts isolated from honey under suitable conditions convert 60% of a 10-20% glucose solution into polyols such as glycerol, Parabitol, erythritol and mannitol (Spencer and Sallans, 1956; Spencer and Shu, 1957; Peterson *et al.*, 1958; Hajny *et al.*, 1960). Glycerol may therefore be considered a fermentation product. A qualitative relation between the number of microorganisms and the quantity of glycerol has been established after studies on over 100 samples of honey. Over 79% of those containing more than 200 mg kg^{-1} glycerol show the presence of microorganisms and spores, whereas only 14% of honeys with less than 200 mg kg^{-1} glycerol contained spores (Laub and Marx, 1987). Recently an enzymatic method to determine glycerol in honey has been reported (Huidobro *et al.,* 1993), the results of which are known to correlate with other methods such as HPLC

and GC. Some compounds such as alcohols, branched aldehydes and furan derivatives are also indicated to reflect the microbiological status of honey (Bouseta *et al.,* 1992).

Contaminants such as mercury from industrial pollution find their way into bees and honey samples. In fact, results have indicated that mercury levels in bees and honey are an effective indicator of mercury loads in the environment (Toporcak *et al.,* 1992). Exposure of the bee hives containing honey to acaricides such as fluvalinate (Sancho *et al.,* 1991; Neri *et al.,* 1992), to drugs used for prevention and treatment of American foulbrood such as sulphonamides (Horie *et al.,* 1992), to chemicals used against parasites such as cymiazole, bromopropylate, coumaphos, flumethrin, malathion, etc. (Cabras and Melis, 1993) or fungicides such as procymidone (Kubik *et al.,* 1991) and dichlofluanid (Kubik *et al.,* 1992) often result in contamination of honey by the said pesticides. Limits for residues in honey have been established in a few countries: 0.05 pprn is the maximum residue limit fixed in the United States for fluvalinate, and 0.10 ppm bromopropylate and 0.01 ppm coumaphos are allowed in Germany. These pesticides are degraded below their detection levels in periods ranging from 1 week for malathion to 28 weeks for fluvalinate (Balayannis and Santas, 1992). Techniques such as first derivative spectrophotometry have been recently used for analysis of pesticides such as amitraz (Berzas Nevado *et al.,* 1991). Antibiotic residues such as tetracyclines in honey are also reported and can be analysed by simple microbiological assay (Jinbo *et al.,* 1992).

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Chapter 8

Spices, Flavourants and Condiments

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Spices, Flavourants and Condiments

8.1 Introduction

Flavour is an essential attribute of foods, both native and processed. Since time immemorial, spices and herbs have been added to processed foods to enhance consumer appeal. These additives serve to mask off flavours as well as to impart appealing aroma. With the objective of preparing flavour concentrates, extracts, oleoresins and volatile essential oil fractions have been prepared from spices. To impart special fruity aromas to certain types of food products, especially ice creams, soft drinks, confections, essential oils of natural origin as well as artificial formulations mimicking these have been in use. Spices, their concentrates, essential oils and flavourants are expensive additives to processed foods and form an important class of food articles of international commerce. The high cost is an inducive to admixing, substitution and adulteration.

Spices and their essential oils have long been used in drug formulations to mask the off taste of drugs and to impart appealing flavours. Standard specifications have therefore been formulated for these and incorporated in pharmacopoeias (United States Pharmacopoeia, 1985; Pharmacopoeia of India, 1985; British Pharmacopoeia, 1993). Characteristics that aid in determining identity, admixture and adulteration based on microscopy, physical properties and chemical components have been studied and included in treatises on pharmacognosy (Kochhar, 1981; Mabey *et al.*, 1988; Tyler et al., 1981; Trease and Evans, 1983). With the active international trade in spices and essential oils, it has been felt necessary to lay down quality specifications for these with a view to ensuring purity and checking adulterations. In the whole spices, adulteration is usually with an inferior variety, an immature dried material, other parts of the same plant, with other plant material of similar appearance and with exhausted spices. Spice powders may be admixed with powders of the above adulterants, other plant materials, grain flours, starch and even sawdust. The essential oils may be adulterated with nature identical synthetic component(s), essential oils from inferior parts of the plants or cheaper plants with similar properties. Oleoresins and extracts may contain vegetable oils and solvent such as ethanol as extenders and solvent residues.

Where the major active constituents responsible for the taste and aroma are known, specifications may be laid down based on their contents, although there usually is a wide range of their concentration in materials from different regions and of different

Table 8.1 An overview of the analytical tests for flavourants

Natural plant materials
(a) General tests: ash, crude fibre, extractive matter, volatile oil, extraneous matter and filth
(b) Tests of limited application: starch content, microscopy, sieve analysis, undesirable plant parts or foreign matter
(c) Specific tests: curcumin in turmeric, ginerine in ginger, capsaicin and colour index in capsicum, piperine in pepper, etc.

Essential oils
(a) General tests: specific gravity, optical rotation, refractive index, solubility
(b) Tests of limited application: boiling point, melting point, flash point
(c) Instrumental: GC, IR, UV
(d) Specific tests: acetals, alcohols, phenols, esters, etc.

Oleoresins
(a) General tests: volatile oil content, solubility, solvent residues
(b) Specific tests: capsaicin, piperine, curcumin, etc.

Dispersed spices
(a) General tests: volatile oil, carrier base, constituents, extractives, microbial examination
(b) Specific tests: same as for oleoresins

Synthetic chemicals
(a) General tests
(i) Liquid: specific gravity, refractive index, optical rotation, solubility, boiling point, flash point
(ii) Solids: Melting point, solubility, freedom from insoluble matter, congealing point
(b) Specific tests: for purity – GLC, IR

Vanilla extract
(a) Specific gravity
(b) Alcohol content
(c) Glycerin content
(d) Vanillin content
(e) Total solids, ash
(f) Neutral lead number
(g) Acidity
(h) Colouring matter
(i) TLC, paper chromatography

varieties. In the case of essential oils certain physical characteristics such as optical rotation, solubility characteristics, molecular refraction, specific gravity, refractive index, congealing, melting or boiling range, evaporation residue and flash point are made the basis of the standards. Chemical properties such as determination of acids, esters, alcohols, aldehydes and ketones, phenols, iodine number, etc. and some specific tests such as flavour tests and tests for halogens are also included in the standards (Guenther, 1972). In the case of oleoresins and extracts, a limit has to be specified for solvent residues especially if the solvent is toxic. An overview of these approaches is as shown in Table 8.1.

An important attribute of flavours is their sensory quality. The past three decades have witnessed impressive advances in 'flavour research' which technically should be called analytical chemistry of volatile compounds, some of which contribute to flavour

Table 8.2 A comparative account of the behaviour of instruments and human judges

Instrumental	Sensory
Separator	Integrator
Univariate	Multivariate
Absolute	Relative
Fast	Slow
Glibratable	Difficult to calibrate
Precise	Subject to drift
Does not fatigue	Fatigues, adapts
No time-order effects	Time order effects
Equal-interval units	Unequal-interval units
Expensive to purchase and maintain	Expensive to hire judges
Cannot measure hedonics	Biased by hedonics
Cannot mimic sensory	Artificial to mimic sensory

Source: Pangborn, 1987 (reproduced with permission).

or aroma. Data on sensory aspects of flavour compounds are often vague, probably because of the inherent variability of the sensory response which require extensive training of judges, adequate replication and detailed statistical analysis of the observations. Sensory aroma research remains a fertile area of innovative investigation, particularly at the interface between the organic and physical chemistry of the aroma stimuli and human perception. Table 8.2 shows a comparative account of the behaviour of the instruments and human judges. The major areas of aroma research include comparison of sensory and gas chromatography analysis data, effect of medium of dispersion, aroma–appearance interactions and cross cultural aroma studies. These quality attributes of flavours which have received so little attention from academicians are of interest to behavioural scientists and marketing personnel (Pangborn, 1987). A combination of chemical and organoleptic analyses achieves the best quality control, but in case of conflict, the organoleptic technique takes precedence (Theile, 1962).

Multiple regression analysis (Aishima, 1979, 1982; Martens, 1985, 1986; Izquierdo and Serra, 1987) and discriminant analysis (Schreier *et al.*, 1978; Noble *et al.*, 1980) have been used to correlate sensory and instrumental data about aromas. Direct sensory estimation of differences (dissimilarities or distances) between samples is a simpler approach and the data can be analysed by multidimensional scaling, a statistical method specifically conceived to handle dissimilarities (Moskowitz and Barber, 1976; Bieber and Smith, 1986; Chauhan and Harper, 1986). This technique has demonstrated that sensory data do not correlate with the absolute concentration of the total volatiles, but rather does correlate to the relative concentrations of all volatiles and to those constituents showing significant changes (95% level) due to storage length or temperature (Velez *et al.*, 1993). Because of the complexities, simple mathematical routines like regression analysis have had limited success in the correlation of organoleptic sensory analysis with instrumental data. Pattern recognition programmes, consisting of mathematical analyses of the data and mathematical modelling can be 'taught' to recognize a pattern(s) by analysing a

Table 8.3 Physical properties of some spice essential oils and flavourants

Spice	Specific gravity (20°C)	Refractive index (20 °C)	Optical rotation (°) (20 °C)	Solubility characteristics	Other remarks
Asafoetida	0.906–0.973	1.493–1.518	–9°0' to +9°18'		Sulphur content, 15.3 – 29%
Allspice (Pimenta) berry oil	1.024–1.055[a]	1.525–1.536	-0°32' to -5–0'	Soluble in 1–2 vols & more of 70% alcohol, occasionally with opalescence to turbidity on dilution	Phenol content, 65 – 89%
Pimenta leaf oil	1.026–1.065	1.530–1.540	inactive to 5°30'	Soluble in 1–2 vols of 70% alcohol	Phenol content, 65 – 96%
Bay oil	0.960–0.985;[a] in oils of lower quality as low as 0.951	1.506-1.520	laevorotatory upto -2°, seldom upto –3°	Freshly distilled oils are soluble usually in 1–2 vols of 70% alcohol; solubility decreases rapidly on storage	Phenol content, 57 – 60%; in oils of poor quality, as low as 40%
Terpenelessbay oil	1.029–1.050'	1.527-1.536	–0°10' to -1°20'	soluble in 2–2.5 vols of 60% alcohol, sometimes even in 6–6.5 vols of 50% alcohol	Phenol content, 82 – 95.5%
Cardamom	0.923–0.941[a]	1.462–1.467	+24°0' to +41°	Soluble in 2–5 vols of 70% alcohol	Acid number, up to 4; Ester number, 92 – 150
Cardamom, wild	0.909'	1.474	+16°30'	Soluble in 1 – 2.5 vols of 70% alcohol	Acid number, 1.1; Ester number, 12
Cinnamon bark oil	1.020–1.030'	1.568–1.535	–1°0' to -2°10'	Soluble in 1–2.5 vols of 70% alcohol, occasionally opalescent to hazy	Aldehyde (calcd. as cinnamaldehyde), 51.8 – 56% Phenol (Eugenol), 14 – 18%

Table 8.3 (cont.)

Cinnamon leaf oil	1.037–1.055[a]	1.529–1.535	-1°36' to 0°40'	Soluble in 1.5 volsor more of 70% alcohol, sometimes with opalescence or paraffin separation	Aldehyde, up to 4% Phenol, 77.3 – 90.5%
Cassia oil	1.055-1.070[a]	1.600–1.606	-1°0' tp +6°0'	Readily soluble in 1–2 vols of 80% alcohol, 2–3 vols. of 70% alcohol	Aldehyde, 75 – 90 %
Clove bud oil	1.043-1.068[a]	1.529-1.537	upto -1°35'	1–2 vols or more o of 70% alcohol with slight turbidity; freshly distilled in 2.5–3.0 vols of 60% alcohol	Eugenol, 78–95%, seldom up to 98%
Clove stem oil	1.040-1.067[a]	1.531-1.538	upto -1°30'	1–2 vols or more of 70% alcohol and 2.5–3 vols of 60% alcohol	Eugenol, 83 – 95%, in exceptional cases higher
Clove leaf oil	1.032-1.067[a]	1.533–1.539	-0°50' to -1°53'	0.9 vols or more of 70% alcohol	Eugenol, 78 – 93%
Ginger	0.877-0.886[a] oils with lower and higher specific gravity have been observed	1.489-1.494	-26°0' to -50°0' lower values observed for oil distilled from old roots stored for a long time	only sparingly soluble in alcohol Up to 7 vols of 95% alc reqd. for solution which is not always clear. In 90% alc., the oils are generally, but not always completely soluble	Acid number, up to 2; Ester number, up to 15; Ester number after acetylation, 24 – 50
Mustard	1.014-1.030	1.527–1.529	inactive	Soluble in 160 to 300 parts of water, 7 – 10 parts of 70% alcohol, 2.5–3.0 vols of 80% ethanol, in 0.5 vols of 90% ethanol, clearly miscible with ether, amyl alcohol, benzene and petroleum ether	Allyl isothiocyanate, 94%; Boiling range at 760 nm, 148 – 154°C
Nutmeg	0.859–0.868	1.469–1.472	+40°48' to +49°48'		Acid number, 1.0–1.3; Ester number, 6.8–7.3

Table 8.3 (cont.)

Mace	0.860–0.892	1.472–1.479	+21°42' to +41°30'		Acid number, 1.5–6.2; Ester number, 2.8-12.8
Oil of Wintergreen	1.180–1.193[a]	1.535–1.536	-0°25' to -1°30'	Clearly soluble in 6–8 vols of 70% alcohol	Ester number, 354 – 365; Ester content, calcd. as methyl salicylate, 96–99%
Onion	1.047–1.098[a]	1.537–1.559	+1°3' to +3°53'	Most oils not completely soluble in 10 vols of 95% alcohol Occasionally soluble in 1–2 vols or more of 95% alcohol	Acid number, 12.0 – 19.8; Carbonyl no., 9.8 – 15.1; Iodine no., 59.9 – 66.2
Pepper oil	0.873–0.916	1.480–1.499	-10°0' to +3°	Not readily soluble in alcohol usually soluble in 10–15 vols of alc; soluble in 3–10 vols of 95% alcohol	Acid no., up to 1.1 Ester no., 0.5 to 6.5 Ester no. after acetylation, 12 – 22.4; Phellandrene test, usually strongly positive
Star anise	0.98–0.99	1.553–1.557	up to -2°; sometimes; upto 0°36'	Soluble in 1.5-3.0 vols of 90% alcohol	Congealing point, +14 – +18°
Ajowan oil	0.910–0.930[a]	1.498–1.504	uptp 5°0'	Soluble in 1–2 vols and more of 80% alcohol	Phenols, 45.0 – 57.0%
Corriander	0.870–0.885[a]	1.463–1.471	+8°0' to +13°0'	Soluble in 2–3 vols of 70% alcohol	Acid number, up to 5.0; Ester no., 3.0 – 22.7
Dill	0.895–0.915[a]	1.481–1.491	+70°0' to +82°0'	Soluble in 4–9 vols of 80% alcohol	Carvone content, 40 – 60%
Anise oil	0.980–0.990	1.552–1.559	upto -1°50'	Soluble in 1.5–3.0 vols of 90% alcohol	

Table 8.3 (cont.)

Fennel seed oil	0.965–0.977[a]	1.528–1.539	+11°0' to +24°0'	Soluble in 5–8 vols of 80% alcohol and in 0.5 vols of 90% alcohol	Congealing point, not below 5°, and as high as 10° in good oils
Celery seed oil	0.872–0.891[a]	1.480–1.484	+65°53' to +76°51'	Turbid in 90% alcohol	Saponification no., 25.1 – 47.6
Caraway seed oil	0.907–0.919[a]	1.484–1.488	+70°0' to +81°0'	Seldom soluble in 70% alcohol soluble in 2–10 vols of 80% alc., clearly soluble in equal vols of 90% alcohol	Carvone content, 50.0 – 60%
Parseley seed oil	1.043–1.110[a]	1.512–1.528	-4°0' to -10°8'	4-8 vols and more of 80% alcohol	Acid No., up to 6; Ester No., 1 to 11, Ester No. after acetylation, 4 to 20
Parseley herb oil	0.902–1.016x	1.509–1.526	+1°16' to +4°30'	Soluble in 95% alcohol	Acid No., up to 1, Ester No., 5 to 14, Ester No. After acetylation, 19 – 68
Lemongrass oil	0.899–0.911[a]	1.485–1.490	-1°10' to -3°10'	2–2.5 vols of 70% alcohol; occasionally opalescent or slightly cloudy. A few lots not clearly soluble in 70% alcohol, up to 10 vols	Aldehyde content, 71.8 – 79.1%
Bitter almond oil	~ 1.050[a]	1.542–1.546	inactive	Soluble in 1–2 vols and more of 70% alcohol	Boiling point 179 °C
Sassafras oil	1.070–1.080[a]	about 1.530	+2°0' to +3°38'	soluble in 95% alcohol, 1–2 vols of 90% alcohol	Acid no., up to 1.0, Ester no., 0.5 – 5.0 Congealing point, 4.5 – 6.9 oC

[a]at 15 °C

Source: Guenther, 1982a, 1982b, 1985; Clevenger, 1935

previously classified set of data. Significant results have been achieved in applying pattern recognition to gas chromatographic data for stress-induced changes of coffee aroma (Roberts and Bertsch, 1987), essential oils from different varieties of hops (Stenroos and Siebert, 1984) and the classification of navel and valencia orange essential oils (Mayfield *et al.*, 1986). Differentiation of good quality orange aromas has been accomplished by the use of SIMCA (statistical isolinear multicategory analysis) analysis of the gas chromatographic data (Lin *et al.*, 1993).

Spices, essential oils, oleoresins, extractives and formulations consisting of these along with synthetic nature identical new related compounds have been accepted as generally recognised as safe (GRAS) additives in food products. Possible toxic effects are only recently being investigated. Toxicity of some constituents like menthol has recently been suggested. Although not much atention has been given to the authenticity and quality aspects of spices, condiments, flavourants in the past, from the point of view of toxicity, quality, adulteration it may be necessary to concentrations of these or certain constituents of these in food products and this underlines the need of foolproof methodology to monitor the concentration of these flavourants.

8.2 Spices as flavourants

Spices are extremely valued in food formulations for the variety of taste/flavour profiles which can make all the difference between the food being acceptable or not. In this section, quality indices of spices are reviewed with particular attention to widely practised, but less often reported types of adulteration. Physical properties such as optical rotation, specific gravity and solubility profile of the various spice essential oils, which have been used as criteria of purity are as given in Table 8.3.

8.2.1 Asafoetida

Asafoetida is an oleogum resin obtained from the thick fleshy roots of an umbelliferous plant, *Ferula foetida* and allied species which are found throughout Afghanistan and Iran. It is the dried latex obtained mainly from living rootstocks or taproots of several species such as *F. foetida*, *F.assafoetida*, *F.narthex*, *F. alliacea*, *F. rubricaulis* and so on. The plants from which the asafoetida of commerce is derived bear massive carrot shaped roots (12.7–15.2 cm diameter) at the crown when they are 4–5 years old. In March and April, just before the plants flower, the upper part of the root is laid bare and the stem cut off close to the crown. The exposed surface is covered with a dome shaped structure made of twigs and earth. A milky juice exudes from the cut surface. After some days, the dried exudate is scraped off. Sometimes the resin is removed along with a slice of the plant. The collection of the resin and the slicing of the root are repeated until this exudation ceases. Asafoetida is acrid and bitter in taste, and emits a strong alliaceous odour. It has been used as a flavouring in a number of food products including non-alcoholic beverages, ice creams, candies, baked goods and condiments.

In India, it is extensively used for flavouring curries, sauces and pickles. In Iran, the natives rub asafoetida on warmed plates before placing meat on them. In commerce, it occurs in three forms, namely tears, resinous mass and paste. Tears constitute the purest form of the resin. Tears are rounded, flattened and are 5–30 mm in diameter. They have a greyish or dull yellow colour. Two types are generally known, one which retains the original colour for years, and another which gradually becomes dark or reddish brown. Mass asafoetida is the common commercial form and consists of tears agglutinated into a more or less uniform mixture with root or earth fragments. The paste also contains extraneous matter.

Asafoetida contains about 40–64% resin, 25% endogenous gum, 10–17% volatile oil and 1.5–10% ash. The resin portion of asafoetida is known to contain asaresinotannols 'A' and 'B', ferulic acid, umbelliferone and four unidentified compounds. Asafoetida hydrolysate is known to contain glucose, galactose, L-arabinose, rhamnose and glucuronic acid. The molecular structure of the gum polysaccharide has been elucidated. The dried gum, when steam distilled yields about 5–15% of an oil which is orange to brown in colour. It has a very pungent, strongly penetrating acrid, sharply alliaceous, garlic like odour, which is very lingering.

The chief constituent of asafoetida oil is secondary butylpropenyl disulphide ($C_7H_{14}S_2$) about 40–45%. The other disulphides are $C_8H_{16}S_2$, $C_{10}H_{18}S_2$, and $C_{11}H_{20}S_2$. A trisulphide, $C_6H_{10}S_3$ is also found and pinene and terpenes are also reported to be present. The disagreeable odour of the oil is mainly reported to be due to $C_{11}H_{20}S_2$.

Asafoetida is often adulterated with gum arabic, other gum resins like colophony resin, rosin, gypsum, red clay, chalk, barley, wheat or rice flour and slices of potatoes. Asafoetida should not contain more than 15% ash and minimum of 50% alcohol-soluble matter (Wealth of India, 1957). As prescribed by the Prevention of Food Adulteration Act (PFA, India 1991), asafoetida should contain total ash of not more than 15%; acid insoluble ash not more than 2.5%; 90% alcohol extractives not less than 12% and starch not exceeding 1%. In some countries 'compounded asafoetida' is permitted to be marketed consisting of one or more varieties of asafoetida blended with gum arabic, edible starches or cereal flour. This must not contain more than 10% ash, more than 1.5% acid insoluble ash and not less than 5% alcoholic extract. It should not contain colophony, galbanum or moriacum or any other foreign resin, coal tar dyes or mineral pigments (PFA, 1991).

8.2.2 Allspice

Allspice, which possesses an aromatic taste and flavour resembling a mixture of cinnamon, clove and nutmeg owes its characteristic odour to an essential oil (3.3–5.5%) , concentrated mainly in the pericarp of the berries of *Pimenta dioica*. Pimento berry oleoresin is produced on a small scale by specialist firms. The volatile oil content can range up to 60–66%. Higher fat contents are obtained with hydrophobic solvents such as petroleum ether. For flavouring purposes, pimenta berry

oleoresin is dispersed on a neutral base, for example salt, sugar, flour, rusk, etc. and is sold weight for weight, equivalent in strength to the dry spice (Purseglove *et al.*, 1981b). The oil contains 65–80% eugenol. Cineole, l-α-phellandrene, caryophyllene, palmitic acid and eugenol methyl ether are the other constituents. Pimenta leaf oil, which also contains eugenol is often used as a substitute or an adulterant for the more expensive berry oil. Clove oil and its fractions are the other adulterants (Wealth of India, 1969). Chemical composition, microscopic identification, GC analysis of the essential oil, adulterants and contaminants, and applications in the food industry of allspice are recently reviewed by Oberdieck (1989). The British Standards Institute (BSI) (BS 494) recommends for whole pimenta a maximum of 12% moisture and on the dry material, maxima of 4.5% ash, 0.4% acid insoluble ash and a minimum of 3.5% v/m of volatile oil. Additional requirements for ground pimenta are maxima of 8.5% non-volatile ether extract and 27.5% fibre and a minimum of 2.8% volatile oil (on dry weight basis).

Pimenta berry oil is used chiefly for the flavouring of all kinds of food products such as meats, sausages, canned goods, table sauces, pickles and confectioneries in which the oil replaces the ground spice to a great advantage, as the oil is of more uniform quality and can be dispensed easily and with greater accuracy.

8.2.3 Ajowan (Trachyspermum ammi)

This aromatic seed spice is used in food and pharmaceuticals. Guenther (1982b) gives *Carum ajowan* or *C. copticum* as its botanical name. The plant is said to be of Egyptian origin, though it is cultivated in India, Afghanistan, Pakistan, Iran and Iraq. The plant is grown mainly on the plains, but flourishes equally well in the hills and at higher altitudes. It is widely used in curries, pickles, certain confectionery and, biscuits as well as in beverages. The essential oil is obtained by steam distillation, the yield varying from 3.0–4.0%, and is used in medicine and is officialy included in the Indian Pharmacoepia (IPC). It is used as a perfume for disinfectant soap. Thymol which is obtained from ajowan is an ingredient of mouth washes, gargles and toothpastes. The oil was the source of thymol for a long time, but synthetic thymol is now used extensively. The other constituents of ajowan oil are reported to be α-pinene, *p*-cymene, dipentene, γ-terpinene and carvacrol. Ajowan oil, both pure and dementholized is employed as an antiseptic, aromatic carminative.

8.2.4 Bay leaves

Pimenta racemosa or bay tree, grown mainly for its leaves, furnishes an essential oil (0.75–1.25%) with eugenol as chief constituent and is used to a limited extent for flavouring culinary preparations, chiefly table sauces.

The constituents of bay oil are eugenol, L-pinene, myrcene, L-phellandrene, limonene and dipentene, cineole and chavicol. Bay oil has been reported to be

adulterated with small quantities of kerosene or alcohol. A skilful form of adulteration which is more difficult to detect is that of clove stem oil, clove leaf oil or their terpenic fractions. Terpeneless bay oil is prepared by removing a greater part of the terpenes from the normal bay oil, usually by fractional distillation. The removal of myrcene which easily polymerizes in the normal oil renders these terpeneless oils not only more soluble but more stable preventing the oil from becoming insoluble on ageing.

8.2.5 Cardamom

Cardamom is used as a spice for flavouring cakes, bread, milk–sugar–starch confections and for several other food applications. It has also been used for flavouring coffee and liqueurs. Medicinally, the oil is often employed as an adjuvant or corrective of tonic, carminative and purgative preparations. Cardamom owes its aroma to a volatile oil present in the seeds (2–8%) with the green variety yielding more oil than the bleached cardamoms. Cardamom oil of commerce is obtained by distillation of whole fruits of *Elettaria cardamomum* (family Zingiberaceae), and is a component of compound cardamom spirit and compound vanilla spirit. An overview on cardamoms with respect to types, characteristics, substitutes and other aspects has been recently published (Oberdieck, 1992). The major constituents identified in the volatile oil are cineole, *d*-α-terpineol and terpinyl acetate. *Elettaria major*, also called as 'long wild cardamom' also yields an essential oil which can be distinguished from *Elettaria cardamomum* by its physicochemical properties, particularly optical density (Table 8.3).

Cardamom seeds are often adulterated with seeds of *Amomum aromaticum* or *A. subulatum* and *A. cardamomum* (Wealth of India, 1948), and the fruits with orange seeds, unroasted coffee seeds as well as small pebbles. A thin layer chromatography (TLC) method has been developed that distinguishes and differentiates between true cardamoms (*Elettoria cardamomum Maton*) and large cardamoms (*Amomum subulatum Roxb.*). Extracted volatile oils are chromatographed on silica gel G with a n-hexane–diethylether (80+20) solvent system and visualized with a saturated solution of antimony trichloride in chloroform. The chromatographic pattern permits the detection of adulteration of one by another at levels as low as 5% (Sen *et al.*, 1977). Camphor which is present in *Amomum cardamomum* but not in *Elettaria cardamomum* can be used as index to detect the admixture. Similarly *Amomum aromaticum Roxb.* can be distinguished from true cardamoms by its physicochemical properties, particularly its negative optical density and solublity behaviour in alcohol. *Amomum aromaticum Roxb.* yields an oil which is clearly soluble in one volume of 80% alcohol. Powdered seeds are adulterated with the powder of hulls (Wealth of India, 1952).

The British Pharmacopoeia (BP) (1993) prescribes the following standards for cardamom seeds: maximum ash 6% (US, India 8%), maximum acid insoluble ash 3.5% (US, India 3%) and minimum volatile oil 4% (India 3 %). The BSI recommends for cardamoms a maximum 13.0% moisture (India 14 %), a maximum of 9.2% ash and

a minimum of 4.0% v/m volatile oil.

Cardamom oleoresins are obtained in about 10% yield with their volatile oil content ranging from 52–58% (Purseglove *et al.*, 1981a).

8.2.6 Capsicum

Capsicum has played a role in the food and pharmaceutical industries (De, 1992) for many centuries (Verghese *et al.*, 1992). In food, capsicum has been used primarily for its pungent taste , the concentration of the pungency factor, capsaicin, varying among cultivars and also among the fruits of the same cultivar. It was discovered during the second voyage of Columbus on the island of Santo Domingo and the fruits of the capsicum family rapidly gained importance and acceptance. Due to wide differences in the growing conditions, many variations of capsicum are available today. Different varieties of capsicums evolved, giving a fruit with a wide range of physical and chemical properties. Thus the small very hot peppers evolving in India, Africa and China became known as chillies. The much larger, less pungent peppers that evolved in Hungary and Spain became known as paprika. Those varieties of capsicum which had pungency contents between chillies and paprika became known as red peppers.

In the fresh green form chilli offers a unique condiment with its characteristic taste and aroma and several condiment formulations are made from these. These may include green chilli sauce, chutney and chillies pickled in vinegar or lime juice. The green colour of capsicum is attributed to the presence of chlorophyll a_1, a_2, b_1, b_2 and pheophytin a (Schandrerl and Denise, 1966). The most abundant carotenoid is lutein with lesser amounts of β-carotene, violaxanthin, foliaxanthin and foliachrome (Curl, 1964). On ripening the colour changes to bright orange and on to dark red. The red colour is mainly due to the carotenoids capxanthin, capsorubin, zeaxanthin, violaxanthin, cryptoxanthin and β-carotene, altogether 37 pigments of which 21 have been identified (De La Mar and Francis, 1969). The major pigment, capsanthin, (35%) mostly occurs as capsanthin dilaurate (Philip *et al.*, 1971). The intense odour of freshly chopped bell pepper is attributed to 2-methoxy-3-isobutylpyrazine. Several other components of the volatiles have been identified (Buttery *et al.*, 1969) including *trans* β-ocimene, limonene, methyl salicylate, linalool, nona-*trans*, *cis*-dienal, deca-*trans*, *trans*, 2,4-dienal, hexa-*cis*-3-enol and a number of potent alkyl thiazoles (Buttery *et al.* 1976).

As world trade grew, a need to monitor the quality of the raw materials from various sources became evident. An organoleptic pungency procedure developed by Scoville (1912) is prone to poor precision in the determination of actual pungency. Colorimetric methods, which were later developed (von Fodor, 1930) are also plagued by problems of lack of specificity.

Adulteration with capsaicin-like pungent compounds soon became a possibility, with the filing of patents for their synthesis.

Many complex separation procedures were also developed to identify such synthetic

substitutes (Suzuki *et al.*, 1957; Schulte and Kruger, 1955; Heath, 1959; 1964; Todd, 1958; Todd and Perun, 1961; Datta and Susi, 1961). It was not until 1975, that a simple, rapid and routine screening method for detecting and estimating adulteration in a wide variety of capsicum products became available (Todd *et al.*, 1975). This method is based on TLC procedures using (i) a two-dimensional reversed phase system and bromination of the sample, (ii) a one-dimensional reversed phase system using silver ion to complex with the unsaturated components or (iii) polyamide plates with silver ion in the developing solvent (Todd *et al.*, 1975).

Chillies contain three capsaicinoids, namely nordihydrocapsaicin, capsaicin and dihydrocapsaicin in a constant ratio of 1.05:1.00:1.02. The Scoville heat strength of chilli or its extract is calculated by determining the concentration of each capsaicinoid, multiplying each of them by the corresponding Scoville heat units and summing. 'Synthetic' capsaicin (*N*-vanillylnonamide) which may be used to spike chilli extracts coelutes with capsaicin and has a high Scoville heat score. A statistical analysis of the ratio of three capsaicinoids and determination of the Scoville heat score can estimate whether or not a sample has been spiked with 'synthetic' capsaicin. The Scoville heat score of *N*-vanillylnonamide is 9.2 million as compared to 16.1 million for capsaicin itself. Its presence would give erroneously high scoville heat units and therefore indicate adulteration (Weaver *et al.*, 1984).

Chromatographic techniques, particularly GC and TLC are adequate for proving the authenticity of spice extracts. This technique also has shown higher stability of spice extracts as compared to natural spices (Wyler, 1967).

The quality assessment of paprika and pepper for use in the meat industry could be monitored by measuring the electrical conductivity of the aqueous extract which is related to ash content as an index of quality (Selmeci *et al.*, 1982). Red peppers from the Capsicum genus have red and yellow carotenoid pigments and their composition can be used to detect possible colouring matters added to commercially derived products (Minquez Monsquera and Fernandes Diez, 1981).

Capsicum oleoresins can be prepared to meet the requirements of processed foods. When a fat-soluble product is required, a water-immiscible solvent, for example. dichloroethane or benzene is chosen, but for a water-miscible oleoresin, acetone, ethanol or any other water-miscible solvent is necessary. Capsaicin oleoresin obtained from the whole fruit contains a considerable amount of fixed oil, originating mainly from the seeds and this is liable to become rancid during storage; hence this oil needs to be removed by repeated extraction with cold ethanol (Purseglove *et al.*, 1981b).

8.2.7 Cinnamon

The bark of a small evergreen tree, *Cinnamonum zeylanicum*, cultivated most extensively in SriLanka, forms the world's principal source of supply of cinnamon. *C. zeylanicum* is a hardy plant which can grow on any soil under a wide variety of tropical conditions. The quality of the bark, however, is greatly influenced by the soil and

Table 8.4 Cinnamaldehyde and eugenol contents of cinnamon oils

Oil % v/v	Cinnamaldehyde % v/v	Eugenol
Cinnamon bark oil	76.5	2.5
Cinnamon bark oil	56.4	6.1
Cinnamon leaf oil	3.1	87.5
Cinnamon leaf oil	2.2	80.7
Cassia oil	80.0	1.3
Cassia oil	81.5	6.3

Source: Ross, 1976 (reproduced with permission).

ecological conditions. The finest quality is obtained from plants grown in white sandy soil. The bark of tender shoots and stem is smooth and pale, whereas on ageing it turns brown and rough. Commercial cinnamon bark is not more than 0.5 cm thick and is a dull pale brown colour, the inner surface is somewhat darker than the outer and is finely striated longitudinally. It has a delicate fragrance and a, 'warm' sweet agreeable taste. Cinnamon is used either in pieces or in the powdered form as a spice or as a condiment. Powdered beechnut husk, aromatized with cinnamic aldehyde is at times marketed as 'pure powdered cinnamon'. A higher content of cinnamic aldehyde is an indication of the fraud.

The term 'cinnamon oleoresin' is a general one and includes oleoresins extracted from any of the species of Cinnamomum. Species mainly differ in the quality of their flavour and aroma and in the content and composition of volatile oil which ranges from 16–60%. The oleoresin yield can vary from 10–12% with ethanol to 2.5–4.3% with benzene. It is marketed as a liquid or dispersed on sugar, rusk or salt (Purseglove *et al.*, 1981b). Cinnamon bark contains about 0.5–1.0% oil, which is light yellow in colour. The colour changes to red on storage. The two main species of cinnamon used commercially for their essential oil are *C. zeylanicum* and *C. cassia*. *C. zeylanicum* yields bark oil and cinnamon leaf oil. Cinnamon leaf oil is used in seasoning blends as an alternative to clove oil to which it resembles in odour. The bark oil is used in high quality seasoning blends and occasionally in natural flavours. It also finds applications in perfumery and cosmetics, soaps, dental preparations and hair lotions. In China, *Cinnamomum camphora* is an important economic trees, yielding essential oils from its leaves, branches, trunks and roots (Shi *et al.*, 1989). The major components of cinnamon oils are phenols (expressed as eugenol) and aromatic aldehydes (expressed as cinnamaldehyde) which can be analysed by high performance liquid chromatography (HPLC) and UV spectrophotometry. The chief constituents, besides cinnamic aldehyde and eugenol, are benzaldehyde, α-pinene, *l*-linalool, phellandrene, esters of isobutyric acid, cinnamyl alcohol and cymene. By using paper chromatography, it has been possible to detect adulteration of Sri Lankan cinnamon oil, cassia oil, cinnamon leaf oil and flavours containing these oils (Guenther, 1965). The cinnamaldehyde and eugenol contents in these oils are as given in Table 8.4. As cinnamon bark oil is by far

Table 8.5 Analytical data and standards for cinnamon

	Range (%)	BPC standards (%)	US standards (%)	Indian standards (%)
Moisture	7.8–10.5	—	—	max. 12
Ash	4.1–5.7	7(max)	5(max)	max. 8
(BP 1932)				
Water soluble ash	1.4–4.6	—	—	
Acid insoluble ash	0.1–0.7		2.0(max)	2.0 (max) max. 2
Volatile oil	0.7–1.4	1.0(min)–whole 0.7(min)–powder		min. 0.5
Fixed oil	1.3–1.7	—	—	
Starch	16–22	—	—	
Foreign organic matter	—	2.0(max)	—	

Source: Pearson, 1976; Prevention of Food Adulteration Act, 1991.

the most expensive of the three, it is frequently adulterated with other oils or with synthetic cinnamaldehyde. This adulteration can be detected by analysis of cinnamaldehyde and eugenol contents (Ross, 1976). Physicochemical properties (shown in Table 8.3) such as specific gravity are altered on extension of cinnamon bark oil with cassia bark oil, and this therefore provides a valuable clue in its detection.

It has also been shown that cinnamon contains less mucilage but gives a higher proportion of ash than cassia. For genuine cinnamon, the former should not exceed 4% and the latter should be at least 17%. Microscopically cassia has broader fibres (diameter over 40 μm), wheras those of cinnamon which are usually under 30 μm. Cassia contains cork and larger starch grains. Table 8.5 gives the analytical data and standards for cinnamon.

Cassia, known as poor man's cinnamon makes a unique contribution in its own right. It forms a major part of the traditional flavour of cola drinks, and is also used in confectionery in conjunction with capsicum oleoresin. Use of cassia oil is restricted to cherry, vanilla and some nut flavours. Adulterants of cassia oil include rosin and kerosene, sometimes with regular addition of cinnamic aldehyde. Benzaldehyde (Dodge, 1939) and benzyl acetate have also been reported to be adulterants of cassia oil.

8.2.8 Clove

Clove, the dried unopened bud of *Eugenia caryophyllus* and cultivated in Tanzania, Madagascar, Indonesia, Srilanka, India and Malaysia is the second most popular spice (after black pepper). Clove oleoresins containing 70–80% volatile oil are generally prepared industrially by acetone extraction. The yield of the oleoresin can, however, vary from 18–22% for benzene to 23–32 % with alcohol. The benzene extract is known to contain about 90 % volatile oil (Purseglove *et al.*, 1981b). Clove oil is used in perfumery, the food industry, dentistry and in the tobacco industry. It is also used for

Table 8.6 Analytical data and standards for cloves

	Range (%)	BPC standards (%)	US standards (%)	Indian standards (%)
Moisture	5.0–11	—	—	max. 12
Ash	4.5–7.0	7 (max)	7 (max)	max. 7
Water soluble ash	2.7–4.2	—	—	
Acid insoluble ash	0–0.3	1.0 (max)	0.5 (max)	max. 0.5
Volatile oil	14.5–20.0	15 (min)–whole 12 (min)– powder	15 (min)–for vol. eth. extract	min. 15
Fixed oil	6.2–10.1	—	—	—
Alcohol extract	13.5–15.5	—	—	
Crude fibre	6–10	—	10 (max)	
Nitrogen	0.9–1.2	—	—	
Foreign organic matter (eg. fruits)	—	1.0 (max)	—	
Stalks for 'stems'	—	5.0 (max)	5 (max)	
Quercitannic acid	—	—	12 (min)	

Source: Pearson, 1976; Prevention of Food Adulteration Act, 1991.

the flavouring of oral preparations (dentrifices, gargles) and chewing gums. Clove oil is used in seasoning blends. It plays an interesting part in other natural flavours, forming an essential part of the character of banana flavour and a useful background note in cherry, blackberry and smoked flavours. Clove stalks seldom contain more than 7% volatile oil but the crude fibre may exceed 20%. Apart from a few stalks, most samples of cloves also contain a few ripe fruits (Mother Cloves) which contain starch. Table 8.6 gives the typical ranges for the analytical figures together with the BPC (British Pharmacopoeia), US and other Indian standards.

Because of the differences in price, clove bud oil is frequently adulterated with stem oil, and particularly the low priced clove leaf oil. Besides eugenol, other chemical constituents of clove bud oil are eugenol acetate, caryophyllene, caryophyllene oxide, methyl-n-amyl ketone and vanillin. Clove stem oil and leaf oil contain traces of naphthalene. Principal component analysis (PCA) and stepwise discriminant analysis (SDA) have shown that the compounds which have a better discriminant power in the differentiation of the bud from stem oils are eugenol, α-cubebene, the oxygenated product 11-terpinyl acetate, (E)-α-bergamotene and caryophyllene (Gaydou and Randriamiharisoa, 1987). Eugenol is sometimes isolated from clove oil. This can be identified by the changes in the optical rotation. Another form of adulteration is the addition of clove terpenes, obtained as by products in the extraction of eugenol from clove oil. Although small quantities cannot be detected, larger quantities increase the optical rotation and decrease the eugenol content, refractive index and the specific gravity. Adulteration with terpineol, dibenzyl or dibenzyl ether is best detected by the odour on a blotting paper after standing for a few days or, even better by the odour of non-phenolic portions of the oil. Acetins too are occasionally encountered in

commercial clove oils and can be detected by washing with a saturated salt solution. A high saponification number of the water soluble material is indicative of acetins. The physicochemical properties of clove bud oil, clove stem oil and clove leaf oil are summarized in Table 8.3.

8.2.9 Curry leaves

Leaves of *Murraya koenigii*, or curry leaves as they are popularly known, belong to the Rutaceae family, and are widely used in the Indian subcontinent as flavouring ingredients in curries and chutneys. They are commonly found in India in the western ghats, along the foot of the Himalayas, Bengal, Burma and the Andaman islands. Fresh leaves on steam distillation yield about 2.6% volatile oil, which finds use as a fixative for perfumes. Direct distillation gives a poor yield of oil, while superheated steam (220 °C) distillation yields a dark coloured foul smelling oil. Refined curry leaf oil is deep yellow in colour with a strong spicy odour and pungent clove like taste. The oil contains 12% cadinol, 18.2% cadinine, 26.3% caryophyllene, 2.7% lauric acid and 3.4% palmitic acid. Young leaves contain more volatile oil and oleoresin than mature leaves. Much work needs to be done on the chemistry and technology of curry leaves to utilize them to their fullest potential.

8.2.10 Garlic

The bulbs or cloves of the common garlic *Allium sativum* of the family Liliaceae have been used since antiquity, particularly in Mediteranean countries, as a popular prophylactic and curative against all kinds of intestinal ailments and even against arteriosclerosis and hyperpnea. The undamaged bulbs of garlic have a weak odour. However, when crushed, the bulbs emit their well known powerful obnoxious odour due to an essential oil consisting chiefly of disulphides. Meagre data is reported on the physicochemical properties of garlic oil. The specific rotation at 15 °C is around 1.046 – 1.057 (Guenther, 1982a). Plants of the genus Allium, including garlic and onion are sources of various organosulphides with anticarcinogenic activity (Block, 1985). The flavour principles are generated by the interaction of allinase, a pyridoxyl phosphate enzyme, and depend on the variety, maturity, cultural and environmental conditions (Verghese, 1992). Compliance markers in garlic are the various volatile non-polar sulphur containing compounds which also have the potential to determine the sensory quality and authenticity in products such as garlic powder. Amongst the compounds reported are allyl mercaptan (Nishimura *et al.*, 1988), allyl methyl sulphide (Nishimura *et al.*, 1971, 1988), allyl methyl disulphide (Brodnitz *et al.*, 1971; Nishimura *et al.*, 1971, 1988; Yu *et al.*, 1989), allyl methyl trisulphide (Brodnitz *et al.*, 1971; Nishimura *et al.*, 1971, 1988; Yu *et al.*, 1989), allyl n-propylsulphide (Nishimura *et al.*, 1971), allyl n-propyl disulphide (Nishimura *et al.*, 1971, 1988; Yu *et al.*, 1989), allyl sulphide (Nishimura *et al.*, 1971, 1988; Yu *et al.*, 1989), allyl disulphide (Brodnitz

et al., 1971; Block *et al.*, 1986; Nishimura *et al.*, 1971, 1988; Yu *et al.*, 1989), allyl trisulphide (Brodnitz *et al.*, 1971; Nishimura *et al.*, 1988; Yu *et al.*, 1989) and allyl tetrasulphide (Block *et al.*, 1986). These organosulphur compounds can be quantitated by solvent extraction followed by gas chromatographic/mass spectrometric analysis. Typical values of the various constituents are methyl sulphide (0.607 $\mu g\ g^{-1}$), methyl trisulphide (0.181 $\mu g\ g^{-1}$), allyl sulphide (2.02 $\mu g\ g^{-1}$), allyl disulphide (0.784 $\mu g\ g^{-1}$), allyl trisulphide (0.795 $\mu g\ g^{-1}$), allyl methyl sulphide (1.64 $\mu g\ g^{-1}$), allyl methyl disulphide (0.411 $\mu g\ g^{-1}$), allyl methyl trisulphide (0.695 $\mu g\ g^{-1}$) and ethyl 2-propenesulfinate (11.4 $\mu g\ g^{-1}$) (Weinberg et al., 1993). Although the prime active component in garlic is alliicin, its degradation compounds particularly the sulphides are also important. Major components of garlic oil responsible for the contribution to flavour have been identified as di(2-propenyl) trisulfide (31.9%), methyl 2-propenyl trisulfide (21.7%) and di(2-propenyl) disulphide (20.7%) (Pino, Rosado and Gonzalez, 1991).ß-Sitosterol has been shown to be the major sterol in the garlic essential oil (Huq, Saha and Begum, 1991). HPLC is known to give a reliable and semi-quantitative measure of the constituents compared with GC (Block *et al.*, 1992a; Block *et al.*, 1992b).

In the garlic health-product market, there are two main types of formulation, a garlic powder formulation mainly containing alliin and possibly small levels of allicin and a garlic oil formulation mainly containing sulphides. Encapsulated garlic flavours with flavour profiles of fresh aromatic top notes, as well as oleoresins where one part is equivalent to 50 parts of fresh garlic are also popular in food processing (Verghese, 1992). Oleoresin yields are generally around 21% using methyl alcohol extraction (Tae-Jin *et al.*, 1993). GC analysis of these sulphur compounds is useful in comparison of the composition of different product formulations (Yan *et al.*, 1993). Diallyl disulphide, allyl methyl trisulphide and diallyl trisulphide, the three main sulphides in garlic oil contribute to 60% in the oil. Oil of garlic has lately come to be appreciated as a valuable flavouring agent, for use in all kinds of meat preparations, soups, canned goods and table sauces.

8.2.11 Ginger

Ginger is one of the oldest of spices and consists of the peeled and sun dried rhizomes of *Zingiber officinale* Roscoe of the family Zingiberaceae. The rhizomes are normally treated with lime before drying. While the appearance of dry ginger is important to fetch a premium price, the essential oil, oleoresin and gingerol which impart flavour, aroma and pungency, respectively, and the starch, protein and crude fibre which make up the bulk of the dry matter are important quality attributes (Govindarajan, 1982). Ginger is subjected to extensive adulteration because of its importance in the drug, food and soft drink industries. The aroma of ginger is mainly derived from the volatile oil (1–3%) which contains n-decylaldehyde, n-nonylaldehyde, cineole, terpenes (*d*-camphene, β-phellandrene and zingiberene), zingiberol, citral and borneol (Brooks,

1916; Soffer *et al.*, 1944; Eschenmoser and Schinz, 1950). The pungency is due to an oleoresin called gingerol. Among the adulterants are capsicum and grains of paradise, both of which are added to give increased pungency, and turmeric which is added to restore colour. Microscopic examination and thin layer chromatography on 90% ethanol extract are reported to detect this adulteration. Adulteration in ginger tinctures can also be detected by a chemical method followed by an organoleptic test. This method is based on the fact that the pungent principles capsaicin and paradol in capsicum and grains of paradol are little affected by alkali treatment, whereas gingerol, the pungent principle in ginger is more readily decomposed with a resultant loss of pungency. This procedure is, however, dependent on the sensitivity of the tastebuds of individuals.

The TLC method affords a reliable and fast means of detecting adulteration even when more than one adulterant is present. The method is also of value in detecting exhausted ginger even when it is fortified with flavour (Osisiogu, 1973). Another adulterant reported is Japanese ginger or *Zingiber mioga*, for which no detection methods are available. Table 8.7 gives the analytical data and BP and BSI standards for ginger. Cold water extract and percentage of the water soluble ash are useful criteria of quality.

Large quantities of ginger oil are used in soft drinks. It is also used in spice blends for bakery and confectionery. Ginger oil may be used in natural flavours such as raspberry. The volatile oil derived from ginger is a mobile greenish to yellowish liquid, possessing a characteristic aromatic odour, but not the pungent flavour (bite) of the spice. The odour of the oil is quite lasting. Table 8.3 gives the physicochemical properties of ginger oil, which can serve as a tentative guideline of the authenticity of ginger oil. The parameters do vary within a small range with the geographical origin of the oil.

The volatile oil of ginger represents only the aromatic volatile constituents of the spice; it does not contain the non-volatile pungent principles for which ginger is so highly esteemed. To obtain all these constituents in a concentrated form, dried and ground ginger is percolated with volatile solvents such as acetone, alcohol or ether and the solvent is then carefully removed. Commercial dried gingers give about 3.5–10 % oleoresin. The oleoresin so obtained has the pungency, and the quantitative composition depends on the solvent used for extraction. Alcohol extraction gives an abnormally high yield, about 20 %, with a relatively low volatile oil and pungency due to dilution by other extractives. Oleoresin of ginger, commercially known as 'Gingerin', comprises mainly gingerol, zingerone and a homologue of zingerone called shogaol.

The yield of quality components in ginger varies with variety and maturity. Correlation between different quality parameters has been reported (Ratnambal *et al.*, 1987).

Table 8.7 Analytical data and BP, BSI and Indian standards for ginger

	BP	Standards BS		Usual ranges for genuine ginger %	Indian standards
		Unbleached	Bleached		
	%	%	%		%
Moisture		12(max)	12(max)	8.4-13.9	max. 13
Total ash	6 (max)	[a]8(max)	[a]12(max)	3.2–7.6	max. 8
Water soluble ash	1.7 (min)	—	—	1.0–3.7	min. 1.7
Acid insoluble ash	—	—	—	—	max. 1
Calcium (CaO)	—	[a]1.1(max)	[a]2.5(max)	—	max. 4
Volatile oil (v/m)	—	[a]1.5(min)	[a]1.5(min)	1.0–3.1	min. 1
Fixed oil and resin	—	—		2.8–7.5	
90% Alcohol extract	4.5 (min)	—	—	4.5-8.1	min. 4.5
Crude fibre	—	—	—	1.7-6.5	
Nitrogen	—	—	—	1.0–1.5	—
Cold water extract	10 (min)	—	—	7–14	min. 10
Starch	—	—	—	48.5–53.0	
Gingerol	—	—	—	0.9–2.5	

[a]On dry weight basis
Source: Pearson, 1976; Prevention of Food Adulteration Act, 1991

8.2.12 Mustard

The seeds of the genus Brassica of the family Cruciferae have been a major global oilseed. The Brassica species are characterized by the volatile oil from the point of view of flavour. The seeds contain glucosinolates from which by the action of the enzyme 'myrosinase', volatile isothiocyanate compounds are released which are responsible for the pungent flavour. Mustard oil is therefore widely used in flavouring all kinds of food products, table sauces, salad dressings, etc. The volatile compound derived from black mustard, *Brassica nigra,* consists .almost entirely of allyl isothiocyanate. This compound can also be prepared synthetically and is often used in imitation mustard flavour. Official standard works in the United States recognize both the natural and synthetic oils as 'volatile oil of mustard'. The physicochemical properties of pure volatile mustard oil are as shown in Table 8.3.

A comparison of physical and chemical properties of the synthetic essence (synthetic allyl isothiocyanate) and the natural essential oil are given by Gupta *et al.* (1960) which reveal that the natural oil samples from Brassica species have the optical rotation -0.8° to 4.8°, while the synthetic oil is dextrorotatory and develops a pinkish colour after storage at ambient temperature.

From white mustard seeds, *B. alba,* a paste made by decortication and grinding called 'prepared mustard' has been used as a culinary condiment in many countries.

Black mustard seeds and flour are reported to be used in a variety of food products. The flour is reported to be adulterated with various seed meals such as linseed meal (Escalante and Liuaga, 1954). Admixture of whole mustard seeds with rapeseed *(Brassica napus)* and turnip seed can be detected by enzymic hydrolysis of the thioglycoside. While mustard yields allyl cyanide, rapeseed and turnip seed yield

butenyl cyanide and pentenyl cyanide, respectively, in addition to allyl cyanide. The sensitivity of the method is 5% (Vangheesdaele and Fournier, 1977).

The US standards specify (i) for both black and white mustard seed maxima for total ash (5%) and acid insoluble ash (1.5%), (ii) for black mustard a minimum for volatile oil (0.6% as allyl isothiocyanate) and (iii) for mustard flour maxima for total ash (6%) and starch (1.5%), and partial removal of the fixed oil is permissible.

8.2.13 Nutmeg and mace

Myrzstica fragrans or the nutmeg tree is the source of two important spices- nutmeg and mace. The harvested ripe fruit of *M. fragrans* with the halves split, discloses the seed with a shell like testa covered by a scarlet fibrous aril. After collection, the pericarp is removed and the seed separated from the aril and dried. Drying is complete when the kernel rattles in the shell. The shells are cracked off with wooden hammers or by suitable mechanical means and the kernels removed and sorted. Dried kernels are the nutmeg of commerce. Mace is the dried fibrous aril covering the testa, which is obtained by separating the arils and drying in the sun after flattening between boards.

East Indian nutmeg is available in three grades (i) Banda nutmeg considered to be the finest for use and containing up to 8% essential oil, (ii) Siauw nutmeg, as good as Banda, but containing 6.5% essential oil, (iii) Penang nutmeg, which is usually wormy and mouldy and suitable only for distillation purposes; Papua nutmeg is derived not from *M. fragrans,* but from the allied spice, *M. argentea.* Bombay nutmeg is derived from *M. malabarica,* which is long and narrow in shape and nearly without aroma. It is used as an adulterant of true nutmeg. Oleoresins in about 34% yield can be prepared from nutmegs by extraction in ethanol (Borges and Pino, 1993). Oleoresins containing a relatively high fat content are obtained by extraction with a non-polar solvent and are preferred for use in flavouring processed foods since they have a greater tenacity and stability to heat (Purseglove *et al.,* 1981 b).

d-Pinene and *d*-camphene are the major constituents of the oil and together account for 80% of the oil (Wealth of India, 1962). The other constituents are dipentene (8%), *d*-linalool, *d*-borneol, geraniol and *dl*-terpineol (together account for about 6%) , myristicin and traces of saffrole, eugenol, isoeugenol and myristic acid esters (Power and Salway, 1907).

Three types of mace are traded (i) Banda mace, considered to be the finest, has a bright orange colour and fine aroma (ii) Tawa estate, golden yellow with crimson streaks (iii) Siauw mace, lighter than banda mace with a less volatile oil. Bombay mace derived from *M. malabarica* is dark red in colour, devoid of aroma, useless as a spice and often used as an adulterant of East Indian mace. Its volatile oil is similar to nutmeg in flavour and composition and is not distinguished in trade. Commercial mace oleoresins are available with volatile oil contents ranging from 10-55%. The yield of oleoresin varies from 27-32% using petroleum ether to 22-27% using hot ethanol. Mace contains negligible amounts of fatty oil or other odourless, flavourless

substances. It is one of the most concentrated forms of nutmeg–mace flavour (Purseglove *et al.*, 1981b). The yield of oil varies greatly with the geographical origin of the spice and with its quality. Therefore the usual physicochemical properties such as the specific gravity, acid number, ester number, optical rotation, etc. are not truly indicative of quality. Data reported by Clevenger (1935) on a number of oils distilled from nutmeg and mace are shown in Table 8.3.

Wormy nutmegs give a much better yield of oil in commercial distillation than do sound nutmegs, for the simple reason that in the former most of the fixed (fatty oil) has been devoured by the worms, while the strongly aromatic volatile oil remains intact. Sound nutmegs on the other hand retain all their fixed oil, and the latter on distillation tend to retain the volatile oil, thus lowering its yield.

Oil of nutmeg and mace are employed for flavouring food products and liqueurs. They are a major component of cola flavours, and this accounts for most of the worldwide production. They are also used in meat seasonings and in spice mixtures for bakery products. The oils find applications also in table sauces, tomato ketchup and all kinds of savoury preparations. Small quantities can be used in natural fruit flavours, where it imparts richness and depth. According to the specifications of the Health Ministry, Government of India, mace shall contain ether extractives not below 20% and not above 30%, crude fibre not above l0%, total ash, not over 3%, and foreign organic and deteriorated matter not above 5%. Table 8.8 gives the US and BPC standards for nutmeg and mace.

Table 8.8 Analytical data and standards for nutmeg and mace

	Nutmeg				Mace		
	Range (%)	BPC (%)	US (%)	Indian (%)	Range (%)	US	Indian
Moisture	4–8	–	–	8 (max)	3.5-7.0	–	10(max)
Ash	1.8–4.5	3(max)	5(max)	5 (max)	1.6–2.5	3(max)	3 (max)
Water soluble ash	1–2	–	–		0.9–1.7	–	
Acid insoluble ash	0–4.3	–			–		1(max)
Fixed oil 30 (max)	30-40	–	25(min)	25(min)	24–33	20-30	20(min)
Volatile oil (v/m)	5–15	5(min) whole 4(min) powder	–	4-15	–		
Alcohol extract		–	–	21.5-25	–		
Crude fibre	2–3.7	–	l0(max)	l0(max)	4.7–7.3	l0(max)	10 (max)
Nitrogen	1.1–1.4	–	–		0.85–1.15	–	
Starch	7.5-12	–	–	–	–		

Source: Pearson, 1976; Prevention of Food Adulteration Act, 1991

8.2.14 Oil of wintergreen

Gaultheria procumbens L. of the family Ericaceae or Wintergreen is one of the oldest and best known American flavours. Its strong characteristic taste was familiar to the

American Indians, who chewed the leaves for their agreeable odour and flavour. It is presently used chiefly as a flavouring agent in candies, chewing gums and certain soft drinks.

The main flavourant, methyl salicylate is not present in the free form but as a glycoside. The plant contains very little volatile oil and only after splitting of the glycoside under the influence of the enzyme premeverosidase can appreciable yields be obtained. Genuine oil of wintergreen is an almost colourless, yellow or reddish liquid of strongly aromatic and very characteristic odour and flavour. The physicochemical properties of oil of wintergreen are given in Table 8.3.

Since natural wintergreen oil consists almost entirely of methyl salicylate, the oil is frequently adulterated with synthetic methyl salicylate. Moderate additions of this ester are most difficult to detect. Large additions result in a slight lowering of the optical rotation of the oils. Formerly synthetic methyl salicylate often contained small quantities of free phenol, and identifying phenol was conclusive proof of the oil being adulterated with methyl salicylate. Presently, methyl salicylate is manufactured in such a pure form that in most cases it does not contain phenol. Modern analytical techniques such as stable isotope ratio analysis or selected-ion-monitoring should be of immense potential in detecting such additions and need to be investigated.

8.2.15 Onion

The bulbs of *Allium cepa* of the family Liliaceae, commonly known as onions have a charcteristic pungent and lasting odour. This is due to a volatile oil, present to the extent of 0.018-0.04% (depending on the variety) which can be distilled to a brownish semi-solid oil. Sulphur-containing compounds, particularly disulphides are the main components of this oil. Although known for a long time, oil of onion has only recently been produced on a commercial scale. The oil is now used as an important ingredient in the flavouring of meats, sausages, soups, table sauces and all kinds of culinary preparations. The physicochemical properties of onions analysed from a genuine batch of onion bulbs has been given by Guenther (1982) and are shown in Table 8.3.

Appraisal of flavour or pungency of alliums such as onions and garlic can be based on either subjective sensory analysis or objective detection of compounds generated by cysteine sulphoxide lyase (C–S lyase: enzyme code EC4.4.1.4) activity after tissue disruption. The typical flavour of alliums is due to the conversion of endogenous alk(en)yl-L-cysteine sulphoxide flavour precursors to pyruvate, ammonia and thiosulfinates by C–S lyase (Nock and Mazelis, 1987). For example, alk(en)yl thiosulphonate products such as I-propyl propanethiosulphate and methyl methane thiosulphinate are primarily responsible for the characteristic fresh flavour of onion tissue (Freeman and Whenham, 1976). The determination of pyruvate as an indicator of pungency is well established (Wall and Corgan, 1992). Pyruvate determination is based on the lactate dehydrogenase (LDH) and NADH coupled reaction or on the 2,4-dinitrophenylhydrazine (2,4-DNPH) derivatization procedure (Schwimmer and

Weston, 1961). The 2,4-DNPH method requires an additional step to correct for background carbonyls since it is non-specific and carbonyl compounds other than pyruvate do react (Lancaster and Boland, 1990). An alternate approach evaluated for determining pungency in alliums has been based on detection of thiopropanal-S-oxide, the lachrymatory compound (Freeman and Whenham, 1975). This method requires hexane extraction and spectrophotometric analysis. Gas chromatography is considered the best to assess the flavour profile of allium tissue, but the contribution of secondary products to the overall pungency of the sample is uncertain (Yu *et al.,* 1989). HPLC also has been used to detect allicin (diallyl thiosulphinate) in garlic (Jansen *et al.,* 1987).

An alternate method for the evaluation of pungency in allium spp. involves the determination of the thiosulphinates (Carson and Wong, 1959; Nakata *et al.,* 1970). The procedure involves derivatizing the thiosulphinates with N-ethylmaleimide and measuring the absorbance of the conjugate at 515 nm. A prototypic simple pungency indicator test for allium spp. based on the application of the N-ethylmaleimide reaction for the sulphonates has been recently reported (Thomas *et al.,* 1992). The efficacy of the test has been confirmed by correlating colour production with the thiosulphinate content (measured spectrophotometrically) and pyruvate concentration in minced onion tissue. Correlation between the thiosulphinate content as absorbance at 515 nm and pyruvate contents are as shown in Figure 8.1. A significant correlation has been obtained (R^2= 0.871; P<0.00l) and found supporting the use of thiosulphinate determination as an indicator of pungency.

In attempts to adapt the N-ethylmaleimide based determination of sulphonates to a reflectance colorimeter method, cotton has been found to be a good matrix for the retention of the colour. Materials such as filter paper and latex sponges are known to give vague results due to leaching of colour of the reaction mixture or failure to produce resolute colour. The correlation between Hunter a values, determined by the reflectance colourimetric procedure and thiosulphinate content (determined spectrophotometrically) is significant ($R^2 = 0.828$; $P < 0.001$). The saturation index, an indicator of the quantity of the colour takes in to account both the Hunter *a* and *b* (yellow) values and better represents the red-orange colour produced by the reaction of the thiosulphinates with *N*-ethylmaleimide. The colour differences between onion samples could be ascertained by using this test. Ideally, the colour produced by this test could simply be compared with a colour-coded chart for an estimation of allium pungency. Further developments in this procedure are in progress (Thomas *et al.,* 1992).

8.2.16 Pepper

The trade distinguishes between two principal types of pepper, namely black and the white, both derived from the same plant *Piper nigrum L.* of the family Piperaceae, a climbing or trailing vine like shrub native to southern India. Black pepper is the dried, whole, unripe fruit of this plant; white pepper consists of the dried ripe fruit from which the dark hull has been removed.

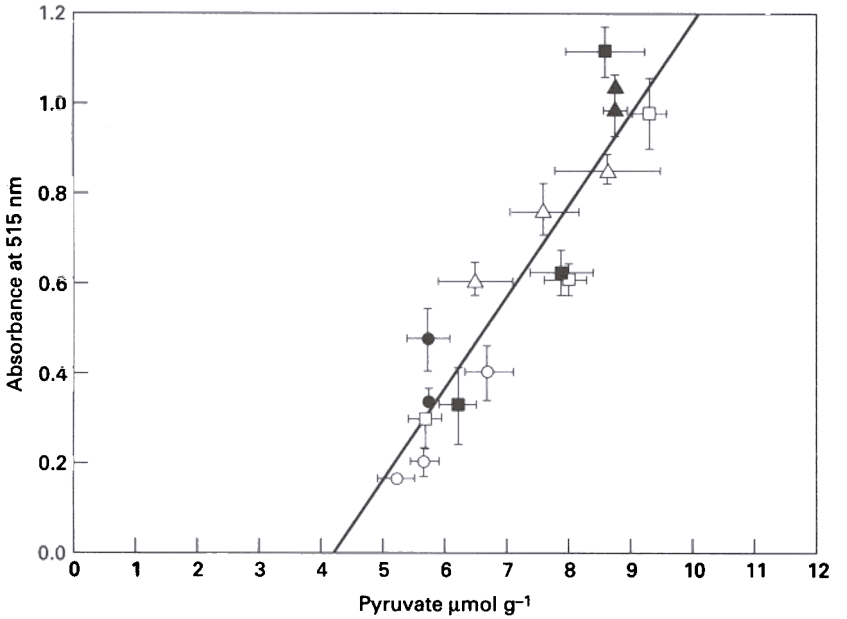


Figure 8.1 Correlation between thiosulphinate and pyruvate contents for each bulb. Pyruvate was determined by the LDH method. Each symbol is the mean ± SD for an individual bulb assayed in triplicate. $R^2 = 0.871$; ○, B8155; ●, B9161; □, Spartan Banner 80; ■, B4535; △, Sweet sandwich; ▲, B9897. (Source: Thomas *et al.*, 1992, reproduced with permission)

Pepper is one of the oldest and most important spices. It was known to the Greeks as far back as the fourth century BC. The Romans valued it highly and imported it in large quantities. On the basis of geographical origin and quality, the trade recognizes a number of grades of black pepper. The most important grade is 'Lampong black pepper' which is produced in the Lampong district of southern Sumatra, Indonesia followed by a closely related Singapore and Penang pepper. Some grades of pepper are of large size and excellent appearance and aroma and therefore very expensive. These include Tellicherry and Alleppi black pepper from the Malabar coast of southern India. With respect to white pepper, the most important type is 'Muntock white pepper' and, to a lesser degree, 'Sarawak white pepper'. The former originates from the island of Banka (off Sumatra) and the latter from the British part of Borneo. White pepper cannot be used for distillation purposes, first because of its high price and because the hulls which contain most of the essential oil have been removed.

White pepper powder is generally adulterated using starch (from maize and/or rice) or even corn flour. The piperine content and K: Ca ratio can be used as indices to check this adulteration. In a white pepper/rice starch mixture, an increase in percentage starch decreases the piperine content and increases the K:Ca ratio. Mixtures containing maize starch showed decreased piperine content but a less rapid increase in K:Ca ratios. Results

are presented in Table 8.9. Undried white pepper should contain not less than 3.5% of *trans-trans* piperine and the ratio of potassium to calcium should not be more than 0.45 on a weight basis. This standard is however not applicable to black pepper (Archer, 1987).

Chillies in black pepper can be detected by HPLC (Weaver *et al.*, 1984). In black pepper, mineral oil is used as a polishing and glazing agent. Furthermore, the storage life of berries is improved by the presence of mineral oil which inhibits insect infestation and prevents growth of fungus on berries. Mineral oil is generally identified as turbidity in Holde's test. The essential oils of black pepper do not undergo saponification by alcoholic potassium hydroxide but produce turbidity that gives a wrong interpretation of the presence of mineral oil. A chromatographic method using TLC can not only detect mineral oil without any interference from unsaponifiables in black pepper as fluorescent spots under UV lamp, but also identify them qualitatively (Chakravorty, 1979).

Crushed black pepper yields about 1.0–2.6% volatile oil on steam distillation, depending on the age of the dried berries. It is a valuable adjunct in the flavouring of sausages, canned meats, soups, table sauces and certain beverages and liqueurs. The oil is also used in perfumery, particularly in bouquets of the oriental type, to which it imparts spicy notes difficult to identify. The main constituents of the volatile oil are reported to be α-pinene, β-pinene, l-α-phellandrene, *d*-limonene, piperonal, β-caryophyllene and dihydrocarveol. Typical physicochemical properties of the pepper volatile oil are as shown in Table 8.3. Pepper oil is occasionally adulterated with low priced and readily accessible terpenes and sesquiterpenes, such as phellandrene, dipentene and caryophyllene. Since these compounds are natural components of the oil, it is most difficult to prove that they have been added to the oil.

The volatile essential oil obtained from steam distillation of dried black pepper represents only aromatic, odorous constituents of the spice; it does not contain the pungent, non-volatile principle for which the spice is highly esteemed. Oleoresins obtained by solvent extraction followed by desolventization overcome this drawback of the volatile oil; the quality depends upon the solvent used. Oleoresin of pepper (varies from 9–15%, depending on the cultivar) generally contains an alkaloid piperine, its isomer chavicine (both of which are piperides of piperidine and chavinic acid), other piperides, a higher homologue of piperine called piperidine (Spring and Stark, 1950), a volatile alkaloid identified as β-methylpyrroline and unidentified resins. The pepper oleoresin, when freshly made is a dark green, viscous liquid with a strong aroma. On standing, crystals of piperine appear, and the oleoresin requires mixing before use to ensure uniformity of consistency.

An oleoresin from the fruits of *Schinus molle* belonging to the family Anacardiaceae, which have a strong peppery odour is used as an adulterant of oleoresin from black pepper, but can be detected by the presence of glucose. This oil had shot into prominence during World War II, when black pepper was in short supply (Wealth of India, 1972). Seeds of *Mirabilis jalapa*, belonging to the family Nyctaginaceae are used as an adulterant of black pepper (Wealth of India, 1962).

Whole black pepper is often adulterated with the fruits of *Lantana camara*, *Vitex*

Table 8.9 Calcium, potassium and piperine content of white pepper, starch and white pepper and starch mixtures

Sample	Calcium (mg kg^{-1}	Potassium mg kg^{-1}	K/Ca (%)	Piperine
Genuine white pepper (10 samples range):	1520 – 3000	340 – 860	0.19 – 0.44	3.64 – 4.35
Mean and std. deviation	1980 ± 470	640 ± 170	0.33 ± 0.07	3.98 ± 0.28
Retail white pepper (15 samples range):	1500 – 3000	510 – 900	0.27 – 0.44	3.72 – 4.46
Mean and std. deviation	1890 ± 460	650 ± 140	0.35 ± 0.05	4.01 ± 0.23
White pepper and starch mixtures:				
10% maize starch	1500	680	.45	3.81
20% maize starch	1380	640	0.46	3.31
30% maize starch	1250	640	0.51	2.92
50% maize starch	880	540	0.61	2.14
70% maize starch	560	480	0.84	1.32
10% rice starch	1500	790	0.53	3.67
20% rice starch	1130	890	0.68	3.33
30% rice starch	1130	930	0.82	2.88
50% rice starch	800	1130	1.29	2.12
70% rice starch	560	1260	2.24	1.28
Rice starch (5 samples range)	30 – 60	1060 – 1800	18 – 45	0
Corn flour (5 samples range)	20 – 140	25 – 60	0.38 – 2.79	0
Black pepper genuine (10 samples range)	15 400 – 20 800	4000 – 7200	2.79 – 3.85	4.33 – 5.28

Source: Archer, 1987 (reproduced with permission).

altissima Linn. or seeds of *Carica papaya* and dried roasted berries of *Schinus molle Linn.* Papaya seeds can be detected from lower crude starch and higher non -volatile ether extract values. A study conducted on 118 commercial samples of ground black pepper for adulteration with papaya seeds has revealed that microscopic inspection detects adulterants at the 1% level, whereas TLC detects only >20% adulterant papaya seeds. It is also suggested in the same study that the permitted upper limit for total ash be reduced from 7 to 4% and that acid-insoluble ash be reduced from 1.5 to 1% (Silveria *et al.*, 1983). A constituent present in the adulterant but not in the pure sample is obviously a more sensitive indicator of adulteration. One such constituent present in seeds of *Carica papaya* is a glucotropaeolin (giving benzyl isothiocyanate) (Harbourne, 1973). This has been made the basis of detecting papaya seeds in pepper (Curl and Fenwick, 1983). Indices to detect other adulterants are surprisingly not as yet available.

Attempts have been made to analyse the sensory quality of pepper by GC fingerprinting for either the number and area of the peaks or ratios of selected peaks (Dutta *et al.*, 1962; Wijeskara *et al.*, 1972), representing mainly the mono- and sesquiterpene hydrocarbons. More meaningful results can be obtained however by concentrating on aroma significant compounds (Pangborn *et al.*, 1971). Coupling of GC with mass spectroscopy (MS) has identified many oxygenated compounds, namely

terpenic, aliphatic, aromatic and carbonyl (Debrauwere and Verzele, 1975). More than 150 compounds, some in very small amounts have been identified in pepper, possibly acting synergistically in their aroma impact, and many more remain to be identified (Artem'ev and Mistryukov, 1979; Lawrence, 1985a). The selection of the relevant GC peaks that would reflect quality therefore becomes very important. The data generated from sensory quality, volatile oil and moisture content reduces dimensionality and helps in arriving at impact attributes, when subjected to principal component analyses. Six small peaks resolved from the oxygenated fraction have been shown to reflect quality as monitored by sensory response, accounting for 93.4% of the information, and are believed to be more meaningful than terpene hydrocarbon peaks of high resolution. Studies on the identitity of these compounds, responsible for impact peaks, which are the determinants of quality are in progress with MS and NMR (Narasimhan *et al.*, 1992b).

8.2.17 Poppy seeds (Papaver somniferum Linn)

Poppy seeds are popularly used in delicacies and cuisine in the Indian subcontinent and the Middle East and to some extent in the Western cuisines as a thickening and flavouring agent. It is generally sprinkled over breads, pastries and chilled soups, in dips and various spreads. Unscrupulous traders generally adulterate the expensive poppy seeds with the cheaper *Amaranthus paniculatas* seeds which closely resemble poppy seeds. This can be detected qualitatively by puffing a sample, wherein any amaranth seed puffs and shows its presence. It can also be detected quantitatively by estimating squalene, an unsaturated long chain hydrocarbon found in large quantities in amaranth seed oil and only in traces in poppy seed oil. This can be easily seen from Figure 8.2. No physicochemical tests have as yet been laid down to check for the authenticity of this widely used spice (Singhal and Kulkarni, 1990).

8.2.18 Sage

The leaves of *Salvia officinalis* or sage have been extensively employed in the food industry as a standard spice in making stuffing for fowl, meats and sausage. It is an important culinary herb. Dried and powdered leaves are mixed with cooked vegetables and sprinkled on cheese dishes, cooked meats and other similar preparations. The young leaves are used for flavouring tea. An essential oil, 1.3–2.6% on dry weight basis is present in the leaves of *S. officinalis* with thujone being the quality determining constituent. The higher the thujone content, the better is the oil. Sage oil finds applications in perfumery. It is used for adulterating rosemary and lavender oils. Sage oil is itself adulterated with American cedar leaf oil which also contains thujone (Wealth of India, 1972).

Sage differs considerably in appearance according to the country of origin. Dried English sage is green, whereas that from Cyprus is a pale-bluish green, hard and

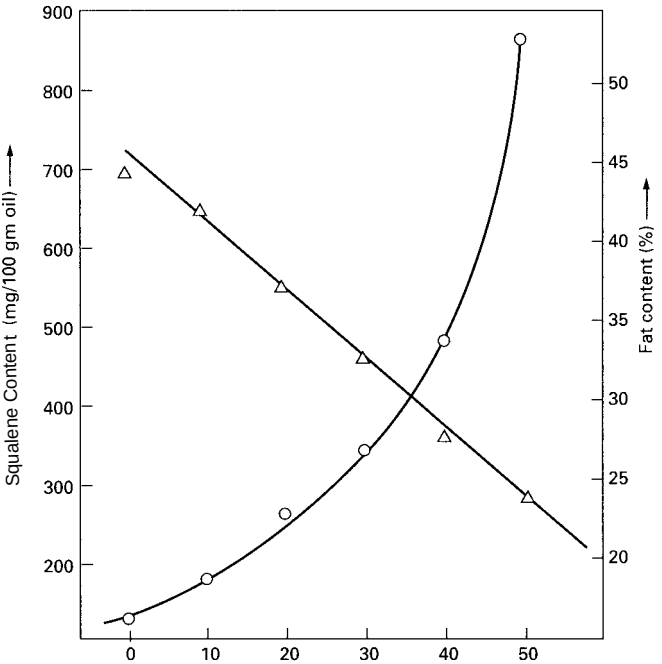


Figure 8.2 Fat and squalene content of khus-khus (poppy seeds) and its admixture with the adulterant rajgeera *Amaranthus paniculatas* △, Fat content; ◯, Squalene content. (Source: Singhai and Kulkarni. 1990)

leathery and the highly esteemed type from Dalmatia is grey and very fluffy. Many samples of English sage tend to give data for the ash and the acid insoluble ash in excess of the US maximums (10.0% and 1.0% respectively). The BP prescribed maxima for foreign organic matter (3%) and ash (8%).

8.2.19 Star anise

Fruits of star anise *(Illicium verum)* of the family Magnoliaceae have an agreeable aromatic sweet taste and pleasant odour resembling anise. The fruit has a star-like shape and exhibits a characteristic anise odour; hence the name star anise. It consists usually of eight boat shaped follicles, or carpels, arranged around a central axis. It is used as a condiment for flavouring curries, confectionery and spirits and also for pickling and perfumery. Star anise fruit is often adulterated with the fruit of *I. anisatum Linn.* syn. *I. religiosum* grown in Japan. The fruit of *I. anisatum* is poisonous, the poisonous principle being hananomin with an empirical formula $C_{14}H_{22}O_{10}$.

Star anise oil of commerce is obtained by steam distillation of fresh fruits of *I. verum* (yield about 2.2-3.5%). It is colourless or pale yellow with the characteristic odour and

aromatic taste of true anise oil (from *Pimpinella anisum*). Anethole is its chief constituent (85–90%) (Wealth of India, 1960) and it also contains *d*-α-pinene, Δ^3-carene, α- and β-phellandrene, *p*-cymene, cineole, dipentene, *l*-limonene, α-terpineol, methyl chavicol, saffrole and some parraffins. On oxidation, anethole is gradually converted to anisaldehyde and anisic acid. Old star anise oils therefore may contain these compounds, the quantity increasing with the age of the oil.

Star anise oil is used in candy, chewing gums, liqueurs and pharmaceuticals as a flavouring agent. The flavour of anise is very popular in Turkish, French, Italian, Spanish and Greek confectionery. However, the use of anethole, or of anise oil (from *Pimpinella anisum*) is preferrable. Animals seem to relish food flavoured with star anise oil and hence its application in all kinds of pet food products. The most important use of star anise oil is for the technical isolation of anethole, which has a much finer odour and flavour than the oil itself. The physicochemical properties of star anise oil are as given in Table 8.3.

Of notable interest is the congealing point, which should be above +15 °C for oils of acceptable quality. The quality of star anise oil, like that of anise oil can be evaluated by its congealing point. In fact, it is possible to estimate the anethole content in the oil from its congealing point. In commercial practise, the star anise oils may be judged as in Table 8.10.

The addition of star anise leaves to the distillation material, and of leaf oil to the fruit oil is often practised. These practices result in a lowering of the congealing point and of the anethole content. Another form of adulteration practised by some traders is the addition of small quantities of mineral oil (kerosene, etc.) or fatty oils. These additions alter the specific gravity, congealing point and solubility in 90% alcohol, and can be demonstrated as in Table 8.11.

8.2.20 Turmeric

Turmeric, the dried rhizome of bulbous root of *Curcuma longa* Linn is probably most subjected to adulteration since it is frequently sold in ground condition. Curcumin is the main colouring principle, and can be measured rapidly by simple spectrofluorometric determination (Jasim and Ali, 1992). Microscopy does detect the adulteration of cheaper vegetable substances in turmeric, but when the adulterants belong to the same genus (Curcuma), the genuineness of the sample is difficult to decipher, even by experts in microscopy. *Curcuma zedoaria* and *Curcuma aromatica* which are extensively used as adulterants however do differ from *Curcuma longa* in the aromatic constituents. The adulterants contain sufficient amounts of camphor and camphene, both of which are absent in *Curcuma longa*, and this forms the basis for differentiation using a simple and rapid TLC technique which has a sensitivity of 5% detection. The detection is by a three step colour sequence and the reader is referred to Sen *et al.* (1974). Table 8.12 gives a profile of different colours and R_f values obtained for genuine *C. longa* and its adulterants. The scheme can also be applied to

Table 8.10 Quality of anise oil from its congedling point

Congealing point (°C)	Quality
18	Best
17	Very good
16	Good
15	Lowest limit
Below 15	Not acceptable

Table 8.11 Physical quantities of adulterated star anise oil

Quality of oil	Specific gravity at 15 °C	Congealing point (°C)	Solubility in 90% alcohol
Pure oil	0.986	+18	1:2.2 and more
5% Petroleum added	0.978	+16.25	Not clearly
10% Petroleum added	0.970	+14.75	soluble in 10 vols of 90% alcohol

check for the presence of *C.caesia* and *C.domestica*, as both the species contain camphor and the former also contains camphene. Adulteration by *C.xanthorrhiza* can be checked by fluorescence and colour reactions (Mitra, 1975). *C. xanthorrhiza* oil contains xanthorrizol (21.5%), a small amount of turmerone and turmerol and no α-atlantone, while *C. domestica* contains large amounts of ar-turmerone, turmerone and turmerol (about 75%) and α-atlantone (2.4%) as a specific constituent. The oil from *C. aromatica* is known to resemble *C. xanthorrhiza* (Zwaving and Bos, 1992). These constituents could be used to detect interspecies blends of turmeric, both in the spice powder as well as in the essential oil.

A very high lead content has been reported occasionally in turmeric due to the use of lead chromate to accentuate the colour. Samples need to be examined for artificial colours and microscopically for the presence of foreign starches (Pearson, 1976).

Turmeric oleoresin is prepared by extracting ground spice with either acetone, ethanol or ethylene dichloride, followed by distilling off the solvent. Acetone extract generally gives a higher yield of oleoresin (7.9–10.4%) with a high curcumin content as compared to ethanol or ethylene dichloride extract. The other constituents of the oleoresin are fatty oil, resin and bitter principles. It is orange red in colour and consists of an upper oily layer and a lower 'crystalline' layer. The pure oleoresin is viscous and difficult to handle and is also relatively insoluble. For commercial use, it is usually mixed with a non-volatile edible solvent such as vegetable oil, propylene glycol or polyoxyethylene sorbitan fatty acid esters in order to disperse the extracted material and to render it free flowing and soluble in aqueous media (Purseglove *et al.*, 1981a, b).

The volatile oil in turmeric ranges from 2–6%. The time required to recover volatile oil is longer for turmeric as compared to pepper or cardamoms, owing to the fact thatturmeric oil contains about 85% of high-boiling sesquiterpenes.

Table 8.12 Colour reactions in the different detection methods (figures in parenthesis are *R* values)

Chromogenic reagent No.1		Chromogenic reagents No.1 and 2	
C.zedoaria and C.aromatica	*C.longa*	*C.zedoaria C.aromatica*	*C.longa*
Violet(0.77)	Bluish violet (0.75)	Violet (0.77)	Bluish violet (0.75)
Pink(0.72)		Orange (0.72)	
Light pink(0.64)		Light pink (0.64)	
Orange(0.60)	Brown (0.60)	Blue (0.60)	Dirty brown (0.60)
Dirty green (0.55)	Bluish fluorescence (0.55)	Deep pink (0.55)	
Greenish violet (0.46)	Light blue (0.46)	Violet (0.46)	Greenish violet (0.46)
Violettish pink (0.40)	Violet (0.40)	Light pink (0.40)	Pinkish brown (0.40)
Bluish violet (0.33)	Bluish green (0.33)	Bluish violet (0.33)	Bluish green (0.33)
Pink (0.23)	Light pink (0.23)	Violet (0.23)	Brown (0.23)

Source: Sen *et al.*, 1974 (reproduced with permission).

8.2.21 Spices of the Umbelliferae family

This family includes many spices of commercial interest. Amongst the spices included are anise, caraway, cumin, corriander, dill, fennel, Indian dill and parsley.

Coriander (*Coriandrum sativum*) seed oil is used in a variety of flavour applications. It is a part of traditional flavouring of a number of alcoholic drinks, especially gin. It is widely used in meat seasonings and curry blends. It provides a very attractive source of linalool in natural flavours, particularly, apricot. Its other constituents are identified as *d*-α-pinene, *dl*-α-pinene, β-pinene, dipentene, *p*-cymene, γ-terpinene and α-terpinene, n-decylaldehyde, geraniol, *l*-borneol, acetic acid and traces of decylic acid. TLC analysis of the sterols has shown β-sitosterol to be the predominant sterol (Adhikari *et al.*, 1991). Geographical and varietal divergence in the raw materials cause variations in the levels of individual constituents in coriander fruit oil. For instance, the organoleptic quality of coriander fruit oil from Russia and Albania has been found to be superior to that from India and Italy, this attributed to the lower *p*-cymene content in the former varieties (Pino *et al.*, 1993). Coriander oleoresin is prepared on a very small scale. It contains volatile oil, fatty oil and some other extractives, but their relative abundance is dependent on the raw material, the processing procedure and the solvent used. Generally about 90% fatty oil and 5% steam-volatiles are present in coriander oleoresin, and the oleoresin extract may be regarded as a solution of volatile oil in the fatty oil (Purseglove *et al.*, 1981a).

The physicochemical properties of oils of coriander seed, dill, anise, fennel, celery, are listed in Table 8.3.

The major use of dill oil is in seasoning blends, particularly for use in pickles.

Besides carvone, which is the major constituent, *d*-limonene, phellandrene, α-pinene, dipentene and dihydrocarvone are also present. Two substances believed to be of special sensory significance are identified as 4-vinyl-2-methoxyphenol with a spicy meat-like note and 4-hydroxy-3-methyl-6-(1-methylethyl)-cyclohex-2-en-1-one with a dill like sweet odour (Nitz *et al.*, 1991). Dill is often adulterated with Indian dill and all kinds of terpenes, for example limonene and those resulting from the preparation of sweet orange oil concentrates. The most common adulterants are the terpenes obtained from the extraction of carvone from caraway seed oil.

Anise or aniseed (*Pimpinella anisum*), is a culinary herb posessing a sweet aromatic taste, and when crushed emits a characteristic agreeable odour. It is reported to be adulterated with exhausted fruits, fine earth and other small seeds and fruits. Ground aniseed is sometimes found adulterated with ground fennel which resembles it in aroma and flavour and is considerably cheaper.

Anise oil containing anethole to the extent of 85–90% is also frequently adulterated with the lower priced star anise oil. Besides anethole, other constituents reported in anise oil are methyl chavicol and *p*-methoxyphenylacetone. Since the congealing point of anise oil gives a good indication of the anethole content, it can be used for a rapid estimation of the percentage of anethole contained in the oil. In India, probably the oil of fennel is sold as a substitute for true anise oil and can be distinguished from the former by its lower anethole content and higher optical rotation (+11 to +20). Other adulterants used are turpentine oil, cedarwood oil, and copaiba and guryun balsam oils. Adulteration with synthetic anethole made from pine oil is also reported (Wealth of India, 1969). Storage of anise fruits under ordinary conditions for a year does not have any noticeable effect on the yield and quality of essential oils. However, longer storage periods lead to progressively decreasing yields, sometimes amounting to only 50% and the quality does not conform to specifications (Georgiev, 1965). Anise oil is used in large quantities in alcoholic drinks. It is also a popular flavouring in confectionery, particularly with a medicinal connotation, and in oral hygiene applications.

Fennel contains up to 4.6% oil, the main constituent of which is anethole (50 – 60%). Other constituents include *d*-α-pinene, *d*-α-fenchone, methyl cavicol, camphene, dipentene, anisaldehyde and anisic acid. Fennel oil is used in confectionery and liqueurs, soups, meat dishes and pickles. Pepper fennel (sp. piperitum) contains estragole as the main component (Dogan *et al.*, 1984; Akgul, 1986), while those from bitter fennel contain relatively high concentrations of α-pinene and fenchone and low concentrations of *trans*-anethone and estragole (Betts, 1968; Karlsen *et al.*, 1969; Lawrence, 1979). The stem, leaf and flowering umbel oils have little value, because of their low yields and low percentages of *trans*-anethole and large amounts of hydrocarbons (Akgul and Bayrak, 1988).

Celery or the dried ripe fruits of *Apium graveolens* are ridged and consist of an ovate, dark brown cremocarp, which is often separated when the spice is purchased. The seeds are highly valued as a condiment and for medicine, either directly or as an extract.

Due to its small size and dark colour, celery seed is liable to be adulterated in different ways. Admixture with extraneous sandy matter and foreign seeds of similar appearance is most common. With a view to framing standards and ensuring the genuineness of celery, Sen *et al.* (1973c) collected some analytical data with respect to moisture, ash, acid insoluble ash and non-volatile ether extract. They suggested neglecting the ash and acid insoluble ash, since they found an unusually high values, attributing them to the probable sandy matter. However cold water extract in conjunction with volatile oil and non-volatile ether extract will check any admixture with exhausted or inferior stuff.

The yield of volatile oil varies from 1.90–2.50% Its main constituents identified include *d*-limonene, selinene, sedanolide, sedanonic anhydride and an unidentified phenol. Celery seed oil imparts a warm, aromatic and pleasing note to food products. It is used in flavouring all kinds of food products such as canned soups and canned sausages. The most frequent adulterants are chaff oil, terpenes, chiefly *d*-limonene resulting from the concentration of sweet orange oil.

The physicochemical properties of celery oil can be easily adjusted and brought within desired limits, therefore properties alone are not conclusive when attempting to detect adulteration in celery seed oil.

Cumin (*Cuminum cuimum*) seeds have an aromatic odour and somewhat bitter taste and are exclusively used as a condiment. They are used as an essential ingredient in all mixed spices and curry powders for flavouring soups, sausages, pickles, cheese, meat dishes and for seasoning breads and cakes. The oil, besides being used in curries and culinary preparations of oriental character, also finds applications in flavouring liqueurs and cordials. Amongst the most annoying adulterant of cumin essential oil is synthetic cuminaldehyde, the presence of which cannot be detected analytically, except that the addition of an excess of synthetic cuminaldehyde would affect the optical rotation of the oil. Other chemical constituents of cumin seed oil are *p*-cymene, *dl*-pinene, and *d*-α-pinene, β-pinene, dipentene, β-phellandrene, dihydrocuminaldehyde and cuminyl alcohol. Modern analytical techniques such as stable isotope ratio analysis (SIRA) and selective ion monitoring (SIM) could be of help in detecting adulteration and blends and deserve attention from food analytical chemists.

Caraway (*Carvum carvi*) is a highly priced spice often adulterated with cumin which closely resembles caraway. The problem in detecting this adulteration is compounded when essential oil is prepared from spice admixtures. Addition of terpenes (chiefly *d*-limonene), obtained as by products from the extraction of carvone, or from orange oil is sometimes encountered. In order to compensate for the deficiency of carvone and for the lowered specific gravity of adulterated oil, other ketones such as piperitone, or certain aromatics such as benzyl alcohol are sometimes employed. The physicochemical characteristics of caraway seed oil are given in Table 8.3.

It possesses a characteristic, aromatic odour and a warm, sweetish, spicy taste. The main use is in flavouring all kinds of food products, for example, meats, sausages and canned goods. It is employed in pickle compounds, confectionery and also in liqueurs.

Table 8.13 Physicochemical properties of volatile oils of cumin from different geographical origins

Property	Mediterranean	Mexican	Persian	Indian	Pakistan
Specific gravity at 15°C	0.917–0.924	0.936	0.911	0.8945	0.9290
Optical rotation at 15°C	+4°22′ to +5°6′	+2°55′	+7°	+3.6°	+4.6°
RI at 20°C	1.501–1.504	1.507	1.498	1.491	1.501
Aldehyde (as cumin-aldehyde) (%)	47.4–51.50	62.70	18.00	16.00	20.00
Solubility in 80% alcohol (vols required)	5 vols	2 vols and more)	—	11 vols	8 vols

Source: Shankaracharya and Natarajan, 1971 (reproduced with permission).

Carvone is the major constituent of caraway seed oil, which also contains *d*-limonene, dihydrocarvone, dihydrocarveol, carveol, acetaldehyde, *d*-dihydrocarveol, *l*-dihydrocarveol, *l*-Neodihydrocarveol, *l*-isodihydrocarveol, *d*-perillyl alcohol and *d*-dihydropinol.

The quality determining component in caraway seeds is carvone, present to the extent of 50-60% in its essential oil (3–6% of seeds), that in cumin seeds is cuminaldehyde, that in coriander seeds is obtained from *Coriandrum sativum* is linalool which is at 70% in the essential oil (1% of the seeds) and that in dill (*Anethum graveolens*) seeds is carvone, 40–60% in the essential oil (2.5–4.0% in seeds) (Hans, 1969). However, adulteration reports using these as indices are surprisingly not available, although adulteration of these seed spices is rampant all over the world, either by extraction of the essential oil or addition of farinaceous substances. A probable reason could be the wide differences of these constituents and other physicochemical properties in spices from different geographical origins. This can be easily seen from Table 8.13 which shows the physicochemical properties of cumin volatile oils from different geographical origins.

Petroselinum sativum commonly kown as Parsley a native of Mediterranean countries, has been cultivated as garden herb since antiquity. The cuisine of France seldom offers a dish without a sprig of parsley. All parts of the plant, particularly the seed, contain a volatile or essential oil which is responsible for the pronounced odour and flavour of parsley. Parsley herb oil, however, has a superior odour and flavour, characteristic of fresh leaves. The main constituents of parsley seed oil are α-pinene, myristicin, apiole and *l*-allyl-2,3,4,5-tetramethoxybenzene. Physicochemical properties of parsley herb and seed oil are as given in Table 8.3.

TLC of the essential oils reveals spots characteristic of the constituents and can qualitatively indicate adulteration. Adulteration can be estimated by determining the concentrations of the characteristic components of either the spice or the adulterant. One such approach is given in Table 8.14. A range of rarer sugars occurs in plant glycosides, one example being the five-carbon branched sugar apiose, present as flavone glycoside in parsley seed (Harbourne, 1973). Screening of this glycoside could be of value in estimating the authenticity of the spice; this approach deserves to be investigated. A more uncommon oligosaccharide is umbelliferose, which is mainly

restricted to members of the Umbelliferae family, and could be a useful indicator of the presence of its members. It is not uncommon in some of the plant families, most notably Umbelliferae, Guttiferae and Rutaceae to encounter species that elaborate 10, 20 or even more coumarins, and many species elaborate four or five coumarins (Thompson and Brown, 1984). Coumarins commonly found in umbelliferous vegetables include bergapten, xanthotoxin, isopimpinellin and psoralen (Erdelmeier *et al.*, 1985; Vo-Dinh *et al.*, 1988; Glowniak *et al.*, 1986; Spencer *et al.*, 1987) and umbelliferone (Anderson and Podersan 1983) The phthalides found in umbelliferous plants include butylidene phthalide (Bohrmann *et al.*, 1967; Gijbels *et al.*, 1982), sedanenolide (Wilson 1970; Lund, 1978), senkyunolide (Gijbels *et al.*, 1982) and 3-n-butyl hexahydrophthalide (Wilson, 1970). Sesquiterpenes such as β-caryophyllene and α-humulene commonly occur in umbelliferous vegetables. These compounds are all possible marker compounds of the umbelliferous members (Weinberg *et al.*, 1993a, 1993b). Analysis of such compounds in spices of this family could give valuable information about their authenticity and needs to be studied.

A report from Tasmania on the relationship between carvone level and dill herb character and market demand is discussed using dill herb constituents as indicators for detecting adulteration with limonene (Clark and Menary, 1984). Fennel fruits (or seeds, as they are known in commerce) vary greatly in quality depending on the variety to which they belong and care bestowed in harvesting and storing the fruit. They often contain sand, stem tissues, stalks and other umbelliferous seeds. They are sometimes adulterated with exhausted or partially exhausted fruits or with immature or mould attacked fruits. According to BP, fennel seeds should contain not less than 1.4% volatile oil, not over 2.0% foreign organic colour and 1.5% acid insoluble ash. IPC permits up to 4.0% foreign organic matter. Powdered fennel should not contain less than 1.0% volatile oil.

Table 8.14 gives ranges for some of the analytical data obtained from the seven umbelliferous fruits which are used as spices, together with US standards and those which have been prescribed in Britain for pharmaceutical purposes (BP and BPC). The limit of 9.5% for ash content in cumin is suggested to be rather high. According to one report, the ash content in cumin rarely reaches 8.0% and a significant number of samples have ash content below 7.5%. It has also been suggested that the upper limit for ash content insoluble in dilute hydrochloric acid be changed to 1.25%, that volatile oil be 2.5% minimum and cold water extract 14% minimum. It has been found that exhausted stuff often shows analysis values within prescribed limits, forcing the declaration of inferior stuffs as genuine (Sen *et al.*, 1973a; Thorpe, 1953). Similar work on coriander seeds also necessitates the inclusion of cold water extract and volatile oil in the prescribed standards, since the ash and acid insoluble ash as quality control parameters allow addition of exhausted stuff to genuine and allow it to pass off as genuine (Sen *et al.*, 1973b). Some light in this regard should also be thrown on other spices by proper analytical studies and if necessary, a change in the standard specifications should be made.

Table 8.14 Analytical data for the umbelliferous fruits

	Aniseed (%)	Caraway (%)	Celery (%)	Corriander (%)	Cumin (%)	Dill (%)	Fenell (%)
Total ash	–	4.8–7.6	about 10	–	about 8	–	–
Total ash (US max)	9	8	10	7	9.5	10	9
Water soluble ash	–	2.0–2.2	–	–	–	–	–
Acid insoluble ash (BP max)	1.5	1.5	2	1.5	–	3	1.5
Acid insoluble ash (US max)	1.5	1.5	2	1.5	1.5	3	2
Crude fibre	–	17.5–22.3	–	–	–	–	–
Foreign organic matter (BP, etc. max)	1	2	1	2	2[b]	2	1.5
Other fruits and seeds (BPC max)	2	–	4	–	–	–	–
Cold water extract	–	20–26	–	–	–	–	22–27
Fixed oil	8–20	8–20	15–30	12–20	10–14	15–18	12–20
Volatile oil	1.5–4.0	2.5–5.9	1.5–3.0	0.3–1.0	2–4	2–4	0.8–4.0
Volatile oil (BP, etc.* min) whole	2	3.5	1.5	0.3	–	2.5	1.2
Volatile oil (BP, etc. *min) powder	–	2.5	1.5	0.2	–	2	1
Major volatile component	–	Carvone	–	Linalool	Cumin-aldehyde	Carvone	Anethole, Anis-aldehyde
Approx. $R_f \times 100$	–	43	–	26	58	43	72, 38

Footnotes: [a]Standards precribed in the present or the past editions of BP or BPC.
[b]US maximum for harmless foreign matter 5%.
Source: Harbourne, 1973; Pearson, 1976.

8.3 Essential oils

Adulteration has been a serious problem for many years in area of essential oils. Undoubtedly the economic incentive to blend synthetic flavourants with the natural oil is too high to resist. Some essential oils naturally contain a single compound at high concentration and often this major component is available synthetically at a low cost. Addition of this single compound to natural essential oils without declaration on the label amounts to adulteration. Such synthetic compounds are also added to processed foods to accentuate the natural flavour. Examples include addition of benzaldehyde to roasted hazelnuts, and 1-(4-hydroxyphenyl)-3-butanone to raspberry extracts and artificial flavours like decadiene esters to apple juice and γ-nonalactone in coconut products (Pfannhauser *et al.*, 1982).

In such cases, selected ion monitoring GC/MS (SIM) is a useful technique. A mass spectrometer usually scans over a range of trace compounds in order to obtain data on every component in a mixture. A mass spectrometer in a SIM mode detects only a few

selected ion masses in order to quantitate the concentration of a single compound in a mixture. The decrease in the number of masses detected using SIM results in a 10-fold to 100-fold increase in detection sensitivity for a single compound. Synthetic flavour compounds contain impurities characteristic of the synthetic route used to produce them. These impurities can be quantitated by SIM in essential oils, and their absence is an indication of the essential oil being natural (Frey, 1988). SIM has been used to detect trace contaminants in food (Startin and Gilbert, 1982) and flavour compounds in blueberries and strawberries (Hirvi and Honkane, 1983).

The mixing of expensive oils with cheaper oils often can be detected by running a GC profile of the oil. One approach is to search for components in the expensive oil which are not commercially available and are unique to the oil. An example is β-selinene in the oil of celery. Good quality oil should contain 7.0–7.5% β-selinene (Straus and Wolstromer, 1974). Oils containing less than 7.0% β-selinene should be suspected of being adulterated.

Some essential oils, their tonnage, major producer countries and their commonly employed adulterants are given in Table 8.15 (Wright, 1991).

These and many other adulterants can be identified by IR, GC, and TLC (Di Giacomo and Calvarano, 1970). Dilution of essential oils with ethanol was checked using refractometric methods which were found to be unreliable (Kaminski and Dytkowska, 1960). TLC has been found to be a simple method of checking adulteration in essential oils of caraway, corriander, parsley and anethum (Hoerhammer *et al.*, 1964).

Table 8.15 Major essential oils; their production and adulterants

Essential oil	Origin	Annual tonnage	Major producer countries	Adulterants employed
Bergamot oil	*Citrus auranticum* (Rutaceae)	115	Italy, Ivory Coast, Brazil Argentina, Spain, Russia	Synthetic linalool and linalyl acetate; orange and lime terpenes
Cassia oil	*Cinnamonum cassia* (Lauraceae)	160	China, Indonesia, Vietnam, Taiwan	Cinnamaldehyde
Cinnamon oil	*Cinnamonum zeylanicum* (Lauraceae)	900 (leaf oil) 5(bark oil)	Srilanka, India	Leaf oil to bark oil and cinnamal-dehyde
Clove leaf oil	*Eugenia caryophyllata* (Myritaceae)	2000	Madagascar, Indonesia, Tanzania, Brazil Srilanka	Clove stem oil
Clove bud oil	*Eugeria caryophyllata* (Myritaceae)	70	Indonesia, Madagascar	Clove stem oil, leaf oil, eugenol, and stem oil terpenes

Table 8.15 (cont)

Coriander oil	*Coriandrum sativum* (Umbelliferae)	100	Russia	Synthetic linalool
Cornmint oil	*Mentha arvensis* (Labiatae)	3500	China, Brazil, India, Paraguay, Taiwan, Thailand, North Korea, Japan	Not a commercially attractive proposition
Dill oil	*Anethum graveolens* (Umbelliferae)	140	US, Hungary, Bulgaria, Russia Egypt	Distilled orange terpenes
Eucalyptus oil	*Eucalyptus globus* (Myrtaceae)	1400	Portugal, S.Africa, Spain, China, India, Austria, Paraguay	–
Garlic oil	*Allium sativum* (Liliaceae)	10	Mexico, Italy, Egypt	Nature identical raw materials
Ginger oil	*Zingiber officinale* (Zingiberaceae)	55	China, India	Not often adulterated
Grapefruit	*Citrus paradisi* (Rutaceae)	180	Brazil, US, Israel, Argentina, New Zealand	Orange terpenes
Lemon oil	*Citrus limon* (Rutaceae)	2500	Argentina, US, Italy, Brazil, Greece, Spain, Australia, Peru	Distilled oil and terpenes
Lemongrass	*Citrus flexuosus* and *C. citratus* (Gramineae)	310	India, China, Guatemala, Brazil, Russia, Srilanka, Haiti, Russia	Synthetic citral
Lime oil	*Citrus aurantifolia* (Rutaceae)	450	Mexico, Peru, Haiti, Brazil Ivory Coast, Cuba, Ghana, Jamaica, China	Synthetic terpineol terpinolene, and other components of lime terpenes
Litsea cubeba oil	*Litsea cubeba* (Lauraceae)	900	China	Synthetic citral
Nutmeg	*Myristica fragrens* (Myristicaceae)	180	Indonesia, Srilanka,	Terpenes and nature identical materials
Sweet orange oil	*Citrus sinensis* (Rutaceae)	16 000	Brazil, US, Israel, Italy, Australia	Adulteration infrequent but higher priced oils diluted with cheaper substitutes
Peppermint oil	*Mentha piperita* (Labiatae)	2200	US, Russia, Yugoslavia, Hungary, France	Cornmint oil, terpenes

Table 8.15 (cont)

Rose oil	*Rosa damascena* (Rosaceae)	15	Turkey, Russia, Bulgaria, Morrocco	Nature identical components such as citronellol and geraniol
Rosemary oil	*Rosamarinus officinalis* (Labiatae)	250	Spain, Morrocco, Tunisia, Russia, Yugoslavia Turkey	Camphor and eucalyptus fraction
Spearmint oil	*Mentha spicata* (Labiatae)	1400	US, China, Italy, Brazil, Japan, France	*laevo*-Carvone
Star anise oil	*Illicium verum* (Magnoliaceae)	90	China, Vietnam, North Korea, Russia	Anethole
Tangerine oil	*Citrus reticulata* (Rutaceae)	300	Brazil, US, Russia, Spain South Africa	Synthetic methyl -n-methyl anthranilate

Source: Wright, 1991; Lawrence, 1985b.

Iodine number has been suggested as a means of detecting adulteration in essential oils (Kartha and Mishra, 1963), but the iodine number has not attained significance in assessing the quality of essential oils, probably due to the unpredictable behaviour of these oils in the presence of solutions commonly employed for iodination. The observation that the iodine monobromide–mercuric acetate reagent brings about quantitative fission of the cyclopropane and cyclobutane rings in essential oils prompted Kumar and Madaan (1979) to make use of such iodine absorption values for this purpose. They found that iodine number could furnish a single criterion for this purpose. Table 8.16 gives the recommended iodine values for pure specimens of various essential oils and isolates. The method could successfully detect adulteration in samples considered to be unadulterated on the basis of conventional analytical procedures.

8.4 Adulteration of spice essential oils

Literature on adulteration of spice essential oils is very scarce. There is an urgent need to devise analytical methods to detect this fraudulent malpractice. Ethyl alcohol represents the main alcohol usually used in moderate quantities to dilute essential oils (Mostafa *et al.*, 1990a). Both edible and mineral oils are often used for adulteration (Nour el-Din *et al.*, 1977). Physical methods such as specific gravity at 25 °C, refractive index at 25 °C, specific optical rotation and ester number have been useful in detecting such adulteration. Table 8.17 gives the critical region (borderline) for detection of adulterated oils by these different methods.

Such physical properties including ester number should be considered as presumptive tests and should be confirmed by other, more specific analysis. Solubility of the essential oils adulterated with cottonseed oil and light paraffin oil also provides a

Table 8.16 Recommended iodine values for pure specimens of various essential oils and isolates

Essential oil/isolate	Recommended iodine value
Oil of ajowan, lab distilled[a]	232–265
Oil of fennel,lab distilled[a]	160–185
Oil of dill, lab distilled[a]	265–307
Oil of clove, lab distilled[a]	232–243
Oil of sandalwood	
(i)lab. distilled from sandalwood powder	283
(ii) government factory (Mysore, India)	288
Oil of cinnamon leaf, lab. distilled	46–52
Oil of citronella[b]	
(i) Srilanka	308
(ii) Bengal chemical works	295
Oil of black pepper, lab. distilled	300–324
Oil of cumin seed, lab distilled	193–195
Oil of geranium (Prima Bourbon)[b]	275
Oil of ylang[b]	175
Oil of lavidin (abrialis)[b]	167
Oil of parsely seeds, lab. distilled [a]	248
Oil of spike lavender[b]	135
Oil of black jeera, lab. distilled[a]	230
Oil of *Curcuma amada*, lab. distilled[a]	266
Oil of *Piper longum*, lab. distilled[a]	265
Oil of dry ginger, lab. distilled[a]	185
Oil of *Pimpenalla anisum*, lab. distilled[a]	296
Vanillin, pure BDH[b]	58
Menthol, pure, lab. distilled	0
Eugenol, pure, lab. distilled	275
Oil of peppermint, dementholized (Japan)[b]	68
Oil of cedarwood (France) [b]	192
Oil of vetiver, lab. distilled	194

[a] Samples collected from different places.
[b] Samples procured from different companies.
Source: Kumar and Madaan, 1979 (reproduced with permission).

valuable clue in their detection (Table 8.17). The differences in the solubility of essences and adulterant castor oil in petrolatum had been made use of in qualitative as well as quantitive determination of castor oil as early as 1948 (Carlos, 1948). Similarly, colorimetric analysis of glycerol can indicate adulteration with edible oils. TLC of the hydrocarbon fraction, GLC and IR are effective in detecting adulterant ethanol, edible oils and liquid paraffins (Mostafa *et al.*, 1990b). The presence of cottonseed oil in different essential oils gave absorption bands characteristic of esters and unsaturated esters (at 1705–1720 cm^{-1}), acetates (at 1245 cm^{-1}) and the carbonyl group (at 1250–1170 cm^{-1}), while the presence of paraffin oil gave a broadened absorption band at 3000 cm^{-1} which characterizes the saturated and unsaturated hydrocarbons.

Aroma constituents of essential oils such as linalool and linalyl acetate can be tracedto various botanical sources such as coriander, lavender, bois de rose, etc. Authentication methods that could trace the botanical and even the geographical origin of such constituents are a challenge to food analytical chemists. Site specific

Table 8.17 Critical region (border line) for detection of adulterated oils by different adulterants *

Properties	Marjoram	Petit grain	Fennel
Adulterants added (%)		bigrade	
Specific gravity at 25°C:	0.94139	0.90820	0.97336
Ethanol	>10	>0.5	>15
Paraffin oil	>10	>0.5	>1
Cottonseed oil	>20	>5	>15
Refractive index at 25°C:	1.44520	1.4919	1.5198
Ethanol	>20	>0.5	>10
Paraffin oil	>0.5	–	>10
Cottonseed oil	>0.5	–	>10
Specific optical rotation:	13.45	3.08	6.39
Ethanol	>5	–	>10
Paraffin oil	>5	>40	>10
Cottonseed oil	>5	40 only	>105
Ester number:	45.16	193.4	17.22
Ethanol	>15	>5	>0.5
Paraffin oil	>20	>10	>0.5
Cottonseed oil	>0.5	–	>2

* Significant at 5% level.
– Not detected.

natural isotope fractionation studied by NMR (SNIF–NMR) combined with molecular isotope ratio determination by mass spectrometry (IRMS) can characterize linalool and linalyl acetate from chemical synthesis or extracted from essential oils of well defined botanical and geographical origins. Chirality can be used as criterion for differentiation between components of natural and nature-identical types (Werkhoff *et al.*, 1991). It can be acheived by using enantioselective capillary GC coupled with stable isotope ratio analysis (Hener *et al.*, 1992). The overall ^{13}C or ^{2}H contents, as measured by IRMS do not constitute an efficient criterion for such identifications. Non-random distribution of deuterium exhibits large variations as a function of the origin of the sample. Discriminant analysis performed over the natural and synthetic families shows that all synthetic samples belong to the same group. Natural linalool is characterized by a strong depletion in the heavy isotope in site 1 and by a relatively heavy enrichment at site 6. Semi-synthetic linalool obtained from pinene can also be distinguished from natural linalool by virtue of its deuterium at site 3 of the sample. The discrimination between linalools of various botanical origins is however reported

to be only 82% effective (Hanneguelle *et al.*, 1992). Similarly, enantiomeric purity of carvone from essential oils of caraway, dill and spearmint can be determined using appropriate columns. While *S*(+)-carvone is detected in herb oils of caraway and dill, spearmint oils from various countries contain *R*(–)-carvone (Ravid *et al.*, 1992).

Most of the literature available in this respect is with either vanilla (Riley and Kleyn, 1989) or citrus (Giacomo, 1977) flavours. Documented literature on adulteration of other flavours is very scarce.

8.5 Citrus essential oils

This group includes essential oils from bergamot, lime, lemon, orange and grapefruit. They have widespread applications in foods. The major flavour use of bergamot oil is in Earl Grey tea flavours, where it is normally the major component. It is also used as a minor component in citrus soft drink flavours and some natural fruit flavours such as apricot. Grapefruit oil is added in flavours to impart a grapefruit character to a wide range of applications. It is sometimes mixed with other citrus flavours, but is not much used outside this field. Besides applications in citrus flavours, lemon oil is also used in other flavours such as butterscotch, pineapple and banana. Lime oil finds applications as a major component in cola flavour. Mandarin and tangerine oils are widely used in soft drink flavours and confectionery, alone and in conjunction with orange flavours. They also find good use in other natural fruit flavours such as mango and apricot.

Blending additives of any kind to orange oils for use in themanufacture of beverages is considered adulteration. Since pressed orange oils frequently have a poor colour, β-carotene is added and its estimation is therefore an essential stage in establishment of adulteration (Benk, 1972). Besides β-carotene, many dyes may sometimes be added, which can be determined electrophoretically after separation by column or TLC (Benk and Bergmann, 1966). High contents of non-volatile components and paraffinic hydrocarbons are indicative of adulteration and addition of esters (Benk, 1972). In some cases, like bitter orange oil, classical analytical data including non-volatile matter and others like density, optical rotation and refractive index, d^{15}, $\alpha^{20}{}_{D}$, $\alpha^{20}{}_{D}$ on 50% distilled head, $\zeta_{20}{}^{D}$ and aldehyde contents are not sufficient to determine the purity. In such cases, the UV spectrum of a genuine sample in ethanol (max at 332 nm) and that of a commercially adulterated sample (max at 325 nm) could provide a clue. The absence of citronellol, and a high ratio of linalyl acatate to linalool in geuine oil compared to adulterated oil are other useful parameters (Di Giacomo *et al.*, 1964). Values of carbonyls, esters and ester to carbonyl ratios are promising for detecting orange oil in bitter orange oil (Calvarano, 1966). Of the three principal citrus essential oils traded internationally, the highest prices are paid for lemon oil, and therefore some risk of adulteration is always present. An early and crude form of this that is no longer encountered, involved the addition of oil of turpentine (Guenther, 1949). Natural lemon oil constituents such as citral, linalool, linalyl acetate, limonene and the residues present in the preparation of terpeneless lemon oil had been implicated as adulterants

(Dugo *et al.*, 1992). Grapefruit oil (Vannier and Stanley, 1958), menthyl salicylate (Stanley, 1959), chalcones (Stanley, 1961) and dibenzyl ether (MacLeod *et al.*, 1964) have also been used for the sophistication of lemon oil since the mid-1960s.

IR and UV spectra have been successful in distinguishing between citrus oils. Citral imparts flavour and aroma to orange and lemon essential oils, and is therefore considered as an indicator of quality. This can be estimated easily by IR spectrophotometry using a first derivative trough-to-peak distance between 1684 cm^{-1} and 1677 cm^{-1} (Lopez-Mahia *et al.*, 1993). The spectrum is independent of the state of maturity of the fruits and varies only in the percentage absorption (Pruthi *et al.*, 1961). The use of a double beam spectrophotometer for recording absorption spectrum curves directly can give more information than a simple UV absorption spectrum and allow detection of adulteration, as has been shown for adulteration of genuine lemon essence with 10–30% synthetic lemon essence and ethyl-*p*-dimethylamino benzoic acid (Giuseppa *et al.*, 1968), even at 0.03% (Ciraolo and Calapaj, 1974a; 1974b).

Other adulterants like phenylsalicylate and chalcones can also be effectively checked in pure lemon essential oils by UV spectrophotometry (Giacomo and Calvarano, 1973). Cold pressed lemon oil is frequently extended by lower quality steam-stripped oil.

Phenylpropanoids are naturally occurring phenolic compounds which have an aromatic ring to which a three carbon side chain is attached. They are derived biosynthetically from the aromatic amino acid phenylalanine and they may contain one or more C_6 to C_3 residues. Among the phenylpropanoids are included hydroxycoumarins, phenylpropenes and lignans. Complex coumarins, for example, furonocoumarins, typified by psoralen, are restricted to only a few botanical families such as Rutaceae and Umbelliferae. Since substances with UV absorption spectra resembling those of coumarins and psoralens are present in cold pressed lemon oil this is added in order to mask adulteration. HPLC has confirmed identification of 7-methoxycoumarin and 5,7-dipropyloxy-4-methylcoumarin in some commercially cold pressed lemon oils. These compounds are only recently discovered in cold pressed lemon oils (McHale and Sheridan, 1988). Benzyl alcohol was first reported as an adulterant in 1963. Gas liquid chromatography on a packed column containing Carbowax 20M as the stationary phase can detect this adulterant in very miniscule amounts (Bradley and Gramshaw, 1980).

Nootkatone, a sesquiterpene, is a secondary metabolite found in grapefruit peel oil (Macleod and Buigues, 1964), pummelo (Sawamura and Kuriyama, 1988; Porras *et al.*, 1991) and in other citrus fruits (Boelens and Jimenez, 1989; Sawamura *et al.*, 1990; Del Rio *et al.*, 1991) and is an important constituent of commercial flavourings and fragrances (Sinclair, 1972). It is frequently taken as an indicator of grapefruit oil quality and can be estimated by HPLC (Schulz *et al.*, 1992) but it only represents a part of the recognizable character. It also varies with the season, storage and maturity of the fruits (Del Rio *et al.*, 1992). The concentration of grapefruit oil in lemon oil can be measured in terms of the characteristic fluorescence.

Terpenes are sometimes added to citrus oils like mandarin, lemon and bergamot. Adulteration of essential oils with terpenes results in reduced stability as well as inferior profile. Fluorescence has been tried as an analytical technique to detect this malpractice. This method does not detect terpenes below 10–20%, but the completely synthetic product can be identified (D'Amore and Corigliano, 1966). A rapid method for determining the percentage of citral and esters in lemon essential oil by IR spectroscopy could be used as a test of adulteration. Analysis of spectrogram data between two limonene bands (at 1781 cm^{-1} and 1646 cm^{-1}) in a known oil which contains citral (1683 cm^{-1}) and ester (1749 cm^{-1}) by a graphical method and using a simple formula allows calculation of these components, which in turn indicate adulteration (Retamar *et al.*, 1975). GC analysis of the geraniol/neral ratio (3.36–3.50:1) and its adulterant, lemongrass citral (2.50:1) to detect adulteration has been suggested (Adolfo, 1962). From the examination of the chromatograms of a large number of industrial lemon essential oils, indices were derived which allowed detection of adulteration of cold extracted oils with distillates from by products by means of the ratios of linalool, α -terpineol and terpinene-4-ol with two unidentified compounds X_1 and X_2 and with citronellol and decanol. Analysis of 40 mixtures of varying proportions showed that 5% of the added distillates could be detected (Dugo *et al.*, 1983a). Analysis of added natural or synthetic citral to genuine lemon essential oils by high resolution GLC showed that differences were less evident at lower concentrations. There is no straight line relationship between citral addition and chromatographic peaks since the response is dependent on the type and concentration of impurities in the synthetic preparations (Dugo *et al.*, 1983b, 1983c, 1984).

Investigations on stable isotope ratio analysis have shown $^{13}C/^{12}C$ to discriminate citral from different sources (Bricout and Koziet, 1976; Barrie *et al.*, 1984; Braunsdorf *et al.*, 1992). Ordinary as well as deterpenated orange oils are not influenced by processing with respect to their $\delta^{13}C$ values (Braunsdorf *et al.*, 1993a). However, the methodology is believed to be of limited use in authenticity determination of flavour material, because most of the commonly cultivated plants belong to the C_3 group of plants yielding very similar values to those of synthetic substances from fossil sources. The ratios are generally obtained by combusting the whole plant tissue. It has already been reported that carbohydrates, lipids and proteins, products of primary metabolic pathways reveal different values of $\delta^{13}C$ compared to flavours, which are secondary metabolites, and thus will be influenced by the isotopic effect. The isotopic effects among genuine monoterpenes are also limited to the influence of enzymatic reactions during secondary biogenic pathways. In the case of special products containing single compounds concentrated up to more than 70%, a shift in the $^{13}C/^{12}C$ isotope ratio has been detected (Braunsdorf *et al.*, 1993a). This can be avoided by the use of an internal isotopic standard, which yields fruit-specific $\delta^{13}C$ values for constituents with a low standard deviation and seems to be more suitable for authenticity control of lemon oils (Braunsdorf *et al.*, 1993b). Bricout and Koziet (1978) demonstrated the utility of ^{14}C analysis in determining the source of citral. They reported 20.1 dpm g C^{-1}

(disintegrations per minute per gram carbon) for citral from lemon grass and 0.25 dpm g^{-1} C for citral synthesized from fossil fuels. There is no significant difference in ^{14}C determinations for synthetic citral synthesized from pinene and that from lemon or lemon grass.

In the case of bergamot oil, different percentages of reconstituted oils are added to the natural products that are sold as genuine (Dugo *et al.*, 1992). Reconstituted bergamot oils are generally obtained by mixing terpenes and distilled oils of different origins, citrus oils other than bergamot, linalool, linalyl acetate and at times small amounts of bergamot natural oil. If the additions are limited in quantity, adulterations of this nature are difficult to detect by traditional analytical methods because of the wide variability of the composition of genuine bergamot oils. The ratio of the two enantiomers of linalool determined by gas chromatography with chiral capillary columns can detect these additions, for example, the presence of (+)-linalool certainly permits detection of 5% addition of reconstituted oils to natural bergamot oil (Cotroneo *et al.*, 1992). Knowledge of the non-volatile residues, which work as natural odour fixatives, can also give useful indications about the genuineness of bergamot oil. Citropten (5,7-dimethoxycoumarin) and bergapten (5-methoxypsoralen) in reconstituted oils have been found to be lower than the lowest values of genuine oils and can be used as genuineness indicators of bergamot oil. Peaks characteristic of lime oil such as 5-geranyloxy-8-methoxypsoralen, 5-isopentenyloxy-7-methoxycoumarin, 5-isopentenyloxy-8-methoxycoumarin, 7-methoxycoumarin and isopinpinellin are indicators of their origin, if found in bergamot oil (Mondello *et al.*, 1993).

Oil of lemongrass is one of the most important essential oils, large quantities being used to isolate citral (75–85%). Citral is the starting material for the manufacture of important ionones, a series of aromatics with a powerful odour of violet. Two types of lemongrass oil are distinguished in trade, namely the East Indian and so-called West Indian oil. The East Indian oil, produced in a small section of the southwestern part of India, near the Malabar Coast, considered superior in quality in comparison to West Indian lemongrass oil, which differs chiefly in possessing a lower solubility. The main constituents of lemongrass oil apart from citral are methylheptenone, dipentene, methyl heptenol, n-decylaldehyde, nerol, geraniol and farnesol. The physicochemical properties of this oil are given in Table 8.3.

In India, adulteration of lemongrass oil with vegetable oils (which can be detected by saponification followed by analysis of the liberated fatty acids), methanol, kerosene and oil obtained from a white variety of grass grown in southern Kerala are reported. These could be monitored by following the changes in physical characteristics and solubility in 70% alcohol (Nair and Vorier, 1952).

Transmittance curves in the range 240–370 nm can differentiate adulterated and genuine mandarin oil. Mandarin oil is regarded as adulterated when the minimum in the transmittance curves lies at a longer wavelength than 335 nm and the transmittance at 295 nm is greater than that at 370 nm (Trifiro,1956).

Addition of >3% orange oil to mandarin oil could be detected on the basis of Δ^3-

carene content, the Δ^3-carene/α-terpinene ratio or the Δ^3-carene/camphene ratio. Detection of this level of addition of orange oil is acceptable, as adulteration with < 5% is not commercially worthwhile (Cotroneo *et al.*, 1987). Bitter orange oil is produced in limited amounts due to inconsistency of demand and low cultivation. The fruits used for the extraction of oil are sometimes mixed with a small number of sweet orange fruits and often the production lines are the same as that used for lemon essential oil. This could lead to contamination of bitter orange oil by sweet orange and lemon essential oils. In addition, sweet orange and lemon terpenes could be deliberately added to bitter orange oil. Δ^3-carene content is present only in traces in bergamot oil and 0.1% in the oil and terpenes of sweet orange. The content and percentage ratios of Δ^3-carene in regard to other components are therefore particularly suited to indicate possible additions of these extraneous compounds. Blends containing 5% terpenes show considerably higher values for Δ^3-carene, Δ^3-carene/camphene and Δ^3-carene/terpinolene than those registered for genuine bitter orange oils. Similarly, α-terpinene is present only in traces in bitter orange oil and about 0.2% in lemon oils and terpene fractions. The content of terpinene and the percent ratios of the latter with other constituents can give useful indications for the detection of possible additions of lemon products. In particular, the ratios, α-terpinene/camphene and α-terpinene/*cis*-β-ocimene can be indicative of levels as low as 3% lemon oil in bitter orange oil (Dugo *et al.*, 1993). The occurrence of characteristic oxgyen heterocyclics, from coumarins-osthol, meranzin, isomeranzin and meranzinhydrate; three psoraless: bergaptan, epoxy beramottin, and epoxy bergamottin hydrate; and four polymethoxy flavouns: tangeretin, 3, 3′, 4′, 5, 6, 7, 8 – heptamethoxyflavourn, nobiletin, and tetra-*O*-methyl seutellarein. Some of these components are going to be of use in identifying mixtures of essential oils encountered in trade (Dugo *et al.*, 1996).

Mandarin oils do not differ from one another to a very great extent in UV characteristics, irrespective of stage of maturity, regional variability, time and temperature of storage except that the content of the major component changes under different conditions (Pruthi *et al.*, Lal and Subrahmanyan, 1960).

Results have suggested that carvone, *p*-ethylacetophenone, limonene hydroperoxides and *p*-cresol could be used as indicator substances for assessment of the deterioration of lemon oil when it is stored under neutral pH conditions. The quality of lemon oil flavourings in acidic foods is also influenced by the formation of *p*-methylacetophenone and *p*-cresol, but in addition by the formation of *p*-cymene and fenchyl alcohol which could also serve as indicator substances (Grosch and Schieberle, 1987).

8.6 Vanilla extract

Vanilla is a tropical epiphytic orchid cultivated for its pleasant flavour. It is the source of natural vanillin and is probably the only orchid having economic importance. Although three species of vanilla are cultivated in different parts of the world, *Vanilla*

planifolia Andrews is the most preferred commercially. This climbing spice crop grows well in tropical climate at a temperature of 25 to 32 °C and mean annual rainfall of 150–300 cm.

Vanilla is used extensively to flavour ice creams, chocolate, beverages, biscuits,cakes, custards, puddings and other confectionery. The fragrance and flavour of vanilla are due to numerous compounds produced during the curing operation. As with other natural agricultural products, the country of origin, agricultural practices, climatic factors and soil types, degree of ripeness at harvesting and method of curing play an important role in the quality and yield of flavour and aroma constituents. In the highly competitive commercial environment which exists in the flavour industry in general and the vanilla trade in particular, a database that would identify geographical source and indicate bean quality would be an expedient in flavour quality control and assurance. Such a database has recently been published (Adedeji *et al.*, 1993).

The most adulterated flavouring material in the world is vanilla (Cabrera, 1950). Pure vanilla is a very expensive product and is often unavailable. Its active ingredient, vanillin, when made synthetically is readily available at minimal cost. Natural variations in vanilla components, accentuated by different processing techniques, make adulteration exceedingly difficult to detect. Vanillin has been known since 1816 and its structure was established as 3-methoxy-4-hydroxybenzaldehyde in 1874. Ethyl vanillin is a closely related compound, 3-ethoxy-4-hydroxybenzaldehyde, which is not found in nature but is prepared synthetically from saffrole. It is 3–4 times more powerful than vanilla as a flavourant, but can give a somewhat harsh 'chemical' character at high dosage levels. In practice, a maximum of 10% vanillin can be replaced by ethyl vanillin without the objectionable note being obvious.

A 'vanilla extract', as defined by a standard of the Food and Drug Administration, is the solution , containing not less than 35% alcohol, of the sapid and odorous principles extracted from one or more units of vanilla constituent. One unit of vanilla constituent is 13.35 oz of vanilla beans containing not more than 25% moisture in 1 gallon of the finished product. No addition of artificial vanillin is permitted in products designated as 'vanilla extract'. Addition of vanillin derived from lignin and synthetic vanillin to low vanillin vanilla extracts are often reported. Pure synthetic vanillin too is reported to be adulterated with acetanilide, benzoic acid and salicylic acid, terpene hydrate and sugar (Yllera Camino, 1974). Studies on qualitative two-phase TLC on chloroform extract of various hybrids of vanilla using chloroform: isopropanol (100:7) as solvent has been shown to be useful in detecting certain crudely adulterated commercial extracts (Oliver, 1973). The source of an unknown compound giving a bluish white fluorescence often found in commercial vanilla extracts that had coumarin contamination had been traced to tonka beans (Sullivan, 1982). 2-Undecylfuran has been proposed as an indicator of tonka beans in a recent paper (Worner and Schreier, 1991). The lead number has been widely used to establish the purity of the vanilla extract (AOAC, 1975). The lead number is directly related to the quantity of the organic acids present in the extract. A qualitative paper chromatographic method has

also been used to detect either gross deficiencies or the addition of acids to the vanilla extract (AOAC, 1975). Various relationships between organic acids found in the extract have been used to trace the geographical origin of the bean (Fitelson and Bowden, 1968). Certain 'identification ratios', based on the analysis of vanillin, potassium, nitrogen and inorganic phosphorus have been calculated, which allow comparison of the sample with that of the authentic vanilla extracts. High ratios of vanillin: N, vanillin: PO_4 and vanillin: K compared to ratios for authentic vanillin extract provide strong evidence of addition of synthetic vanillin. When the absolute values for vanillin, K, N, and PO_4 of vanilla extracts of questionable character are divided by the authentic values, it is possible to obtain a good indication of the strength of the extract, that is, one-fold, two fold etc. When the absolute values are low, it is a possible indication that the beans have not dried sufficiently or that an insufficient amount of bean is present. Table 8.18 shows the concentration of the 'identifying factors' and identification ratios of a single strength authentic vanilla extract obtained from Madagascar beans. However, these identifying compounds and identification ratios are dependent on the geographical origin of the bean (Table 8.19) and could find applications in attempts to trace the geographical origin of the vanilla extract itself. The values of K, N, and PO_4 fall within a very narrow range, although the vanillin values do differ for different bean types. In general, the vanillin values obtained from the Tahiti, Comores and Mexican beans are lower than in Madagascar beans, and Java beans contain vanillin below the threshold level of detection (50 ppm). This technique has proved quite useful to the Bureau Of alcohol, Tobbaco and Firearms as a regulatory tool (Martin *et al.*, 1975).

Addition of synthetic vanillin to natural vanilla could be detected using a new instrument developed on the principles of GC and IRMS with precision and accuracy (Freedman *et al.*, 1988). Compounds extracted from a plant material have an intrinsic ratio of ^{13}C: ^{12}C due to their specific biological origin. Vanillin isolated from extracts of Madagascar, Java, Tahitian and Mexican vanilla beans; made from lignin, clove oil eugenol and coal tar guaicol give $\delta\ ^{13}C$ units expressed as parts per thousand, calculated as

$$1000 \times \frac{(^{13}C/^{12}C)\ \text{sample}}{(^{13}C/^{12}C)\ \text{standard}} - 1$$

with good reproducibility (Bricout and Koziet, 1978). With a confidence of 99%, it has been recommended that a sample with $\delta^{13}C$ value more negative than –21.0 indicates vanillin from a source other than vanilla beans (Anon, 1979). This is also detectable by SIRA, and as carbon atom SIRA which can be circumvented by addition of (carbonyl ^{13}C) vanillin to approximate the $\delta^{13}C$ of natural vanillin. The $^{13}C/^{12}C$ ratio and hence $\delta^{13}C$ for natural vanillin, lignin vanillin and lignin vanillin modified by the addition of (carbonyl-^{13}C) gave $\delta^{13}C$ and carbonyl-$\delta^{13}C$ values which make such adulterations easily recognizable and should be useful in quality control of vanilla extracts (Krueger and Krueger, 1985). This technique also reliably distinguishes between natural vanillin and simulated vanillin and mixtures (Krueger and Krueger, 1983). However, this method can be confounded if vanillin is synthesized by methylation of 3,4-dihydroxy-

Table 8.18 Concentration (ppm) of identifying factors and identification ratios for authentic single strength vanilla extracts from vanilla beans

Sample	Vanillin	K	N	PO_4	Vanillin: N	Vanillin: PO_4	Vanillin: K	PO_4: N	K: N	K: PO_4
1	782	1291	140	183.5	12.72	9.71	1.39	1.31	9.22	7.04
2	2155	1513	182	254.1	11.84	8.48	1.42	1.40	8.31	5.95
3	1435	1533	235	191.6	6.10	7.49	0.94	0.82	6.52	8.00
4	1610	1355	158	124.0	10.18	12.98	1.19	0.79	8.57	10.93
5	1824	1264	202	130.4	9.03	13.99	1.44	0.65	9.69	9.69
6	1733	1340	211	182.2	8.21	9.51	1.29	0.86	6.35	7.35
7	1361	1340	228	188.8	6.12	7.21	1.02	0.83	5.87	7.10
8	2131	1488	236	280.5	9.02	7.60	1.43	1.19	6.31	5.30
Mean	1754	1390	199	191.9	9.15	9.62	1.27	0.98	7.61	7.67
Standard deviation	289.3	104.9	36	53.8	2.40	2.57	0.20	0.28	1.50	1.86
Coeff. of variation	16.49	7.54	18.09	28.03	26.22	26.71	15.74	28.57	19.71	24.25

Source: Martin *et al.*, 1975 (reproduced with permission).

Table 8.19 Concentration (ppm) of identifying factors and identification ratios for authentic single strength vanilla extract from beans from various geographical areas

Sample bean	Vanillin	K	N	PO_4	Vanillin: N	Vanillin: PO_4	Vanillin: K	PO_4: N	K: N	K: PO_4
Java	<50	2087	205	294.3	–	–	–	1.43	10.18	7.09
Java	<50	2627	324	417.6	–	–	–	1.29	8.10	6.29
Comores	1162	1398	210	246.1	5.53	4.72	0.83	1.17	6.66	5.68
Mexican	968	1833	184	294.6	5.26	3.29	0.63	1.60	9.96	6.22
Tahiti	507	1098	139	165.5	3.64	3.06	0.46	1.19	7.90	6.63
Tahiti	652	1174	128	166.0	5.09	3.93	0.56	1.29	9.17	7.07
Comores	1773	1283	217	233.4	7.99	7.60	1.38	1.08	5.91	5.50
Tahiti	820	1164	129	162.0	6.36	5.06	0.70	1.26	9.02	7.18

Source: Martin *et al.*, 1975 (reproduced with permission).

benzaldehyde enriched in ^{13}C. This can be prevented by demethylation of vanillin, followed by measurment the $\delta^{13}C$ values of 3,4-dihydroxybenzaldehyde. Measurements on authentic samples of natural and synthetic vanillin showed that $\delta^{13}C$ values were unaffected by demethylation, and studies on synthetic vanillin enriched in ^{13}C showed that the determination of aldehyde gave a correct indication of synthetic origin which is not given by analysis of vanillin itself (Bricout *et al.*, 1981). Recently a combination of stable isotope ratio analysis and HPLC is shown to be a good method of determining the authenticity of vanilla extracts (Lamprecht *et al.*, 1994).

Adulterators have kept pace with new procedures for detecting adulteration (Heath and Reineccius, 1986). One of the recent methods for the detection of adulteration of vanillin is via stable isotope ratios as described above. Unfortunately, adulterators have now synthesized a ^{13}C enriched vanillin which is added in trace amounts to boost the

^{13}C level up to that normally found in natural vanillin. The ^{13}C enrichment is typically done in the aldehyde group of the vanillin molecule. Splitting the aldehyde group from the vanillin and determining the ^{13}C in the remainder of the vanillin molecule can detect adulteration, but the technique is quite tedious and each step adds error which limits adulteration detection limits.

The determination of ^{14}C levels in food flavourings has also been used to monitor adulteration. All living plant tissues contain ^{14}C due to ^{14}C in carbon dioxide in the atmosphere (nuclear testing) The only way for a flavour compound to show no ^{14}C is if it has been synthesized from petroleum sources. ^{14}C has a half-life of about 5730 years. Unfortunately this technique can therefore only be used to determine whether a pure chemical has been synthesized from petroleum based chemicals or from recent plant metabolism. A further limitation of this technique is the requirement of large quantities of pure sample (about 1 g), and is therefore useful only for the analysis of pure ingredients, not finished flavours or flavours in food products

8.7 Mint flavours

Mint oils are amongst the important flavourants which are used in food products. There are considerable differences in the composition of the various mint oils, determined by species and environmental conditions during growth, harvesting and postharvest handling. These oils can be conveniently considered into two groups peppermint and spearmint.

Peppermint (*Mentha piperita*) and cornmint (*M. arvensis*) oils have much in common, the principal constituents of each being menthol, menthone, menthyl acetate and other esters. It is the ratio of these components which determines their distinctive profiles. Menthofuran, which is present in *M. piperita* oils is absent in *M. arvensis* oil. Colorimetric tests based on this observation have been proposed, but the distinguishing colour is produced in blends containing as little as 15% *M. piperita* oil. It is the final balance of menthol/menthone which is the major determinant of the flavouring quality of distilled essential oil. Smith and Levi (1961) have proposed certain compositional ratios as measures of quality. Those of particular interest include the following terpenes: other constituents; menthone: isomenthone; limonene: cineole; menthofuran: menthone related constituents; neomenthol: menthyl acetate; menthol related compounds: neomenthol; and finally menthone related compounds: menthol related compounds (i.e. menthofuran + menthone + isomenthone: neomenthol + menthol menthyl acetate).

Peppermint oil contains sabinene hydrate at the 1% level. Cornmint does not contain this component, nor is it commercially available. Analysis of sabinene hydrate can therefore indicate adulteration of peppermint oil with cornmint oil. Recently Indian standards have established requirements and methods of sampling and tests for whole as well as dementholized peppermint oil (Indian Standards Institution,1989).

One of the approaches for detecting adulteration is via determination of individual

optical isomers in a flavour. Considerable progress has been made towards separating the D and L isomers of optically active flavour compounds (Tressl and Engel, 1984, 1985; Chang, 1986; Mosandl and Heusinger, 1984; Konig, 1984). While naturally occurring flavours are of only one isomeric type, chemical synthesis produces racemic mixtures. To cite an example, plants typically produce D-limonene, and the presence of L-limonene is an indicator of the molecule being chemically synthesized, and hence the flavour being artificial. Use of chiral chemistry to detect adulteration of peppermint oil with cornmint oil or synthetic peppermint oil has been attempted, but without success (Chang, 1986).

The main use of peppermint oil is to give a peppermint flavour to a wide range of products. It is frequently blended with spearmint oil and also with many other common flavourings, including eucalyptus oil, methyl salicylate and anethole.Triacetin is another adulterant of peppermint oil, which can be determined by hydroxyl number (Weber, 1952). Small quantities can be used to give subtle effects in natural fruit flavours. The main use of spearmint oil is to give spearmint flavour to chewing gum and oral hygiene products. Most cornmint oils are used to give a cheap peppermint flavour in a wide range of applications, often blended with true peppermint oil. It is more frequently used than peppermint oil because of its price advantage.

8.8 Saffron

The high cost involved in the production of saffron (*Crocus sativus*) acts as a positive temptation to adulteration (Angeletti, 1927). Although very costly, saffron in filaments is still being used as a spice for delicacies. Even in the ancient world, saffron was of great importance as spice, colouring agent and a medicinal drug. Worldwide, about 50 000 kg of dried goods are produced annually. The main cultivating area is Spain. Natural saffron, (saffron, stigma with style), coupe saffron (pharmaceutical quality) as well as saffron powder are available on the market. The raw material prices fluctuate, depending on quality.

Adulterants of commercial samples of saffron or its products are synthetic colourants like azo dyes, safflower flowers or corn silk suitably dyed, meat fibre, marigold (Fromme, 1914), red sandalwood (Angeletti, 1927), flowers of *Onopordon acanthium* (Nestler, 1914) and turmeric. Inorganic adulterants of saffron include sodium sulphate (Fromme, 1914; Wattiez, 1928), potassium nitrate, ammonium salts (Darling, 1915), magnesium sulphate, barium sulphate (Fromme, 1914) and borax. The detection of borax, potassium nitrate and magnesium sulphate in saffron is based on the appearance of their peculiar or characteristic crystalline forms, when an aqueous extract of these is evaporated and the residue examined under microscope (Nestler, 1913, 1914).

The main azo dyes detected include tartrazine, ponceau 4R, sunset yellow, amaranth and orange GG (Krogh and Akerstrand, 1981). TLC analysis of alcoholic extracts on

silica gel G using pyridine: benzene (21:29) as solvent distinguishes genuine saffron from its adulterant acidic dyes (Dhar and Suri, 1974). Adulterated samples containing safflower, marigold and turmeric can be detected by their characteristic fluorescence under ultraviolet light which are distinct from those given by pure saffron (Castiglioni, 1933). Besides chromatography, spectrophotometry can also be used to assess the purity of saffron. An aqueous solution of saffron has a maximum absorption at 435 nm and one of its adulterants, safflower has a maximum absorption at 405 nm (Parvaneh, 1972).

Carotenoids are an extremely widely distributed group of lipid soluble pigments, found in all kinds of plants from simple bacteria to yellow-flowered composites. Amongst the 300 known carotenoids is a rare glycoside, water soluble crocin, the gentiobiose derivative of an unusual C_{20} carotenoid crocetin which is the yellow pigment of saffron, *Crocus sativa* (Harbourne, 1973). The purity of saffron could be ascertained by analysis of crocetin. This has however not caught the attention of food analytical chemists. Recently a French standard has classified saffron into four quality grades depending on its physicochemical specifications; moisture + volatiles, total ash, HCl-insoluble ash, cold water soluble extract, colouring capacity expressed in terms of crocin, bitterness expressed in terms of picrocrocin, safranal, total nitrogen and cellulosic insoluble material (French Standard, 1991a). The techniques for preparation of samples for testing and methods for analytical parameters as listed above have also been spelt out (French Standard, 1991b). A quality classification of saffron available in Spain, Italy, India, Greece, Pakistan, Iran and Morroco is based on shape, size and colour of the filament, and is variously called select, mongra, mancha, etc. Two parameters used in grading are contents of floral waste and extraneous matter. Portions of dried yellow stamens, pollen, parts of the gynecium and other parts of the flower form the floral waste, while leaves, chaff and stems are considered as extraneous matter. More advanced investigations of the microscopic structure are also employed occasionally to identify parts of the flower. The most outstanding quality of saffron however is its sensory quality, which has not been defined by different grades by any standards, local or international. Advances in new postharvest practises have resulted in saffron with different proportions of stigma and style (Sampathu *et al.*, 1988), while the use of tissue culture from half ovaries with proliferated growth of stigma like structures has acheived morphological and biochemical similarity to the secondary metabolites of the intact stigma (Hyota and Konosuke, 1987; Sarma *et al.*, 1990). On the other hand, patenting synthetic saffron (Kuhn and Wendt, 1936; Torri *et al.*, 1977) has also necessitated a clear understanding of the sensory quality of saffron. A study undertaken to select a method of flavour analysis for saffron and an understanding of the psychophysics of the delicate saffron flavour has established saffranal as the principal flavour component. The dose–response relationship with saffranal has been confirmed by liquid and headspace GC and GC-MS, and from bioassay at the exit port by sniffing (Narasimhan *etal.*, 1992a). This study also observed a saffranal peak for style and tissue culture grown samples of saffron, but with a very low area.

Saffron has been recently discussed with respect to its origin, composition, applications, quality specifications, microscopic and chemical analysis, and detection of contaminants and adulterants (Oberdieck, 1991). Limits are being placed in the food laws and pharmacoepias.

8.9 Almond oil

Bitter almond oil is the oil derived by steam distillation from the dried ripe fruits of *Prunus amygdalus* or from other kernels containing amygdalin. Oil of bitter almond is a very important flavouring agent, used particularly in baked goods, cakes, confectionaries and candies. However for scenting cosmetics, soaps, and technical preparations, much lower priced synthetic benzaldehyde is employed almost exclusively. In modern industrial production of bitter almond oil, the kernels of the bitter almond tree have for been some time replaced by the lower priced and higher yielding kernels of other fruit trees which contain amygdalin and yield an essential oil that cannot be distinguished from that of bitter almonds. The essential (volatile) oil is not present as such in the kernels, but is in the form of a cyanogenetic glucoside, namely amygdalin, which is decomposed into a molecule of benzaldehyde and two molecules of glucose and hydrocyanic acid under the influence of the enzyme, emulsin. Benzaldehyde, the chief constituent of bitter almond oil is quite soluble in water. The oil as such contains between 2% and 4% hydrocyanic acid which can be removed by shaking with milk of lime and vitriol (ferrous hydrogen sulphate) as an insoluble precipitate of calcium ferrocyanide. The colour of the bitter almond oil turns yellow on ageing. The physicochemical properties useful in ascertaining the authenticity of almond oil (from which hydrocyanic acid has been removed) are as given in Table 8.3.

At one time, bitter almond oil was occasionally adulterated with nitrobenzene, but this crude form of adulteration is no longer practised. Benzyl alcohol is reported to be yet another adulterant. A much more annoying adulterant is synthetic benzaldehyde, since it cannot be detected readily. In past years, the synthetic benzaldehyde used sometimes contained chlorine as a result of improper purification. Thus detection was possible by a halogen test, but presently synthetic benzaldehyde is produced in a high state of purity and therefore the halogen test has lost its importance. Stable isotope ratio analysis by MS has also been used to identify benzaldehyde of natural as opposed to synthetic origin. Isolation of benzaldehyde from a number of natural C_3 plant sources including bitter almonds and kernels of plums, peaches and apricots as well as marzipans and pepsipans, as well as from two synthetic sources and from ice-cream raw material has shown the futility of using $\delta^{13}C$ values to differentiate beween them. The mean values for each were reported to be -29.7 ± 0.5 and -26.8 ± 0.4 ppt, respectively. Strong evidence is also available wherein the total ^{14}C content of petrochemical benzaldehyde is compared with that of the botanical material (Culp and Noakes, 1990). Values of $\delta\ ^2H$, however, are distinctive, mean values being -125 ± 14 for natural sources, -40.1 ± 21 for the synthetic sample obtained by benzal chloride

hydrolysis and -777 ± 20 ppt for the synthetic sample obtained by catalytic oxidation of toluene. Analysis of benzaldehyde from ice-cream raw material had a $\delta\ ^2H$ value of +764 ppt indicating it to be of synthetic origin from toluene. Synthetic benzaldehyde is enriched with ^2H in the formyl group. Degradation of benzaldehyde on air exposure also leads to ^2H enrichment of the formyl group so that foods should be analysed fresh and unspoiled to avoid possible confusion between natural and synthetic benzaldehyde. If this precaution is taken, ^2H analysis of benzaldehyde should be a secure means of identifying the natural origin of the flavour compound; artificially ^2H-depleted benzaldehyde is not available and therefore adulteration is impossible (Butzenlechner *et al.*, 1989).

Site-specific deuterium distribution data has been reported for many botanical and petrochemical materials such as anethole, camphor, 3-carene, citral, eugenol and geraniol, ethanol, limonene, α-pinene and vanillin. The distribution of deuterium among the aromatic sites provides a means of differentiating benzaldehyde and detecting adulteration of bitter almond oil. The aromatic distribution ratio (ADR) value (f[*ortho*]/f[*meta* + *para*]) can be readily calculated from the ^2H NMR spectrum and provides an indication of authenticity. An ADR value greater than 0.6084 for an unknown sample indicates 10% or higher adulteration of bitter almond oil with other commercial sources of benzaldehyde. The absolute level of *o*-2H benzaldehyde is also a unique discriminant of bitter almond oil, the proportion of deuterium at the *ortho* site (f[*ortho*]=0.3177, ADR=0.5906) being the lowest in bitter almond oil (Hagedorn, 1992).

8.10 Oil of sassafras

One of the oldest and best known essential oils is that of sassafras (*Sassafras albidum*), belonging to the family Lauraceae. The essential oil occurs in the underground parts of the tree, especially in the bark of the roots which yields 6–9% oil.Besides being used for therapeutic purposes, its most important applications are in flavours. Its taste is aromatic. Some of the most typical American soft drinks, for example, root beer and sarsaparilla owe their characteristic flavour largely to sassafras oil. Some types of candies and chewing gums are also flavoured with sassafras oil. The main constituents of the oil are reported to be α-pinene, phellandrene, saffrole (which is the chief constituent at 80%), eugenol and *d*-camphor.

Sassafras oil is a yellow to reddish–yellow liquid with a characteristic odour and taste of sassafras. Its physicochemical properties are listed in Table 8.3.

Artificial sassafras oil is the common adulterant of sassafras oil, and consists of certain fractions of camphor oil, produced in very large quantities in Japan and Formosa. Since the physical and chemical constituents are similar, simple tests cannot distinguish the adulterant from the genuine sassafras oil. However, Brazilian sassafras oil, another adulterant of North American or true sassafras oil has different physicochemical properties, permitting its easy detection.

8.11 Vinegar

Vinegar is the product of fermentation of alcoholic beverages by Acetobacter species under highly aerobic conditions wherein the ethanol is oxidized to acetic acid. The common species employed is *Acetobacter aceti*. The product, apart from acidity or sour taste due to acetic acid, has the flavour of the starting alcoholic beverage. Malt vinegar for instance is derived from malted barley without intermediate distillation, distilled vinegar is derived from distillation of the alcoholic and acetous fermentation of any saccharine material, spirit vinegar is the product of acetous fermentation of a distilled alcohol. Artificial or imitation vinegar contains acetic acid, not wholly derived from alcoholic and subsequent acetous fermentation.

The main problem in the vinegar industry is its adulteration with diluted acetic acid. Occasionally diluted solutions of acetic acid coloured with caramel have been marketed as vinegar and are highly cost competitive.

Detection of added synthetic acetic acid is difficult. Distinction between malt, spirit and artificial vinegar has been based on oxidation value, ester value and iodine value. A vinegar derived principally from grain which has been converted by acid hydrolysis will probably have a relatively high ash rich in sulphates and low in its alkalinity. Similarly a vinegar brewed from molasses and glucose will have a low ash, while the presence of unfermentable matter may result in a vinegar of high gravity. Similarly vinegar prepared from maize gives a high figure for total solids, and low figures for phosphoric acid and nitrogen compared to those for malt (barley) vinegar. The presence of pleasant tasting bodies such as ethyl acetate is also characteristic of malt vinegar, while the presence of potassium tartrate is an indication of genuineness of wine vinegar (Nicholls, 1952).

Methods such as the determination of ^{14}C content, ratios between various fundamental components (Mecca, 1967), for example volatile acidity: dry extract, volatile acidity: fixed acidity, volatile acidity: ash, fixed acidity: ash and dry extract: total ash and determination of substances which are specific and characteristic of both the initial wine and the final vinegar, for example tartaric acid, polyphenols, proline and other amino acids (Fukano *et al.*, 1976,) have been reported as indicators of the genuineness of vinegar. Determination of substances produced during acetic fermentation of wine such as glycerol, lactic acid, acetoin and volatile compounds (Llaguno, 1977) are also useful. Relative diminution of fixed acidity, dry extract, ashes and other minor components such as esters, aldehydes, acetone, 2,3-butylene glycol, tartaric acid, amino acids, etc. are promising indices to detect these practises.

In wine vinegars, certain quantities of fixed acids existing in wines must be present. The most typical of these acids is tartaric acid in grape wine. No plant contains a higher quantity of tartaric acid than the Vitis family. It is clear that if the content of tartaric acid is less than 1 g l^{-1}, its origin could be doubted (Llaguno, 1977). Distinction between genuine and fraudulent wine vinegars is possible by subjecting the results of chemical analysis to linear discriminant functions calculations. The free amino acids

and total nitrogen content is higher in wine vinegars than in cider and sugar cane alcohol vinegars, with quantitative differences also appearing among the wine vinegars. Therefore, the differences between vinegars of known authenticity and artificial or those suspected to be adulterated were seen in terms of an exceptionally low total amino acid content, mainly proline and hydroxyproline in the latter. Analysis of free amino acid, therefore serves as an index for detection of adulteration of vinegar (Polo *et al.*, 1976; Seppi and Sperandio, 1980) as well as distinguishing fermented and synthetic vinegar (Schanderl and Staudenmayer, 1956). Table 8.20 shows free amino acids and total nitrogen in wine vinegar samples (Morgantini, 1963). Wine vinegars show proline contents of 150–300 mg l^{-1}. The following values have been reported for proline concentration: wine vinegars, superior quality, 149.5–359.7 mg l^{-1}; medium quality, 2.8–233.0 mg l^{-1}; ungraded, 35.0–225.6 mg l^{-1}; cider vinegar, 0; cane alcohol vinegar, 14.0 mg l^{-1}. Other amino acids such as alanine and valine also appear in substantial quantities. If a sample has less than 80 mg l^{-1} proline, suspicions may be drawn about its origin.

Wine contains polyphenols which must be present in authentic wine vinegar instead of caramel or other added colourants; the UV absorption spectrum determines the identity and at 280 μ the concentration of polyphenols and related substances (Webb and Galetto, 1965). The *o*-diphenol content is higher than 50 mg l^{-1} in genuine vinegars. A relation has been found between glycerol content and vinegar quality, in the sense that a higher quantity of glycerol corresponds to a more balanced and better quality vinegar. No such correlation exists for lactic acid (Suarez *et al.*, 1976).

A number of easily oxidizable volatile compounds are produced; their total value is established by the so-called 'oxidation index'. This index has been used to detect adulteration in cider vinegars (Michael, 1951; Michael and Williams, 1952).

The identification of a particular type of vinegar can be done through the relative concentration of volatile constituents, especially alcohols and esters (Jones and Greenshields, 1969, 1970, 1971; Kahn *et al.*, 1972). Recently, a new constituent hereto unreported, has been identified as 1,3-propanediol by ^{13}C-NMR in cider vinegar (Kawai *et al.*, 1991). Studies are needed on whether this compound is specific to cider vinegar, to enable its use as an index of authenticity of cider vinegar. Genuine malt vinegars can be distinguished from synthetic products by using the 'albuminoid ammonia value', typical values being 200–420 ppm for malt vinegar and 0–4 for artificial ones (Mitra, 1955). The pH value can also be significant. According to White (1971), pH values below 2.75 are to be considered as suspicious, since the values in genuine vinegars range between 2.80–3.20. Similar values are applicable to wine vinegars. All vinegars can be differentiated from non-brewed condiments by osmotic pressure/freezing point depression methods (Kearsley and Gibson, 1981). Table 8.21 shows the results of osmotic pressure and freezing point depression measurements on vinegars and non-brewed condiments.

The data for the non-brewed samples appear to be completely different from the data for the brewed samples since in the former the mean is much smaller and the

Table 8.20 Free amino acids and total nitrogen in wine vinegar samples

Amino acid	Range[a]	Mean[a]
Aspartic acid	7.6 – 35.9	25.69
Threonine	7.0 – 54.9	18.21
Serine	2.9 – 69.9	22.27
Glutamic acid	11.7 – 61.5	28.99
Proline	149.5 – 316.7	279.84
Glycine	4.3 – 36.0	19.81
Alanine	4.7 – 100.7	49.44
Valine	6.3 – 72.6	30.88
Cystine	< 1 – 10.0	
Methionine	< 1 – < 2	
Isoleucine	3.3 – 26.5	12.7
Leucine	6.4 – 49.8	25.94
Tyrosine	3.6 – 10.3	5.97
Phenylalanine	9.1 – 23.1	15.02
γ-Aminobutyric acid	5.7 – 90.6	41.64
Ornithine	2.9 – 69.6	14.71
Lysine	10.5 – 38.5	22.59
Histidine	1.9 – 11.8	4.98
Arginine	6.4 – 18.4	13.87
Total nitrogen	157.0 – 351.0	235.37

[a] Range and mean are of nine samples.
Source: Llaguno, 1977 (reproduced with permission).

extreme spread much less. All except spirit vinegar can be differentiated using UV absorption method (Kearsley, 1981; Kearsley and Gibson, 1981). Measurements of the absorbance of the neutralized distillate at 200 nm readily distinguishes malt, cider and wine vinegars, but not spirit vinegars from the non-brewed condiment. Spirit vinegars presumably contain a lower concenration of minor compounds, which absorb strongly at 200 nm, than the other brewed samples, and the range overlaps with that for the non-brewed condiment (Kearsley, 1981).

A rapid and sensitive distinction of genuine wine vinegar from products adulterated with acetic acid, and differentiation between vinegars from alcohol fermentation and synthetic acetic acid is a polarographic method. The method is based on the optical properties of actaldehyde, acetoin and diacetyl, which are present in genuine vinegars and absent in their synthetic counterparts (Sandoval and Hidalgo, 1975). The determination of volatile reducing substances based on reduction of $KMnO_4$ can become invalid in the case of wine vinegars because sulphur dioxide is used as preservative in many countries (Haguno, 1966). Biogenic acetic acid and oxidation number are other indices of adulteration (Fukano *et al.*, 1976). Malic acid detection could indicate cider vinegar in wine vinegar (Armandola, 1954). TLC identification of phloroglucinol and phloroglucinol carboxylic acid rather than tannins is suggested to indicate repeated use of grape residues in fermentation to produce wine vinegar. This index however cannot be applied to grapes infected with *Botrytis cinerea* (Berger and Herrmann, 1971).

Table 8.21 Results of osmotic pressure and freezing point depression measurements on vinegars and non-brewed condiments

Malt vinegar		Wine vinegar		Cider vinegar		Spirit vinegar		Non-brewed condiment	
OP	FPD	OP	FPD	OP	FPD	OP	FPD	OP	FPD
27	0.075	61	0.140	50	0.100	51	0.112	0	0.004
19	0.060	49	0.120	33	0.080	25	0.059	0	0
40	0.120	68	0.155	7	0.070	15	0.065	0	0.003
32	0.088	42	0.128	41	0.102	16	0.065	0	0.001
18	0.049	36	0.090	26	0.054	20	0.063	0	0
21	0.060	28	0.065	23	0.052			1	0.005
23	0.050	12	0.032	23	060			1	0
24	0.061	73	0.175	42	0.105			2	0.003
50	0.100	25	0.071	56	0.112			3	0.007
25	0.065	17	0.030	15	0.022				
28	0.060	13	0.020	25	0.070				
22	0.048	29	0.072						
15	0.053								
41	0.095								

OP = osmotic pressure (mosmol kg^{-1}), FPD = freezing point depression (°C).
Source: Kearsley, 1981 (reproduced with permission).

The applicability of specific ^{3}H radioactivity determination to distinguish between synthetic and biogenic vinegars has been tested and the difference in ^{3}H content between two types of vinegars has been shown to be 80–100 tritium units. The values should be used cautiously since the level of activity is subject to annual fluctuations. This can be overcome to a certain extent by using a series of synthetic and biogenic comparison samples (Schmid *et al.*, 1978). The ^{14}C content can be determined using scintillation methods which measure number of disintegrations per minute per gram carbon (dpm gc^{-1}). Genuine wine vinegars have ^{14}C in the range 22.4–23.4 dpm gc^{-1} while mineral acetic acid has no ^{14}C radioactivity. An admixture of genuine vinegar with 5% acetic acid can be detected. Samples showing <21 disintegrations dpm gc^{-1} are taken to be undoubtedly adulterated (Francesco *et al.*, 1970; Simon *et al.*, 1968; Mecca and Vicario, 1969). A sample of Spanish wine vinegar showing 19.1 dpm g C^{-1} to which synthetic acetic acid was added to 10%, 25% and 50 % concentration the exhibited activity of 17.6, 14.8 and 10.1 dpm g C^{-1}. respectively the synthetic acetic acid showed zero activity (Llaguno, 1977).

Stable isotope carbon analysis has also been used to evaluate the composition of various vinegars. Measurements of ^{13}C values of acetic acid indicated that the acetic acid derived from rice vinegar could be distinguished from that derived from cane sugar. The ^{13}C value for acetic acid from rice vinegar is about –2.44% compared to -1.21% from cane sugar. Using this technique, it has been shown that some commercial vinegars labelled as rice vinegars were not produced exclusively from rice (Kanno *et al.*, 1989). The price difference between vinegar from apple cider stock and from grain alcohol stock makes it financially advantageous to extend cider vinegar with grain-

based vinegar. Pure cider vinegars have been shown to have a $\delta^{13}C$ range of -24.1 to -27.2%, while corn vinegar gives values near or more positive than –10.0%. Results have indicated that as little as 10% added corn vinegar may be detected in cider vinegar. On the basis of natural variability of cider vinegar $^{13}C/^{12}C$ ratios, it has been recommended that samples yielding results more positive than -22.0% be classified as not pure cider vinegar. This method has been accepted as a first action by AOAC International (Krueger, 1992).

8.12 Miscellaneous

In the commercial production of frozen concentrated fruit juices, volatile fractions are added back to the product during processing. Determination of the combination of fractions and the proportions required to simulate the aroma of concentrated juices to that of the fresh fruit relies on the judgment of the flavour expert. Any mismatched judgements, more often than not are treated as suspicious by some consumers and consumer fora. Amongst the many techniques available for optimization, simplex optimization is one which can be successfully applied to find the best blend ratios of concentrated juices. A simplex is a geometric figure defined by a number of points equal to one more than the number of space dimensions. In two-and three-dimensional spaces, the simplexes are triangles and tetrahedrons respectively. In higher dimensional factor spaces, the simplexes cannot be visualized due to figures in hyperspaces. For more detail, the reader is referred to Morgan and Deming (1974) and Aishima *et al.* (1987).

Artificial flavouring in coffee extracts can be detected spectrophotometrically in the ultraviolet region (Van den Dool, 1957). The natural flavour has a λ_{max} at 270 nm, whereas the artificial flavour has a far more pronounced maximum at 270 nm and two smaller ones at 315 nm and 370 nm. The method is sensitive at 0.5% addition of artificial flavour (Seris, 1954). Other methods include specific weight or pyknometry, optical density, refractive index, bound residues and solubility characteristics (Arena, 1980).

Soy sauce or soybean sauce, commonly called 'kicap' is used as a condiment in chinese food and is a clear salty brown liquid prepared from the fermentation of the soybean (*Glycine max*). There have been allegations that some admixtures of soy sauce with acid hydrolysed plant protein (HPP) have taken place. Methods to detect HPP in sauce should normally be based on an indicator compound that is found in HPP but not in the sauce. Laevulinic acid, originating from the action of acids on carbohydrates in defatted soya are found at levels of 900–1100 mg g^{-1} of total nitrogen under conditions employed in the hydrolysis (Manley and Fagerson, 1970; Chei and Dun, 1976). The acid is very stable (Okuhara and Yokotsuka, 1963) and is not found in fermented soy sauce (Yeh-Chen and Hsu, 1985), so that it appears to be a suitable indicator of the presence of HPP in soy sauce (Yamashita *et al.*, 1972). It can be determined by spectrophotometric measurement after reaction with hydrazine

(Shilling and Hunter, 1965), colorimetry after reaction with vanillin-sulphuric acid (Nomura *et al.*, 1966), gas chromatography (Hirose and Fukuzaki, 1975) and liquid chromatography (Yeh-Chen and Hsu, 1985). A recent study has shown that the apparent HPP calculated from the laevulinic acid content of soy sauces deemed to have been produced wholly by fermentation were found to be 3.4–21% and blended sauces to have corresponding figures of 60%. A tentative maximum of 20% has been suggested as normal for genuine sauces (Lam and Hee, 1991).

A contaminant of protein hydrolysates produced by hydrochloric acid hydrolysis is 3-chloro-1,2-propanediol. This compound is specific only to protein hydrolysates (Velisek *et al.*, 1992), and could be used along similar lines as levulinic acid to detect HPP in soy sauce. This approach needs experimental evidence.

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Chapter 9

Tea, Coffee and Cocoa

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Tea, Coffee and Cocoa

9.1 Introduction

Tea, coffee and cocoa are entrenched in our dietary regimen as stimulant beverages rich in caffeine or theobromine or confections containing theobromine. The species or varieties, environmental conditions of soil, rainfall and altitude and the methods of processing especially fermentation, drying or roasting ultimately influence greatly the quality parameters so that the sale prices show wide differences. High grown tea is thus highly priced. High grown coffee exhibits superior sensory properties. Cocoa or chocolate from different regions exhibit grossly distinguishing quality features. It is therefore necessary that proper methods are available to distinguish their botanical and geographical origins, the processing methods employed and the quality of raw material used in the manufacture. In the past the problem of adulteration of these food materials has received extensive attention from food analysts though adulterators are always ahead in innovativeness and hence newer challenges are continually being posed. There is thus a great scope for sharpening the tools of the analyst to be ready to shoulder these challenges.

9.2 Tea

Tea essentially signifies two or three leaves and the apical bud of the plant *Camellia sinensis* var. *sinensis*, *Camellia sinensis var. assamica*, and allied species belonging to the genus Camellia. Crosses between these and other species such as *Camellia taliensis* and *C. irrawadiensis*, account for most of the tea under cultivation. The leaves are picked at various stages of their growth, the earlier pickings being considered the best.

9.2.1 Processing of tea

Tea is processed differently to give black (fully fermented), green (not fermented) or oolong tea (partly fermented). Green tea constitutes about 20% of the tea manufactured in the world, oolong tea about 2% and black tea forms the major part. The degree of fermentation involved in their manufacture is the primary cause of their differences.

The process of manufacture of black tea involves four stages: withering, rolling, fermentation and firing. Withering is done in both open and closed troughs. The

leaves are spread on a wire mesh at about 0.55 kg m^{-2} on a withering trough over which hot air is circulated. The time of withering is more important than the thickness of the layer. Generally the suitable period of wither is 16–24 h to obtain the best colour, strength and briskness. Longer withering time improves the colour and strength and decreases the briskness. The degree of wither is determined by the extent of moisture removal to a specified hygroscopic difference between the dry and wet bulb temperatures in a defined period of time. Subjective judgement is generally applied to decide the end point. It has been recommended that an integrated computer-based monitoring and control system for withering could be applied to both the open and closed trough systems. The system configuration can aim at on-line sensing, monitoring and controlling the temperature of the hot and ambient air, the moisture content of the withered leaf and the relative humidity of the air flowing beneath (open trough) or above and beneath (closed trough). It could also incorporate additional parameters such as the type of leaf processed, climatic conditions, transit time between plucking and start of the withering process, desired withering time and withering profile entered through a database (Kapur, 1993).

Rolling in orthodox leaf processing is done by eccentric rolling under pressure, while in crushing–tearing–curling (CTC), it is done by two differential speed rollers moving in opposite directions. Because of the large friction and shearing strain, the leaf is heated up, in turn affecting the quality. It is therefore desirable to monitor and control the heat which develops during rolling.

Fermentation is carried out by spreading the leaf on the floor or by feeding to a continuous fermentor. This process is catalysed by endogenous enzymes and lasts for 3–4 h. The end points are judged manually by an apple-type odour and a copper brown colour. Biochemical changes initiated during withering continue more briskly during fermentation. The four factors, quality, briskness, strength and colour reach their optima at different times and are affected differently by time and temperature. A large amount of unchanged catechin in tea results in a pale coloured infusion. If there is too great a proportion of high molecular weight polymers derived from polyphenolic compounds, the liquor from an infusion would be cloudy. In Russia, fermentation is checked by the degree of cell damage in rolling as well as by the content of tannins at the end of fermentation. Cell damage is determined by dipping the leaf in a solution of potassium dichromate and comparing the colour developed with known standards. Cell damage at 75% is considered optimum. The index for the degree of fermentation is the percentage of theaflavins which, apart from other parameters depends on the temperature and humidity in the fermentation room. The theaflavin content increases as the fermentation proceeds and starts declining after reaching a maximum. The time corresponding to achievement of the maximum theaflavin level could be taken as the optimum fermentation time (Ramasamy and Raju, 1993). The reactions also cause a change in colour, dielectric properties and refractive index.

After fermentation, in the next stage of firing, the leaf is either dried in conventional chain driers or in fluidized bed driers. The temperature of the inlet and the outlet air

has to be monitored and controlled. Electronic temperature meters have been developed, but no other parameters are measured and the operation is manual. The moisture content at the end of this stage is about 2–3%.

Fired tea is graded with a series of oscillating screens of appropriate size. The frequently produced grades in descending particle size are orange pekoe (OP), broken orange pekoe (BOP), broken orange pekoe fannings (BOPF) and dust. Dust, debris and some fibre are removed by winnowing. Stalky materials are somewhat more moist than the fired leaf and may be removed with an electrostatic sorter. Traditionally tea has been packed in foil lined plywood chests of dimensions 40×50×60 cm holding about 60 kg each (Graham, 1983).

9.2.2 Changes during tea processing

Numerous changes occur during each stage of the process. Withering causes protein breakdown leading to an increase in free amino acids, soluble carbohydrates and caffeine. Changes in organic acids also take place. It is during this process that aroma and colour precursors such as carotenoids, amino acids and flavanols are formed. Rolling triggers many other chemical changes and permits, among other things, contact of the polyphenols with oxidases, a prerequisite of fermentation. During fermentation, primary oxidation occurs and the leaves assume an agreeable odour and colour.

Bacteria originating from the leaf itself can sometimes survive the early stage of tea manufacture. Some bacteria can produce soft teas. They can affect the fermentation by increasing the rate and extent of oxidation of polyphenols in the surface juice, by forming alkaline substances which can induce the formation of dark leaf and liquors and by the production of substances of unpleasant taste or odour giving a 'taint' to tea. Firing involves the inactivation of enzymes, the elimination of water and the loss of part of the aromatic substances. Secondary oxidation and the full development of the characteristic odour of black tea take place during firing (Santhanakrishnan, 1993).

9.2.3 Sensory quality of tea

Two important criteria for tea are quality and origin. The 'tea taster' forms an indispensable part of the tea industry and determines the market value of tea on a purely subjective basis. It is believed that chemical and microscopic analysis may show deterioration or adulteration of tea, but organoleptic tests alone can determine its quality (Asselin, 1959). The chemical tests based on the method of brewing used by tea tasters are only auxilliary to quality determination (Khocholava and Kobakhidze, 1958). The tea taster plays an important role in purchasing and blending tea. His goal is usually to establish and maintain a chosen standard of tea under rapidly changing conditions. It becomes necessary to include teas from many countries and many gardens within a country in a single blend to ensure constancy of colour, flavour and

price over a long period of time. An experienced tea taster takes only a few seconds to look at and taste the sample and mentally correlate his findings and give a valuation. The tea taster has developed a language of his own as a result of appraising commercial teas. A glossary of such teas is given by Harler (1963) and Eden (1976). Despite the multiplicity of terms, the important characteristics that determine tea values are limited to only five of these: flavour, colour, strength, quality and briskness.

Attempts have been made for a long time to find a correlation between the tea taster's results and the constituents of tea as determined by chemical analysis. The quality of green tea has been shown to be positively correlated to the tannin content (Dzhemukhadze, 1950). In fact, phosphorus fertilizer in high doses is known to improve the quality of tea leaves by increasing the content of tannins and extractable matter (Guseinov and Agalarova, 1965). Low molecular weight tannins, particularly phloroglucinol and esters of gallic acid are believed to give tea of the highest quality and could be used as an objective index of quality (Kursanov and Brovchenko, 1950). Fractionation of tannins by precipitation using ammonium sulphate and extraction with ether have shown certain fractions to be more closely related to tea quality. For instance, the fraction not precipitated by ammonium sulphate and extracted with ether has the most pronounced effect on taste and aroma qualities, followed by ethyl acetate extractable fraction (Bradfield and Penney, 1945). The precipitable tannins exhibit bitter taste, while the polyphenol–catechol fraction gives fruitiness (Bokuchava and Movozhilov, 1946). Desirable quality characteristics are also correlated to high theaflavin and thearubigin content in liquors (Roberts and Smith, 1961; Owuor *et al.*, 1986a; Nosek, 1964). The theaflavin content as well as total and acid soluble oxidizable substances are positively correlated to the altitude at which the tea is grown (Ramaswamy, 1963, 1964). It can therefore be stated that higher the altitude at which the tea is grown, the better is the quality as obtained by organoleptic evaluation. The ratios of polyphenol to theaflavin and thearubigin give quality criteria which cannot be used to assess market value, but are suitable for comparing teas during manufacture, and for selecting conditions for the best brew (Roberts, 1962a).

No correlation between the tea taster's results and flavonol glycoside patterns could be found. The total flavonol glycoside content in black teas ranges from 0.38–1.7% with kaempferol being present in largest amounts, followed by the physiologically active rutin. It is believed that the flavonol glycoside pattern is a promising parameter to establish the geographical origin of a tea sample (Engelhardt *et al.*, 1992).

The relationship between colour and strength of tea liquors in terms of chemical compounds was studied by Roberts (1962b) who concluded that apart from small contributions by flavonotropolones, triacetidin and possibly products of non-enzymic browning, the colour is due to theaflavins and thearubigins. Roberts and Smith (1963) and Nakagawa (1969) found a positive correlation between theaflavin content and theaflavin/thearubigin ratio and the tea taster's assessment of quality, colour and strength of liquors. A high sensory score for tea samples has been shown to be correlated with high total polyphenols content in the fresh tea shoots, and it can be

Table 9.1 Correlation coefficient between catechins, theaflavins and astringency

	Correlation coefficient (r)	Significance level		
Catechins				
Catechin	0.5050	$r_{0.05}$	=	0.5139
Epicatechin	0.6903[b]	$r_{0.01}$	=	0.6411
Epigallocatechin	0.6378[a]	$r_{0.001}$	=	0.7603
Epicatechingallate	0.7615[c]			
Epigallocatechingallate	0.6723[b]			
Total catechins	0.8672[c]			
Theaflavins				
Theaflavin	0.3001	$r_{0.05}$	=	0.5139
Theaflavin-3′-gallate	−0.1084	$r_{0.01}$	=	0.6411
Theaflavin-3-gallate	0.1160	$r_{0.001}$	=	0.7603
Theaflavin-3,3′-digallate	−0.0741	Total Theaflavins		−0.0067

[a] Weakly significant.
[b] Significant.
[c] Highly significant.
Source: Zhang *et al.*, 1992 (reproduced with permission).

used to identify potentially high quality clonal teas. Polyphenol oxidase was evaluated in a similar manner, but failed to show any correlation, and is therefore not a suitable parameter to assess the quality potential of clonal teas (Obanda *et al.*, 1992). A similar observation of a positive correlation between concentration of theaflavins and thearubigins and quality is reported (Takeo, 1974; Takeo and Osawa, 1973a, 1973b). A significant correlation between astringency and content of total catechins and individual catechins, except catechin itself has been observed and is shown in Table 9.1. The correlations between these two parameters and astringency conform more closely to a logarithmic function, $Y = 0.21296 + 2.7742 \ln X$ for total catechins and $Y = 4.3382 + 2.0091 \ln X$ for epicatechin gallate (Y = sensory score and X = total catechins or epicatechin gallate in g/100 g dry matter). However no correlation between astringency and theaflavins could be found (Table 9.1) (Zhang *et al.*, 1992). Significant correlation between price and theaflavin content has, however, been reported by several investigators (Wood and Roberts, 1984; Millin *et al.*, 1969; Hilton and Palmer-Jones, 1975). Theaflavins affect the tea taster's results due to their contribution to colour, brightness and formation of 'cream' in black tea brews, but their concentration in black tea brews is very low and below the threshold values for astringent taste.

Water-soluble oxidized matter which is primarily responsible for the colour of black tea infusion showed a negative correlation with quality. The ratios of optical density at 380 nm of theaflavins and thearubigins with highly polymerized substances (HPS) were found to be effective in evaluating the quality of black tea.

In a statistical evaluation of North-East Indian plain teas (Biswas and Biswas, 1971; Biswas *et al.*, 1971; Biswas *et al.*, 1973) it was found that the total oxygen uptake, the epigallocatechin in unprocessed tea shoots and theaflavins and thearubigins, and the theagallin content of processed black tea determined the cash valuation which in turn

is dependent on the quality and/or briskness. This has been recently shown with tea samples from nine countries, wherein multiple regression analysis indicated a relationship between tea taster's score and chemical constituents comprising five resolved thearubigins and one unresolved thearubigin hump (McDowell *et al.*, 1995a; 1995b). A neural network obtained by computer simulation of the brain's abilities such as speech and image recognition has also affirmed the correlation between the black tea score and phenolic composition (Tomlins and Gay, 1994). Thearubigin and theagallin content of black teas beyond a certain concentration was found to be detrimental to the quality as assessed by tea tasters. Hilton and Palmer-Jones (1975) have shown negative correlation for East African teas with respect to total colour and the quality is predominantly associated with theaflavin. For North East Indian plain teas, however, theaflavin content and total colour were positively related. Total colour value was as good a predictor of price as theaflavin content. A regression equation relating theaflavin and total colour to market price has been given but the extent to which the selling price depended on theaflavin content and the total colour varied according to the geographical area of origin.

The ability of brew to cream on cooling along with the colour of the cream formed is one of the criteria used by tea tasters to judge the quality of black tea. The major constituents of cream are theaflavins, thearubigins and caffeine which are responsible for brightness, briskness and colour of the tea infusion.

A study of the contribution of non-volatile compounds of black tea to the character of the beverage (Millin *et al.*, 1969) by tasting pure compounds and various fractions isolated from black tea liquors has been reported. Caffeine, but not phenolics and amino acids, contributed significantly to the taste of the beverage. Theaflavin and other oxidation products of intermediate molecular weights were astringent and together with caffeine influenced briskness and strength of tea liquor.

Discrimination of commercial black tea samples for sensory profile can also be done by using near infrared (NIR) reflectance spectroscopy at four wavelengths; 1660 nm assigned to aromatic groups, 1720 nm and 2300 nm assigned to the methylene group and 2050 nm assigned to the hydroxyl functional group. The success rate with this method is about 91% (Osborne and Fearn, 1988).

A blend of several black tea aroma constituents is the primary determinant of flavour quality. Gas chromatographic regions consisting of ionones, linalool and dimethyl sulphide have been reported to be positively related to quality (Vuataz and Reymond, 1970). The volatile flavour compounds (VFC) of black tea made from the young tender shoots of *Camellia sinensis* are known to contribute towards the quality of the beverage. Many studies have eported the composition of VFC in black tea without indicating how the varying levels of the compounds affect the quality (Yamanishi *et al.*, 1966, 1972; Bondarovich *et al.*, 1967; Renold *et al.*, 1974; Aisaka *et al.*, 1978; Cloughley *et al.*, 1982; Takeo, 1983, 1984; Takeo and Mahanta, 1983; Mick *et al.*, 1984; Horita and Owuor, 1987). It has been recognized that some compounds contribute negatively whereas others contribute positively towards black tea flavour. No studies have

attempted to quantify how the quality of black tea changes with the varying levels of VFC. In an attempt to overcome this problem, several quantitative methods have been examined (Wickremasinghe *et al.*, 1973; Yamanishi *et al.*, 1978; Yamanishi *et al.*, 1989; Baruah *et al.*, 1986; Owuor *et al.*, 1986, 1988; Mahanta *et al.*, 1988). These studies have also used ratios of gas chromatographic peak areas of compounds perceived to be beneficial to the black tea quality to those which are thought to lower the quality. Wickremasinghe *et al.* (1973) and Yamanishi *et al.* (1978) used the ratio of the sums of gas chromatographic peak areas of compounds eluting before linalool to those eluting subsequently. This ratio, refereed to as Wickremasinghe–Yamanishi ratio assumes that the compounds with retention times shorter than linalool are deleterious to quality whereas linalool and the VFC with longer retention times were desirable for black tea quality. Thus smaller the ratio, the better is the quality. The VFC were later classified according to their odour characteristics (Owuor *et al.*, 1986b). The compounds imparting a green grassy smell were classified as Group I VFC, while those with desirable sweet flowery aroma were classified as Group II VFC. The ratio of the desirable:undesirable VFC or Group II:Group I VFC was referred to as Owuor's flavour index and abbreviated as FI (Owuor *et al.*, 1986b, 1988). FI measures directly and linearly the aroma quality, thus the larger the ratio the better the aroma quality. Again linalool oxides *cis* (*cis*-2-methyl-2-vinyl-5-hydroxyisopropyl tetrafuran) and *trans* (*trans*-2-methyl-2-vinyl-5-hydroxyisopropyl tetrafuran) furanoid and benzaldehyde, which have desirable aroma (Yamanishi *et al.*, 1968) were classified into the correct group. Apart from these compounds, the rest of the classification of the compounds remained the same as those of Wickremasinghe *et al.* (1973) and Yamanishi *et al.* (1978).

A ratio based on the sum of the gas chromatographic peak areas of terpenoids to non-terpenoids (Baruah *et al.*, 1986; Mahanta *et al.*, 1988), the Mahanta ratio was also formulated. The terpenoids were assumed to be desirable and non-terpenoids undesirable for tea quality. Yet another ratio, based on the gas chromatographic peak areas of linalool and E-2-hexenal which ignored all the other VFC has also been developed (Yamanishi *et al.*, 1989). This ratio, called as the Yamanishi–Botheju ratio, has been shown to have a relationship with prices at an auction of Srilankan orthodox black teas.

Yamanishi–Botheju ratios which are reported to be significant for Srilankan Black teas are not so for Kenyan clonal black teas. This can be explained by the fact that this ratio ignores too many volatile flavour compounds which may be contributing to the aroma quality of tea. The rationale for the use of Yamanishi–Botheju ratio requires that linalool and E-2-hexenal occur in large amounts in all teas and therefore have a dominant effect. Many black teas contain large amounts of hexenal, linalool oxides, phenylacetaldehyde and methyl salicylate (Owuor *et al.*, 1986b, 1987, 1988; Owuor *et al.*, 1989). The Yamanishi–Botheju ratio (Yamanishi *et al.*, 1989) is therefore unlikely to be a useful index in aroma classification, especially for Kenyan black teas and their equivalent. Some clones of black Kenyan tea have shown a significant relationship to

the Mahanta ratio, while others have not. This is because some non-terpenoids in black tea such as benzaldehyde (Yamanishi *et al.*, 1968), phenylacetaldehyde (Motoda, 1979), methyl salicylate (Aisaka *et al.*, 1978; Howard, 1978) and benzyl alcohol (Aisaka *et al.*, 1978) have been demonstrated to have a desirable aroma, and their presence in large amounts in some clones of black tea concurrent with their inclusion in the group of compounds imparting inferior flavour to black tea (Baruah *et al.*, 1986; Mahanta *et al.*, 1988) may be erroneous. A significant inverse relationship has also been observed between the Wickremasinghe–Yamanishi ratio and the tasters evaluation of clones of Kenyan black tea. The compounds with retention times less than linalool are products of lipid degradation, and during tea manufacture these compounds impart a green grassy flavour to black tea. The FI, developed as an improvement over the Wickremasinghe–Yaminishi ratio does have a significant regression with the tea taster's evaluation, except that the regressions are linear and positive. The only difference between these classifications is the inclusion of linalool oxides (*cis* and *trans*-furanoid) and benzaldehyde in the group of VFC imparting sweet flowery aroma to black tea (Yamanishi *et al.*, 1968). Although the FI is sensitive to the methods of tea manufacture, it does plateau off above a certain value. Also it should be recognized that the relative concentration of volatiles as detected by gas chromatography (GC) following steam distillation may not necessarily reflect the relative concentration above a cup of black tea. These data must also be used cautiously; they should be treated as indicative, since the olfactory perception limits of different VFC are variable. It must also be remembered that the contribution of each VFC to flavour is not proportional to the gas chromatographic area. It is known that the relationship between the stimulant concentration (represented by the GC peak area) and neural response (perceived flavour intensity) is not linear. It is therefore felt that the methods of aroma quantification need to be improved so that data from different laboratories can be compared (Owuor, 1992).

The ratio of linalool and its oxides to geraniol and phenylethanol in the essential oil which is responsible for the flavour of black tea could distinguish between samples from different geographical regions like India, Peru, Formosa and Japan (Yamanishi *et al.*, 1968). Similarly, the ratio of linalool to linalool with geraniol, defined as the terpene index (TI), represents the aroma of black tea. The value of TI varies between 0.1 and 1.0. Assam type clones show TI values of about 1.0, whereas in the China type of tea clones, it ranges between 0.9 and 1.0. It is on this terpene index that attempts have been made to trace the dispersion route of the tea plant in China and surrounding areas (Satyanarayana and Sharma, 1993). The essential oil from fresh green and fermented tea leaves can be distiguished by thin layer chromatography (TLC) of the terpenes and aromatic alcohols. While fresh leaves contain cinnamyl and benzyl alcohols and geraniol and citronellol in small amounts, fermented leaves contain phenylethanol and linalool in addition to these (Gogiya, 1966a).

Distinction between the fresh and wilted tea leaf can be made on the basis of the free amino acids leucine and phenylalanine which are present in wilted, but not in fresh

leaf. These amino acids undergo oxidation later to give the tea aroma (Bokuchava *et al.*, 1954). The sodium salts of C_1–C_6 fatty acids in tea essential oil also give a clue to its origin, that is, from fresh, dried, withered or fermented leaves. While fresh leaves contain only acetic acid, withered leaves contain in addition butyric acid and fermented leaves contain two further constituents, namely caproic and caprylic acids (Gogiya, 1966ab).

9.2.4 Adulteration of tea

Tea adulterants reported in the literature are exhausted tea leaves, mineral and organic matter, leaves of other species such as dried bilberry leaves (*Vaccinium myrtillus*) (Kemal, 1943) willow, elder, sloe, hawthorn and beech leaves (Nicholls, 1952). Tea is sometimes adulterated with *Stachytarpheta jamaicensis*, which is said to be sold as Brazilian tea. It may be detected by its strong violet fluorescence in ultra-violet light. Added colourants, substances to increase astringency such as catechu, borax and sodium carbonate, and non-tender stalks and leaves are also known adulterants.

Exhausted tea leaves are generally detected by lower percent extractives and tannins. These can also be detected by measurement of the electrical conductivity of tea infusions prepared according to a specified procedure at 20 °C. The value for genuine samples is $110–135 \times 10^{-6}$ mho and values beyond the given range indicate a definite adulteration (Roy and Mitra, 1955). Added colourants in black tea can be examined by the absorption spectrum in the IR and UV regions. The colour intensity in the visible region increases appreciably, while little change is produced in the UV region (Mohamed, 1980).

The criteria for defining adulteration used, that is total ash (4–8%), percentage of water-soluble ash (<40%) and extractives (<30%) are insufficient for detecting many adulterants. It has been suggested that caffeine and manganese contents also be included as criteria of tea adulteration (Chatterji and Ganguly, 1950). In teas containing >5% leaf stems and veins, there is a decrease in the yield of extract and tannins and an increase in cellulose, which coincides with a notable impairment of organoleptic quality (Khoperiya, 1957). The presence of non-tender stalks at >10% levels does not affect the amount of total and soluble ash, total extractives, caffeine and tannin but does affect the value of crude fibre and lowers the quality with respect to flavour and taste of infusion. A crude fibre content >13.5% is indicative of woody stalks in tea and is practically useful in the analysis of dust tea where even physical methods cannot detect such addition (Mitra and Roy, 1953).

9.2.5 Herbal teas

The origin of herbal teas, their most popular ingredients, health standards, laboratory examination, cleaning and processing and creation of tea blends for expanding markets have been discussed by Signore (1979). Health benefits have been claimed for many

herbal teas which in many cases are questionable. For instance, 'Ipe Roxo' tea made from the bark of the plant *Tecoma impetiginosa* or *T. conspicua* contains the toxic 1,4-naphthaquinone derivative lapachol, although its low concentration in Ipe Roxo tea may not pose a significant hazard (Fuchs *et al.*, 1990). Increasing consumption of herbal teas is also suggested to contribute to the intake of pyrrolizidine alkaloids in the USA (Huxtable, 1980) which can cause liver damage (Roitman, 1983).

Lovage roots are used in herbal tea mixtures. An indicator which would allow quantitative detection of lovage roots in multicomponent mixtures is an obvious neccessity. Ligustilide, which is present in quantities ranging from 0.08–0.17% in various samples of lovage roots, but absent in other plants (also used in herbal teas) is one such indicator. A high performance liquid chromatography (HPLC) method of great sensitivity which determines the amount of lovage roots down to 0.5% has been recently reported (Segebrecht and Schilcher, 1989).

Herbal tea infusions such as that of orange blossom, peppermint and mallow blossom teas are many times contaminated with dithiocarbamate fungicides, which is converted to ethylenethiourea (Wuthrich *et al.*, 1984). However, the daily consumption of ethylenethiourea is very low, even if the legal limit of dithiocarbamates is heavily exceeded. Organochlorine and organophosphorus pesticides from the herbs may be transferred to the tea infusion. The transfer is independent of the pesticide concentration in the teas and also of the tea essential oil content. It is mainly governed by the solubility in water according to the equation, $Y + 100/(5.62 + L^{-0.42} + 1)$, where L is the solubility of the pesticide in water at room temperature and Y is the transfer ratio, given as the amount of pesticide in the infusion as a percentage of the total pesticide (Zimmerli and Blaser, 1982)

Although herbal teas have been reported to be regularly contaminated with microorganisms such as *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus* and Salmonella species, infusion of such teas reduces bacterial counts by 3–4 log cycles, showing that consumption of herbal teas does not constitute a health hazard on this count (Yde *et al.*, 1981).

9.3 Coffee

The term 'coffee' comprises not only the consumable beverage obtained by extracting roasted coffee with hot water, but also a whole range of intermediate products starting from freshly harvested coffee cherries. After oil, it is reckoned to be the most widely traded commodity in the world and also provides employment for some twenty million people. The coffee tree is indigenous to Ethiopia, and was introduced in Europe around 1600.

The two main coffee species of commerce are *Coffea arabica* and *Coffea canephora* (also known as *Coffea robusta*) belonging to the natural order Cinchonaceae. *Coffea canephora* today accounts for about 20% of the world exports. Some hybrids of the *C. arabica* and *C. robusta* varieties have been developed, the most important being *C.*

arabusta. It was the result of attempts to develop a variety with good cup quality with high disease resistance. Besides *C. arabica* and *C. robusta*, *C. liberica* and *C. excelsa* varieties are also known. However liberica cherries are pulpy and difficult to dry, while excelsa cherries drop to the soil when dry. In both these cases, the fermentation is uncontrolled giving rise to an unclean taste. These do not have much commercial value.

Infinite care is taken to cultivate coffee from the time that the seed is sown till the bushes are ready for yielding. The crop has to be protected against bad weather, insects and diseases. Agronomic treatments can cause changes in coffee quality. Nitrogenous fertilizers can cause a poorer, thinner and lighter cup quality, while spraying with benzene hexachloride (BHC) will produce beans with a 'bricky' flavour.

9.3.1 Composition and processing

The ripe fruit resembles a small black cherry the pulp of which usually contains two berries enclosed in a hard membrane-like pericarp known as parchment. The berries are freed from the pulp and parchment by fermentation after which the pulp can be washed away with water. Pulping and fermentation are somtimes combined in the 'aquapulper', a pulper demucilager, where the pulp is rubbed off the parchment mechanically.

Caffeine, the physiologically active component of coffee ranges from 0.58–1.89% total dry matter (DM) in *C. arabica*, 1.16–4.0% DM in *C.canephora*, 0.23–0.51% DM in *C. eugenioides* and about 1.7% DM in *C. stenophylla* (Charrier and Berthaud, 1975). It can be analysed by HPLC (Internal Organization for Standardization, 1992; French Standard, 1992) by near infrared spectrometry (Guyot *et al.*, 1993) or by UV spectrometry (Cepeda *et al.*, 1990), and can be used as an indicator of coffee in blended coffee containing chicory (Ferreira *et al.*, 1987). Another alkaloid 'coffearine' is also present in coffee beans in very small amounts. Besides alkaloids, dried coffee bean contains about 50% carbohydrates, 8–18% lipids, up to 13% proteins and amino acids and about 5% minerals. These components form the typical coffee flavour during the subsequent roasting process. Decaffeinated coffee is popular with many consumers. Decaffeination is carried out on green beans at a moisture content between 30–65%, wherein caffeine is diffused out of the cell walls and solubilized as the caffeine–potassium permanganate complex (Viani, 1986).

The International Coffee Organization (ICO), a body formed by the coffee producer and consumer countries in close cooperation with the United Nations defines 'green coffee' as 'all coffee in the naked form before roasting', 'roasted coffee' as 'green coffee roasted to any degree' and 'soluble coffee' as 'dried water-soluble solids derived from roasted coffee' (International Coffee Organization, 1983). Other international bodies such as the International Standards Organization (ISO), have given equivalent definitions and coded impurities or 'defects' such as wood, sticks, husks, parchment or whole cherries which may be present (International Standards Organization, 1984).

Most countries have fixed the maximum number of defects tolerated in commercial coffee. An automatic tool which can recognize colorimetric and dimensional characteristics of single green coffee beans, sort defective beans into classes, classify coffee lots according to a suitable criterion or to international standards, and also can store the data generated in a database has been reported (Suggi, 1992).

9.3.2 Detecting blends of coffee species

In view of the higher price commanded by arabica beans, it is important to identify and quantify the species of the various coffee products. Arabica and robusta beans are easy to distinguish by their size, but this visual difference is eliminated by processing. Efforts have been made to distinguish between the two species by chemical analysis. Unroasted arabica coffee beans contain high concentrations of sucrose, while robusta is especially rich in the reducing sugars, glucose and fructose. Similarly, the content of free amino acids is higher in the unroasted robusta variety compared to arabica (Tressl *et al.*, 1982). Alkaline and heterocyclic amino acids are also more abundant in robusta than in arabica. However, both amino acids and sugars are degraded during roasting via Strecker degradation to various flavour ompounds such as pyrroles, pyrazines and pyridines, furans, sulphur-containing compounds, etc. These can therefore only be used to detect blends in unroasted coffee.

It is recognized that arabica and robusta coffees differ in their unsaponifiable constituents, kahweol being absent or present only in traces in robusta coffee. Kahweol occurs at a concentration of 1.2–2.1 mg kg^{-1} in arabica and only 0.1 mg kg^{-1} in robusta, and can be measured quantitatively by a colour reaction. Processing raw coffee, such as steaming or decaffeination, leads to a varying degree of reduction of kahweol colour absorption (Wurziger, 1977). TLC separation of the unsaponifiables has also shown a constituent, later identified by high-resolution MS as 16-*O*-methylcafestol, to be present in robusta but not in arabica (Speer and Mischnick, 1989). Raw robusta and arabusta coffees contain 0.6–1.2 mg kg^{-1} DM and 0.8 mg kg^{-1} DM 16-*O*-methylcafestol, respectively. A level of 0.1 g kg^{-1}, determined by HPLC in an unknown roast implies a 7–13% robusta content, with almost 80% certainity. Roasting does not cause a significant decrease in the content of 16-*O*-methylcafestol. This constituent aids in detecting as low as 2% added robusta coffee in arabica coffee (Speer and Montag, 1989), even in soluble coffees (Speer *et al.*, 1992). Robusta coffees are also known to contain a higher amount of of Δ^5-avenasterol compared to arabica (9–15% and 2–5% of the desmethylsterol fraction respectively) and could be another approach to detecting blends (Picard *et al.*, 1984). Multivariate methods based on characteristic sterols such as campesterol, stigmasterol, β-sitosterol and Δ^5-avenasterol can permit distinction between arabica and robusta coffees (Consiglieri *et al.*, 1991). Mid-infrared spectroscopy, particularly in the region of 1100 cm^{-1} and a sharper brand at 1744 cm^{-1}, is proposed as a rapid alternative to existing authentication methods, which are often time consuming or difficult to implement successfully. The former occurs in the

spectral region associated strongly with carbohydrates, and could be due to differences in the polysaccharide composition of arabica and robusta varieties. The feature at 1744 cm^{-1} has been found to be relatively more intense in arabica than robusta, and arises from the lipid content of the beans. Although this can identify the variety, blends have not yet been characterized by this method (Kemsley *et al.*, 1995). Fourier transform infrared (FTIR) spectroscopy has also been shown to be promising in discriminating between arabica and robusta, the differences being attributed to different contents of chlorogenic acid and caffeine (Briandet *et al.*, 1996).

A diterpene glycoside of the furokaurane type has been isolated from the seeds of the caffeine-free coffee species, *Coffea pseudozangubariae* belonging to the section Mozambicoffea. This bitter tasting compound named mozambioside is considered to replace caffeine with respect to chemical defence (Prewo *et al.*, 1990), and could probably serve as a marker compound of its origin in blends with other species. Similarly, *N*-caffeoyltyrosine has been found in many green robusta coffee beans from many origins, but particularly of those from Angola (Clifford *et al.*, 1989). The presence of this compound which survives medium roasting temperatures (Correia *et al.*, 1995) and quantification of its levels could indicate not only the species, but also the geographical origin of the coffee. Work in this direction could be useful.

The application of the headspace technique is an important tool for an aroma specific differentiation between arabica and robusta coffees. Roasted arabica variety contains less alkylated pyrroles and more furfurylpyrroles than robusta (Tressl *et al.*, 1981). The concentrations of 2-methylbutanal, 3-methylbutanal and 2-methylfuran reach typically higher levels in freshly roasted robusta than in arabica (Piringer, 1983). These compounds and mathematical/statistical relations thereof could provide valuable information in detecting blends in roasted coffee. A 99:1 mixture of arabica:robusta can be differentiated from pure arabica coffee by measuring the amount of sulphur-containing compounds present (Nurok *et al.*, 1978). Computer aided discrimination of 13 compounds in the headspace allows exact differentiation between classes such as arabica/freshly roasted and arabica/10 days old, as well as robusta/freshly roasted and robusta/10 days old. Methanethiol has the biggest influence on the discriminant analysis and therefore is valuable in distinguishing arabica from robusta varieties (Holscher and Steinhart, 1992). Flavour differences between the two types of coffee are mainly due to the predominance of the enoloxo compounds sotolon, abhexon, furaneol, 3,4-dimethylcyclopentenol-1-one in arabica coffee and of 3,5-dimethyl-2-ethylpyrazine, 2,3-diethyl-5-methylpyrazine, 4-ethylguiacol and 4-vinylguiacol in robusta coffee. Brewing enhances flavour differences between the two types and could be used as an aid to detecting blends (Blank *et al.*, 1992).

Some 99% of world coffee is produced on an internationally agreed quota system and marketed as having a specific origin, and robusta coffee beans from 34 origins account for about 25% of this. Concern has been expressed regarding the possibility of fraud perpetrated by smuggling non-quota coffee into a quota area and its subsequent

disposal under a falsely described origin. In cases of dispute or uncertainity, an objective indicator of geographic origin would be of value. Minor chlorogenic acid-like components can be separated from the major chlorogenic acid-rich fraction that also includes caffeoylquinic acid, feruloylquinic acid and dicaffeoylcaffeic acid by chromatographic procedures to reveal patterns characteristic of the geographical origin of the coffee. In particular Angolan coffee can be very easily distinguished from others on the basis of seven chlorogenic acid-like spots which are unidentified as yet (Clifford and Jarvis, 1988).

9.3.3 Processing quality of coffee

Before roasting, the raw coffee beans have to be graded, either on the basis of size, density, colour or by cupping. Grading by colour can be accomplished by hand picking, electronically with monochromatic light to sort out the black beans, biochromatically to eliminate brown and bleached beans or fluorimetrically to eliminate 'sinker' beans. Grading by cupping is preferred in the consumer countries and is done after roasting a representative sample. Good quality is denoted by neutral and clean, while defective quality gives a harsh, rubbery and earthy odour.

Roasting is generally carried out at 200 °C, during which the coffee beans develop a brittle structure, a dark brown colour and characteristic flavour and aroma. Browning reactions, doubling of the volume and release of some gases such as carbon dioxide and some carbon monoxide are the visually observed effects of roasting. Reactions such as decarboxylation, dehydration of quinic acid moeity, lactonization, isomerization, polymerization and reactions with sugars occur during roasting. The degree of roast is important since it determines the sensory attributes, and roasting is terminated when these are at the maximum desired level. Generally chlorogenic acid contents are indicative of the degree of roast. The values range from 6.9–0.2% for the raw and dark roast arabica variety, and 8.8–0.2% for the raw and dark roast robusta variety (Trugo and Macrae, 1984).

9.3.4 Sensory quality of coffee

Coffee has been known to be adulterated with coffee berry skins, coffee berry parchment and other cellulosic materials such as twigs. These cause changes in the organoleptic properties, the change being linear with the amount of adulterant material. Serious impairment of organoleptic properties has been observed in coffee containg 20–40% coffee berry skins, 50% coffee parchment and 30–40% cellulosic material (Miya and Shirose, 1977). Fungal contamination by *Cladosporium*, *Penicillium* and *Fusarium* is also concurrent with lower cup quality (Wellman, 1961). Amateur tasters can easily distinguish 'high grown' coffee from a 'low grown' one, since the former beans are harder, smaller, firmer and the crease is tighter in appearance. The latter beans are softer, usually a little larger, not quite firm and more open in the crease.

Sensory evaluation of coffee is often done by tasters who use all the possible sense spots. The back of the mouth and the nasal passages record impressions and assist in 'aroma' classification. The back of the tongue is sensitive to bitter and freshness flavours, while the sides can successfully detect staleness. There was no correlation of either aroma or quality with chemical determinations such as hydrogen ion concentration, alkalinity or acidity, mineral content, caffeine content, amount of proteinaceous components, colour intensity, or contents of fats and oils. Sulphur compounds, that is mercaptans in combination with ketones, diketones, acetic and isovaleric acids, histidine and certain phenols together simulate coffee aroma. Trigonelline, a bitter tasting substance is also of great importance in coffee flavour and aroma (Wellman, 1961). The ratio of monochlorogenic to dichlorogenic acids is related to the cup quality of coffee, the value being lower in robusta compared to arabica beans. The ratio of dicaffeoylquinic acid:monocaffeoylquinic acid is statistically correlated to the maturity of coffee beans. Sensory analysis has indicated that dicaffeoylquinic acid confers a disagreeable flavour to coffee beverage, and the addition of monocaffeoylquinic acid can mask it. Hence inclusion of partially green berries negatively affects beverage flavour due to their lower ratios (De Menezes, 1994). Good correlation between titratable acidity of the roasted coffee, to which phosphoric acid also contributes, and the acid taste of coffee brew is reported (Viani, 1986).

The relationship between GC profiles and sensory responses have been analysed in 31 arabica coffees by multivariate analysis. Aroma profiles arising from GC determination appear to be close to those deduced from sensory evaluation (Wada *et al.*, 1989). Based on this data, coffees could be classified into six groups by principal component analysis of GC data. The relationships between principal components and sensory data have been reported to be linear, according to a 'quantification theory' (Osajima, 1989). While arabica coffee contains more dimethyl sulphide, 2-methyl propanal, butanedione, 2,3-pentanedione, 2-furaldehyde and 3-methylbutanal, robusta contains more phenol, toluene, 1-methylpyrrole, thiophene, 2-hydroxyphenol, 2,5-dimethylpyrazine and furyl acetate. These discriminations are responsible for sensory discrimination observed between them. Robusta coffee has less cocoa and brown sugar, but more earthy, papery and burnt odour characteristics than arabica (Leino *et al.*, 1992b).

The freshness of roasted coffee has long been an indicator of quality. Staling on storage has been of interest to the industry as well as the consumer, and can be widely observed using 'aroma indices'. The headspace technique has since long been applied as an objective analytical approach to determine the freshness of roasted coffee beans for a long time because of its simple handling and good reproducibility (Kallio *et al.*, 1990; Shimoda and Shibamoto, 1990). Staling of coffee has been correlated with the generation of n-hexanal after an initiation phase of seven weeks storage in air. This alone, however, cannot explain the effect completely, since a certain loss of odour intensity is already perceptible after 8–10 days with a significant loss in cup quality. 2-Methylfuran, 2-butanone and methanol are also reported to be common indicator

Table 9.2 Definitions of the age determination of coffees ($n = 5$)

Storage time indicator, y[c]	a[c]	b[c]	r[d]
Ground coffees[e]			
Acetone:propanal	0.0107–0.0116	3.2728–10.162	0.74–0.97[g]
Thiophene:propanal	0.0004–0.0005	0.1325–0.1888	0.87[e]–0.91[f]
Thiophene:butanedione	0.0001–0.0007	0.0763–0.1132	0.70–0.94[f]
Butanedione:2-methylfuran	−0.0002 to −0.0016	0.6174–1.3720	−0.79 to −0.89[f]
Acetone:butanedione	0.0021–0.0207	1.9146–6.2612	0.90[f]–0.98[g]
Coffee beans[d]			
Acetone:propanal	0.0066–0.0079	3.0871–4.3367	0.40–0.96[f]
Thiophene:propanal	0.00054–0.0008	0.1842–0.2699	0.41–88[e]
Thiophene:butanedione	0.0002–0.0003	0.0663–0.0685	0.83[e]–0.92[f]
Butanedione:2-methylfuran	−0.0013 to −0.0015	1.1367–1.2805	-0.91[f] to -0.93[g]
Acetone:butanedione	0.0021–0.0207	1.9146–6.2612	0.90[f]–0.98[g]

[c] Range for three varieties
[d] Range for two varieties.
[e] $p < 0.10$;
[f] $p < 0.05$;
[g] $p < 0.01$.
[e] see text.
[f] r is the correlation coefficient between x and y (see text).
Source: Leino *et al.*, 1992a (Reproduced with permission).

compounds of freshness (Arackal and Lehmann, 1979; Kwansy and Werkhoff, 1979; Vitzthum and Werkhoff, 1979). Several different ratios have also proved useful in measuring staling. Methanethiol has been identified as having a strong impact on aroma freshness (Steinhart and Holscher, 1992). In three weeks of storage, it declines sharply. The loss is recognizable one day after roasting and decreases to about 10–20% relative to the starting value (Holscher and Steinhart, 1992).

Indices of storage period or indices of ageing of coffee are not necessarily similar to indices of staling. For instance, the ratio of 2-methylfuran:2-butanone as well as 2-methylfuran:methanol are commonly used as a measure of age (Vitzthum and Werkhoff, 1979), but have not been identified to be good indicators of staling of coffee (Leino *et al.*, 1992a). The ratios of acetone:butanedione, acetone:propanal, thiophene:propanal, thiophene:butanedione, 2-methylfuran:propanal and butanedione:2-methylfuran have been found to be useful indicators of ageing of coffee (Kallio *et al.*, 1990). Table 9.2 gives linear correlations of the various ratios versus actual age ($y = ax + b$, where y is the ratio of chemical constituents of and x is the age a and b are constants). It can be seen that butanedione:2-methylfuran is a good indicator of age of roasted coffee. The age of roasted coffee can also be determined on the basis of reduction in volatile reducing substances (Pekkarinen and Porkka, 1963).

9.3.5 Coffee substitutes and adulterants

Consumer preferences, religious beliefs and health grounds are the factors that have promoted the development and marketing of various coffee substitutes. The

substitutes used are devoid of or very low in caffeine, but generate coffee aroma on roasting. Substitutes such as cereals, figs and chicory enjoy substantial sale along with pure coffee in some consuming countries. Thus 'coffee' made from dandelion roots has been available from health food shops for many years. Other roots such as carrots and parsnips, turnips and mangold wurzel have also been used (Nicholls, 1952).

The most important coffee substitute is undoubtedly chicory, *Cichorium intybus*. Its chemical composition and beneficial properties as a beverage have been reviewed by Boussard (1982). Chicory itself is reportedly adulterated by roasted beetroot. The microscopic characteristics of the two roots are so similar that it appears impossible to differentiate between them. A number of cereals, particularly barley, malt, rye, wheat and maize have been used. The grain may be roasted as such, or may be ground to a flour which is then pasted, cooked, dried and coarsely milled, the milled fragments then being roasted. Leguminous seeds which have been used include chickpeas, peanuts, soybeans, other beans and lupins. The substitutes are usually blended with coffee either singly or in combination, for example coffee, chicory, barley and malt. The list of possible substitutes is endless. 'Viennese coffee', for instance, is a blend of roasted and ground coffee, with roasted figs, whilst dates, cocoa beans, acorns, cola nuts, sweet potatoes, sugar cane, cashew nuts, carobs and even beetroots have been encountered (Smith, 1985). Percent total solids in 5% w/v extract has been recommended as a basis for estimating the proportion of chicory and coffee in a mixture. The values of percent solids for pure coffee and chicory are 1.6% and 3.85%, assuming 32% and 77% extractable material, respectively (Lyons and Co., 1955). Unlike chicory, date seeds contain less extractives than coffee. Husk, coats and stems, sugar, soil, sand and spent coffee are among the major adulterants (Lopez, 1974). Extensive adulteration of coffee with hulls could also be determined by this method.

Besides the content of volatile matter, trimethylxanthine, soluble and insoluble fixed mineral residues and alkalinity of the ash are also valuable chemical indicators to detect such fraudulent practices (Ferraz de Menezes, 1955). Caffeine content along with supplementary tests with ferric chloride, chloramine number, reduction by Fehling's solution and testing with iodine solution and lead acetate can determine whether 0–10%, 10–35% or >35% coffee is present (Streuli, 1942). Unroasted chicory roots contain caffeoylquinic acid and dicaffeoylquinic acid at much lower levels than green coffee beans, but roasted products give only 5-caffeoylquinic acid consistently and 4-caffeoylquinic acid occasionally at levels approximately two orders of magnitude lower than in the corresponding roasted coffee products. Roasted substitutes such as figs, wheat, malted barley, soybeans and dandelion roots are manifested as 5-hydroxymethyl furfural, characterized by their presence in 80–90% of the total chromatogram area (Clifford *et al.*, 1987).

In addition to chemical methods, low power microscopy (20×) can be used to detect these adulterants (Lopez, 1983). Dried or roasted carob powder in coffee powder can be estimated by IR spectrophotometry and X-ray diffractometry which can distinguish between different crystal forms of sucrose. Carob powder contains

considerable amounts of sucrose in the crystalline form, which becomes amorphous on roasting (Yagasaki and Kato, 1987).

Several chemicals have been used at some time or other in adulteration and sophistication processes. Soft forms of lead and certain other metals have been employed as bean polishers, leaving a sheen on the coffee grain to make it attractive. Inedible fats and paraffins have been employed to leave a shine on the beans and even to brighten the chicory bits. Chicory itself was adulterated with venetian red (Wellman, 1961).

9.3.6 Detection of adulteration in instant or soluble coffee

This detection is of special importance, not only because such adulteration is in most cases economically exploiting the consumer but also because numerous coffee substitutes have been permitted, subject to correct labelling. The substitutes may be added before or after the extraction process, in some cases even after drying. Microscopy or other physical methods as an aid to detecting these adulterants are ruled out for obvious reasons. A collaborative trial on the use of insoluble matter content of instant coffee has been shown to be of limited importance as a statuatory procedure, although it would discriminate gross adulteration of samples by insoluble material (Reynolds *et al.*, 1983). Methods based on mineral (Ferreira *et al.*, 1971) or caffeine contents (Smith, 1981; Newman, 1981) are also of limited value due to the wide variability of pure coffee. Other methods based on the determination of monosaccharides have been developed. In pure soluble coffee, arabinose is indicated as the main free sugar (0.4–2.5%), followed by galactose (0.1–1.0%) and mannose (0.2–0.9%). Fructose and glucose are less important (0–0.5%). Xylose and ribose are found only in traces (Kroplien, 1974). Free fructose and glucose have been considered to measure declared values of chicory (Newman, 1981; Promayon *et al.*, 1976; Kazi, 1979; Bheema Rao *et al.*, 1986), barley (Lutman, 1982), figs (Kazi, 1979) and glucose syrups (Newman, 1981). Total xylose and mannitol (Davis *et al.*, 1990) have been recognized as good tracers for fraudulent addition of coffee husks and parchments, while a high maltose and total glucose content are indicative of addition of maltodextrins (Blanc *et al.*, 1989). The total fructose and glucose contents of three samples of unadulterated and those deliberately adulterated with various proportions of chicory, caramel, malted barley and cereal blend extracts are given in Table 9.3. Values of 0.8% for total fructose and 1.8% for total glucose have been derived as reasonable limits above which an instant coffee can be considered to be adulterated (Berger *et al.*, 1991).

Table 9.3 Total fructose and glucose contents of adulterated instant coffee (%)

Coffee	Fructose[a]	Glucose[b] robusta
Unadulterated	0.1	0.51
+5.0% Chicory	3.25	0.96
+5.0% Caramel	0.39	3.04
+5.0% Cereals	<0.1	4.45
+5.0% Malted barley	<0.1	4.39
+5.0% Cereals + chicory	0.70	2.86
+2.5% Cereals	<0.1	2.39
Blended		
Unadulterated	<0.1	0.64
+5.0% Chicory	3.24	1.00
+5.0% Caramel	0.46	2.84
+5.0% Cereals	<0.1	3.98
+5.0% Malted barley	<0.1	4.17
+5.0% Cereals + chicory	0.78	3.10
+2.5% Cereals	<0.1	2.18
Decaffeinated and blended		
Unadulterated	0.27	0.82
+5.0% Chicory	3.14	1.22
+5.0% Caramel	0.61	3.29
+5.0% Cereals	0.24	4.59
+5.0% Malted barley	0.25	4.08
+5.0% Cereals + chicory	0.96	3.09
+2.5% Cereals	0.25	2.83

Results are the mean values of two determinations:
[a] Determined after weak hydrolysis.
[b] Determined after strong hydrolysis.
Source: Berger *et al.*, 1991 (reproduced by permission).

9.4 Cocoa and cocoa products

Cocoa is the prepared seed of *Theobroma cacao*. The seeds are contained in a pulpy substance in a pod. Cocoa pods are of two basic types, *criollo* which is native to Spanish America and *forestaro* or foreign. The seeds are separated from the pulp by fermentation and dried in the sun or in ovens, after which they are roasted either batchwise or in a continuous process. The continuous process is preferred since there is less breakage and also minimum loss of valuable cocoa butter. Roasting is essential for full flavour development. The husks are then separated by cracking them in machines, while the shell and nib are ground together If the nibs alone are wanted the crushed beans are passed through a winnowing machine which separates the nibs. If no cocoa butter has been removed, the product is called cocoa mass (minimum 50% fat content on dry weight basis). Cocoa powder is made from partly defatted cocoa mass by breaking and pulverizing cocoa press cake obtained from the mass. It finds extensive use in the food industry for producing fillings, coatings, desserts, ice creams, cocoa and chocolate beverages, chocolate confections, etc. Cocoa butter is obtained exclusively by compression or mechanical extraction in a heated press from cocoa nibs or cocoa mass, and is collected as a pale yellow oil. Chocolate contains ground cocoa nibs with or

without sugar and vanilla or other flavouring. It can be divided into milk-free chocolates, milk chocolates, cream chocolates and white chocolates depending on the ingredients included therein. The processing steps consist essentially of mixing and kneading the raw materials, disintegration, prerefining to enclose sugar in cocoa mass or cocoa butter, tempering, final refining, plasticizing, storage and moulding.

9.4.1 Cocoa adulterants and contaminants

Studies have been conducted on methods for qualitative and quantitative determination of adulteration of cocoa powder such as with cocoa shell flour, hazelnut shell flour, carob flour or soy flour at 10–60% levels using concentrations of alkaloid, carbohydrate, protein, amino acid, crude fibre and ash as indices. HPLC determination of theobromine gives efficient detection of adulteration, while caffeine concentration is of little value. Fructose, sucrose and total carbohydrates could be used for detection but not for quantitative determination of carob powder in cocoa powder. Protein content is useful for soy flour. Glutamic acid and proline are valuable for detecting cocoa shell flour and soy flour in cocoa powder, while fibre is useful for detecting cocoa shell flour and hazelnut shell flour. Although ash content is of no use for quantitative estimation, along with pH it is useful for qualitative detection. Sensory analysis can detect 10% soy flour or cocoa shell flour, 20% carob flour or 30% hazelnut shell flour (Altug, 1987).

Modern cocoa processing leads to iron contamination, the origin being the grinding tools of the hammer and impact mills and, agitator blades and ball fillings of the rotating ball mills. This can, however, be easily separated by magnetic separators (List and Thiede, 1987).

Husk content in cocoa and chocolate products can be determined by hydrolysing the sample, extraction of the sample in several portions of chloroform followed by mixing with glycerol in a 1:1 ratio and then examination under a microscope having a special equipment for counting the spirals. Using regression equations, husk content can be calculated from the total number of spirals per 10 mg sample (Tapodo, 1973).

9.4.2 Assessment of degree of fermentation

The degree of fermentation of cocoa beans is important from the sensory angle, and governs taste and flavour. Fermentation of the pulp containing beans is carried out in heaps or in boxes when wild lactobacilli grow and degrade the pulp components. These are converted into several organic acids in the predominantly anaerobic conditions, acetic and lactic acid being the main acids. These compounds enter the nib during the subsequent drying step and may interact with the endosperm components. These products of bacterial metabolism are largely responsible for the characteristic acid, astringent taste of the nib. The interactions may be accentuated during roasting. During fermentation and drying the endogenous oxidative enzymes react with their

substrates causing typical colour changes mostly due to polyphenolics–polyphenol oxidase reactions, hence the degree of fermentation needs to be measured. Assessment of degree of fermentation can be done by dividing the beans into four categories: fully fermented, partly brown–partly purple, fully purple and slaty. Slaty beans are unfermented and are a defect of processing. The fully fermented beans category includes fermented beans even though the colour may not be completely brown. In the second category comprising partly purple and partly brown beans, the beans are manifest as a purple, blue or brown colour on the exposed surface, while fully purple beans include beans showing a completely blue, purple or violet colour. Overfermentation can be revealed by a dull dark appearance when cut. Such beans give an unpleasant smell arising due to breakdown of proteins and production of ammonia (Wood, 1975).

Fully purple beans are similar to with slaty beans with repect to their cheesy texture and should not be used in cocoa processing. Since it is not possible to prepare a sample with 100% fermented beans, partly brown and partly purple beans up to 20% are generally permitted with 30–40% being acceptable and above 50% being objectionable since it would give rise to bitter, astringent taste.

The purple colour is due to anthocyanins and its measurement could provide an index of fermentation of cocoa beans. This can be done by extracting the sample in ethyl alcohol and hydrochloric acid in the ratio 97:3, determining the spectral bands corresponding to catechins and anthocyanins in the extract and calculating the degree of fermentation against a reference value. The peak intensity can be measured by fluorescence with excitation wavelengths for the catechins and anthocyanins of 360 nm and 380 nm and emission wavelengths of 460–470 and 580–590 nm, respectively (Timoshkin *et al.*, 1990).

9.4.3 Quality of chocolate

The manufacturer's main criterion of quality of all types of chocolate is the flavour. Since flavour is essentially a subjective judgement, physical examination of the beans, especially their appearance when cut is of importance. The criteria used in the cut test are more readily expressed than flavour, and have therefore attracted more emphasis. The cut test can reveal mouldy beans, slaty beans and infested beans. Mouldy beans result due to attack by fungi such as *Botryodiplodia theobromae* and *Phytophthora palmivora*. Twenty eight fungal species have been identified as infesting cocoa beans, although only 13 of these occur frequently. The mould attack can result during fermentation, drying or even during storage, particularly under a humid atmosphere. Their presence causes a mouldy off-flavour which is unaffected by the manufacturing process. Slaty beans result from drying before the initial changes associated with fermentation take place. Such beans have none of the precursors of the chocolate flavour. Chocolate made from such beans has a bitter, astringent taste and an unpleasant flavour. Infested beans result due to attack by storage pests such as *Cadra*

cautella, *Araecerus fasciculatas*, *Lasioderma serricorne* and *Tribolium castaneum* (Wood, 1975). These pests pose a hazard to other foodstuffs and to the chocolate factory, where it is relatively easy to find conditions suitable for breeding.

Bean size affects the shell percentage, fat content and the initial roasting process. All these are in turn affected by distribution of rainfall during the period of development of the crop. The manufacturers prefer beans with lower shell percentage and higher fat content. Generally these parameters do not change above a bean weight of 1 g. An average bean weight of 1 g is therefore specified by the manufacturers.

9.4.4 Cocoa butter: quality criteria

Cocoa butter is an important raw material of commerce. Continuously operated expeller presses are used to obtain cocoa butter from whole cocoa beans or from low quality or impaired cocoa beans. Expeller fat is of inferior quality compared with that pressed from cocoa mass which has to be filtered to remove cloudiness.

9.4.4.1 Cocoa butter substitutes

The large demand for cocoa butter which far outstrips its limited availability has led to a search for substitutes and also to adulteration with many other fats. The characteristic fatty acid and triglyceride composition, melting behaviour, unique cooling/solidification properties, and the many other textural and sensory properties of cocoa-based products have posed challenges to oil technologists trying to find substitutes from vegetable fats available. (Meursing, 1992a, 1992b; 1993). Hazelnut oil, milk fat, coconut oil, palm oil, palm kernel oil, sal fat, tenkawang fat, haco fat, and fractions or formulations derived from these and named as choclin, coberine and kaobien are some of the substitutes used for cocoa butter. These can be readily detected on the basis of contents of indicative triglycerides, percentage unsaponifiables, sterols, methylsterols and terpenes (Homberg and Bielefeld, 1982), and fatty acid and sterol ratios (Kanematsu *et al.*, 1978). Shea fat, another adulterant of cocoa butter, can also be detected by TLC separation of methylsterols from the unsaponifiable fraction. The methyl sterols can be identified by UV fluorescence. The sensitivity of the method is 0.03 mg shea fat methyl sterols equivalent to about 1% shea fat in cocoa butter or 3% shea fat-based cocoa butter substitute (Fincke, 1975). Isooleic acid in cocoa butter can be used as an index of substitution by hydrogenated fats in chocolate products (Pelz, 1958). Gas chromatography (Fincke, 1970) of fatty acid methyl esters is recommended to detect adulteration of cocoa butter with as low as 5% of various fats, for example coconut oil (Woidich *et al.*, 1960). It is particularly sensitive when the foreign fat contains fatty acids absent in cocoa. GLC analysis of fatty acids and identification of caprylic, capric and lauric acids can indicate the presence of fats of palm kernel and 'babasu' (*Orbignya speciosa L.*) in chocolate (Badolato and Almeida, 1977). However GC as well as column chromatography is suitable only when the

adulterant is a fat of medium or short chain fatty acids, which are present in cocoa butter only in traces. Fatty acids of cocoa butter and its adulterant 'coberine fat' can be determined after separation by crystallisation into a liquid fraction using 1:10 acetone or 1:20 methanol and analysing for lauric acid in the liquid fraction. The values are <0.05% for cocoa butter, and 2.6–3.3% for all coberine samples (Bonar, 1965). Illipe butter samples, obtained from *Shorea macrophylla*, *S. mecistopteryx* and *S. singkawang*, alone and in combination with cocoa butter do have potential as replacers of cocoa butter, as demonstrated from studies of the fatty acid composition and solid fat content (Nesaretnam and Razak bin Modh Ali, 1992). As an adulterant of cocoa butter, Illipe butter can be distinguished by its higher oleodistearin and less oleodipalmitin compared to cocoa butter. The reverse is the case with coberine butter (Steiner and Bonar, 1965a). *Rhizopus arrhizuz* lipase catalysed interesterification of the mid-fraction of palm oil to a cocoa butter equivalent fat has been reported. Under optimal conditions, acyl exchange occurs mainly between the palmitoyl group from the palm oil mid-fraction and the stearoyl group from the reaction mixture, giving an interesterified product whose fatty acid composition is similar to cocoa butter (Mojovic *et al.*, 1993).

UV absorption of steroid-free unsaponifiable matter is also of value in identifying cocoa butter in admixed fats from inferior and shell-containing cocoa raw materials (Wurziger, 1962). Kokum fat, obtained from seeds of the kokum tree, *Garcinia indica*, and mango kernel fat have been reported to be used as adulterants. A TLC method has been developed to detect such admixtures. The results of TLC spots under UV light on pure samples of cocoa butter and kokum fat, and mixtures of 5%, 7% and 10% kokum fat in cocoa butter have shown blue/green fluorescence at an R_f value of 0.5–0.6, the intensity of which decreases with a lowering in the percentage of kokum fat. No spots are obtained with pure cocoa butter. The solvent system benzene:ethyl acetate:acetic acid in the ratio 96:4:1 has been found to be the most effective (Deotale *et al.*, 1990).

Amongst the physical methods, the IR spectrum is suitable for detection of partially hydrogenated extraneous fats (Luck *et al.*, 1961a, 1961b). Dielectric measurements have limited value since the constants for cocoa butter and adulterant fats differ by a very small margin (Luck *et al.*, 1961a). The measurement of increased plasticity with respect to that of pure cocoa butter from the solidity index, that is, the ratio of dilation change in the time unit, is useful for detecting adulteration with hydrogenated peanut oil (Janicek *et al.*, 1962). Low temperature crystallization of cocoa butter has also been reported to be useful in the detection of non-permissible foreign fats (Fincke, 1962). The melting point, cooling curves and viscosity have been pointed out as means of identifying adulteration of cocoa butter with other oils like sesame or olive (Finke, 1940). Melting curves of cocoa butter samples obtained by differential scanning calorimetry can be used to classify the confectionery fats with respect to blends and minor constituents (Cebula and Smith, 1992) and to predict the tempering behaviour of cocoa butter and whether it will cause processing difficulties during industrial

chocolate manufacture (Merken *et al.*, 1982).

Milk fat in milk chocolates can be calculated from the Reichert–Meissl value in the absence of coconut and/or palm oil, and by the butyric acid value in their presence (Hadorn and Jungkunz, 1950). The amount of milk fat can be calculated from the volume of 0.1 N sodium hydroxide required to neutralize the volatile fatty acids present in 5 g of the fat and from the amount of fat soluble in sulphuric acid saturated with potassium sulphate and caprylic acid (butyric acid number, I). Milk fat has an average butyric acid number of 20. Coconut oil as well as milk fat can be determined in mixtures between (I) and the 'rest number' (II). The rest number is obtained by determining the lower fatty acids present in 5 g of fat after precipitation of the higher fatty acids with magnesium sulphate and subtraction of the butyric acid number. The formula for percentage milk fat in total fat is $5.09 \times I - 0.12 \times II$; for coconut oil it is: $2.76 \times II - 2.07 \times I$ (Hadorn and Jungkunz, 1952; Schetty and Vaucher, 1953).

9.4.4.2 Processing quality of cocoa butter

Correct tempering of chocolate mass is important to provide the cocoa butter in the stable β-polymeric form (Vaeck, 1951, 1952, 1960). It involves cooling, heating and stirring. Large differences in the crystallization behaviour of pure unadulterated cocoa butter are well known in chocolate industry. Some batches crystallize with difficulty and need longer tempering. Distinction between 'normal' and 'difficult' cocoa butter has recently been shown to be possible by differential scanning calorimetry using a heating rate of 10 °C min^{-1} between 26 °C and 40 °C. Gas chromatography of the triglycerides does not distinguish between cocoa butters with different crystallization behaviour. A correlation between the industrial tempering behaviour and the relative contribution of the β-form to the melting enthalpy has been observed (Figure 9.1). It can be seen that the points corresponding to samples of poor tempering behaviour (points 8, 9, 10) lie to the left of a line, while normal cocoa butters correspond to points on the right of the line. This simple technique uses only a 3 mg sample of cocoa butter (Merken *et al.*, 1982). This method has been confirmed and is recommended as a means of routine quality control to measure the degree of tempering in precrystallized chocolate mass (Schuster-Salas and Ziegleder, 1992).

9.4.4.3 Geographical origin of cocoa butter

The size and shell percentage of cocoa beans varies with the geographical origin. For instance the shell percentage of beans from Ghana and Nigeria is about 11–12%, while beans from Trinidad and New Guinea contain about 15–16% shells. Fermentation techniques, flora and conditions vary traditionally in different regions. These differences are responsible for extensive variations in taste, flavour and aroma of the chocolate formed. On the basis of these aspects the beans from Ghana and Nigeria fetch higher prices than those from Brazil, Malaysia, Srilanka and India. Therefore

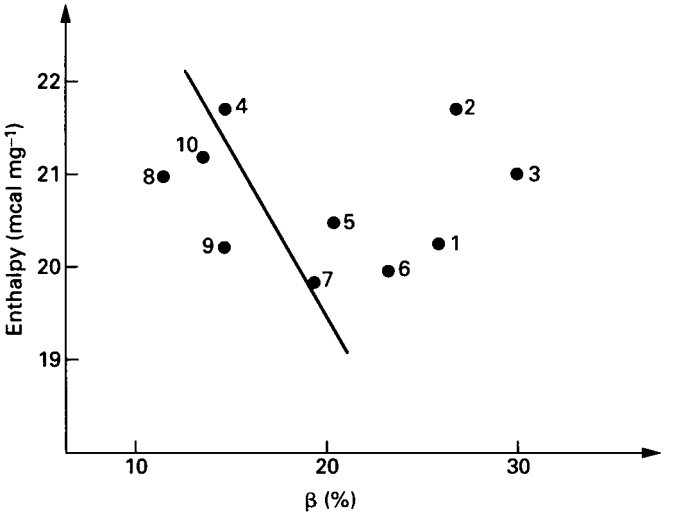


Figure 9.1 Relationship between total melting enthalpy and the percentage contribution of the b-form for 10 cocoa butters. (Source: Merken et al., 1982, reproduced with permission)

methods need to be evolved to identify the geographical origin of bean samples.

There are virtually no reports available in this area, presenting an exciting challenge to analytical food scientists. Volatile components in cocoa powder from Ghanian or Cuban cocoa beans are useful for correct classification using statistical techniques such as stepwise linear discriminant analysis (Pino *et al.*, 1992). The different compositions of the fatty fraction of cocoa beans together with discriminant analysis is useful for identifying samples from different regions (Hernandez *et al.*, 1991). A plot of PPO, where 'P' and 'O' represent palmitoyl and oleoyl residues in triglycerides, versus the mean number of double bonds in the triglycerides enables geographically similar cocoa bean samples to be grouped (Figure 9.2). This method could distinguish 28 samples of cocoa beans obtained from five main geographical areas. For instance, a Bahia sample which had significantly lower monounsaturated and higher diunsaturated triglyceride content, was separated distinctly from other areas of the plot (Podlaha *et al.*, 1984). Brazilian cocoa butters have been distinguished from African samples on the basis of diunsaturated glycerides, which are present in the former at twice the level of the latter (Steiner and Bonar, 1965b). The hardness of cocoa butter, as measured by the iodine value and content of POO and SOO, and the area under the polymorph II endotherm from differential scanning calorimetry (DSC) data can provide a new approach for determining the geographical origin of cocoa butter. For instance, South American cocoa butters which are the softest have an iodine value of 37.03, a total of 9.1% POO and SOO, and a 24.4% area under the polymorph II endotherm. In contrast, cocoa butters from Asia and Oceania have been found to be the hardest, and have an iodine

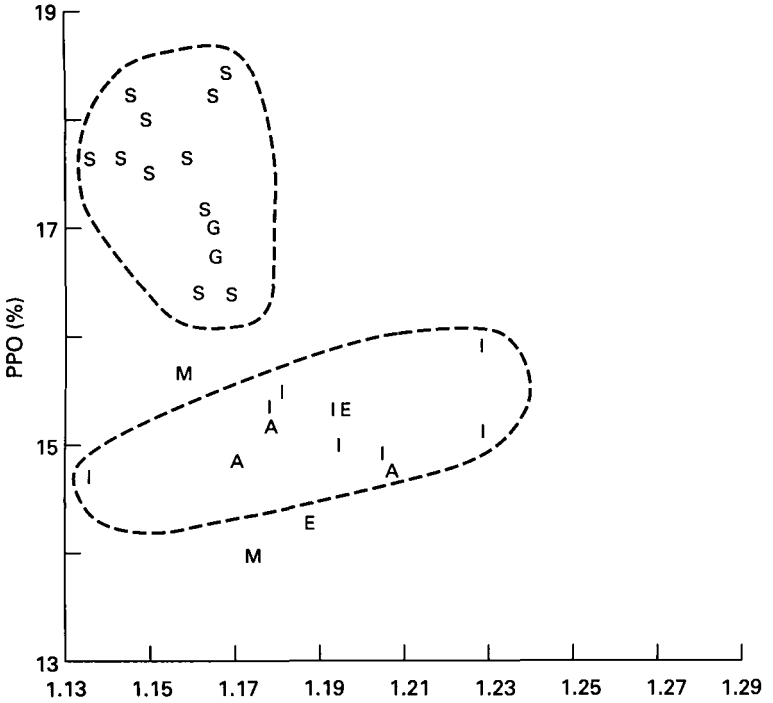


Figure 9.2 Plot of PPO content and mean numbers of double bonds (NDB) in cocoa butters. A = Africa, not Ivory Coast; E = Ecuador; G = Grenada; I = Ivory Coast; S = Samoa. (Source: Podlaha *et al.*, 1984, reproduced with permission)

value of 34.74, a total of 4.1% POO and SOO, and a 35.65% area under the polymorph II endotherm. North and Central American and African cocoa butters have intermediate hardness characteristics and intermediate values for POO and SOO contents and area under the polymorph II endotherms (Chaiseri and Dimick, 1989).

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Chapter 10

Indicators of Processing of Foods

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Chapter 10

Indicators of Processing of Foods

10.1 Introduction

Food commodities used in the manufacture of food products undergo varied types of handling, transport, storage, short time preservation and other treatments. This history of raw or semi-processed food commodities is of far reaching significance in determining the quality of food products. Is the milk used for the manufacture of milk products like cheese, pasteurized? Is the milk used for making ice cream, fresh liquid milk or reconstituted? Is the meat or fish used for the production of processed products fresh or frozen-stored? In a processing method for a food product how severe was the thermal treatment? Such questions often arise in the food industry where the detailed procedure may depend on the history of the raw material. The wholesomeness of the product often depends on the product history. Food scientists have, therefore, been trying to explore the changes taking place in the commodities during handling. Was storage under ambient conditions or chilled or frozen? Were thermal treatments such as pasteurization, blanching, sterilization applied? Was the commodity subject to irradiation or fumigation? Food scientists would like to correlate the changes with the quality parameters of the food products. Such studies have revealed important alterations in the chemistry of the products which can often be linked with the conditions of treatment. These can be used as markers or indicators of the treatment. Much information of this nature has been emerging from research investigations on the physicochemical, morphological, histological and microbiological changes occurring during processing. Such information has great potential in the development of novel approaches to the analysis of foods. In this chapter a survey has been undertaken of such indicators of processing that have been identified and the changes that have potential use as indices. Work on major food commodities has been included in the previous chapters.

10.2 Thermal processing

Thermal treatment of foods is one of the most common and important techniques used in the food industry and in culinary practices for the preservation of foods and for rendering the product acceptable to the consumer. The primary goal is to inactivate food spoilage microorganisms to obtain a commercially sterile food with an acceptable shelf life and retention of quality attributes to the maximum extent. Process

calculations based on the Z value (change in temperature (°C) required to effect a ten-fold change in decimal reduction time) of the target organisms, derived from the time–temperature data have been used in thermal processing (Lenz and Lund, 1977; Ball and Olson, 1957). These can be safely applied in the canning industry, but applications in continuous processing of particulate foods have been limited. The complex heating behaviour of foods (Pflug and Odlaug, 1978) makes it difficult to determine the time–temperature history curve and to identify the coldest point in moving particulate foods in rotating retorts and continuous systems. In case of heat sensitive food products such as tomato and apple juices, the holding or sterilization time is of vital importance. Methods based on ion selective electrodes have of late been satisfactorily developed (Nagy, 1981). Protein solubility of muscle extracts after heating in water or salt solutions has been used as a measure of heat denaturation of protein (Hamm and Deatherage, 1960). Water soluble components are differently insolubilized, as has been shown with beef, pork and chicken muscle (Caldironi and Bazan, 1980; Lee *et al.*, 1974). This change is a function of time of heating, end point temperature and initial quantities of soluble protein in muscle (Davis and Anderson, 1984). Similarly the ratio of creatine:creatinine in meat is indicative of the preceeding heat treatment during meat processing. The sum of creatinine and creatine, however, remains practically constant during such processing (Dvorak, 1961). Attempts have been made to correlate end-point temperature with internal cooked colour and expressible juice colour in ground beef patties but were found to be affected by maturity and fat content of the patties (Hague *et al.*, 1994).

A bioindicator can provide additional information and could be used as a tool to evaluate more accurately the impact of a thermal process on the food. The uniformity of the sterilization effect within the retort can best be monitored with the help of bioindicators.

10.2.1 Sterilization

10.2.1.1 Microorganisms as indicators of sterilization efficiency

Spores of Bacillus (Pflug *et al.*, 1980; Sastry *et al.*, 1988) or Clostridia (Brown *et al.*, 1984) have been used as biological indicators of wet heat sterilization. The use of immobilised yeast *Zygosaccharomyces bacilli* in a peach/alginate matrix at 60–65 °C, and of *C. butyricum* at 95–100 °C as bioindicators under conditions of continuous processing have been reported (Holdsworth, 1989). Variable results are obtained on correlation between theoretically calculated integrated lethalities from the time–temperature curve and that from the bioindicator.

The use of microorganisms as bioindicators has associated problems such as differences in the Z value of the target organism, time needed for analysis (2–10 days), which is too long for process control and the possibility of contamination in the food. A quick, non-harmful bioindicator with the same Z value as the target organism (Hayakawa, 1978) is obviously of interest.

10.2.1.2 Enzymes as indicators of sterilization efficiency

Peroxidase is a markedly heat stable enzyme and its thermal characteristics are well studied. It has been proposed as an indicator to evaluate thermal processes under conditions of pasteurization (60–85 °C) (Hendrickx *et al.*, 1992) as well as to evaluate processes for particulate foods (Weng *et al.*, 1991, 1992). The lethality values, F_{bio} obtained from this bioindicator coincide with the conventional method.

The need for an analytical method to determine the adequacy of thermal treatment of meat and poultry products, like patties, nuggets, meat balls, thin meat strips, loaves, etc. has been acutely felt. Residual enzyme activity after heat processing has been evaluated. For instance, a direct relationship between phosphatase activity and survival of the vegetative forms of microorganisms has been demonstrated (Avakyan, 1956). A method for the assesment of core temperature in canned hams based on residual acid phosphatase activity has been developed. Based on the earlier work by Kormendy and Gantner (1960, 1967), this method has been accepted by the United States Department of Agriculture (USDA) (Lind, 1987). The suitability of this method has been confirmed by several workers (Cohen, 1969; Finogerova *et al.*, 1973). However, there are two problems identified with the acid phosphatase assay. First, there is a need to increase the sensitivity and reproducibility of the assay and second, ways must be found to substitute the core temperature regulation of USDA with the correct heat treatment equivalent (F_c) concept which can take into account the integral heat treatment in the centre of the can (Kormendy *et al.*, 1992). Adequate blending of the sample, increased substrate concentration and working at the pH optimum are recommended to improve the sensitivity of the acid phosphatase assay. A rapid fluorometric analysis of acid phosphatase activity in cooked poultry meat that requires only 3 minutes has been proposed as a sensitive analytical method for monitoring end-point temperature (Davis and Townsend, 1994).

According to USDA regulations, a core temperature of at least 69 °C (156 °F) has to be reached during pasteurization of these products (USDA–APHIS, 1982). The rate of heating is known to profoundly influence the levels of residual enzyme activity. For instance, Kormendy *et al.* (1987) reported that canned hams heat processed at a fast rate to an end-point temperature of 69 °C could have higher levels of residual acid phosphatase than when heated slowly to the same end point. Similar results have been reported for heat processed pork samples (Townsend and Blankenship, 1987a).

A number of enzyme systems have been evaluated as potential indicators of effectiveness of thermal treatment in meat and poultry products (Pfeiffer *et al.*, 1969; Hamm, 1977; Davis *et al.*, 1988; Townsend and Blankenship, 1987b); many of them are available as biochemical test kits. One such kit is the combination transaminase (ALT/GPT and AST/GOT; ALT = alanine amino transferase, AST = aspartate amino transferase) test system. The abundance of transaminase in muscle tissue hasbeen extensively reported (Hamm *et al.*, 1969; Bogin and Sommer, 1976).

Table 10.1 Glutamic oxalacetic transaminase (GOT) activity and percentage loss in activity in ground beef as influenced by the rate of heating (0.35 °C min^{-1} and 3.5 °C min^{-1}), end-point temperature, dwell time and extraction medium

		Enzyme extraction medium					
End-point temp[a]	Dwell time[b]	Deionized water		0.9% NaCl		pH 7.0 buffer	
(°C)	(min)	(U/L)[c]	%Loss[d]	(U/L)	%Loss	(U/L)	% Loss
57.2	0	47.6(24.6)[e]	0.0(0.0)	50.4(27.7)	0.0(0.0)	44.3(61.9)	0.0(0.0)
71.1	0	30.2(16.8)	36.6 (31.7)	36.3(24.5)	28.0 (11.6)	1.2(52.8)	97.3 (14.7)
79.4	0	0.9(15.8) (6.0)	97.0	0.4(2.4) (90.0)	98.9	0.9(3.2)	99.9 (93.9)
57.2	30	53.2(39.4)	0.0(0.0)	54.7(69.3)	0.0(0.0)	37.9(38.7)	0.0(0.0)
71.1	30	17.9(15.2) (61.4)	66.4	9.7(43.8) (36.8)	82.3	9.9(33.6)	73.9 (13.2)
79.4	30	0.4(0.5) (96.7)	98.0	0.5(1.1) (97.5)	95.0	0.4(2.2)	96.0 (93.5)

Figures within parentheses are values for a heating rate of 3.5 °C min^{-1}.

[a]50 g of extra lean ground beef placed in glass tubes and heat processed in a water bath at rates of 0.35 C min^{-1} and 3.5 °C min^{-1} to end-point temperatures of 57.2 C, 71.1 °C and 79.4 °C.

[b]Dwell time of the beef sample at the desired end-point temperature.

[c]U/L = International units per litre.

[d]Percent loss of activity as end-point temperature increased from 57.2 °C to 71.1 °C to 79.4 °C, respectively.

[e]Each value is reported to be the mean of three determinations.

Source: Townsend and Davis, 1992 (reproduced with permission).

Mitochondrial glutamic oxalacetic transaminase enzyme (GOTm) and a sarcoplasmicisoenzyme (GOTs) in skeletal muscles of pigs and cattle have also been reported (Kormendy *et al.*, 1965; Hamm *et al.*, 1969). The GOT activity decreases only slightly during frozen storage at 0–4 °C. Although the heat stability of AST/GOT is established (Hamm, 1977), it has only recently been suggested as a means of determining end-point temperature in ground beef (Townsend and Davis, 1992) and processed poultry (Senter *et al.*, 1995). AST/GOT has been concluded to be the most suitable enzyme marker for determining the adequacy of heat treatment of turkey muscle. Table 10.1 gives the GOT activity and percentage loss in activity in ground beef as influenced by the rate of heating (0.35 °C min^{-1} and 3.5 °C min^{-1}), end-point temperature, dwell time and extraction medium. Table 10.2 gives similar information about the glutamic pyruvic transminase (GPT) activity in ground beef.

GOT assay could possibly be used for determining the adequacy of cooked beef which must be heat processed to 79.4 °C.

Lactate dehydrogenase activity has been measured in heated extracts of cooked bovine muscle tissue to evaluate the potential for developing a rapid accurate assay to verify cooking end-point temperature (Collins *et al.*, 1991a, 1991b; McCormick *et al.*, 1987; Stadler *et al.*, 1991) in beef. A sandwich enzyme-linked immunosorbent assay (ELISA) has also been developed to detect lactate dehydrogenase as a marker protein to verify end-point cooking of uncured poultry products. Results have suggested that

Table 10.2 Glutamic pyruvic transminase (GPT) activity in ground beef as influenced by rate of heating (slow and fast), end-point temperature, dwell time and extraction medium

End-point temp[a] (°C)	Heating rate[b]	Dwell time[c] (min)	Enzyme extraction medium		
			Deionized water (U/L)[d]	0.9% NaCl (U/L)	pH7.0 buffer (U/L)
57.2	slow	0	4.9[e]	5.8	3.2
71.1	slow	0	0.9	3.7	0.8
79.4	slow	0	0.9	0.4	2.2
57.2	slow	30	2.6	0.9	5.3
71.1	slow	30	0.7	0.7	1.0
79.4	slow	30	0.7	0.7	1.1
57.2	fast	0	3.4	2.7	3.7
71.1	fast	0	0.5	0.5	0.4
79.4	fast	0	0.9	0.2	1.3
57.2	fast	30	0.8	2.9	3.0
71.1	fast	30	0.6	0.5	0.9
79.4	fast	30	0.6	0.8	2.2

[a]50 g sample of extra lean ground beef placed in glass tubes and heat processed in a water bath to end-point temperatures of 57.2 °C, 71.1 °C and 79.4 °C, respectively.
[b]Slow heating rate was 0.35 °C min^{-1} and fast heating rate was 3.5 °C min^{-1}.
[c]Dwell time of the beef sample at the desired end-point temperature.
[d]U/L = International units per litre.
[e]each value is reported to be the mean of three determinations.
Source: Townsend and Davis, 1992 (reproduced with permission).

this technique should be applicable in determining whether turkey breast rolls are processed to the required USDA end-point temperature of 71.1 °C (Abouzied *et al.*, 1993). Processing treatments other than curing and heating show virtually no effect on lactate dehydrogenase activity. Ageing results in a marked increase in activity, while freeze–thawing lowers the activity. A major portion of the activity is lost on heating to 63 °C, and only marginal activity is detectable at 66 °C. Extractable protein decreases with increasing temperature. Pyruvate kinase (Davis *et al.*, 1988), malate dehydrogenase, adenylate kinase, isocitrate dehydrogenase, creatine kinase, aspartate aminotransferase, fructose-1,6-diphosphate aldolase, citrate synthase and glutamate oxaloacetate transaminase activities were monitored in cured ham muscle heated to 64.5 °C and 68.8 °C. No appreciable enzyme activity was detected in heated or cured samples. None of the enzymes appeared to be useful as indicators of end point temperature during processing of hams (Collins *et al.*, 1991b). A study on twenty-four enzymes in bovine, porcine and avian muscles has shown triosephosphate isomerase (TPI) to have a sharp inactivation point near the USDA regulated end point of 68.8 °C for imported pork and pork products, and could be a marker of heating end-point (Smith, 1992). Similarly, an improved method that enables catalase-related activities has been proposed as a biochemical marker for estimating cooking end-point temperature of chicken meat in the broad range of >68 °C and <72 °C (Ang *et al.*, 1994).

Immunochemical determination of degree of denaturation of sarcoplasmic proteins

such as lactate dehydrogenase M4, myoglobin, albumin, immunoglobulin G and transferrin has been shown to be a useful technique for assessment of the intensity of heat treatment to which the proteins have been exposed. Lactate dehydrogenase M4 has been reported to give the best results in the temperature range of 55–60 °C, whereas albumin gives good results in the temperature range 65–70 °C (Levieux *et al.*, 1990). The use of haemoglobin–myoglobin activity is a potential test for evaluating heat treatment of turkey breast meat (Bogin *et al.*, 1992).

10.2.1.3 Indicators of sterilization of milk

The quality and suitability of dried milk for end use is largely dependent on the severity of heat treatment it has undergone. Overprocessing brings about nutritive losses in milk, and generates a cooked/UHT flavour. It is therefore often necessary to ascertain the heat treatment undergone by the sample of milk powder. The number of free -SH groups are known to correlate with UHT flavour and could be used to assess heat treatment, however, they are dependent on the oxygen concentration and storage temperature (Andersson and Oste, 1992). Evaluation of colour components L^*, a^* and b^* (the tristimulus values on the CIE colour system, L for lightness, a for red or green and b for yellow or blueness) on milk samples subjected to direct and indirect UHT sterilization has shown heat treatment to cause colour changes, but the change is dependent on storage conditions and the fat content in the milk. This as well as the wide variability in colour changes has precluded its use as a means of distinguishing commercial samples of UHT and in-bottle sterilized milk (Rampilli and Andreini, 1992). Changes based on the whey proteins or disaccharide fractions in milk have been made the basis of assessing the severity of heat treatment (Fox, 1989).

Most of the methods developed for heat classification are based on direct measurements of the undenatured whey proteins. Under simulated high temperature short time (HTST) conditions (73 °C, 15 s), the percentages of denaturation in whey proteins are α-lactalbumin 20%, β-lactoglobulin 25% and bovine serum albumin 39%; while under simulated UHT conditions (135 °C, 6 s), the corresponding percentage values are 46%, 89% and 100%, respectively. Due to the parallel relationship between heat treatment and whey protein denaturation (Lucisano *et al.*, 1994a, 1994b), rocket immunoelectrophoresis could be used to evaluate the extent of heat treatment of fresh milk (Lo *et al.*, 1989).

When milk is heated to 80 °C or above, all the albumin becomes denatured and in the presence of inorganic salts or acids precipitates with the casein. Two well separated turbidity ranges can be distinguished. The first range covers values less than 50 nephelometric turbidity units (NTU) and the second covers the range greater than 75 NTU. A turbidity of 50 NTU can be considered as the lowest limit for measuring the quality of UHT treated milk (Moermans and Mottar, 1984). In the test proposed by Aschaffenburg for sterilized milk, the sample is shaken with ammonium sulphate,

Table 10.3 Effect of heat on milk constituents

	High heat			Medium heat		
	x_n ($n = 6$)	maximum (mg g^{-1})	minimum	x_n ($n = 6$)	maximum (mg g^{-1})	minimum
UMSPN[a]	1.32	1.50	1.20	2.34	3.10	1.60
UMSPN[b]	1.05	1.50	0.77	2.34	3.21	1.53
β-Lactoglobulin	0.19	0.31	0.11	0.57	0.88	0.22
Serum albumin	3.05	4.59	2.09	4.51	5.72	3.18
α-Lactalbumin	3.47	4.83	2.65	9.83	13.93	5.68
Galactose	1.17	1.42	0.89	1.10	1.43	0.99
Lactulose	0.38	0.44	0.30	0.15	0.20	0.09

[a]By turbidimetry.
[b]By PAGE (polyacrylamide gel electrophoresis).
Source: Olano *et al.*, 1989 (reproduced with permission).

filtered and the filtrate is then heated. Any albumin which is still present in solution due to insufficient heat treatment during manufacture will reveal itself as turbidity in the heated filtrate. The test is not prescribed for UHT milk which gives a slight turbidity (Pearson, 1976). In general, based on the turbidimetric determination of undenatured milk serum nitrogen (UMSPN), the dried milk could be classified as low heat (>6.0 mg UMSPN/g), medium heat (5.99–1.51 mg UMSPN/g) and high heat (<1.51 mg UMSPN/g). Other methods include measurement of the cysteine number (De Koning *et al.*, 1976; Mrowetz and Klostermeyer, 1977) and high performance liquid chromatogaphic (HPLC) determination of whey proteins (Kneifel and Ulberth, 1985; Reimerdes *et al.*, 1984). Table 10.3 shows the UMSPN and β-lactoglobulin, α-lactalbumin, galactose and lactulose content of dried milk samples.

Enzymes which appear in milk as a result of bacterial growth can degrade proteins, mainly k- and β-caseins (Fairbaim and Law, 1986). The inactivation of these enzymes depends not only on the heat treatment intensity but also on the number and types of bacteria present in milk before heat processing (Mottar, 1981). The regulation of limits for lactulose and undenatured whey proteins would contribute to avoiding overprocessing and could be an incentive for producers to improve the quality of their products. The detection of proteolysed caseins in heat-processed milk can be an indicator of the initial microbiological quality of milk. This has been confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophopesis (SDS–PAGE) analysis of casein fractions which allows the separation of intact casein and high molecular weight degradation products based upon their relative molecular mass. Although proteolysis of casein in UHT milk can be attributed to the action of native milk proteinases, mainly plasmin, and bacterial proteinases, the k-casein fraction is resistant to native proteinases (Grufferty and Fox, 1988). The presence of *para*-k-casein in UHT milk samples therefore indicates the presence of thermostable bacterial proteinases which are able to attack k-casein as well as β-casein (Fairbaim and Law, 1986). Thus, if it is suspected that UHT milk has been produced from raw milk of poor microbiological quality, SDS–PAGE analysis of the casein fraction can give an

indication of raw milk quality (Lopez-Fandino *et al.*, 1993).

Severe heat treatments of food proteins are known to induce extensive racemization of amino acids (Hayashi and Kemada, 1980; Friedman *et al.*, 1981; Bunjampamai *et al.*, 1982; Liardon and Hurrell, 1983; Tovar and Schwass, 1983). Attempts to quantify free and bound D-amino acids in milk as a function of range of heat treatments such as pasteurization, sterilization and UHT treatment failed to show any correlation (Gandolfi *et al.*, 1992). The formation of unnatural amino acids such as histidinoalanine and lysinoalanine in amounts proportional to the severity of heat treatment has been demonstrated (Henle *et al.*, 1993) and might be indicative of UHT treatment. This approach could be worth investigating.

Lactose undergoes two types of reactions during heating of milk; isomerization (Lobry de Bruyn-Alberda van Ekenstein rearrangement) and amino-sugar condensation (Maillard reaction). The aldose–ketose isomerization has been extensively studied, and the formation of lactulose (Adachi, 1958; Adachi and Patton, 1961; Martinez-Castro and Olano, 1980), galactose (Olano and Martinez-Castro, 1981; Calvo and Olano, 1989) and tagatose from galactose (Adachi, 1958; Richards, 1963) has been reported. Lactulose (Andrews, 1986; Geier and Klostermeyer, 1983; Martinez-Castro and Olano, 1980), epilactose and galactose have been used as indicators of the heat treatment conditions to which milk has been subjected. HPLC determination of lactulose has been reported (Mathur *et al.*, 1992). Tagatose is formed from galactose and its concentration in milk increases with the severity of heat treatment. The formation of lactulose, epilactose and galactose in heated milk proceeds according to the first order reaction kinetics (Olano and Calvo, 1989). The formation of tagatose takes place only under sterilization conditions (Troyano *et al.*, 1992). The amount of galactose increases during storage of dried milk samples (Richards, 1963). The Maillard reaction in which lactose reacts with protein-bound lysine to form protein-bound lactosyllysine (Berg and Boekel, 1994), its degradation product, formic acid, and its acid hydrolysed product, furosine as well as ratio of lactulose to furosine (Corzo *et al.*, 1994) (Bergaentzale *et al.*, 1994) have all been correlated to the extent of heat treatment in milk.

It has also been recognized that no single heat classification method is likely to be suitable for all purposes (ISO, 1985). Therefore it could be useful to take the determination of milk serum proteins as well as the formation of lactulose in dried milk into account.

The kinetics of the release of the glycosylated and carbohydrate-free forms of the caseinomacropeptide (CMP) from renneted raw and UHT milk has shown a 40% decrease in the glycosylated forms in UHT milk compared to raw milk (Ferron-Baumy *et al.*, 1992), and could probably be used to classify samples according to heat treatments. Similarly, the formation of lactosan, 4-*O*-β-D-galactopyranosyl-1,6-anhydro-β-D-glucopyranose, which is obtained from the pyrolysate of lactose subjected to heat treatment could be considered as an indicator of heat processing of lactose containing foods (Suyama *et al.*, 1987). Lactosan can be formed by three

possible routes. One route is dehydration of glucosyl residue in lactose, another is the liberation of the lactose residue as a lactosan from a polysaccharide formed by heating the lactose, and the third route is condensation of the levoglucosan with galactose. Lactosan can be easily polymerized by heat treatment, especially in the presence of an acid catalyst. Characterization of these polysaccharides could offer newer clues to the estimation of prior heat treatment of a milk-based product.

10.2.2 Indicators of pasteurization

The processing time and temperature to be employed for the pasteurization of milk are well known. Although it is accepted that efficient pasteurization makes milk safe, there is no assurance that the procedure has been properly implemented. With the development of the phosphatase test, a simple sensitive analytical method first became available (Kay and Graham, 1933), that could give fairly substantial assurance. Underpasteurization or recontamination with as little as 0.1–0.2% of raw milk or cream gives a positive test. The method devised by Aschaffenburg and Mullen (1949), using disodium *p*-nitrophenyl phosphate as substrate of phosphatase is now recognized as official. The possibility, however, of reactivation of phosphatase in milk or cream at high temperature has posed some concern (Johns, 1959). The Rutgers test, based on the release of free phenolphthalein from the substrate, phenolphthalein monophosphate, which gives a red colour gives an idea of residual alkaline phosphatase, and can differentiate between residual and reactivated phosphatase activity (Kleyn, 1977). The phosphatase test is also useful to check pasteurized cheese (Blasi, 1956) or butter (Olivo and Venturi, 1956; Francetic and Jemeric, 1957). Cheese made from raw milk, especially cheddar and its modifications, has been responsible for numerous outbreaks of enteric diseases. Regulations usually require that cheddar cheese to be sold within 60 days of production must be made from pasteurized milk. The Sanders and Sager method (1945) is a modification of the phosphatase test designed to overcome the strong buffering capacity of cheese and the presence of interfering substances. That of Kosikowski (1949) is another. While the phosphatase test may be applied to freshly churned butter to determine whether or not it has been made from pasteurized cream, stored butter samples have exhibited unsatisfactory results (Kay and Graham, 1933; Parfitt *et al.*, 1940). Flavours and colouring compounds in ice cream which possess phenolic rings may give false positive reactions. Citrus phosphatase activity is also useful as an index of juice pasteurization (Bernard, 1947). The half life of citrus phosphatase decreases rapidly with an increase in temperature (4 min at 147 °F (64 °C), pH 3.38). Increased pH decreases the sensitivity to heat. It may be a useful indicator for citrus juices held under chilled or frozen conditions.

Liquid egg may contain Salmonellae derived from a small proportion of infected eggs. Such organisms can be destroyed by pasteurization at 64.4–65.5 °C (148–150 °F) without coagulating the egg or impairing the baking qualities (Knight, 1963).

Shrimpton *et al.* (1962) showed that the reduction in activity of α-amylase by heat could be made the basis of a simple routine test for assessing the adequacy of heat treatment. For the test, the sample is incubated at 44 °C with starch. Liquid egg (pasteurization) regulations require that liquid egg be heated to 64.4 °C for at least 2.5 min and the cooled product should comply with the α-amylase test.

10.2.3 Indicators of blanching

Blanching is a step prior to processing of vegetables by canning, dehydration, freezing as it inactivates enzymes, reduces microbial load and also deaerates the material. Inadequately blanched vegetables develop 'off' flavours and colours during subsequent freezing, owing to continued enzyme activity. A disturbance in the normal glycolytic reactions is believed to be involved in the production of off-flavours in the tissues of underscalded vegetables during frozen storage (Joslyn, 1949; Joslyn and David, 1952). Arighi *et al.* (1936) observed acetaldehyde to be an index of such off-flavours. The product is of inferior quality when acetaldehyde exceeds a certain level (Gutterman *et al.*, 1951; Lovejoy, 1952). However acetaldehyde is not the cause of the off-flavour but appears to be a by product. Unlike in asparagus and peas, ethyl alcohol rather than acetaldehyde is believed to serve as an objective criterion of underscalding in frozen broccoli (Buck and Joslyn, 1953).

Peroxidase and catalase have been used as an index of adequate blanching in frozen vegetables (Joslyn, 1953). Residual lipoxygenase, another oxidative enzyme was shown to be linked to off-flavour of leguminous vegetables (Wagenknecht and Lee, 1958; Williams *et al.*, 1986). In contrast, residual peroxidase activity had little effect on the quality of frozen vegetables (Bottcher, 1975; Burnette, 1977). A recent comparative study of these two enzymes has demonstrated lipoxygenase to be more suitable as a blanching index than peroxidase (Sheu and Chen, 1991).

10.2.4 Indicators of parboiling of rice

Parboiling is a process in which the rice has been precooked in paddy form before drying. This treatment alters the product making quality, eating quality, nutritional quality as well as the hydration and cooking behaviour. A series of elegant tests that can differentiate not only parboiled rice from raw rice, but also parboiling of different severities have been developed on the basis of recognized physicochemical properties. Amongst the tests are protein solubility, grain colour, degree of grain expansion acheived on heating, ratio of water uptake at 60 °C to that at 96 °C which increases from about 5% for raw rice to about 50% for severely parboiled rice, the equilibrium moisture content attained by rice soaked in water at room temperature which increases from about 28% for raw rice to about 50% or more for severely parboiled rice, the ratio of dissolved amylose at 50 °C to that at 96 °C which increases from 2% for raw rice to 15% or more for severely parboiled rice, the degradation of the rice kernels in dilute

alkali which is one of the simplest and quickest tests, and viscosity of a cold water slurry and gel volume. All of these tests can distinguish parboiled rice from raw rice, but not the severity of the parboiling.

A practical test for cooked rice texture has been reported based on extrusion. The amount of cooked rice extruded through a screen bottom under a given pressure is inversely proportional to the hardness of the cooked rice and hence to the severity of the parboiling process.

A possible index to distinguish between parboiled rices produced by cold soaking (20–40 °C) and hot soaking (60–70 °C) is the discoloration produced in them upon reheating the milled rice at 100 °C. The discoloration is greater in the hot soaked method because of the greater amount of reducing sugars from enzymic action. However, soaking at a very high temperature (80 °C or higher) will inhibit the discoloration due to enzymes being inactivated (Bhattacharya and Ali, 1985).

10.2.5 Indicators of degree of roasting

Roasting has a determinant role in obtaining an organoleptic quality in foods such as cocoa. During this process, while the Maillard reactions occur, many chemical compounds which are responsible for flavour are formed. In fact, the principal aroma compounds are formed by interaction between reducing suars and amino acids (Vernin, 1980; Galois, 1984; Humbert and Sandra, 1988). These reactions produce particularly the alkylpyrazines, whose concentrations are variable depending on the time and temperature (Reineccius *et al.*, 1972). The possibility of using these substances as indicators of roasting has been tested successfully (Ziegleder, 1982). Mono-, di-, tri- and tetramethylpyrazine are known to occur in unroasted cocoa beans. During the roasting, the methylpyrazines increase though weakly; only tetramethylpyrazine reaches a maximum peak of concentration (Table 10.4). The ratio of tetra-2,5-dimethylpyrazine and tetra-/trimethyl pyrazine of 1.0 is recommended as the optimum degree of roast of cocoa (Chaveron *et al.*, 1989). Similar indices for the degree of roast for other food products are needed to aid in control of the industrial process.

In the case of coffee, the ratio of water soluble melanoidins and aromatic substances soluble in organic solvents has been proposed as an end point quality criterion, as judged by sensory analysis (Obretenov *et al.*, 1989). Isomers of quinic acid and quinides are generated during roasting. A quotient, the degree of isomerization, has been defined which closely relates to the colour and thus the degree of roasting of coffee (Scholz-Bottcher and Maier, 1992). Organic roasting volatiles (ORV) can be calculated with high precision using regression equations involving the determination of relative contents of both enantiomers of alanine, leucine, phenylalanine and glutamic acid. ORV is an indirect method of determination of the degree of roast, and is also independent of roasting temperature, roasting process duration, variety of coffee plant and steam pretreatment of green coffee (Nehring and Maier, 1992). Yet

Table 10.4 Evolution of methylpyrazine during industrial roasting of cocoa beans (mg kg^{-1} of cocoa)

Pyrazines	Roasting time (min)							
	0	5	10	15	20	25	30	32
Monomethyl	270	360	460	700	890	1150	1360	1590
2,5-Dimethyl	260	340	440	810	990	1450	1970	2450
2,6-Dimethyl	270	310	450	540	800	1220	1730	2050
2,3-Dimethyl	260	360	460	530	600	850	1090	1400
Trimethyl	870	1120	1310	1760	2120	3090	3320	3480
Tetramethyl	1770	2200	2890	3330	4300	3620	3200	2400

Source: Chaveron *et al.*, 1989 (reproduced with permission).

another indicator of degree of roast of coffee is the chemiluminescent determination of small amounts of hydrogen ion originating in extracted essence from roasted coffee beans. A linear relationship between chemiluminescence intensity and hydrogen ion concentration in coffee essence in the range of 6.8 μM–2.5 mM has been reported, and is readily applicable to roast process control and quality control of coffee beans (Ishii *et al.*, 1989).

Similar investigations need to be performed in foods subjected to extrusion cooking, baking, oil-frying, puffing and popping and other forms of heat treatment.

10.2.6 Chemical markers of heat processing

Monitoring chemical changes within the food, which involves compounds either indigenous to or added to the food, offers an alternative route for assessing the integrated time-temperature exposure of the food particulate. Pertinant data in the literature are scarce for thermal processing. The products which are formed in food reactions are comparatively more useful than the compounds which disappear as a result of processing. Recently three chemical compounds have been identified as markers of aseptic processing, and have been designated as M_1, M_2 and M_3. M_1, identified chemically as 2,3-dihydro-3,5-dihydroxy-6-methyl-(4*H*)-pyran-4-one, has been detected in heated broccoli, chicken meat, ham, other vegetables and fruits as well as in glazed samples processed aseptically. M_3 is identified as 5-hydroxymethylfurfural from aseptically processed juice drinks and from heated fruits and vegetables. M_2 is associated with protein and is observed primarily in heated meats. Fructose is believed to be the precursor of M_1 and M_3. These compounds can be rapidly and reproducibly determined through separation by anion exclusion chromatography and detection by UV spectrophotometry. These markers could be utilized to validate the thermal processing of a particulate food, and could work for a wide range of meats, fruits and vegetables and are ideally suited for the high temperature short time profiles associated with conventionally and ohmically heated aseptic processing and microwave heating (Kim and Taub, 1993).

10.2.7 Instrumental methods to monitor heat exposure

Instrumental methods such as near-IR reflectance spectroscopy (NIR) and near-IR transmittance spectroscopy (NIT) have been used to detect the maximum temperature of exposure of cooked beef (Ellekjaer and Isaksson, 1992). Application of the principal component analysis on NIR reflectance spectral data can permit discrimination between extrudates produced under moderate, intermediate or severe conditions of extrusion cooking. The study of protein quality through solubility experiments, PAGE, determination of available basic amino acids and brown colour measurement have shown that under moderate conditions of extrusion cooking, protein is only slightly affected and can be easily restored after solubilization in phosphate buffer containing a detergent and a reducer of disulphide bonds. However, under severe conditions, the extrudates exhibit a relatively intense dark brown colour and all protein fractions are implicated in non-disulphide covalent bonds and also undergo some macromolecular degradation. The spectral determination between 1100 nm and 2500 nm can be related to the processing parameters and also to the degree of transformation of these extrudates (Ben-Hdech *et al.*, 1993).

Monoclonal antibodies against β-conglycine from soya bean could be effectively used to probe thermally induced changes and markers to monitor heat exposure (Plumb *et al.*, 1995). Caution must be excercised before interpreting the data from electrophoretic patterns of proteins in processed foods. Frozen storage itself, prior to any kind of heat processing alters the electrophoretic pattern. This has been shown with fish mince where species and the season in which the fish was caught influence the electrophoretic pattern (Huidobro and Tejada, 1995). Although, both NIR and NIT under the current stage of development, are not suitable for regulatory purposes (Townsend and Blankenship, 1989), they show promise as a reliable screening method. A more accurate but time consuming method to determine the maximum cooking temperature of beef employed is differential scanning calorimetry (DSC) (Parson and Patterson, 1986), which has also been used to investigate the denaturation of meat proteins (Ledward, 1978; Stabursvik and Martens, 1980). The onset of denaturation, determined from the first slope of the thermogram and its intersection with the baseline has been used to detect the maximum cooking temperature to which the meat has been exposed. A positive correaltion between maximum cooking temperature and onset of denaturation has been found. However, the DSC thermograms are affected by the sample mass, pH and content of connective tissue (Stabursvik and Martens, 1980; Parson and Patterson, 1986; Findlay and Barbut, 1990). Figure 10.1 shows the correlation between cooking temperature used for beef and that computed from DSC data (Ellekjaer, 1992). A prediction error of 0.6 °C has been reported with 17 samples treated within the temperature range 50–72 °C when multiplicative signal correction (MSC) of the thermograms was done prior to calibration (Martens and Naes, 1989). The main improvement achieved by the use of MSC seems to be a better fit to a multivariate linear model and improved regularity by correcting for some of the

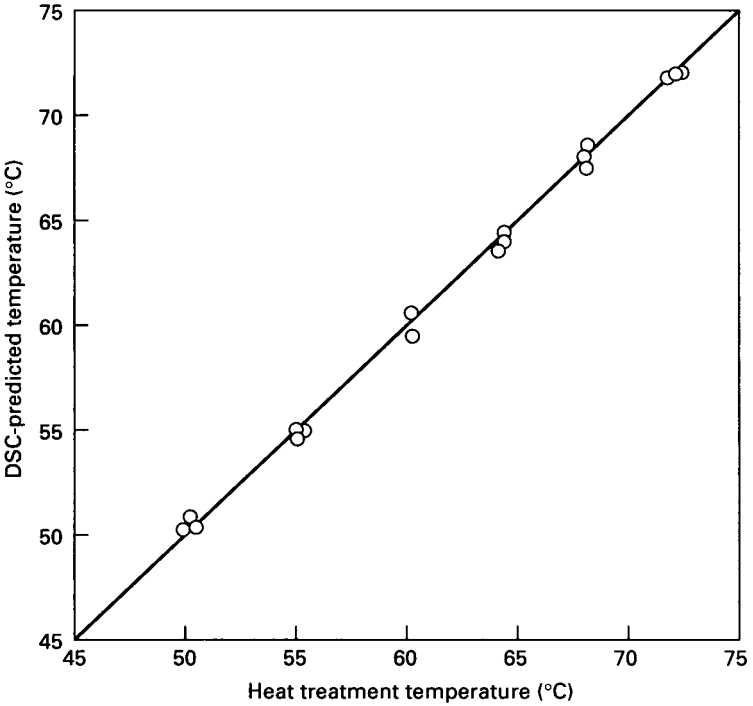


Figure 10.1 DSC determined maximum cooking temperatures of heat treated beef plotted against the actual laboratory heat treatments. A least square regression line is drawn and the correlation coefficient (R) is 0.999. Partial least squares (PLS) is on MSC dQ/dr (differential heat input) with six factors in the model. (Source: Ellekjaer, 1992, reproduced with permission)

discrepancies in the thermograms caused by natural variations in the random samples and incomplete baseline subtractions.

Meat colour is one of the major criteria by which commercial cooking procedures are evaluated. Conventional methods for the measurement of the colour of poultry meat include the reflectance spectrophotometry of relatively large, uniformly coloured samples and the absorbance spectrophotometry of extracted meat pigments (Francis and Clydesdale, 1975). Both these methods are difficult to apply to routine measurement or quality control. A very strong peak at 560 nm due to myoglobin (Swatland, 1982) is generally seen (Figure 10.2), which is markedly decreased by cooking treatments such as baking and drying. The decrease is evident at all wavelengths beyond 500 nm. It appears that fibre optic systems might be used to measure the colour and thereby to assess colour changes due to cooking (Swatland, 1983). Physical texture measurements using maximum shear/compression force have shown a good correlation with processing conditions for canning low-acid artichoke hearts (Rodrigo *et al.*, 1992).

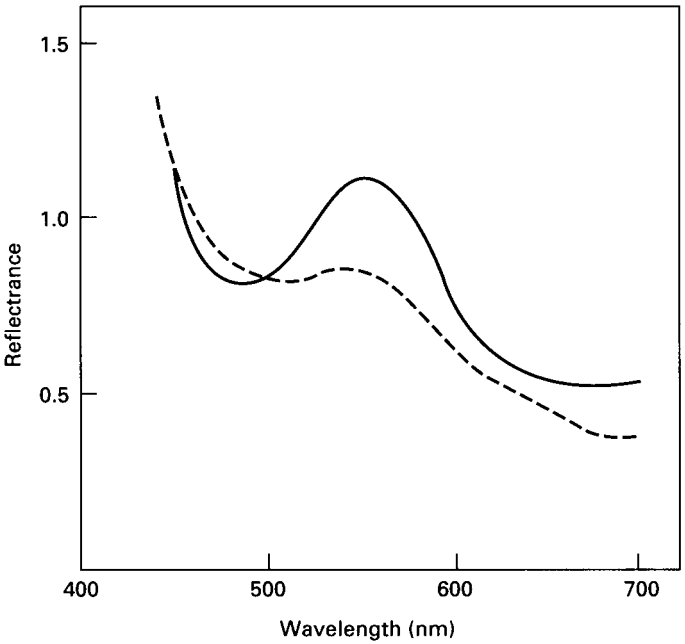


Figure 10.2 Effect of cooking on the colour of red poultry meat from the leg, –, raw meat; –, cooked meat. (Source: Swatland, 1983, reproduced with permission)

The electrical conductivity of meat during thermal treatment as an aid to quality control has been evaluated. However, this parameter is influenced by the temperature at the centre of the sample, the contents of sodium chloride, phosphates, calcium plus magnesium and fat (Zilaf and N'Oft, 1978).

10.3 Indicators of processing quality of beans

Damaged beans can be a problem because cracked seed coats allow the cotyledons to separate on soaking or canning. In the can, broken beans and exposed cotyledons will slough, allowing starch to dissolve into the liquid portion of the pack. A common procedure for characterizing canning quality of dry beans is to measure and compare water uptake (Morris *et al.*, 1950; Estevez and Luh, 1985). The damage that the beans sustain during handling and storage is revealed in the rate of water uptake during the first phase of soaking as the middle lamella dissolves and the seed coats are released. It would be valuable to have physical property measurements that identify bean lots suitable for canning, that is the ability to predict the amount of damage that beans will sustain during a canning process. Such measurements may also serve plant breeders in developing new bean varieties which have better processing qualities.

The extent to which beans can swell and adversely affect the integrity would be

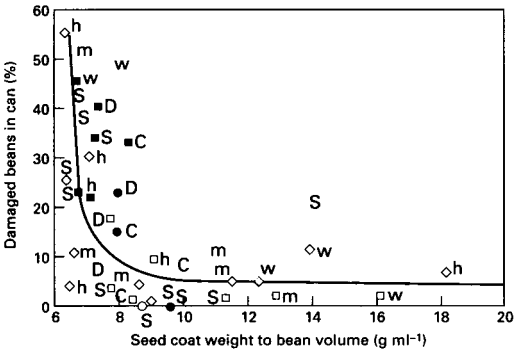


Figure 10.3 Relationship of the proportion of damaged beans in the can to the ratio of seed coat weight to bean volume. (Source: Heil *et al.*, 1992, reproduced with permission)

generally related to the compactness and contents (starch granules, etc.) of bean cells, and the inability of seed coats to accommodate the volume of a fully processed seed. Density, an estimate of compactness and composition, and the weight of skin per unit volume of the bean are, therefore, not surprisingly the two parameters related to canning quality. However, the proportion of damaged beans after thermal treatment in the can is a function of density. Figure 10.3 shows the relationship between the proportion of damaged beans in the can and the ratio of seed coat weight to bean volume; the proportion of damaged beans in the can after thermal processing increases dramatically when the ratio of seed coat weight to bean volume is less than 10 g ml^{-1}. This method is believed to be superior to the soak test method. Studies need to be carried out to explore the utility of this test with beans of other species, cultivars and regions (Heil *et al.*, 1992).

Fluorescence microscopy has been used to measure the intensity of fluorescent emanation from the cut surface of uncooked bean (*Phaseolus vulgaris*) cotyledons. This parameter is known to correlate significantly with the hardness of the soaked cooked beans, determined by compression force on whole beans or probe force on cotyledons. The relationship is independent of the cause of the hardness. A significant correlation has been found between fluorescence intensity of the isolated cell wall material and bean hardness. It is suggested that the increase in fluorescence intensity in hard beans may have been due to accumulation of phenolic compounds within the cell wall. This procedure has potential as a simple and quick way of determining bean hardness (Stanley and Plhak, 1989). The results of a recent study indicate that soaking the beans in acetate buffer (pH = 4.0, 37 °C, 4–7 h) might be useful for screening new bean varieties and selecting those prone to hardening defect, using either a modified Mattson bean cooker or a puncture force method (Reyes-Moreno *et al.*, 1994).

10.4 Fresh versus frozen–thawed foods

The acetaldehyde content of various frozen vegetables shows promise of being a measure of the off-flavour associated with underblanching. It has been claimed that in the same package one pea or one spear of asparagus is likely to differ from another in enzyme activity and consequently in the acetaldehyde content that eventually develops. A stricter control of the industrial blanching operation is thus essential. Differentiation between fresh and frozen–thawed products such as vegetables, fish and meat is often desirable. In the case of meat, methods based on amino acids released during the autolytic process during and after defrosting (Massi, 1958) or the release of mitochondrial enzymes into the sarcoplasm, have been proposed in the literature (Hamm, 1979; Gottesmann and Hamm, 1983, 1984; Chen *et al.*, 1988). The process of freezing and thawing of animal tissues leads to the disruption of cellular organelles (Karvinen *et al.*, 1982) like mitochondria and lysosomes, causing release of enzymes bound to these structures. Mitochondrial enzymes such as cytochrome c oxidase (Voet and Voet, 1990; Barbagli and Crescenzi, 1981), glutamate aspartate aminotransferase (Salfi *et al.*, 1985a; Salfi *et al.*, 1986), succinate dehydrogenase (Frigerio *et al.*, 1980), lysosomal enzymes such as aryl sulphatase, β-glucuronidase, acid phosphatase and acid proteinase (Goldemberg *et al.*, 1987) as well as α-glucosidase (Rehbein *et al.*, 1978; Cattaneo *et al.*, 1982) and β-*N*-acetylglucosaminidase have been used to differentiate fresh and frozen–thawed fish types. This is reflected in an increased enzyme activity in the tissue.

The requirements of specific techniques or long processing times makes many assays difficult. An assay of β-hydroxyacyl-Co A-dehydrogenase (HADH) by a colorimetric method has been reported (Hamm and Gottesmann, 1982; Gottessmann and Hamm, 1983); however, the main problem is the reliability of the test when the meat is frozen at −12 °C or below. This enzyme has recently been shown to differentiate unfrozen and frozen crawfish (*Procambarus clarkii*), trout (*Salmo gairdneri*), kuruma prawns (*Penaeus japonicus*) (Hoz *et al.*, 1993) and frogs (*Rana esculenta*) legs (Pavlov *et al.*, 1994). For instance the mean values of activity were found to be 7.9 and 78.9 units and 12 and 70 units for unfrozen and thawed trout and crawfish, respectively (Hoz *et al.*, 1992). An 'Apizym' system (Api system, Montalieu-Vercieu, France), which can measure 19 enzymes simultaneously in a semi-quantitative manner has been tested for differentiation between fresh and frozen–thawed pork (Toldra *et al.*, 1991). Only three enzymes, esterase–lipase, β-glucuronidase and α-glucosidase show significant differences between fresh and frozen–thawed pork. This system constitutes an easy method to differentiate between fresh and frozen–thawed meat for a wide range of freezing temperatures, −10 °C to −60 °C.

Fish muscle enzyme assay has been employed to differentiate fresh fish and frozen–thawed fish (Salfi *et al.*, 1985b). β-*N*-Acetylglucosaminidase (Kitamikado *et al.*, 1990; Yuan *et al.*, 1988) and acid proteinase activity (Prathapchandra *et al.*, 1988)

have been proposed as indices. Glutamic oxalacetic transaminase is another suitable enzyme to detect whether the ground beef (Visacki and Rudja, 1968) has been frozen (Stalder, 1969). The enzyme, β-*N*-acetylglucosaminidase is found in fish red blood cells in the cytosol, is inactive in intact cells, but becomes activated when the cells are disrupted by freezing and thawing. The analysis of this enzyme can be done by a simple fluorimetric method. Samples from unfrozen fish appear red, while samples from frozen–thawed fish appear blue. A rapid paper test has been developed based on this principle (Kitamikado *et al.*, 1990). Both the routine and test paper methods are applicable for testing most common edible fish (Kitamikado *et al.*, 1988). For fresh and chilled fish kept for 1–3 days at 4 °C, the blood shows little fluorescence, while for frozen fish stored at -20 °C to -40 °C for 1 day or longer, the blood shows a strong fluorescence. The method is applicable to most commercial species, both fresh and marine water.

Marine and fresh water fish are likely to be different in their enzymatic activities. Recently ATPase and lactate dehydrogenase have been proposed as indices of cold storage deterioration in a variety of fresh water and brackish water fish. A highly significant negative correlation between enzyme activity and frozen storage period has been obtained with mullet, pearlspot, milk fish and tilapia. Significant linear correlations have been obtained between the decrease in enzyme activities and other biochemical indices and sensory scores. In general, loss of activities of ATPase and lactate dehydrogenase in muscle is closely related to early changes in quality of frozen stored fish prior to bacterial spoilage (Nambudiri and Gopakumar, 1992).

Physical methods such as measurement of electrical resistance using a fish tester, examination of the opacity of the eye lens (Ciani and Salerni, 1965) and of the erythrocytes have all been studied and reviewed (Rehbein, 1992) to distinguish between fresh and thawed fish–meat products. A non-destructive method, Magnetic Resonance Imaging (MRI) which can investigate histochemistry and structure of plant materials has shown promise in distinguishing between blueberries before and after freeze–thaw processing. Freeze–thaw results in the rupture of water retaining membranes within discrete locations. This causes a change in the ratio of motion modified water (i.e. hydrogen bonded or chemically exchanged) to unmodified (i.e. mobile and not chemically exchanged) in those regions, as well as a concomitant change in sugar concentration, due to diffusion to other tissues. These changes can be mapped by MRI (Gamble, 1994).

10.5 Indicators of storage quality of foods

The quality of foods and food ingredients that is optimized at the processing plant, should ideally extend to the point of storage. However, the control of the manufacturer over temperature is usually minimal once the product gets into the distribution network. During distribution, the storage temperature conditions are often less than ideal and abuses do occur. The reaction rates leading to quality loss are strongly

dependent on temperature, even when other factors such as moisture gain are controlled. Without knowledge of the temperature conditions, estimation of the shelf life is difficult and open date labelling (such as use by date) is often meaningless. An effective system of monitoring the temperature conditions of individual products throughout distribution and indicating their remaining shelf life would be useful. Time–temperature indicators (TTI) are such a system. Excellent reviews on this topic have been recently published (Hendrick *et al.*, 1995 Van Loey *et al.*, 1996).

A TTI is a device that can show an easily measurable, time–temperature dependent change that reflects the temperature history of a food product. It can be used to help manufacturers improve the delivery of perishables and ensure that the product is perfect at the point-of-purchase and point-of-use (Farquhar, 1983; Fields, 1990). TTI operation is based on mechanical, chemical, enzymatic or microbiological systems that change irreversibly from the time of their activation. The rate of change is temperature dependent. The change is usually expressed as a visible response in the form of mechanical deformation, colour development or colour movement. A variety of TTIs based on different physicochemical principles have been described (Byrne,1976; Singh and Wells, 1986). The quality of a number of perishable refrigerated and frozen products has been statistically correlated to TTI (Tinker *et al.*, 1985; Chen and Zall, 1987; Wells and Singh, 1988; Taoukis *et al.*, 1991).

Examples of TTI are time–temperature dependent diffusion of a dyed fatty ester along a porous wick (Manske, 1983), colour change caused by a pH decrease due to controlled enzymatic hydrolysis (Blixt, 1983) of a lipid substrate (Blixt and Tiru, 1977; Yoon *et al.*, 1994) or urea (Jalinski, 1993), and a TTI based on solid state polymerization of a thinly coated colourless acetylinic monomer that changes to a highly coloured polymer (Fields, 1985; Zall, 1986). The colour change of an indicator based on the enzyme substrate (Mistry and Kosikowski, 1983) and corrosion and liquid crystallization (Selman, 1991), which in turn depend on time–temperature, are other examples. Catalase activity has been used as an indicator of grain drying efficiency (Zdzsilaw and Irena, 1958).

A device indicating temperature abuse during storage of foods and other perishables at temperatures >0 °C has been based on a sealed capsule containing freeze-dried lactic acid bacteria embedded in a non-toxic matrix melting at the desired temperature, and a suitable sugar-containing culture medium containing a pH indicator. If the device reaches the specified temperature, the matrix melts and the lactic acid bacteria are releasedand, in turn, ferment the sugars in the medium resulting in acidification which may be detected via a colour change of the indicator (Fanni and Ramet, 1992). A correlation scheme for *Pseudomonas fragi* growth has been developed with the TTI response, and has applications for dairy products (Bin *et al.*, 1991). Growth of microorganisms recovered at 18 °C and 42 °C from ready-to-cook whole broiler carcasses have been made the basis for detecting temperature abuse (Russell *et al.*, 1992). Yet another indicator for refrigerated or frozen foods is based on a sealed outer container, inside which an ampoule containing a coloured liquid and an absorbent

material are placed. The seal of the ampoule is broken by thermal expansion of the liquid and the absorbent material becomes coloured by the released liquid (Berrebi and Toporenko, 1992a). A clearly visible indicator which could fall due to gravity under temperature abuse is also reported (Micault, 1992). A modification of this system consists of a set of tubes containg coloured liquids such as frozen brine (Bertrand, 1992) at different freezing points. The liquid in the tubes is frozen so that when the frozen liquid in the appropriate tube melts, there will be a visible change in the position of the liquid in that tube (Loustaunau, 1991). Brightly coloured fluorescent material with selected freezing points could also be used for such systems (Berrebi and Toporenko, 1992b). Another device is constructed with an activator tape, an indicating tape and an optional barrier matrix between the two which is a pressure sensitive adhesive. The activating composition, for example, an organic acid such as citric acid, diffuses through the barrier and/or indicating matrix to make contact with the indicating composition continuously, for example, an acid–base dye indicator, and to produce a visible colour change at the temperature being monitored. The colour intensifies with time and temperature (Patel, 1991a, 1991b). Quality loss in sweetened products due to aspartame degradation, degradation of limonene in natural lemon flavour and formation of epoxide in encapsulated orange peel oil, all serve as TTIs of the respective products in which they are used (Taoukis *et al.*, 1989).

In some cases, changes in the chemical constituents can reveal the storage time. Thus the ratio of several pairs of compounds in the headspace such as thiophene:butanedione and 2-methylfuran:butanedione increases with storage time of ground roasted coffee packed in impermeable bags. The changes in these ratios can be modelled to predict the storage time.

10.6 Indicators of irradiation of foods

Irradiation has now been accepted as a treatment of foods for sprouting inhibition in tubers, delayed ripening of fruit, insect disinfestation and shelf life extension, which are all applications of low dose irradiation. The report of the joint FAO/WHO/IAEA Expert Committee (1981) on wholesomeness of irradiated food concluded that there was no toxicological hazard associated with food irradiated up to a dose of 10 kGy. Concern has however been expressed by several consumer organizations (Report (Australian Government), 1988) that there is no reliable method for detection of overirradiated food.

The changes occurring in irradiated foods are small and often are identical to those produced by other treatments such as heating. Although it has proved difficult to devise a method to detect irradiated food exclusively, considerable progress has been made and a number of approaches based on physical, chemical, microbiological and biological changes have been attempted (Delincee and Ehlermann, 1989; Grootveld and Jain, 1989; Delincee *et al.* , 1988) and reviewed (Glidewell *et al.*, 1993).

The properties which are likely to demonstrate linearity with the radiation dose

include the physical properties: electrical impedance, viscosity, wetability; chemical properties including proteins, lipids, carbohydrates, nucleic acids, vitamins and volatiles; changes in histological or morphological characteristics; and changes in microflora and formation of free radicals visualized by chemiluminescence, thermoluminescence or by electron spin resonance (Delincee and Ehlermann, 1989).

There is an interest in the use of rapid screening methods that give a preliminary indication that a food material has undergone irradiation. The suspicion can then be confirmed by more definitive methods. Amongst the techniques evaluated are analysis of DNA damage which would indicate exposure to ionizing radiation; detection of radiation induced pigment changes in fruit; methods based on protein changes in the food; determination of changes in enzyme activities such as ethylene forming enzyme, phenylalanine ammonia lyase, pectin methyl esterase and peroxidase; analysis of volatile components in vegetables; and observations of changes in microbial flora (Mitchell, 1990).

10.6.1 Changes in histological/morphological characteristics

Plant and animal tissues are composed of cells which have many essential features likely to be affected by ionizing radiation. Inability to develop the sprout is an index of such an effect, but the effect of ionizing radiation is mostly irreversible. Recent studies on the tissues of bud eyes by transmission electron microscopy have revealed rupture of the nuclear envelope and mitochondrial membrane in irradiated but not in unirradiated potatoes. Chromosomal aberrations in root tip cells have been described for irradiated wheat and rice (Atsumi and Matano, 1973). Cytological abnormalities in the cells of primary roots of germinating seeds have been observed in irradiated strawberries. Recent results have indicated that changes in germinated seeds could be used for identification purposes (Kawamura *et al.*, 1989).

It has been suggested that morphological indices such as the behaviour of spores could indicate a radiation treatment in mushrooms, since in irradiated mushrooms the spores differ in number and refractive index.

10.6.2 Changes in physical properties

Radiation-induced chemical changes may be manifested by a change in physical properties such as damage to the cell membrane and its associated effects, for example membrane transport of ions and hence a change in electrical current. Electrical impedence and conductivity might thus serve for integral characterization of unirradiated and irradiated tissues and cells. This has been shown for potatoes (Hayashi, 1988) and fish (Ehlermann, 1972).

The penetration of solvents depends on cell permeability and is apparent as viscosity of homogenates and suspensions of comminuted biological materials such as sea foods (Rhee, 1969), spices and condiments (Mohr and Wichmann, 1985; Farkas *et*

al., 1987; Farkas *et al.*, 1988; Heide *et al.*, 1988; Heide and Bogl, 1988) and onion powder (Kominato and Nishimi, 1988).

10.6.3 Changes in microflora

Since irradiation is used in food processing to reduce or eliminate microorganisms, detection of changes in the microbial population and the loss of known radiation sensitive types could indicate that irradiation has taken place. It has been possible using both an aerobic plate count (APC) and a direct epifluorescent technique (DEFT) to devise a method that gives an indication of whether a food has been irradiated. This approach measures the levels of both viable and non-viable organisms. In spices, a DEFT count that is 4 log units higher than the APC count indicates that a sample has been irradiated (Sjoberg *et al.*, 1991). In a collaborative study of spices and herbs average values of the differences between DEFT and APC in samples irradiated with 5 kGy and 10 kGy were found to be 5.1 and 6.1 logarithmic units, respectively (Wirtanen *et al.*, 1993). Since other techniques such as ethylene oxide treatment or heat give similar results, the microbiological techniques are not specific and the results need to be confirmed by another technique such as thermoluminescence. A similar approach has been investigated in irradiated chicken; the amount of endotoxin measured by the limulus amoebocyte lysate (LAL) test is compared with the number of gram-negative bacteria present (McWeeney *et al.*, 1990).

Results from different sea foods tested indicate that irradiation at doses of 1 kGy, 3 kGy and 5 kGy destroys Pseudomonas spp. present. Moraxella spp. seem to be radiation resistant even up to 5 kGy, and could therefore be used diagnostically to determine whether sea foods have been irradiated or not (Mitchell, 1990). This method, however, requires a lengthy incubation period of 5–6 days (Van Sprekkens and Toepoel, 1978).

A method proposed for identifying irradiated fish based on the production of total volatile acids (TVA) and total volatile basic nitrogen (TVBN) by bacteria in irradiated and unirradiated fish is quite simple and rapid (Alur *et al.*, 1991). Study on the growth of a few species of bacteria in five sea food varieties irradiated and unirradiated (0–5 kGy), revealed that the growth of bacteria is not different in irradiated fish, however, the formation of volatile TVA and TVBN is drastically reduced by 40–50% in irradiated fish (Alur *et al.*, 1991). This observation suggested a means of distinguishing irradiated from unirradiated fish. Table 10.5 shows the formation of TVA and TVBN in control and irradiated meats seeded with *Aeromonas hydrophila* immediately after irradiation, after storage at 0–3 °C for 7 days and storage at −11 °C for 15 days.

It has been observed that invariably the bacterium produced 50–60% less TVA and TVBN in irradiated meats irrespective of the radiation dose applied and the type of meat, in comparison to non-irradiated samples. Incubation of the chicken meat at 37 °C for 6–7 h with bacteria followed by estimation of volatile acids and bases suggests a possible method for detecting irradiated samples (Alur *et al.*, 1992).

Table 10.5 Formation of TVA and TVBN in non-irradiated and irradiated meats seeded with *A.hydrophila* immediately after irradiation, after storage at 0–3 °C for 7 days and storage at −11 °C for 15 days

Meats	Radiation dose (kGy)	TVA number	TVBN (mg %)
Beef	Control	210 ± 27.30[a]	147.30 ± 26.57[a]
		264 ± 35.42[a,c]	264.40 ± 33.21[a,c]
		280 ± 31.21[a,d]	162.40 ± 16.24[a,d]
	1	68.5 ± 9.25[b]	85.47 ± 18.14[b]
		100 ± 11.23[b,c]	90.51 ± 12.64[b,c]
		112 ± 12.56[b,d]	30.2 ± 4.05[b,d]
	3	74.66 ± 9.70[b]	53.20 ± 12.65[b]
		72.00 ± 7.43[b,c]	53.22 ± 11.29[b,c]
		88 ± 8.23[b]	33.6 ± 3.56[b,d]
	5	66.66 ± 6.5[b]	53.20 ± 8.47b
		72.00 ± 6.58[b,c]	53.10 ± 12.53[b,c]
		72 ± 7.44[b,d]	28 ± 2.95[b,d]
Chicken	Control	177.6 ± 25.61[a]	175.3 ± 33.6[a]
		204.0 ± 25.52[a,c]	288.4 ± 61.1[a,c]
		240 ± 25.09[a,d]	145.6 ± 15.0[a,d]
	1	65.6 ± 20.78[b]	74.66 ± 12.6[b]
		80.0 ± 9.53[b,c]	84.75 ± 11.6[b,c]
		120 ± 12.13[b,d]	28.0 ± 2.25[b,d]
	3	54.6 ± 21.71[b]	48.53 ±23.13[b]
		40.0 ± 5.96[b,c]	48.74 ± 21.36[b,c]
		72 ± 7.54[b,d]	22.4 ± 2.22[b,d]
	5	49.3 ± 19.2[b]	48.3 ± 24.1[b]
		32.0 ± 4.67[b,c]	48.5 ± 23.1[b,c]
		88 ± 9.83[b,d]	28 ± 2.52[b,d]
Mutton	Control	215.0 ± 28.0[a]	216.6 ± 44.5[a]
		372 ± 40.3[b,c]	304.6 ± 52.3[b,c]
		288 ± 31.2[b,d]	184.8 ± 20.5[b,d]
	1	78 ± 23.9[b]	124 ± 27.7[b]
		126 ± 11.2[b,c]	121.4 ± 31.4[b,c]
		162 ± 17.3[b,d]	44.8 ± 5.4[b,d]
	3	68.0 ± 11[b]	94.2 ± 32.5[b]
		76.0 ± 8.43[b,c]	97.6 ± 33.4[b,c]
		70 ± 7.15[b,d]	28.0 ± 3.8[b,d]
	5	53.3 ± 20.6[b]	84.0 ± 23.3[b]
		40.0 ± 4.2[b,c]	80.2 ± 27.2[b,c]
		70 ± 6.4[b,d]	44.8 ± 3.54[b,d]
Pork	Control	232.6 ± 29.3[a]	269.6 ± 55.3[a]
		288.0 ± 30.1[a,c]	232.5 ± 61.3[a,c]
		320 ± 30.4[a,d]	168.0 ± 17.1[a,d]
	1	74.0 ± 16.7[b]	48.5 ± 13.6[b]
		86.0 ± 9.23[b,c]	55.3 ± 14.3[b,c]
		168 ± 17.3[b,d]	16.8 ± 2.13[b,d]
	3	52.0 ± 12.1[b]	33.6 ± 14.2[b]
		48.0 ± 5.84[b,c]	35.2 ± 11.2[b,c]
		166 ± 10.0[b,d]	22.4 ± 2.52[b,d]
	5	41.3 ± 19.9[a]	30.8 ± 9,68[a]
		40.00 ± 4.48[b,c]	30.7 ± 10.3[b,c]
		88 ± 9.75[b,d]	22.4 ± 2.74[b,d]

Table 10.5 (cont.)
[a,b]Averages of six individual experiments. Values in same columns for each sample of meat not followed by the same letter are not significantly different ($P < 0.01$).
[c]Storage at 0–3 °C for 7 days.
[d]Storage at -11 °C for 15 days.
Source: Alur *et al.*, 1992 (reproduced with permission).

10.6.4 Changes in protein constituents

10.6.4.1 Electrophoretic methods

A study on 8 week old chicken carcasses (washed, eviscerated and packaged in polyethylene bags) held for 6 h at 2 °C, irradiated with doses of 0 (control), 6 kGy, 10 kGy and 20 kGy and then stored at 4±1 °C showed differences in the protein bands resolved by SDS–PAGE electrophoresis. Irradiation produces changes in the electrophoretic patterns of the pectoralis major muscles including disappearance of some bands, weakening of others, induction of new bands and changes in the molecular weight. The extent of these changes is correlated to the irradiation dose. Changes also occurred in the electrophoretic patterns during storage, the rate of protein breakdown decreasing with increasing radiation dose. It has been concluded that SDS–PAGE can be used to identify irradiated chicken (Hassan, 1990).

10.6.4.2 Use of hydroxyl radical biomarkers

Methodology has been developed to identify the irradiated status of foods using hydroxyl radical biomarkers. Irradiation of phenylalanine or proteins containing phenylalanine yields *o*-, *m*-, and *p*-tyrosines, the former two having been proposed as indicators for radiation processing (Dizdaroglu *et al.*, 1985; Simic *et al.*, 1983; Szekeley *et al.*, 1992). Since *o*-tyrosine is easier to separate by chromatographic methods from *p*-tyrosine, it was chosen as a potential indicator of radiation processed foods (Chuaqui-Offermanns and McDougall, 1991a; Karam and Simic, 1988a, 1988b; Pedersen and Fuhlendorff, 1991; Zoller *et al.*, 1991), and its suitability assessed (Chuaqui-Offermanns and McDougall, 1991b; Chuaqui-Offermanns *et al.*, 1993; Meier *et al.*, 1988a; Willemot *et al.*, 1989). This marker could also be used to determine the absorbed dose in chicken meat irradiated with gamma rays or electrons. The levels of *o*-tyrosine are independent of the storage time and temperature after irradiation. They can be determined by solvent extraction and removal of the free tyrosine, which is present in the unirradiated tissue, followed by acid hydrolysis of the bound tyrosine in the proteinaceous residue and measurement of the cleaved residue by high performance liquid chromatography (HPLC) with fluorescence detection. This method eliminates a confounding influence to discriminate between irradiated and unirradiated chicken tissue (Ibe *et al.*, 1991).

When raw meat, which comprises >50% water, is exposed to ionizing radiation, the incoming radiation first interacts with molecular water, splitting it into hydroxyl

radicals and hydrated electrons which in turn interact with amino acids in proteins. 2-Hydroxyphenylalanine (2-HPA), a product of hydroxylation of phenylalanine (Karam and Simic, 1986) has been chosen as the internal dosimeter to measure the dose of irradiation absorbed. The amount of 2-HPA in irradiated samples was determined by comparing the integrated areas of its fragmentation ion peaks with the corresponding peaks of 4-hydroxyphenylglycine (4-HPG) used as an internal standard. Also 2-HPA may be used as a biomarker for .OH radical generation *in vivo* in the absence of any radiation (Karam and Simic, 1988b).

The early optimism concerning the use of *o*-tyrosine as an indicator and as an internal dosimeter for irradiated chicken meat has been dampened by the detection of *o*-tyrosine also in unirradiated meat (Karam and Simic, 1988a, 1988b) and as product of photolysis (Hasselmann and Laustriat, 1973). At the dose approved for treating chicken (3 kGy), the expected levels of *o*-tyrosine based on experimentally determined radiation dose should be 1.8 ± 0.1 ppm, which is much above the background level of 0.43 ± 0.08 ppm.

Since irradiation of proteins also yields crosslinked products, possibly some of these could function as indicators (Garrison, 1981; Karam *et al.*, 1984; Gajewski *et al.*, 1984; Dizdaroglu *et al.*, 1984; Simic *et al.*, 1985). However, until now no practically useful methods have been published.

It has been concluded that it is unlikely that any single method will produce a satisfactory result and that a number of tests to establish a 'profile' of a food will be necessary.

10.6.5 Volatile compounds from lipids

Volatile compounds from lipids may be used as indicators of irradiation (Chiang and Hau, 1992). Most foods contain some lipids, so a method based on changes in this food component should have applications in a wide range of products. Although proteins also contribute to the formation of volatile products on irradiation, their contribution is relatively small (Merritt and Taub, 1983; Delincee, 1983). Irradiation has shown negligible changes in the profile of fatty acids, esters and triacylglycerols in neutral lipids of muscles or fatty acyl residues of skin lipids (Dubravcic and Nawar, 1968; Champagne and Nawar, 1969; LeTellier and Nawar, 1972a; Nawar, 1978; Maxwell and Rady, 1989). Phosphatidic acid, lysophospholipids, fatty acids, the phosphoryl base and volatile hydrocarbons are phospholipid radiolytic products of edible oils such as peanut (Bancher *et al.*, 1972) and soybean (Hafez *et al.*, 1989) and also of lecithin (Coleby, 1959). However, phospholipid radiolysis in aqueous suspensions is a complex process. No clear correlation has been shown for the susceptibility of the different phospholipid classes to gamma-radiation or the effect of gamma-radiation on the saturated as opposed to unsaturated phospholipids. Two predicted radiolysis products, phosphatidic acid and the lysophospholipids which have been quantitated and identified in aqueous suspension are also the endogenous lipids in food. Thus neither

lipid can be used effectively to develop an analytical method for the detection of irradiated food (Tinsley and Maerker, 1993).

10.6.5.1 Long chain hydrocarbons

The changes induced by irradiation of fatty acids found in foods into hydrocarbons suggested the use of this as the basis for the identification of irradiated foods containing fat (Nawar and Balboni, 1970). The hydrocarbons of interest are those with one carbon less, or two carbons less and one double bond more than the parent fatty acid (Ammon *et al.*, 1992). The most appropriate markers suggested are tetradecaene, hexadecadiene and heptadecene, the amounts of which increase with increasing radiation dose (Nawar *et al.*, 1990), and which can be detected by HPLC–GC–FID (flame ionization detector) in products such as soup mixes, spices and fish and shrimps (Biedermann *et al.*, 1992). Pentadecene, tetradecadiene, heptadecadiene and hexadecatriene, produced from palmitoleic acid and linoleic acid have also been suggested as indicators of irradiation in chicken, since the contents of the two parent fatty acids are high in chicken fat (Nawar, 1990). Similarly nonane and hexadecadiene may be used as markers of irradiated bacon (Singh *et al.*, 1989). The irradiation detection method based on the analysis of volatile radio-induced hydrocarbons also seems well adapted to Camembert cheese. Four hydrocarbons, namely tridecane, 1-dodecene, 1-tetradecene and 1-hexadecene formed by the radiolysis of myristic, palmitic and stearic acids, never appear in non-irradiated cheeses, either during ripening or during storage. In fact, the 1-alkene to alkane ratio remains constant at 1.3 and is independent of the fatty acid precursor. Quantification would be possible if a reference unirradiated Camembert cheese was available (Bergaentzle *et al.*, 1994). The method involves isolation of the volatile compounds by vacuum distillation followed by identification by gas chromatography (Tuominen *et al.*, 1991; Spiegelberg *et al.*, 1991). Nonane has recently been shown to be present in irradiated bacon, and absent in unirradiated bacon (Singh *et al.*, 1993).

Analysis of hydrocarbons by GC/MS can identify eggs irradiated up to 3 kGy (Helle *et al.*, 1992c). Comparable results by different laboratories on irradiated chicken have been obtained, despite the differences in analytical methodologies (Meier and Stevenson, 1992). While the utility of GC/MS in qualitative detection of irradiated food has been confirmed (Sjoberg *et al.*, 1992), it also has potential for quantitative detection of irradiated foods. The absorbed radiation dose calculated from the radiolytic hydrocarbons resulting from irradiation of whole chicken thighs and breast quarters is also known to agree with electron spin resonance (ESR) spectroscopy within experimental error (Morehouse *et al.*, 1993; Morehouse and Ku, 1992). However, results show no linear relationship between dose and the amount of hydrocarbons formed although further work is necessary.

The effects of processing conditions influencing the formation and stability of long chain hydrocarbons also needs to be investigated. Applications are possible in three

possible commercial situations, the irradiation of avocado pears, fresh pilchards and poultry meat. Amongst the hydrocarbons produced, alkanes and alkenes are useful in identifying irradiated avocado pears (for doses above 0.5 kGy) and poultry meat, however numerous volatile compounds are already present in fresh pilchards before irradiation (Lesgards *et al.*, 1993).

10.6.5.2 2-Alkylcyclobutanones

A series of cyclic compounds, 2-alkylcyclobutanones, with the same number of carbon atoms as the parent fatty acid have been isolated following irradiation of pure triglycerides at high doses (LeTellier and Nawar, 1972b). One such compound, 2 dodecyl cyclobutanone is formed from palmitic acid in chicken meat irradiated at doses below 10 kGy (Stevenson *et al.*, 1990; Boyd *et al.*, 1991). This compound has not been detected in unirradiated fresh or microbiologically spoiled chicken meat, and is not generated by heat treatment (Crone *et al.*, 1992a); thus it appears to have potential as a specific marker for irradiated chicken meat (Stevenson, 1992). Doses as low as 1 kGy can be detected by this marker compound. The presence of 2-tetradecylcyclobutanone is formed by irradiation of stearic acid has also been confirmed in irradiated chicken meat.

Similar investigations have shown 2-dodecy cyclobutanone and 2-tetradecyl-cyclobutanone in irradiated whole liquid egg (Crone *et al.*, 1993) and pork (Stevenson, 1992). These compounds are promising, even when irradiated eggs are used as an ingredient in processed foods such as baked products (Pfordt and Von Grabowski, 1995). In all cases the compounds are not detected in unirradiated samples thereby confirming the specificity of this method for the identification of irradiated foods. The compound persists on freeze drying and long storage periods of the order of 12 years (Crone *et al.*, 1992b). Studies are in progress on the validity of using these hydrocarbons and 2-dodecylcyclobutanone as indicators of irradiation of chicken meat.

10.6.5.3 Cholesterol oxides

Cholesterol oxides, formed under the influence of oxidizing conditions (Smith, 1981) are also formed when cholesterol in aqueous dispersions is exposed to ionizing radiation (Kucera *et al.*, 1984; Sevanian and McLeod, 1987; Lakritz and Maerker, 1989). These products are similar to those formed by autoxidation (Smith, 1987; Maerker, 1987), but the relative amounts formed by the two processes are substantially different (Maerker and Jones, 1991). Specifically, the ratio of 7-ketocholesterol to total epoxides generated by irradiation is generally less than unity, whereas this ratio in autoxidation is greater than 10. Free radicals generated by radiolysis presumably interact with cholesterol in the aqueous phase to give the observed products. The products include α- and β-epoxides, 6-ketocholestanol and 7-ketocholestenal. The

last two are not known to occur in any food and are not formed on autoxidation. If their occurrence in irradiated chicken or meat is confirmed these may be good indicators of irradiation (Maerkar and Jones, 1992).

Ring oxidation products of cholesterol have been known to occur in mammalian tissues and are recently demonstrated to be produced in significant quantities in liposomes during the irradiation of cholesterol (Maerker and Jones, 1993), However, differences are observed in the behaviour of cholesterol in model systems and in meat, probably due to the complexity of the meat system.

Methods of detecting cholesterol oxidation products in meat systems have been reported (Park and Addis, 1985, 1987; Higley *et al.*, 1986; De Vore, 1988; Csallany *et al.*, 1989; Sander *et al.*, 1989; Nourooz-Zadeh and Appelqvist, 1989; Pie *et al.*, 1991; Zubillaga and Maerker, 1991). Some of these methods have limited sensitivity and are inadequate for measurement of compounds of interest. Chloroform–methanol–water extraction followed by solid phase extraction, column separation, thin layer chromatography and gas chromatography have recently been shown to recover 78–88% of the cholesterol oxidation products, and detection is possible at levels of 10 ppb or higher (Hwang and Maerker, 1993).

10.6.6 Changes in DNA

Exposure of DNA to ionizing radiation produces single strand breaks, double strand breaks (Deeble *et al.*, 1990) or lesions in the bases (purines and pyrimidines), breaks in the sugar phosphate backbone of the molecule, and both DNA–DNA and DNA–protein crosslinks. Determination of damage to animal or plant cell DNA therefore seems a possible approach to detecting irradiated foods (WHO, 1987). Measurement of DNA content by flow cytometry and the DNA index have been shown to be promising for differentiating irradiated and unirradiated onions (Selvan and Thomas, 1995). Thymine glycol (5,6-dihydroxydihydrothymine) is one of the major base damage products formed upon radiolysis of DNA *in vitro* (Teoule *et al.*, 1977; Breimer and Lindahl, 1985) and is present in DNA extracted from irradiated cells (Leadon, 1987). This was examined as a potential marker for identifying irradiated wheat (Jabir *et al.*, 1989), cod, shrimps and chicken breast (Pfeilsticker and Lucas, 1987, 1988). The fluorimetric assay used for detecting thymine glycol is based on its instability in alkaline solution and undergoes fragmentation to produce acetol, which can then be condensed with *o*-aminobenzaldehyde (*o*-ABA) to form the fluorescent compound 3-hydroxyquinaldine. However, the assay was reported to be subject to interference from one of the reagents, *o*-aminobenzaldehyde, and lacks sensitivity (Ewing and Stepanik, 1992). Other techniques that have the potential to detect DNA base damage products in irradiated foods are the enzyme immunoassay (Jabir *et al.*, 1989), gas chromatography–mass spectrometry with selected ion monitoring (Fuciarelli *et al.*, 1989, 1990) and HPLC with electrochemical detection (Grootveld and Jain, 1989; Grootveld *et al.*, 1990; Park *et al.*, 1989; Berger *et al.*, 1990).

These techniques appear to be capable of providing the sensitivity and specificity necessary for a practical test for identifying irradiated food. The main drawback is that the changes induced cannot be distinguished from those occurring in other treatments such as freezing.

A method of detecting DNA fragments by microelectrophoresis (Ostling and Hofsten, 1988) of single cells followed by staining with acridine orange and estimation under a fluorescence microscope has been investigated (Cerda *et al.*, 1992). The migration of the DNA fragment is seen as a 'tail', while intact DNA can be seen as a small, bright dot. Although repeated freezing and thawing also damages DNA, the pattern observed following electrophoresis is different from that seen with irradiated samples. So far this rapid and simple screening method has been shown to be applicable to chicken, onions and potatoes.

Another approach that shows promise is the analysis of mitochondrial DNA, which is protected against enzymatic reactions that cleave nuclear DNA during storage. In addition, mitochondrial DNA appears to be resistant to freeze–thaw cycles, so that detection of strand breaks in the DNA will be a specific indication that irradiation has taken place (Marchioni and Hasselmann, 1991).

It is possible to detect differences in samples if they are examined both before and after irradiation. It is not possible to examine a single sample and say whether or not it has been irradiated without the benefit of a control non-irradiated sample from the same source. Trials are continuing to gauge the variation in DNA patterns within different species and the changes associated with normal food handling practices such as storage and freezing.

10.6.7 Formation of free radicals

Amongst the various approaches, thermoluminescence, electron spin resonance and volatile compounds from lipid containing foods offer promising possibilities. Studies organized by the UK Ministry of Agriculture, Fisheries and Food (Scotter *et al.*, 1990), the Commission of European Community Bureau of Reference (BCR) and the International Atomic Energy Agency are in progress. In particular, BCR has made a significant contribution towards the validation of the methods through active collaborational trials within Europe. The techniques are individually discussed as follows.

10.6.7.1 Thermoluminescence

Thermoluminescence (TL) is the emission of light upon release of free radicals from their traps by heating. It is an established method in radiation dosimetry (Mahesh and Vij, 1985). The low levels of TL emitted by unirradiated foods are magnified many times on irradiation to permit differentiation between irradiated and unirradiated foods. The work began with a whole range of herbs and spices (Heide and Bogl, 1987;

Heide *et al.*, 1989a; Sanderson, 1990), but the considerable variation found in individual herbs and spices makes unequivocal identification difficult. In addition, TL fades during storage which further limits the usefulness of the technique. Earlier the origin of TL was thought to be from organic compounds, but later it was clearly demonstrated that the mineral debris adhering to the spices and herbs is responsible for the signal (Sanderson *et al.*, 1989a, 1989b). The process generally consists of heating a 5–25 mg spice sample in TL apparatus to a maximum temperature of 250 °C, at a heating rate of 100 °C in 10–15 s; integral light emission over 30s is determined. Five replicate analyses are conducted on each sample. The highest and lowest values are discarded, and a TL index is calculated. This value is compared with a reference value to assess whether the sample has been irradiated. For the result to be considered positive, the TL value must exceed the reference value by a factor of three (Federal Republic of Germany, 1989b). The discriminatory signals obtained can be further improved by subjecting the samples to reirradiation and comparing the two responses. Positive identification of foods given 1–10 kGy doses depend on the composition of the minerals and the time span between irradiation and analysis. Too low a mineral content does not permit detection by this method (Autio and Pinnioja, 1990). At present the technique is used mainly for qualitative identification of herbs and spices, but efforts are underway to use the technique to detect irradiated fruits, vegetables (Federal Republic of Germany, 1989a) and shell fish (Raffi and Agnel, 1989; Agnel *et al.*, 1992). The highest TL intensity measured on strawberries has been found to be 0.06 nC in unirradiated samples, whereas the levels in samples exposed to a 3 kGy dose were three times higher. Irradiated samples show a TL intensity of >0.2 nC even after washing, and the method is therefore considered suitable for detecting whether or not samples have been irradiated (Heide *et al.*, 1989b). The TL intensity does not decrease markedly over a period of three years (Pinnioja *et al.*, 1993).

Encouraging results have been obtained from an interlaboratory trial of the TL method (Stevenson, 1992; Schreiber *et al.*, 1995). This method has been applied to the import control of irradiated foods (Pinnioja *et al.*, 1993) in Finland.

10.6.7.2 Chemiluminescence

Chemiluminescence (CL) is the emission of light during a chemical reaction, mostly by the addition of an aqueous solution to the dry material thereby inducing chemical reactions involving the free radicals present. It has extensively been utilized for radiation dosimetry due to the observation that many irradiated solid substances emit light on dissolution in a reproducible manner (Ettinger and Puite, 1982). This light can be amplified by some enhancing substances such as luminol. The efficacy of CL as a means of identifying irradiation has been studied in black pepper and garlic as a function of moisture content. An increase in moisture content decreases the CL value. CL values of irradiated samples (10 kGy) increase by 3% and 693% in corriander and juniper berries, respectively (Meier *et al.*, 1988b). Decreases in TL and CL intensity

during storage need to be considered. There is also a poor reproducibility between irradiation and CL value. However, a high CL value and low bacterial count may indicate sample irradiation, but lower bacterial counts can also be due to chemical fumigation. Results have shown that CL does not provide a reliable indication of irradiation of all spices (Heide and Bogl, 1988) and dried vegetables (Meier *et al.*, 1989). Hydrogen peroxide-stimulated CL measurements have however shown promise as a means of screening fresh fruit for irradiation (Lewin *et al.*, 1993). With spices containing unsaturated fatty acids, for example sesame seeds, lipid oxidation may lead to an increased CL (Bogl and Heide, 1985; Heide and Bogl, 1985).

10.6.7.3 Electron spin resonance

When food is treated by ionizing radiation, free radicals are formed. Generally these are very short lived and therefore cannot be used to identify irradiated food. However, if they are trapped in hard, relatively dry components of food, the radicals are sufficiently stable to be detected by ESR spectroscopy. The technique has been applied to a number of foods, including those containing bones, some fruits, foods that contain shells and several spices (Helle *et al.*, 1992b; Stevenson and Gray, 1990) as well as in eggs through irradiation specific free radicals in the cellulose component of egg packaging (Helle *et al.*, 1992c). It has also been found to be useful in estimating the degree of irradiation in milk protein concentrate powder irradiated at 2–20 kGy. However, added ferrous ions have been shown to have a quenching effect on ESR. The technique is affected by storage period and correlates well with TL measurements (correlation coefficient, $r = 0.973$) (Kispeter *et al.*, 1992).

A co-trial organized by the Community Bureau of Reference on the use of ESR in irradiated foods in 21 laboratories using a variety of foodstuffs has shown a good correlation for meat, fish, dried raisins and papaya. The need for a kinetic study of different species in the case of fish bones has been advocated. However, pistachio nuts also presented difficulties because changes in the spectrum are too small and are dependent on the instrumental variables (Raffi *et al.*, 1992).

10.6.7.3.1 Food containing bones

When a food material containing a bone is irradiated, the free radicals are trapped in the crystalline lattice of the bone. The radiation induced free radicals give an ESR signal with a characteristic shape; no such signal is found in unirradiated bone or bone processed by any other method (Federal Republic of Germany Standard, 1991). This phenomenon should be independent of the species, although most research has concentrated on chicken bone (Stewart *et al.*, 1991; Dodd *et al.*, 1988). Since the number of free radicals increases on increasing the dose of irradiation, it appears feasible that the technique could be used to estimate the dose of radiation (Stevenson and Gray, 1989; Desrosiers and Simic, 1988). The free radicals formed are dependent on the nature of the bone, temperature of irradiation and gaseous environment,

although not on dose rate, water content or type of radiation, either gamma or electrons. Cooking before irradiation greatly enhances the yield of radicals, while cooking after irradiation causes little or no destruction of the radicals. The presence of carbon dioxide in the atmosphere, and higher temperatures greatly increase the radiation-induced signal (Dodd *et al.*, 1992; Stevenson and Gray, 1989; Gray and Stevenson, 1989a, 1991). The crystallinity of bone may influence the number of free radicals present and consequently the intensity of the signal. This was confirmed using bones from chickens of different ages (Gray *et al.*, 1990) and from different animals (Goodman *et al.*, 1989). Some research workers have constructed a 'dose response curve' for determining the dose absorbed by a food sample (Raffi, 1992). It is believed that there should be no difficulty in detecting low, medium or high doses of irradiation. Detection of irradiation in a secondary food product such as mechanically recovered meat (MRM). It is possible to detect the characteristic radiation-induced ESR signal in bone fragments extracted from irradiated MRM (Gray and Stevenson, 1989b). A calibration curve using ESR batch behaviour up to 10 kGy has been recommended to give a reliable evaluation of the radiation dose in chicken drumstick bone samples taken randomly from the market (Bordi *et al.*, 1993). The ESR signal strength decreases with the length of storage in the case of chicken bones, the decline in signal intensity is approximately 20% after storage for 14 days at 4 °C (Gray and Stevenson, 1991). However by changing the measurement conditions to low microwave power, additional lines appear on both sides of the main signal (believed to originate from cellulose) only in irradiated spices, which are stable after long periods of storage and could be more useful in quantifying irradiation (Helle *et al.*, 1992d). International trials on the use of this technique to detect irradiated meats were successful, and a protocol for electron paramagnetic resonance (EPR) is provided on this basis (Desrosiers *et al.*, 1994).

10.6.7.3.2 Food containing shells

Radiation-induced signals in Norway lobsters are similar in irradiated and unirradiated samples and show the characteristic Mn^{+2} ESR signal (Stewart *et al.*, 1992). However, the software available can isolate the signal induced by irradiation and use it for qualitative detection of irradiated foods. Freeze drying gives a larger signal than air drying. The signal intensity increases with the radiation dose, but decreases during storage (Stewart *et al.*, 1993). There is evidence that the nature of the free radicals formed and hence the shape of the ESR signal differs between prawn (Desrosiers, 1989) and shrimp species (Morehouse and Desrosiers, 1993). This may complicate interpretation of the ESR signal from an irradiated prawn of an unknown background.

The part of the exoskeleton from which the sample for ESR measurement is taken also plays an important role. Relative intensities differ, although the spectra remain the same. In Norway lobster, claw samples give the most intense signal while walking legs give the weakest (Stewart *et al.*, 1993). The spectrum is rather complex consisting of a

number of radicals not assignable to the general bone radical. Some of the radicals could be due to manganese (Desrosiers, 1989), others to chitin components, as estimated by the effect of boiling and X-ray diffraction measurement.

The results of an intercomparison of ESR techniques (Raffi *et al.*, 1993) carried out by the BCR have been very promising.

10.6.7.3.3 Fruit

ESR spectroscopy has been used to detect irradiation of fruits by the detection of relatively stable radiation-induced free radicals that are trapped in achenes, pips or stones (Raffi *et al.*, 1988; Desrosiers and McLaughlin, 1989). The responses observed are characteristic of the fruit. For example, a multicomponent signal thought to be due to radicals induced in the sugars glucose, fructose and sucrose is present in irradiated papaya; this signal is quite different from that found in unirradiated samples. Under such circumstances, identification is quite easy. On the other hand, the ESR signal from some fruits like strawberries is much more complex, and relatively weak and is thought to be due to cellulose (Raffi *et al.*, 1988). With citrus fruits, although ESR can distinguish irradiated samples from unirradiated ones, the constituents giving different signals remain unidentified and have been named as Features A, B, C and D (Tabner and Tabner, 1994). Identification of irradiated dried fruit in routine control should be possible by this technique in the near future, particularly as storage and heating of the samples show almost no effect on the ESR signals (Helle *et al.*, 1992a).

10.6.8 Other methods

10.6.8.1 Pigment changes

Trials have been undertaken to determine the extent of radiation induced pigment changes in mangoes and red capsicums at doses up to 600 Gy (Mitchell *et al.*, 1990). While some significant changes have been recorded it is too early to say whether these changes will provide a reliable indicator of irradiation treatment as in some cases natural variation in product composition appears to be greater than any change due to radiation treatment.

10.6.8.2 Changes in enzyme activities

The production of ethylene which stimulates the ripening of fruits is suppressed by irradiation. The ethylene forming enzyme (EFE) activity is measured by the production of ethylene from fruit slices shaken with the substrate 1-aminocyclopropane-1-carboxylic acid. Contrary to expectation the EFE activity is substantially increased in irradiated pawpaws, even at low dose used for insect disinfestation. The activity of phenylalanine lyase (PAL) in papayas, mangoes and potatoes is enhanced by irradiation. This results in a high content of phenolic

compounds which presumably prevents rotting and extends the shelf life of the fruit. The deterioration in texture which often follows irradiation may result from changes in the activity of pectin methyl esterase. The charge properties of the isoenzymes of horseradish peroxidase are extensively modified by irradiation. All these enzyme systems offer potential to detect irradiated foods (Mitchell, 1990).

10.6.8.3 Carbon monoxide gas as a probe

Carbon monoxide is one of the major radiolytic gases arising from irradiated foodstuffs (Pratt and Kneeland, 1972; Simic, Merritt and Taub, 1979). It is also retained in irradiated frozen products. Recently Furuta *et al.* (1992) proposed a novel method of detecting radiolytic CO formed within irradiated deboned frozen products. Figure 10.4 shows CO content within irradiated frozen chicken, beef and pork after 50 days and 1 year of storage as a function of irradiation dose. It has been observed that the yield of CO increases with increasing doses of irradiation. The change in CO levels between the two storage periods is unexpectedly small. The validity of this method for the identification of irradiated frozen meat and poultry is demonstrated by the small background in non-irradiated samples. The method is comparable to the ESR method with respect to sensitivity, and has an added advantage of being useful for boneless products and therefore has more general applicability (Furuta *et al.*, 1992). The efficacy of CO as a probe for quantifying irradiation in pepper berries has also been established. However, for other spices such as allspice, cumin and cinnamon, as well as for grains such as rice and wheat, only qualitative distinction was possible. In the case of several spices, the CO content dropped to the same level as the non-irradiated ones in a few days (Furuta *et al.*,1995).

10.6.8.4 Hydrogen as a marker

Besides CO, considerable amounts of other low molecular weight gases such as H_2, and CO_2 and CH_4 are produced by radiolysis of organic compounds in irradiated foods. Hitchcock (1993) demonstrated that irradiation of water (>0.1 kGy) generated H_2 gas that could be quantified by headspace gas analysis using an electric sensor, but perceived its potential only for packaged or solid foods because of the rapid diffusion of H_2. However, H_2 shows promise for distinguishing between irradiated and unirradiated frozen foods such as chicken. Although positive detection of hydrogen provides conclusive evidence of irradiation, no definite conclusions can be drawn from a failure to detect hydrogen (Hitchcock, 1995).

Several processes used in the food industry have received scant attention so far with respect to chemical changes. Thus, possible differences between quick and slow freezing, blast freezing and individual quick freezing have as yet not been scrutinized. Bakery, oil frying, extrusion cooking, roller, spray and freeze drying have not been

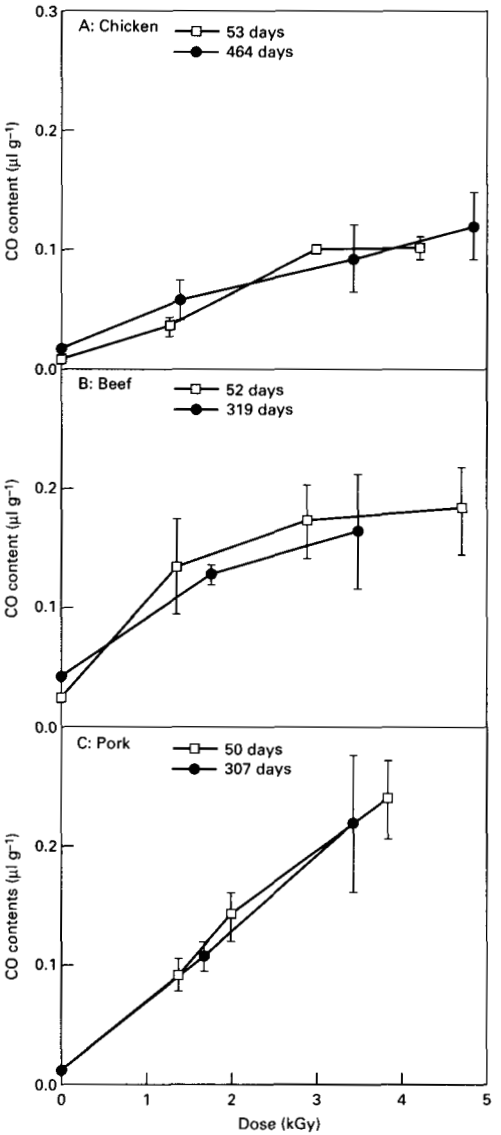


Figure 10.4 CO contents within irradiated frozen (A) chicken, storage: □, 53 days; ●, 46 days, (B) beef, storage: □, 52 days; ●, 319 days, (C) pork, storage: □, 50 days; ●, 307 days, after about one year of storage as a function of dose. The amounts of CO are expressed in terms of the volume of CO at 25 °C and 1 atm liberated from 1 g of frozen sample. Error bars depict 1 standard deviation calculated from three measurements. CO contents within non-irradiated samples after each storage period are indicated at 0 kGy in each graph. (Source: Furuta *et al.*, 1992, reproduced with permission)

studied from this angle. Processing methods employed in Asian, African and South American culinary practices have yet to receive scientific scrutiny. Vast work remains to be done.

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