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Detecting allergens in food

Edited by Stef J. Koppelman and Sue L. Hefle

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Preface

With food allergies on the increase in Western societies, and new legislation on allergen labeling in both Europe and the USA, the food industry is confronted with the issue of providing safe foods for the food-allergic consumer. While most food-allergic reactions occur after ingestion of non-packaged food products, the food industry has been subjected to increasing scrutiny of its allergen controls; the resulting impact on the industry has been remarkable. In the past 15 years, the food industry has made significant investment, effort, and improvements in allergen control. In the past eight years, tests for some allergenic foods have been commercialized and have proven useful to the industry in controlling allergens, and also to regulatory agencies investigating food-allergic consumer complaints. There are many strategies food manufacturers can exercise in controlling allergens in their plants, from changing raw materials to improving cleaning procedures and using precautionary labeling indicating allergens that might be present. However, measuring the content of particular allergenic residues on processing equipment, in ingredients or in final products, provides information that can also be used for risk assessment, enabling the food industry to provide the food-allergic consumer with practical information. The inspiration for this book originated in the early years of this millennium when increasing numbers of commercial test kits for measuring allergenic food residues were launched. The purpose of the book is to serve as a resource for experts from different backgrounds such as biochemistry, food chemistry, food legislation, and allergology with regard to the technical possibilities and limitations of food allergen detection methods.

The first part of the book deals with the nature of food allergy and introduces the reader to the range of allergenic foods. What causes an individual to be at risk from food allergy? What makes a protein an allergen? How much allergenic residue is too much for a food-allergic individual? What is the nature of the immune response to food that causes allergic reactions? These questions will be discussed as a basis for the remainder of the book.

Part II deals with methods designed for measuring food allergens, based

on either immunochemical or DNA techniques. How specific and sensitive does a method need to be, and does one need quantitative ability, or just qualitative? Should a method detect the allergen itself or marker/indicator molecules? What is a technically feasible detection limit, and what is a practically useful detection limit? When do we need high throughput screening, and how much time will analysis take? And what will allergenic residue detection methods look like in the future?

Part III deals with published detection methods and those that are commercially available at the time this book went to print. Methods for the main food allergens such as peanut, milk, egg, tree nuts and seeds, fish and crustacean shellfish, soy and cereals are discussed by experts in those particular areas. Detection limits are discussed in view of clinical food allergen threshold data as well as case reports of allergic individuals who experienced reactions after inadvertent ingestion of a certain amount of allergen.

Part IV of the book deals with the challenges and issues facing the food industry in the struggle to deal with allergenic food residues in production environments. Methods for allergen detection are available, with certain specificities and sensitivities, and practical limitations. New ingredient-labeling legislation is pending in many countries, and the outcome of these efforts may in the future give rise to even more specific legislation on inadvertent allergen contamination of food products. The food industry often uses shared equipment and facilities for the production of processed foods with a variety of formulations. This is often out of necessity rather than just fueled by economic reasons. What are the tools and information available to the industry in risk assessment, and where do detection methods fit into these discussions?

In the concluding chapter, we will summarize the problems involved in the detection of allergenic residues in food, and will provide an outlook for the future of allergen detection as we envision it.

We wish to express our gratitude to all of the contributors to this book for their excellent work and for their cooperation and patience during the reviewing process. We are proud of the result, and we hope many colleagues will enjoy using this book.

Stef Koppelman and Sue Hefle

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Part I

The basics of food allergy

1

The nature of food allergy

S. Taylor, University of Nebraska, USA

1.1 Introduction: defining food allergy

While eating is necessary to sustain life, most people find eating to be an enjoyable experience given the variety and abundance of food available to them. However, for individuals with food allergies and related illnesses, consuming certain foods can be a debilitating, and possibly even life-threatening, experience. Consequently, the joy of eating is diminished by the ever-present fear of consuming a food or food ingredient that will cause an adverse reaction. Food-allergic consumers must assiduously avoid the offending foods and/or food ingredients because strict avoidance diets are the only available preventive strategy. For such consumers, food selection often becomes a tedious task requiring meticulous reading of ingredient lists on labels, dependence on food manufacturers to maintain accurate labels, and a continual search for more knowledge about food composition. For these individuals, food preparation requires careful attention to detail, cooking 'from scratch', and seeking alternative recipes for many dishes. Because very small amounts of the offending food can elicit allergic reactions in some affected individuals, these consumers live in constant fear that, despite their caution, trace amounts of the offending food, sufficient to elicit an adverse reaction, might still exist in the foods that they consume. They are concerned about ingredients derived from the offending food because such ingredients might contain residual allergenic proteins from the source food. This fear is compounded by the fact that declaration of the source of ingredients used in foods is not always required on food labels.

1.1.1 Definition and classification

Food allergies can be defined as adverse, immune-mediated reactions to foods that occur in certain individuals. Often, the public and even some within the medical community categorize all individualistic reactions to foods as food allergies. However, true food allergies should be restricted to those individualistic reactions to foods that are mediated by the immune system. The term 'food sensitivity' can be used to refer to all types of individualistic adverse reactions to foods. These food-related illnesses are individualistic because they affect only a few people in the population. Food intolerances are individualistic adverse reactions to foods that occur through non-immunological mechanisms. Knowing the difference between *immunological* food allergies and *non-immunological* food intolerances is critical to proper management of these illnesses. Food intolerances are often controlled by limiting the amount of food eaten; with food allergies, much more strict avoidance of the offending food is usually necessary. Table 1.1 provides a classification scheme for the various illnesses that are known to occur as individualistic adverse reactions to foods.

Food allergy is an abnormal *immunological* response to a food or food component; food allergens are almost always proteins.[1] Examples include allergic reactions to common foods such as peanuts and milk. Within this category are immediate hypersensitivity reactions where symptoms ensue within minutes to an hour after ingestion of the offending food and delayed hypersensitivity reactions where the onset of symptoms occurs 6–24 or more hours after ingestion of the offending food.

Immediate hypersensitivity reactions are mediated by immunoglobulin E (IgE) antibodies. Exercise-induced food allergies are a subset of food allergies involving immediate reactions that occur only when the specific food is ingested just before or after exercise,[2] although many cases of exercise-induced allergies are not associated with foods.[3]

Delayed hypersensitivity reactions are cell-mediated, normally involving sensitized immune cells in the small intestine, usually lymphocytes, that are sensitized to the specific substance that triggers the reaction.[4] The ultimate result is tissue inflammation often restricted to certain sites in the body with

Table 1.1 A classification scheme for food allergies and intolerances

Food allergy	IgE-mediated food allergy (immediate hypersensitivity)
	e.g. peanut allergy, cows' milk allergy
	Cell-mediated food allergy (delayed hypersensitivity)
	e.g. celiac disease
Food intolerance	Anaphylactoid reactions
	e.g. strawberry-induced urticaria (unproven)
	Metabolic food disorders
	e.g. lactose intolerance
	Idiosyncratic reactions
	e.g. sulfite-induced asthma

symptoms appearing on a more delayed basis, as much as 24 or more hours after consumption of the offending food.

Food intolerances, in contrast to true food allergies, do not involve abnormal responses of the immune system.[5] Anaphylactoid reactions involve the release of the chemical mediators (mostly histamine) of allergic reactions into the body without the intervention of IgE antibodies. Foods such as strawberries and chocolate are thought to allegedly induce such reactions, but definitive proof for this type of food intolerance does not exist. Metabolic food disorders are genetically determined metabolic deficiencies that result in adverse reactions to a food component. Lactose intolerance serves as a good example of a metabolic food disorder. In lactose intolerance, the affected individual has a deficiency of the intestinal enzyme, β-galactosidase, which is essential for the metabolism of the lactose in milk.[6] Consequently, lactose cannot be absorbed from the intestinal lumen leading to bacterial fermentation of the lactose in the colon with resultant flatulence and frothy diarrhea. Food idiosyncrasies are adverse reactions to foods or a food component that occur through unknown mechanisms. Examples include sulfite-induced asthma and tartrazine-induced asthma.[7] In many cases, the cause-and-effect relationship between the food or food component and the particular adverse reaction remains unproven; this would be the situation with tartrazine-induced asthma. Psychosomatic illnesses are included in this category.

Allergy-like intoxications are worth some mention here because these illnesses can be confused diagnostically with food allergies. Unlike food allergies, everyone in the population is probably susceptible. This reaction occurs as a result of the ingestion of histamine, one of the primary mediators of allergic disease. Histamine is released from cells within the body in true food allergies and anaphylactoid reactions but is ingested in the case of allergy-like intoxications. Histamine poisoning (also known as scombroid fish poisoning) is commonly associated with the ingestion of spoiled tuna, mackerel, mahi-mahi, and other fish and also occasionally with cheese.[8] The symptoms mimic some of the most common symptoms encountered in true food allergies.

1.1.2 Prevalence and health impacts

The overall and worldwide prevalence of IgE-mediated food allergies is not precisely known. In the US, the overall prevalence of IgE-mediated food allergies would be estimated at 3–4% of the population. The prevalence is highest in infants and young children ranging from 4–8% in that age group,[9] while that in adults is probably about 2–3%. Recently, the estimate of the prevalence of IgE-mediated food allergies in adults in the US has been increased, based upon a survey that indicates that 1.9% of the total population has shrimp/crustacean shellfish allergy and 0.4% of the total population has fish allergy;[10] both fish and crustacean shellfish allergies affect many adults. These percentages are added to earlier estimates that peanut and tree nut

Table 1.2 Symptoms of IgE-mediated food allergies

Gastrointestinal	Nausea, vomiting, diarrhea, abdominal cramps
Respiratory	Asthma, wheezing, rhinitis
Cutaneous	Urticaria (hives), eczema or atopic dermatitis, pruritis, rash, angioedema
Other	Anaphylactic shock, hypotension, swelling of the tongue, laryngeal edema, oral allergy syndrome

allergies affect an estimated 1.1% of the US population, including many adults.[11] Cows' milk, eggs, and peanuts are among the most common allergenic foods for infants and young children,[12] while crustacean shellfish, fish, and peanuts are among the most common allergenic foods for adults in the US,[10,11] although the situation can vary in other countries depending upon dietary eating patterns.

IgE-mediated allergic reactions involve numerous symptoms ranging from mild to life-threatening (Table 1.2).[13] Food-allergic individuals experience quite varied symptoms and it is likely that no one suffers from all of the symptoms mentioned in Table 1.2. The nature and severity of the symptoms may also vary from one occasion to another in the same individual as a result of the amount of the offending food ingested and the length of time since the last previous exposure. Anaphylactic shock is the most severe symptom for IgE-mediated individuals. Fortunately, comparatively few food-allergic individuals are susceptible to such severe reactions. Systemic anaphylaxis involves many organ systems and numerous symptoms including those also noted in Table 1.2 together with cyanosis, chest pain, and shock. Anaphylactic shock and asthma are the most common causes of death in the occasional fatalities associated with true food allergies.[14,15]

Although severe, life-threatening reactions are definitely the most worrisome manifestations of IgE-mediated food allergies, mild symptoms are much more likely to occur. One of the more common and typically most mild forms of IgE-mediated food allergy is the so-called oral allergy syndrome (OAS).[16] In this condition, symptoms are usually confined to the oropharyngeal area of the mouth and throat and include pruritis, urticaria, and angioedema. OAS is most frequently associated with the ingestion of various fresh fruits and vegetables.[16] Even though fresh fruits and vegetables typically contain low amounts of proteins, OAS is an IgE-mediated reaction to specific proteinaceous allergens present in these foods.[16] These fruit and vegetable allergens are apparently quite susceptible to digestive proteases in the gastrointestinal tract.[17] Systemic reactions are not typically encountered in OAS, but can be with certain patients and certain foods, e.g. celery. These allergens are also apparently heat-labile,[17] since the heat-processed versions of these foods are not typically involved in initiation of OAS. With OAS, affected individuals are initially sensitized to pollens in the environment, such as birch and mugwort pollens, that cross-react with related proteins

found in the fresh fruits and vegetables.[18] Apparently, sensitization to these pollens can increase the likelihood of sensitization to specific foods.

Occasionally, food allergies occur in conjunction with exercise.[2] The prevalence of exercise-associated food allergies is unknown. In these cases, the exercise must be preceded or followed by the ingestion of specific foods in order to elicit an allergic reaction. Shellfish,[19] wheat,[20] and celery[2] are among the foods that have been incriminated in food-dependent, exercise-associated anaphylaxis. The symptoms in this type of food allergy are individualistic and similar to those involved in other food allergies. With increased awareness of the existence of this syndrome and the emphasis on physical fitness in many countries, reports of this condition may continue to increase.

1.2 Mechanisms of food allergy

Two types of allergic or hypersensitivity reactions occur as basic immunological mechanisms involved in food allergies.[5] IgE-mediated reactions, also known as immediate hypersensitivity reactions, involve the formation of IgE antibodies that specifically recognize certain allergens in foods. IgE-mediated reactions are the most important type of food allergy because these reactions involve a wide variety of different foods and the reactions can be severe in some individuals. IgE-mediated mechanisms are also responsible for allergic reactions to pollens, mold spores, animal danders, insect venoms, and certain drugs; only the source of the allergen differs. Cell-mediated reactions or delayed hypersensitivities probably play an important, although as yet undefined, role in food hypersensitivity.[5] Celiac disease, which will be discussed later, may be a form of cell-mediated delayed hypersensitivity.[21]

1.2.1 IgE-mediated allergic reaction (immediate hypersensitivity)

Although Hippocrates was the first to document the occurrence of food allergies,[22] the involvement of the immune system in food allergies was not recognized for many years thereafter. Prausnitz and Kustner[23] were the first clinicians to recognize that the blood contained some substance that conferred allergic hypersensitivity. They subcutaneously injected a non-allergic individual with a fish extract and noted no adverse reaction. However, when this same normal individual was first inoculated, under the skin, with serum from a fish-allergic person and then subsequently injected with the fish extract, there was an inflammatory skin reaction at the serum-injected site. This passive sensitization experiment provided the first evidence that the blood contained some substance that sensitized the allergic individual to the fish. For some years thereafter, this blood factor remained unidentified and was referred to as reaginic factor. The reaginic factor involved in allergic reactions was first recognized as an antibody in 1966, when Ishizaka *et al*.[24,25]

demonstrated that this reaginic activity was associated with a unique immunoglobulin and tentatively called this protein γE. The protein was officially named immunoglobulin E or IgE by the World Health Organization in 1968. The identification of IgE as a reaginic antibody provided immunochemical approaches to analyze the mechanisms involved in hypersensitivity reactions.[26] Immunoglobulin E is one of five classes of antibody that are present in the human immune system (the others being IgG, IgM, IgA, and IgD).

The normal function of IgE antibodies is protection from parasitic infections. Although all humans have low levels of IgE antibodies, individuals predisposed to the development of allergies are most likely to produce IgE antibodies that are specific for and recognize certain environmental antigens.[5] These antigens are typically proteins, although only a few of the many proteins in nature are capable of stimulating the production of specific IgE antibodies in susceptible individuals.[27] The allergens eliciting IgE antibody formation can be found in pollens, mold spores, venoms, dust mites, and animal danders in addition to foods.[28]

The mechanism involved in IgE-mediated reactions is now well understood and is depicted in Figure 1.1.[5] In IgE-mediated food allergies, allergen-specific antibodies are first produced in response to stimulus of the antibody-forming B cells in response to the immunological stimulus created by exposure of the immune system to a specific food allergen, usually a naturally-occurring protein present in the food. The immune response in the small intestine which is responsible for the dominance of the IgE antibody generation is quite complex and involves T helper type 2 cells, interleukin-4 (IL-4), and other factors.[29] The IgE antibodies bind to the surfaces of mast cells in the tissues or basophils in the blood in a process known as sensitization. The sensitization phase of the allergic reaction is symptomless. In fact, sensitization can occur without the development of clinical reactivity[29] so the demonstration of IgE antibodies directed against a particular food in human blood serum is

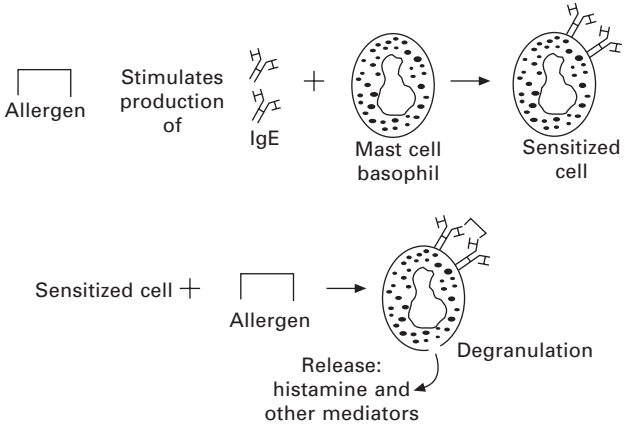

Fig. 1.1 Mechanism of IgE-mediated allergic reaction.

insufficient evidence for the diagnosis of a food allergy unless it is coupled to a strong history of food allergy or a positive double-blind, placebo-controlled food challenge.

Once sensitized, exposure to the same food allergen on a subsequent occasion can result in an allergic reaction.[5] When this happens, the allergen associates with the mast cell- or basophil-bound IgE, and cross-links two of the IgE molecules. A series of biochemical events is initiated which causes cell membrane disruption and the release of a variety of mediators contained within granules existing in the mast cells and basophils. The granules in mast cells and basophils contain most of the important mediators of the allergic reaction.[30] While several dozen substances have been identified as chemical mediators emanating from mast cell and basophils, histamine is responsible for most of the immediate effects of allergic reactions. The histamine-related effects include inflammation, pruritis, and contraction of the smooth muscles in the blood vessels, gastrointestinal tract, and respiratory tract.[31] Other important mediators include a variety of prostaglandins and leukotrienes; these particular mediators are associated with some of the slower-developing responses observed in some cases of food allergy (e.g. late-phase asthmatic reactions).[22]

While those individuals prone to the development of food allergies may form specific IgE antibodies upon dietary exposure to a particular food, other individuals will not. Even among those individuals predisposed to allergies, exposure to food proteins does not usually result in formation of allergen-specific IgE. Food-allergic individuals will normally be sensitive to only a few of the wide variety of foods in the typical human diet. In normal individuals, and even in those who are susceptible to the development of food allergies, exposure to a food protein most often results in oral tolerance through induction of T-cell anergy, deletion of reactive T cells, the generation of suppressor T cells, the formation of protective secretory IgA antibodies, and other immunological responses.[29,32] Heredity and other physiological factors are significant in predisposing individuals to the development of IgE-mediated food allergies and also other environmental allergies.[33] Approximately 65% of patients with clinically documented allergy have first degree relatives with allergic disease.[33] Conditions that increase the permeability of the intestine to macromolecules, such as viral gastroenteritis, premature birth, and cystic fibrosis, may increase the risk of development of food allergy.[22] Although food allergies may also involve other types of immunological mechanisms, the IgE-mediated mechanism is, by far, the most well-documented and understood.

1.2.2 Exercise-associated food allergies

The mechanism for food-dependent, exercise-induced anaphylaxis is unknown, but enhanced mast cell responsiveness to physical stimuli may be involved.[3] IgE sensitization appears to be involved in many cases.[34]

1.2.3 Cell-mediated reactions (delayed hypersensitivity)

In contrast to IgE-mediated reactions, the symptoms of cell-mediated allergic reactions do not begin to appear until 6–24 hours after ingestion of the offending food.[5] These reactions develop slowly, reaching a peak at approximately 48 hours and subsiding after 72–96 hours. The mechanisms of cell-mediated food allergies are not nearly as well understood. They involve an interaction between specific food allergens and sensitized T lymphocytes. Lymphocyte stimulation initiates the release of cytokines and lymphokines which produces a localized inflammatory response.[35] Antibodies are not involved in these reactions.

T lymphocytes are a major component of the gut-associated lymphoid tissue.[36] Except for celiac disease, evidence for the involvement of cell-mediated immune reactions in food allergies remains incomplete. However, cell-mediated reactions appear to be involved in some cases of cows' milk allergy occurring especially in infants and with symptoms confined primarily to the gastrointestinal tract.[37] No estimates of the prevalance of cell-mediated food allergies have been made.

Celiac disease, also known as celiac sprue or gluten-sensitive enteropathy, appears to be an example of a cell-mediated reaction.[21] Celiac disease is a malabsorption syndrome occurring in sensitive individuals upon the consumption of wheat, rye, barley, triticale, spelt, and kamut.[5,38] The role of oats in celiac disease is much less well-defined. Apparently, oats that are totally free of wheat, rye, and barley are safe for celiac sufferers to consume.[39] The consumption of wheat or other offending grains or products made from these grains elicits inflammatory damage to the absorptive epithelial cells in the small intestine.[38] The absorptive function of the small intestine is compromised as a result. Nutrients are not properly absorbed and water can leak out. The loss of absorptive function along with the ongoing inflammatory process results in a severe malabsorption syndrome characterized by diarrhea, bloating, weight loss, anemia, bone pain, chronic fatigue, weakness, muscle cramps, and, in children, failure to gain weight and growth retardation.[13,40] The inflammatory mechanism involved in celiac disease is mediated by intestinal T lymphocytes.[21] The gliadin fraction of wheat and related fractions in barley and rye are associated with initiation of celiac disease in susceptible individuals.[40]

1.3 Avoidance diets and treatment for IgE-mediated food allergies

Typically, the prevention of IgE-mediated food allergies primarily involves avoiding the offending foods.[41] Certain drugs, such as epinephrine (adrenaline) and various anti-histamines, can be used to treat the symptoms that develop during an allergic reaction if inadvertent ingestion of the offending food

occurs.[42] However, no pharmaceutical approaches are presently available for the prevention of food allergies. Anti-IgE therapy may hold some promise as initial trials have resulted in an increase in tolerance to peanuts among peanut-allergic individuals.[43]

With the lack of pharmaceutical approaches, specific avoidance diets remain the preventive strategy of choice for most food-allergic individuals. Thus, food-allergic individuals are forced to become avid label readers in an attempt to avoid their offending foods and certain ingredients derived from these foods. Their efforts are fraught with difficulty because individuals with IgE-mediated food allergies can react to mere traces of the offending food in their diet.[44,45] The construction of safe and effective avoidance diets and the difficulties faced by consumers who must adhere to such diets have been extensively reviewed elsewhere.[46,47]

Consumers with IgE-mediated food allergies often face several challenging questions as they attempt to implement a safe and effective avoidance diet.

(1) Will trace levels of the food elicit reactions or increase sensitization?
(2) Do all foods and food ingredients made from the offending food contain the allergens?
(3) Are cross-reactions likely to occur between closely related species?

Clear answers to these questions are not always available, in part because of a lack of research data and in part because of the lack of reliable analytical information. For example, many ingredients are derived from commonly allergenic foods; soybeans alone are used to generate hydrolyzed soy protein, soy sauce, soy oil, soy lecithin, soy isoflavones, tocopherol (vitamin E), soy phytosterols, soy fiber, and others. As noted later in Chapter 15, not all of these ingredients will be expected to contain the responsible allergens but clinical data on safety are often lacking. Also, from an analytical perspective, detection of proteins or allergens can be particularly challenging in some of these ingredient matrices. For example, the detection of protein residues in oils can be difficult. Until recent years, reliable methods for the detection of residues of allergenic foods in other foods or in such ingredients did not exist. Even now, the ability of existing methods to assess the allergenic potential of ingredients containing hydrolyzed or partially hydrolyzed proteins is problematic.

1.3.1 Threshold doses (minimal elicting doses)

Practical experiences indicate very clearly that trace levels of the offending food can elicit adverse reactions. Anecdotal reports exist of reactions from touching utensils or bottles contaminated with the offending food, kissing the lips of someone who has recently eaten the offending food, opening packages of the offending food, the inhalation of vapors from cooking of the offending food, and the transfer of food allergens from lactating mothers to breast-feeding infants.[48] Although the amount of the offending allergen involved in eliciting such reactions must be rather low, analytical information on

minimal eliciting doses from such experiences is not available. However, a few episodes of reactions to residues of allergenic foods hidden in other foods have been well investigated and lend credibility to the anecdotal reports.[44,45] For all practical purposes, complete avoidance must be maintained.

Analytical evidence can be used, in part, to obtain clues to the minimal eliciting doses for various allergenic foods. However, great care must be taken in the use of this information to establish such doses. First of all, existing evidence suggests that food-allergic individuals may vary in their minimal eliciting doses by several orders of magnitude.[49,50] This fact complicates the investigation of case reports because the affected individual will not necessarily be representative of other individuals in the population allergic to the same food. A second uncertainty factor surrounds whether or not a representative food sample can be obtained for analysis. On many occasions, several foods are consumed, and the affected consumer may not necessarily incriminate the food that actually caused the reaction. If the analyst finds a very low level of allergenic residue in an incriminated food, it is very important to determine if this food was the only one consumed at the implicated meal because other foods might have even higher levels of the particular allergen. Additionally, the consumer may have consumed all of the incriminated food sample or discarded the remainder. In those situations, the analyst can attempt to obtain similar samples from the marketplace, but the analysis of these samples creates uncertainties due to the questions about whether this sample is representative of the actual product that may have caused the illness. Analysis of samples from actual episodes is always complicated if the allergen contamination may not have been uniform. The bite that the consumer eats may have higher levels of allergen contamination than the remaining portion in some cases. Finally, until recently, reliable analytical methods for the detection of residues of allergenic foods were not readily available. Even today, it is uncertain whether analysis of the same sample by several different methods would yield a similar result. When assembling data from the existing literature, the analytical results presented in case reports may be somewhat questionable and direct comparison with results from current methods may be difficult.

Despite uncertainties involved in the investigation of consumer complaints, threshold doses or minimal eliciting doses do exist below which allergic individuals will not experience adverse reactions.[49,51] These minimal eliciting doses are likely to be very low and variable from one allergic individual to another.[49] For example, the minimal eliciting doses for peanut needed to provoke mild, objective adverse reactions in one particular group of peanut-allergic individuals ranged from 2 to over 50 mg.[50] Although a rather large number of low-dose challenge trials have now been conducted for peanut, milk, and egg in particular, consensus does not yet exist on the minimal eliciting dose for the most sensitive individuals. For example, other investigators have occasionally identified individuals with lower minimal eliciting doses than 2 mg.[52] While consensus on minimal eliciting doses has not been achieved,

dose-response modeling for peanut, milk, and egg (where the largest number of challenges has been obtained) indicate that only 1% or less of the affected population would react to residual levels of the allergenic food of 0.26 mg for egg, 0.76 mg for peanut, and 7.76 mg for milk.[51] Additional controlled challenge studies conducted with standard challenge protocols will likely reduce the uncertainty of these estimates in coming years.

Existing test methods for residues of allergenic foods appear to be in the ideal range to detect potentially hazardous residues of undeclared allergens in foods. Current immunoassay methods have lower limits of about 1 ppm (1 mg/kg). If minimal eliciting doses are 200 μg or higher and typical serving sizes are 50–100 g or less, then potentially hazardous foods would be easily detected with these immunoassay methods. If a highly sensitive individual ingested a typical 50 g serving of a food containing 200 μg of undeclared residues of an allergenic food, that food would have contained 4 ppm of the offending food. Previously, the advice given was that foods containing less than 10 ppm of undeclared residues of an allergic food would present little risk to food-allergic consumers.[53] That advice remains fairly sound now that much more evidence exists on minimal eliciting doses. The development of analytical procedures that are more sensitive than the current ones does not seem to be justified.

Food-allergic consumers, especially those susceptible to comparatively low threshold levels, can occasionally experience reactions to packaged food products even when the ingredient list indicates that the particular item should be safe. In manufacturing situations, foods may become contaminated with trace amounts of other foods through various means including the use of rework and the use of shared equipment.[54,55] No avoidance diet provides absolute safety, but careful adherence to an effective avoidance diet will minimize the chances of a reaction.

1.3.2 Allergenicity of ingredients derived from known allergenic sources

Many ingredients are derived from sources that are known to be allergenic. Very common examples would include peanut and soybean oils, soybean lecithin, and hydrolyzed milk, wheat, or soybean proteins. Less well known examples would include fish gelatin used to encapsulate certain vitamins and xanthan gum, a fermentation product made using wheat or soybean substrates. When considering ingredients derived from an allergenic food source, the presence of the allergenic protein is an important consideration.

Refined edible oils contain extremely low levels of residual proteins.[56] Clinical trials have documented that refined peanut and soybean oils are safe for individuals allergic to the source food.[57,58] However, less highly refined oils including cold-pressed peanut oil[59] and sesame seed oil[60] can contain sufficient protein residues to elicit allergic reactions in individuals sensitive to the source foods.

Lecithin can be derived from several sources, although soy lecithin is probably the most commonly used commercially. Soy lecithin definitely contains residual amounts of soy protein and detectable soy allergens.[61,62] Extensive surveys of the amount of protein in commercial soy lecithin have not been conducted although some variability is likely to exist because several different commercial processes are used. Soy lecithin does not appear to elicit adverse reactions in the majority of soybean-allergic individuals, although clinical trials have not been conducted to critically examine the allergenicity of soy lecithin to such individuals.

If the proteins have been extensively hydrolyzed, the allergenicity of the protein is likely to be eliminated.[17] For example, extensively hydrolyzed casein serves as the basis for hypoallergenic infant formulae recommended for milk-allergic infants. However, protein hydrolysates used in the food industry can vary widely in terms of degree of hydrolysis, and only those that are extensively hydrolyzed would be safe for individuals allergic to the source food. Of course, hydrolysis of the protein can dramatically affect the ability to detect residual proteins, especially using immunochemical techniques, even in cases where residual allergenicity may remain.

In other cases, the nature of the protein may have some impact on allergenicity. Although fish gelatin is derived from fish, this ingredient is primarily composed of fish collagen while the fish allergens are primarily parvalbumins.[63] Recently, fish gelatin derived from codfish skins was documented to be safe for the vast majority of codfish-allergic individuals at levels up to 3.61 g of cumulative intake.[64] If foods derived from allergenic sources contain detectable protein residues, the safety of these foods must be established by clinical trials in sensitive individuals. Alternatively, the foods should be labeled to declare the source of the ingredient.

1.3.3 Cross-reactions

Ubiquitous statements cannot be made about cross-reactions between closely-related foods. Cross-reactivity to closely-related foods occurs in some situations, but not others. For example, individuals with a shrimp allergy are often sensitive to all species of shrimp and to other crustacean shellfish such as crab and lobster.[65] Tropomyosin is a pan-allergen existing in all crustacean shellfish[66] and possibly also in some species of molluscan shellfish.[66] Fish have a distinctly different pan-allergen, parvalbumin, that exists in all species of fish,[63] although fish may also contain certain other minor allergens and cross-reactivity has not been universally observed.[63] Some peanut-allergic individuals are allergic to other legumes, such as soybeans,[67] although this is not a frequent occurrence. Clinical hypersensitivity to one legume, such as peanuts or soybeans, does not warrant dietary elimination of the entire legume food family unless allergy to each legume is individually confirmed by double-blind, placebo-controlled food challenges (DBPCFC).[68] In contrast, it is known that cross-reactions commonly occur between different species of avian eggs[69] and between cows' milk and goats' milk.[70]

As noted previously, cross-reactions also occur between some types of pollens and certain foods. These include ragweed pollen and melons (watermelon, cantaloupe, honeydew), mugwort pollen and celery, and birch pollen and various foods such as carrots, apples, hazelnuts, and potatoes.[71–74] Cross-reactions also occur between latex and certain fruits, particularly banana, chestnut, and avacado.[74] Patients with a history of allergic reactions to latex should be aware of the potential for allergic reactions to certain fruits.

The clinical observation of cross-reacting IgE antibodies is occasionally unexpected and confusing, but does not invariably imply the existence of an allergy to each food. For the interpretation of IgE antibody assays, it is important to appreciate that finding IgE antibodies to an allergen does not imply that the patient has ever been exposed to that allergen or that they will react after ingestion of that food.[75]

1.4 Future trends

Research in food allergies has been rapidly expanding in recent years with the recognition that the prevalence and severity of food allergies is increasing. Research is progressing on multiple fronts. From the clinical perspective, research will continue on the prevalence of food allergies including specific food allergies; improved diagnostic methods, particularly focusing on strategies that circumvent the need for DBPCFC in all cases; an improved understanding of the role of delayed hypersensitivity reactions in food allergy; and better treatment options for food allergy, ranging from anti-IgE therapy to vaccines to 'cure' allergies to probiotic approaches that will aid in the prevention of food allergies in infants. However, perhaps the most important research will be to better define threshold doses. While this is clinical research, there is currently no more important research than this for the food industry. How much is too much? That is a critical question that will drive food industry labeling and sanitation (and other allergen control) strategies as well as improved clinical approaches oriented toward the needs of individual patients. From the food industry perspective, research should also continue on assessment of the potential allergenicity of ingredients derived from allergenic sources and on the effectiveness of various sanitation practices. How clean is clean enough? That question is also related to the threshold issue and cannot effectively be answered until more data are available on minimal eliciting doses.

From an analytical perspective, more research is needed to develop methods for the detection of residues of allergenic foods that are not commercially available yet. Such analytical methods will provide important tools for the food industry and for regulatory agencies. With respect to regulatory agencies, the detection of allergenic food residues can be of tremendous assistance in their role as protectors of the health of food-allergic consumers. Obviously,

these methods will be important quality assurance tools for the food industry because they will allow the validation of sanitation practices on shared equipment. Shared equipment is an economic necessity in the food industry and is common in a wide variety of food industry sectors (e.g. ice cream, confectionary, bakery, pasta). Proper sanitation of that shared equipment is critical to mitigation of potential allergen hazards, and analytical tools will facilitate the validation of sanitation practices. Clearly, analytical methods must be specific, allowing the detection of an allergenic food in the presence of others, and must be appropriately sensitive. The ideal range of sensitivity of the methods should be driven by knowledge of minimal eliciting doses. The goal should be to protect allergic consumers while allowing them the greatest variety of foods in their diets rather than the detection of miniscule levels of allergenic food residues that have no adverse health consequences on even the most sensitive individuals.

1.5 Sources of further information and advice

The latest clinical information on food allergies is often available from medical societies such as the American Academy of Allergy, Asthma and Immunology (www.aaaai.org) and the European Academy of Allergy and Clinical Immunology (www.eaaci.org). Consumer groups can be sought out for information from the consumers' perspective, but some care must be taken to assure the accuracy of this information. Consumer groups do not exist in all countries. The Food Allergy and Anaphylaxis Network in the US (www.foodallergy.org), the Anaphylaxis Campaign in the UK (www.anaphylaxis.org.uk), Nederlands Anafylaxis Network in the Netherlands (www.anafylaxis.net), Anaphylaxis Australia (www.allergyfacts.org.au), Allergy New Zealand (allergy.org.nz), and Association Quebecoise des Allergies Alimentaires in Quebec Province of Canada (www.aqaa.qc.ca) serve as examples of such consumer groups. Regulatory agencies should be consulted for information on labeling and other regulations that may exist in various locales to protect food-allergic consumers. Regulations vary greatly from one country to another. The Codex Alimentarius Commission (www.codexalimentarius.net) and its Committee on Food Labeling are a good source for general guidance. For regulations specific to individual countries, the websites of the countries of interest should be examined – for Europe, the European Food Safety Authority (www.efsa.ev.int) would be the most appropriate agency. Specific advice can also be sought from various research groups including the University of Nebraska (Lincoln, Nebraska, USA) Food Allergy Research and Resource Program (www.farrp.org).

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2

Classifying food allergens

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2.1 Introduction

Plant tissues including those used for human consumption contain thousands of different proteins. Proteins are clustered together into families if they have residue identities of 30% and greater, or if they have lower sequence identities but their functions and structures are very similar.[1] Families whose proteins have low sequence identities but whose structures and functional features suggest a probable common evolutionary origin are placed together in superfamilies.[1] The Structural Classification of Proteins (SCOP) database (http://scop.mrc-lmb.cam.ac.uk/scop/count.html) to date includes 2164 protein families and 1232 protein superfamilies.

However, most plant food allergens are included in only a few protein families and superfamilies. They belong to the cupin superfamily (7S and 11S seed storage proteins), the prolamin superfamily (2S albumins, non-specific lipid transfer proteins (nsLTPs), alpha-amylase/trypsin inhibitors, prolamin storage proteins of cereals), or the papain superfamily of cysteine proteinases (Table 2.1). The pathogenesis-related (PR) proteins represent a heterogeneous collection of 14 plant protein families that are all involved in plant resistance to pathogens or adverse environmental conditions.[2] Many plant food allergens are homologous to such PR proteins (Table 2.2).[3,4] Storage proteins are the cause of well-known allergic reactions to peanuts and cereals.[3] PR proteins are responsible for pollen–fruit or latex–fruit cross-reactive syndromes.[3,4] In addition, there are some structural and metabolic plant protein families which harbour allergenic proteins such as the profilins or isoflavone reductase homologues (Table 2.3).[3] In animal-derived food products one finds a much smaller variety of allergens. Seafood contains

Table 2.1 Allergenic cupins, prolamins and cystein proteinases

Protein superfamily/family	Representative allergenic proteins
Cupin superfamily	
Vicilins (7S seed storage proteins)	Ara h 1 (peanut), alpha-subunit of beta conglycinin (soybean), Jug r 2 (English walnut), Len c 1 (lentil), Ana o 1 (cashew), Ses i 3 (sesame)
Legumins (11S seed storage proteins)	Ara h 3 and Ara h 4 (peanut), glycinin subunits (soybean), Cor a 9 (hazelnut), AMP (almond)
Prolamin superfamily	
2S albumins	Sin a 1 (yellow mustard), Ber e 1 (Brazil nut), Jug r 1 (English walnut), Ses i 2 (sesame), Ara h 2, Ara h 6, Ara h 7 (peanut)
Non-specific lipid transfer proteins	Pru p 3 (peach), Mal d 3 (apple), Pru ar 3 (apricot), Cor a 8 (hazelnut), Aspa o 1 (asparagus), Lac s 1 (lettuce)
Alpha-amylase/protease inhibitors	Hor v 15 (barley), Sec c 1 (rye), RAPs (rice allergenic proteins)
Cereal prolamins	Tri a 19 (wheat), Sec c 20 (rye), Hor v 21 (barley)
Papain superfamily of cysteine proteases	
Papain-like cysteine proteases	Act c 1 (kiwi), papain (papaya), bromelain (pineapple), P34/Gly m Bd 30K (soybean)

Table 2.2 Allergenic pathogenesis-related proteins

Plant pathogenesis-related protein families	Representative PR-like allergens
PR-2 (beta-1,3-glucanases)	beta-1,3-glucanases from banana, potato, and tomato
PR-3 (class I chitinases)	Pers a 1 (avocado), Cas s 5 (chestnut)
PR-4 (hevein- and Win-like chitinases)	Bra r 2 (turnip)
PR-5 (thaumatin-like proteins)	Pru av 2 (cherry), Mal d 2 (apple), Cap a 1 (bell pepper),
PR-9 (peroxidases)	Tri a Bd 36K (wheat)
PR-10 (Bet v 1 homologues)	Mal d 1 (apple), Pru av 1 (sweet cherry), Api g 1 (celery), Cor a 1.04 (hazelnut), Gly m 4 (soybean)
PR-14 (non-specific lipid transfer proteins)	Pru p 3 (peach), Mal d 3 (apple), Pru ar 3 (apricot), Cor a 8 (hazelnut), Aspa o 1 (asparagus), Lac s 1 (lettuce)

Table 2.3 Allergenic members of other plant protein families

Other protein families	Examples of allergens
Profilins	Mal d 4 (apple), Pru av 4 (sweet cherry), Apig 4 (celery), Cor a 4 (hazelnut), Lyc e 1 (tomato), Ara h 5 (peanut), Gly m 3 (soybean), Ana c 1 (pineapple)
Kunitz-type protease inhibitors	20 kDa Kunitz soybean trypsin inhibitor, Sola t 2, Sola t 3, Sola t 4 (potato)
Lectins	31 kDa peanut agglutinin
Patatin-like proteins	Sola t 1 (potato)
Phenylcoumaran benzylic ether reductases	Pyr c 6 (pear)
Oleosins	peanut oleosin
Beta-fructofuranosidases	Lyc e 2 (tomato)
Subtilisin-like serine proteases	Cuc m 1/cucumisin (melon)
FAD[1] containing oxidases	Api g 5 (celery)

[1]Flavin adenine dinucteotide

basically either the calcium-binding parvalbumins or the tropomyosins that play a key regulatory role in muscular contractions, and avian and mammalian food products contain the well-characterized egg and milk allergens (Table 2.4).

2.2 Plant food allergens

2.2.1 The cupin superfamily

The cupins form a large and functionally highly diverse superfamily of proteins whose evolution can be traced from bacteria to eukaryotes including animals and higher plants.[5] The term cupin (from the Latin term *cupa* for a small barrel or cask) has been given to a beta-barrel structural domain identified in these proteins.[6] These proteins have been divided into two categories, one being the single-domain cupins that contain only one conserved cupin domain. The two-domain bicupins that include the 7S and 11S globular seed storage proteins, major components of the human diet, are thought to have evolved from the duplication of this single microbial sequence.[7] Seed storage globulins from various legumes and tree nuts are highly abundant proteins comprising up to 50% of the total seed protein. They provide resources for the growth of the seedling. The globulins can be divided into two groups based on their sedimentation coefficient, the 7S vicilin-type globulins and the 11S legumin-type globulins. They have been studied in the most detail in legumes, particularly in soy.

Table 2.4 Animal-derived food allergens

Protein family	Examples of allergens
Calcium-binding EF hand proteins	
Parvalbumins	Gad c 1 (cod), Gad m 1 (cod), Sal s 1 (salmon), Cyp c 1 (carp)
Tropomyosins	Pen i 1 (Indian shrimp), Par f 1 (Taiwanese shrimp), Pen a 1 (brown shrimp), Cha f 1 (common crab), Pan s 1 (spiny lobster), Hom a 1 (American lobster), Cra g 1 and Cra g 2 (Pacific oyster), Tur c 1 (a gastropod), Tod p 1 (squid), Per v 1 (tropical green mussel)
ATP:guanido phosphotransferases	
Arginine kinases	Par f 1 (*Parapenaeus fissurus* shrimp) Pen m 2 (*Penaeus monodon* shrimp)
Glycoside hydrolase family 22	
Alpha-lactalbumins	Bos d 4 (cows' milk)
Lysozymes	Gal d 4 (hen's egg)
Lipocalins	
Beta-lactoglobulins	Bos d 5 (cows' milk)
Serum albumins	Bos d 6 (cows' milk and meat) Gal d 5 (hen's egg and chicken meat)
Immunoglobulins	Bos d 7 (bovine IgG)
Alpha/beta-caseins	Bos d 8 (cow's milk)
Transferrins	lactoferrin (cow's milk) Gal d 3 (ovotransferrin, hen's egg)
Kazal-type serine protease inhibitors	
Ovomucoids	Gal d 1 (hen's egg ovomucoid)
Serpins	
Ovalbumins	Gal d 2 (hen's egg ovalbumin)

Vicilins

Molecular characteristics

The mature 7S globulins are homotrimeric proteins of about 150–190 kDa but may undergo reversible aggregation into hexamers, depending on the ionic strength of the ambient medium.[8] The molecular weights of the subunits range from about 40–80 kDa. Vicilins lack cysteines and, therefore, contain no disulfide bonds. Their detailed subunit compositions vary considerably due to differences in proteolytical processing and glycosylation.[9] Among the vicilins are two major variants in size, the regular vicilins with subunits of about 50 kDa and the large vicilins of the convicilin and beta-conglycinin type which have additional N-terminal insertions of about 80–190 residues in length.[10] The three-dimensional structures of three 7S globulins have been determined: canavalin from jack bean,[11,12] phaseolin from French bean,[13] and the beta subunit of beta-conglycinin from soybean.[14] These structures show that the trimeric vicilins are disk shaped.

Allergenic vicilins

Probably the best characterized allergenic vicilin is the major peanut allergen Ara h 1, one of the main storage proteins of the peanut, and recognized by serum IgE from over 90% of peanut allergic patients.[15] Its linear IgE-epitopes have been mapped and shown to consist of 23 independent binding sites.[16] Mutational analysis of the immunodominant epitopes revealed that single amino acid changes within these peptides had dramatic effects on IgE-binding characteristics. The 63.5 kDa Ara h 1 forms highly stable homotrimers, a characteristic that may be important for its allergenic properties.[17] The majority of the IgE-binding epitopes are located in the area of the subunit–subunit contacts. These sites are protected from protease degradation, indicating that the protein structure may play a significant role in overall allergenicity.[18] Ara h 1 purified from peanuts and subjected to dry heat treatments at different temperatures exhibited IgE-binding properties similar to those found for native Ara h 1, indicating that the allergenicity of Ara h 1 is heat-stable although the conformation of native Ara h 1 undergoes significant heat-induced change.[19] However, compared with dry roasted peanuts, the relative amount of Ara h 1 was reduced in fried and boiled preparations as practised in China which resulted in a significant reduction of IgE-binding intensity.[20] Clinically relevant cross-reactivity between pea and peanut does occur. Vicilin homologues in pea and peanut (Ara h 1) are the molecular basis for this cross-reactivity.[21]

Beta-conglycinin, a glycoprotein of 180 kDa, is a major soybean globulin that comprises about 50% of the 7S fraction. It forms trimers that are composed of three subunits, alpha, alpha′, and beta. These have molecular weights of 76, 72, and 53 kDa, respectively.[22] Beta-conglycinin is a heterogenous mixture of different molecular species resulting from the various combinations of the three subunits. One of the major allergenic proteins in the soybean 7S-globulin fraction was identified as the alpha subunit of beta-conglycinin, also known as Gly m Bd 60K.[23]

To date, several other 7S globulin family members have been identified as food allergens. These include Jug r 2, a 47 kDa allergenic vicilin from English walnuts;[24] Len c 1, a gamma-vicilin subunit from lentils;[25] Ana o 1 from cashew;[26] and Ses i 3 from sesame seeds.[27]

Legumins

Molecular characteristics

The mature 11S globulins that are initially assembled as intermediate trimers are hexameric.[28] They comprise six subunit pairs that interact non-covalently and are arranged in an open ring structure of approximately 360 kDa. Each subunit pair consists in turn of an acidic 30–40 kDa polypeptide linked by a disulfide bond to a basic polypeptide of ~ 20 kDa. Each subunit pair is synthesized as a single precursor that is post-translationally cleaved after disulfide bridge formation into an acidic and a basic polypeptide chain. The basic or C-terminal chain of the 11S legumins is related to the C-terminal

region of the 7S vicilins. Legumins are rarely glycosylated. The three-dimensional structure of proglycinin from soybean, an 11S globulin precursor, has been determined.[29]

Allergenic legumins

Two important peanut allergens belong to the 11S legumin-like seed storage proteins. The 14 kDa Ara h 3 represents the N-terminal part of a 57 kDa peanut glycinin subunit. A cDNA clone encoding the full-length protein was isolated and the linear IgE-binding epitopes of Ara h 3 were mapped.[30] The recombinant Ara h 3 was expressed in *E. coli* and was recognized by serum IgE from about 50% of a peanut allergic patient population.[31] The cDNA clone of Ara h 4 encodes a protein of 35.9 kDa with a sequence similarity of 70% to the glycinin family of proteins.[32] The structure of Ara h 3 is similar to that of soybean glycinin, and both the basic and the acidic subunit can bind IgE from peanut allergic individuals.[33]

The 11S fraction of soybean proteins consists almost entirely of glycinin, the principal soy globulin. Soybean glycinin is a major seed storage protein that makes up more than 20% of the soybean seed by dry weight and 35–40% of total soy protein. Native glycinin is a 350 kDa hexamer composed of six non-identical subunits. The five known subunits (G1–G5) of soybean glycinins are encoded by a small gene family. IgE epitopes for the soybean glycinin G1 acidic chain have been determined and found to be similar to IgE epitopes of the peanut glycinin Ara h 3.[34] Moreover, each basic chain from the five glycinin subunits reacted with IgE from soybean allergic individuals to a similar extent.[35] The linear IgE epitopes of the basic chain of the glycinin G2 subunit were mapped and a three-dimensional model was developed using the known structure of the 7S phaseolin.[36] Additional 11S allergenic plant food globulins were identified as Cor a 9 in hazelnut,[37] in coconut and walnut,[38] and as AMP (almond major protein) in almond.[39]

2.2.2 The prolamin superfamily

The prolamin superfamily includes the major storage proteins of cereal grains, with the exception of oats and rice (in which the major storage proteins are 11S globulin-like), and also several groups of low molecular mass cysteine-rich proteins. Prolamins were originally characterized by their solubility in alcohol–water mixtures and their high content of proline and glutamine, hence the name prolamin. Allergies to prolamins in the strict sense do not occur very frequently. Gluten is the protein component of wheat flour. It consists of numerous proteins. Two different types are responsible for different physical properties of dough: the glutenins, which are primarily responsible for the elasticity, and the gliadins, which contribute to the extensibility.[40] The glutenins are of two different types, termed low (LMW) and high molecular weight (HMW) glutenins. Although IgE reactivity to LMW glutenin exists, coeliac disease is not an IgE-mediated type I allergy but a genetically-

determined chronic inflammatory intestinal disease induced by wheat gluten.[41] The interested reader is referred to Chapter 14 by F.W. Janssen. Alpha-gliadin, gamma-gliadin and low molecular weight subunits have been shown to bind IgE from patients with dietary allergy to wheat.[42,43]

While true prolamins are restricted to grass seeds, they form part of a larger protein superfamily that contains proteins with much wider distribution. The broader definition of the prolamin superfamily now includes several groups of proteins that contain many important plant food allergens: 2S albumin seed storage proteins of dicotyledonous plants, ns LTPs and inhibitors of alpha-amylase and trypsin from cereal seeds. All of these proteins can be described as low molecular weight cysteine-rich proteins. They have similar three-dimensional structures that are rich in alpha helices and are defined in the SCOP structural database as an all alpha class of proteins with a structure made up of four helices, a folded leaf, and a right-handed superhelix (scop.mrc-lmb.cam.ac.uk/scop).[1] Interestingly, all three protein families also have defensive roles against pathogens or pests, at least in some species. The soybean hydrophobic protein that is responsible for respiratory allergy to soybeans possesses the characteristic eight cysteine residue skeleton forming four intra-chain disulfide bridges, illustrating the relationship to the similar folds of the 2S albumins, nsLTPs and the cereal trypsin/alpha-amylase inhibitors.[44,45]

2S albumins

Molecular characteristics

The 2S albumins constitute a family of structurally related homologous proteins and form a major group of storage proteins in many dicotyledonous plant species.[46] Many 2S albumins possess high levels of sulfur-containing amino acid residues. Typical 2S albumins, such as the napins from the Brassicaceae or the Brazil nut 2S albumin, are heterodimeric proteins consisting of two polypeptide chains with molecular weights of about 4 and 9 kDa. They are synthesized as single precursor proteins that are proteolytically cleaved with the loss of a linker peptide and short peptides from both the N- and the C-terminus.[47] These proteins are rich in alpha-helices and are held together by four disulfide bonds involving eight conserved cysteine residues.[46] The major role of 2S albumins is as seed storage proteins for the developing seed. They appear to be stable to proteolysis and can bind lipids. In addition, antifungal activity against a number of plant-pathogenic fungi has been shown for the napins from radish.[48]

Allergenic 2S albumins

Water-soluble allergenic 2S albumin storage proteins occur in many cultivated species of dicotyledonous plants and include Sin a 1 from yellow mustard seeds (*Sinapis alba*),[49] Bra j 1 from oriental mustard seeds (*Brassica juncea*),[50] the napin BnIII/Bra n 1 from oilseed rape (*Brassica napus*),[51] Ber e 1 from Brazil nut (*Bertholletia excelsior*),[52] Jug r 1 from the English walnut (*Juglans*

regia),[53] and SFA-8/SSA from sunflower seeds (*Helianthus annuus*).[54] For Jug r 1 one major linear IgE epitope and its critical core amino acid residues could be identified.[55] Allergenic 2S albumins were also identified in cashew nuts[56] and as Ses i 2, the clinically most important allergen of sesame seeds.[27,57] Among the peanut allergens, the 17.5 kDa glycoprotein Ara h 2,[58] the 14.5 kDa Ara h 6,[32] and the 15.8 kDa Ara h 7[32] belong to the conglutin protein family that is related to the 2S albumin family of seed storage proteins. Ara h 2 has significant homology to trypsin inhibitors and bifunctional trypsin/alpha-amylase inhibitors. It was shown that Ara h 2 functions as a trypsin inhibitor and that dry roasting caused a 3.6-fold increase in this activity.[59]

Non-specific lipid transfer proteins (ns LTPs)

Molecular characteristics

The LTP family is made up of low molecular weight (7–9 kDa) monomeric proteins. They are able to catalyze the transfer of lipids between liposomes and membranes *in vitro* and there is increasing evidence that their *in vivo* role may be in cutin biosynthesis.[60] LTPs usually accumulate in the outer epidermal layers of plant organs, thus explaining the stronger allergenicity of peels as compared to the pulps of Rosaceae fruits. LTPs are made up of a bundle of four alpha-helices with a lipid binding cavity in the center. LTPs are resistant to harsh pH changes or thermal treatments and can refold to their native structure on cooling. Plant LTPs have been identified as important food allergens. LTPs have also been listed as family 14 of the pathogenesis-related proteins.[2] Their common structural features, such as eight conserved cysteines forming four disulfide bridges, basic iso-electric point, and high similarity in amino acid sequence, are the basis of their allergic clinical cross-reactivity.[61] LTPs are potentially severe food allergens because of their high resistance to pepsin digestion.[62]

Allergenic nsLTPs

The nsLTPs have an even wider distribution than the 2S albumins with sequences being available from seeds, fruits, and vegetables. In the Mediterranean area, Rosaceae fruits, particularly peach, are among the most frequent causes of food-induced allergic reactions. LTPs represent major plant food allergens in populations in these regions that are virtually free of Fagales trees, in particular birch. LTPs have been identified as major peach (Pru p 3),[63,64] apple (Mal d 3),[65] and apricot (Pru ar 3)[66] allergens in Mediterranean populations. In general, the LTP allergens of the Prunoideae subfamily of the botanical family of the Rosaceae whose sequence identities are between 88 and 98% are highly cross-reactive. In addition to peach and apricot, this subfamily includes sweet cherry and the European plum, whose LTPs have been described as the allergens Pru av 3[67] and Pru d 3,[68] respectively. LTPs seem to be able to sensitize subjects who have not been previously sensitized by pollen allergens. The full spectrum of cross-reactivity with different foods containing LTPs has yet to be investigated. Some of the

patients sensitized to peach LTP are also clinically allergic to tomato and corn, whereas the hazelnut LTP Cor a 8 was described as highly cross-reactive with the peach LTP.[69] The chestnut LTP, Cas s 8, was found to share only some IgE epitopes with the corresponding peach allergen.[70] The list of allergenic nsLTPs is continuously expanding, encompassing more seeds (Zea m 14 from corn),[71] vegetables (Aspa o 1 from asparagus),[72] and fruits (Vit v 1 from grape).[73] Although allergic reactions to lettuce are not frequently reported, its nsLTP was shown to cause anaphylaxis in susceptible individuals and, therefore, received the nomenclature designation Lac s 1.[74]

The cereal superfamily of alpha-amylase and protease inhibitors

Molecular characteristics

Inhibitors of this family are present in a range of cereals including wheat, barley, rye, rice, and corn. They generally have subunits of about 12–16 kDa, exist in monomeric, dimeric, and tetrameric form, and possess inhibitory activity against trypsin or various types of alpha-amylases from insects and fungi, or they may be bifunctional.[75] These allergens are capable of sensitizing susceptible atopic patients by ingestion or by inhalation.[76] The inhibitors of this family present in wheat, barley, and rye are selectively extracted in chloroform/methanol (CM) mixtures which is reflected in their designation as CM proteins. They include monomeric inhibitors of trypsin and dimeric and tetrameric inhibitors of alpha-amylase.

Allergenic alpha-amylase/trypsin inhibitors

Allergens within the cereal superfamily of inhibitors include the glycosylated subunits of the tetrameric CM16* (CM, soluble in chloroform/methanol; 16, molecular weight in kDa; *, glycosylated) inhibitor from wheat,[77] the homologous glycoproteins CMb* (b, barley),[77] Hor v 15 (Hor v 1/BMAI-1; barley monomeric alpha-amylase inhibitor),[78] and BDP (barley dimeric protein)[79] from barley, and three members of the alpha-amylase/trypsin inhibitor family from rye flour, Sec c 1,[79] RDAI-1 (rice dimeric alpha-amylase inhibitor), and RDAI-3.[80] The best characterized allergens of this group are the alpha-amylase inhibitors of rice.[81,82] cDNA and genomic clones coding for 14–16 kDa rice allergenic proteins have been obtained.[83–85]

Cereal prolamins

The ethanol-soluble cereal prolamins, gliadins in wheat, secalins in rye, and hordeins in barley, are the major storage proteins found in the endosperm of cereal grains. They are unusually rich in proline and glutamine. Several sulfur-rich members of the prolamin superfamily were identified as allergens from wheat. The highest IgE reactivity was found for low molecular mass (LMM) glutenin followed by alpha-gliadin and gamma-gliadin.[86] The allergenic LMM glutenin was shown to contain a number of pentapeptide repeat motifs, Gln-Gln-Gln-Pro-Pro.[87] Alpha-gliadin, gamma-gliadin, and LMM glutenin

subunits have similar sequences comprising a repetitive N-terminal domain and a non-repetitive C-terminal domain that contains the conserved cysteine residues typical for the prolamin superfamily. In contrast to the sulfur-rich alpha- and gamma-gliadins, omega-gliadins consist almost entirely of repeats and are characterized by a low content of sulfur-containing amino acid residues and a lack of cysteine residues. Nevertheless, omega-5 gliadin (Tri a 19) has been shown to be a significant allergen in young children with immediate allergic reactions to ingested wheat.[88] Omega-5 gliadin from wheat cross-reacts with gamma-70 and gamma-35 secalins from rye (Sec c 20)[89] and with gamma-3 hordein from barley (Hor v 21).[89] In addition to IgE-mediated type I allergy, cereal prolamins are also of crucial importance in coeliac disease. Coeliac disease is a T-cell mediated chronic inflammatory bowel disorder with an autoimmune component. This disease is precipitated by the gluten storage proteins of wheat. The alcohol-soluble fraction, gliadin, has been most studied, but most or all gluten proteins are likely to be involved, as are similar proteins of barley (hordeins) and rye (secalins).[41]

2.2.3 Papain superfamily of cysteine proteinases

This superfamily of proteins contains three groups of cysteine proteases that are distantly related: the papain group, the bleomycin hydrolase group, and the calpain group.[90] Proteases of the papain superfamily possess a nucleophilic cysteine residue in their active site that attacks the peptide bonds of proteins to be cleaved. They are synthesized as preproenzymes and are located in lysosomes or analogous organelles. These proteases appear to have arisen early during eukaryotic evolution.

Peptidases C1A, papain-like cysteine proteases

Molecular characteristics

Peptidases are grouped into clans and families. Clans are groups of families for which there is evidence of common ancestry. Families are grouped by their catalytic type, the first character representing the catalytic type, so C stands for cysteine. Cysteine proteases are divided into clans that consist of proteins which are evolutionary related.[91] Clan CA contains the families of papain (C1), calpain (C2), streptopain (C10), and the ubiquitin-specific peptidases (C12, C19). The subfamily C1A of cysteine proteases consists of papain and related plant proteinases such as chymopapain, caricain, bromelain, actinidin, ficin, and aleurain. These proteases occur widely in plants where they display a variety of functions including digestion of seed storage proteins, ageing of flowers and leaves, or processing of enzyme precursors.[92] Cysteine proteases have also been associated with resistance to lepidopteran pests.[93] The high levels of cysteine proteases in fruits is consistent with such a protective role. The most widely studied cysteine protease is papain from the papaya fruit. Mature cysteine proteases have a molecular weight of about 25–35 kDa. They are typically stabilized by three disulfide bonds and tend to be stable against denaturation.

Allergenic papain-like cysteine proteases
The family of cysteine proteases includes several proteolytic plant enzymes such as papain from papaya, ficin from fig, bromelain from pineapple, and actinidin from kiwi. Patients with allergic reactions to kiwi fruit produced IgE that was also reactive to bromelain and papain.[94] The cross-reactivity of papain-like allergens was further shown by cross-inhibition experiments including ficin and papain.[95] Actinidin, a thiol protease of kiwi (*Actinidia chinensis*) that accounts for about 50% of the soluble protein of the fruit, was identified as its major allergen and designated Act c 1.[96] P34/Gly m Bd 30K, a soybean seed storage vacuole protein, belongs to the plant thiol protease family and is an outlying member of the papain superfamily. P34 has lost its protease activity and as a glycine has replaced the cysteine residue in the active site. It has been shown to be associated with the storage vacuoles of soybean.[97] P34 has been identified as a major soybean allergen in patients with atopic dermatitis and food allergy.[98] As P34/Gly m Bd 30K is a major soybean allergen, it was also referred to as Gly m 1 by several research groups.[99,100] However, the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies (www.allergen.org) lists a LMW respiratory allergen from soybean hull as Gly m 1.[101,102] The activity of Gly m Bd 30K as a cysteine protease could not be demonstrated and an alternative function of binding syringolide elicitor has been proposed.[103]

2.2.4 Plant pathogenesis-related proteins

Pathogenesis-related proteins (PRs) are defined as proteins that are encoded by the host plant and induced specifically in response to infections by pathogens such as fungi, bacteria, or viruses, or by adverse environmental factors. PRs do not form a superfamily of proteins but represent a collection of unrelated protein families which function as part of the plant defense system. To date, PRs have been classified into 14 families.[2] Many plant food allergens are homologous to proteins that are included in PR families (Table 2.2).

PR-2: beta-1,3-glucanases
Molecular characteristics
O-Glycosyl hydrolases are a widespread group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety.[104] A classification system for glycosyl hydrolases, based on sequence similarity, has led to the definition of 85 different families.[105] Plant beta-1,3-glucanases are monomers with a molecular weight in the 25–35 kDa range. Most plant beta-1,3-glucanases are endoglucanases with the potential to partially degrade fungal cell walls by hydrolyzing the beta-1,3-glucan fibers of the growing hyphae of filamentous fungi.

PR-2-like allergens

The latex–fruit syndrome describes allergy to *Hevea brasiliensis* latex products and an associated allergy to certain plant foods, especially avocado, banana, chestnut, fig, and kiwi.[106] It is speculated that the inhalation of PR-2 proteins is one of the causes for the cross-reactivity of latex with plant foods. Homologous proteins present in these fruits and vegetables are regarded as responsible for the observed cross-reactivity. The basic beta-1,3-glucanases from *Hevea* latex were recognized by specific IgE from food-allergic patients suffering from hypersensitivity to banana, potato, and tomato – rather than to latex products.[107] Certain isoforms of the allergenic beta-1,3-glucanases from *H. brasiliensis* are glycosylated. The carbohydrate moieties seem to harbour the major IgE epitopes and may be responsible for the observed cross-reactivities to beta-1,3-glucanases from fruits and vegetables.[108] Banana is a commonly reported cross-reactive food included in the latex–fruit syndrome. An abundant beta-1,3-endoglucanase was isolated from ripe bananas and its cDNA was cloned.[109] Its allergenic potential has not yet been determined.

PR-3: class I chitinases

Molecular characteristics

Chitinases are enzymes that catalyze the hydrolysis of the beta-1,4-N-acetyl-D-glucosamine linkages in chitin polymers. Chitinases belong to glycoside hydrolase families 18 or 19.[105] Chitinases of family 19 (also known as classes IA or I and IB or II) are endochitinases from plants that degrade chitin, a major structural component of the exoskeleton of insects and of the cell walls of many pathogenic fungi.[110] Class I chitinases contain an N-terminal so-called hevein domain with putative chitin-binding properties.[111]

PR-3-like allergens

Relevant allergens of chestnut and avocado have been identified as class I chitinases.[112] Pers a 1, an endochitinase and major allergen from avocado, was cloned and expressed in the yeast *Pichia pastoris*.[113] Two major IgE-binding proteins from banana were identified as class I chitinases.[114] Class I chitinases have an N-terminal domain that is homologous to hevein, one of the major allergens of latex. Hevein (Hev b 6.02) occurs in *Hevea* latex cleaved from prohevein and shares high sequence similarities with the hevein domain of class I chitinases. Hevein contributes to the increased prevalence of fruit allergies in individuals allergic to latex. Sensitization to Pers a 1 seems to be caused by hevein in the majority of patients allergic to latex.[115] Class I chitinases are now regarded as the major pan-allergens in fruits associated with the latex–fruit syndrome. These enzymes are inactivated by heat treatment which may explain why only freshly consumed fruits are associated with the latex–fruit syndrome. The hevein-like domains of allergenic class I chitinases seem to include all the main IgE epitopes.[116]

PR-4: Hevein- and Win-like chitinases

Molecular characteristics

The 4.7 kDa hevein (Hev b 6.02), a small antifungal protein that is found in high concentrations in the latex of *Hevea* trees, is one of the most important latex allergens.[117] The wound-induced proteins Win1 and Win2 from potato were found to include a hevein-like domain and revealed high similarity to the hevein precursor prohevein.[118] Two PR-4-type proteins were isolated from tobacco mosaic virus infected leaves[119] and found to constitute another family of chitinases.[2] Their primary structure indicated 50% sequence similarity to Win1 and Win2. However, these tobacco PR-4 proteins lack the N-terminal hevein-like domain of the Win proteins and Hev b 6.[120]

PR-4-like allergens

Wounding and chemical treatment of turnip (*Brassica rapa*) plants induced the expression of an 18.7 kDa allergen that was recognized by IgE of 80% of 60 sera of natural rubber latex allergic individuals.[121] It seems likely that prohevein and hevein were the primary sensitizers and the IgE binding to the turnip allergen was a result of allergen cross-reactivity. Peptide sequencing of the turnip allergen revealed 70% identity to prohevein and high similarities to wound-induced proteins from tomato (74%) and potato (71%). Cloning of a hevein-like fruit protein from mature elderberry (*Sambucus nigra*) fruits demonstrated the occurrence of a hybrid gene encoding a protein consisting of the N-terminal hevein-like domain of PR-4 proteins and the C-terminal domain of class V chitinases.[122] The allergenic properties of this elderberry protein remain to be investigated.

PR-5: thaumatin-like proteins (TLPs)

Molecular characteristics

The family of PR-5 proteins comprises unique proteins with diverse functions. Due to the sequence homologies between PR-5 proteins and thaumatin, an intensely sweet tasting protein isolated from the fruits of the West African rain forest shrub *Thaumatococcus daniellii*, members of this family of proteins are referred to as thaumatin-like proteins (TLPs). TLPs were classified by Dudler *et al.*[123] into three groups: (i) those produced in response to pathogen infection, (ii) those produced in response to osmotic stress, also called osmotins; and (iii) antifungal proteins present in cereal seeds. TLPs are generally resistant to proteases and pH- or heat-induced denaturation. This may be due to the presence of 16 conserved cysteines that form eight disulfide bridges. The crystal structure of three PR-5-type proteins, PR-5d from tobacco,[124] zeamatin from *Z. mays*,[125] and thaumatin from *T. daniellii* [126], are known to date.

PR-5-like allergens

Mal d 2 is an important allergen of apple that is associated with IgE-mediated symptoms in apple-allergic individuals. The cDNA sequence of Mal d 2 was

expressed in *Nicotiana benthamiana* plants using a recombinant tobacco mosaic viral vector.[127] Purified recombinant Mal d 2 displayed ability to bind IgE from apple-allergic individuals equivalent to natural Mal d 2. In addition, the recombinant thaumatin-like Mal d 2 exhibited antifungal activity against *Fusarium oxysporum* and *Penicillium expansum*, implying a function in plant defense against fungal pathogens. In sweet cherry (*Prunus avium*), a 23.3 kDa thaumatin-like protein was identified as a major allergen, designated Pru av 2, and its cDNA was cloned.[128] The N-terminal sequence of a 23 kDa bell pepper allergen was found to be identical to a corresponding portion of the osmotin-like protein P23 from tomatoes[129] and the complete coding sequence of Cap a 1 was obtained (EMBL Accession No. AJ297410).[130] Recently, a TLP was identified as a minor allergen of grape with an amino acid sequence highly similar to Mal d 2 and Pru av 2,[131] and a TLP from kiwi was described as the allergen Act c 2.[132]

PR-9: peroxidases

Molecular characteristics

Peroxidases are heme-containing enzymes that utilize H_2O_2 for a series of oxidative reactions. Heme peroxidases include two superfamilies, one found in bacteria, fungi, and plants, and the other found in animals. The first one consists of three major classes.[133] Class III includes the secretory plant peroxidases that are monomeric glycoproteins and contain four conserved disulfide bridges and two calcium ions. They share the same architecture, two all alpha domains between which the heme group is located. Peroxidases have been thoroughly researched in higher plants where their activities are correlated with a large number of growth, developmental, and defense processes. Specific lignin-forming peroxidases, induced by pathogens and involved in plant defense against pathogens, have been designated PR-9.[134]

PR-9-like allergens

A prominent 36 kDa allergen was isolated from wheat flour and identified by N-terminal and internal amino acid sequences as a peroxidase.[135] This wheat allergen, also referred to as Tri a Bd 36K, was characterized as a glycoprotein. The carbohydrate moiety seemed to be at least partially involved in the IgE binding.[136] IgE of food-allergic patients bound to a glycosylated peroxidase from tomato whose cDNA coded for seven potential N-linked glycosylation sites.[137] In general, the role of glycosylation in the allergenicity of plant peroxidases has not yet been elucidated.

PR-10: proteins homologous to Bet v 1

Molecular characteristics

The PR-10 family is a ubiquitous group of defense-related and intracellular proteins that are over expressed after fungal or bacterial infections. Bet v 1, the major birch pollen allergen, was the first allergen described that was homologous to PR-10 family members.[138] PR-10 family members have been

identified in a large number of plant species, both mono- and dicotyledons.[139] Many of the allergenic PR-10 proteins exist in various isoforms that differ in their IgE-binding capacities.[140–142] Crystal structures have been determined for Bet v 1.2801,[143] the hypoallergenic isoform Bet v 1l (also designated Bet v 1.1001),[144] and for Pru av 1, the Bet v 1 homologue from cherry.[145] These proteins function most likely as plant steroid hormone transporters.[144,145]

PR-10-like allergens

The oral allergy syndrome (OAS), an association of food allergies to fruits, nuts, and vegetables in patients with pollen allergy, is the most frequent clinical syndrome caused by cross-reactive IgE antibodies. In the majority of the cases, OAS in individuals allergic to tree pollen is caused by IgE cross-reactivity between the major birch pollen allergen Bet v 1 and its homologous proteins. Bet v 1-homologous food allergens have been identified in fruits of Rosaceae species (Mal d 1 in apple[146], Pru av 1 in sweet cherry[147], Pru ar 1 in apricot[148], Pyr c 1 in pear[149]), or somatic tissues, vegetables of Apiaceae species (Api g 1 in celery[150], Dau c 1 in carrot[151], pcPR1 and pcPR2 in parsley[152]). Additional Bet v 1-homologous proteins capable of binding anti-Bet v 1 IgE were described as pSTH-2 and pSTH-21 from potato.[152] Recently, the Bet v 1-related major allergen of hazelnuts Cor a 1.04 has been characterized in detail.[153] The Bet v 1 homologous allergen SAM22/Gly m 4 of soybean was found to be responsible for inducing an OAS of extraordinary severity and severe systemic reactions in some birch pollen allergic individuals.[154]

PR-14: non-specific lipid transfer proteins

See prolamin superfamily, pp. 28–29.

2.2.5 Allergens from other plant protein families

Profilins

Molecular characteristics

The actin cytoskeleton of a cell is composed of a network of actin filaments whose organization is regulated by a number of actin-binding proteins. One of these proteins is profilin, a 12–15 kDa monomeric actin-binding protein. The allergenic nature of profilin was first discovered by the ability of the birch pollen profilin Bet v 2 to bind IgE from birch pollen-allergic individuals.[155] Today, profilins are well-known ubiquitous cross-reactive plant allergens.[156]

Allergenic profilins

In most cases, patients who are sensitized to pollen profilins characteristically react with a wide range of profilins from nutritive allergen sources. Profilins are involved in the celery–mugwort–spice syndrome.[157] A hazelnut profilin, Cor a 2, was identified as a relevant IgE-binding protein for a minority of pollen–nut allergic individuals.[158] Grass pollen profilin-allergic patients also reacted to homologues in celery and carrots, Api g 4 and Dau c 4, respectively.[159]

IgE of food-hypersensitive tree pollen-allergic individuals cross-reacted with profilins from apple (Mal d 4), pear (Pyr c 4), carrot (Dau c 4), celery (Api g 4), and potato.[158] An anaphylactic reaction to lychee fruit was mediated by a profilin, Lit c 1.[160] Furthermore, profilins were identified as causing allergic reactions to tomato (Lyc e 1)[161] and to pumpkin seeds.[162] The cDNAs coding for profilins from peanut,[32] soybean,[163] and celery[164] were cloned, produced as recombinant allergens, and designated Ara h 5, Gly m 3, and Api g 4, respectively. Furthermore, the allergenicity of profilins from pear, cherry (Pr u av 4), and celery,[165] and from banana (Mus xp 1) and pineapple (Ana c 1)[166] have been studied in detail.

Kunitz-type protease inhibitors

Molecular characteristics

The soybean trypsin inhibitor (Kunitz) family is one of the numerous families of proteinase inhibitors.[167] It comprises plant proteins which have inhibitory activity against serine proteinases from the trypsin and subtilisin families, thiol proteinases, and aspartic proteinases, as well as some proteins that are probably involved in seed storage. All the actively inhibitory members contain two disulfide bridges. The Kunitz family of trypsin inhibitors is present in a range of legume species and has been characterized in most detail from soybean. The soybean inhibitors have a molecular weight of about 21 kDa and a single reactive site for trypsin. Soybean trypsin inhibitor inhibits the growth of lepidopteran and coleopteran larvae.[168]

Allergenic Kunitz protease inhibitors

The 20 kDa Kunitz soybean trypsin inhibitor bound IgE of 20% of soy challenge positive patients, indicating that this protein is a minor allergen.[169] However, the Kunitz soybean trypsin inhibitor was also reported to induce food anaphylaxis.[170] IgE-binding potato proteins with molecular weights from 16–20 kDa were identified as protease inhibitors belonging to the family of soybean trypsin inhibitors and designated Sola t 2, Sola t 3, and Sola t 4.[171]

Lectins

Molecular characteristics

Lectins, also known as plant agglutinins, are a class of proteins that bind to specific sequences of sugar determinants on glycoproteins. Lectins are found in seeds, especially those of legumes. Some lectins react unspecifically with the carbohydrate moieties of IgE, induce histamine release and can thus induce allergy-like symptoms.[172]

Allergenic lectin

The 31 kDa peanut agglutinin was identified as a lectin that is specifically recognized by IgE from a minority of peanut allergic patients.[173]

2.2.6 Emerging plant food allergens

Several of the now well-accepted allergen families, such as the class I chitinases, the nsLPPs or, more recently, the thaumatin-like allergens have started out with descriptions of a single allergenic member of the respective protein family. Over time, evidence has been collected that resulted in properly establishing these allergen families. As the field of allergen identification progresses, more allergens are discovered. Whether these allergens will remain the subject of case studies or serve as the building blocks for further allergen families, only time will tell. Sola t 1, a patatin storage protein, was described as a novel allergen of potato tuber.[174] Pyr c 6, a Bet v 5-related food allergen from pear, was identified as a phenylcoumaran benzylic ether reductase.[175] A peanut oleosin that belongs to a family of proteins involved in the formation of oil bodies was suggested as a new allergen.[176] Lyc e 2, a glycosylated allergen from tomato, was characterized as a beta-fructofuranosidase.[177] Cuc m 1/cucumisin, a subtilisin-like allergenic serine protease of melon, was proposed as a potential pan-allergen.[178] Api g 5, a glycoprotein allergen from celery with homology to flavin adenine dinucleotide-containing oxidases, was used to show that cross-reactive carbohydrates were capable of eliciting allergic reactions *in vivo*.[179] Genetically modified plants may become a potential source of allergens.[180] The classic example that has received extensive publicity was that of the introduction of a Brazil nut 2S albumin allergen in transgenic soy.[52]

2.3 Animal derived food allergens

Seafood allergy is a serious food allergy, especially in coastal regions and fish processing communities where seafood is a common constituent of the diet. Besides peanuts and tree nuts, fish and shellfish are among the most frequent causes of IgE-mediated allergic reactions in adolescents and adults.[181] The major allergen of crustaceans such as shrimp, crab, and lobster, has been identified as the muscle protein tropomyosin.[182] Invertebrate tropomyosins are pan-allergens with significant sequence similarity identified in seafood and insects such as storage and house dust mites and cockroaches.[183] Consequently, tropomyosins are also found as aeroallergens, which raises the possibility of sensitization by the respiratory route.[182] Parvalbumins have been identified as the major allergens of fish that are recognized by IgE of more than 95% of fish-allergic individuals.[184]

Cows' milk is the first foreign antigen source ingested in large quantities in early infancy. Consequently, cows' milk allergy (CMA) is a common disease of infancy and childhood. The incidence of CMA in infancy is approximately 2–3% in developed countries.[185] Normally, children outgrow their milk allergy. After acidification or chymosin treatment, cows' milk proteins can either be found in the lactoserum (whey) or the coagulum (curd). Whey contains essentially globular proteins, the major ones being beta-

lactoglobulin, alpha-lactalbumin, and bovine serum albumin. The curd fraction comprises the caseins.[186] Beef allergy is rare. A higher prevalence of beef allergy might be expected among children allergic to milk, because both foods contain bovine serum albumin, bovine gamma globulin, and other proteins in significant quantities. Patients clinically reactive only to rare beef had decreased IgE binding to beef fractions compared with patients reactive to well-cooked beef. Therefore, patients reacting only to rare beef would not necessarily have to maintain a complete beef elimination diet.[187]

Hen's egg is another food most frequently reported to elicit allergic reactions in children.[188] Egg white protein contains 23 different glycoproteins. Ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), and lysozyme (Gal d 4) have been identified as the major allergens, but ovomucoid has been shown to be the dominant allergen.[189,190] Cross-reactions among various avian eggs have been described[191] and reactions to duck and goose egg in the absence of hen's egg allergy have also been reported.[192] Reports of allergy to bird meats are not common. Most cases have been observed in patients with 'bird-egg syndrome' that is based on the presence of alpha-livetin in egg yolk, feathers, and serum (chicken serum albumin).[193] A subset of the patients with 'bird-egg syndrome' are also allergic to chicken meat possibly due to the serum proteins present in the meat.[194] Another category of patients is allergic to chicken meat but not to egg.[195] Patients allergic to one bird's meat may be allergic to others, including game birds. However, allergy to chicken meat is quite rare and allergy to turkey is even less common.

2.3.1 Calcium-binding EF-hand proteins

Many calcium-binding proteins belong to the same evolutionary family and share a type of calcium-binding domain known as the EF-hand.[196] This type of domain consists of a twelve residue loop flanked on both sides by a 12-residue alpha-helical domain.

Parvalbumins

Parvalbumins constitute a class of calcium-binding proteins characterized by the presence of several helix-loop-helix (EF-hand) motifs. They are present in the white muscle of many fish species in relatively high amounts of up to 5 mg/g fresh weight. Parvalbumin is assumed to be important for the relaxation of muscle fibers by binding free calcium in the cells.[197] Parvalbumins are heat stable and resistant to denaturation and proteolytic digestion. The major allergenic protein of cod (*Gadus callarias*) is a 12.3 kDa parvalbumin. It was named allergen M or Gad c 1 and has been intensively studied both structurally and immunologically.[198] Recently, two distinct parvalbumin transcripts were identified in Atlantic cod suggesting that isotypic variants are generally present in fish.[199] Allergenic parvalbumins have also been described from a second cod species *Gadus morhua* as Gad m 1,[200] from the salmon *Salmo salar* as

Sal s 1,[201] from the carp *Cyprinus carpio* as Cyp c 1,[202] from tuna,[203] from the edible frog *Rana esculenta*,[204] and from mackerel.[205]

2.3.2 Tropomyosins

Tropomyosins are a family of closely related proteins present in muscle and non-muscle cells.[206] Tropomyosin plays a key regulatory role in muscle contraction together with actin and myosin. In non-muscle cells, tropomyosin is believed to play a role in the regulation of cell morphology and motility. In muscle cells, two alpha-helical tropomyosin molecules are wound around each other forming a parallel dimeric alpha-helical coiled-coil structure. Tropomyosins have a sequence (284 amino acid residues long in most isoforms) with a seven-residue ('heptad') repeat of the form *a-b-c-d-e-f-g*, where *a* and *d* are generally apolar residues. Most isoforms have been shown to have an unbroken series of 40 continuous heptads. Tropomyosin molecules bond head-to-tail with a short overlap to form an unbroken coiled-coil cable that winds around the actin helix.

Allergenic tropomyosins

Two invertebrate groups, Crustacea and Mollusca, are generally referred to as shellfish and are common constituents in the diet of many populations. Tropomyosins are heat stable cross-reactive food allergens present in crustaceans and molluscs. Tropomyosins were identified as the major allergens of shrimp by several laboratories. They include the allergenic tropomyosins Pen i 1 from the Indian shrimp *Penaeus indicus*,[207] Par f 1 from the most common Taiwanese shrimp *Parapenaeus fissurus* ,[208] Pen a 1 from the brown shrimp *Penaeus aztecus*,[209] and Met e 1 from *Metapenaeus ensis*.[210] Additional allergenic crustacean tropomyosins were described as Cha f 1 from the common crab *Charybdis feriatus*,[211] as Pan s 1 from the spiny lobster *Panulirus stimpsonii*,[212] and as Hom a 1 from the American lobster *Homarus americanus*.[213] Allergenic tropomyosins are also common in molluscs including Cra g 1[214,215] and Cra g 2[216] of the Pacific oyster *Crassostrea gigas*, Tur c 1 of the gastropod *Turbo cornutus*,[217] Tod p 1 from the squid *Todarodes pacificus*,[218] Per v 1 from the tropical green mussel *Perna viridis*,[219] Hel as 1 from the brown garden snail,[220] and the allergenic tropomyosins of the abalone *Haliotis diversicolor* and the scallop *Chlamys nobilis*.[221]

2.3.3 ATP:guanido phosphotransferases

ATP:guanido phosphotransferases are a family of structurally and functionally related enzymes that reversibly catalyze the transfer of phosphate between ATP and various phosphogens.[222] The enzymes belonging to this family include arginine kinases.

Arginine kinases
Arginine kinases catalyze the transfer of phosphate from ATP to arginine. An arginine kinase of the shrimp species *Parapenaeus fissurus* was discovered as a major and novel allergen of crustaceans and named Par f 1.[223] The homologous allergen was also isolated from the crab *Portunus trituberculotus*.[223] Another allergenic arginine kinase was identified from the shrimp species *Penaeus monodon* by two-dimensional immunoblotting, designated Pen m 2, and its cDNA was cloned.[224] Additional allergenic crustacean arginine kinases from the sand shrimp *Metapenaeus ensis*, the lobster species *Homarus gammarus*, the crawfish species *Metanephrops thomsoni*, and the crab species *Scylla serrata* were described.[224]

2.3.4 Glycoside hydrolase family 22

O-Glycosyl hydrolases hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. Glycosyl hydrolases have been classified into 85 different families based on sequence similarity.[105,225,226] Glycoside hydrolase family 22 comprises alpha-lactalbumins and lysozymes of the type C. Lysozymes type C and alpha-lactalbumins probably evolved from a common ancestral protein.

Alpha-lactalbumin, Bos d 4
Lactalbumin, which attaches to beta-galactosyltransferase to create the lactose synthetase complex, is essential for milk production.[227] Alpha-lactalbumin is a monomeric globular calcium-binding metalloprotein of 14.4 kDa, with four disulfide bridges.[228] The calcium is bound in a loop that is superficially similar to the classic EF-hand motif. Bovine alpha-lactalbumin is a major cows' milk allergen. Alpha-lactalbumin and hen's egg white lysozyme are closely related, having evolved from a common ancestral gene.[229]

Lysozyme, Gal d 4
Lysozyme is a muramidase that catalyzes the hydrolysis of beta-1,4-links between N-acetyl-muramic acid and N-acetyl-D-glucosamine in the peptidoglycan of bacterial cell walls. Egg white lysozyme, Gal d 4, is one of the main allergens of hen's egg.[230]

2.3.5 Lipocalins

The lipocalins are a diverse family of proteins comprising extracellular ligand-binding proteins with high specificity for small hydrophobic molecules.[231] These proteins transport pheromones or nutrients, control cell regulation, or play a role in cryptic coloration or the enzymatic synthesis of prostaglandins. The crystal structures of several lipocalins have been elucidated and show a novel eight-stranded anti-parallel beta-barrel fold well conserved within the family.

Beta-lactoglobulin, Bos d 5

Beta-lactoglobulin (Bos d 5) is the major whey protein in the milk of ruminants and many other mammals. It binds a wide variety of hydrophobic ligands, but its function remains unknown. Bos d 5, a major cows' milk allergen, is absent from human breast milk. Bos d 5 occurs as a 36 kDa dimer. Each subunit consists of 162 amino acid residues and possesses two disulfide bonds and one free cysteine. The relative resistance of Bos d 5 to acid hydrolysis as well as to proteases allows some of the protein to remain intact after digestion. There are two main isoforms which differ only in two amino acid positions.[232] The crystal structure of Bos d 5 has been determined, confirming its membership of the lipocalin protein family.[233]

2.3.6 Serum albumins

Albumins are the main proteins of plasma. They bind water, cations (such as Ca^{2+}, Na^{+}, and K^{+}), fatty acids, hormones, bilirubin, and drugs. Their main function is to regulate the colloidal osmotic pressure of blood. Structurally, the serum albumins are similar, each of the three homologous domains containing five or six internal disulfide bonds. The three-dimensional structure of human serum albumin has been determined by X-ray crystallography to a resolution of 2.8A.[234] Bovine serum albumin, also referred to as Bos d 6, although present in milk in low quantities, reacted with IgE from 50% of milk allergic patients.[235,236] Chicken serum albumin (Gal d 5, alpha-livetin) contains three albumin domains and has been implicated as the causative allergen of the bird-egg syndrome.[237] Gal d 5 is a partially heat-labile allergen.

2.3.7 Immunoglobulins

IgE directed against bovine IgG, Bos d 7, was detected in raw beef in 83% of beef-allergic subjects but in only 24% of beef-tolerant subjects. Complete inhibition of the IgE reactivity to the bovine IgG was obtained with lamb, venison, and milk. Bovine IgG appears to be a major cross-reacting meat allergen that could predict beef allergy.[238] The role of Bos d 7 in cows' milk remains to be studied.

2.3.8 Alpha/beta-caseins

Caseins are the major protein constituent of milk. The biological function of the caseins is considered as modulating the precipitation of calcium phosphate from solution.[239] The major allergens of the casein fraction are the calcium-sensitive alpha-s1-, alpha-s2-, and beta-caseins (whole casein fraction = Bos d 8).[240,241] Alpha-s1 and alpha-s2 caseins from cows, goats, and sheep share 87–98% identical amino acids. The alpha-caseins from these animal species are highly cross-reactive and consequently milk of sheep and goat is not a suitable alternative for individuals suffering from cows' milk allergy

(CMA).[235] Oral challenge studies clearly showed that goats' milk is not an appropriate cows' milk substitute for children with IgE-mediated CMA.[236] In addition, IgE from children allergic to cows' milk were capable of recognizing most of the milk proteins from ewe, goat, and buffalo while no serum contained IgE that reacted with camels' milk proteins.[242] Interestingly, mares' milk can be regarded as a good substitute for cows' milk, having produced a positive oral challenge in only 1/24 children with CMA.[243]

2.3.9 Transferrins

Transferrins are eukaryotic iron-binding glycoproteins that control the level of free iron in biological fluids. The proteins have arisen by duplication of a domain, each duplicated domain binding one iron atom. Members of the family include milk lactotransferrin (lactoferrin) and egg white ovotransferrin (conalbumin). The structural comparison of allergenic sites in alpha-lactalbumin and beta-lactoglobulin with the structure of lactoferrin has shown that lactoferrin also possesses allergenic sites.[244] Lactoferrin reacted with IgE from 45% of patients allergic to cows' milk.[241] Ovotransferrin from hen's egg, also known as Gal d 3 or conalbumin, has been identified as another major egg white allergen.[245]

2.3.10 Kazal-type serine protease inhibitors

Kazal inhibitors, which inhibit a number of serine proteases (such as trypsin and elastase), belong to a family of proteins that includes pancreatic secretory trypsin inhibitor, avian ovomucoid and elastase inhibitor. These proteins contain between 1 and 7 Kazal-type inhibitor repeats.[246] The structure of the Kazal repeat includes a large quantity of extended chain, two short alpha-helices and a three-stranded anti-parallel beta sheet.[247]

Ovomucoids

Avian ovomucoids contain three Kazal-like inhibitory domains.[248] Chicken ovomucoid has been shown to be the dominant hen's egg white allergen Gal d 1.[190] Gal d 1 comprises 186 amino acid residues that are arranged in three tandem domains (Gal d 1.1, Gal d 1.2, Gal d 1.3). Each domain contains three intradomain disulfide bonds. Gal d 1.1 and Gal d 1.2 contain two carbohydrate chains each, and about 50% of the Gal d 1.3 domains contain one carbohydrate chain.[189] Significantly more IgE from hen's egg-allergic patients reacts with the second ovomucoid domain. It has been further suggested that conformational B-cell epitopes play a significant role in ovomucoid allergenicity and that the carbohydrate moieties have a minor affect on allergenicity.[189]

Serpins

The serpins (serine proteinase inhibitors) and related proteins constitute one

of the earliest described protein superfamilies.[249] Serpins are a group of structurally-related proteins of high molecular weight (400–500 amino acids), and they are extracellular irreversible serine protease inhibitors. The ovalbumin family of serpin proteins includes both active protease inhibitor molecules as well as molecules lacking this activity, such as ovalbumin.[250]

Ovalbumin, Gal d 2
It has become clear that ovomucoid is the immunodominant protein fraction in egg white and that the use of commercially purified ovalbumin has led to an over-estimation of the dominance of ovalbumin as a major egg allergen in human beings.[190]

2.4 Future trends

The presently ongoing classification of food allergens by protein families provides an instrumental framework for the characterization and analysis of allergenic proteins. In addition, a protein can be evaluated in terms of its association with a certain protein family that is already known to contain a high number of allergens such as the cupin or prolamin superfamily. This knowledge is useful for the safety assessment of genetically modified organisms, especially genetically engineered crops.

Plant biotechnology and food technology, albeit by different means, aim to reduce the allergenicity of foods by modification of the allergen structure or the complete removal of the allergen. An antisense RNA strategy was used to markedly reduce the mRNA and protein content of a family of 14–16 kDa rice allergens that belong to the alpha-amylase/trypsin inhibitors.[251] Transgene-induced gene silencing was also applied to completely suppress the expression of Gly m Bd 30 K, a major soybean allergen and member of the plant thiol protease family.[252] The Gly m Bd 30 K silenced plants and their seeds were developmentally, structurally and compositionally identical to the control plants. Future developments in plant biotechnology may allow targeted gene mutation or a broader application of gene silencing or replacement. This technology may be limited by the presence of multiple allergens. Moreover, the long-term stability of these systems needs to be evaluated.

The use of certain processing methodologies to reduce the allergenicity of foods so far has been largely empirical. One needs to differentiate between thermolabile allergens and allergens that are more resistant to heat treatment, decrease in pH, or proteolytic degradation. It is not possible to describe the full range of literature available on the effects of processing on the allergenicity of certain proteins within this chapter. Generally, the different allergens of the pollen/fruit syndrome such as members of the Bet v 1 family (PR-10 proteins) are less resistant to heating and digestion, a fact that is connected to their ability to cause only oral allergy symptoms. Sensitization occurs predominantly via inhalation of the pollen allergens. Heating reduces the

allergenicity of apple, hazelnut, and celeriac.[253,254] However, the Bet v 1 homologous allergen SAM22/Gly m 4 of soybean was able to induce severe systemic reactions in birch pollen-allergic individuals.[154] Allergens of the cupin, prolamin or cysteine protease superfamilies predominantly sensitize via the gastrointestinal tract. Their stability to heating and digestion is attributed to the presence of several disulfide bonds in the molecules, as has been shown for example for nsLTPs[255] or for the 2S albumin-related conglutin Ara h 2.[256] The increase in the knowledge of allergen and epitope structures will in the future allow the design of more rational and efficient processing strategies. Currently the only available therapy for food-allergic individuals is dietary exclusion. This is the reason why reliable detection methods for food allergens are of extreme importance to allow accurate food labeling. Therefore, in the search for recombinant food allergy therapeutics several molecular biology techniques are being explored.[257–260] In general, these genetically engineered immunotherapeutics will need to possess greatly reduced IgE epitopes while preserving sequences necessary for T-cell recognition and for induction of IgG antibodies reactive with the natural allergen.[261]

2.5 Sources of further information and advice

There are several databases of allergen sequences on the internet. The Allergen Nomenclature Subcommittee of the International Union of Immunological Societies (IUIS) makes the official list of peer-reviewed allergens available on the net (www.allergen.org). The Allergome (www.allergome.org) is based on the literature published since the early sixties and provides information on allergenic molecules for people working in allergy and immunology. The Food Allergy Research and Resource Program (FARRP) at the University of Nebraska-Lincoln contains a list of publicly known allergens (www.allergenonline.com). The biotechnology information for food safety database contains accession numbers for food allergens, non-food allergens, and wheat gluten proteins (www.iit.edu/~sgendel/fa.htm). The Structural Database of Allergenic Proteins (SDAP, fermi.utmb.edu/SDAP/index.html) is a web server that integrates a database of allergenic proteins with various bioinformatics tools for performing structural studies related to allergens and characterization of their epitopes. Nine specialized allergen databases have been critically reviewed and the need for a centralized allergen reference database has been pronounced.[262]

PROTALL is a European network of scientists with expertise relevant to studying the problems of food allergy. The PROTALL database contains biochemical and clinical information about plant food allergens (www.ifr.bbsrc.ac.uk/protall). The SAFE EU project studies apple allergy in great detail (www.akh-wien.ac.at/safe). InformAll (www.informall.eu.com), a concerted action project funded under the 5th Framework programme of the EU, was created to promote the provision of visible, credible food allergy

information sources to consumers, the agri-food industry, allergic consumers, health professionals, and regulators.

The information that led to the structure- and function-based classification of food allergens can be found in several databases that specialize in protein structure, families, and superfamilies. The Protein Databank (PDB) currently holds almost 32 000 structures of biological macromolecules including crystal and solution structures of many important allergens (www.rcsb.org/pdb/). SWISS MODEL is a fully automated protein structure homology-modeling server that makes protein modeling accessible worldwide (www.expasy.org/swissmod/SWISS-MODEL.html). The PredictProtein server is a service for sequence analysis and structure prediction. After submission of a protein sequence, PredictProtein retrieves similar sequences in the database and predicts aspects of protein structure (www.embl-heidelberg.de/predictprotein/predictprotein.html). The CATH protein structure classification (www.biochem.ucl.ac.uk/bsm/cath/) uses a novel hierarchical classification of protein domain structures, which clusters proteins at four major levels, class (C), architecture (A), topology (T) and homologous superfamily (H). The SCOP database aims to provide a detailed and comprehensive description of the structural and evolutionary relationships between all proteins whose structures are known (scop.mrc-lmb.cam.ac.uk/scop/). Pfam, a protein family database, is a large collection of multiple sequence alignments covering many common protein domains and families (www.sanger.ac.uk/Software/Pfam/index.shtml).

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Part II

Types of detection method

3

Antibodies

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3.1 Nature of antibodies

The basic role of the humoral immune system is to eliminate foreign invaders. This is accomplished through the production of antibodies, proteins produced by the immune system in response to the presence of a foreign antigen. In order for this to occur, it is necessary that the immune system recognizes a large range of non-self antigens but responds well to foreign antigens while not over-responding to self or switching to IgE responses (allergy).

Antibodies can be divided into five classes: IgG, IgM, IgA, IgD and IgE, based on the number of Y units and the type of heavy chain. IgG is most often used in immunoassays. The basic structure of an antibody (immunoglobulin) was discovered by Porter in 1959 (Porter, 1959); using the enzyme papain, he isolated one fragment from rabbit 7S immunoglobulin that was subsequently crystallized and named Fc, for 'fragment crystallizable'. The two remaining fragments (which bound antigen) were designated Fab, or 'fragment antibody binding'. Based on these initial studies, in this same research, Porter (1962) described a four-chain structural model for immunoglobulins; antibodies exist as one or more copies of a Y-shaped unit, and the typical structure is as shown in Fig. 3.1. Each Y contains two identical copies of a 'heavy chain' (about 50 kD each), and two identical copies of a 'light chain' (about 25 kD each), named for their relative molecular weights. The four chains are held together by non-covalent forces and disulfide bonds. Both heavy and light chains contain peptide sequences which show little dissimilarity between antibodies, and which are termed the 'constant' regions; other regions vary considerably among individual immunoglobulins, and thus are called the 'variable' regions. The Fc region comprises the 'tail' of

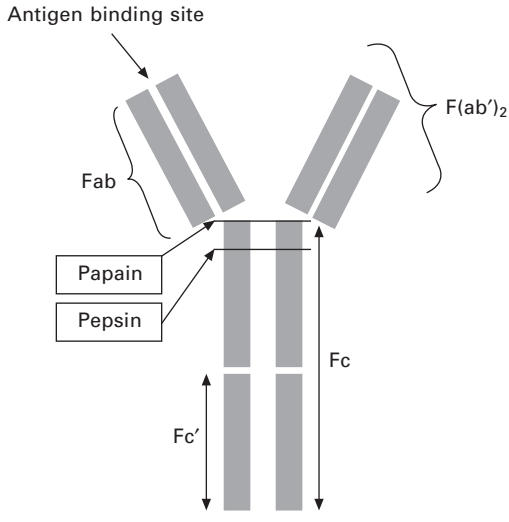

Fig. 3.1 Antibody (immunoglobulin) structure showing heavy and light chains. Papain and pepsin cleavage sites are also indicated. Digestion by papain gives Fc and Fab fragments. Digestion by pepsin separates the molecule into two parts, a bivalent fraction called $F(ab')_2$ that contains both antigen binding sites, but also the Fc into small parts called Fc′.

the antibody and plays a role in immune response, as well as serving as a useful 'handle' for manipulating the antibody during some immunochemical procedures. Digestion by pepsin separates IgG into two parts, a bivalent fragment that contains both antigen binding sites, F(ab′)2, but also the Fc into small parts called Fc′.

Antibodies are produced by plasma B lymphocytes (B cells). They are flexible molecules that on the specific ends can bind their antigens and on the other end can bind to effector cells (phagocytes) of the immune system; the antigen–antibody complex is then removed by phagocytosis, thereby eliminating invaders (Deshpande, 1996a). The specific ends display a great deal of flexibility and thus can operate independently: each monomeric antibody unit contains two antigen-binding sites and is thus bivalent. The primary job of immunoglobulins is to bind antigen, but they can have other effects depending on the type of antibody involved, and these can include complement activation, placental transfer, reactivity with staphylococcal proteins, and other functions (Deshpande, 1996a).

Antigen–antibody interactions occur through multiple non-covalent bonds between the antigen and the amino acids in the binding site of the antibody, including van der Waals forces, electrostatic attractions, and hydrophobic and hydrogen bonds. Individually, these forces are weak compared to covalent bonds (Deshpande, 1996b). However, together they form a tight bond, leading to stabilization. The strength of this bond is called the antibody's 'affinity' (Harlow and Lane, 1999). Typical affinity constants are 10^{-5} to 10^{-12} M (Feldkamp and Carey, 1996; Harlow and Lane, 1999).

Hydrogen bonding is the result of hydrogen bridges being created between atoms, and does not often play a role in the primary binding of the antigen with the antibody (Ag–Ab binding), although it does significantly assist in secondary binding (Jefferis and Deverill, 1991). Electrostatic forces consist of the attraction between oppositely-charged groups on proteins, and cause primary Ag–Ab binding (Davies and Padlan, 1990). Van der Waals forces occur by the interaction of electron clouds of two polar groups and contribute to primary Ag–Ab binding. Hydrophobic interactions are based on the association of non-polar, hydrophobic regions or side chains of amino acids. They have a high stability owing to structural alteration of the aqueous environment when these groups come into contact, excluding water (Deshpande, 1996b). Hydrophobic interactions strengthen existing primary and secondary Ag–Ab bonds, and the act of excluding water from the bond increases the binding energy considerably, serving to further stabilize the bond. While both electrostatic and van der Waals forces are factors in primary Ag–Ab bonding in the early phases (first few milliseconds), they contribute only a small portion of the total (Jefferis and Deverill, 1991). Secondary Ag–Ab binding involves the creation of hydrogen bonds and hydrophobic interactions, continuing over a longer time-frame, and contributes significantly to the stabilization of the Ag–Ab bond. The overall stability of the Ag–Ab complexes is referred to as 'avidity' (Harlow and Lane, 1999).

For forces to become substantial, the interacting moieties must be in close proximity to each other. Compared to the attractive forces described above, steric repulsion is much more sensitive (Steward and Steensgaard, 1983). Repulsive influences between non-bonded atoms come from the interpenetration of their electron clouds, and determine the level of 'fit' between antigen and antibody. A better 'fit' of the electron clouds between the antigenic determinant and the binding site of the antibody will lower the repulsive force, and vice versa. Non-complementarity will result in high repulsive forces, and minimize any small forces of attraction, resulting in poor 'fit'. Because of the varied nature of the forces involved in Ag–Ab bonding, electrostatic forces are very dependent on the pH. Outside the range of pH 6–8, Ag–Ab binding can be affected adversely (Hughes-Jones *et al*., 1964). Temperature and ionic strength can also affect binding in a negative way (Deshpande, 1996b).

There are two types of antibodies used in immunoassays; monoclonal and polyclonal. The productions of both types is discussed at length later in this chapter; however, basic definitions are made here. Polyclonal antibodies are produced in animals in response to injections of a substance that is capable of inducing the immune system (antigen) to produce antibodies. Since several antibody-producing B cell clones in the immunized animals synthesize these polyclonal antibodies, polyclonal antibodies recognize a variety of specific binding sites (epitopes) on the antigen. Monoclonal antibodies are usually produced using rats or mice, and are created by the fusion of immunized spleen cells and myeloma cells; the spleen cells convey antibody secretion

and the myeloma cells impart immortality. Since monoclonal antibodies allow for individual B cells to combine with a myeloma cell, antibody very specific for a certain part of the antigen can be selected and produced.

3.2 Immunogens and antigens

Traditionally, the term 'antigen' was used to describe any molecule that produced a specific immune response such as antibody production, tolerance, or cell-mediated immunity (Deshpande, 1996a). Nevertheless, to differentiate molecules that make an immune response and those that are antibody-binding targets, 'immunogen' is used for the former and 'antigen' for the latter (Catty, 1988). Molecules that can act as immunogens are normally > 1 kD and usually > 5 kD in molecular mass. Antibodies do not recognize the whole antigen, merely portions of it. These portions are called 'epitopes' or 'antigenic determinants'. Each individual antibody is specific for a particular antigenic determinant of the antigen. Even when a single pure protein is used as an immunogen, the resulting polyclonal antibodies may be heterogeneous and possess a range of different affinities (Hawcroft *et al*., 1987). Antibodies are produced by injection of immunogens that are different from self antigens. For allergens, antibodies can be produced against any protein or peptide that has at least one antigenic determinant (generally 4–6 amino acids) (Deshpande, 1996a). Like small chemicals, synthetic peptides can serve as haptens with carrier proteins as immunogens. Once an immune response to an antigen is made, the plasma B cells make large amounts of antibody. For proteins, T cells must also be involved in order to get antibody response, and for these T-dependent responses, at least two antigenic determinants are required (one for the T cells and one for the B cells) (Raff, 1970).

Because antibodies themselves can act as immunogens, 'second antibodies' – or, antibodies that detect antibodies – can be produced. Second antibodies are made against specific sections of the antibody of interest and they bind without destroying the binding of the primary antibody. Some of these are available commercially (examples include rabbit anti-goat IgG, goat anti-rabbit IgG, rabbit anti-mouse IgG, and mouse anti-human IgE second antibodies), with or without enzyme- or radio-label.

3.3 Antibody production

3.3.1 Choice and form of immunogen

The choice of immunogen, and also the form of the immunogen, is particularly important for success in making antibodies for allergenic residues. This is beyond a simple choice of raw or heat-processed immunogen. Every aspect of the immunogen preparation process, from the choice of variety to the

washing of the starting materials to remove agricultural contaminants, contributes to the selectivity and usefulness of the resulting antibodies. One can manipulate the immune response by injecting processed or denatured antigen, but parameters around how to process and how much to process are still mostly determined by trial and error and previous experience. Processing can result in alteration of proteins such as partial or complete denaturation, aggregation, and formation of Maillard reaction products, some of which can serve as immunodominant determinants. Even age-old recommendations such as defatting may not be advisable for production of antibodies for allergenic residues, as important oil-body associated proteins can be lost in the process. A severely underestimated cause of cross-reactivity in polyclonal antiserum preparations is agricultural contamination, which is more often the norm rather than the exception, even with unrelated materials; simple extensive washing of the source materials can circumvent this problem. The debate of using raw or processed source material for producing antibodies for allergens continues; a good approach to start with is to use processed immunogen in trying to make antibodies that can detect processed forms of the allergenic food; however, success depends on many factors and properties beyond this solitary consideration.

While the approach of using a crude extract of allergenic food to make antibodies has been criticized for having the potential to result in highly cross-reactive antisera, high-quality monospecific polyclonal antisera against peanut and other allergenic foods have been very successfully produced using crude extracts (Yeung and Collins, 1996; 'Holzhauser and Vieths, 1999; Hlywka *et al*., 2000). In fact, many of the successful commercial enzyme-linked immunosorbent assay (ELISA) kits for allergenic residue detection use antibodies generated against crude extracts of allergenic foods, and the methods suffer few cross-reactivity issues. It is possible to make antibodies against a single isolated protein, but processing or denaturation of that protein can mean reduced activity. In most cases the food industry really only cares about whether any peanut protein is present, not just if one allergenic peanut protein is present. The view of regulatory agencies may differ from this in that they may need to prove a hazard exists, and hence the presence of the allergen, but indicator proteins can be a successful approach. In fact, the commercial peanut protein test kits can detect the major peanut allergens Ara h 1 and Ara h 2 (Nogueira *et al*., 2004).

3.3.2 Adjuvants

Often the use of adjuvants is necessary for antibody production. The function of an adjuvant is to enhance the immunogenicity of the antigen by increasing the immune response in several ways; it increases the efficiency of antigen presentation and the number of antibody-secreting B cells, acts as an immunogen depot (for extended antigen stimulation), protects the immunogen from rapid removal and catabolism, and increases the affinity and avidity of

the antibody response (Spier and Griffiths, 1985; Catty, 1988; Roitt *et al*., 1989). Adjuvants are available in many forms (Harlow and Lane, 1988). The best known adjuvant is complete Freund's adjuvant (CFA), a suspension of killed mycobacteria in mineral oil which was first described over 50 years ago. Incomplete Freund's adjuvant (IFA) is the mineral oil minus the bacteria; sometimes IFA can be used successfully by itself (Campbell, 1996). There are many other types of adjuvants that can be used besides CFA and IFA (Hefle, 1995; Campbell, 1996).

3.3.3 Route of administration

The route of administration of immunogen varies according to the volume being injected, the buffers and other components that will be injected with the immunogen, and how quickly the immunogen should be released into the circulation (Harlow and Lane, 1988). The choice of route used also depends on the physical nature of the immunogen, the species, and the stage of immunization (Deshpande, 1996c). Larger volumes are usually given to animals via the subcutaneous route, but in rodents, intraperitoneal injection is the most-used method. Intravenous injections can be used, but care is necessary, as there is a risk of both anaphylaxis and also pulmonary embolism. Immunogens which contain particulate matter or adjuvants should not be given intravenously. Intramuscular or intradermal injections are used for slower immunogen release. Subcutaneous and intramuscular routes are most often used with Freund's adjuvant. Both of these are proficient in forming depot sites and slow immunogen release. Subcutaneous injection releases immunogen slower than other routes, and is also preferred for booster injections, due to a decreased chance of anaphylaxis. Adjuvant can be safely used with intraperitoneal injections, but should not be used with intravenous injections. Subcutaneous and intradermal routes are often used in rabbits, but intradermal or intramuscular routes are used in larger animals.

3.3.4 Dose

The dose of immunogen depends on the its nature and also on the animal being used. Large doses may induce tolerance, but in some cases hyperimmunization schedules utilizing a relatively high amount of immunogen have been successful (Cordle *et al*., 1991). The desired result is an antibody with high avidity and affinity; therefore, use of the lowest amount of immunogen necessary to achieve antibody production is usually the best approach. A typical dose for rabbits is 100 μg for the primary injection, with 50–100 μg used for booster injections (Harlow and Lane, 1988), although amounts well above that have been reported and can be used. For larger animals, 500–1000 μg is usual to start with. For booster injections, the traditional amount used is 10–50% of the primary dose. Smaller booster doses promote clonal selection for high-affinity Ab. For antigens in precious supply, one or two

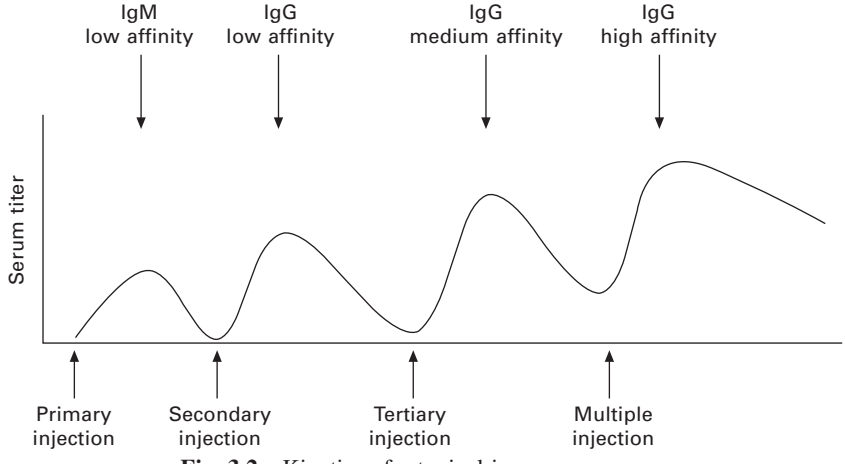

Fig. 3.2 Kinetics of a typical immune response.

injections with a low dose to prime the animals followed by a larger booster injection can be successful (Harlow and Lane, 1988).

A specific antibody in serum can usually be detected about a week after the initial immunization. The first type of antibody produced is IgM with high avidity but low affinity (Deshpande, 1996c), but then a switch to IgG of lower avidity, but higher affinity, occurs (Fig. 3.2). Responses to booster injections are an increase in titer; as the time of immunization extends, antibodies of the IgG class dominate. In general, a three to four week interval between the primary injection and a booster injection is recommended (Deshpande, 1996c). Usually, high titer is attained in about two to four weeks after booster injections. The immune response matures, producing higher-affinity and avidity antibodies as time goes on. For this reason, it is possible to pool polyclonal antibodies after a certain amount of time, rather than including early bleeds. Although intervals between booster injections can be varied, there should be adequate time allowed for the circulating antibody level to drop low enough to prevent prompt clearance of the injected immunogen. For IgG, the half life is 20–25 days in the blood circulation, and the clearance is 10–15% per day (Cruse and Lewis, 1999). As a result, to avoid this problem, booster injections should be done every four to six weeks.

The quality of the antiserum is more important in immunoassays than in other techniques. During immunization, antibody production and quality (titer) needs to be monitored. Serum samples are collected 7–14 days after booster injection, which corresponds to peak IgG production rate. For polyclonal antibody production, test bleeds (small volume) are taken until sufficient titer is reached, at which time production bleeds (larger volume) are done. Serum samples from individual animals should not be pooled in the initial stages; once a good titer and affinity have been developed, individual bleeds can then be pooled to form a larger quantity of homogeneous antiserum. Titers are usually monitored using indirect ELISA, in which the antigen is

coated onto microtiter plates and the antiserum is diluted serially and added to plates. Titer can be defined in many ways, but one common method is the mid-linear point of the titration curve. Antigen-specific IgG of approximately 8–14 mg/mL serum can be obtained.

Antibody affinity can also be ascertained using indirect ELISA, but using the analyte of interest as an inhibitor. The dilution of antibody used in the ELISA is the dilution identified by the titer. The horizontal displacement of the inhibitor curve is an indirect measure of the affinity of the antibody (Morris, 1985), as the greater the displacement, the greater the affinity of the antibody. Cross-reactivity of the antibody can also be determined using a similar technique; the curve generated from other substances should be able to be superimposed on the antibody dilution curve.

3.3.5 Engineered antibodies

Progress in phage antibody display technology has revolutionized the ability to select and engineer monoclonal antibodies. Usually these are human monoclonal antibody fragments with desirable specificities that have therapeutic potential. They are selected from extremely large libraries consisting of engineered phage particles, each expressing an antibody fragment with a unique specificity, such as for a tumor cell. Antibody fragments with relevant binding are converted into intact IgG or IgA antibodies and could have future potential in therapy for some types of diseases. While engineering production of antibodies for therapeutic purposes is a promising new field, the costs for such an approach preclude the use of this technique for routine production of specific antisera for many applications.

Tolerance to a particular immunogen can occur for several reasons. Certain conditions promote tolerance, and should be avoided in the production of high-affinity, quality antibodies (Howard, 1979). Tolerance is mounted against specific antigenic determinants, not the antigen as a whole. T-independent immunogens (those which induce antibody synthesis, usually only IgM, in the absence of lymphokines released by T-cells) may act as tolerogens if injected at a high dose (100–1000 × normal) (Parks *et al.*, 1979). In contrast, low doses may cause immature B-cell clones to terminate, and immature B cells are more prone to tolerance than mature B-cells. Excess T-independent immunogen and absence of T-cell assistance prevent plasma B cells from their normal function and, if repeatedly injected, can function to remove functional B-cell clones. In addition, excess T-independent immunogen can hinder secretion of antibody by plasma B cells. Weak immunogens may induce tolerance if given in very small doses. Slowly released immunogens preserve tolerance longer than those that are quickly catabolized.

3.3.6 Cross-reactivity

Antigen–antibody reactions show a high degree of specificity when the binding sites of the antibodies are not complementary and thus do not recognize or

cross-react with the determinants of another antigen. However, the specificity of an antiserum echoes the many specificities of its component antibodies (Catty, 1988). A polyclonal antiserum does have the capability to monospecifically bind to an antigen if the range of its constituent specificities is only to epitopes that are restricted to that antigen. A cross-reactive antigen combines with antibodies induced in response to a different antigen but which has shared antigenic determinants. Cross-reactivity comes to pass either from the binding of structurally different determinants of the same antigen by the same antibody, or because of the existence of common epitopes (Deshpande, 1996b).

It is important to assess cross-reactivity with many food ingredients when developing immunoassay methods for detection allergenic residues, due to the vast variety and type of foods and food ingredients used in the manufacture of packaged food products. Simply reporting cross-reactivity of low levels of, or diluted, ingredients, is not sufficient, and test substances for cross reactivity should be extracted in concentrated form (at full extraction concentration) and analyzed; in other words, cross-reactivity should be approached such that testing substances are assumed to comprise 100% of future samples.

3.3.7 Production of monoclonal antibodies

The ability of each individual B lymphocyte to produce an individual, unique antibody was discovered in the 1950s. It is estimated that a typical BALB/c mouse that has 2×10^8 B-cells can produce $10–40 \times 10^6$ distinct clonotypes (Klinman, 1972); therefore, for any given animal, hypothetically, several different clonotypes could be produced. Kohler and Milstein (1976) were the first to produce monoclonal antibodies. Antibody-producing lymphocytes are isolated and then fused with myeloma cells (mutant cells that produce no antibodies) to produce perpetual hybridomas, which produce many copies of the same antibody. Detailed procedures for production of monoclonal antibodies can be found elsewhere (Goding, 1986; Harlow and Lane 1988). There are five major parts to monoclonal antibody production; immunization of the rodents, fusion of immunized spleen cells with myeloma cells, selection of appropriate clones, cloning, and production of large amounts of antiserum (Fig. 3.3). The hybrid cells are selected using selective culture media and then screened for the specific antibody produced. The result is a cell line clone that only produces one type of monoclonal antibody. These cells can be maintained over long periods of time. Large amounts of antiserum can either be collected through the production of ascites *in vivo* or by *in vitro* cell culture. Ascites is intraperitoneal fluid obtained from mice that have been injected intraperitoneally with a hybridoma clone, causing tumors in the gut that produce the monoclonal antibody. In the past, ascites generation was most often used to collect large amounts of antiserum from monoclonal cell lines, but due to animal welfare concerns, production of ascites is banned or severely limited in many countries today. Ascites fluid can contain up to 5–

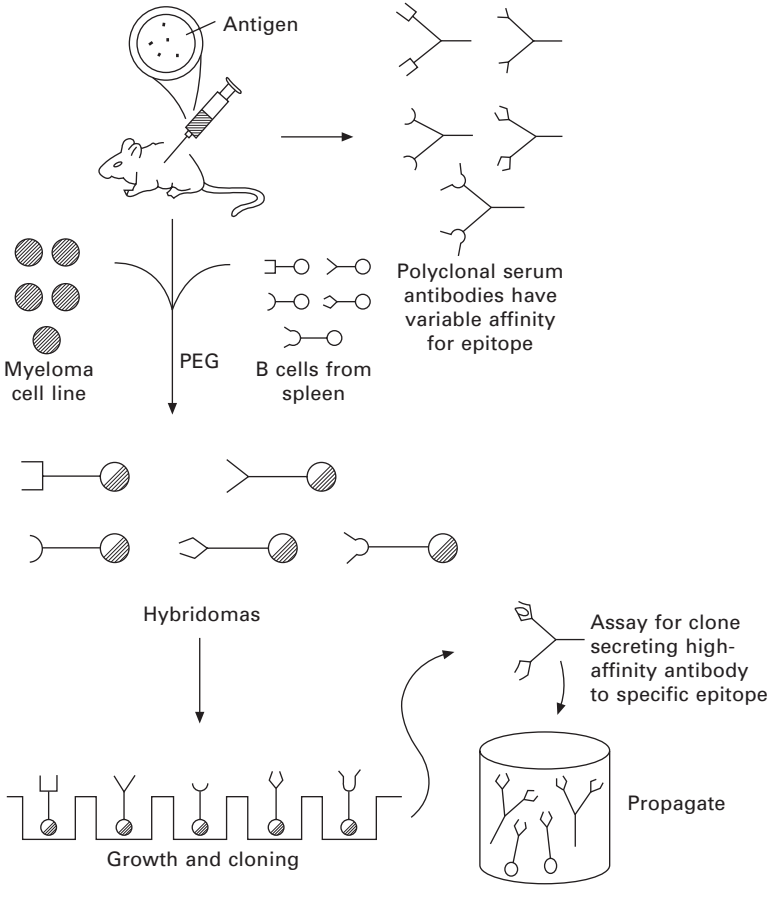

Fig. 3.3 Monoclonal antibody production.

15 mg/mL of the monoclonal antibody (Deshpande, 1996c), and as much as 40 mL can be obtained per mouse. Alternatives to ascites include recovery of the antibodies from stationary cell culture supernatant solutions, use of roller bottles or stirred tanks, gas-permeable tissue culture bags, mini-fermentors, and airlift (packed-bed) reactors. Spinner flasks are usually not used for antibody harvest becaue the shear forces can damage the antibodies. The process of producing hybridomas can take from two months to up to a year. Methods for ascites and cell culture production of monoclonal antibodies are can be found elsewhere (Goding, 1986; Harlow and Lane, 1988).

3.3.8 Purification

Antiserum either from polyclonal antiserum or monoclonal culture supernatant or ascites can be further purified. Often, crude serum or semi-purified serum can be used, but in some cases further purification is recommended or necessary.

For polyclonal antiserum, antibodies can be semi-purified using as crude a scheme as enriching the IgG content through ammonium sulfate precipitation, or as sophisticated as using antigen immunoaffinity to purify it. The source material, nature of possible contaminants, and the final application of the antiserum are all considerations for the choice of purification approach (Deshpande, 1996c). While the total immunoglobulin concentration in a crude polyclonal antiserum preparation can be as high as 10 mg/mL, the antibody of interest may comprise only about 10% of this (Deshpande, 1996c). Monoclonal antibodies in culture supernatant are fairly dilute (5%) and therefore usually need concentration before use; one can use ammonium sulfate precipitation for this, although substances in the culture media can be co-precipitated, such as fetal calf serum proteins. Ascites can contain 0.9–9 mg/mL specific antibodies, but may also have other mouse antibodies present (Harlow and Lane, 1988). Approaches for purification of antibodies are reviewed elsewhere (Goding, 1986; Harlow and Lane, 1988; Deshpande, 1996c).

3.4 Choice of producing monoclonal or polyclonal antibodies

An ideal antibody will possess high titer and also high affinity and specificity for the analyte of interest. Affinity is the main factor in achieving a low detection limit for an immunoassay (Deshpande, 1996b). For commercial interests, large scale production of antibody is a major consideration; while monoclonal antibodies are good from this aspect, they are expensive to initially produce and may not be as good as polyclonal antibodies at detecting processed forms of the antigen. Usually, a specific polyclonal antibody, once produced, is available in quantities that are large enough for commercial or research interest, and if a goat or sheep is used, large amounts of antiserum are available. With antigens that are good immunogens, producing more antiserum is ordinarily not a problem.

Polyclonal antibodies often recognize multiple epitopes, making them more tolerant of small changes in the nature of the antigen. Therefore, polyclonal antibodies may be preferred when the antigen of interest is denatured or altered in some way, such as in food processing (Goding, 1996). Antigen–antibody bonds are usually stable under pH conditions of 4–9 and salt concentrations of 0.1–1 M NaCl, although occasionally, monoclonal antibodies can be separated from their antigen under these mild conditions (Hermann and Mescher, 1979). By definition, a monoclonal antibody binds with only one epitope on the antigen. The popular myth is that monoclonal antibodies have an advantage over polyclonal antibodies because they are supposedly not cross-reactive; however, monoclonal antibodies can possess cross-reactivity to unrelated antigens (Lane and Koprowski, 1982). In one report, monoclonal antibodies were reported to cross-react with two muscle proteins, tropomyosin

and vimentin (Blose *et al.*, 1982). Lane and Koprowski (1982) have suggested two possible causes for monoclonal antibody cross-reaction; that the antibody detects structural similarities in the two antigens, or that the binding site of the antibody could combine with unrelated antigens in a multispecific way, and recognize two or more entirely different epitopes (Richards *et al.*, 1975). An antigen–antibody complex occurs whenever there are enough and the right strength of inter- (and intra-) molecular forces. Goding (1996) states that it is not surprising that monoclonal antibodies can have multispecificity and, indeed, Hefle *et al.* (1994) noted cross-reactivity among their developed monoclonal antibodies, to different peanut proteins. Unusual cross-reactions are almost never seen with polyclonal antibodies because the odd cross-reaction is 'diluted out' in the enormity of the rest of the response and the 'consensus' of different clones (Goding, 1996). Specificity also depends on this consensus and polyclonal antibodies bind to determinants that cover almost the entire external surface of the antigen (Benjamin *et al.*, 1984). Therefore, minute changes in the structure of the antigen due to genetic polymorphism, heterogeneity of glycosylation, or slight denaturation will usually have little or no effect on polyclonal antibody binding. A subset of polyclonal antibodies will usually bind to antigen that has been modified or denatured, even if this was not the form of the immunogen used for the immunization (Burnette, 1981). In contrast, monoclonal antibodies most often bind to a single unique epitope – if for any reason this site is altered (due to denaturation, unfolding, aggregation, formation of Maillard reaction products, etc.), the monoclonal antibody may not bind (Goding, 1996). Another potential drawback to monoclonal antibodies is that they may only be able to cross-link antigen molecules with two or more binding sites (Goding, 1996), although this requirement is usually not an issue for allergenic proteins. If cross-reactivity is present with monoclonal antibodies, it is hard to remove, unlike for polyclonal antibodies. Monoclonal antibodies have sensitivity to only part of the total antigenicity of the immunogen, resulting in poorer performance compared to that of polyclonal antibodies in many applications. Polyclonal antibodies may be generated in a great variety of species, including rabbit, goat, sheep, donkey and chicken, giving the immunoassay developer many choices in design of assay; this is in contrast to monoclonal antibodies, which are usually only produced in rodents due to practical considerations.

It can be difficult to obtain polyclonal antisera with identical properties from different animals. Even from the same animal, polyclonal antisera collected at different times can have different properties. These limitations of polyclonal antibodies can justify producing monoclonal antibodies, as the latter have identical physical, biochemical and immunological properties. Due to their specificity, monoclonal antibodies are good for use as a primary antibody in immunoassays. In addition, the specificity of monoclonal antibodies spurs efficient binding of antigen in a mixture of related molecules, and this property can be exploited in such techniques as affinity purification. A disadvantage of monoclonal antibodies is that their preparation can be time-

consuming and expensive. In addition, they can be difficult to label (Deshpande, 1996c).

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4

Allergen-specific human IgE antibody-based analysis of food

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4.1 Introduction

Allergens are foreign proteins or immunogens that, when introduced in an immunocompetent and predisposed host, elicit the formation of IgE antibodies. Once induced, IgE circulates in the blood and binds on to high-affinity IgE Fcε receptors on mast cells and basophils. This process leads to a state of sensitization that is evidenced by the detection of allergen-specific IgE antibody on skin mast cells and blood basophils. Upon further allergen exposure by ingestion, injection or inhalation, allergen can cross-link receptor bound IgE antibody, causing degranulation of vasoactive mediators from mast cells and leading to a spectrum of allergic reactions. Allergen-specific IgE antibody is thus the key analyte in both *in vivo* – skin test, basophil meditator release – and *in vitro* radioallergosorbent (RAST)/fluorescent enzyme immunoassay (FEIA)/enzyme-linked immunosorbent assay (ELISA) – immunoblot – inhibition assays that permits qualitative identification and quantitative measurement of allergens in complex biological substances such as foods.[1]

In vivo, allergen that is injected into the skin of a sensitized individual induces a wheal and flare reaction that can be measured and used to identify the presence and quantify the allergen's potency. Alternatively, controlled ingestion of foods in a double-blind, placebo-controlled manner allows *in vivo* food challenges which many allergists consider a definitive diagnostic test for an individual's sensitization to a particular food allergen. Due to its relative hazard, however, this method is rarely used to identify or quantify the levels of allergenic protein in particular foods. *In vitro*, allergen can competitively inhibit the binding of allergen-specific IgE antibody to solid phase allergen in the RAST[2] and its newer non-isotopic counterparts such as

the FEIA-based CAP System™ (Pharmacia Diagnostics AB, Kalamazoo, MI)[3,4] and the Immulite® System (Diagnostic Products Corporation, Los Angeles, CA).[5] This chapter focuses on the design, performance characteristics and strengths and weaknesses of allergen-specific IgE antibody-based *in vivo* and *in vitro* assay methods.

4.2 IgE antibody-based *in vivo* assay

The allergenic potency of an extract of raw or cooked food can be measured by the ability of allergenic proteins in this extract to elicit a reaction in the skin of an individual who is known to be sensitized to that food. Administration of allergen into the sensitized skin produces a wheal (sharply circumscribed localized area of edema) and flare (area of erythema surrounding the wheal). As briefly noted above, the principle of this *in vivo* assay involves food allergen-specific IgE antibodies that are bound to Fcε receptors on skin mast cells. They are cross-linked by allergen once it has been injected into the skin of a sensitized individual. Within 15 minutes of this event, the cells are activated to secrete histamine, leukotrienes and other mediators that produce the observed wheal and flare reaction in the skin. By injecting different dilutions or doses of calibrated diagnostic reference allergen extracts of known potency into the skin, a dose-response curve can be generated and the relative potency of a test food extract can be determined. The larger the skin reaction, the more allergen is present in the extract.

4.2.1 Reagents

Food allergen extracts

While virtually any food can cause allergic symptoms, relatively few foods reportedly induce allergic reactions with any frequency in humans. The commonly implicated allergenic foods include cows' milk, egg, wheat, soy, peanut, tree nuts, fish and crustacean shellfish.[6] Diagnostic skin test extracts are commonly prepared from fresh or cooked foods in a defined weight versus physiological extraction buffer ratio (e.g. 1:20 w/v). They are sometimes defatted, concentrated and put into 50% glycerin to minimize degradation during storage. Except in the case of recombinant and some purified research food allergens, diagnostic food allergen extracts tend to be crude allergenic mixtures that display multiple protein and glycoprotein bands on polyacrylamide gel electrophoresis and isoelectric focusing. Food allergens contain linear allergenic epitopes that bind to IgE antibody and appear highly resistant to denaturation by heat and proteases and conformational epitopes that require three-dimensional folding of the allergenic protein to permit binding to IgE antibodies. A comprehensive listing of food allergen specificities together with their genus and species is presented elsewhere.[7]

Reference materials

Since there are few established diagnostic food allergen reference preparations, most materials that are used as reference or calibration reagents in IgE-based quantitative assays have been through a previous allergenic potency assessment by the *in vivo* or *in vitro* methods described in this chapter. They are often first characterized by separation and identification of their component proteins using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and isoelectric focusing. Their total protein concentration is also determined by one of several assays. They are then analyzed in an *in vitro* inhibition assay for the purpose of determining the relative allergenic potency of test allergen extracts, presumably with the same allergenic protein profile.

4.2.2 *In vivo* assay design

The US Food and Drug Administration has adopted the 'parallel line bioassay skin test method' to assay the allergenic potency of diagnostic skin test extracts *in vivo*.[8] In this assay, 18 serial three-fold dilutions of reference and test extracts are prepared from a concentrated extract (0 dose). A volume of 0.05 mL of each extract dilution is then injected intradermally or applied by puncture onto the skin (volar area of the arm and/or back) and monitored at 15 minutes for swelling and redness. The circumferences of the wheal and erythema are outlined with a pen and transferred onto tape for a permanent record. The sum of the longest and midpoint orthogonal diameters of the erythema and wheal is then used to construct titration curves. Preliminary skin tests on the arm are used to determine the doses that result in response with a sum of the erythema diameters from 0–120 mm. Responses at the first four dilutions that produce a positive reaction are used to construct dose–response curves on the back that exhibit steep slopes and good correlation coefficients. The relative potency (RP) of a test extract is interpolated from the dose–response curve of the reference extract that has been calibrated in either mg of total protein, protein nitrogen units or arbitrary biological RP units. While the parallel line bioassay skin test method is useful in assessing the potency of diagnostic skin test reagents, it is not practical for the routine quantification of the amount of allergen that may be present in an extract prepared from a food.

Figure 4.1 illustrates the typical immediate-type hypersensitivity skin reaction that occurs 20 minutes following the administration of four ten-fold dilutions of an allergen extract into the skin of an individual sensitive to the allergen. The diameters of both the wheals and erythemas get larger with increasing concentrations of the allergen. The highest concentration of allergen (1:10 dilution) produces the largest wheal and erythema response. The figure illustrates the importance of administering the different skin test doses at sufficient distances from each other to ensure that the erythema from the individual skin tests do not overlap and thus become difficult to measure accurately. At five hours, a late-phase skin reaction associated with cellular

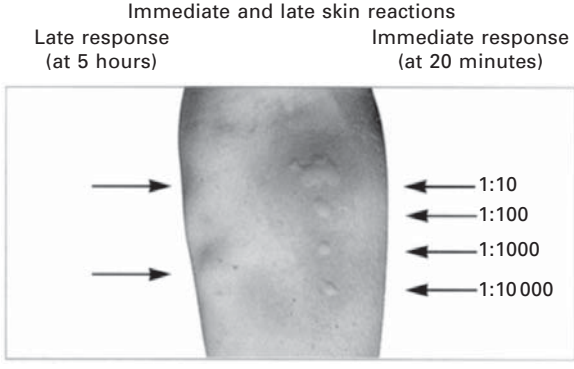

Fig. 4.1 Intradermal skin test titration in which the photograph was taken 20 minutes (right side) and five hours (left side) after the administration of four ten-fold concentrations of allergen to the forearm of a sensitized individual. The swelling (wheal) and redness (erythema) on the right side of the arm after 20 minutes identify the titer or the most dilute concentration of allergen that produces a positive skin reaction in comparison to a saline control. Reproduced with permission from Roitt *et al.*[25]

infiltrates can also be observed; however, this is generally overlooked in the assessment of diagnostic skin test extracts for allergen potency.

4.2.3 Human subject considerations

Atopic subjects participating in the assessment of the *in vivo* potency of diagnostic food allergen extracts must first have a definitive sensitivity to the allergenic food stuff in question, based minimally on a positive clinical history of food allergy and a positive skin test. Second, they must provide informed consent. Where possible, the subject should have a positive double-blind placebo-controlled food challenge[9] to confirm their sensitivity to the particular food(s) being evaluated. Approximately 30–50% of individuals with a clinical history of an adverse food reaction and a positive puncture skin test to the identified food have a positive confirmatory oral food challenge.[10,11] At the time of the study, the individual should not have ingested medications such as antihistamines that might interfere with a wheal and flare response and they should not be on immunotherapy. Atopic individuals who are chronically exposed to allergen will often produce IgE antibody to different allergens within a given complex protein mixture. Thus, to obtain a reliable estimate of the relative allergen content of test extracts, it is preferable to perform a potency bioassay on more than 20 sensitized individuals.[8]

4.2.4 Calibration strategy

The relative potency of an allergen extract is determined by interpolation of test extract response data from a dose–response curve generated with a reference

extract of defined allergenic potency. Often the allergen content in the reference material is reported in arbitrary units such as AUs (allergen units), BAUs (biological allergen units) or RP units. Occasionally, the reference extract's total protein has been quantified using the modified ninhydrin assay[12] or the primary allergen content of the extract. Allergen levels in the test extracts are interpolated and reported in relationship to these arbitrary units in the reference extract.

4.2.5 Quality control

The controls used in this assay include histamine as a positive control to rule out medication (anti-histamine) interference or suppression of the wheal and flare reaction and a negative diluent control such as saline to rule out dermagraphism that can occur from skin trauma. Intradermal injections are performed in duplicate to maximize repeatability and reproducibility.

4.2.6 Performance characteristics

Analytical sensitivity

The analytical sensitivity of the *in vivo* bioassay is dependent upon the overall sensitivity of the subject for the allergen specificity of interest and the criteria used for defining a positive skin test result (e.g. 3 mm above the saline control). Sensitivity ultimately depends upon the amount and specificity of IgE antibody bound to mast cells and the releasability of the individual's mast cells following allergen challenge. The reported sensitivity in the parallel-line skin test assay using common ragweed (*Ambrosia artemisiifolia*) as a model antigen was 5×10^{-5} μg/ml of Amb a 1 [AgE] for subjects sensitive to labile antigens and 3×10^{-6} μg/ml of Amb a 1 for subjects sensitive to stable allergens.[8] Using erythema data, the intradermal skin test application required an average of 30 000-fold lower dose of allergen than the puncture skin test application to obtain a comparable skin test response.

Analytical specificity

The allergenicity of a substance depends on the extent of its foreigness (size, stability, 1°, 2°, and 3° protein structure), the extent of the individual's exposure (concentration) and the genetic (atopic) predisposition of the exposed individual. Plant-derived food allergens have been classified into discrete allergen groups that explain the molecular basis of the cross-reactivity seen among different food groups[13] (see Chapter 2). Some individuals, for instance, who develop an IgE antibody response to proteins in natural rubber latex from *Hevea brasiliensis* (Hev b) trees also exhibit an oral allergy syndrome when they eat certain foods such as avocados, kiwi, bananas and chestnuts. This has been called the latex–fruit syndrome and it stems from cross-reactivity of IgE antibodies to proteins in these foods and natural rubber latex[14]. For example, the class I chitinases from the pathogenesis-related (PR) group 4

have been shown to be involved in the IgE antibody cross-reactivity observed between avocado, chestnut and natural rubber latex.[15] The consequence of this cross-reactivity is that individuals who are selected for allergen potency assessment by *in vivo* bioassay will need to have their IgE antibody tested for confounding cross-reactivities to structurally similar allergenic epitopes in other foods.

Variability
Both repeatability (intra-assay) and reproducibility (inter-assay) should be maximized by applying each skin test dilution in duplicate. The reproducibility reported in the parallel-line skin test assay using the intradermal method of application was 14.2% coefficient of variation (mean +/– 2SD = 102 +/– 14.8 RP).[8] Since the slope of the puncture skin test dose–response line is significantly flatter than the intradermally applied skin test results, there is increased variability of an assay using the puncture skin testing application method.

4.2.7 Dynamic range and data analysis

The dynamic range of this bioassay extends over 4–5 logs depending upon the concentration of allergen-specific IgE and the overall skin sensitivity of the test subjects. Figure 4.1 illustrates the increasing size of the wheal and erythema with increasing allergen concentrations from 1:10 000 to 1:10 at 20 minutes following administration of the allergen to skin. By administering the reference and test extracts each at four dilutions in duplicate, side by side in the same subjects, the mean wheal and erythema data for the test extract can be interpolated from the reference curve. In the original report, Turkeltaub *et al.*[8] performed a best-fit linear regression of the sum of the erythema or wheal diameters obtained from four intradermal three-fold serial dilutions near the endpoint. Their original expectation was a correlation coefficient of > 0.85 and slopes of the reference and test extract regression lines that did not significantly differ from each other.

4.2.8 Assay limitations and applicability

Because of a number of limitations associated with the *in vivo* potency assessment of food allergen extracts, methods such as the parallel-line bioassay are rarely performed today for allergen quantitation. First, the bioassay involves direct challenge of the skin of sensitized individuals with allergen. This places an allergic individual at risk for a possible severe systemic allergic reaction, especially when test extracts of unknown potency are being administered. The possibility of a systemic reaction makes obtaining informed consent most difficult. Second, when the bioassay is performed, it is limited to sensitized adults. Since there is a loss of symptomatic reactivity to most food allergens over time in adults who avoid exposure, it is difficult to obtain

a number of sufficient individuals with the appropriate skin reactivity to evaluate the potency of food extracts. This can even be the case with food allergen sensitivities such as peanut, tree nut, fish and shellfish that extend into adulthood. Third, even if an individual is willing and has the appropriate skin reactivity, the individual's actual degree of sensitivity is often not sufficient to permit the bioassay to actually be performed. Fourth, there is a general concern that a potency assessment dependent upon skin reactivity does not accurately reflect the actual potency of food allergens that are ingested and adsorbed through the gastrointestinal (GI) tract. Finally, the quality of the bioassay can be compromised by prior anti-histamine use, dermagraphism, the variability of skin test procedures and the diverse criteria used for defining positive results. These limitations, especially the dependence on consented human subjects with sufficient sensitivity to the food specificity of interest, have forced investigators interested in food allergen potency assessment toward the use of human IgE antibody-based *in vitro* assays.

4.3 IgE antibody-based *in vitro* assay for food allergen detection

The radioallergosorbent test or RAST was the first immunoassay developed to detect allergen-specific human IgE antibody.[2,16] It employed a paper disc-based solid-phase allergen or allergosorbent to bind specific antibody from human serum and radiolabeled anti-human IgE to detect bound IgE antibody. A minor modification involving pre-incubation of the IgE antibody containing human serum with an allergen preparation (such as food extract) prior to the addition of the allergosorbent produced a concentration-dependent inhibition of the IgE antibody binding to the allergosorbent. The quantitative nature of this inhibition permitted the amount of allergen in the extract to be assessed. This competitive inhibition assay format (RAST inhibition assay) rapidly became a widely used tool for identifying the presence and quantifying the amount of allergen in a test extract. More recently, a plate-based enzyme immunoassay (EIA) version of the RAST inhibition assay has been developed as discussed elsewhere in this volume. Since allergen is typically adsorbed on plastic surfaces of microtiter plate wells, the EIA inhibition assay works best with purified allergens. Its IgE antibody binding capacity is generally more limited than assays that use allergens covalently coupled to an allergosorbent since less than 1 microgram of total protein typically adsorbs on the plastic well surface. Both the RAST and EIA inhibition assays are especially useful for assessing the allergenic potency of extracts containing mixtures of allergenic proteins because the allergosorbent can be prepared in a manner to ensure that all the different proteins in the mixture are insolubilized in molar excess concentrations to IgE antibody levels in serum. However, as with the *in vivo* method, the *in vitro* competitive RAST inhibition assay still

depends on human serum with sufficient quantities and appropriate specificities of the allergen-specific IgE antibody.

4.3.1 Reagents

There are a number of required reagents that are needed to perform the competitive RAST inhibition analysis. These include the allergosorbent that is composed of a solid-phase matrix and an allergen component, allergen-specific IgE antibody containing human serum, a radioisotope-, enzyme- or fluorophor-labelled anti-human IgE detection reagent and characterized allergen calibrators.

Allergen

There are several hundred food allergen specificities that are known to induce IgE antibody responses in humans. Of these, there are a handful of allergen specificities that produce much of the food allergy symptoms involving the GI tract (vomiting, diarrhoea), respiratory tract (pneumonitis, asthma) and skin (chronic urticaria, atopic dermatitis). These include cows' milk, chicken egg, wheat, soybean, fish, crustacean shellfish, peanuts, tree nuts, cereal grains and citrus fruits. *In vitro* assay manufacturers have classified them as a group with the letter F (for foods) and a sequential number that has no significance other than when it was identified as an allergen source. For instance F13 is the peanut specificity that is prepared from raw and shelled *Arachis hypogaea*. Within the food group, the allergens can be segregated into subgroups, such as dairy, fish, grains/grass, seed/nut, legume, crustacean, vegetable, meat, fowl, mollusk, fungi, fruit, spice and stimulant (chocolate, coffee). A number of complex food allergen extracts have been further characterized with immunochemical methods so that individual allergenic proteins have been identified. For instance, five allergens have been identified in chicken egg white: ovomucoid (Gal d 1), ovoalbumin or conalbumin (Gal d 2), ovotransferrin (Gal d 3), lysozyme (Gal d 4) and chicken serum albumin (Gal d 5).[17,18]

For the purposes of this discussion, food extracts should be considered complex protein mixtures. They are prepared by selecting fresh or cooked food, grinding it up and preparing a physiological extract that is sometimes defatted and then centrifuged and sterile filtered. Initial quality control of the crude extract begins with analysis in one of several immunochemical methods (isoelectric focusing immunoblot, SDS-polyacrylamide gel electrophoresis separation followed by Western blot analysis, competitive binding immunoassay) to ensure that it contains allergenic proteins. Allergen extracts are subject to contamination, inherent biological variation and laboratory misclassification. The use of fresh or cooked food, the precise method and buffer used in extraction and the subsequent processing (column chromatography, precipitation steps) can lead to highly variable extracts. Stability during storage and following chemical manipulations such as coupling onto a solid support or labelling with biotin can add to the heterogeneity.

Allergosorbent

The solid phase allergen or allergosorbent is one of the principal components of the competitive RAST inhibition assay. The earliest RAST assay used paper discs that were activated with cyanogen bromide to covalently couple proteins. Because the paper disc was a defined size that fit into a 12×75 mm test tube, it was difficult to vary the allergen amount bound to the disc. This led researchers to covalently couple allergenic proteins to a variety of mobile cyanogens/bromide-activated carbohydrate-based particles such as cellulose (Avicel®/FMC BioPolymer, Philadelphia, PA) and cross-linked agarose (Sepharose™ CL-4B; GE Healthcare AB, Uppsala, Sweden).[19,20] Allergenic proteins have also been coupled to cellulose threads, immobilon or nitrocellulose membranes or more recently to a high binding capacity immobile sponge that is used in the Pharmacia CAP System™.[3] Diagnostic Products Corporation has chosen to biotinylate allergen mixtures and then insolubilize them on an avidin-coated solid phase.[5] In each of these cases, the covalent binding process has permitted the immobilization of proteins of widely varying molecular weights and isoelectric points to a single solid phase. Ideally, the allergen concentration on the solid phase should be in molar excess to the amount of allergen-specific IgE antibody present in the human serum.

Some commercially-available allergosorbents have also been prepared with food allergen mixes. For instance, the F × 5E from Pharmacia contains major allergens from the five foods (chicken egg white, cows' milk, fish [*Gadus morhua*], wheat [*Triticum aestivum*], peanut [*Arachis hypogaea*] and soybean [*Glycine max*]) that are known to produce most food allergies in young children. While these food allergen mixes containing allergosorbents are useful in screening children for confirmation of sensitization to foods, they are not generally useful in inhibition assays for assessing allergenic potency of food extracts and thus they are not discussed further in this chapter.

A different allergosorbent preparation approach has been used in microtiter plate-based non-isotopic assays. While the inhibition format of ELISAs is discussed elsewhere in this volume, it is important to note here that adsorption of proteins onto a plastic microtiter plate surface has severe limitations for complex protein mixtures such as those present in food extracts. Adsorption is a random process in which proteins of select isoelectric points and molecular weights adhere to the plastic surface by weak ionic and hydrophobic interactions. This adsorption process in allergosorbent preparation functions best for recombinant or native allergens that have been purified from other 'irrelevant' proteineous material in the extract. Unfortunately, it has not been possible to ensure the binding of all the relevant allergens from crude allergen extracts, especially those prepared from foods. Therefore, it is recommended that a covalent coupling method be employed if mixtures of proteins from foods are being used to prepare the allergosorbent.

Allergen-specific IgE antibody source

The second critical reagent in the competitive allergosorbent inhibition assay is the allergen-specific human IgE containing serum. Most atopic individuals produce IgE antibodies to multiple allergen specificities. Only a small number of these specificities are relevant to any given RAST inhibition assay for food allergen detection and quantification. For this reason, pools containing serum from 10–100 individuals who are naturally sensitive to the allergen of interest are the most useful in competitive inhibition assays. Use of individual sera in inhibition assays is uncommon (unless units of plasma are available) because IgE antibody is typically present in ng/ml levels and this generally requires the use of undiluted serum. The individuals providing serum should have a history of allergic symptoms following ingestion of the food in question. Ideally, the subjects should also have a double-blind placebo-controlled food challenge to verify their sensitivity to the food specificity in question. Use of serum pools increases the volume of serum available and the probability that the IgE antibody-containing serum will be representative of the major allergen specificities relevant to the particular allergen that is being detected in the inhibition assay. Use of a serum pool also minimizes the possibility of an over-representation of idiosyncratic minor IgE antibody specificities. These theoretical advantages are, however, sometimes offset by the fact that mixing many sera tends to concentrate the most common IgE antibody specificities and dilute out the minor IgE antibody specificities.

When human serum containing specific IgE antibodies is in short supply, well-characterized polyclonal antibodies from hyper-immunized animals or monoclonal antibodies that are known to bind to epitopes on defined food allergens may be attractive alternatives. These can be used either individually or in mixtures to replace human IgE containing serum; however, care should be taken in their use to make sure that these antibodies bind specifically to allergen epitopes and not just antigenic proteins in the food extract.

Anti-human IgE reagent

Bound IgE antibody in the RAST or its non-isotopic counterpart may be detected with I^{125} radioisotope or enzyme-labelled anti-human IgE Fc. A number of labelled polyclonal anti-human IgE reagents are commercially available. Alternatively, biotinylated monoclonal antibodies specific for human IgE Fc such as clone HP6061, murine IgM and clone HP6029, murine IgG1 are available from companies such as EMD Biosciences (La Jolla, CA). These polyclonal and monoclonal antibodies are usually prepared by immunization of animals with the Fc fragment of a human IgE myeloma and screened for IgE reactivity with a second intact IgE myeloma. Specificity for human IgE needs to be confirmed using well-characterized human immunoglobulin-related proteins, including IgE, IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgD and kappa and lambda light chains and secretory component. The material safety data sheet for the anti-human IgE should indicate its immunizing antigen source, species, clonality (polyclonal or

monoclonal), pH, preservatives, label type, buffer, shelf-life, optimal temperature for storage and percentage cross-reactivity to human polyclonal and monoclonal myeloma proteins in comparison to its reactivity with human IgE. This can be performed by dilutional analysis using the consensus protocol from the National Committee on Clinical Laboratory Standards.[7]

Calibrators, standards and controls
Commercially-available standardized allergen extracts in the US are limited to short ragweed pollen, Hymenoptera venom, house dust mites and cat hair, dander or pelt. There are a number of purified food allergen reagents available from researchers: peanut (Ara h 1, 2 and 3), chicken egg (Gal d 1 [ovomucoid], Gal d 2 [ovalbumin], Gal d 3 [ovotransferrin] and Gal d 4 [lysozyme]), cows' milk (α-casein, β-lactoglobulin, α-lactalbumin), Brazil nut (Ber e 1), rice (Ory s 1: α amylase inhibitor), codfish (Gad c 1 [antigen M]), shrimp (tropomyosin, Pen a 1, Pen i 1, Met e 1). No standardized crude food allergen extracts are available for use as reference proteins. Thus, any calibrator that is to be used in the competitive inhibition assays to study a food extract will have to have a previously defined potency from researchers in the area. It can be used to assess the relative potency of new food extracts or to interpolate competitive inhibition data for purposes of identifying the presence and quantifying the amount of the food allergen in a test extract.

Preparation of reference allergen calibrators is challenging, especially when the extracts being testing are prepared from foods with widely different biomass matrices. For instance, highly purified chicken egg white lysozyme (Gal d 4) can be weighed out and used to prepare a precise calibrator for a Gal d 4 allergosorbent-based inhibition assay. However, the matrix into which the calibrator lysozyme is dissolved must vary as a function of the matrix of the material being tested. For instance, various lots of red wine, extracts of cheese and xanthan gums may each be tested for the presence of Gal d 4 that is occasionally used in processing steps for these foods. However, the biomass matrix of wine, cheese and 0.25% xanthan gum preparations vary in pH, lipid, protein and carbohydrate content and general consistency from each other. Thus each will have a different non-specific binding profile that can lead to non-parallelism in the inhibition assay unless the calibrator has the same matrix. This issue is addresed by preparing appropriate calibrators with known quantities of Gal d 4 in each of the different matrices using predefined wine, cheese and xanthan gum preparations that are known not to contain Gal d 4 in any of the processing steps.

4.3.2 Assay design

The general assay protocol involves the addition of 0.05 mL of fluid phase test or reference allergen (inhibitor) at several three-fold dilutions into their respective 12 × 75 mm plastic test tubes containing 0.1 mL of the human IgE antibody serum pool, 0.4 mL of assay buffer (phosphate buffered saline with

1% bovine serum albumin) and 0.5 mL of particulate allergosorbent (typically 1–3% v/v). The tubes are capped and rotated overnight at room temperature. The following day, the tubes are uncapped and centrifuged (1000 × g, 10 minutes, room temperature) and the supernatant is aspirated. The pelleted allergosorbent–antibody complexes are washed three times with assay buffer followed by centrifugation and aspiration of the supernatant. For the RAST assay, radiolabeled anti-human IgE is added to the allergosorbent–antibody pellet (0.5 mL, typically 100 000 cpm, immunoreactivity 50–60%). The tubes are re-capped and rotated (one hour or overnight), uncapped and washed four times followed each time with centrifugation and supernatant aspiration. The amount of radiolabeled anti-human IgE bound to the allergosorbent is determined in an automated gamma spectrometer. Tubes are counted to statistical accuracy (at least 10 000 counts).

A number of quantitative, second and third generation, non-isotopic RAST-type assays for allergen-specific IgE antibody have been introduced commercially. The most widely used assay is the Pharmacia ImmunoCAP System™ that employs a sponge matrix onto which allergenic proteins are covalently coupled and an enzyme-labelled anti-human IgE to detect bound IgE antibody.[3,4] The CAP System™, like most of the other new generation RAST-type assays, is calibrated with a total serum IgE dose–response curve so IgE antibody results are reported in kIU/L, based on a World Health Organization Total Serum IgE reference preparation. Moreover, the CAP™ allergosorbents tend to be stable. Most are cleared by the US Food and Drug Administration; however, there are some that are labelled as 'analyte-specific reagents' because they have not been FDA-cleared due to the unavailability of sufficient numbers of sera from individuals sensitized to that food. Each new batch of allergosorbent is quality controlled by the manufacturer with IgE-containing sera from subjects with documented allergic disease. Prior to coupling, the food allergen extract is generally subjected to immunochemistry studies by the manufacturer that involve Western blot analysis. Thus, the performance of the allergosorbent (and the IgE antibody assay) remains highly reproducible and potent for most allergen specificities, especially when testing challenging crude food allergen extracts. For this reason, use of these commercial allergosorbents and newer generation IgE antibody assays has become an attractive alternative in food allergen detection and quantification to the use of research-based particle allergosorbents that have been routinely used in the past in RAST-type assays.

4.3.3 Data analysis

All analyses are performed in replicates. The extent of non-specific binding is assessed by the analysis of sera from non-food allergic (atopic and non-atopic) individuals instead of the food-specific IgE antibody-containing serum pool. The extent of maximum IgE antibody binding (B_0) is determined by the addition of buffer (0 dose) instead of test or reference allergen extracts

(inhibitor). The percentage of inhibition is computed by subtraction of the non-specific binding from the total binding obtained with the test or reference extract inhibitor and this is then divided by B_0.

The percent inhibition (linear scale) is plotted on the ordinate versus the quantity or volume of inhibitor ($\log_{10}$ scale) on the abscissa. Since the test allergen extract has been analyzed at multiple dilutions, least squares regression lines for each of the test extract results from 10–90% inhibition should be plotted. Semi-log plots in this region should be linear. Adolphson, Yunginger and Gleich[21] proposed the following criteria for validity: a minimum of four inhibition points; these inhibition points must bracket the 50% inhibition point; the linear correlation coefficient for each curve is > 0.9; and the slope of the test extract inhibition curve must not differ from that of the standard inhibition curve at $p < 0.01$, as determined by a t-test.

Identity of the allergen distribution between two extracts may be surmised if the inhibition curves are non-overlapping or parallel. Parallel but displaced curves would suggest identity of the allergen distribution but differences in allergen quantity (potency). Non-parallel (intersecting) curves suggest partial identity and differential quantities of allergens. Inhibition curves with a slope of 0 or a negative slope would suggest no identity or cross-reactivity.

4.3.4 Assay quality control

Quality control is performed by analyzing dilutions of several control extracts that have previously determined potency estimates. This permits the computation of a precision profile in which the coefficient of variation (CV) of the interpolated dose is plotted against the allergen dose. The precision profile plot may be used to statistically define the dynamic range of the allergen assay that provides the desired precision (10–15% CV). It can also be used to determine the sensitivity of the assay or the minimum detectable concentration of allergen inhibitor that can be reproducibly and statistically distinguished from the variance of the background noise produced in the absence of allergen inhibitor. The upper limit of the dynamic working range of the assay is that concentration of inhibitor that saturates the system and above which the assay estimates of allergen quantity become increasingly imprecise.

4.4 Applications

A number of studies provide unique insights into the practical use of the RAST inhibition assay in the assessment of foods for the presence and relative quantity of allergens. As an illustration, the RAST inhibition assay has been used to investigate the allergenicity of commercially-available peanut-containing foods and to identify peanut contamination of non-peanut derived foods.[19,21,22] In one such protocol, 100 gm of each test product is defatted

once with 250 mL of acetone and then five times with 250 ml of ethyl ether. The residual, defatted product is then dried and mixed for 20 hours at 25 °C with 300 mL of 0.1 M ammonium bicarbonate adjusted to pH 8.0. The extract is clarified by centrifugation of the supernatant for 30 minutes at 24 000 × g at 2 °C and sterile filtration. A total protein is performed using the Folin phenol reagent described by Lowry *et al.*[23] Only the peanut oil was tested directly without defatting or extraction.

In these studies, the competitive RAST inhibition analysis was performed using a human IgE antibody pool comprising sera from multiple peanut allergic individuals with highly positive peanut specific IgE antibody levels. The peanut allergosorbent was prepared by coupling crude raw peanut extract to cyanogen bromide-activated microcrystalline cellulose particles. RAST inhibitory activity of the test extracts was compared to the competitive inhibition reference curve produced by a reference peanut extract.

Qualitatively, 17 of the 20 peanut products tested were shown to contain peanut allergen by the competitive RAST inhibition assay. Two were raw peanuts and the others were processed foods containing peanut that had involved shelling, blanching, dry roasting, oil roasting, toasting, grinding, defatting, extracting and/or combining them with other ingredients. Three figures have been reproduced from the Nordlee *et al.* paper[19] to illustrate several points. Figure 4.2 displays similar RAST inhibition curves produced

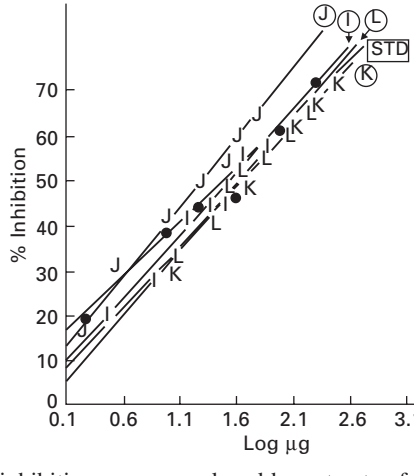

Fig. 4.2 RAST inhibition curves produced by extracts of four peanut butter products. The percent inhibition of the binding of peanut-specific IgE-containing serum to raw peanut extract allergosorbent that is induced by the various extracts is plotted as a function of the log of the protein concentration in the extract in micrograms. Extract I = peanut butter powder; extract J = peanut butter; extract K = peanut butter syrup; extract L = peanut butter flavoured chips. The inhibition curves are essentially parallel, with slopes that are analogous to the RAST inhibition curve slope produced by the standard raw peanut extract. Only the slope of the peanut butter extract (J) differs significantly from the standard curve ($p < 0.025$) and extract J was more potent than extracts from the other peanut butter products that contained a mixture of peanut and whey proteins. Reproduced with permission from Nordlee *et al.*[19]

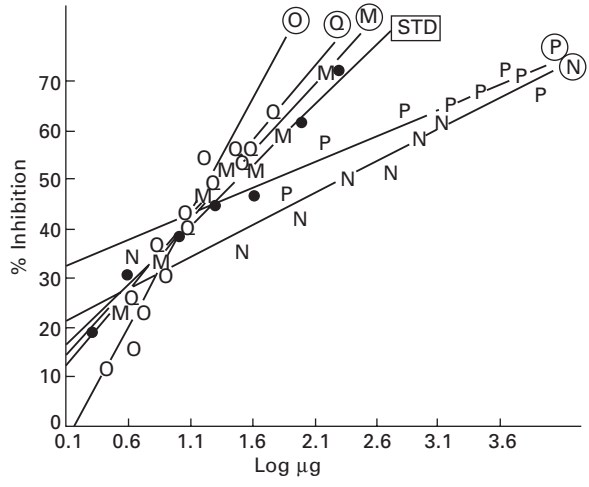

Fig. 4.3 RAST inhibition curves produced with raw and roasted peanut extracts. Extract M = deflavored peanuts; extract N = raw Virginia peanuts; extract O = oil roasted Virginia peanuts; extract P = raw Florunner peanuts; extract Q = dry roasted Florunner peanuts. These curves suggest differences in the allergenic potency among the different varieties of peanuts. Moreover, other factors are most likely involved because the inhibition curves produced by extracts of the deflavored peanuts (M) and dry roasted Florunner peanuts (Q) had approximately the same degree of allergenicity in comparison to the standard peanut extract. Reproduced with permission from Nordlee *et al.*[19]

with the peanut butter product extracts that appear to have a similar quantity and specificity distribution of peanut allergens. In contrast, Fig. 4.3 displays inhibition curves with significantly different slopes suggesting that the extracts from Florunner peanuts are less allergenic than the deflavored and dry roasted peanuts. This also may suggest varietal differences in the nature of the allergenic determinants among the different sources of peanuts. Figure 4.4 illustrates RAST inhibition curves for peanut oil and the acid hydrolyzed peanuts that contained no detectable peanut allergen. The negative slope of the inhibition curve with the peanut oil suggests non-specific interference in the RAST inhibition assay. In Fig. 4.4, the peanut hull flour contained low levels of peanut allergen in comparison to the reference peanut extract as evidenced by the large shift of the inhibition curve (albeit parallel) from the reference curve. The authors conclude that peanut hypersensitive individuals should avoid all peanut-containing products with the exception of peanut oil and possibly acid hydrolyzed peanut where the allergens appear to have been destroyed. The authors also caution that some non-peanut foodstuffs can become cross-contaminated with peanut allergens as detectable by competitive RAST inhibition due to inadequate cleaning of common processing equipment.[21]

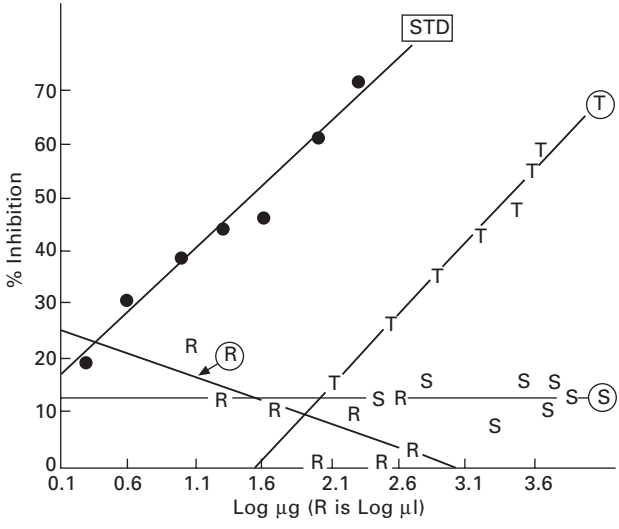

Fig. 4.4 RAST inhibition curves produced by a variety of peanut containing foods and peanut oil. Extract R = peanut oil (analyzed per microliter volume of oil); S = hydrolysed peanut protein; extract T = peanut hull flour. The flat or inverse slope of extracts S and R, respectively indicate that these materials do not contain any detectable peanut allergen. In contrast, the peanut hull flour extract contains only small amounts of peanut allergen as evidenced by the parallel slope that is displaced extensively to the right. Reproduced with permission from Nordlee *et al.*[19].

4.5 RAST inhibition assay strengths and weaknesses

There are a number of assay protocols that employ human IgE antibodies in a competitive inhibition format to detect and quantify the presence of residual food allergens in aqueous extracts. These methods differ in minor aspects, such as in the solid-phase matrix to which the allergen has been coupled (covalent coupling to particles or nitrocellulose paper versus adsorption to plastic microtiter plates) and the type of label used in the assay (radiolabel versus enzyme with colormetric or fluorescent substrates). Each of these protocols has their strengths and weaknesses.

4.5.1 Strengths

The competitive RAST inhibition's strength is its ability to detect and quantify cross-reactivity among allergenic proteins from structurally similar allergens in different foods and between pollens and food.[24] Because allergen-specific human IgE antibody is used, clinically relevant allergens are detected that may induce human IgE antibody responses in some clinically sensitive individuals. The slope and displacement of the test extract inhibition curve from the reference curve provides useful information about the relative allergenic potency and specificity distribution of complex protein allergen mixtures, especially when specific allergens have not yet been identified.

4.5.2 Weaknesses

All human IgE-based methods suffer from a dependence on the unique allergenic specificity of the IgE antibody that is naturally present in individual sera or serum pools. Second, inhibition assays that use IgE antibody containing human sera consume large amounts of serum because the IgE antibody is normally present in small (ng/mL) quantities. For this reason, IgE antibody-based inhibition assays are rarely used in food manufacturing facilities where IgE containing sera are not readily available. Third, the heterogeneity of the IgE antibody specificity among different sensitized individuals can be a problem for reproducibility of the RAST inhibition assay. Cross-validation of serum pools is important to verify consistency of the IgE antibody specificity and quantity in new serum pools which are being prepared for use. Fourth, radioisotopes such as I^{125} in the RAST assay need special care and skill and specialized laboratory licences ensuring appropriate surveying programs to permit their handling. Some laboratories prefer to use non-isotopic methods such as enzymes or fluorophors that are safer and easier to handle and dispose of after the assay is completed. This has been largely addressed by the development of commercial RAST-like assays that employ an enzyme label and colorimetric or fluorescent substrates to detect IgE bound to the allergosorbent following the serum incubation step.

4.6 Future trends

The competitive inhibition immunoassay format that uses human serum containing allergen-specific IgE antibody will remain a principal tool for detecting and quantifying food allergen residues in extracts of test foodstuffs, especially in cases where the major food allergens have not been identified. In fact, in the case of food containing fermented ingredients or hydrolyzed proteins, the competitive inhibition assay may be the only assay that can adequately detect and quantify residual allergenic proteins. The use of the human IgE antibody provides an element of specificity to the assay since it detects clinically relevant allergenic proteins that can elicit allergic symptoms in sensitized individuals. However, once the major food allergens have been characterized, polyclonal or monoclonal animal antisera, specific for known (often cloned and sequenced) allergenic epitopes, will begin to be used in two-site immunoenzymetric assays to replace the use of human IgE antibody containing sera in competitive inhibition assays. Future trends will also involve the increased use of non-isotopic labels coupled to the anti-human IgE reagent, improved and better characterized reference allergen extracts and the use of solid phases that increase the binding capacity of allergosorbents.

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5

Immunoblotting in allergen detection

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5.1 Introduction

Since its introduction in health sciences (Burnette, 1981; Glass, Briggs and Hnilica, 1981; Karcher *et al*., 1981; McMichael, Greisiger and Millman, 1981; Reiser and Wardale, 1981; Symington, Green and Brackmann, 1981), immunoblotting or Western blotting has proven to be a very powerful research tool and one that has spread rapidly. A search in PubMed using the keywords 'immunoblotting', 'food' and 'allergens' resulted in almost 500 hits, of which the first go back to the second half of the 1980s (Theobald *et al*., 1986; Enberg *et al*., 1987; Burks, Jr, Brooks and Sampson, 1988; Bush *et al.*, 1988; Enberg, McCullough and Ownby, 1988; Vallier *et al*., 1988 Naqpal *et al*., 1989;). In most cases, immunoblotting (Kurien and Scofield, 2003) is used in conjunction with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Fig. 5.1).

Separation of proteins prior to electrophoretic transfer to nitrocellulose or polyvinylidene difluoride (PVDF) membranes can also be two-dimensional, in which case SDS-PAGE is preceded by isoelectric focusing (Celis and Gromov, 2000; Lilley, Razzaq and Dupree, 2002). Two-dimensional electrophoresis allows separation of proteins with similar molecular mass but different pI. In food allergen research, immunoblotting has been used for many different purposes. Most frequently, the technique has been applied for qualitative purposes, i.e. the identification of allergenic (IgE-binding) molecules and the establishment of IgE cross-reactivity between foods or between foods and inhalant allergens (Bauer *et al*., 1996; Teuber and Peterson, 1999; Kazemi-Shirazi *et al.*, 2000; Asturias *et al*., 2002; Lüttkopf *et al*., 2002; Reindl *et al*., 2002; Miguel-Moncin *et al*., 2003; Wensing *et al*., 2003; Bolhaar

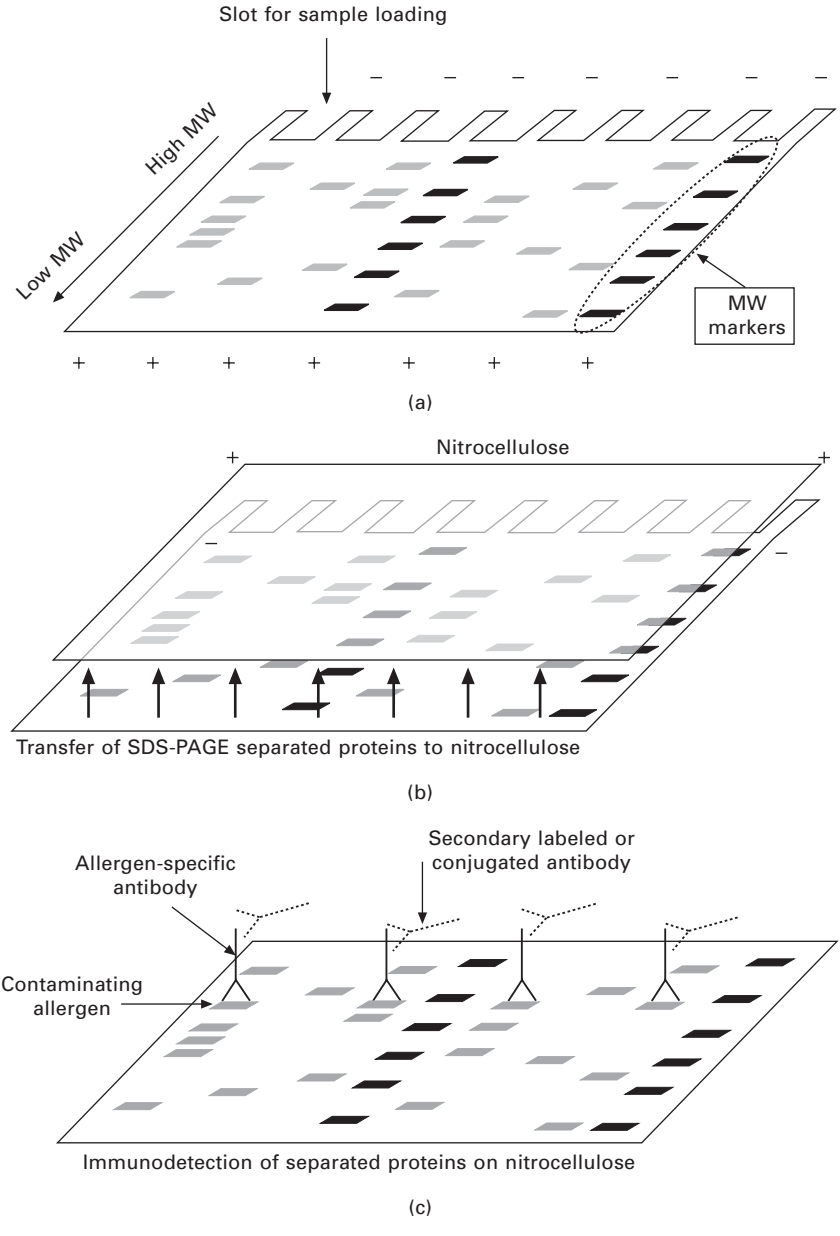

Fig. 5.1 Schematic representation of SDS-PAGE in conjunction with immunoblotting: (a) Proteins in extracts of food samples are separated by SDS-PAGE (MW = molecular weight); (b) Separated proteins are electroblotted from the gel onto nitrocellulose (or PVDF) membrane; (c) By incubation with allergen-specific antibody reagents, contamination can be traced.

et al., 2004; Comstock *et al*., 2004). In the former case, serum samples of food-allergic patients are used to identify proteins that are recognized by IgE antibodies. A critical issue in such analyses is of course patient selection. In the latter case, IgE binding to immunoblots is inhibited by allergen extracts or purified natural or recombinant allergens with suspected cross-reactivity to the allergens on immunoblot. Immunoblot inhibition with purified allergens as an inhibitor is a powerful tool to identify or confirm the nature of an allergen recognized on immunoblot.

Detection of allergenic molecules in food products is another area where immunoblotting can be applied. Specific monoclonal antibodies (mAb) or monospecific polyclonal antibodies against the major peanut allergen Ara h 1 could, for example, be used to trace contamination of chocolate products with peanut. Also human sera from allergic patients have been used for the detection in chocolate of trace amounts of allergens from, for example, hazelnut and almond (Scheibe *et al*., 2001). Prerequisite for such an application is selective recognition of the food contamination under investigation. Although antibodies do not necessarily have to be directed to allergens (any protein will do), we will limit ourselves to allergen-reactive antibody reagents.

5.2 Mono-specific antibody reagents

Which antibodies should be used for detecting allergens in food products? Most food-allergic patients have IgE antibodies that are far from food-specific, let alone mono-specific. Even when they are clinically allergic to a single food, their IgE antibodies almost always cross-react to some other foods. This property makes IgE antibodies less suitable for specific detection of traces of allergens in food products. Mouse mAbs against many important food allergens have been produced but are usually not commercially available (Kahlert *et al*., 1992; Tsuji *et al*., 1993; Burks *et al*., 1994, 1995; Fahlbusch *et al*., 1995; Gonzalez De La Pena *et al*., 1996; Miyazawa *et al*., 1996; Reese *et al*., 1996; Becker 1997; Bando *et al*., 1998; Karamloo *et al*., 2001; Yamashita *et al*., 2001; Duffort *et al*., 2002; Restani *et al*., 2002; Rozenfeld *et al*., 2002; Pomes *et al*., 2003; Pomes, Vinton and Chapman, 2004). These antibodies can potentially be used to detect food allergens in extracts of food (products). An example is given in Fig. 5.2. A mAb raised against the major birch pollen allergen Bet v 1, mAb 5H8, was found to be cross-reactive to the major allergen in apple, Mal d 1 (Akkerdaas *et al*., 1995). Semi-quantitative detection of Mal d 1 in extract of apple is possible using a standard curve of purified Mal d 1. Similar approaches can be used for many other food allergens.

Polyclonal antisera raised against purified food allergens can be used instead of mAbs. Usually rabbits are used for this purpose, but also guinea pigs, sheep, goats and even chicken are sources for mono-specific antibody reagents. The advantage of mono-specific polyclonal antisera is that they usually have higher affinity than mAbs, resulting in a lower detection limit.

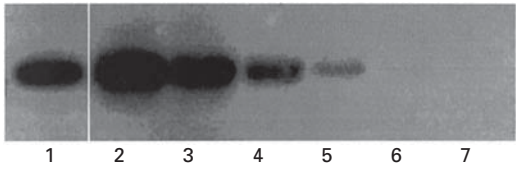

Fig. 5.2 Semi-quantitative detection of Mal d 1 in apple. Monoclonal antibody 5H8 raised against Bet v 1 was used for the detection of Mal d 1 in extract of Golden Delicious apple. Lanes 2–7 contain a standard curve of affinity purified Mal d 1 (highest concentration 30 µg/ml; two-fold dilutions), lane 1 an apple extract. On the basis of this experiment a concentration of ~ 10 µg/ml was assigned to the apple extract.

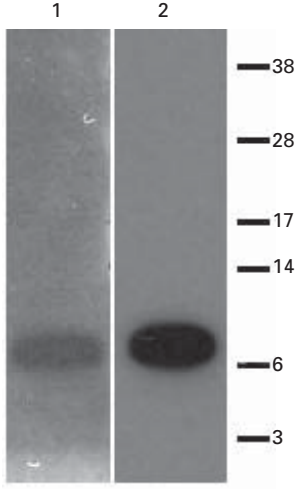

Fig. 5.3 Detection of apple LTP (Mal d 3): monoclonals versus polyclonals. Mouse monoclonal antibodies (lane 1) and rabbit polyclonal antibodies (lane 2) raised against purified Mal d 3 both exclusively detect the 9 kDa LTP band, demonstrating their mono-specificity.

In addition, loss of reactivity as a result of processing steps used in highly-processed foods is more likely for mAbs. Moreover, production of such antibodies is much cheaper and easier than mAbs. Most commercial enzyme-linked immunosorbent assay (ELISA) kits for allergenic residue detection utilize polyclonal antibodies. Of course, polyclonals lack the infinite availability of mAbs. In Fig. 5.3 rabbit antibodies and mAbs against Mal d 3 have been used to detect this non-specific lipid transfer protein (LTP) in extract of apple. Both reagents exclusively detect the same band in apple extract.

5.3 Critical assessment of (mono-)specificity

As described above, mAbs reactive with a major food allergen like Mal d 1 are very useful tools to (semi-)quantify such allergens in extracts of the food itself, in this case, apple. What if they are used for tracing the presence of a food (allergen) in compound food products like candy bars, desserts or cookies? Mal d 1 is member of a large family of so-called pathogenesis-related (PR) proteins, i.e. the PR10 proteins (Breiteneder and Ebner, 2000). mAb 5H8 was raised by immunization with Bet v 1, the birch pollen representative of the PR10 protein family, resulting in mAbs that are cross-reactive to Mal d 1 (Akkerdaas *et al*., 1995). This cross-reactivity is, however, not limited to apple, but covers a broad spectrum of fruits and nuts (Fig. 5.4). This implies that detection of a protein band with mAb 5H8 in an extract of a compound food product establishes the presence of a PR10-like allergen, but its origin remains unclear due to cross-reactivity of the antibody reagent. Similarly, rabbit polyclonal antibodies against apple LTP (Mal d 3) recognize LTPs in several related fruits and nuts.

In the above described examples, cross-reactivity results in detection of related allergens. IgE antibodies in food-allergic patients tend to have similar degrees of cross-reactivity. A patient with severe LTP-induced peach and apple allergy (Fernandez-Rivas *et al*., 2003) will most likely want to know whether a food product contains fruit or nut LTP in general, even though not every LTP will induce clinical allergy (Asero *et al*., 2002). Antibodies reactive to a family of related allergens therefore certainly have their value. Polyclonal antisera can, however, also be (cross-) reactive across the border of protein

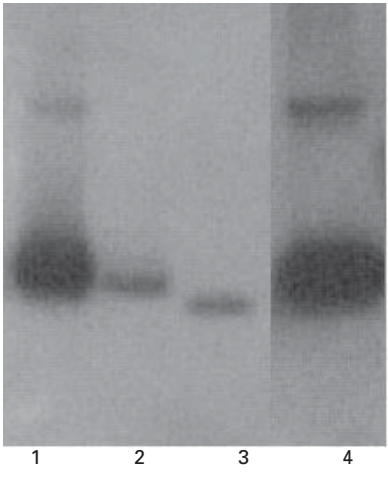

Fig. 5.4 Detection of Bet v 1 homologues in different fruits. Mouse monoclonal antibody 5H8 detects Bet v 1 homologues in a broad spectrum of fruits. Lane 1: control (birch pollen extract), lane 2: sharon fruit extract, lane 3: jackfruit extract, and lane 4: apple extract.

families. In that case, the interpretation of immunoblot data becomes more complex. Rabbits or other laboratory animals are not always immune-naïve when they are immunized to a food allergen, since they may have been (orally) exposed to foods before. Serum samples taken at the start of an immunization protocol frequently contain IgG antibodies that react to the food from which the immunogen originates. If this reactivity causes recognition of proteins in the same molecular mass range as the immunogen, the antiserum will have to be affinity-purified using the same immunogen before it can be used for its specific detection in immunoblotting. Alternatively, binding to immunoblot can be inhibited by the purified immunogen to confirm specificity of the interaction.

Another source of disturbing cross-reactivity resides on the immunogen itself. Immunization of rabbits with plant glycoproteins usually results in an immune response that is partly directed to plant carbohydrate moieties, in particular complex N-glycans (Kaladas, Goldberg and Poretz, 1983; Prenner *et al*., 1992; Faye *et al*., 1993; Wilson *et al*., 1998; Bardor *et al*., 2003). Plant complex N-glycans carry two substitutions that are not seen in mammals, a fucose linked α(1,3) to the proximal N-acetylglucosamine and a xylose linked β(1, 2) to the core mannose (Lerouge *et al*., 1998, 2000; Fotisch and Vieths, 2001; van Ree, 2002). These carbohydrate moieties are, therefore, highly immunogenic in mammals. Since these typically linked fucose and xylose are found on most plant glycoproteins, antisera raised against them tend to recognize a broad spectrum of mainly high molecular mass glycoproteins in virtually any plant food. This is illustrated in Fig. 5.5, where an antiserum against the major grass pollen allergen Lol p 1 is used in immunoblots of several foods. There is a relatively simple way to remove these cross-reactive antibodies that severely lower specificity, namely absorption with a source rich in plant N-glycans. Essentially, any plant (food) extract coupled to a solid phase, for example Sepharose™ (GE Healthcare AB, Uppsala, Sweden),

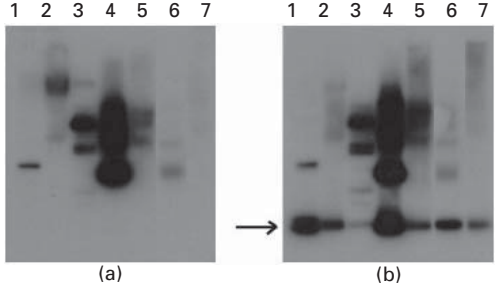

Fig. 5.5 Lack of specificity of polyclonal rabbit antisera. Rabbit antisera against two grass pollen allergens, Lol p 1 (a) and Lol p 12 or profilin (b) detect multiple bands in plant food extracts. Although anti-Lol p 12 indeed detects profilin (indicated by an arrow) this antiserum is far from monospecific. The fact that both antisera recognize very similar (glyco)proteins suggests that these antibodies were already present prior to immunisation and are directed to highly cross-reactive (carbohydrate?) structures.

can be used for depletion. It is important to choose a plant (food) that is phylogenetically not too closely related to the source from which the immunogen originates. This will decrease the risk that antibodies against the peptide backbone of the immunogen will also be depleted on the basis of cross-reactivity. Alternatively, protease-digested plant (food) extracts can be used for depletion. This is efficiently achieved using proteinase K (van Ree *et al*., 2000). Finally, purified plant-derived glycoproteins that carry the typical plant N-glycans are candidates for use as immunosorbent. A good example of such a glycoprotein is pineapple stem bromelain (Bouwstra *et al*., 1990).

5.4 Food processing and antibody specificity

An alternative to depletion of the antiserum is confirmation of the specificity of the interaction by immunoblot-inhibition with a non-glycosylated recombinant version of the allergen obtained by expression in *E coli*. Many food products undergo processing steps like heating, fermentation or high pressure before they are marketed. These processes potentially impact on the integrity of food proteins. It is, for example, well established that PR10 proteins like Mal d 1 lose their allergenicity during processing (Björksten *et al*., 1980; Asero *et al*., 2000). During extraction, polyphenol oxidases and peroxidases modify the protein in such way that IgE antibodies lose their reactivity. Subsequent heating will also result in loss of structural integrity (Breiteneder and Ebner, 2000). In contrast, lipid transfer proteins like Mal d 3 are extremely stable and survive most processing steps in a fully immune-reactive fashion (Asero, *et al*., 2000; Scheurer *et al*., 2004). When using antibodies for detection of allergens in processed foods, these reagents will have to be evaluated for their reactivity after processing of food. One could of course argue that an allergen that loses its reactivity after processing is not worth being detected because it poses no allergenic risk anymore. It should be realized though that if detection of an allergen is used as a marker for the presence of a certain food as an ingredient of a compound food, processing-sensitive allergens are not the optimal marker proteins. A choice for stable allergens such as LTP makes more sense. Alternatively, antibodies can be raised against processed allergens.

5.5 Future trends

Provided that the right precautions are taken considering specificity of antibody reagents, immunoblotting for the detection of allergens in food will remain a powerful qualitative and semi-quantitative tool. With the increasing availability of purified recombinant food allergens, it will become easier to produce

good mono-specific reagents. Disadvantages for a broad application of immunoblotting in a production setting are the requirement for specialized equipment and trained staff, and special handling if used in combination with human serum. For accurate quantitative analysis other techniques such as sandwich ELISA seem more appropriate. Although the same precautions apply for antibodies used in ELISA, the technique is more rapid and better adapted to use in a food processing environment.

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6

Enzyme-linked immunosorbent assays (ELISAs) for detecting allergens in foods

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6.1 Introduction

Immunoassays such as Enzyme-Linked Immunosorbent Assays (ELISAs) have been found to have considerable application in clinical diagnostics. They are in fact the method of choice, but have had thus far little impact on food analysis. However, since 1990 there have been an increasing number of reports on the use of ELISA techniques for environmental contaminants in foods. Unfortunately, only a few have been reported for food allergens.

Until 1988, when Yunginger *et al.*[1] reported a series of eight accidental deaths due to food-induced anaphylaxis, allergic reactions to food were not considered a significant public health risk. Subsequent reports of fatalities and severe allergic reactions[2–4] due to food allergies heightened the awareness of both regulators and the food industry that a small segment of the population could experience serious reactions to food allergens. With the increased attention to allergic reactions to food and, especially, allergen-related recalls, there was a need for quick and accurate methods to detect allergenic residues in food.

Prior to the current emphasis on immunochemical methods for the detection of allergens, most of the detection used organoleptic techniques, such as subjective sensory (smell and taste) tests, or chromatographic fatty acid profile analyses. Unfortunately, these sensory and chromatographic methods were neither specific to the allergenic proteins, nor sensitive enough to test for allergen residues. Methods currently used to test for allergens, including commercially available test kits, are primarily based upon immunochemical procedures, although some polymerase chain reaction (PCR) kits have also been developed. These allergen detection methods have recently been reviewed by Besler[5] and Poms *et al*[6]; an update is provided in the second part of this book.

6.2 Principles of ELISAs

Immunochemical methods for the analysis of environmental contaminants are relatively new in the analytical chemistry arena.[7,8] These methods are based on the use of a specific antibody as a detector for the analyte of interest, such as a food allergen. In this context, we do not differentiate between allergen and antigen, and they may be used interchangeably. Immunoassays are rapid, sensitive and selective, and are generally cost-effective. Immunoassays can be designed as rapid, field-portable, semi-quantitative methods, or as standard quantitative laboratory procedures. They are well suited to the analysis of large numbers of samples, and often do not require lengthy sample preparation as is the case in chromatography.

ELISA tests are based on the use of an enzyme linked to an antibody to detect the binding of antigen (Ag) and antibody (Ab). The enzyme converts a colorless substrate (chromogen) to a colored product, indicating the presence of Ag:Ab complex. In the food industry, ELISA tests are usually used to detect antigens such as allergens, pesticides, mycotoxins or pathogens in a sample. There are two techniques for antigen measurement, the 'sandwich technique' and the 'competitive technique.' Almost all commercial allergen ELISA test kits use the sandwich technique.

6.2.1 Antibodies

Immunoglobulins (as discussed in Chapter 3) are a group of closely related glycoproteins composed of 82–96% protein and 4–18% carbohydrate. An antibody is an immunoglobulin, a protein produced by the immune system in response to the presence of an antigen. Antibodies exist as one or more copies of a Y-shaped unit, composed of four polypeptide chains. Each Y contains two identical copies of a 'heavy chain' (H), and two identical copies of a 'light chain' (L), named as such by their relative molecular weights, linked together by inter-chain disulfide bonds (Fig. 6.1a). Intra-chain disulfide

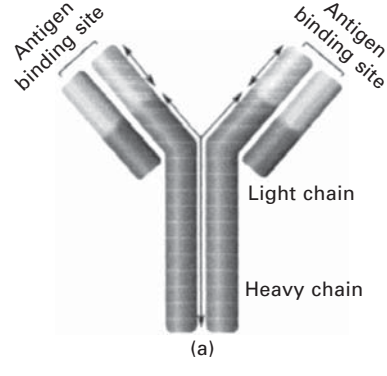

Fig. 6.1 (a) Antibody structures. (b) V_H and V_L regions on an antibody. (c) Pepsin and papain digestion of an IgG antibody.

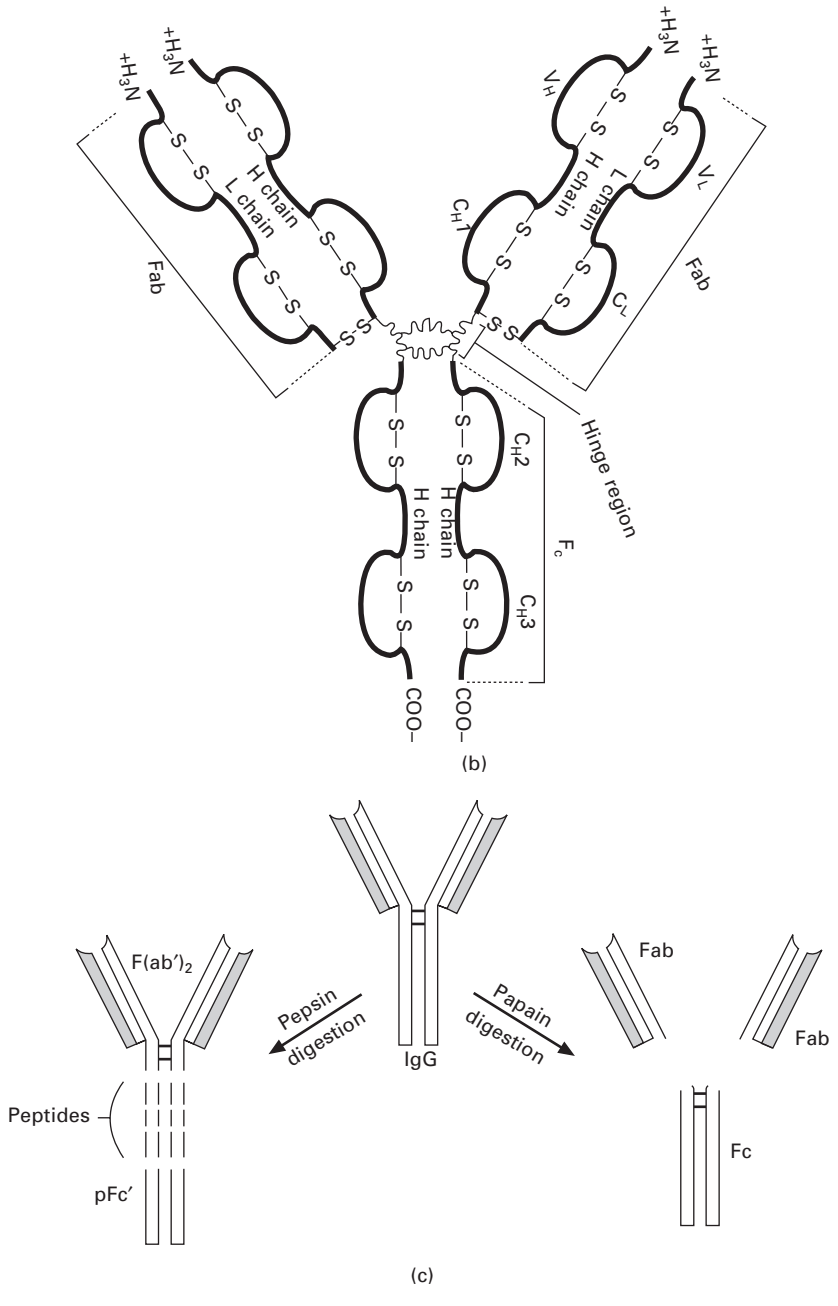

Fig. 6.1 continued.

bonds are responsible for the formation of loops, leading to the compact, domain-like structure of the molecule. The amino terminal portions of the heavy and light chains, characterized by a highly variable amino-acid composition among different antibodies, are referred to as V_H and V_L, respectively. The constant parts of the light chain are designated as C_L, while the constant parts of the heavy chains are further divided into three distinct subunits: C_H1, C_H2 and C_H3 (Fig. 6.1b). Functionally, the V regions are involved in antigen binding. The C regions interact to hold the molecule together and are involved in several biological activities, such as complement binding and binding to cell membranes.

Antibodies are divided into five major classes: IgM, IgG, IgA, IgD and IgE, based on their constant region structure and immune function. The most commonly used antibody is IgG, which can be cleaved into three parts, two F(ab) regions and one Fc, by the proteolytic enzyme papain, or into two parts, one $F(ab')_2$ and one Fc by the proteolytic enzyme pepsin. While both F(ab) and $F(ab')_2$ fragments can be used in immunoassays to enhance sensitivity, $F(ab')_2$ has higher avidity (Fig. 6.1c). When designing procedures, it is important to differentiate between monoclonal and polyclonal antibodies, as these differences are the foundation of both advantages and limitations for their use.

6.2.2 ELISA formats

Sandwich ELISA

Sandwich ELISA is a sensitive test that can detect and quantify the concentration of specific soluble proteins. A sandwich ELISA can be more specific because antibodies directed against two or more distinct epitopes are required. The basic sandwich ELISA method uses excess of highly purified, specific antibodies (capture antibodies), which are adsorbed or 'coated' onto plastic microwell plates. The immobilized antibodies serve to specifically capture their corresponding antigens, such as food allergens, that are present in samples. After washing away unbound material, the captured antigens are detected using enzyme-conjugated antibodies (detector antibodies). Following the addition of a chromogenic substrate-containing solution, the level of colored product generated by the bound, enzyme-linked antibodies can be conveniently measured spectrophotometrically using an ELISA-plate reader at an appropriate wavelength. While sandwich ELISA results can be quantified against a standard curve, the intensity of the color change is roughly proportional to the concentration of allergen in the sample, i.e. the more intense the color change, the higher the amount of allergen in the sample (Fig. 6.2). Alternatively, qualitative results can be visualized against the standard used.

Standard curves are generally plotted as the standard protein concentration versus the corresponding mean optical density (OD) value of replicates. The concentrations of the analyte-containing samples can be interpolated from the standard curve. This process is made easier by using a computer software

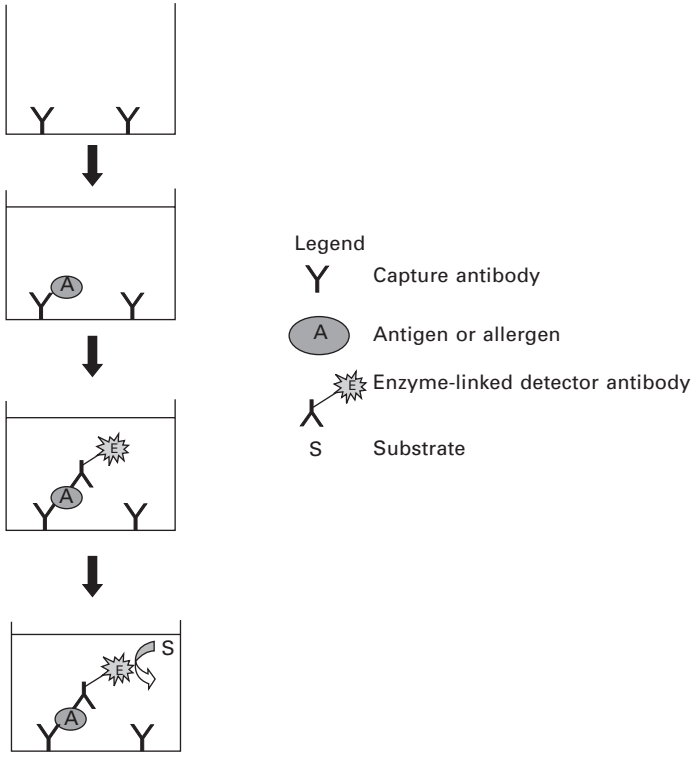

Fig. 6.2 A typical sandwich ELISA.

curve-fitting program, which is either a stand-alone or part of the ELISA reader operating software. Although a typical dose–response curve or standard curve is sigmoid shaped, depending on the design of the ELISA, investigators may choose to apply different curve-fitting analysis to their data, including linear regression, point-to-point, linear-log, logit log, four-parameter logistic or cubic spline transformations.[7,8]

Competitive ELISA

The competitive inhibition ELISA is a technique that uses a one-epitope approach for the Ab to recognize allergenic residue in a sample. In a competitive assay, the Ag is coated on the wells, and a solution containing a limited amount of first Ab along with the Ag or analyte is added. The assay is based on the principle that an Ag in the sample will bind to an Ab and then compete for the binding of the Ag coating on the wells. After the unbound Ab is washed off, a second Ab-enzyme conjugate is used to detect the bound Ag:Ab complex in the wells. Then a substrate of the enzyme is added (Fig. 6.3). In this format, the color produced is inversely proportional to the concentration of the analyte, i.e. the higher the color the lower the concentration of the protein.

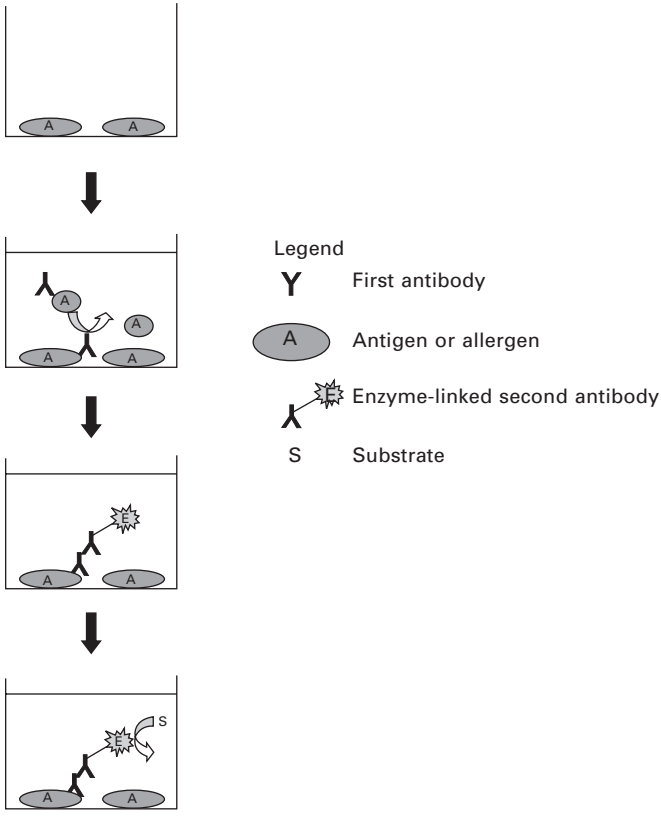

Fig. 6.3 A typical competitive inhibition ELISA. First antibody reacts with bound antigen, and a labelled second antibody reacts with the primary antibody.

General characteristics of ELISA platforms

Given the formats described previously the critical components needed to build a good immunoassay system to test for the presence of the antigen include: a supply of the appropriate antigen (used to raise antisera and as an antigen standard for the assay); a supply of high-quality antigen-specific antisera or antibodies; a stable, high-turnover enzyme detection system; and a substrate for the reaction. The single most effective way to enhance sensitivity in ELISA is to find a higher-affinity Ab, although increasing the amount of captive Ab in a sandwich ELISA may be possible. On the other hand, decreasing the amount of high-affinity first Ab in a competitive ELISA is an option. For quantitative ELISA, since the signals of unknown samples are compared to those of a standard curve, it follows that standards must be run with each assay to ensure accuracy.

A trained analyst in a modestly equipped laboratory can do a typical sandwich ELISA test in about 30 minutes–4 hours, while a competitive inhibitory ELISA takes about 4–8 hours to complete. It should be noted that

it is more complicated to develop a sandwich ELISA than an inhibitory ELISA. Sandwich ELISA requires a large amount of purified captive Ab and a successful conjugation of an enzyme to the signal Ab items are not necessary for inhibitory ELISA. The common buffers used for proteins, including Ab and Ag, in both ELISA systems are usually phosphate buffer or Tris buffer. Tris is temperature-sensitive, while phosphate is ionic strength-dependent. Although a number of enzymes have been used with immunoassays, alkaline phosphatase and horseradish peroxidase have received the most attention. The latter is usually preferred because of its low cost. Common substrates used with peroxidase include *o*-phenylenediamine (OPD) and 3,3′5,5′-tetramethylbenzidine (TMB) base. Both substrates require the use of different filters for reading the respective stopped and non-stopped OD values. Unlike OPD, TMB is not considered a hazardous material.

Advantages and disadvantages of ELISA
Advantages:

- ease of use, simple, fast, and can be automated
- convenient and standardized 96-well format, which may come in strips of microwells
- sensitive (in low ppm range)
- selective to the allergenic residues
- availability of labeled reagents
- rapid data reduction
- low initial cost
- portability

Disadvantages:

- lengthy developmental time
- cross-reactivity possible
- matrix effects
- potential false positives from noise or matrix
- confirmation requirement for positives
- no multi-residue analysis yet
- difficult to diagnose problems when assay does not meet the quality assurance specifications.

6.3 Applications

6.3.1 Methodologies

Numerous immunochemical methods for the detection of allergens have been described in the literature[9–46] and are discussed in detail elsewhere in the book. These include ELISAs, dipsticks (see Chapter 10), biosensors (see Chapter 9) and immunoblot (see Chapter 5) and immunoaffinity columns.

6.3.2 Commercial allergenic residue test kits

An allergenic residue test kit is a packaged system of the key components for detecting or measuring specific allergen or marker proteins in a food matrix within a laboratory or non-laboratory environment. The key components include standard calibrators, antibody-coated wells, antibody-enzyme conjugate, color substrate, stopping solution, extraction buffer and washing buffer that may be readily prepared by the user of the kit. Test kits include directions for use and are often self-contained, complete analytical systems, but they may also require additional supplies and equipment.

Availability

In the mid-1990s, commercial kits came to the market offering laboratories the opportunity to analyze peanut proteins in processed food products or in samples taken from various places in a processing facility, and others followed. Currently, most of the available allergen test kits are ELISA-based methods, but PCR-type kits are emerging. At the present time, there are five allergen kit manufacturers (Table 6.1); it is expected that in the near future more manufacturers will be producing a wider range of allergenic residue kits for use by the food industry. Since detection limits declared by manufacturers change in time, especially with the change of regulatory climate, readers are referred to their respective kit manufacturers for details.

6.3.3 Strengths and weaknesses

Issues with commercial kits

Allergenic residue test kits are simple yet sensitive and specific assays conveniently packaged in one box. However, a major difficulty facing the manufacturers of allergenic residue test kits is the extent of assay validation necessary. The intended use of a test kit is usually not defined for specific matrices, but rather is given in general terms such as ‘to detect peanuts in foods.’ However, the potential uses of kits by the food industry, regulatory agencies and research community are extensive, and they may be used for tests associated with a variety of complex food matrices. This makes full validation covering all possible applications virtually impossible. In instances where a defined market need is known, specific support studies can be performed, e.g. validation studies on the detection of peanuts in chocolate. Otherwise, the responsibility for validating the kit in a specific food matrix is left to the investigator. Furthermore, even if the kit is properly validated, end users should still do their own in-house evaluation of the limit of detection (LOD), limit of quantification (LOQ), recovery, precision and ruggedness, according to AOAC Research Institute recommendations (http://www.aoac.org/testkits/app_packet_3/Ops.pdf). Users should be aware that immunoassay kits intended for research, including allergenic residue test kits, do not require licensing by the US Food and Drug Administration or other national bodies. Some national bodies are in the process of carrying out validation trials on the test kits themselves.

Table 6.1 Commercially available kits for food allergen testing (at time of printing)

	ELISA Systems	r-Biopharm	Neogen Corporation	Pro-Lab Diagnostics	Tepnel BioSystems
ELISA	Almond Crustacea Egg Hazelnut Milk (casein and β-lactoglobulin) Peanut Sesame Soy	Almond B-lactoglobulin Egg Gluten (wheat, rye, and barley) Hazelnut Peanut	Almond Egg Gluten (wheat, rye, and barley) Milk (casein and whey) Peanut	Peanut Egg	Egg Gluten (wheat and rye only) Milk (casein, B-lactoglobulin, BSA) Peanut Seasame Soy
Dipstick		Gluten	Peanut		Gluten (wheat and rye only) Peanut
PCR		Almond Hazelnut Peanut Soy			Milk Peanut Soy
PCR-ELISA		Almond Hazelnut Peanut Soy			

Contact:

ELISA Systems	www.elisasystems.net
Neogen Corporation	www.neogen.com
Pro-Lab Diagnostics	www.pro-lab.com
r-Biopharm	www.r-biopharm.com
Tepnel	www.tepnel.com

Other than the newly introduced PCR kits, all five commercial kit manufacturers use a similar ELISA technology for the detection of allergenic residues; hence, they all share the same pitfalls. Using peanut as an example, one manufacturer claims a detection limit of 0.5 ppm peanut proteins while the other four claim detection limits ranging from 1–5 ppm peanuts. Please note, the reporting units are not equivalent. Since the LOD can vary greatly depending on food matrix interferences, extraction efficiency, specificity of antibodies and variation of peanut protein standards, should be using proper validation suitable for its intended use is essential. However, none of the commercial methods have been published in a peer-reviewed journal and only test kits for peanuts are validated by inter-laboratory collaborative studies to date, although work on this is ongoing at the time of writing.

In the real world, analytical sensitivity has limited practical value if uncertainty is not defined, since precision decreases very rapidly as concentration decreases. A question that remains to be answered is how sensitive the detection methods should be. It is commonly recognized that the required analytical sensitivity will vary, depending on the purpose of use of the assay result. Allergen test results are ultimately used to protect allergic consumers. In the absence of *de minimis* threshold levels for food allergens, sensitivity of allergen methods should aim at detecting any amount of allergenic food sufficient to elicit objective allergic reactions in allergic individuals established by double-blind placebo-control food challenge (DBPCFC) studies. Although allergen threshold levels are finite, and not zero,[47] unfortunately, regulatory guidance on action levels on undeclared allergens is still lacking.

Any positive ELISA result can only be treated as a presumptive positive, and confirmation or further testing has to be performed. There is an urgent need for confirmatory tests for the presence of allergenic residues in food. The only unambiguous confirmatory test for peanut ELISA has demonstrated the presence of a major peanut allergen, Ara h 1, in ice cream using liquid chromatography/tandem mass spectrometry (LC/MS/MS). To identify potential Ara h 1 biomarkers, Shefcheck and Musser[48] digested the peanut proteins with trypsin into their component peptides and then identified the four abundant peptides that are unique to Ara h 1 as specific peptide biomarkers.

Finally, there is no consensus agreement on acceptable reference materials by the analytical community, even though some standard reference materials are commercially available, e.g. National Institute of Standards and Technology (NIST), https://srmors.nist.gov/pricerpt.cfm; or The Institute for Reference Materials and Measurements (IRMM), http://www.irmm.jrc.be/. Because there is no uniform peanut protein standard known, kit manufacturers use their own peanut protein extracts as standards and as immunogens. As a result, quantitative results of identical samples will differ when they are analyzed by different manufacturers' kits. For example, our data showed that when 10 ppm of commercial medium roasted peanut flour was added to melted milk chocolate and mixed to homogeneity, results obtained from four different commercial kits ranged from 2 to 40 ppm of peanuts or peanut

Table 6.2 Quality control samples analyzed by different commercial peanut kits

Commodities	Spikes (ppm flour)	Tepnel	Neogen	Pro-Lab	r-Biopharm
Chocolate	0	nd	nd	nd	nd
	10	positive[1]	26	2.3	9.2
	50	positive	207	18.8	27.8
Cookies	0	nd	nd	nd	nd
	10	positive	10.9	0.9	4.8
	50	positive	160	7.0	13.1
Cereal	0	nd	nd	nd	nd
	10	positive	43.5	2.3	6.7
	50	positive	171	24.9	44.8
Ice cream	0	nd	nd	nd	nd
	10	38.5	40.2	2.0	8.7
	50	154	222	7.5	56.0
Detection limit (ppm)		1.0	2.5	0.5	2.5

nd: below the detection limit
[1]As defined by manufacturer at the time of analysis, i.e ≥ 1 ppm

proteins according to the reporting unit of the test kit used. Similar variations were also observed in different matrices in our quality control samples that were prepared similarly (Table 6.2). In order to resolve questions regarding commercial test kit methods, reference standards must be available globally from a recognized body such as the NIST or IRMM, reporting units need to be harmonized and collaborative validation studies should be done. Without reference standards, other issues such as false positives, false negatives, sensitivity, matrix interference and recovery cannot be adequately assessed.

Sampling

Even when the best analytical methodology and quality assurance schemes are in place, large errors can be introduced into allergenic residue analysis by inadequate sampling. This is due, in part, to the inadvertent presence of allergens being unevenly distributed in solid samples, such as finished food products. Therefore, obtaining a representative sample is a way of minimizing false results and increases the chances of accurate determination of allergens in a batch or lot.

Different organizations/analysts develop different sampling protocols to ensure that 'representative' samples are taken. Some ensure that 'false negative' results are minimized; others are more effective at reducing 'false positive' results. The ideal sampling plan minimizes the risks associated with both these errors to protect consumer safety and reduce unnecessary product waste. Nevertheless, it is important to have effective sampling plans to satisfy both government and industry acceptable limits.

6.4 Future trends

6.4.1 Rapid methods and new technologies

Automation and rapid methods are dynamic areas of interest for the detection of allergens in foods, both in the laboratory environment and in food processing plants. Assays can be automated to reduce hands-on manipulations in conventional tests, speed up analyses and reduce human errors. The difficulty in adapting test kits for use in food plants is to enable the assays to be conducted quickly and accurately under operational conditions and with non-technical staff. Nevertheless, several rapid methods have been developed that can be used to analyze allergenic residues with accuracy, simplicity of operation and acceptable operational speed. Rapid methods usually do not require a laboratory and results can be obtained on site. This type of procedure may be vital for allergen screening at remote sites, such as food processing plants. The results from rapid tests can be displayed immediately and also archived for documentation. Such tools can be of great benefit to the food processor, enabling management decisions to be made early in the production cycle.

Dipstick technology

A promising approach in rapid food allergen detection is the use of a dipstick format (discussed at length in Chapter 10). Such tests are inexpensive, rapid and can be done anywhere. They can be used on-site rather than in the laboratory, hence permitting early detection of potential problems, such as the ineffective cleaning of equipment. Antibody-based dipsticks (lateral flow devices) for peanut and gluten are now commercially available. Similar developments for other allergens are expected to emerge shortly.

Biosensors

Biosensors offer the possibility of in-line detection of allergens. These devices provide real-time data and require only minimal technical training to operate. Biosensors are based on the coupling of two components: a sensor chip containing a bioactive receptor such as an Ab that captures the analyte of interest, and a transducer that converts the biochemical recognition step into a quantifiable optical signal.[49] For example, surface plasmon resonance (SPR) biosensors use a direct sensing technique that can detect refractive index changes that occur in the vicinity of a thin metal film surface where Ab complexes with Ag. The change in refractive index is proportional to the concentration of the analyte under investigation. For this type of assay, the whole process takes less than five minutes. The SPR-based biosensor has been successfully used for the determination of proteins, mycotoxins, drug residues, pesticides and peanut proteins.[50,51] It should be possible to use SPR technology for the detection of all food allergens since they are all proteins.

6.5 Conclusions

Immunoassays, such as ELISA, have clearly had a major impact on food allergen detection, but their potential in other areas of allergen research is only now just beginning to be realized. ELISA-based methods can help manufacturers to validate their allergen prevention programs, including sanitation Standard Operation Procedures (sSOP), to prevent cross contact in food processing operations and help government agencies to support regulatory actions.

While allergenic residue testing techniques are continuing to improve with respect to accuracy, reliability and speed, it should be stressed that their performance is strongly affected by sampling strategies. It is important to harmonize internationally accepted sampling protocols, validated methodologies and approved reference standards in this global economic climate. Validation and harmonization of quantitative ELISA is needed to address regulatory compliance for food processing and food services industries to ensure the effectiveness of their food safety programs.

There is an urgent need for the development of ELISA-based rapid methods, such as dipsticks or biosensors and multi-residue procedures, which may provide attractive tools for field monitoring of the integrity of good manufacturing practices (GMP), whereby non-specialized personnel can employ them in a cost-effective manner. Such tests can be used on-site rather than in the laboratory, hence permitting early detection of potential problems, such as the ineffective cleaning of line equipment before shifting to another product. This early availability of critical data translates into early management decisions to avoid the inadvertent introduction of allergens into the production lines due to cross-contact.

Clearly, ELISA is a very powerful tool in food allergen detection. In fact, it is the method of choice. The field of ELISA technology shows dynamic growth driven by growing interest in allergenic residue analyses, and increasing legislative and regulatory attention to the health risk of food allergens. As a technical platform, current ELISA technology offers a significant improvement in versatility and performance advantages over other detection technologies.

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7

Polymerase chain reaction (PCR) methods for the detection of allergenic foods

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7.1 Introduction

For verification of allergen labeling of foods and in order to identify hidden allergens in processed food it is important to provide analytical methods that are able to detect very low amounts of allergenic residue in processed foods. In general, these methods are based on the detection of species-specific proteins by enzyme-linked immunoassay (ELISA).[1–8] In addition, the detection of species-specific DNA molecules by the polymerase chain reaction (PCR) for the identification of allergenic foods at trace levels has been demonstrated.[9–18]

PCR technology has already been established as the DNA-based method for the identification of genetically modified organisms, pathogens and food-related plant and animal species. Furthermore, PCR has been widely used for many approaches in the field of molecular biology and routinely applied in clinical chemistry. Since the mid 1990s this methodology has attracted increasing attention for the detection of allergen traces in food products; this is reflected by an increasing number of published and commercially available PCR assays (summarized by Poms *et al.*[19]). This chapter will review published data on the applicability of PCR as a possible alternative to, and as a tool for the verification of, ELISA methods and discuss the potential and restrictions of PCR used in the detection of allergenic foods.

7.2 PCR principles

Each PCR analysis comprises three consecutive steps: the DNA extraction and purification, the amplification of a specific DNA sequence, and the

detection of the amplified PCR products (amplicons). This section highlights the particular steps in PCR with special regard to the detection of allergenic foods.

7.2.1 DNA extraction and purification

For analytical methods that detect hidden allergens, a sensitivity of 10 ppm (mg/kg) or less of the allergenic food in a composed food has been suggested.[20] The need for detection of allergenic foods at this level is supported by double-blind placebo-controlled food challenge (DBPCFC) studies that have shown that as little as 100 µg of peanut can elicit a mild allergic reaction in peanut-sensitive individuals.[21,22] To reach an appropriate sensitivity in PCR, the DNA extraction method is extremely important because an insufficient purification procedure can drastically reduce the assay sensitivity. A variety of compounds in animal and plant tissues, such as proteases and polyphenols, can slow down or even inhibit the amplification reaction in PCR. Hence, DNA preparations of high purity are necessary. A common and approved method for the extraction of PCR-grade DNA is a combination of the CTAB (cetyltrimethylammonium bromide) or the GTC (guanidine thiocyanate) protocol and a subsequent purification of the extracted DNA with commercially available silica columns.[23,24] An alternative to this relatively time-consuming method is the use of commercially available kits that usually provide a silica-based DNA extraction and are optimized for the analysis of processed foods. Figure 7.1 displays the principle of a silica-based DNA extraction and purification.

7.2.2 General principle of PCR

The basis for PCR is a thermo-stable DNA polymerase which is able to amplify a specific DNA fragment that is flanked by two specific oligonucleotides (primers). In most applications the Taq DNA polymerase from *Thermus aquaticus* is used. In general, the PCR is a temperature-dependent reaction that consists of a series of 25–45 cycles with each cycle consisting of three phases, the denaturation, the annealing, and the extension phase (Figure 7.2). All phases are performed in one reaction vial at defined temperatures.

During the denaturation phase the reaction temperature is increased to 94–96 °C for 30–60 seconds to melt double-stranded DNA into single strands by breaking up hydrogen bonds. During the annealing phase the temperature is decreased to 45–65 °C for 30–60 seconds depending on the melting temperature of the primers. In this phase the primers hybridize to their complementary sequences on the single-stranded template DNA. A suitable annealing temperature depends on the length and the nucleotide composition of the primers (for details, see Newton and Graham[25]). A wrong temperature during the annealing process can result in the occurrence of unspecific PCR

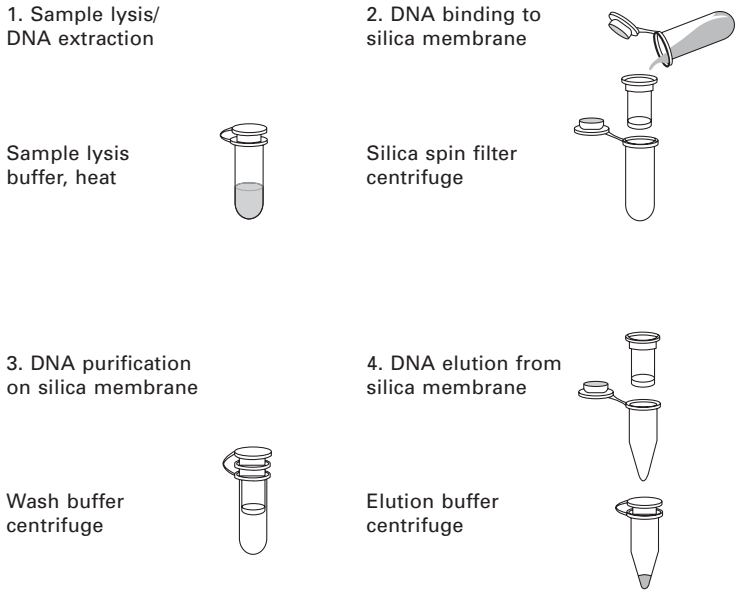

Fig. 7.1 DNA extraction and purification based on DNA-binding silica membranes: characteristic steps in DNA-isolation and necessary reagents and consumables.

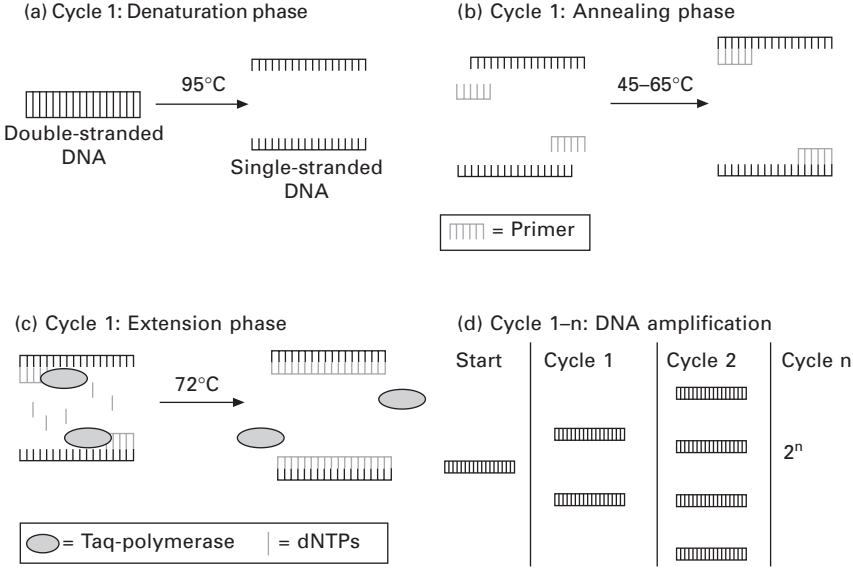

Fig. 7.2 Principle of the PCR. After heat denaturation of the genomic DNA (a), sequence-specific primers bind to the single-stranded DNA during the annealing phase (b). Subsequently, the Taq DNA polymerase extends the primers by filling up the missing strand with desoxynucleotidetriphosphates (c). The exponential amplification of the PCR products is displayed in (d).

products if the annealing temperature is too low, or in a failure of the whole reaction if the temperature is too high. The extension phase is performed at 72 °C, the optimum temperature for the enzymatic activity of the Taq DNA polymerase. During this step the polymerase binds at the 3′-OH end of the primers and fills up the missing strands with desoxynucleotidetriphosphates (dNTPs) which are part of the PCR reaction mix. With a well-optimized and highly efficient PCR, the amount of the generated PCR product is ideally doubled within each cycle which results in an exponential amplification.[25,26] Subsequently, the PCR product can be visualized and its sequence verified as described in Section 7.2.3.

7.2.3 Detection of PCR products

With special regard to the verification of the amplified sequence, the commonly used size-dependent detection of the PCR product by agarose-gel electrophoresis is not sequence-specific and may cause false positive results as described below. Therefore, the German Institute for Standardization (DIN) and the American Society for Testing and Materials (ASTM) have emphasized the sequence verification of a PCR product if used for analytical purposes. This can be done either by Southern blotting with sequence-specific hybridization probes, by nucleotide sequencing, or with endonuclease restriction cuts.[27,28] However, these methods are very time-consuming and can be overcome by the use of a real-time PCR or a PCR-ELISA if the sequence of the generated PCR product is verified by a detectable probe that binds sequence-specific to the amplified sequence.

Agarose gel electrophoresis

The classical method for the detection of amplified PCR products is agarose-gel electrophoresis that provides a size-dependent separation of the negatively charged DNA molecules in an electric field where small amplicons migrate faster than larger ones. After electrophoresis the gel is stained with a fluorescent dye, e.g. ethidium bromide or Sybr®Green (Molecular Probes Inc, Eugene, OR), that binds to nucleic acids, and the PCR products can be visualized on a UV transilluminator. The amplicon size can be roughly determined by comparison with a marker DNA that contains DNA fragments of defined sizes. For analytical approaches such as the detection of hidden allergens in food, it is advisable not to use this procedure as the only method of detection because false positive results can be obtained from PCR products of comparable size that may be caused by a cross-reactivity. Consequently, the identity of the PCR products should be verified by a sequence-specific method. Another disadvantage of agarose-gel electrophoresis is the use of fluorescent dyes that are potentially mutagenic because of their ability to intercalate with DNA.

Detection of PCR products by PCR–ELISA

After PCR amplification, the products are detected sequence-specific with an ELISA-like technique. This method requires a modification of the PCR protocol. Several protocols are available of which the following is an example of a recently published hazelnut-specific PCR–ELISA.[13] Biotinylated primers are used in the PCR which leads to biotinylated PCR products that are coupled onto a streptavidin-coated microtiterplate. Subsequently, the PCR product is denatured resulting in a surface-bound single-stranded DNA that is accessible for the binding of a sequence-specific DNA probe. The probe, which is linked to a label is detected by an enzyme-conjugated antibody that is directed against the label. A colorimetric reaction is catalyzed by the enzyme after the addition of a chromogenic substrate. The light absorption of the developed color product is measured in a microplate reader. The principle of this post-PCR detection of amplicons is shown in Figure 7.3. Even though the ELISA-like detection of amplicons can be used in a quantitative manner, the PCR–ELISA can only be used as a qualitative test for the absence or presence of the allergenic food within the validated range of detectability. The reason for this is the qualitative nature of the PCR as an endpoint method.

In comparison to the agarose-gel electrophoresis, a higher sample throughput can be achieved with the PCR–ELISA, and the amplicon sequence is verified with a sequence-specific probe. Further benefits of the PCR–ELISA are the avoidance of mutagenic dyes such as ethidium bromide and cheaper equipment compared to real-time PCR applications. As in agarose-gel electrophoresis, the PCR–ELISA makes it necessary to perform manipulations with amplified PCR products which can lead to DNA contaminations and therefore to the occurrence of false positive results in future PCR experiments, especially if adequate safety precautions are not complied with (see page 133).

Detection of PCR products in real-time

The principle of this state-of-the-art method for quantitative detection of amplifiable DNA is based on the measurement of a fluorescent signal that is increased during the amplification of PCR products. For sequence-specific real-time detection of PCR products, a fluorogenic probe that is designed to bind to the PCR product is used. Several probe formats are described such as 'TaqMan'™ Probes (Roche Molecular Systems Inc, Pleasanton, CA), 'LC Hybridization®' Probes (Roche Diagnostics, Indianapolis, IN, and Idaho Technology Inc, Salt Lake City, UT) 'Scorpions'® (DxS Ltd, Manchester, UK) and 'Molecular Beacons'. Most of the commonly used probe formats are based on a physical process called fluorescence resonance energy transfer (FRET). Details of the various probe formats and their characteristics are given elsewhere.[29] Due to the proportional relation between the fluorescent signal and the amount of the generated PCR product it is possible to record a PCR amplification plot. Based on the so-called threshold cycle (C_t), the PCR cycle number at which the fluorescent signal can be distinguished from

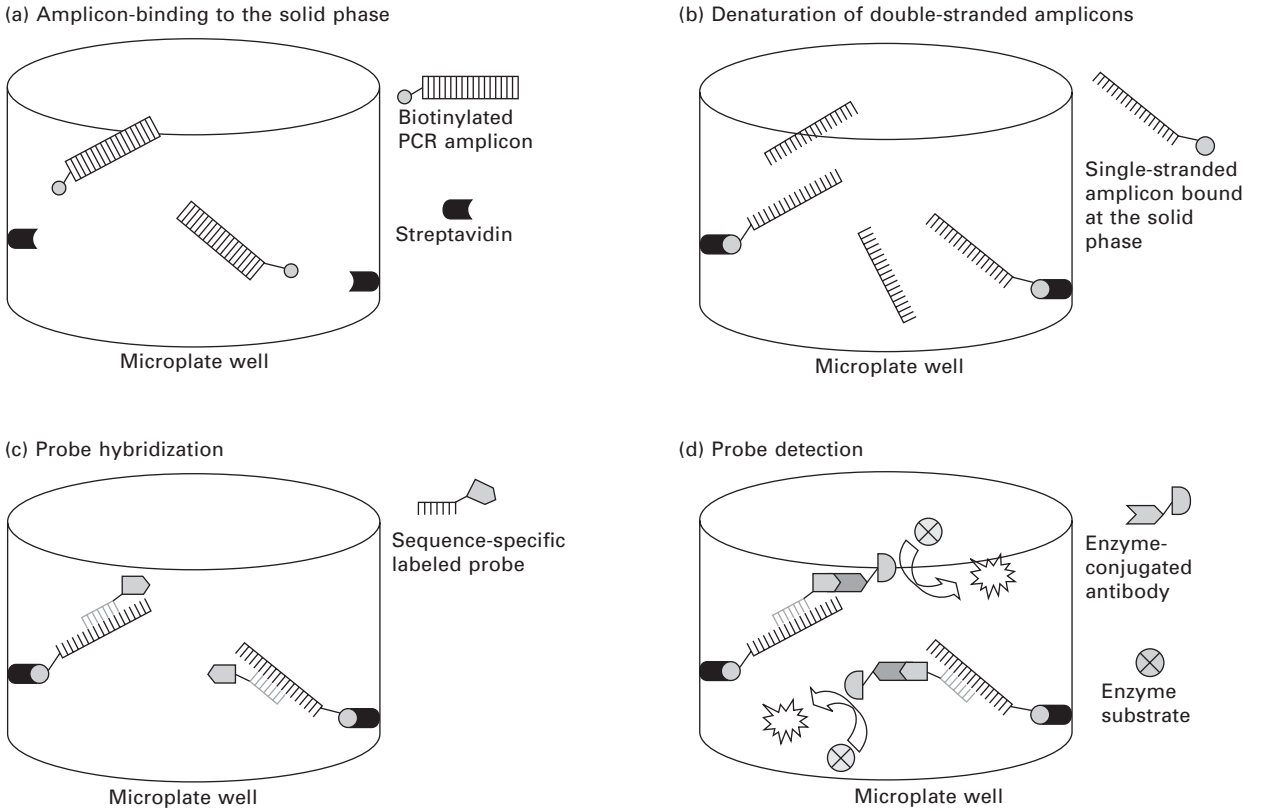

Fig. 7.3 Principle of the PCR-ELISA. Biotinylated PCR amplicons are bound to streptavidin-coated wells in a microtiterplate (a). After denaturation of the double-stranded amplicons (b) a sequence-specific labelled probe can hybridize (c). The detection of the probe is done by an enzyme-conjugated antibody specific for the probe label (d). The color development is measured as absorbance in a microplate reader.

the background noise, it is possible to quantify the number of DNA copies present in the sample before amplification. For the determination of the DNA copy number in the unknown sample, the C_t value from a sample is compared with the C_t values from a DNA standard calibration curve, similar to the protein standards in ELISA tests (Figure 7.4). In the case of the quantitative analysis of allergenic foods in composed food products, the availability of standard reference materials is essential to cover the influence of matrix and processing effects.

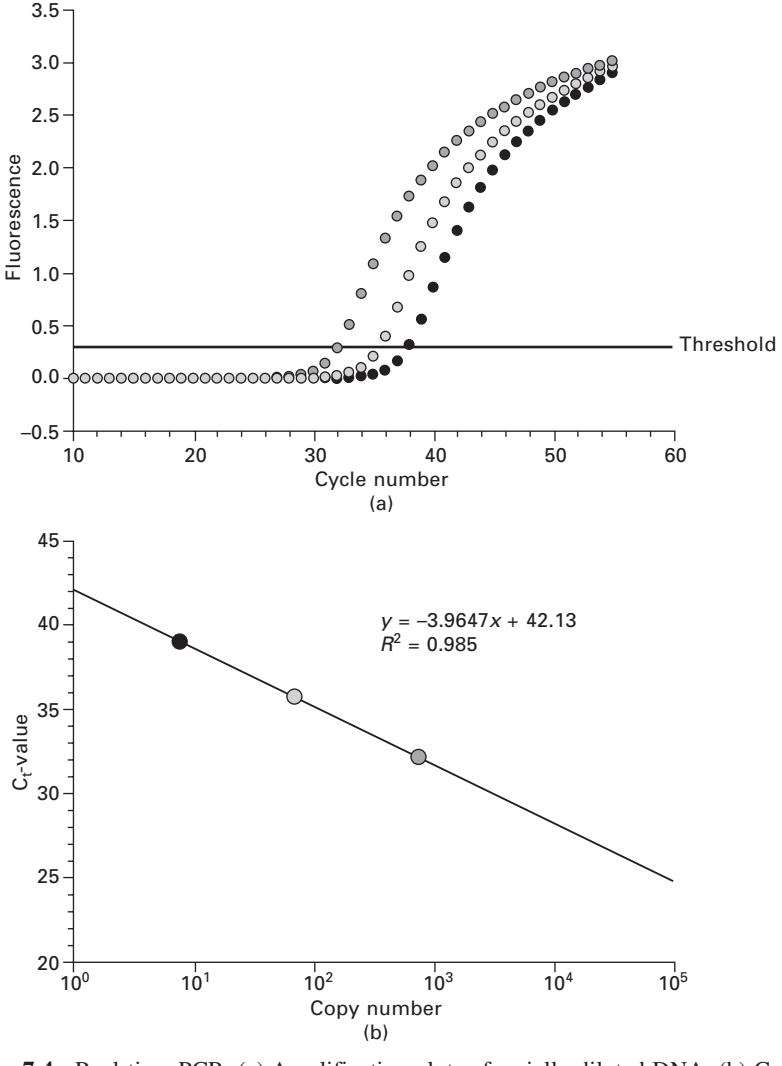

Fig. 7.4 Real-time PCR. (a) Amplification plots of serially diluted DNA. (b) Cycle threshold (C_t) values of the amplification plots from (a) plotted against the logarithm of the copy numbers of amplifiable DNA prior to PCR analysis. These result in linear correlation.

Besides the possibility of DNA quantification, real-time PCR methods do not need further post-PCR steps, which overcomes problems encountered with post-PCR contaminations. However, it has to be considered that the equipment for this PCR technology is still expensive (> $30 000). For the quantification by PCR, competitive PCR assays with amplicon detection in agarose gels have also been applied. An example is the quantification of wheat, barley, and rye in gluten-free food for coeliac patients.[14] However, because of easier handling, a lower risk of post-PCR contamination, and a larger dynamic range of quantification, real-time PCR assays favoured.

7.2.4 Particular considerations in PCR

Choice of primers

The choice of the target gene and the design of the primers have a great impact on the sensitivity and the specificity of a PCR method. DNA is often degraded in processed food. As a consequence, for PCR to detect hidden allergens, the amplified DNA fragment that is defined by the position of primers on the target gene should have a relatively small size of the some 60–150 basepairs.[28] Moreover, the primer pair needs to be tested for possible cross-reactivity with the most important food matrices and with all relevant food ingredients that are related to the analyte. For example, a peanut-specific PCR should not detect relevant legumes, nuts, cereals, milk, and cocoa to allow analysis of chocolate, cereals, and cookies.

Furthermore, very sensitive PCR assays can be established if the primer target is a multicopy gene (e.g. mitochondrial genes, chloroplast genes) as was described for the quantitative detection of wheat, barley, and rye in gluten-free foods.[14] However, due to highly conserved nucleotide sequences of these genes in different species, strong cross-reactions can occur.[15] For this reason, most of the PCR assays for identification of allergenic foods have been based on the amplification of the more characteristic, and thus more specific, single-copy genes. With regard to the detection of allergenic foods, a detection of the gene encoding an allergen is not necessary. Any gene that allows an unequivocal identification of the allergenic food species can be used as an indicative marker.

No matter which gene sequence is chosen for amplification, it is essential that the primers bind specifically to the target sequence to avoid unspecific by-products and a reduction of PCR efficiency. Hence, the primers and probes have to be designed and optimized to meet these needs. Several rules for primer and probe design have to be considered.[25,26] Briefly, the annealing temperature of the primers should be within the range 45–65 °C and the content of the pyrimidine bases guanine and cytosine should be between 35% and 65%. Self-complementary sequences should be avoided, especially in the 3′ region of the primers, to avoid the formation of hairpins or primer dimers that can drastically reduce the efficiency of the amplification reaction.

Contamination and how to avoid it

The very high sensitivity of the PCR is one of the major advantages of this method, but at the same time it is a great challenge because of the risk of persistent DNA contamination that may lead to false positive results in future PCR experiments. Theoretically, one copy of amplifiable DNA can be multiplied and detected. For this reason, negative controls are extremely important. Post-PCR steps are, in particular, very problematic. Significant copy numbers of amplified DNA fragments can be dispersed in the laboratory due to the formation of aerosols when handling the PCR products. Consequently, post-PCR steps need to be performed in a dedicated room that is separated from the other PCR working areas. To minimize the risk of carry-over contamination, further precautions, such as the use of separate pipettes, aliquoting of reagents, use of filter-tips, and frequent changing of gloves, should be considered.[30] Moreover, the use of a chemical decontamination reaction during each PCR run helps to avoid carry-over contamination. This system is based on an enzyme called uracil-DNA-glycosylase (UNG) that catalyzes the removal of uracil from single- and double-stranded DNA. If dTTP is substituted by dUTP in the dNTP mix, amplicons that contain uracil will be generated in PCR. If amplicons that contain uracil are carried over into a following amplification reaction, these amplicons will be degraded by UNG in contrast to the natural DNA template that contains thymidine.[31]

7.3 Application of PCR for the detection of allergenic foods

7.3.1 Published PCR applications

So far five PCR assays for the detection of hidden hazelnut and peanut traces at a level of 10 mg/kg have been published in the scientific literature.[12,13,15,16,18] A PCR for the detection of celery as an allergen was presented[11], and another one recently reported.[32] Furthermore, a PCR specific for soy was developed with special regard to meat adulteration, but has potential to be applied in the detection of allergenic soybean in composed foods.[10] In addition, for the detection of cereals that are toxic for subjects with coeliac disease, a PCR for wheat,[9] and a competitive PCR for the quantification of the gluten-containing cereals wheat, barley, and rye in gluten-free food[14] were published, and recently a real-time PCR with melting curve analysis for the detection and discrimination of wheat, barley, and rye was developed and applied to a selection of final food products.[17]

The first qualitative PCR method for the detection of hazelnut as an allergen in processed foods was published in 2000.[12] The primers were specific for the gene of Cor a 1.0401, the major hazelnut allergen, and the 182 bp PCR product was detected in agarose-gel electrophoresis. The LOD was determined as 0.001% (10 mg/kg) in the investigated food matrices (chocolate, cookies, cereals/muesli, potato snacks, puffed corn) and detection of hazelnut

traces correlated well with a previously published and in-house validated hazelnut-specific ELISA. Cross-reactivities with 33 relevant foods were not observed. The specific hazelnut PCR was further improved and developed as a PCR–ELISA application.[13] Herein, a significantly shorter DNA extraction method was used and led to a shortened total assay duration of approximately six hours in contrast to the previously described method. For the PCR–ELISA, the sensitivity was also determined at a level of 0.001% or 10 mg/kg of hazelnut in various foods. In this study the PCR–ELISA was used to analyse 41 food products for the presence of undeclared hazelnut traces and was compared with an in-house validated hazelnut-specific sandwich ELISA having a limit of detection of < 1 mg/kg of hazelnut protein (corresponding to < 10 mg/kg hazelnut). Both tests showed a very good correlation for foods having ≥ 1 mg/kg of hazelnut protein as was quantified by ELISA. Sporadic discrepancies in the detection of hazelnut were only found for foods having < 1 mg/kg of hazelnut protein (Fig. 7.5).

Recently, another hazelnut-specific PCR was published that is based on a primer set specific for an intron of the mitochondrial encoded gene *nad1*.[15] The specificity of this method was tested with a variety of closely related plants. Cross-reactivities were found with *Carpinus turczaninovii* (rock hornbeam) and *Ostrya carpinifolia* (hop hornbeam), belonging to the Betulaceae family as does hazelnut (*Corylus* sp.). The sensitivity of this method was reported as 10 mg/kg of hazelnut in foods.

The first peanut-specific PCR assay was published in 2003.[16] With this real-time PCR using the TaqMan™ format, the authors were able to detect peanut down to 2 mg/kg spiked into a biscuit. However, the PCR results

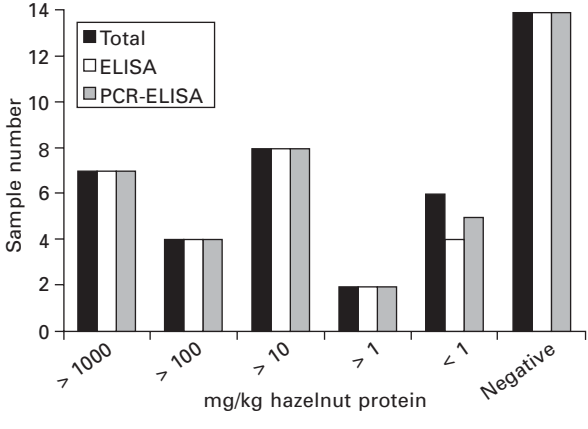

Fig. 7.5 Investigation of commercial food samples for hazelnut residue with hazelnut specific protein sandwich-ELISA and DNA PCR-ELISA.[13] Of the 41 investigated chocolate, creme desert, breakfast flakes, cereal bars, and cookie samples, only three discrepancies were observed for samples having less than 1 mg/kg hazelnut protein (two samples were negative in ELISA but positive in PCR; one sample was positive in ELISA but negative in PCR).

were not compared with those of a validated immunological assay, nor was an unspiked biscuit analyzed as negative control. As reported by the authors, the peanut PCR may be used for analysis if reference standards for peanut become available.

The performance of a new peanut-specific real-time PCR was compared with an in-house validated peanut ELISA.[18] Thirty-three different food samples were analyzed for the presence of peanut traces with both methods allowing a detection of peanut at a level of ≤ 10 mg/kg. The results of ELISA and PCR were in good correlation. There was only one discrepancy found in a chocolate that was negative in ELISA (< 0.5 mg/kg of peanut protein), but positive in PCR. The major characteristics of the published PCR methods are summarized in Table 7.1.

7.3.2 PCR as possible alternative for immunological methods

So far, the results of several different PCR assays specific for wheat, soya, hazelnut, gluten-containing cereals (wheat, barley, and rye), and peanut have been compared with the results obtained from in-house validated or commercial ELISAs.[9,10,12–14,17,18] The data presented indicated that both DNA-based and protein-based assays are applicable for the detection of hidden wheat, soy, hazelnut, gluten-containing cereals, and peanut traces in the investigated matrices, and that there is a good correlation of the results from both methods.

Furthermore, it was shown that some matrices may be more suitable for PCR methods than for ELISA analysis and vice versa. In particular, two creme deserts containing hazelnut, as stated in the list of ingredients, were tested negative for hazelnut by protein ELISA but not by PCR–ELISA.[13] This phenomenon was supposed to be related to an acidic or microbial degradation of the proteins that had no or little influence on the DNA stability. By contrast, a white chocolate containing only 0.5 mg/kg of hazelnut protein tested negative by the PCR–ELISA but positive by the protein-ELISA.[13] The analysis of bread, pastry, and baby food for gluten-containing cereals demonstrated that some food matrices may inhibit the PCR and therefore lead to false-negative results. By contrast, the applied ELISA seemed to be less sensitive in other food samples.[14] Studying the detection of wheat, barley, and rye in gluten-free oat products, the results of PCR and a gliadin-specific ELISA were in complete concordance above a level of 50 mg/kg gluten.[17] However, the ELISA was slightly more sensitive in some foods. In another study, a commercial soya-specific ELISA was compared with a soybean-specific PCR. The PCR was able to detect a soya product having approximately 70 mg/kg of soya protein, whereas the ELISA was negative with a limit of detection of 3500 mg/kg of soya protein,[10] which may be not sensitive enough for the detection of soybean as an allergen. Similar findings were obtained by comparing a gluten-specific ELISA and a more sensitive wheat-specific PCR for the detection of wheat adulterations in foods with special regard to coeliac disease.[9] Moreover, it should be mentioned that the PCR assays for

Table 7.1 Major characteristics of published PCR methods suitable for food allergen detection

Food allergens	See reference	Target gene	Amplicon size [bp]	Sensitivity		
				pg DNA	Gene copies	mg/kg allergenic food
Hazelnut	12	*Cor a 1.0401*	182	n.p.	n.p.	≤ 10
Hazelnut	13	*Cor a 1.0401*	152	2–4	1–2	≤ 4
Hazelnut	15	*Nad1* (mitochondrial)	294	30–300	n.p.	10
Peanut	16	*Ara h 2*	66[3]	n.p.	n.p.	2
Peanut	18	*Ara h 2*	86	n.p.	10	≤ 10
Soy[1]	10	*Lectin Le1*	118	n.p.	n.p.	n.p.
Wheat[1]	9	*RNA gene interspacer between 25S and 18S* (ribosomal)	109	1	n.p.	< 300[4]
Gluten-cereals wheat, barley, rye[1]	14	*trnL* (chloroplast)	201 (wheat, rye) 196 (barley)	2–20	n.p.	< 200[4]
Gluten-cereals wheat, barley, rye[1]	17	*Wheat ω-gliadin* *Rye ω-secalin* *Barley hordein* *Oats avenin*	181 (wheat, rye) 164 (barley) 104 (oats)	< 50	≤ 5	100–1000[5]
Celery	32	*Api g 1*	145	30–250	n.p.	n.p.

n.p. not published
[1]the soybean and gluten-cereals specific PCR methods were not developed for food allergen detection but for soy adulteration and gluten analysis, respectively
[2]validated versus an ELISA method
[3]derived from published sequences
[4]derived from positive sample with lowest detected amount of wheat
[5]derived from a detection level of approximately 50 mg/kg gluten

soy and wheat were not optimized for sensitivity as the aim of the studies was either the identification of meat adulterations at the percent level or the detection of gluten-containing wheat at a level of 200–400 mg/kg (10–20 mg/kg gliadin). Hence, even better sensitivities may have been achieved with optimized PCR protocols. Most of the published and commercial immunological methods for soya have been developed for the detection of meat adulteration with soybean products having high limits of detection of approximately 1000 mg/kg of soy protein (summarized by Poms *et al.*[19]). In contrast to the numerous publications about the detection of peanut, which is a legume like soybean, only two scientific references about the detection of soybean with ELISA at a suitable level of 1–2 mg/kg have been reported so far.[33,34]

One reason why soya has been difficult to target at the protein level using ELISA methods is the great number of different soya products, such as defatted soybean flakes, texturized soybean protein, protein isolates and concentrates, and protein hydrolysates. The differences in processing can result in a variety of altered peptides and protein breakdown products with differing structural properties and therefore differing detectabilities. Therefore, soya detection by PCR methods may be a possible alternative to immunological methods if the detection of soybean DNA correlates with the presence of soybean protein and peptides, respectively. So far, no celery-specific and sensitive ELISA has been published. One reason for this may be the difficulty in generating a celery-specific antiserum that allows sensitive detection of celery in foodstuffs without cross-reacting to other species of the Apiaceae family. By contrast, the PCR has great potential for the differentiation between phylogenetically closely related species. In this context, celery, fish, crustaceans, and various tree nuts may be interesting candidates to detect with PCR methods in addition to the application of PCR for verification of ELISA results or even as a substitute for ELISA.

For hazelnut, peanut, wheat, soya, and celery, PCR has already been shown to be an alternative to ELISA assays. However, more investigations have to be done to support these findings. Additionally, the applicability of both methodologies has to be thoroughly investigated for each allergenic food and in the most important food matrices. Thereafter, a final conclusion can be drawn about the potential use of both techniques depending on the allergenic food and the food matrix.

7.3.3 Commercially available PCR assays

Besides the published DNA-based methods for the detection of hidden allergens in processed foods, several applications of PCR–ELISA and real-time PCR are commercially available (R-Biopharm, Darmstadt, Germany; Eurofins Scientific, Memphis, TN; Tepnel Biosystems Ltd, Deeside, UK; Genetic ID NA, Fairfield, IA). Currently, assays for the detection of peanut, almond, hazelnut, soy, wheat, chicken (for hen's egg), beef (for cow's milk), fish,

celery, sesame, and mustard are available (summarized by Poms *et al.*[19]). The detection limit of the tests was specified by the manufacturers to be between 10 and 20 DNA copies. However, without having standard reference samples, a determination of copy numbers may not be correlated with the actual amount of the allergenic food that is present in the investigated sample. Detailed studies that show the performance characteristics of most of these assays have not been published, and publications that investigate the applicability of these methods are mainly lacking.

7.4 Advantages and disadvantages of PCR compared to ELISA

The majority of commercial and published allergen-specific assays are protein-based, whereas only a few DNA-based assays have been developed so far. Reasons for this are quick performance and easy handling, and the rather simple equipment which is required for ELISA. Furthermore, experience in the development of ELISA techniques has been gained over more than 30 years, whereas the analysis of foods by PCR began only in the mid 1990s. However, the PCR methodology presents several interesting features for the application in routine analysis of food allergens. In contrast to ELISA tests that are based on polyclonal antibodies, the whole PCR chemistry and recombinant thermostable DNA polymerase can be synthesized and manufactured in unlimited amounts and constant quality. Furthermore, the specific reagents, such as primers and probes, that are needed for PCR can be designed to meet the needs for specificity and sensitivity. Similarly, the use of monoclonal antibodies in ELISA can overcome these limitations of availability and consistency, but ELISA based on monoclonal antibodies may be more susceptible to matrix effects because of a lower complexity in protein-specific recognition sites. This can lead to false-negative results if the target protein or the target epitope is affected during food processing or because other proteins that are not detected are important allergens for a subgroup of allergic individuals. Moreover, the antigenic epitopes that are detected with animal antibodies do not necessarily reflect the allergenic epitopes that are recognized by the allergic individual. Similarly, the PCR cannot detect the allergenic molecules because the amplified and detected target molecule is the DNA. Therefore, neither currently available ELISA tests nor the PCR applications can be considered to be direct detection methods. Both methodologies are mainly based on the detection of specific markers that allow extrapolation for the presence of the allergenic food of interest.

Nevertheless, the identification of protein from an allergenic food gives some more evidence for allergenic potential as does the DNA analysis by PCR. Hence, it is a prerequisite in allergen detection with PCR to verify a positive correlation of the marker DNA in comparison to the presence of potentially allergenic food proteins. As DNA can be found in any tissue of

plant or animal origin, the species-specific detection of DNA gives evidence for the presence of the allergenic food if food technological processing does not separate the marker DNA from the allergenic protein fraction. Consequently, allergenic residues in food ingredients such as protein isolates, sugars, starch, aromas, and highly-processed vegetable oils may be difficult to detect and results obtained by PCR difficult to interpret. However, residues in sugars, starch, aromas, and highly-processed vegetable oils may also be difficult to detect with ELISA tests. Moreover, the sensitivity of the PCR strongly depends on the amount and on the quality of the template DNA. In some cases the DNA isolated from food products can be highly degraded, making amplification of a specific PCR product impossible. Especially in products having a low pH, such as soured or fermented foods, the relevant DNA can be hydrolyzed, which makes a PCR analysis very difficult. This matrix effect was obvious when celery DNA from celery juice could not be amplified and detected with a 241 basepair celery-specific PCR.[11] However, such matrix effects can also occur with protein-based methods if the target proteins are susceptible to degradation, as was described for hazelnut.[13]

Other problematic matrices for PCR analysis are foods or food products having low amounts of DNA, such as milk, egg white, vegetable oils, or animal fats. In these cases, protein ELISA may be more suitable than PCR assays. Moreover, the PCR is not able to distinguish between egg and chicken meat, or cow's milk and beef, because DNA is not tissue-specific. Nevertheless, PCR offers unparalleled specificity in species identification making it a favourable method for the detection of allergenic food components of animal or plant origin with high homology to other species used in food manufacture. By the use of a single protocol, DNA from various species is extracted simultaneously from a complex food matrix. Therefore, a multi-analyte detection that is based on the same sample preparation is possible. For this reason, PCR has great potential to become a screening method because currently available ELISAs are still single-analyte tests and require optimized extraction conditions for each allergenic food that is to be detected. However, it has to be considered that the performance of PCR, at least with current technology, does still need specialized laboratories. Besides the issues with possible cross-contamination, PCR does not seem to be practical for direct analysis in an average food production line, whereas dipstick ELISA or other rapid ELISAs are favoured because of easier and faster handling. Obviously, it is very important to consider the pros and cons of both technologies before starting to analyse foods for the presence of hidden allergens. Moreover, any method used for the detection of allergens in foods needs to be validated for its sensitivity, specificity, and detectability in the food matrix of interest to guarantee its applicability. Ideally, the correlation between the presence of allergenic proteins and the successful detection of the allergenic food by protein ELISA or PCR should be demonstrated.

So far, validation on allergen-specific tests has been carried out mainly with peanut-specific ELISAs. Taking into account the results of these studies,

it was shown that commercially available peanut-specific ELISAs have the potential for correct qualitative and semi-quantitative but not yet for quantitative analysis.[35–37] Similarly, the detection of peanut with real-time PCR methods indicated that the relative C_t values obtained by the real-time PCR reflected the relative amount of peanut present in a sample.[16,18] Moreover, the comparison of a real-time PCR and an ELISA specific for peanut revealed that the relative C_t values obtained by the real-time PCR are correlated to the amount of protein measured by the sandwich ELISA.[18] This underlines the potential of PCR to be used as a semi-quantitative method for the detection of peanut traces in processed foods if suitable reference materials with defined amounts of peanut are available. Currently, several approaches to generating such standard material and to improve the standardization of allergen specific assays are in progress (European Institute of Reference Materials and Measurements, IRMM; German Institute for Standardization, DIN; European Committee for Standardization, CEN; US Food and Drug Administration, FDA). In spite of the availability of standard reference materials, it will nevertheless not be possible to quantify the exact amount of an allergenic food in processed foods because of unknown processing grades (e.g. different roasting times and temperatures for nuts) of the foods and the vast number of different food matrices that lead to a varying detectability of the biological target molecules protein and DNA. However, the generation of standard reference materials is necessary to make the results of allergen-specific assays comparable and to achieve reproducible inter-laboratory results.

7.5 Future trends

A few scientific studies suggest that DNA-based methods can be applied to the analysis of hidden allergens in finished food products and can complement the application of ELISA tests. Some allergenic foods and food matrices may be more suitable for PCR and others more suitable for ELISA analysis. In both cases the developed methods have to be thoroughly validated with particular regard to their applicability in the target food matrices. For example, tests developed for peanut or tree nut need to perform well in food matrices such as cookies or chocolate, whereas a crustacea-specific assay needs to be validated for products potentially containing fish or crustacea residues. ELISA tests may be advantageous for the detection of eggs and milk as allergenic foods whereas the differentiation between phylogenetically closely related tree nuts, fish, and crustaceans may be easier to achieve with PCR-based methods. In the future, the development of methods to isolate amplifiable DNA at high yields and of high purity will further help to improve the applicability of PCR in the detection of hidden allergenic foods. Moreover, with the use of a single DNA extraction, several allergenic food compounds can be screened at once. This makes PCR technology an ideal multi-analyte screening tool.

In conclusion, the development of sensitive and specific analytical methods for the quantification of allergens in finished food products is still a great challenge due to the lack of suitable standard reference materials. The availability of either DNA or protein standards will help to make quantification with protein- and DNA-based assays comparable. With the availability of standard reference materials, it is to be expected that the development of PCR assays for allergen detection will further proceed, and the development of quantitative PCR assays will be made possible.

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8

Proteomic assessment of allergens in food

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8.1 Introduction

Proteomics has recently received much attention. The word 'proteomics' encompasses a family of technologies with which to determine the complement of proteins in a cell, tissue or organism. Currently, these technologies include multi-dimensional separations via electrophoresis or liquid chromatography; various methods for protein identification including mass spectrometry; arrays to map protein–protein interactions; and bioinformatics to analyze massive data sets.

Proteomics is evolving from its roots in two-dimensional electrophoresis. However, at present, the electrophoretic approach still represents a valuable technique for separating proteins. Proteomics is especially useful for the identification of IgE-binding proteins for several reasons. First, conditions for solubilization of proteins are very aggressive, involving combinations of urea, thiourea, non-ionic detergents and strong reducing agents. These reagents increase the likelihood of detecting allergens. Second, spots in two-dimensional separations are more likely to be homogenous compared to protein bands in one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separations. In addition, two-dimensional separation provides substantial information in the form of isoelectric point (pI), molecular weight and heterogeneity (charge and/or size variants) of the IgE-binding component. This information makes it possible to substantially narrow the search for the protein's identity. Finally, the value of a proteomics approach can be seen in the number of newly discovered allergens (discussed below). While the number of reports using proteomics to investigate IgE-binding proteins is low, most have uncovered 'novel' allergens.

8.2 Key issues in proteomic assessment of allergens

8.2.1 Separations

Two-dimensional electrophoresis (2-DE) consisting of isoelectric focusing (IEF) followed by SDS-PAGE separation represents the core of proteomics technology since its establishment a quarter century ago (O'Farrell, 1975). Recent advances have greatly improved the efficiency of resolution, speed and reproducibility of the technology. Most notable has been development of immobilized ampholine technology. Strips containing immobilized ampholines (IPG strips) result in greater resolution by enabling the application of higher electric field strengths while also preventing cathodic drift of the pH gradient.

IEF power supplies with vastly improved performance are now available. These units are capable of routinely running at field strengths of 8–10 kV. They also employ efficient cooling systems to control Joule heating and apply power in a programmed sequence. The new generation of IEF power supplies can run 8–12 samples at once. Typically, IEF separations are conducted for a specified number of volt-hours (Vh), e.g. 50 kVh. Thus relatively high sample throughput can be achieved when compared with older equipment.

IEF separation

Complex mixtures of proteins can be separated by IEF. The unique balance of positive and negatively charged amino acids determines a protein's isoelectric point (pI). A protein's pI corresponds to that pH at which the positive and negative charges sum to zero. When the pH is equal to a protein's pI, it is electrically neutral and its migration in an electric field is halted. Typically, IEF is conducted in the presence of high concentrations of urea (6–8 M), non-ionic detergents (e.g. (3-[3-cholamidopropylammonio]-1-propanesulfonate – CHAPS) and reducing agent (e.g. dithiothreitol – DTT) to promote solubility. A pH gradient is established typically from 3.0–10.0 using immobilized ampholines in a polymer-based IPG strip.

The second dimension separation is usually performed via SDS-PAGE following appropriate equilibration. The combination of separations based on different mechanisms of selectivity (pI vs relative mass) results in enhanced resolution of component proteins. Thus 2-DE has distinct advantages for characterizing the IgE-binding components in complex mixtures of proteins.

8.2.2 Quantitation

Significant improvements in staining methods and computer-driven image analysis routines have greatly improved the ability to quantitatively assess differences between samples. However, while the image analysis packages may provide numerical evaluations, careful use is needed to ensure accuracy (Miller *et al*., 2001; Miura, 2001).

Quantitation of stained proteins in gels requires several steps to establish the validity of the measurement. Determination of linear range of detection

is an often overlooked evaluation criterion. The linear dynamic range can be established by comparing (plotting) response measurements for a series of protein standards. Differential dye absorption should also be expected. Proteins can vary significantly in the amount of dye absorbed per unit of protein and this results in values that over-or under-estimate the amount present. Additionally, accurate quantitation requires control over separation parameters such as: voltage, separation time, temperature and gel staining/destaining procedures. Finally, appropriate analytical protocols such as triplicate sampling and determining the variability (standard error) of measurements, should be employed.

Image analysis programs have advanced features that facilitate recording and measuring the spot position and intensities. No two gels (especially 2-D) will have identical x-y coordinates for all component spots. Most image analysis programs can adjust patterns such that highly accurate overlays and subtractive analyses can be performed. In addition, these programs usually contain statistical evaluation routines to aid in the accuracy of the assessment.

Image analysis programs generally determine the amount of protein in a spot based on measurement of its size and intensity. The intensity of a spot is determined from a Gaussian representation of its optical density (OD). The size of the spot is determined from appropriate area integration algorithms. Spot values are then expressed as OD times area units. This value is called spot quantity in some image analysis programs. In applications where the goal is to compare the amount of an individual component between two or more samples, spot quantities are usually expressed as a percentage of the total. In this way, small differences in load amounts are normalized. Thus, image analysis programs can be useful for determining overall differences in the protein profile of samples. For example, image analysis can be used to assess whether there has been alteration in potential allergen content between conventional and genetically modified (GM) varieties.

8.2.3 Methods of detection and identification

Proteins in 2-DE separations are commonly visualized by staining with Coomassie Blue (R250). This tried and true standard has one major disadvantage, namely its lack of sensitivity. The limit of detection is approximately 0.1 μg for most proteins. Enhanced sensitivity can be achieved by use of silver staining. There are several slightly different silver stain chemistries that result in varied colors (brown to black) but most have a 10-fold lower limit of detection. Recently, fluorescent dyes such as SYPRO® (Molecular Probes Inc, Eugene, OR) with even lower (1–10 ng) detection limits have become available. Staining with SYPRO® is much faster than silver staining and has a greater dynamic range of measurement in image analysis (Berggren *et al*., 2000; Lopez *et al*., 2000; Nishihara and Champion, 2002). Fluorescent dye is also more compatible with subsequent mass spectrometry (MS) analysis procedures than is silver stain.

Proteins in 2-DE separations can be identified following transfer to polyvinylidene difluoride (PVDF) membrane via immunoblotting or Edman-based N-terminal sequence analysis. However, the amount of material required for Edman analysis and/or the presence of N-terminal modifications, often limits its use for identification of proteins separated by 2-DE. Immunoblotting can also be used to assess the level of specific IgE-binding proteins in a sample, but this of course requires prior knowledge of the protein's identity and an appropriate detection antibody.

Mass spectrometry has become the method of choice for the detection and identification of proteins in 2-DE gels because of its greater sensitivity and ability to determine sequence information. Mass spectrometry can also be used to characterize protein modifications such as phosphorylation and/or glycosylation. This section will only briefly describe some of the mass spectrometry techniques that are useful in the application of allergen identification. The reader is referred to the recent review by Mann *et al.* (2001) for a wider survey of various MS analytical approaches and their mechanism of operation.

Most MS analyses are performed on proteins after they have been digested with trypsin into constituent peptide fragments. In this analysis, a gel plug containing the protein is excised, destained and digested with an excess amount of trypsin. The resultant peptides are extracted and can then be analyzed by several MS techniques.

The most commonly used MS analytical approach is matrix-assisted laser desorption ionization (MALDI). In MALDI analysis, the mixture of peptides is combined with an excess of matrix compound such as α-cyano 4-hydroxycinnamic acid and 1–2 μL is spotted onto a plate. The spots are irradiated by nanosecond laser pulses, during which excited matrix molecules donate protons to the peptides. The charged molecules are differentially accelerated (based on mass/charge ratio) in a strong electric field. This results in lighter ions arriving at the detector before heavier ones. This technique for differentiation is referred to as time-of-flight (TOF) analysis. Accurate masses obtained from each peptide are collected into a mass profile that is compared to theoretical mass profiles based on predicted trypsin cleavage sites of known protein sequences. Thus the identity of an unknown protein can be established from its mass profile. Difficulties in the MALDI–TOF method of identification (called mass fingerprinting) are encountered when digestion of the protein is incomplete or when peptide recovery is low. Hydrophobic proteins often present this type of problem. Some Arg and Lys residues may be inaccessible to the enzyme and/or peptides containing a high proportion of non-polar residues which are poorly soluble (Mann *et al.*, 2001).

A second major type of MS analysis employs the use of tandem mass spectrometers to fragment an individual peptide and obtain its sequence (MS/MS analysis). In this technique, a peptide is selected from the mixture in the first mass spectrometer and is then dissociated by collision with an inert gas. Collision-activated dissociation causes a single cleavage to occur

randomly and predominantly at various amide bonds in the peptide. The resulting fragments (a series differing by the mass of one amino acid) are analyzed in the second mass spectrometer. Tandem mass spectrometry also employs other techniques to refine the analysis. For example, the range of the masses to be analyzed can be selected by using a magnetic sector (quadrupole) coupled with a time-of-flight analyzer. The MS/MS approach has several advantages in that multiple sequences can be obtained for the protein thus enabling more precise identification. Additionally, MS/MS analysis can also characterize protein modifications such as phosphorylation and glycosylation.

Mass spectrometry analysis can also be employed in combination with liquid chromatography (LC) separation. The LC-MS/MS approach provides information in the form of peptide sequences and represents an excellent tool for the detection and identification of allergens in foods. Shefcheck and Musser (2004) recently demonstrated the usefulness of this approach for detecting the peanut allergen Ara h 1 in ice cream. In this report, extracts of ice cream matrix were enzymatically digested followed by LC separation tandem MS analysis. The authors found the method to be capable of detecting as little as 10 ppm of Ara h 1 in spiked samples. Thus MS shows promise for being an important tool for the detection and characterization of allergens.

8.2.4 Limitations of the technology

Two-dimensional electrophoresis still represents the major separation technology used to analyze complex samples. There are numerous examples in which this technology has enabled characterization of the complement of proteins in plant and animal tissues. Databases can now be accessed to provide presumptive identification of proteins based on their positions (pI & M_r) in the separation (http://www.lecb.ncifcrf.gov/EP/table2Ddatabases.html).

However, 2-DE also has a number of limitations that must be considered. First, unlike SDS-PAGE, the technique requires more operator skill and time. Second, almost every sample system presents unique challenges for development of appropriate preparation and separation protocols. The optimization of resolution, i.e. reduction of horizontal and/or vertical streaking, must be achieved by empirical adjustment of variables such as surfactant type and concentration and reductive alkylation reagents. Also, run conditions such as pH range employed and volt-hours of separation in IEF must be optimized.

Third, the nature of the proteins to be analyzed impacts 2-DE separations. Large and/or very hydrophobic proteins do not resolve well in IEF. Typically, proteins with M_r greater than 100 kD are not observed in 2-DE separations. While the advent of IPG strip technology has greatly enhanced the efficiency and reproducibility of IEF separations, hydrophobic proteins remain problematic. Thus, membrane-associated proteins represent a very difficult group of proteins to resolve via 2-DE.

Fourth, low abundance proteins represent a challenge to 2-DE-based analyses. This is of concern to investigation of IgE-binding proteins because major allergens may be minor components of the total protein complement. Enhanced detection of proteins in 2-DE separations can be achieved by use of silver or fluorescent (e.g. SYPRO®) staining protocols. Typically, a 10–100 fold increase in sensitivity can be achieved by these methods. However, lower protein level in the spot also represents a challenge to protein identification, even by MS. Most MS methods require the protein to be digested to peptides. However, the extent of digestion may be low and the subsequent recovery of peptides (especially those with a significant percentage of non-polar residues) may be very low. Thus the ability to identify a protein diminishes significantly as its level in the gel decreases.

A common strategy to overcome the issue of low abundance is to fractionate the sample prior to separation. This approach is referred to as a targeted proteome and may employ a number of methods such as differential sedimentation (e.g. 2S, 7S & 11S fractions) or 'pull downs' using affinity probes such as antibodies. The compromise in a targeted proteome approach is that other proteins of interest may be excluded from the analysis.

8.3 Applications of proteomics for detection of allergens

While the number of reports using a proteomics approach for the investigation of allergens is small, substantial growth is expected as the advantages of the technology become known. The examples discussed below describe some of the relevant applications of note.

8.3.1 Wheat allergens

Investigation of wheat proteins associated with baker's asthma represents one of the first applications of proteomics. Two-dimensional gel electrophoresis and immunoblotting demonstrated numerous IgE-binding proteins in wheat (Weiss *et al*., 1993; Posch *et al*., 1995; Weiss *et al*., 1997).

Two-dimensional electrophoretic separation and detection of IgE-binding proteins was performed on fractionated wheat flour. Specifically, the albumin/globulin fraction was the focus of investigation because it was judged to contain a majority of the IgE-binding proteins (Weiss *et al*., 1993). The study used sera from a pool of four patients (bakers) experiencing asthmatic symptoms for immunoblotting of 2-DE separations. Approximately 30 polypeptide spots were observed to bind IgE from these allergic patients. Major IgE binding was observed for a cluster of spots in the 27 kD region. This group contained four to five charge variants with approximately equal IgE-binding intensity. Additional IgE-binding polypeptides were observed in the separation including groups in the 15–20 kD and 30–35 kD regions.

Subsequent proteomic investigations of IgE-binding wheat proteins associated with baker's asthma implemented N-terminal sequencing for identification (Posch *et al*., 1995). Nine major IgE-binding spots in the 2-DE separation of the globulin fraction were selected for analysis. The 27 kD family IgE-binding proteins were identified from Edman sequencing data as isoforms of Acyl-CoA oxidase. Identification of IgE-binding spots with M_r in the 29–35 kD range was not successful. Finally, two IgE-binding spots of 14–18 kD were identified from their N-terminal sequence as isoforms of α-amylase inhibitor.

Most recently this group used a collection of 20 sera from bakers experiencing asthmatic symptoms to further characterize the profile of IgE-binding wheat proteins (Weiss *et al*., 1997). The salt-soluble fraction of several wheat cultivars containing albumins and globulins was the focus of this study. Numerous IgE-binding proteins were again observed. IgE binding was found for albumins/globulins in the 70, 55, 35, 26–28 and 14–18 kD regions. However, major IgE binding was found to 26–28 and 14–18 kD proteins. IgE-binding spots corresponding to these spots were identified as Acyl CoA oxidase, and α-amylase inhibitors, respectively.

8.3.2 Hazelnut allergens

Hazelnuts represent a significant cause of allergic reactions ranging from oral allergy syndrome to anaphylaxis. The source of oral allergy syndrome may be linked to cross-reactivity of the profilin protein, termed Cor a 1 in hazelnuts (Hirschwehr *et al*., 1992). However, most other IgE-binding proteins, thought to be responsible for more severe reactions, have not been characterized. Recently, a major allergen was identified using a proteomic approach (Beyer *et al*., 2002a). Two-dimensional electrophoresis and immunoblotting with sera from 14 hazelnut-allergic individuals was used to detect IgE-binding proteins. While IgE binding was observed to numerous spots, a 40 kD polypeptide was recognized by 12 of the 14 allergic patient sera. The 40 kD protein exhibits several isoforms. The protein corresponding to this spot was excised, digested and the sequence of two fragments obtained by the Edman method.

The sequences were found to have significant homology with the legumin precursor of English oak and pruning 2 precursor of almond. Both proteins are 11S seed storage proteins. The identity of this protein as an 11S globulin was confirmed by expressing the sequence-specific clone from a hazelnut cDNA library. The full-length clone was found to encode for a 59 kD protein. Protein expressed by this clone was positive for IgE-binding. Like other 11S globulins of soybean and peanut (glycinins), the 40 kD portion represents the post-translationally processed acidic subunit that has also been shown to be an allergen in these species (Rabjohn *et al*., 1999; Xiang *et al*., 2002).

This 11S globulin (glycinin acidic chain) represents a novel IgE-binding protein in hazelnuts and has been designated in allergen nomenclature as Cor

a 9. This protein shares a considerable sequence similarity with other allergens, based on it its origin in the cupin superfamily of proteins. Interestingly, when sequences were aligned with the corresponding peanut allergen (Ara h 3) IgE epitope, nine of 15 residues (from G237 to A251) were found to be identical (Beyer *et al*., 2002a).

8.3.3 Sesame seed allergens

There has been a rise in the number of reported cases of allergic reaction to sesame seed (Sporik and Hill 1996). The incidence of sesame allergy is higher for young children. It is thought that the incidence of sesame allergy is linked to increased consumption of sesame seed used in desserts and as toppings on baked goods. Numerous proteins with M_r from 8–80 kD bind IgE from sesame-allergic patients. Among these proteins, a 2S albumin was reported to be immunodominant and has been designated in the allergen nomenclature as Ses i 1 (Pastorello *et al*., 2001).

Recently Beyer *et al*., (2002b) used 2-DE in combination with MS and Edman methods of analysis to investigate IgE-binding proteins in extracts of sesame. This study used sera from 20 sesame-allergic patients to characterize the IgE-binding protein pattern in both 1-D and 2-DE separations. IgE-binding proteins ranged from 7–78 kD. Four IgE-binding regions at 7, 34, 45, and 78 kD were recognized by most (> 50%) of the allergic-patient sera used in these immunoblots of 2-D separations The spots corresponding to these IgE-binding polypeptides were excised, digested and sequenced.

Peptides from 78 and 34 kD spots were identified as embryonic abundant protein (soybean) and seed maturation protein (glucose and ribitol dehydrogenases from soybean), respectively. These findings represent novel allergens, not previously described in the literature. Their significance will have to await future investigation.

The 45 kD IgE-binding polypeptides were identified from sequence analysis as a 7S vicilin-type globulin. Comparison of the full-length protein sequence for this allergen with the much more allergenic peanut vicilin (Ara h 1) showed only 36% homology. Interestingly, examination of the sequence in the region corresponding to a reported IgE epitope of Ara h 1 revealed a much closer match with the 45 kD sesame protein. Seven of 10 residues corresponding to T294 to P303 were identical. The newly discovered 7S sesame vicilin has subsequently been designated as Ses i 3 (Beyer *et al*., 2002b).

The 7 kD polypeptide was found to be a 2S albumin. Several examples of 2S albumin allergens have been characterized from Brazil nut (Ber e 1) and walnut (Jug r 1). They are heterodimeric proteins with approximately 8 and 14 kD subunits, linked by disulfide bonds. The sequence of the polypeptide isolated by Beyer *et al*. (2002b) showed a high degree of homology (38 and 40% respectively) to Ber e 1 and Jug r 1. A comparison of the deduced amino acid sequences of the sesame 2S albumin reported by Beyer *et al*. (2002b)

with that reported by Pastorello *et al*. (2001) shows differences sufficient for them to be regarded as distinct allergens. The sesame 2S albumin reported by Beyer *et al*. (2002b) is higher in sulfur-containing amino acids and is deemed to be a novel allergen. It has been designated in the allergen nomenclature as Ses i 2.

8.3.4 Shrimp allergens

Shellfish (shrimp) are a common cause of food allergy. The major IgE-binding protein in shellfish has been designated in the allergen nomenclature as Pen a 1. This allergen has been extensively researched and is an invertebrate form of the contractile protein tropomyosin (Reese *et al*., 1999). Allergic reactions to this protein have been noted in cockroaches and dust mites as well as crustaceans (Asturias *et al*., 1998; Santos *et al*., 1999).

Recently, proteomics has been applied to further characterize the IgE-binding proteins from shrimp. In particular, the black shrimp (Penaeus mondon) of southeast Asia was the focus of this investigation (Yu *et al*., 2003). A crude shrimp extract was separated by 2-DE and immunoblotted using a pool of six shrimp-allergic patient sera. Numerous IgE-binding polypeptides were observed in the 2-DE separation using a pool of six shrimp-allergic sera. Approximately 10 IgE-binding spots were found in the 20–40 kD (pI 4.0–7.0) M_r range. The strongest IgE binding was found at 40 kD (pI 6.0) and 34 kD (pI 4.6). They were excised, digested with trypsin and the resultant peptides analyzed by MS. Spots 2 and 3 were, not surprisingly, identified as the well-known allergen, tropomyosin (Pen a 1). However, analysis of spot number 1 revealed a sequence that matched the enzyme arginine kinase, a novel finding. To further examine the allergenicity of arginine kinase, the enzyme was purified from other crustaceans (crab, lobster and crawfish), tested for IgE binding, and analyzed by dot blot and competitive ELISA using 13 shrimp-allergic sera. Arginine kinase from the other crustaceans showed significant cross-reactivity, suggesting that it is also a common seafood allergen. Arginine kinase was designated in the allergen nomenclature as Pen m 2.

8.3.5 Bird's nest allergen

Consuming the Chinese delicacy known as bird's nest is a long practised tradition. While some claims have been made for its medicinal value, it has more recently become a suspect cause of food-induced anaphylaxis, especially in children (Shek and Lee, 1999). Proteomics was used to analyze the proteins from an aqueous extract of this material (Ou *et al*., 2001). The 2-DE separation contained relatively few spots (< 30). Immunoblotting with the sera from a bird's nest-allergic patient showed that a single family of spots with a M_r of approximately 66 kD was positive for IgE binding. Mass spectrometry analysis of several internal peptide fragments corresponding to the 66 kD protein

resulted in its identification as a Kazal-type serine protease inhibitor, analogous to egg white ovoinhibitor (Ou *et al*., 2001). Ovoinhibitor is a well known egg-associated allergen and therefore linked to adverse reactions resulting from consumption of this food.

8.3.6 Monitoring genetic modification

Agricultural biotechnology is well recognized for its potential benefits to both producers and consumers. *Bacillus thuringiensis*-transformed varieties express a Bt protein that inhibits insect damage. This transformation offers the potential for increased yield while reducing chemical application. Similarly, enhanced nutritional value is possible in golden rice, a variety that produces the vitamin A precursor, beta carotene.

However, agricultural biotechnology has also come under intense criticism because of the perceived potential for causing unintended consequences. A major concern is that insertion of a novel gene or removal (silencing) of an existing gene may alter the expression of undesirable components such as allergens. Thus in many parts of the world, genetically modified crops are not allowed in food production.

Proteomics represents an excellent technology for objectively assessing the changes in crop varieties resulting from genetic modification or conventional breeding as well. Recently, Herman *et al*., (2003) used proteomic analysis together with immunoblotting and localization to assess the effectiveness of gene silencing technology on the expression of a major soy allergen. The allergen targeted for silencing in soybean was Gly m Bd 30K, also referred to as P34. This low-abundance protein has been shown to be a major allergen (Helm *et al*., 1998, 2000). P34 protein is associated with other globulins and can be found after processing as a component of soy protein isolates, a widely used food ingredient. Thus silencing the expression of this protein would remove a major allergen from soybean.

Herman *et al*., (2003), used transgene-induced gene silencing to produce soybeans that expressed little or no P34 protein. Two-dimensional electrophoresis and image analysis of control and P34-silenced seed lines showed very few changes. A comparison of control and silenced lines revealed only five polypeptide spots with significantly altered expression. These spots were down-regulated in the transgenic line two of the polypeptides with positions in the gel corresponding to the pI and M_r range of P34 were positively identified by MS analysis as the P34 allergen. This report serves as an example of how proteomics can used to provide objective evaluation of changes (or the lack thereof) induced by genetic modification. In this case, proteomic analysis clearly showed that silencing technology had achieved its desired objective of allergen reduction. The proteomic approach also demonstrated that no unintended consequences had occurred.

8.3.7 Detection of IgE-binding proteins in foods

In general, a proteomic approach might not be suited to routine identification and quantitation of IgE-binding proteins in foods because of the amount of time required to perform the analyses. Additionally, appropriate protein extraction protocols would be needed for food matrices. However, there are examples when the time and effort required by the proteomic approach are of importance.

Once such case can be found in the food ingredient lecithin. Lecithin, derived from soybeans, is composed principally of phosphatidyl choline, phosphatidyl inositol and phosphatidyl ethanolamine. Phospholipids are excellent emulsifiers and are often used for that function in food and pharmaceuticals. However, soybean lecithin has been shown to contain protein at levels ranging from 50–1000 ppm. Several of these proteins also bind IgE from soy-allergic patients (Awazuhara *et al*., 1998; Muller *et al*., 1998; Gu *et al*., 2001).

Previous studies using 1-D SDS-PAGE and immunoblotting with soy-allergic sera identified several IgE-binding bands (Awazuhara *et al*., 1998; Muller *et al*., 1998; Gu *et al*., 2001). Recently Gu *et al.* (unpublished) used 2-DE, image analysis and immunoblotting to further examine the profile of IgE-binding proteins associated with lecithin.

The presence of IgE-binding proteins in the separation was determined by immunoblotting with the same soy allergic sera pool as used by Gu *et al*., 2001. Numerous spots exhibited IgE binding and are summarized as follows. Several spots in the 57 kD range (pI 4.2–5.0) bound IgE but were low in abundance. Little or no protein can be seen in the 2-DE separation. However, IgE binding was detected. Based on their pI and M_r, these spots were tentatively identified as conglycinins.

All spots in the 39 kD range were strongly IgE-binding. They represented a significant proportion of the total lecithin-associated protein. Collectively the 39 kDa spots (pI 4.2–5.2) constitute about 23% of the sample protein. However the 39 kD protein is a novel IgE-binding protein whose function and location is unknown (Gu *et al*., 2001). The 39 kDa protein may be associated with oil bodies as our recent findings with a polyclonal antibody suggest (Xiang *et al*., unpublished).

The most diverse group of proteins was found in the 20 kD region. At least eight individual polypeptides with pIs ranging from 4.25–9.7 were observed with this M_r. Collectively, polypeptides in the 20 kD group make up the largest fraction of lecithin-associated proteins. They comprise about 37% of the total sample protein. The IgE-binding, 20 kD spot (pI 4.4) was tentatively identified (based on pI and M_r) as soybean Kunitz-type inhibitor (SKTI).

Strong IgE-binding spots can also be found at 12 kD. They range in pI from 4.38–5.3. The 12 kD (pI 4.38) spot is much greater in abundance than the others in this M_r range and represents approximately 26% of the sample protein. This 12 kD spot was tentatively identified (based on pI and M_r) as

a soybean 2S albumin (napin type) protein. This work further illustrates advantages associated with proteomic analysis for allergen identification. The approach provides uniquely definitive information in regard to the profile and amount of proteins present.

8.4 Future trends

The future promises to bring many changes for proteomic technology. A major focus of developmental effort is to replace 2-DE gels with other separation technologies. Microfluidic devices are currently receiving considerable attention for achieving this goal. Microfluidic devices are small platforms constructed from glass or polymeric substances, typically equivalent in size to a microscope slide. Separation channels with nanoliter internal volumes are etched into the surface. Microfluidic separations are driven electrophoretically because of separation efficiency and the difficulty in developing miniaturized mechanical pumps. Separation times vary with the analyte but are typically accomplished in seconds. Detection can be via laser-induced fluorescence or MS. It is anticipated that a complete separation device may eventually be handheld, i.e. the size of a pocket calculator. Numerous applications for microfludic separations of proteins, peptides, nucleic acids, drugs, etc. have been reported (Figeys and Pinto, 2001; Verpoorte 2002).

The separation of peptides from tryptic digests of complex (tissues or cells) samples represents an immediate goal for development. A model for the direction of future separation technology can be seen in ongoing research of multi-dimensional CE-based methods (Chen *et al*., 2002; Shen and Smith, 2002). These separations employ various combinations of IEF, reversed-phase, affinity or ion exchange to achieve resolution of complex peptide mixtures. A further goal of the technology is to couple separation with MS analysis to obtain several types of information, including profile (mass fingerprint), sequence and modifications of the parent protein. This analytical approach generates enormous amounts of data, requiring development of appropriate bioinformatic programs.

8.5 Conclusions

Proteomics is an increasingly valuable tool in allergy research. Presently, it offers enhanced potential for the detection and identification of IgE-binding proteins in complex mixtures. All of the examples included in this review showed that proteomic analysis resulted in new and more detailed information regarding the profile of allergens in the materials examined. Proteomics was also shown to be of value in the quantitative assessment of allergen content in genetically modified soybeans. In the future, the development of new

separation (microfluidics) and detection (aptamers) technologies will further enhance the value of proteomics to allergen investigations.

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9

Detecting food allergens with a surface plasmon resonance immunoassay

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9.1 Introduction

During recent years, awareness of food allergens has increased, resulting in a growing demand for reliable, rapid and sensitive analytical methods to detect them (Poms *et al*., 2004). Tracing and quantifying allergens in food is often a challenging task. Allergens can be present in a wide variety of food types, with different compositions and chemical properties. Some food components may interfere with the assays. Food processing of different kinds makes the extraction difficult. The sensitivity requirement is also high since even very low doses may elicit allergic reactions. Tracing allergens in food could very much be compared to finding a needle in a haystack. To be able to reliably analyze as many allergens as possible in as many sample matrixes as possible, a whole battery of methods is needed. Different analytical techniques may complement each other.

As a research project, the Swedish National Food Administration has started to investigate a novel analytical technique based on a surface plasmon resonance (SPR) biosensor for food allergen detection. The objective is to develop rapid and easy-to-use assays for common food allergens that would complement existing techniques already employed, such as rocket immunoelectrophoresis (RIE), enzyme-linked immunoassays (ELISAs) and real-time PCR. Besides enhanced sensitivity, increased sample throughput is a major driving force behind the development of the automated platform. The high speed, ease-of-use and high degree of automation should also be attractive features for food manufacturers who want to analyze ingredients or final food products.

This chapter summarizes the literature on SPR technology related to food

allergen detection and describes the work performed so far on developing SPR biosensor immunoassays on the Biacore™ SPR system. (Biacore AB, Uppsala, Sweden).

Section 9.2 gives a short introduction to biosensor technology, followed in Section 9.3 by a description of the process of setting up an allergen SPR assay. Published methods are summarised in Section 9.4 and then in Section 9.5 details of the assay are given together with a description of the specific analysis conditions used and presentation and discussion of the results obtained.

9.2 Biosensors and SPR technology

Biosensors are analytical devices consisting of a biological recognition element (e.g. cells, proteins or oligonucleotides) in direct contact with a transducer that produces a signal, which is further processed to give an output that is proportional to the concentration of a specific analyte. Transducers can be optical, amperometrical, potentiometrical or acoustical. Surface plasmon resonance employed in this study is an affinity-based optical transduction principle that detects the binding between molecules through local changes in the refractive index close to a surface. Currently, food analysis using SPR is a rapidly growing field. So far most development has been in the area of vitamins and veterinary drug residues, with ready-to-use kits commercially available. Examples of protein SPR food immunoassays are detection of Staphylococcal enterotoxin B (SEB) (Rasooly, 2001; Medina, 2003), Salmonella antibodies in chicken (Jongerius-Gortemaker *et al*., 2002) and fungal aflatoxin B_1 (Daly *et al*., 2000). Feriotto *et al*. (2002, 2003) developed an assay for genetically modified soy oligonucleotides. Detection of various milk proteins has been reported by several groups (Haasnoot *et al*., 2001; Indyk and Filonzi, 2003; Nygren *et al*., 2003; Müller-Renaud *et al*., 2004). Analysis of peanut allergens using a miniaturized SPR system has been demonstrated by Mohammed *et al*. (2001).

SPR instruments are available from different commercial sources. Approximately 90% of all work published in the optical biosensor field has used instruments from Biacore (Myszka, 1999; Rich and Myszka, 2000, 2001, 2002). In Biacore's SPR technology, detection takes place in a flow cell on an interchangeable sensor chip (Fig. 9.1). To construct a SPR biosensor, the recognition element is immobilized on the surface which consists of a gold film deposited on a glass support. The surface is covered by a dextran layer which provides covalent binding sites when chemically activated and a protein-friendly hydrophilic environment (Löfås, 1995). Samples are introduced through an integrated microfluidic cartridge (Sjölander and Urbaniczky, 1991). Built-in microvalves are used to accurately switch between sample and buffer flow. Opposite the flow cell side of the sensor surface, a prism is optically coupled to the sensor surface. Polarized light from a light-emitting diode is reflected in the glass and detected by a charge-coupled diode (CCD) array.

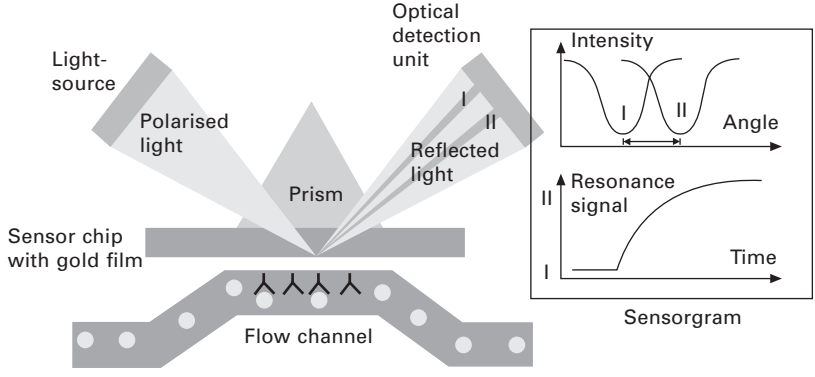

Fig. 9.1 Principles of surface plasmon resonance detection. Due to the SPR phenomenon the reflected light will have an intensity minimum at a specific angle that is continuously monitored. Binding of biomolecules to the sensor surface results in a shift in the resonance angle.

At a specified resonance wavelength and angle, surface plasmons, i.e. clouds of free electrons in the metal film, interact with the photons resulting in a drop in the reflected light detected by the CCD. The resonance angle is sensitive to the refractive index close to the surface. Liedberg *et al.* (1983) first showed that biomolecules binding to the surface result in a refractive index change, which in turn alters the resonance angle. The shift is proportional to the mass bound to the surface. In the Biacore instrument, the resonance angle is continuously monitored and converted to 'resonance units' (RU). One RU corresponds to an angular shift of 0.0001° (Jönsson *et al.*, 1991). Plotting the resonance angle against time yields a sensorgram that describes the interaction taking place on the surface in real-time.

Typically in SPR immunoassays samples are analyzed serially. Each sample cycle consists of sample injection followed by a regeneration pulse that washes away any bound sample molecules making the surface ready for the next cycle (Fig. 9.2). Between injections a continuous flow of buffer is maintained. In the food allergen assays developed, an extra injection of antibodies was usually added after each sample injection. Binding of the antibodies confirms the identity of the sample and can also be used to determine the amount bound indirectly. This is similar to sandwich ELISA, but with the great difference that all steps can be followed in real-time, without the need to label any of the interactants. In contrast to ELISA, the regeneration, however, allows the same antibody coated surface to be used for about 100–200 times.

9.3 Developing a food allergen SPR immunoassay

This section describes the major steps in developing an SPR immunoassay for food allergens.

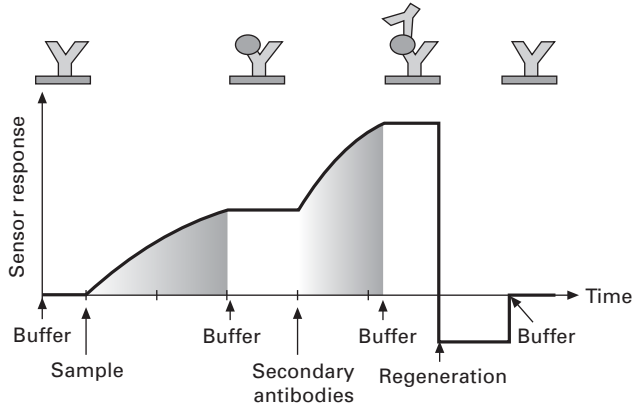

Fig. 9.2 Sensorgram for one analysis cycle. This example shows a typical injection sequence for a sandwich format assay, with secondary antibody injection after the sample injection. Switching of solutions is indicated by arrows. The pictures above illustrate schematically the surface coverage at different stages.

9.3.1 Preparation of the sensor surface: immobilizing the antibody

The first step in developing an SPR food allergen immunoassay is to prepare a sensor surface coated with specific antibodies that bind the allergen in question. Immunopurification on an affinity column coupled with the allergen is highly recommended in order to obtain high responses and a specific assay. In Biacore instruments, preparation of a surface covered by antibodies is easily performed through a standardized and automated procedure that covalently couples the antibodies via free primary amine groups to the carboxylated dextran layer on the sensor surface with the sensor chip mounted in the instrument (Löfås *et al.*, 1995). With SPR detection the entire immobilization procedure can be monitored in real-time, and the immobilized level of antibodies can be read out as the increase in sensor response. For a sensitive assay, high levels of immobilization are required. The dextran layer on the Biacore CM5 chip extends the surface into a third dimension and enhances the surface binding capacity, compared to a bare gold surface. In the allergen assays developed, immobilization levels of 10 000–13 000 RU of antibodies were typically achieved on the standard CM5 chip, which corresponds to surface densities of 10–13 ng mm^{-2}. For many of the experiments a sensor chip CM3, with a thinner dextran layer than CM5, was used. For CM3 chips the immobilization level was between 3000 and 5000 RU. These levels were sufficient to allow detection in the ng mL^{-1} range.

9.3.2 Binding and regeneration

When an antibody-coated sensor surface has been prepared, the activity can be checked by injecting a solution of the allergen. An increase in sensor response of several hundred RU should be observed during the injection of

food allergens (two minutes, 10 μg mL^{-1}). The next essential step is to find a suitable regeneration solution able to remove all the bound analyte without damaging the surface. This solution is injected after each sample. When a good regeneration solution has been found, the same surface can be used to analyze 100 or more samples, resulting in a very economical use of antibodies and sensor chips. In the allergen assays developed so far, 0.1 M hydrochloric acid has been proved to regenerate the surface quite well and can be used initially for newly-developed assays. To further optimize the regeneration for the specific antibody used, it is advisable to test the regeneration efficiency with 10 mM glycine-HCl with pH ranging from 3 down to 1.5 in a series of analysis cycles run on a newly immobilized surface. In each cycle, a high concentration of allergen (e.g. 10 μg mL^{-1}) is injected followed by the regeneration solution. The mildest regeneration solutions (highest pH) should be run first, and each condition should be repeated for five cycles before switching to the next. A plot showing the response increase during the allergen injection versus cycle number can then be used to determine the optimal regeneration solution. The regeneration solution that gives the highest and most stable analyte response should be chosen for the assay. Software guides are available for this type of assay development in the Biacore Q system.

9.3.3 Sandwich assay

To enhance sensor signal and increase the specificity of the assay, a sandwich format can be employed. In this assay format an injection of secondary antibodies is performed after the injection of sample and before the regeneration step (see Fig. 9.2). The regeneration step removes both the second antibody as well as the allergen and allows repeated use of the sensor surface. Binding of an allergen-specific antibody, readily seen as an increase in the sensor output during injection, confirms the identity of the bound material on the surface. Specific binding of allergen can in this way be distinguished from non-specific binding from the sample matrix. With SPR the secondary antibody needs no labelling to be detected. Furthermore, with polyclonal antibodies it is possible to use the same antibody as for the immobilization. This convenient strategy was found to work well for all tested allergens. Complete removal of allergen during the regeneration is especially crucial in the sandwich assays, because any remaining allergens on the surface in one cycle can bind secondary antibody in the next. The secondary antibody injection step should therefore be included when regeneration optimization is performed.

9.3.4 Calibration of the sensor response

For the determination of the concentration of allergens in unknown samples, the sensor output has to be calibrated before unknown samples are run. This is achieved by injecting a series of standard solutions with known concentrations of allergens. Each standard is run exactly the same way as samples with

unknown concentrations, with regeneration of the surface at the end of the cycle. For direct measurements the increase in response during the sample injection is used for quantification. The response increment is measured after the end of the injection when buffer flow has been re-established and non-bound sample has been washed away. In this way the measurements become independent of the refractive index of the sample extract. In the sandwich assay the response increment during injection of the secondary antibody is used instead. The calibration curves in the food allergen assays were generally linear for low concentrations ($< 1\ \mu g\ mL^{-1}$) but flattened out for higher concentrations due to saturation of binding sites on the surface. A four-parameter non-linear function was used to fit the points in the calibration curve.

9.3.5 Recovery studies

During assay development, recovery studies with spiked samples can be undertaken to study the effect of the sample matrix on the assay. Spiking, i.e. addition of known amounts of allergen, can be done in different stages depending on the purpose of the experiment. In the development of the SPR allergen assays two types of recovery studies were carried out: spiking of extracts and spiking of food before extraction. Spiking of blank extracts, made from samples known to be free from the allergen, was used to reveal possible matrix inhibition of the binding to the surface. In general, matrix inhibition turned out not to be a problem. However, for a dark chocolate extract the specific binding to an anti-hazelnut protein surface was reduced by 50%. The subsequent binding of secondary antibodies was also inhibited by 50% resulting in a recovery of only 25% in the sandwich assay. Apparently, this extract contained something, probably tannins, that bound to the hazelnut proteins and inhibited the binding to the antibodies. When spiking was performed before the extraction, the recovery of hazelnut proteins from dark chocolate was even lower, indicating precipitation by tannins. In the hazelnut assay, addition of 1% polyvinylpyrrolidone (PVP) during extraction was found to restore the recovery in dark chocolate to 97–104%.

9.3.6 Analysis of food products

Once the sensor output has been calibrated, single samples can be analysed very rapidly, within minutes. In the Biacore Q software the calculations of concentration in unknown samples, including any dilutions made, are performed in real-time and the results are presented immediately as soon as the sample cycle is finished. The instrument can also be programmed to analyze up to 180 samples (two microplates) subsequently without any need of human interaction. Control samples and recalibrations can be included at intervals. In this way it is convenient to load the instrument in the evening, let the instrument analyze the samples overnight and on the following day all results are summarized in a software-generated report, ready to be printed out.

9.3.7 Advantages of real-time detection

SPR detection is not dependent on radioactive, fluorescent or enzyme labels. All binding events on the surface can thus be followed in real-time, which is very useful during assay development as well as during routine analysis. Compared to endpoint analysis such as ELISA and rocket immunoelectrophoresis, analysis by SPR gives additional information. For instance, the amount of antibodies immobilized on the surface of a chip can be quantified. ELISA does not offer similar direct control of the coating levels. Although only a single point is used to construct the calibration curve and to determine the concentration, the reviewing of the binding curves for each sample cycle is an important quality control operation. Operational errors can be identified by reviewing the response curves. For instance, an empty vial will result in injection of air, which is readily revealed as a great shift in the response during the injection period. As discussed below, non-specific binding to the surface can be a problem for certain types of food samples. Such conditions can often be identified by analyzing the binding curve. Generally, non-specifically bound material dissociates faster than allergens specifically bound to the antibodies. Thus, an unusually high negative slope after injection is a warning signal of non-specific binding. A response not returning to the base level after regeneration is another warning that not only allergens have been bound to the surface. A trend plot of the baseline response levels in the beginning of each cycle during run is thus an efficient tool to reveal assay anomalies.

9.4 Published methods

No fully validated method for food allergen detection with SPR detection has yet been published. Besides the results presented in Section 9.5, Mohammed *et al*. (2001) have demonstrated food allergen analysis with SPR. They carried out detection of peanut proteins on a miniature SPR system that could be used to provide immediate, on-line detection and quantification during food production. The limit of detection was 0.7 $\mu g\ mL^{-1}$ of purified peanut protein in buffer solution, which is about 100 times higher than that obtained for the peanut assay developed on the Biacore (see below).

A few other SPR food protein assays have been published to date. Haasnoot *et al*. (2001) developed a rapid (five minutes per sample) SPR immunoassay for the detection of soy, pea and soluble wheat proteins. Although these analytes are allergens, the main purpose with this assay was to reveal adulteration of dairy products with non-milk proteins. A four-channel system was employed, enabling simultaneous detection of all three species (the fourth channel was used as a reference). The detection limits for the three plant proteins in solution were 20–50 $ng\ mL^{-1}$ and below 0.1% of plant protein in total milk protein content. Prepared sensor chips could be used for more than 650 sample injections, resulting in a very economical use of antibodies.

Rasooly (2001) reported a sensitive SPR immunoassay for the detection of SEB, which employed a sandwich format. SEB was detected down to 10 ng mL^{-1} within five to eight minutes per sample. Little interference from the food matrix was observed.

A quantitative SPR assay for beta casein in milk and cheese was developed by Muller-Renaud *et al*. (2004). A two-step sandwich approach was used involving secondary antibodies binding to the casein protein was used. In this way native casein could be discriminated from its degradation products. The analysis time was less than 10 minutes per sample and the same surface could be used for more than 250 determinations. The detection limit was 85 ng mL^{-1}.

9.5 Experimental data

This section presents the results from the development of SPR food allergen assays at the National Food Administration, Sweden.

9.5.1 Assay details

Instrumentation and Reagents

The SPR system Biacore Q from Biacore AB was used in most analyses. However, the initial experiments with beta-lactoglobulin, hazelnut and ovomucoid were performed on a Biacore 2000.

CM5 (Research Grade) and CM3 sensor chips, Surfactant P20, HBS-EP buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20), Amine Coupling kit containing 100 mM NHS (N-hydroxy-succinimide), 400 mM EDC (N-ethyl-N′-(3-ethylaminopropyl) carbodiimide) and 1 M ethanolamine, pH 8.5, were all bought from Biacore AB. Polyvinylpyrrolidone (PVP, 150 kD) was obtained from Sigma (Sigma-Aldrich, Stockholm, Sweden). All other chemicals were of analytical grade.

Protein fractions from hazelnuts, peanuts and sesame seeds were purified at the National Food Administration according to Klein *et al*. (1985) and crab protein according to Byun *et al*. (2000). Beta-lactoglobulin and ovomucoid were purchased from Sigma. Polyclonal rabbit antibodies against beta-lactoglobulin and ovomucoid were obtained from Pharmacia Diagnostics (Uppsala, Sweden). Rabbit antibodies against hazelnut proteins, peanut proteins, sesame seed proteins and crab tropomyosin were produced for the National Food Administration by Agrisera AB (Vännäs, Sweden).

Surface preparation

Allergen-specific rabbit IgG from the immune sera was isolated by immunoaffinity chromatography on a NHS-activated HiTrap™ column (GE Healthcare, Uppsala, Sweden) coupled with the respective allergens (affinity purification). In some of the initial experiments total IgG, as obtained

by purification with Protein G (GE Healthcare, Uppsala, Sweden), was used.

The affinity-purified antibodies were covalently bound to the carboxymethylated dextran layer of a CM5 or a F1 chip using the amine coupling method pre-programmed in the instrument (Löfås *et al.*, 1995). A flow rate of 5 μL min^{-1} was used during immobilisation. The sensor chip surface was activated with a mixture of NHS and EDC (1:1 v/v) for seven minutes prior to incubation of the ligand solution. The ligand solution, consisting of purified antibodies, diluted to a concentration of about 50 μg mL^{-1} in 10 mM sodium acetate, pH 4, was injected for seven minutes, followed by a seven minute injection of ethanolamine to block remaining active sites. Newly prepared surfaces were washed with three short pulses or more of regeneration solution (0.1 M HCl) prior to use.

Sample extraction and analysis

Between 0.5 and 3.0 g homogenized sample was extracted in 10 mL extraction buffer (20 mM Tris, pH 8.7, 0.5 M NaCl) in an ultrasonic bath at 45 °C for one hour, followed by centrifugation at 30 800 g for 10 minutes at 4 °C. One percent (1%) PVP was added to the extraction buffer when analyzing dark chocolate. The supernatant solutions were collected in 1.5 mL vials. Immediately before analysis, extracts were centrifuged to remove particles.

Biosensor analysis was performed using a running buffer composed of the extraction buffer with addition of 0.05% Surfactant P20 to reduce the non-specific binding. Samples were injected for two minutes at a flow rate of 40 μL min^{-1}, followed by a two minute washing period with running buffer. Secondary antibodies, diluted to about 20 μg mL^{-1} in running buffer, were injected for 60 seconds at a flow rate of 10 μL min^{-1}. The surface was typically regenerated with a 60 second pulse of 0.1 M hydrochloric acid.

9.5.2 Results and discussion

Evaluation of the SPR system for food allergen analysis has been made with several important food allergens, including egg ovomucoid, milk beta-lactoglobulin, hazelnut proteins, peanut proteins, sesame seed proteins and crab tropomyosin, a common allergen in crustaceans. The results obtained so far are briefly summarized below.

Initial proof-of-principle experiments with ovomucoid, beta-lactoglobulin and hazelnut proteins

Ovomucoid, beta-lactoglobulin and hazelnut proteins were included in the first round of tests to evaluate the versatility of the method. Antibodies against ovomucoid, beta-lactoglobulin and hazelnut protein were successfully immobilized on sensor surfaces. These antibodies were the IgG fraction from antiserum purified on a Protein G affinity column. Figure 9.3 shows the calibration curves for the three allergens based on total binding of pure

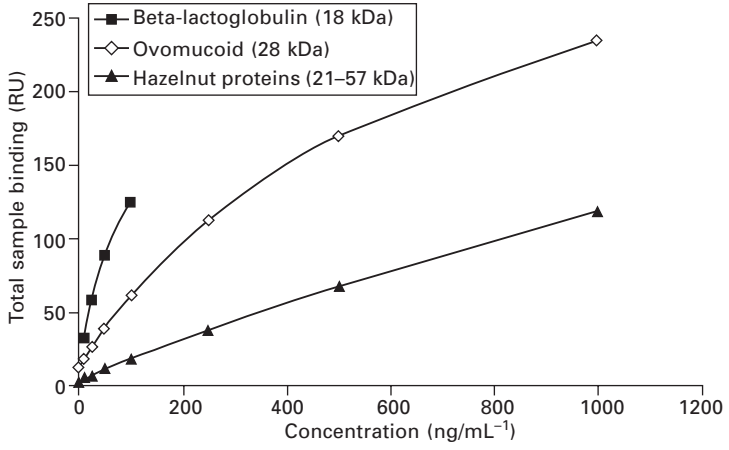

Fig. 9.3 Calibration curves for beta-lactoglobulin, ovomucoid and hazelnut proteins. Two-minute injections were used.

solutions. This initial investigation showed that the sensitivity of the SPR detector is sufficient for detection of food allergens. Compared to rocket immunoelectrophoresis using the same antibodies the sensitivity was at least ten times more sensitive.

Analysis of food extracts, however, showed that the assays were not yet specific enough. Some types of food extracts bound non-specifically to the surface. Since everything that binds to the surface will contribute to the sensor signal in SPR, non-specific binding will result in false-positive results in measurements based on total sample binding. By comparing the response on surfaces with or without immobilized antibodies, it was shown that most of the non-specific binding occurred to the antibodies, not to the surface. The dextran-coated surface was quite inert. Extracts from bread and other bakery products never gave high non-specific binding. Chocolate extracts, on the other hand, were very important for the hazelnut assay, often bound to a very high degree making sensitive measurements of hazelnuts impossible.

One reason for the non-specific binding to the immobilized antibodies is that the Protein G-purified antiserum contains a mixture of IgG towards all food proteins that rabbits have encountered during their life. One way to enhance the specificity is to isolate only specific antibodies (see below).

Fighting non-specific binding

In the further development of the hazelnut assay, many different means of reducing the non-specific binding were tested (Jonsson and Hellenäs, 2001). Firstly, the sensor surface was made more specific by isolation of antibodies on an affinity column coupled with hazelnut proteins. This resulted in 50% higher sensor response, at the same level of immobilization, compared to antibodies purified on the Protein G column. Switching from the most common type of sensor chip (CM5) to a variant with a thinner dextran layer (CM3) decreased the non-specific binding of a dark chocolate extract by 55% while

the specific binding of hazelnut proteins remained almost the same. Still, the non-specific binding was too high for several types of sample extracts.

Increasing the concentration of salt and increasing the pH of the buffer further reduced the non-specific binding from chocolate extracts. However, these buffer modifications also reduced the specific binding. Increasing the amount of surfactant (P20) reduced the non-specific binding for dark chocolate extracts (Jonsson and Hellenäs, 2001). In contrast, increasing the surfactant concentration increased the non-specific binding of milk chocolate extracts. The best way of reducing the high non-specific binding for dark chocolate extracts was found to be addition of PVP during the extraction. This also greatly increased recovery of hazelnut proteins in spiked samples.

This examination indicates that it is possible to optimize the assay conditions in the SPR assays so that sensitive measurements can be performed even in food matrices that contain many interfering substances. When the composition of the analyzed food extract is known and similar from time to time, this can be used to obtain a very rapid analysis of samples. For instance, this could be used in continuous monitoring of a manufacturing process. In most situations, however, the compositions of the samples are not very well known and differ much from sample to sample. Under such assay circumstances, it is very difficult to find conditions that will give very little non-specific binding for all types of sample extracts. The best way of circumvent this problem was found to be extending the assay slightly by adding an injection of antibodies after the sample, creating a 'sandwich' format.

Increasing the specificity through the sandwich format

In the sandwich format, injection of a secondary antibody is used to increase the specificity of the assay. Binding of the secondary antibody is used to confirm the identity of the material bound during the sample injection. As SPR detects everything that binds to the surface, the secondary antibodies do not have to be labelled in any way to be detected. Binding of the secondary antibody can be followed second by second, just like the binding of samples. The binding will be proportional to the amount of the allergen bound on the surface during the sample injection. Specific antibodies will not bind to non-specifically bound material from the sample extracts. Thus, using the response increase during injection of the secondary antibody instead of the increase during sample injection to determine the concentration will give a much more specific assay.

Table 9.1 shows a comparison of cross-reactivity obtained in the hazelnut assays utilizing both direct and indirect measurements. While virtually all samples cross-reacted to some degree in the direct binding assay, only a few species cross-reacted in the sandwich assay using affinity-purified antibodies. The minor cross-reactivity that was observed in the latter approach was towards walnut, coconut, cashew and pecan, similar to what was earlier described for affinity-purified antibody-based sandwich ELISA for hazelnut (Koppelman *et al*., 1999). These results show that very specific SPR assays can be achieved by using a second antibody in a sandwich approach.

Table 9.1 Cross-reactivity expressed as ppm hazelnut protein in solid food sample. All samples were diluted a hundred times before analysis. Concentrations assigned as >100 indicate that the level was above the measurement range when normal assay conditions were applied. Only hazelnut extract was further diluted to allow higher concentrations to be determined.

Sample	Direct binding assay	Sandwich assay
Hazelnut	98 700	97 200
Walnut	> 100	2.2
Pecan	> 100	2.9
Cashew	n.d.	1.3
Pistache	55	n.d.
Brazil nut	76	n.d.
Nutmeg	>100	n.d.
Almond	35	n.d.
Sesame	54	n.d.
Peanut	45	n.d.
Coconut	83	n.d.
Pine kernel	24	n.d.
Dark chocolate	0	n.d.
Milk chocolate	10	0

n.d. = not detected (< 10 ppm)

Precision and sensitivity for the hazelnut assay

Replicate injections of different concentrations of hazelnut proteins showed that the response was repeatable. For concentrations between 0.05 and 1.0 μg mL^{-1} the variation (relative standard deviation, four replicates) was less than 1.5%. Based on the response of buffer injections, the limit of detection in purified solutions of hazelnut proteins was calculated to 5 ng mL^{-1} (three standard deviations above the average response for buffer injections). This is the same order of magnitude that was achieved using hazelnut ELISA (Holzhauzer and Vieths, 1999; Koppelman *et al*., 1999). Compared with a previous report of analysis of peanut proteins on a miniaturized SPR system, this represents a hundredfold improvement (Mohammed *et al*., 2001). However, analysis of purified allergens in buffer is an idealized situation. The actual limit of detection will be determined by the background response of real blank food extracts. The dilution of extracts also influenced the sensitivity. Preliminary results for the hazelnut assay showed that the limit of detection is about 0.5 mg protein per 100 g in chocolate (5 ppm), when samples were diluted 100 times before analysis. Later results with a peanut assay showed that it is possible to analyze samples that were diluted only ten-fold. This could improve the limit of detection even more. As the response was still increasing when the sample injection was ended, the maximum surface binding capacity had not been reached. The limit of detection may therefore also be improved by increasing the contact time. In the present study two minute injections were used to optimize the assay for speed. Compared to rocket immunoelectrophoresis assays

currently used for routine analysis at the Swedish National Food Administration, the SPR hazelnut assays were ten-fold more sensitive. The limit of detection was in the same range as commercially available kits for hazelnuts.

Assay for peanuts

A peanut assay was established using the same conditions as for hazelnut. For comparison between the SPR immunoassay and the RIE and commercially available enzyme immunoassays (EIAs), model chocolate samples, spiked with peanut butter, were analyzed. The model chocolates were made by melting different amounts of peanut butter in a peanut-free milk chocolate at 70 °C. The peanut butter contained about 25% protein according to the ingredient list. Chocolate samples with peanut protein levels from 12 500 down to 0.375 ppm were produced (Table 9.2). The RIE assay (Malmheden Yman *et al*., 1994) detected peanut proteins down to 70 ppm. The SPR immunoassay extended the measurement range down to 1 ppm, giving a response significantly above the response for blank samples. The Ridascreen® enzyme immunoassay (R-Biopharm, Darmstadt, Germany) had a similar sensitivity, while the BioKits™ peanut assay (Tepnel BioSystems, Deeside, UK) was even more sensitive. A good correlation between the SPR assay and both the ELISA kits was seen. The recovery of the SPR assay was similar to that of the Ridascreeen assay, but lower than that of the BioKits™ assay.

Egg protein assay

Conalbumin (ovotransferrin) (Sigma-Aldrich, Sweden) was used for immunization of a rabbit. The protein was also coupled to a HiTrap™ column as described above for hazelnut, for the immunopurification of specific rabbit IgG to conalbumin. The purified IgG was immobilized on a CM3 chip. Pasta samples were extracted with Tris-HCl buffer, pH 8.7. The extracts were further diluted in HBS-EP buffer, before being applied to the chip. The result for positive samples ranged from 0.29 to 6.8 ppm conalbumin. The results were compared to those obtained by analyzing the same samples for another egg protein, ovalbumin, by RIE. All samples positive with the RIE were also positive in the SPR assay. Due to the low level of ovalbumin in the pasta samples, near or below the quantification limit of RIE, a quantitative comparison between the RIE and the SPR assay was not possible.

Sesame seed assay

Antiserum to purified sesame seed proteins was produced in a rabbit. The antiserum achieved was not mono-specific towards a single sesame seed protein, but showed reactivity to several proteins with molecular mass ranging from 10 up to 70 kDa. The dominating bands had a molecular mass of 21 and 30 respectively, as revealed by immunoblotting. The antiserum was immunoaffinity purified on a HiTrap™ column, coupled with the protein. Analysis by SPR in an indirect (sandwich) assay showed high sensitivity and selectivity for sesame seed proteins. Sesame seed protein could be detected

Table 9.2 Comparison of four different antibody-based methods for peanut protein analysis

Sample	Peanut butter	Chocolate	Calculated amount of peanut protein (ppm)	RIE peanut protein (ppm)	SPR immunoassay peanut protein (ppm)	BioKits™ EIA peanut protein (ppm)	Ridascreen EIA peanut protein (ppm)
1	0.5 g	9.5 g	12 500	13 000			
2	0.1 g	9.9 g	2500	3250			
3	0.05 g	9.95 g	1250	1630			
4	0.025 g	9.975 g	625	740			
5	0.013 g	9.9875 g	310	350		226	
6	0.0062 g	9.9938 g	150	70	75	133	80
7	5 g of sample 6	5 g	75		38	53	42
8	5 g of sample 7	5 g	37.5		17	26	19
9	1 g of sample 8	5 g	3.75		1.0	2.35	1.7
10	1 g of sample 9	5 g	0.375			0.125	

at a level of 0.125 μg mL^{-1}, corresponding to 12.5 ppm in solid food in both a direct assay as well as in a sandwich assay. In comparison, the immunoblotting technique used could detect 8 ppm sesame seed protein in purified solution. However, the latter technique is quite laborious and time-consuming. It is only semi-quanitative. The established RIE, based on the precipitation between the antigen and the corresponding antibody, gave very diffuse and faint staining rockets with the antiserum produced and was regarded as not suitable for the purpose of analyzing sesame seed protein in food samples.

Tropomyosin assay

Recently, crab tropomyosin was purified and antisera raised in a rabbit as well as in eggs (egg yolk) by immunizing two hens. The rabbit IgG as well as the IgY from egg yolks was immunopurified on a HiTrap™ column, coupled with tropomyosin. Rabbit IgG or hen IgY was coupled to CM3 chips according to the established procedure. The chips were tested both in a direct and in a sandwich assay with different solutions of tropomyosin, with crab, shrimp and model foods, composed of surimi containing different amount of crab meat. The lowest concentration detected in a tropomyosin solution was 0.2 μg mL^{-1}. In model products, a level of 0.063% crab meat in surimi could be detected, corresponding to a tropomyosin concentration of 13 ppm tropomyosin in solid food. The response both in the direct as well as in the sandwich assay was virtually the same. By using IgY bound to the surface and rabbit IgG as the secondary antibody, the non-specific binding to the chip surface was reduced.

9.6 Conclusions

The SPR technique has proven to be a useful tool for the analysis of allergens in food. Analysis of six important food allergens has been demonstrated. SPR provides non-destructive detection that requires no labeling or any other type of modification of the analytes detected. This gives unsurpassed control during assay development and routine analysis, not available in established methods of food allergen detection. Limits of detection in the low ppm level can be obtained and with the sandwich format, very specific assays can be achieved.

The main advantage of the SPR methodology is the short analysis time per sample. This is important for large series of samples and for real-time process monitoring. In the future, SPR analysis of food allergens may be extended to analyze several allergens in the same extract simultaneously by injection over several surfaces immobilized with antibodies raised against different species.

9.7 Sources of further information and advice

- More information on SPR analysis in general, and food analysis in particular, can be found on the Biacore company homepage www.biacore.com/food.
- FoodSense was a EU sponsored project with the goal of exploring the capability of SPR biosensors in the food industry. More information on the FoodSense project can be found on the project's homepage www.slv.se/foodsense

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10

The use of lateral flow devices to detect food allergens

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10.1 Introduction

In recent years, the number of solid phase-based immunoassays available has further increased. Most of these are enzyme-linked immunosorbent assays (ELISAs). Among the newly introduced tests, a significant number have been developed either to identify the presence of allergenic substances in e.g. food preparations, such as peanuts or hazelnuts in cookies, or to measure in sera the antibodies (IgE) that patients may have developed in response to repeated exposure to such allergens. These types of tests show a very high specificity and a high sensitivity.

In general, an analytical methodology is sought where the detection of offending foods or allergenic proteins needs to be managed with high specificity, sensitivity and rapidity. In the following, typical demanded characteristics for the methodology are listed.

- **Sufficient specificity** – should allow the detection of the analyte in question in a wide variety of matrices including processed foods.
- **Satisfactory sensitivity** – detection in the range of 1–5 mg/kg is necessary.
- **Rapid detection of contamination** – short reaction time for correction before large volumes of mislabelled products have been made or to give the consumer a yes/no decision within an adequate time span.
- **Ease-of-use** – no specially trained employees needed or less experienced persons can perform the assay.
- **For industrial purposes** – continuous sampling could be advantageous.

Most of the immunochemical methods mentioned above are considered rapid, but there is still great demand for easy-to-use immunochemical tests

for the detection of traces of allergenic proteins in food. The faster commercial ELISA kits for allergens on the market take only 30 minutes, and some of them (qualitative determinations) do not require a high level of expertise, as they have dropper bottles and visual comparisons. However, the more sensitive, quantitative tests take a trained technician to carry them out; the desire to improve on speed and ease-of-use, especially in non-research settings, has prompted researchers to look for faster and simpler procedures. Thus, new rapid test platforms have been developed, such as the chromatographic test strip (lateral flow test). This is widely used for example in so-called 'home tests', such as hCG measuring pregnancy tests, which do not require assistance from trained technicians, but can be carried out by the consumers themselves. The first generation of such tests was more or less based on variations on the classic 'dot blot' test. From the experience gained with these early rapid assays, much was learned regarding attainable sensitivities, and suitable read-out systems. However, they did not take full advantage of the increased kinetics, due to close contact of high concentrations of the analyte with antibody. More recently, a second generation of immunomigration media has been developed which exploit the micropores in nitrocellulose or nylon membranes. The success of this new generation of so-called 'lateral flow' tests is based on the fast flow of fluids running through the membrane enabling the application of various immunoreactants at different locations along the membrane strip. There are currently four dipstick-type tests commercially available, two for detection of peanut and two for detection of gluten – these will be discussed later in the chapter.

10.2 Antibodies

10.2.1 Antibody requirements

There is more to designing one-step tests than meets the eye. Components proven to be useful in ELISA are not necessarily adequate for one-step tests. The environment in which they have to bind in ELISA is totally different from that in one-step tests – in the latter all components are acting simultaneously and within a very short time-frame. Thus, selecting antibodies for a one-step test requires their affinities to be sufficient and yet mutually tolerant so that they will allow each other to participate in forming a visible complex within the short time that they flow across the test and control lines. Also, it is more critical that they do not compete for the same epitopes. In addition, gold labeling has a greater interference with antibodies, where binding affects overall charge and secondary and tertiary structure more than labeling with enzymes or biotin. As an example, rabbit antibodies do not retain gold for more than a few days, while gold-labeled goat antibodies are very stable.

10.2.2 Subclass influences

Suitable antibodies can be found among all IgG subclasses. In contrast, IgM-type antibodies may be suitable as capture antibodies on the test or control lines (see Section 10.3 and Figs 7.1 and 7.3); however, attaching gold label to them is cumbersome and, due to their pentameric structure, they tend to envelope the allergen tested, preventing their capture at the test and control lines.

10.3 Constructing a lateral flow device (LFD)

A lateral flow test comprises five different basic components.

1. **Sample filter**. The sample filter is a paper-like material with two main functions: filtering out any solid material (food or serum particles), and buffering the sample after extraction/pretreatment (see Fig. 10.1).
2. **Conjugate pad (gold pad)**. The conjugate pad is a fibreglass-like pad. This can either be sprayed by, or bathed in, gold-, latex- or carbon-conjugated solutions:

   - spraying gives a well-defined, reproducible amount applied per mm^2;
   - bathing gives a non-controllable amount applied per mm^2, which results in variable intensities of both test and control lines.

   The conjugate particles can be of various sizes (5–200 nm), which will influence test sensitivity.
3. **Membrane**. The membrane is composed of nylon or nitrocellulose, usually glued upon a backing material (made of polypropylene, polyethylene or

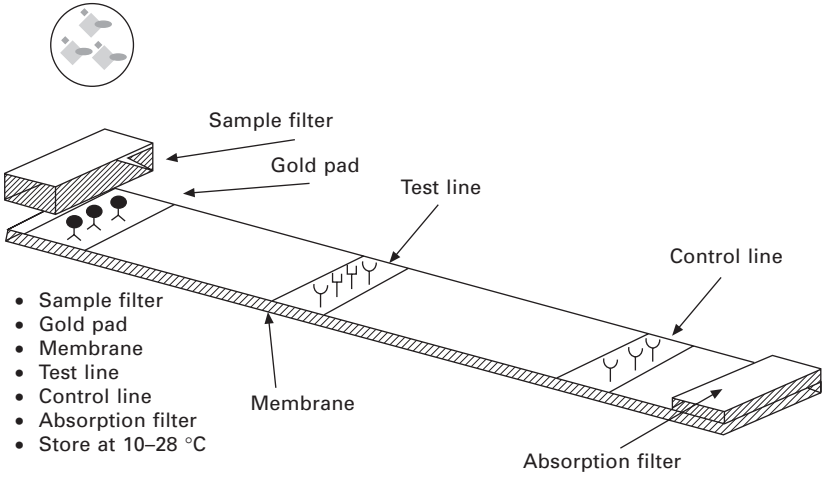

Fig. 10.1 Components of the one-step assay (store at 10–28 °C).

polystyrene). These membranes are made to display a maze (fishnet) structure with various pore sizes. The size of the pores will influence both the speed and sensitivity of the test.

4. **Reservoir (absorption pad)**. The reservoir has only one purpose: to adsorb the liquid at the end of the strip. It is usually made of paper-like material. The material can basically be any material with high wetting capacity. The adsorption capacity of the reservoir causes the sample to continue to flow over the nitrocellulose or nylon membrane.
5. **Test line and control lines**. The test and control lines are sprayed onto the nitrocellulose/nylon membrane with an instrument delivering a precise volume per mm^2. The system and speed used determine the quality of the lines.

10.4 Running a sample

A sample, suspected to contain the analyte in question (extracted or not), is applied to the sample filter, housed inside the device casing. The sample runs through the *sample filter* and *conjugate pad*. This conjugate pad contains the affinity-purified and labelled (gold/latex/carbon) antibody, specific for the analyte under test. The analyte, if present, will form a complex with the conjugate and migrate further along the membrane to the *test zone*. This test zone contains an immobilised antibody, specific for the analyte, but preferably not competing with the conjugated antibody for the same or adjacent epitopes. This *test line* will thus capture the migrating analyte–conjugate complex. The intensity of the test line correlates well with the amount of analyte in the sample.

Thus these tests can also be read semi-quantitatively by use of reader equipment, based on change-coupled diode (CCD) cameras and analysing software. These readers use calibration curves to calibrate and calculate the concentration of the analyte in the sample. To ensure that the test has run to completion a control system is included in it. This will normally be a control line, immobilised at the end of the membrane (*control zone*), usually composed of anti-conjugate antibodies, e.g. in the case where mouse monoclonal antibodies are used as the basis for the conjugate, antibodies to mouse immunoglobulins will be used for the control line at the end of the strip.

In general, the following results will be obtained (Fig. 10.2):

- **positive result** – two coloured lines will become visible;
- **negative result** – only one, the control line, will become visible;
- **invalid result** – all other possibilities (no line, only test line).

In this type of test several test formats can be used (Fig. 10.3):

- **antigen detection** – measuring the presence of the analyte as described above;

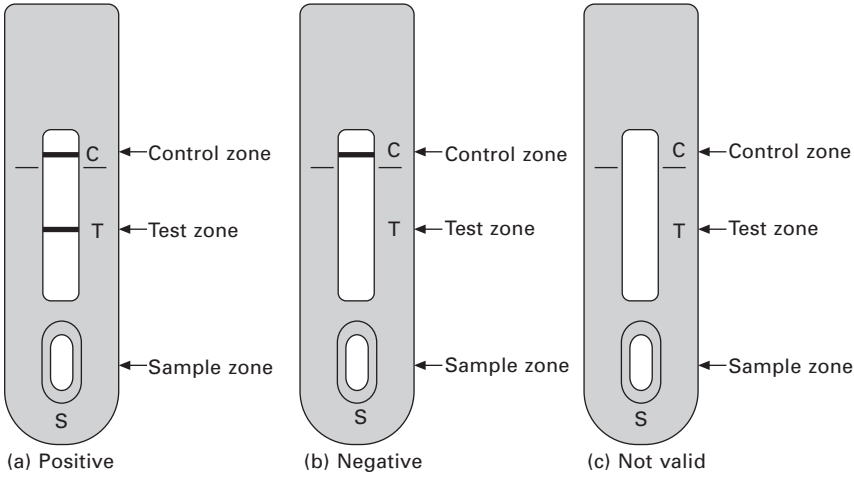

Fig. 10.2 One-step assay results (in general).

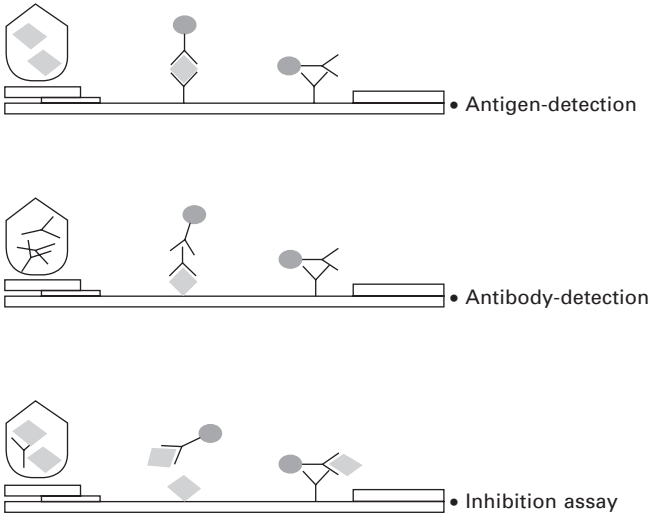

Fig. 10.3 Assay principles.

- **antibody detection** – measuring the antibodies reacting with a certain antigen (e.g. IgE against peanut or wheat proteins);
- **inhibition assay** – usually used to detect small analytes (hormones, pesticides), but proven useful in some cases for larger molecules like allergens. In this type of test only one control line means a positive result (the analyte is present in the sample and blocks the reaction), two lines means a negative result. Some commercially available LFDs for allergenic residues use this format.

10.5 Methods available

At the time of writing, there is little information and published data to be found concerning commercially available dipsticks for use in the food industry. In Europe, the ability to detect peanut residues is in great demand. With the combination of rabbit anti-conarachin IgG, biotinylated rabbit anti-conarachin IgG, avidin-horseradish peroxidase and a paddle-type dipstick, a rapid test was achieved with a detection limit of 0.01% of unprocessed peanut in marzipan (Mills *et al*., 1997), which corresponds to a detection limit of 0.0007–0.001% of conarachin. A commercially available LFD is the Gluten Home Test Kit, a dry-strip immunochemistry format from Tepnel Biosystems Ltd (Deeside, UK) for the qualitative determination of ω-gliadin. Detection limits of 50–200 ppm were claimed to be reached in cakes for example and 200–1200 ppm in highly-processed flour. Also, R-Biopharm AG (Darmstadt, Germany) has an LFD device (RIDA®QUICK Gliadin) for gluten detection that has a detection limit of 5 ppm gluten (corresponding to 2.5 ppm gliadin).

Additionally, two LFDs for the detection of peanut residues were launched in 2004, the first by Neogen Corporation, Lansing, MI (Reveal® for Peanut), followed some months later by Tepnel Biosystems (BioKits® Rapid Peanut). Both tests work according to the single-step lateral flow immunochromatographic principle. The Tepnel method uses antibodies against conarachin and the Neogen antibodies are directed against multiple peanut proteins. In terms of detection limits or working ranges, both tests refer to a low ppm level. The Neogen Reveal® test screens samples at 5 ppm peanut and can be used for all foods, rinse waters, etc., whereas the instructions for the Tepnel method indicate that the test can be conducted in defined matrices – milk and dark chocolate, cereals, biscuits, ice cream and nuts. Although much academic research is currently directed towards the development of dipsticks for the determination of allergenic residues, only the few mentioned above are commercially available. Even though fast test systems make it a lot easier to monitor adulteration and unintentional inclusion or contamination, with the exception of the gluten 'home' test, they should be restricted to manufacturing/industrial use or food labelling enforcement testing – this is also indicated in the test kit inserts. It is not the test itself or the handling which make at home or restaurant use risky; rather, it is problems of accurate sampling and efficient extraction of the protein in question from food that make this infeasible.

10.6 Future trends

Many commercial systems that allow fully automated testing, mostly based on ELISA, are already on the market and commercial LFDs for allergenic residue detection are now starting to appear. The use of new recombinant

antibodies and molecular imprinting techniques will improve the sensitivity and versatility of the technique.

In summary, immunoassays are in general specific, sensitive and rapid methods which enable the detection and quantification even of trace amounts of allergens in raw materials as well as in intermediate and finished food products, the success of which always depends on how the antibodies were raised. However, routine application is still limited to the detection of only some of the allergenic residues of concern. Only a few test kits have been validated in third-party ring trials (AOAC, 2003). Moreover, standardised tests according to CEN (European Committee for Standardisation) are completely lacking. There is still a great need for cost-effective screening methods which can rapidly detect ppm amounts of allergenic residues.

There are three main reasons for using lateral flow testing devices:

- in order to test outside the laboratory (food processing line, rinse waters, home testing);
- when results have to be reported quickly;
- if a qualitative result is desired, LFDs are preferred because they are packaged individually and so there is no need to use an entire strip of micro-wells for just a few samples.

However, in some cases LFDs are not suitable, for example, if quantitative results are required or if an extract is too viscous to be processed as is the case with cereals or food gums. Here ELISA remains the method of choice.

In the future, these single-step tests will be used more and more in allergen control programs, such as in validating sanitation procedures (line cleaning in food production), checking for absence of contaminants in raw materials and in-process products and, in the clinical arena, checking for presence of diseases (doctor's office/airports/hospitals).

10.7 References

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Part III

Detection methods for particular allergens

11

Methods for detecting peanuts in food

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11.1 Introduction: peanut allergy

Peanuts are the most allergenic food known and, in sensitive individuals, can cause adverse reactions ranging from mild urticaria to life-threatening anaphylaxis (Yunginger *et al*., 1988; Bock and Atkins, 1990; Sampson *et al*., 1992). The estimated prevalence of food allergy in the population is estimated to be 4% (Sampson, 2004), while the number of people allergic to peanuts and tree nuts has been estimated at 0.6% in the US (Sicherer *et al*., 1999) and 0.48% in the UK (Emmett *et al*., 1999). A recent report indicated that peanut allergy doubled in the years between 1999 and 2003 in children in the US (Sicherer *et al*., 2003). While the threshold level necessary to cause a reaction is not known with certainty, minute amounts may trigger anaphylaxis (Hourihane *et al*., 1997; Wensing *et al*., 2002); most studies to date have shown that low mg amounts (1–3 mg) of peanut protein are sufficient to cause objective reactions in peanut-allergic subjects (Taylor *et al*., 2002; Morisset *et al*., 2003). Therefore, trace amounts of undeclared peanut present in food can be hazardous to peanut-allergic individuals if consumed. Persons suffering from food allergy carefully avoid consuming their allergenic food, but inadvertent ingestion still occurs. Bock *et al*. (2001) found that peanut was responsible for more than half of 32 food anaphylaxis fatalities they investigated, and it is recognized as the most common cause of food-induced anaphylactic death (Ewan 1996; Burks *et al*., 1999).

11.2 Allergenic peanut proteins

Peanut seeds contain an average of 29% protein (Freeman *et al*., 1954). Approximately 20% of the total protein can be attributed to the major allergen Ara h 1 (vicilin-like protein, molecular weight 63–64 kD), and about 10% is made up of another major allergen Ara h 2 (conglutin-like protein, molecular weight about 17 kD) (Burks *et al*., 1998; Koppelman *et al*., 2001). Ara h 2 is emerging as the most important allergen in terms of a link to severity of reactions and number of peanut-allergic individuals that possess IgE for it (Clarke *et al*., 1998; Koppelman *et al*., 2004), but other allergens in peanut include Ara h 3 and 4 (glycinin proteins) (Bannon *et al*., 2000; Koppelman *et al*., 2003; Scurlock and Burks, 2004), Ara h 5 (profilin), 6 and 7 (conglutin-like proteins; Scurlock and Burks, 2004), and Ara h 8 (Mittag *et al*., 2004). There are several excellent overviews on peanut allergens that the reader can consult for a full review (Burks *et al*., 1998; Sampson, 2004; also Chapter 2).

The major peanut allergens have been found to be resistant to food processing techniques and stable to digestion (Burks *et al*., 1998). Koppelman *et al*. (1999) showed that roasting had no effect on allergenicity of Ara h 1, while Malecki *et al*. (2000) found in another study that roasting enhanced allergenicity of Ara h 1. A key factor in the success of immunodetection methods for peanut residue is how well animal-derived antibodies recognize roasted/heat-treated peanuts, since these are the most common forms consumed.

11.3 Peanut detection methods

Several types of methods have been used over the years to detect peanut residue in other foods, including radioallergosorbent assay (RAST) inhibition, IgE immunoblotting, rocket immunoelectrophoresis (RIE), enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) methods. These methods are described more fully in other chapters of this book.

11.3.1 Rocket immunoelectrophoresis (RIE)

Rocket immunoelectrophoresis was first used by Barnett and Howden in 1984 (Barnett and Howden, 1984) to detect heat-treated peanut protein, and this marked the first time an immunological method was used to detect peanut protein. The authors at the time stated that the results of their work 'may extend the usefulness of immunological techniques to include the detection of peanut protein in processed foods.' They used RIE to detect peanut protein in a vegetarian meat loaf, but no levels were reported. Rocket immunoelectrophoresis was also used by Malmheden-Yman *et al*. (1994) to detect the undeclared presence of peanuts in a small number of food products. The detection limit of the method was approximately 30 ppm, and no cross-

reactivity evaluations were reported. Holzhauser *et al.* (1998) a few years later used a commercially available anti-raw peanut antibody in developing a RIE method and were able to obtain a 10 ppm detection limit. The method was optimized for the detection of peanut in chocolate, achieving recoveries of 85–101% in this matrix. More than 20 types of foods were analyzed in cross-reactivity studies, with no observed issues. Successful use of a commercial antibody and also attaining a 10 ppm detection limit in a difficult matrix like chocolate were significant achievements of this work. One disadvantage of this technique was that the amount of antiserum used in the gel has to be optimized; this is dependent upon whether or not the sample contains a large or small amount of peanut, details of which are seldom known in advance. The investigators also performed a limited survey of 11 commercial food samples that did not declare the presence of peanut as an ingredient but four of which contained appreciable amounts (0.0006–1.5% peanut). Notably, these investigators used model chocolates with specific amounts of peanuts manufactured into them under industrial conditions, an approach that shows the true extraction efficiency of a method, as compared to simple 'spiking'.

11.3.2 Radioallergosorbent assay (RAST) inhibition

The first report of RAST inhibition being used to detect peanut contamination of a non-peanut-containing food was described by Yunginger *et al.* (1983). The technique was used to document the presence of peanut butter in sunflower butter (likely made on shared production lines) in investigating a reaction in a peanut-allergic consumer. The investigators found peanut butter at levels ranging from 0.3–3.3% (3000–33 000 ppm) in six of eight samples of sunflower butter from one company. The protein used on the solid phase and for standards was a crude extract of raw unsalted peanuts.

This method was expanded upon by Keating *et al.* (1990). In contrast to the work discussed above, a roasted defatted peanut meal was used for the solid phase and standards in a RAST inhibition method. However, test samples required a long extraction and handling process, including lyophilization. The investigators reported that their recoveries for peanut protein were 31–94% and the detection limit was 87.5 ppm peanut; most experts agree at this time that allergen detection methods should have an ability to detect down to at least 10 ppm. These investigators also showed that peanut allergens can be transferred to clean vegetable oil after roasting, an important point that has not been studied by any other group to date. While one case report has linked transfer of allergens in cooking oil to a fatality in a fish-allergic person using RAST (Yunginger *et al.*, 1988), this area will remain a concern until more work is done to demonstrate the true risks from this type of processing.

11.3.3 IgE-immunoblotting

An IgE-dot-immunoblotting method using peanut-allergic sera was described by Schappi *et al*. (2001) to detect the presence of peanut proteins in foods. The standards were made from roasted peanuts. The detection limit was approximately 50 ppm, too high for practical allergen management. The use of human sera and of urea in the extraction procedure limits the practical use of this type of method in food-processing facilities. The authors found that 19 of 46 commercial food products had undeclared peanut residues, ranging from 0.05 – >1% peanut (500 ppm – >10 000 ppm).

While IgE-based techniques such as RAST and enzyme allergosorbent test (EAST inhibition) and IgE-immunoblotting are useful techniques that can be used in many clinical and research settings (see Chapters 4 and 5), it must be stressed that they require well-characterized peanut-allergic human sera, a substance which needs special handling, and the use of this reagent is precluded in food processing environments. Also, the specificity of IgE from allergic individuals can vary considerably, making standardization difficult.

11.3.4 Enzyme-linked immunosorbent assays (ELISAs)

The first ELISA method for detection of peanut residues was described by Hefle *et al*. (1994a). The method used monoclonal antibodies against selected peanut proteins with molecular weights [in sodium dodeyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)] of 14–44 kD (Hefle *et al*., 1994b) as capture antibody and a polyclonal anti-peanut rabbit antibody as detector. The rabbit antibody was raised against a crude roasted peanut extract. The method could detect < 40 ppm peanut in most food matrices. Dry-roasted peanut was spiked into vanilla ice cream at levels of 0.01–5.0%, then tested in the ELISA, but also skin prick tested in seven peanut-allergic subjects. In 6/7 patients, the skin test was more sensitive than the ELISA at the lowest levels tested (0.01% = 100 ppm). The samples were also analyzed by RAST inhibition using the sera from the seven peanut-allergic individuals. The ELISA and the RAST inhibition results were proportional and correlated well (r value = 0.95). This new method opened the door for other ELISA formats with increased sensitivity (Poms *et al*., 2004). These authors suggested that the use of amplification systems, improved antibodies, and assessment of food matrix interference problems could result in increased sensitivity, and this transpired in the form of many ELISA methods for peanut being developed and published since the mid-1990s.

In 1996, both Yeung and Collins (Yeung and Collins 1996) and Koppelman *et al*. (1996) published ELISA methods for detection of peanut proteins in foods. Yeung and Collins developed a competitive format ELISA using a rabbit polyclonal antibody raised against a mixture of defatted, roasted, raw, denatured, and unfolded raw peanut. Their 'denatured' raw immunogen was prepared by treatment with sodium dodecyl sulfate and dithiothreitol at 4 °C for 60 minutes. In the described ELISA, roasted peanut was used to coat the

plate, and the authors indicated that they could store coated plates for 24 months. Yeung and Collins also spiked food samples at 2.5, 5, 10, and 20 ppm; the spiking resulted in observed recoveries of 68–90%. They reported no cross-reactivity but tested samples for this in very low protein amounts (< 40 ug/mL). They found that their rabbit anti-roasted peanut antibody produced the most effective ELISA. They analyzed 15 non-peanut chocolate bars and three types of sesame crackers and found that none of them had undeclared peanut residues. This ELISA method reportedly had a detection limit of 400 ppb.

Koppelman *et al*. (1996) described a sandwich-type ELISA using rabbit polyclonal antibodies against partially purified Ara h 1 derived from raw peanuts. This sandwich ELISA utilized the same antibody for detector and capture; part of the antibody was labeled with horseradish peroxidase to be the detector antibody. In studies, it was observed that fried peanuts were somewhat less reactive in the ELISA, but the method did not show cross-reactivity with the majority of legumes and other foods tested. The highest concentration of almond and cashew tested (500 ug/mL) showed a slightly positive signal, but a ten-fold dilution removed it. Recoveries in various matrices ranged from 35–75%. The investigators also found that of 25 non-peanut foods surveyed, none had detectable peanut content. The assay was capable of measuring raw or roasted peanut, and the reported detection limit was 0.1 ppm.

Much later, Pomes *et al*. (2003) published another sandwich ELISA based on monoclonal antibody against Ara h 1. In this study, Ara h 1 could not always be recovered in spiked chocolate samples nor always detected in the presence of chocolate. However, it was found that in general, Ara h 1 content correlated with a commercial ELISA method (Veratox® for Peanut – Neogen Corporation, Lansing, MI), but the results were not compared to any other commercial ELISA kits. The investigators 'spiked' ground chocolate with ground peanut flour, but without using industrially manufactured standards, the true extraction efficiency of the method cannot be truly ascertained. The best extraction of Ara h 1 from chocolate was found to be with 5% non-fat dry milk in phosphate-buffered saline for 2.5 hours at room temperature, an approach that is too long for the rapid testing needs of the food industry, but may be applicable elsewhere.

Keck-Gassenmeier *et al*. (1999) used a commercial sandwich-type ELISA kit for peanut (Cortecs, now Tepnel Biosystems, Deeside, UK) and found that adding 12.5% fish gelatin to the extraction buffer allowed recoveries in chocolate of 60–90% and other food products of 48–88%; foods other than chocolate in most cases did not require the use of the fish gelatin for improved recoveries. A detection limit of 2 ppm could be obtained in chocolate. The commercial test was qualitative only, but the investigators could make it semi-quantitative by making their own standards; dark chocolate was melted and then spiked with peanut butter. Cross-reactions were only assessed for almonds, hazelnut, and milk and dark chocolate. Raw peanuts were found to

have a higher response in the assay. The detection limit of the modified assay for detection of peanut proteins in dark chocolate was better than that claimed by the manufacturer for the commercial kit.

Newsome and Abbott (1999) coupled an immunoaffinity column to capture peanut proteins from foods, with a subsequent ELISA. The antiserum used was raised against roasted peanuts. The antiserum was coupled to a commercial gel preparation and they then did an ELISA according to the protocol of Yeung and Collins (1996). The authors said that spiked chocolate gave recoveries of 72–84%, and that the minimum detection limit was 0.1 ppm, but they did not provide many details on the spiking.

Holzhauser and Vieths (1999) also developed an indirect ELISA method using commercial anti-raw peanut rabbit antisera (absorbed to remove possible soy reactivity) and ground roasted peanuts as a standard. This method was reported to have a detection limit of 2 ppm and showed no cross-reaction concerns with the food ingredients tested. In five of 17 commercial food products not declaring peanut, levels of peanut ranging from 2–18 ppm were found.

Later, the same research group (Stephan and Vieths, 2004) reported both a sandwich ELISA and a PCR method for detection of peanut residues. Both sheep and rabbit anti-peanut antibodies were used in the development of the ELISA and, while the authors did indicate that the antibodies were raised against peanut extract, it was not reported whether the peanuts used were raw or roasted. The methods were developed primarily for confection products, and so cross-reactivity with some types of food ingredients, such as spices, was not done. Industrially-manufactured standards of 10, 40, and 200 ppm peanut were made in semi-sweet and whole milk chocolate. For the sandwich ELISA, recoveries from whole milk chocolate were 80.6–141.9%, and for semi-sweet, 64.3–110.9%. Fish gelatin was added to the extraction buffer as recommended by Keck-Gassenmeier *et al*. (1999); however, interference still was observed with chocolate even when this was employed. In a survey of 33 products, two were found to have undeclared peanut residue, in the range of 6.3–74 ppm.

A cloth-based immunoassay was described by Blais and Phillippe (2000). The method utilized polyester cloth as the solid phase and the capture and detector (peroxidase-labeled) antibody was chicken anti-peanut IgY. Peanut immunogen prepared according to the Yeung and Collins (1996) method was used to immunize chickens, followed by a fairly lengthy purification of IgY from egg yolks. The authors felt that the cloth solid phase provided the advantages of conferring a high surface area for rapid immunoreactions and ease of washing between steps. Food samples connected with consumer illnesses were analyzed using the cloth-ELISA; of 11 samples not declaring peanut, nine contained peanut residues and were positive in the cloth-ELISA. Levels were reported to be 1.2–116 ppm (determined using the Yeung and Collins (1996) quantitative ELISA method). Cross-reactivity data was not shown; chocolate seemed to give significant interference in the method. The

investigators found that skim milk powder alleviated the problem, but some samples were positive when analyzed in a commercial peanut kit but not detected using the Yeung and Collins (1996) method – however, results from the cloth-ELISA and the commercial peanut kit did match. The authors reported that the detection limit of the cloth-ELISA was less than 1 ppm peanut protein for some matrices.

As an outgrowth of Koppelman *et al*.'s (1996) work (discussed above), R-Biopharm AG (Darmstadt, Germany) has developed a faster version of their peanut ELISA assay (RIDASCREEN®), which can be completed in about 30 minutes (Immer *et al*., 2004). Other commercial kit companies have also reduced the assay time of their peanut ELISA peanut kits to a similar level.

11.3.5 Dipsticks/lateral flow methods

The first peanut dipstick method was described by Mills *et al*. (1997). Dipstick or lateral flow methods (described in Chapter 10) are only semi-quantitative or qualitative in nature. In this study, polyclonal rabbit antisera were raised to conarachin, the 7S globulin of peanut, which had been isolated from defatted raw peanuts. The same serum was used as both capture and detector antibody (detector was labeled with biotin). No cross-reactions among several foods and food ingredients were noted but, since the antibody was made against a purified protein, no cross-reactions were necessarily expected. The detection limit was reported as being 100 ppm in marzipan and 1000 ppm in chocolate; roasted peanut could be detected down to 1000 ppm in marzipan and chocolate. The format used, that of using cryotubes coated with antisera, limited matrix interference issues with confection products. Only one food product of six tested gave a positive result in the study; roasting did not appear to affect the assay a great deal. The detection limit of this assay (0.01% of raw peanut in marzipan) is much too high for allergen concerns.

Stephan *et al*. (2002) developed a dipstick method for the detection of trace amounts of peanut and hazelnut in foods. The reported detection limit was 1 ppm protein. These tests took 3–4 hours, which is long for dipstick methods (the current commercial dipsticks on the market are performed within 10 minutes). Various food samples were tested using the dipstick and results were compared with a previously described validated quantitative ELISA for peanut (Holzhauser and Vieths, 1999). Dipsticks are a useful tool for rapid and convenient testing of food and environmental samples. The investigators reported some cross-reactivity with some food ingredients with this method (walnut in particular). Also employed was the use of 10% fish gelatin in the extraction buffer, which improved the extraction of the peanut from chocolate-containing foods. Of 28 foods examined in this study that did not declare peanut on their labels, eight tested positive for residues. There are two commercial lateral flow devices on the market for peanut, made by Neogen (Reveal®) and Tepnel Biosystems (Biokits® Rapid Peanut

Test), and other diagnostic kit companies are planning to produce kits in this type of format also.

Two multiplex methods have been described, based on earlier work by the authors. Ben Rejeb *et al*. (2002) developed a multi-analysis semi-quantitative ELISA for the detection of peanut, almond, cashew, Brazil nut, and hazelnut. The reported detection limit was 2 ppm for all allergens. Blais *et al*. (2003) continued with their use of IgY and cloth in a multiple immunoassay method (as described above) for peanut, Brazil nut, and hazelnut. The claimed detection limit for peanut for this multiplex method was 0.1 ppm. The multiplex approach offers the advantages of simple and rapid testing for various allergens simultaneously, allowing for an initial preliminary screening to identify which allergenic residues are present, to be followed up with a confirmatory test, such as a specific ELISA method. The practicality of and need for this type of approach for food manufacturers have yet to be evaluated fully.

In the only study to date addressing simply chocolate contamination, Vadas and Perelman (2001) performed a limited survey of retail chocolate bars using the Neogen Veratox® for Peanut test kit; results showed that eight of 46 samples contained undeclared peanut residues, ranging from 7.9–69.6 ppm peanut.

11.3.6 Comparisons/validation of commercial peanut ELISA kits

Hurst *et al*. (2002) compared commercial test kits for peanut on samples of dark and milk chocolate. Dark and milk chocolate samples were made in the levels of 0, 10, 40, and 200 ppm peanut, although details of how this was done and how homogeneity was determined were not disclosed. Four commercial test kits for the detection of peanut – Neogen Veratox®, Pro-Lab Prolisa Peanut Pak® (Pro-Lab Diagnostics, Richmond Hill, Ontario), Tepnel Biokit®, and R-Biopharm RIDASCREEN®) were evaluated using the chocolate samples. In general, the kits varied in their ability to quantitate the levels of spiked peanut. However, with no details given on the nature of the peanut material used to make the spiked chocolate, etc., it is difficult to truly compare one kit to another. In addition, refinements have been made to most of these kits since this work was published.

In one study, Koch *et al*. (2003) compared spiked samples using some commercial peanut ELISA kits and IgE-immunoblotting using peanut-allergic sera. Samples of peanut-free cookie matrix were spiked with various levels of ground raw or roasted peanuts. The protein extractions for blotting were done with urea, a powerful disrupting agent. However, when samples were analyzed in the commercial ELISAs, manufacturer instructions were followed, and none of these include urea in their extraction buffer. Dot blotting was used to estimate the amount of peanut in the samples, whereas IgE-immunoblotting was used to analyze the extracts further and identify specific peanut allergens. The levels of peanut spiked ranged from 500–2500 ppm, well above allergen concern levels. In addition, extracts of spiked samples

were diluted in extraction buffers to 10 ppm in the comparison of the three kits; it must be pointed out that this is not how the kits are designed to be used. The authors made some assumptions that test kit companies used raw peanuts as immunogen to explain their results showing that raw peanuts were detected at higher levels, but in fact some kit manufacturers use roasted peanuts as immunogen for producing their antibodies. The authors also concluded that roasted peanuts were less detectable than raw peanuts in commercial ELISA kits, but only used one of the three commercial kits to test this hypothesis. A direct comparison of IgE-blotting using urea extraction and use of commercial test kits (with their non-urea extraction methods) casts some doubt on the validity of some of the conclusions made in this study.

The Association of Official Analytical Chemists (AOAC) – Research Institute and the US Food and Drug Administration has undertaken a performance evaluation of three (Neogen Veratox®, Tepnel Biokits®, and r-Biopharm RIDASCREEN®) commercial peanut quantitative ELISA kits; matrices of breakfast cereal, cookies, ice cream, and milk chocolate were 'spiked' at levels of 0 and 5 ppm peanut all three kits have passed the performance evaluation (Park *et al*., 2005).

In another effort, the analytical quantitation performance of five different peanut ELISA kits was validated in an international collaborative study by the Joint Research Centre of the European Commission. The study involved industrial dark and milk chocolate samples containing peanut in the low ppm range (Poms *et al*., 2003, 2005).

Using dark chocolate 'manufactured' to contain known amounts of peanut in preliminary work for a more in-depth study, we evaluated five commercial peanut detection kits. Individual reference dark chocolate standards were made in an industrial setting (Barry Callebaut, St Hyacinthe, Quebec, Canada). Dark chocolate standards containing 0, 0.5, 1, 2, 10, 20, 50, and 100 ppm peanut were prepared. The chocolate was prepared using chocolate liquor, butter oil, cocoa butter, soy lecithin, sugar, and vanilla. Tempered chocolate standards were thoroughly mixed (Hobart Corp., Troy, OH) before molding. Kits were purchased from four manufacturers: R-Biopharm (RIDASCREEN® Peanut); Pro-Lab Diagnostics (Prolisa Peanut Pak®), Neogen Corporation (Alert® and Veratox® for Peanut) and Tepnel BioSystems (Tepnel BioKits® Peanut Assay), and run on the peanut-in-chocolate dark chocolate model food (three trials, triplicates for each trial) strictly according to manufacturer instructions. In general, four of the five kits performed well in their ability to quantitate the levels of peanut in the dark chocolate (Table 11.1), even though their target analytes are different (some are for total peanut, some for peanut protein). However, one commercial kit did not perform well – this kit was purchased twice, six months apart, and the result was the same both times. Many more studies like this one using 'manufactured', real-life model foods is needed, not only for peanut, but also other allergenic foods (see Section 11.6).

Table 11.1 Comparison of four commercial quantitative peanut kits using manufactured peanut in dark chocolate samples

Peanut in dark chocolate	Neogen Veratox® for Peanut	R-Biopharm RIDASCREEN® Peanut	Tepnel Biokits Peanut	Pro-Lab Prolisa Peanut Pak®
0 ppm	< 2.5 ppm	< 2.5 ppm	0.33 ± 0.1 ppm	0 ppm
0.5 ppm	< 2.5	< 2.5	0.93 ± 0.25	0.16 ± 0.02
1 ppm	< 2.5	< 2.5	1.57 ± 0.29	0.11 ± 0.11
2 ppm	< 2.5	< 2.5	2.82 ± 0.38	0 ± 0
10 ppm	12.87 ± 5.6	6.67 ± 0.31	12.1 ± 2.8	0.16 ± 0.1
20 ppm	23 ± 6.1	12.0 ± 0.67	25.1 ± 7.1	0.27 ± 0.1
50 ppm	52.5 ± 16.7	66.4 ± 15.0	63.5 ± 9.7	1.62 ± 1.4
100 ppm	121.0 ± 27.6	117.4 ± 34.2	127.1 ± 28.5	3.65 ± 3.9

11.3.7 Non-immunological methods for detection of peanut

A recent publication by Shefcheck and Musser (2004) describes the detection/confirmation of Ara h 1 in a food matrix using a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method. This technique provides confirmation of allergenic proteins in food systems as a confirmatory test for immunoassays, which has been identified by some regulatory agencies as a significant need. The method can be adapted for use with any well-characterized protein in almost any matrix, and does not require specific antibodies. It does, however, require specialized equipment and training, and it is likely to be best suited to regulatory agencies or academic labs rather than food manufacturers. It is felt that these types of methods could possibly have detection limits approaching 1 ppm protein.

In addition to protein/immunochemical methods, some PCR (discussed in Chapter 7) methods for peanut have been described. Two types of formats are currently commercially available; real-time PCR and DNA-ELISA. Reported detection limits are below 10 ppm. Hird *et al*. (2003) published a real-time PCR method for detection of peanut residues. The amplification consisted of a part of the DNA encoding Ara h 2, a major peanut allergen. Several commercial DNA extraction methods were evaluated. The investigators reported that the method could detect 2 ppm lightly roasted peanut flour spiked into cookies. Raw peanuts were spiked into pastry, sausage, chocolate, or cake at a 10% level (much higher than is of concern for allergen residue detection) and, to attempt to mimic cross-contact, a single piece of peanut was tumbled with these negative food matrices for 30 minutes, then analyzed. The spiked peanut was detected in the foods as was the presence of peanut after being tumbled with the foods. Stephan and Vieths (2004), as noted above, also developed a PCR method for the detection of peanut. A commercial DNA extraction method was used and the primer was also specific for a coding region on Ara h 2. This real-time PCR method was able to detect down to 10 ppm peanut in semi-sweet chocolate and whole milk chocolate. In analyzing selected products for undeclared peanut, the PCR method detected

one more positive sample than their sandwich ELISA did. The authors concluded that PCR and ELISA gave similar results for detection of peanut residues in foods, but pointed out that in some cases, such as pickled foods, oils, and canned foods, the results will not be comparable due to DNA degradation during processing. There are two commercial PCR methods currently on the market for the detection of peanut residues (Table 11.2).

Indirect approaches such as PCR-based methods detect a marker for the allergenic fraction rather than allergen itself. For food ingredients that are only slightly processed, such as peanuts as topping on cookies, these indirect approaches can be useful. However, when food ingredients undergo fractionation steps such as concentration of the protein fraction as is done for defatted peanut meal, indirect approaches run the risk that the outcome of the analytical results could be underestimated. On the contrary, for peanut PCR, results may be overestimated for food products from which the protein fraction is removed, such as refined, bleached, deodorized peanut oils, which are essentially devoid of protein, but can have DNA potentially present.

11.4 Appropriate detection limits for peanut methods

As discussed in Chapter 1 of this book, existing methods appear to be in the ideal range to detect potentially hazardous residues of undeclared allergens in foods. Detection limits based on clinical relevance are important; good manufacturing practices and regulatory limits imposed by governmental agencies should be based on knowledge of clinical elicitation levels. Poms *et al*. (2003) indicates that detection limits for peanut allergens probably need to be 1 ppm or lower, but support for this level is not borne out by double-blind clinical oral threshold studies. Morisset *et al*. (2003), based on their threshold study data, recommends a detection limit for peanut of 24 ppm for foods and 5 ppm for oil. The action level of 1000 ppm put forth by Schappi *et al*. (2001) is certainly not appropriate, and could leave some factions of the peanut-allergic population at great risk. At this time, it appears that methods with detection limits of 1–5 ppm are best for peanut, and there is currently no need to take detection abilities below this level. The currently commercially available methods have detection limits in this range, or somewhat higher (Table 11.2).

11.5 Future trends

With so many peanut detection systems already developed, one particular need in this area is the production of peanut reference materials for multi-laboratory validation trials. This work is of particular importance due to the plethora of types of peanuts and the wide variety of processing methods used

Table 11.2 Commercially available peanut residue detection kits

Company	Method	Name of kit	Type	LOD[1]	LLOQ[2]	Time[3]	Contact information
ELISA Systems	ELISA[4]	Peanut Protein Residue	Quantitative	0.5 ppm	–	30 min	www.elisasystems.net
ELISA Systems	ELISA	Peanut Residue Mass	Quantitative	1 ppm	2.5 ppm	60 min	www.elisasystems.net
Neogen	ELISA	Veratox®	Quantitative[5]	1 ppm	2.5 ppm	30 min	www.neogen.com
Neogen	ELISA	Alert®	Qualitative	5 ppm	–	30 min	www.neogen.com
Neogen	LFD[6]	Reveal®	Qualitative	5 ppm	–	10 min	www.neogen.com
Pro-Lab Diagnostics	ELISA		Quantitative				www.pro-lab.com
R-Biopharm	ELISA	RIDASCREEN® Peanut	Quantitative	< 2.5 ppm	3.3 ppm	1.5 hr	www.r-biopharm.com
R-Biopharm	ELISA	RIDASCREEN® FAST Peanut	Quantitative	1.5 ppm	2.5 ppm	30 min	www.r-biopharm.com
R-Biopharm	PCR[7]	SureFood® Peanut PCR-ELISA	Quantitative	< 10 ppm	–	2–3 hr	www.r-biopharm.com
R-Biopharm	PCR	SureFood® Peanut Real-time PCR	Qualitative	10–50 ppm	–	30 min	www.r-biopharm.com
Tepnel BioSystems	ELISA	*BioKits* Peanut Assay	Quantitative	< 0.1 ppm	1 ppm	1.25 hr	www.tepnel.com
Tepnel BioSystems	LFD	*BioKits* RAPID Peanut Test	Qualitative	Low ppm	< 50 ppm	6 min	www.tepnel.com
Tepnel BioSystems	PCR	*BioKits* Peanut PCR Mastermix Pod	Qualitative	< 10 ppm	–	< 2 hr	www.tepnel.com

[1]LOD = limit of detection; LOD is defined in different ways (whole food or protein); check with manufacturers for specifics
[2]LLOQ = lower limit of quantitation
[3]Time = excludes sample and reagent preparation times
[4]ELISA = enzyme-linked immunosorbent assay
[5]Any quantitative ELISA can be run qualitatively or semi-quantitatively
[6]LFD = lateral flow device
[7]PCR = polymerase chain reaction

Notes: Claims are as specified by manufacturers; all test performance is sample-dependent
Units of measurement are different for different kits; please check with manufacturers for specifics

world-wide. Another important research need is for a comparison of different methods with regard to their ability to detect peanuts in a variety of matrices using industrially-manufactured standards. Simple 'spiking', i.e. putting a peanut extract into a food extract, or peanut flour into a dry food powder, is no longer an appropriate means of assessing performance other than for the purpose of initially probing possible matrix interference. Making model foods according to pilot plant or true industrial conditions is not necessarily an easy task, but some research groups (as discussed in Chapter 17) are doing work in this area, and these materials will be key to assessing peanut detection methods now and in the future. A listing of the currently available commercial peanut detection methods is given in Table 11.2.

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12

Detecting tree nuts and seeds in food

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12.1 Introduction

Food allergy is an increasing health problem, and tree nuts belong to the 'big eight' food allergens related to 90% of the food allergies (FAO, 1995). Tree nut allergens are stable towards food processing and digestion. Similar stable allergens have been identified in several seeds, and both seeds and nuts are often used by the food industry owing to their taste. Food product diversification since the 1990s has led to a vast number of recipes with nuts and seeds. This was further stimulated by the observation that nuts have the beneficial health effect of lowering cholesterol levels (Hu and Stampfer, 1999). Products that do not contain nuts or seeds may be manufactured in facilities that also process nuts and seeds, potentially forming a risk of cross-contact. Traces of these allergens present in assumed nut- or seed-free products have led to several severe allergic reactions. This indicates that methods to detect nut and seed allergens are important tools for the food industry to control their manufacturing and cleaning procedures. This chapter deals with the prevalence of nut and seed allergy, threshold levels of allergens, the types of allergens found in nuts and seeds, the effect of food processing on allergenicity, and with tools available for detecting nuts and seeds. An overview of individual methods will be given as well as a list of commercially available methods that are presently used by the food industry. Since hazelnuts have been used for a long period of time, especially in Western Europe, a lot of effort was put into hazelnut detection. Pros and cons of the hazelnut detection methods will be discussed, and we will look ahead to how detection of nut and seed allergens may help the food industry in the future.

12.2 Prevalence of nut and seed allergies

In 1996, Tariq *et al*. published a cohort study that included 1218 newborns of the Isle of Wight in the UK on the prevalence of tree nut allergy (Tariq *et al*., 1996). 1.2% of the cohort was IgE-positive for tree nuts as demonstrated by skin prick test, and 0.16% reacted clinically. This is in accordance with the study of Sicherer *et al.* who estimated the prevalence of tree nut allergy at 0.5% using a random digit dial telephone survey that included over 12 000 individuals in the US (Sicherer *et al*., 1999). Nut-induced allergic reactions vary from mild, local reactions like oral allergy syndrome, to severe, systemic reactions, and a number of fatal and near-fatal reactions have been described (Yunginger *et al*., 1988; Bock *et al*., 2001). The consumption of nuts differs between the US and Europe, and this may have consequences for sensitization patterns. In Europe, hazelnut allergy is common and often associated with birch pollen allergy (Andersen and Lowenstein, 1978) whereas in the US walnut leads, followed by cashew, almond, pecan, and pistachio (Sicherer *et al*., 1999). Other nuts that are considered as important allergens are Brazil nut and chestnut. Since Brazil nuts are rich in sulfur-containing amino acids, one of its proteins was cloned into transgenic soy in order to improve the nutritional value for animal feed. The fact that this transgenic soy was allergenic for nut-allergic individuals (Nordlee *et al*., 1996) stopped the development and marketing of this crop. A nut that is gaining increasing attention is the pine nut and cases of pine nut-induced allergic reactions have been reported (Beyer *et al*., 1998). Some other, more exotic nuts have been reported to be allergenic, for example the Micronesian nut Nangai (Sten *et al*., 2002), although these cases are rare.

Table 12.1 summarizes the tree nuts involved in allergic reactions. Also included in Table 12.1 are seeds that allergenic. Because of similarities in protein composition, stability, and area of application, allergenicity of tree nuts and seeds are often discussed together. Peanuts are legumes rather than nuts and therefore discussed elsewhere in this book. Sesame seed and sunflower seeds have been reported as causing allergic reactions (Axelsson *et al*., 1994; Kanny *et al*., 1996; Sporik and Hill, 1996; Asero *et al*., 1999) while reaction

Table 12.1 Allergenic nuts and seeds

Nuts	Seeds
Almond	Celery
Brazil nut	Linseed
Cashew	Mustard
Chestnut	Poppy seed
Hazelnut	Rape seed
Macadamia	Sesame seed
Pecan	Sunflower seed
Pine nut	
Pistachio	
Walnut	

to poppy seed, rape seeds, and linseed are less frequently described (Axelsson *et al*., 1994; Kolopp-Sarda *et al*., 1997; Lezaun *et al*., 1998; Frantzen *et al*., 2000; Galloway, 2000). Reactions vary for seeds, as for tree nuts, from mild to severe, with anaphylaxis often described. Interestingly, the majority of studies on the allergenicity of seeds and reports of allergic reactions come from Europe. The European Commission published data on the prevalence of seed allergies in 1998, and seeds were involved 1–2% of food-induced anaphylaxis. There are no studies reporting prevalence of seed allergy in a cohort of randomly selected individuals as has been done for tree nuts.

12.3 Thresholds

A lot of cases of unintentional ingestion of tree nuts and seeds have been described, and traces of nuts and seeds are able to induce allergic reactions. In some cases, the amount of offending allergen has been estimated. However, double-blind, placebo-controlled food challenge studies are required to judge how much allergen is too much (Hefle and Taylor, 2002). For hazelnut, several researchers have investigated threshold in this way. Ortolani *et al*. (2000) described thresholds of 168 mg–1.8 g hazelnut protein (Ortolani *et al*., 2000) and Wensing *et al*. (2002) found thresholds as low as 1 mg hazelnut protein (Wensing *et al*., 2002), indicating a considerable difference between patients. The published thresholds are in line with case reports concerning allergic reaction to hazelnut traces; milligram amounts of hazelnut protein have been found to be enough to induce allergic reactions (Malmheden Yman *et al*., 1994; Wensing *et al*., 2001a). Wensing *et al*. (2001b) showed that the threshold for roasted hazelnuts was higher compared than that for raw hazelnuts (Wensing *et al*., 2001b). This was further investigated by Hansen *et al.* (2003) who found that only five of 17 hazelnut-allergic patients that reacted clinically to raw hazelnut reacted to roasted hazelnut. Differences in threshold levels were not determined (Hansen *et al*., 2003). Table 12.2 gives a brief overview of threshold data for some nuts and seeds obtained using double blind, placebo-controlled food challenges. Note that for most tree nuts and seeds, thresholds have not been established. Considering thresholds in the mg range, and an average food intake of 50 g, as in the case of a chocolate bar, a relevant limit for food allergen detection is in the order of 10 ppm (Taylor and Nordlee, 1996). It should be noted, however, that there may be food-allergic individuals who react to smaller amounts of allergenic food. Clearly, there is an urgent need for threshold data, and the Food Allergy Research and Resource Program of the University of Nebraska (FARRP) coordinates multi-center studies on this topic (Hefle and Taylor, 2002; Taylor *et al*., 2002). A standardized protocol for performing threshold studies has also been developed (Taylor *et al*., 2004). An overview of threshold studies and the progress thereof is described in Chapter 1.

Table 12.2 Thresholds for nuts and seeds determined with double-blind, placebo controlled food challenges

Allergen	Reported thresholds (amount of protein)	Reference
Hazelnut	1 mg–1.8 g	Wensing *et al*., 2002
	168 mg–1.8 g	Ortolani *et al*., 2000
	10–18.2 g (roasted) hazelnut	Hansen *et al*., 2003
	> 30 mg (roasted)	Wensing *et al*., 2001b
Pecan	93 mg	Bock *et al*., 1978
Pistachio	88 mg	Bock *et al*., 1978
Sesame	18 mg–1.8 g	Kanny *et al*., 1996
	18 mg–1.8 g	Kolopp-Sarda *et al*., 1997

12.4 Allergenic proteins in nuts and seeds

It is clear that allergenic compounds in nuts and seeds lie in the protein fraction, as in virtually all food allergies. However, the carbohydrate moieties on proteins may play an important role as well, especially for plant-derived allergens (van Ree, 2002). Allergenicity of nut oils has been described, but the allergen in these oils is residual protein that is present in a concentration that depends on the refining process (Teuber *et al*., 1997; Crevel *et al*., 2000). Since the 1990s, a number of allergens from nuts and seeds have been identified using IgE-immunoblotting combined with N-terminal protein sequencing and cloning techniques. An excellent review on plant allergens was published by Breiteneder and Ebner (2000). An important aspect of nut and seed allergens with respect to clinical reactions is related to IgE cross-reactivity with pollen from the corresponding tree or plant (Andersen and Lowenstein, 1978; Caballero and Martin Esteban, 1998; Etesamifar and Wuthrich, 1998). For example, allergens homologous to pollen allergen Bet v1 often lead to oral allergy syndrome, while the more severe reactions are associated with other allergens (Schocker *et al*., 2000; Beyer *et al*., 2002). In the case of hazelnut, this phenomenon was investigated in detail and reviewed recently (Pastorello *et al*., 2001). In particular, storage proteins which are abundant in nuts and seeds have been identified as allergens. Because of the often high stability towards heating and digestion, storage proteins can be potent allergens (Astwood *et al*., 1996).

12.5 Effect of food processing on allergenicity

Heat treatment is often applied in food processing for obtaining better taste and smell (roasting, frying) or to extend shelf-life (pasteurization). Typical processes for nuts and seeds are drying at ambient temperatures, roasting at 140–170 °C and frying at around 180 °C, and further processing in the

preparation of final consumer products, like baking for cookies. Wigotzki *et al.* (2000) investigated the IgE-binding properties of hazelnut proteins after heating and found no effects up to 155 °C. At 170 °C and higher some IgE-binding was lost, but other allergens remained able to bind IgE (Wigotzki *et al*., 2000). In another study, pollen-related hazelnut allergens were found to be heat-labile, resulting in strongly diminished IgE-binding using serum from pollen-allergic individuals co-sensitized for hazelnut (Muller *et al*., 2000). Using sera from hazelnut allergic subjects who recognized non-pollen related allergens, on the other hand, showed that part of the hazelnut allergen fraction was stable to heat treatment (Schocker *et al*., 2000). True allergenicity can only be judged *in vivo*, and data from Hansen *et al.* (2003) show that roasted hazelnuts elicited reaction in fewer subjects compared to raw hazelnuts (Hansen *et al*., 2003), and Wensing *et al.* (2001b) showed that thresholds for roasted hazelnuts were higher compared to raw hazelnuts, indicating a heat-labile nature of hazelnut allergens.

For the majority of allergenic nuts and seeds, the effect of heat treatment on allergenicity has yet to be established. Tree nuts and seeds are applied both in raw and heat-treated form. It is therefore fair to say that the majority of nut- or seed-allergic individuals will also react, to some extent, on the heated form of the allergen. It is not expected that food processing will lead to hypoallergenic foods containing nuts and seeds. Although the general belief is that heating reduces allergenicity to some extent, there is one report that describes the contrary. An atopic child that tolerated raw pecan nuts experienced an anaphylactic shock after ingestion of pecan nuts in cookies (Malanin *et al*., 1995). Apparently, baking the cookie resulted in the formation of neo-allergens or neo-epitopes, probably due to Maillard cross-linking of sugars to protein (Berrens, 1996). However, this remains the only report of its kind.

Performing oral challenges and comparing minimum provoking doses is the best approach to studying the effect of heat treatment on nut and seed allergens. However, it is difficult to compare such studies from different medical centers since there are often small deviations in experimental design. In some studies, it is difficult to discern whether raw or heat-treated nuts or seeds are used (Hefle and Taylor, 2002).

12.6 Detecting nut and seed residues in food: selecting a method

Reports of severe allergic reactions in nut- or seed-allergic individuals have led to the development of methods for detecting residues of specific nuts and seeds in other foods. The techniques that can be used for developing such methods are described elsewhere in this book. The next section will start with an overview on the development of hazelnut detection methods, chosen

because hazelnuts are widely used in food manufacturing. Following this, an overview of described methods for the detection of other nuts and seeds will be given. The percentage of hazelnut used in a recipe is considered to be a mark of quality, for example in chocolate, chocolate products, and nougats. The first studies on hazelnut detection were therefore related to quantification of content of hazelnuts rather than to detecting traces.

12.6.1 Detecting hazelnuts

Product adulteration has always been a matter of concern for the food industry and retailers, especially where it is suspected that expensive ingredients have been replaced by cheaper alternatives. Hazelnuts in chocolate and chocolate products are appreciated by many consumers, and the hazelnut content determines the cost price of certain consumer products to a large extent. Typically 5–15% hazelnut is added in order to get the desired hazelnut flavor. In the mid 1980s, the first method for quantifying the amount of hazelnut in food products was reported. The method was based on electroimmunodiffusion (Laurell, 1972) using antiserum from laboratory animals and detected 0.01–0.1% of hazelnut in food products corresponding to 100–1000 ppm (Klein *et al*., 1985). The investigated matrix was nougat for which the recovery was > 95% (Klein *et al*., 1985). Since roasting hazelnut is a common practice to improve the flavor, the reactivity of roasted hazelnuts was investigated with this method as well. Up to 135 °C, the reactivity decreased to 70–80% of the original value, and above 160 °C, the reactivity was too low for proper analysis (Klein and Guenther, 1985).

A similar technique was used by Malmheden-Yman *et al*. (1994) who analyzed several foods for undeclared presence of hazelnut. This was the first time that hazelnut detection was used in relation to the safety of hazelnut-allergic individuals. A complaint sample of chocolate, supposedly free of hazelnut, contained 0.2% of hazelnut, leading to an asthmatic reaction (Malmheden Yman *et al*., 1994). Thresholds for hazelnuts were not known in the 1990s, but for peanut, thresholds as low as 100 μg were reported (Hourihane *et al*., 1997). Using this figure, our group aimed at a detection limit of 1–10 ppm when developing a new immunological assay for the detection of hazelnuts. A polyclonal antibody was raised in rabbits, and its reactivity was compared to a pool of serum from hazelnut-allergic patients. The rabbit serum detected proteins on immunoblots that bound IgE, indicating that relevant proteins were detected (Koppelman *et al*., 1999). To improve the sensitivity of the method, several ELISA formats were evaluated, and a sandwich ELISA using the rabbit antiserum gave the best results. Around 30 ng/mL hazelnut protein could be detected (both raw and roasted) and, taking into account the required extraction and dilution steps, this detection limit corresponds to 0.1 ppm in food samples. As shown in Fig. 12.1, the assay detected both raw and roasted hazelnuts. This assay was applied to the analysis of several suspected samples, and one case report on an allergic reaction to chocolate

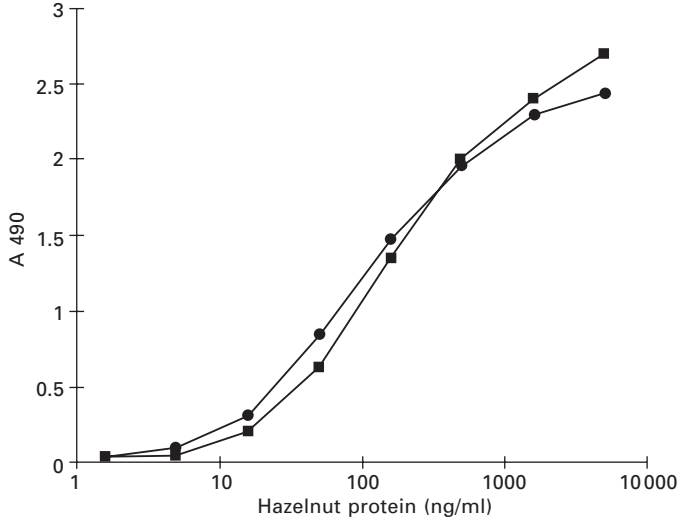

Fig. 12.1 Detection of hazelnut proteins by sandwich ELISA using polyclonal antibodies. Circles: dilutions of an extract of raw hazelnuts; squares: dilutions of an extract of roasted hazelnuts. Reprinted with permission from the *Journal of Immunological Methods*, Koppelman *et al*., 1999).

spread that inadvertently contained hazelnut was recently published (Wensing *et al*., 2001a). Holzhauser published a similar sandwich ELISA, with a comparable sensitivity (Holzhauser and Vieths, 1999). Since 1999, several other immunochemical assays for the detection of hazelnut have been reported. In addition to ELISA-based methods, dipstick immunoassays, IgE and IgG immunoblots, and PCR techniques have been described. Table 12.3 gives an overview of the methods published to date.

Although hazelnut detection via DNA is reasonably sensitive, one should keep in mind that such an approach is indirect. It is not the allergenic fraction but a marker molecule which is measured. Hazelnuts are often used as whole nuts, with DNA and proteins probably present in a constant ratio, but when hazelnut oil, hazelnut meal, or extracted hazelnut flavor are used, it is not clear whether DNA and protein remain together; test results may become false positive or, worse, false negative. An advantage of detection via DNA is the specificity of the assay. Primers can be chosen that uniquely react with hazelnut DNA (Holzhauser *et al*., 2000), while immunoassays dependent on antibodies can show some degree of non-specificity. Specificity is of particular importance when assessing the content of hazelnut in products like hazelnut-containing chocolate, or nougats (Klein *et al*., 1985), related to product adulteration. On the other hand, hazelnut-allergic consumers are often also sensitized to other tree nuts (Roux *et al*., 2003). False positive test results of hazelnut-free products caused by the presence of other tree nuts or peanuts will not change the conclusion of the hazelnut-allergic consumers, because

Table 12.3 Detection methods for hazelnut residues (in order of publication date)

Analyte	Technique	Key reagent	Detection limit	Reference
Hazelnut protein	Electroimmunodiffusion	Polyclonal animal serum	100–1000 ppm	Klein and Guenther, 1985; Klein *et al*., 1985; Malmheden Yman *et al*., 1994
Hazelnut allergens	Immunoblot	Hazelnut-allergic serum	Not reported	Teuber *et al*., 1997
Hazelnut protein	Immunoblot	Polyclonal rabbit serum	300 ppm	Koppelman *et al*., 1999
Hazelnut allergens	Inhibition ELISA	Hazelnut-allergic serum	0.1 ppm	Koppelman *et al*., 1999
Hazelnut protein	Sandwich ELISA	Polyclonal rabbit serum	0.1 ppm	Koppelman *et al*., 1999
Hazelnut protein	Sandwich ELISA	Polyclonal rabbit serum	0.2 ppm	Holzhauser and Vieths, 1999
Hazelnut DNA	PCR-hybridization	Specific DNA primers	10 ppm	Holzhauser *et al*., 2000
Hazelnut protein	Dot blot	Egg yolk IgY	1 ppm	Blais and Phillippe, 2000
Hazelnut protein	Immunoblot	Polyclonal rabbit serum	5 ppm	Scheibe *et al*., 2001
Hazelnut protein	Sandwich ELISA	Egg yolk IgY	1 ppm	Blais and Phillippe, 2001
Hazelnut protein	Biacore immunoassay	Polyclonal rabbit serum	2 ppm	Jonsson and Hellenas, 2001
Hazelnut protein	Dipstick (sandwich)	Polyclonal rabbit serum	1 ppm (only qualitative)	Stephan *et al*., 2002
Hazelnut 2S albumin	Basophil histamine release	Hazelnut-allergic serum	< 0.1 ppm	Akkerdaas *et al*., 2002a
Hazelnut protein	Sandwich ELISA	Polyclonal rabbit serum	< 0.1 ppm	Akkerdaas *et al*., 2002b

food products that contain nuts other than hazelnut will also be avoided by hazelnut-allergic consumers. Nevertheless, specificity is an important aspect and, since tree nuts are phylogentically related, immunochemical cross-reactivity leading to non-specificity is expected. The theoretical explanation for immunochemical cross-reactivity is found in the antibodies recognizing specific parts of the target proteins. Amino acid sequences of nut proteins are sometimes very similar and homologous to a high extent as in the case for the family of 2S storage proteins (Ampe *et al*., 1986; Teuber *et al*., 1998). Antiserum may recognize epitopes on the immunogen as well as on homologous proteins in other species. Cross-reacting epitopes usually have lower affinities compared to the original epitopes, and the recognition of other species can be orders of magnitude less. It is interesting to note that four different research groups who raised hazelnut antisera independently describe the same cross-reacting species; walnut appeared to cross-react in all raised antibodies, while almond and cashew cross-reacted with three and two antisera, respectively (Holzhauser and Vieths, 1999; Koppelman *et al*., 1999; Blais and Phillippe, 2001; Scheibe *et al*., 2001). One antiserum recognized pumpkin seed, and another reacted with pine nut and peanut, although the latter two were a 100 000 fold less compared to hazelnut (Koppelman *et al*., 1999) and therefore considered to be negligible. Other tree nuts, seeds, and legumes were tested for only two of the sera, and appeared to be negative (Holzhauser and Vieths, 1999; Koppelman *et al*., 1999), while for the other sera an extensive investigation of cross-reactivity was not performed (Blais and Phillippe, 2001; Scheibe *et al*., 2001). One antiserum was not tested for possible cross-reactivity (Jonsson and Hellenas, 2001).

Recovery of analytes, in this case hazelnut protein, from food matrices is sometimes difficult because of food processing steps. Heat treatment especially can denature proteins, thereby decreasing the solubility. This aspect is discussed in detail elsewhere in this book. For hazelnut detection, the main areas of application are chocolates and bakery products. Extraction from chocolate can be particularly difficult due to binding of tannins to proteins, limiting the protein solubility. Holzhauser and Vieths investigated the recovery of hazelnut protein from various spiked samples as analyzed with their assay. They found an average recovery of 106 ± 17%, although for samples spiked with lower amounts of hazelnut, the recovery varied between 62 and 132% (Holzhauser and Vieths, 1999). Recoveries of commercial kits are optimized by adding tannin-binding components such as skimmed milk powder or fish gelatin (Keck-Gassenmeier *et al*., 1999). Another aspect that may influence the quantification of hazelnut in food is roasting. Roasting of hazelnut has two effects on immunochemical analysis: (i) changing the immunochemical properties of the target protein, for example by protein denaturation and Maillard reactions; and (ii) limitation of solubility. It is therefore important to assess the effect of roasting on the quantitative determination of hazelnuts. One report shows that dilution curves of raw and roasted hazelnut protein were almost identical with an estimated maximum difference of about 10%

(Koppelman *et al*., 1999), and another report shows a difference between raw and roasted of about 20% (Holzhauser and Vieths, 1999). For the IgY-based assay it was shown that roasting did not significantly change the reactivity (Blais and Phillippe, 2001). For the PCR method it was demonstrated that the PCR led to amplified DNA for both raw and roasted hazelnut, but the possible differences were not quantified (Holzhauser *et al*., 2000). In the case of detecting hazelnut residues related to food safety of hazelnut-allergic consumers, true quantification is not necessary. Differences between detection of raw and roasted hazelnut would be important if the roasted form is only recognized with a sensitivity that is orders of magnitude less, or if roasted hazelnuts are not detected at all, but this is not the case in the published methods.

In summary, there are a number of methods published for the detection of hazelnuts in foods. The majority are based on specific antibodies, and all have rather similar characteristics with respect to sensitivity and specificity. A commercial method for detection of hazelnut is available, and several laboratories offer an analytical service. A list with website addresses for commercially available methods for tree nuts and seeds is provided in Table 12.5 on p. 214.

12.6.2 Overview of methods for other tree nuts and seeds

While a number of assays have been described for hazelnut, the published methods for other nuts and seeds are very limited. Radioallergosorbent assay (RAST) inhibition techniques with defined patient sera may provide a tool for allergen-specific measurement when no animal sera are available (see Chapter 4). The section below describes published methods for specific nuts and seeds.

Almond

For almond, polyclonal antibodies were raised in rabbits, and successfully applied in immunoblot and competition ELISA (Acosta *et al*., 1999). The reactivity was strongly influenced by heat treatment and pH treatment, and some cross-reactivity with other nuts and legumes was observed (Acosta *et al*., 1999). The same group reported an optimized ELISA for almond and recovery from different matrices, and in addition the effect of heat treatment was studied (Roux *et al*., 2001). The detection limit of the method was 5 ppm in processed foods, and neither blanching, roasting, nor autoclaving markedly decreased the reactivity (Roux *et al*., 2001). Meanwhile Hlykwa *et al.* published a method to detect almond residues in food as well (Hlywka *et al*., 2000). This sandwich ELISA detected down to 1 ppm, and did not cross-react substantially with other food ingredients, although some reactivity was observed for sesame seeds, and to a lesser extent for walnuts and macademia, (Hlywka *et al*., 2000). The assay was used in a survey of 20 brands of dry breakfast cereal, and in these products no almond cross-contact was observed.

Interestingly, some products did not contain detectable amounts of almond although the manufacturer included almond on the ingredient list. It is likely that the reason for labeling was that the manufacture could not exclude the possibility of cross-contact with almonds (Hlywka *et al*., 2000). The recovery of almond from difficult matrices such as chocolate was 86–100%, possibly owing to the heating step during extraction (Hlywka *et al*., 2000).

Brazil nut

Blais *et al*. (2002) developed an ELISA for the detection of Brazil nut. This assay was based on egg yolk antibodies (IgY) and reached a sensitivity of well below 1 ppm. Cross-reactivity with other ingredients was investigated and appeared to be negligible for peanut, hazelnut, sunflower seed, cucumber seed, and castor beans (Blais *et al*., 2002). The antibody was later applied in a multiplex enzyme immunoassay for the detection of peanut, hazelnut, and Brazil nut with a sensitivity of around 1 μg protein per gram (1 ppm) (Blais *et al*., 2003). The advantage of this test is that important food allergens that are often used in chocolate manufacturing are assayed simultaneously. Clemente *et al*. (2004) developed an inhibition ELISA for Brazil nut 2S albumin, with a limit of detection of 1 ppm. Cross-reactivity with other nuts and legumes including peanuts was negligible, and the food products with Brazil nut declared were judged positive (Clemente *et al*., 2004).

Walnut

An ELISA for walnut detection has been described by Niemann and Hefle (2003). The antibodies were raised against a mixture of several varieties of walnut, both raw and roasted, in sheep and rabbits. Levels of 1 ppm were detected, and recovery from different food matrices including chocolate was between 85 and 96% even when low concentrations of walnut were spiked. Of a set of 50 main food ingredients, only pecan which is a close relative of walnut cross-reacted significantly (Niemann and Hefle, 2003).

Cashew

Recently, the first assay for cashew nut detection was published. Antibodies were raised in rabbits and goats against major storage protein. The resulting antisera were immunoadsorbed with immobilized protein of several nuts, legumes, and seeds in order to decrease possible cross-reaction (Wei *et al*., 2003). Immunoblotting studies demonstrated that the polyclonal sera recognized proteins similar to those recognized by IgE from cashew nut-allergic patients. Cross-reactivity of other food ingredients was low, although pistachio and sunflower seed showed significant cross-reaction. A sensitivity of 0.02 ppm was reached and recoveries from divers food matrices varied from 64 to 136%, while heating did not affect the reactivity of cashew in the test (Wei *et al*., 2003).

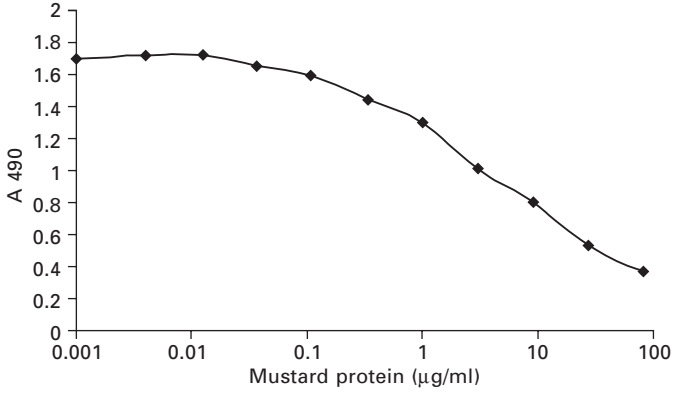

Fig. 12.2 Calibration curve of mustard seed protein in an inhibition ELISA for mustard using rabbit polyclonal antibodies. The line represents serial dilutions of mustard seed protein.

Sesame

Brett *et al*. (1998) purified the 13S protein fraction of sesame seeds and raised polyclonal antibodies in rabbits towards it. IgG-immunoblots indicated that the serum was specific although the authors did not provide a complete list of tested food ingredients. Using ELISA, detection of sesame was possible (no detection limit described) (Brett *et al*., 1998).

Mustard

EU legislation on labeling of food allergens also includes mustard and products thereof (EU, 2003). Up to now, no methods are either commercially available or published. Recent work from our group led to a polyclonal antiserum for mustard seed proteins, and an inhibition ELISA was constructed. The test performance has been sorted out for some food applications, and Fig. 12.2 shows a calibration curve for mustard seed protein. Mustard seed protein at a concentration of around 50 ng/ml results in significant inhibition. Taking into account the necessary dilution steps, the sensitivity of this ELISA is around 1–2 ppm.

Table 12.4 gives an overview of the published methods for nuts and seeds.

12.7 Conclusions

For hazelnut detection a large number of (ELISA) tests has been described, and there are test kits commercially available. For other nuts and seeds, there remains a lot of work to do. Some tests are already commercially available and used by the food industry (Table 12.5). The assays described up to now have detection limits around 1 ppm. This is suitable for allergen detection, as clinical case reports and threshold studies indicate that allergen levels of

Table 12.4 Detection methods for nuts and seeds other than hazelnut

Analyte	Technique	Key reagent	Detection limit	Reference
Almond protein	Inhibition ELISA	Polyclonal rabbit serum	5 ppm	Acosta *et al*., 1999; Roux *et al*., 2001
Almond protein	Sandwich ELISA	Polyclonal rabbit and sheep serum	1 ppm	Hlywka *et al*., 2000
Brazil nut protein	Sandwich ELISA	Egg yolk IgY	< 0.1 ppm	Blais *et al*., 2002
Walnut protein	Sandwich ELISA	Polyclonal rabbit and sheep serum	1 ppm	Niemann and Hefle, 2003
Cashew storage protein	Sandwich ELISA	Polyclonal rabbit and goat serum	< 0.1 ppm	Wei *et al*., 2003
Sesame 13S protein	Inhibition ELISA	Polyclonal rabbit serum	Not specified	Brett *et al*., 1998

Table 12.5 Commercially available detection methods for nut and seed allergens[1]

Target allergen	Type of method	Kit manufacturer	Sensitivity	Web site
Hazelnut	Sandwich ELISA	R-Biopharm	10 ppm	www.r-biopharm.com
Almond	Sandwich ELISA	Neogen	2.5 ppm	www.neogen.com
Almond	Sandwich ELISA	R-Biopharm	1.7 ppm	www.r-biopharm.com
Sesame	Sandwich ELISA	Tepnel Biosystems	2.5 ppm	www.tepnel.com

[1]Note that peanut detection is described in Chapter 11 and is therefore not included in this table

10 ppm are relevant (discussed in Chapter 1). A major problem with test development for allergen detection from nuts and seeds is that nuts and seeds are closely related phylogenetically. Therefore, raising antibodies that are specific is a challenge. On the other hand, products tested for certain analytes, for example traces of hazelnut, should in most cases also be free of other nuts. In the case that nut speciation is desired, PCR techniques may be helpful as they are usually more specific compared to immunochemical techniques.

Commercially available ELISA kits have been improved with respect to performance. Most optimized tests use three incubation steps of 10 minutes only (Immer *et al*., 2004). Compared to sample preparation which may take considerable time (milling, mixing, heating, and centrifugation) the test itself is no longer the rate-limiting step. We foresee a future for ELISA-based methods in the detection of nut and seed allergenic residues. With new polyclonal antibodies produced and characterized by several groups around the world, the availability of new kits will increase. It is, however, difficult to predict which nuts and seeds will be considered the most important allergens in the future as clinical reactions for a wide variety of nuts and seeds have been described. For the nuts and seeds that are common food ingredients in Western diets, we believe that a complete set of ELISA-based kits will be available in the coming years.

12.8 References

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13

Detecting dairy and egg residues in food

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13.1 Introduction

The major sources of food allergens having animal origin are milk, egg (especially egg white), crustaceans and fish. Allergies to meat are less frequent. Crustaceans and fishes are less often used as processed ingredients, except in surimi, flavors and spices using shrimp powders. They are generally consumed as clearly recognizable and labelled ingredients. Moreover, they are usually eaten later in life. In contrast, milk and egg are widely consumed as basic food products, as milk is the first food for babies. They constitute a good source of animal protein for a modest financial cost. They are also traditionally used around the world as food ingredients due to their useful technological properties such as foaming, jellification, thickening, emulsifying and binding. They are used in bakery and cakes, delicatessen and processed meat products, soups, dressings and sauces, among other applications.

Food allergies to cows' milk and hen's egg white are frequent in man, and they are the two prevalent food allergies in children. Symptoms of egg and milk allergy are identical to those for other food allergies (see Chapter 1). Many studies have been published concerning the prevalence of egg and milk allergies; results may differ depending on the selection (food-allergic patients or unselected population) or on the age of the population studied, but they show that egg and milk allergies are the most prevalent among food allergy. In studies on unselected (general) populations, milk allergy could represent about 2.0% in Australia (Hill *et al*., 1997, 1999), 1.9% in Finland (Saarinen *et al*., 2000), 3.2% in France (Kanny *et al*., 2001), 3.9% in Germany (Schäfer *et al*., 1999), 4.0% in Japan (Iikura *et al*., 1999) and 1.4% in the US (Rhim and McMorris, 2001) and egg allergy could represent about 3.2% in

Australia (Hill *et al*., 1999) and 2.8% in Germany (Schäfer *et al*., 1999). Allergic reactions due to the presence of egg white proteins in vaccines were also shown by Davies and Pepys (1976). Pichler and Campi (1992) reported an allergy case due to the presence of lysozyme contained in vaginal suppositories.

The amount of allergenic food that can provoke allergic reactions is not well known. There is variability in patient sensitivity and in allergen specificity. Food ingredients can be enriched with allergens due to the production methods or processing. Heat treatment may decrease or increase the allergenic potential of food allergens. For all these reasons, according to studies performed by clinicians, it is considered that detection techniques for allergenic sources should be around 1–10 ppm, i.e. 1–10 mg/kg (Moneret-Vautrin *et al*., 2003; Morisset *et al*., 2003; Osterballe and Bindslev-Jensen, 2003). Most commercial methods have detection limits encompassing this range.

Taken together, these reasons indicate the need for quick, sensitive, economical and available detection methods for milk and egg. Analyses using these methods are carried out in order to ensure the fairness of commercial transactions and the protection of food-allergic consumers.

13.2 Milk

13.2.1 Cows' milk allergy

Cows' milk allergy (CMA) is a common disease of infancy and early childhood, with a prevalence of 8% reported in a population of 1121 people with food allergies (Kanny *et al*., 2001). CMA affects 2.5% of children under two years of age, but about 80% become clinically tolerant within the first three years of life (Høst, 1997). Symptoms of CMA are the usual symptoms of food-allergic reactions. They are mainly urticaria (76%), atopic dermatitis (28%), vomiting (9%) appearing in less than two hours (68%) at cumulative reactive doses below 20 mL (59%) and between 20 mL and 200 mL (32%) (Saarinen *et al*., 2000).

More recent studies have tried to evaluate the threshold doses of clinical reactivity to milk allergens. Clinical allergists have published existing data on threshold doses for several food allergens such as milk, egg, peanut, fish and mustard (Hefle and Taylor, 2002; Taylor *et al*., 2002; and Taylor *et al*., 2004). For milk, the lowest amount which provoked symptoms by double-blind placebo-controlled, single-blind or open challenge ranged from 0.02–5 mL corresponding, respectively, to 0.6–180 mg of protein. Morisset *et al*. (2003) presented results of 59 positive oral challenges to milk. A low threshold inferior or equal to 0.3 mL of milk was observed in 1.7% of milk allergies and a cumulative reactive dose inferior or equal to 0.8 mL of milk characterized 5% of milk allergies. The lowest reactive dose found was 0.1 mL of milk. Sicherer *et al*. (1999) reported that 25% of milk allergics reacted to doses of 100 mg.

13.2.2 Cows' milk allergens

The protein content of cows' milk is about 30–35 g/L, divided into 20% whey and 80% caseins (see Table 13.1). Caseins precipitate under acidic pH (pH 4.6) or with the action of the chymosin and are the major components of cheese. Caseins (allergen nomenclature: Bos d 8) are a family of related proteins, divided into α, β, κ and γ caseins and ranging from 19–33 kDa. Casein subunits associate in solution forming complexes and ordered aggregates of micelles. Sequence homologies between caseins αS1 and αS2 are about 22%. One study of 58 children with CMA showed that 85% possessed IgE against each casein with different IgE levels: αS1 > β >> αS2 = κ (Bernard *et al*., 1998). Casein is one of the major allergens responsible for CMA and has been shown to play an important role in persistent allergy. Using 96 overlapping synthesized decapeptides representing the entire length of αS1-casein, Chatchatee *et al*. (2001) identified two amino acid regions (AA 69–78 and AA 173–194) recognized by the majority of patients over nine years of age with persistent CMA. None of children under three years of age who are likely to outgrow CMA recognized these epitopes.

β-lactoglobulin (Bos d 5) is the most abundant protein from the whey fraction. It is a protein of 18 kDa belonging to the lipocalin family (which bind retinol). The amino acid sequence of bovine β-lactoglobulin shows 90% homology with ovine β-lactoglobulin. This allergen is not present in human milk, but in one study was recognized by approximately 60% of 92 CMA patients (Wal *et al*., 1995).

α-lactalbumin (Bos d 4) is composed of two variants of 14.2 and 13 kDa. It belongs to the family of glycosyl hydrolase (lysozyme c superfamily) and shows 95% sequence homology with α-lactalbumin from goat and sheep and 78% with α-lactalbumin from human milk. α-lactalbumin also shows 46% sequence homology with egg lysozyme. Frequency of sensitisation to α-lactalbumin was reported to range from 0–80% of patients (Besler *et al*., 2002a).

Bovine serum albumin (Bos d 6) is a 67 kDa molecule containing nine disulfide bridges. It is the main plasma protein and binds water, ions, fatty acids and hormones and regulates osmotic blood pressure. Sequence homology with mammalian serum albumins ranges from 74–79%. Recognition frequency was reported as ranging from 0% in six children using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and IgE immunoblotting (Restani *et al*., 1999) to 75% with 21 children using crossed radio-immunoelectrophoresis (CRIE) (Høst *et al*., 1992). Bovine immunoglobulins and lactoferrin have been described as minor allergens. Maillard reaction products, which are glycan-protein conjugates, may also act as allergens (Wal, 1998, 2001).

13.2.3 Hydrolyzed infant formulae

Breast-feeding is certainly the best way to prevent children from becoming

Table 13.1 Cow's milk allergens

	IUIS nomenclature	Relative content	kDa	pl	Sequence database SwissProt	Structure (post-traductional modifications)	Function/ family
Whey		**20%**					
α-lactalbumin	Bos d 4	5%	14.2	4.8	P00711	4 disulfide bonds glycosylated	Glycosyl hydrolase
β-lactoglobulin	Bos d 5	10%	18.3	5.3	P02754	2 disulfide bonds, dimer, not present in human milk	Lipocalin Retinol binding protein (RBP)
Bovine serum albumin	Bos d 6	1%	67	4.9–5.1	P02769	9 disulfide bonds	Plasma protein
Immunoglobulins	Bos d 7	3%	160	6–8			
Lactoferrin		< 1%	80	8.7	P24627		Iron transport
Caseins	Bos d 8	**80%**					
αS1-casein		32%	23.6	4.9–5	P02662	Phosphorylated	Calcium transport
αS2-casein		10%	25.2	5.2–5.4	P02663	Phosphorylated + 1 disulfide bond	
β-casein		28%	24.0	5.1–5.4	P02666	Phosphorylated and glycosylated	
κ-casein		10%	19.0	5.4–5.6	P02668	Phosphorylated + 1 disulfide bond	Miscelles stability
γ-casein							

sensitized to cows' milk proteins. However, cows' milk proteins have been demonstrated to be present in breast milk (Axelsson *et al*., 1986). An increasing population of infants receives hydrolyzed cows' milk derived formulae for the prevention and treatment of CMA. However, for primary prevention in non-sensitized infants and protection from CMA, partially hydrolyzed hypoallergenic formulae can be recommended. These formulae have reduced allergenic activity (Restani *et al*., 1996).

Evaluation of the residual antigenicity and allergenicity of cows' milk substitutes was performed using several *in vitro* tests (Docena *et al*., 2002). Immunoenzymatic methods such as enzyme-linked immunosorbent assay (ELISA) using anti-cows' milk protein antibodies were used to detect the presence of residual antigenic epitopes. Direct EAST (enzyme allergosorbent test) enables the detection of a potential decrease of IgE-binding to peptides from moderately and extensively hydrolyzed milk preparations. SDS-PAGE followed by IgE-immunoblotting using sera from CMA patients or cows' milk protein-specific IgG antibodies showed that extensively hydrolyzed preparations do not contain residual components from cows' milk proteins. While extensively hydrolyzed milk substitutes produced from whey or caseins were reported to be safe for some CMA consumers (Halken *et al*., 1993; Martin-Esteban *et al*., 1998), adverse reactions were also reported (Ragno *et al*., 1993; Nilsson *et al*., 1999; Sotto *et al*., 1999; Carroccio *et al*., 2000). In these cases of extreme sensitivity, an elemental amino acid formula should be used.

13.2.4 Milk protein denaturation

However heat stable milk proteins are, heat treatments cause modifications in their structure. Heat destroys conformational epitopes and can thus reduce the allergenicity of some proteins. Boiling milk for ten minutes eliminates skin prick test reactivity to bovine serum albumin and β-lactoglobulin, whereas caseins were found to be heat stable after testing in eight CMA patients (Norgaard *et al*., 1996). When using CRIE, Gjesing *et al*. (1986) showed a reduction of IgE binding to α-lactalbumin (about 50%), to the casein fraction (> 66%) and abolishment of IgE binding to β-lactoglobulin, bovine serum albumin and bovine immunoglobulins after ten minutes of boiling. γ-irradiation was shown to reduce IgE reactivity to α-lactalbumin and β-lactoglobulin (Lee *et al*., 2001).

13.2.5 Hidden milk allergens

Cows' milk proteins have often been described as hidden (undeclared or contaminated) in food products (Gern *et al*., 1991; Laoprasert *et al*., 1998; Giovannacci *et al*., 2004) and even in human milk due to ingestion of cows' milk by the mother. By radioimmunoassay, detectable amounts of β-lactoglobulin (from 5–800 ng/mL) were found in 93 out of 232 (40%) human

milk samples (Axelsson *et al*., 1986). On the other hand, Restani *et al*. (2000) did not detect any β-lactoglobulin or casein in human milk from breast-feeding mothers using SDS-PAGE, immunoblotting, and β-lactoglobulin or caseins-specific monoclonal antibodies. However, monoclonal antibodies might not be able to detect the proteins due to proteolytic changes.

Using RAST inhibition, α-lactalbumin was found to contaminate food-grade lactose at levels of 1–5 ppm (Frémont *et al*., 1996). A 30 year old woman with CMA and without fish allergy experienced anaphylaxis after ingestion of reconstituted salmon containing undeclared casein, which was used as a gelation agent combined with microbial transglutaminase, which serves to cross-link casein to meat proteins for restructuring (Koppelman *et al*., 1999).

Cows' milk proteins can be found in other types of products. Ylitalo *et al*. (1999) detected the presence of cows' milk casein in 13 of 30 commercial natural latex glove brands by rocket immunoelectrophoresis (RIE) and RAST inhibition with casein-specific IgE from pooled human sera. Caseins can also be an aero-allergen: a case of occupational asthma and rhinoconjunctivitis has been described in a chocolate candy worker in contact with dried cows' milk. α-lactalbumin was implicated as the cause of the sensitization (Bernaola *et al*., 1994). Mäkinen-Kiljunen and Mussalo-Rauhamaa (2002) showed the presence of caseins in 90 out of 91 house dust samples. The median casein level was 30 μg/g (range 3–3300 μg/g), which was higher than those of pets or mites (0.11 μg/g of Fel d 1 and < 0.01 μg/g of Der p 1 or Der f 1). The authors explain these findings by the use of casein in indoor plasters to improve their handling properties in practice since the 1960s. Some paints can also contain casein proteins. In one case, inhalation of milk proteins was reported to cause death (Bosetti *et al*., 1997).

13.2.6 Cross-reactions

Cross-reactions between ovine (sheep or goat) and bovine milk have been studied by several authors (Dean *et al*., 1993; Sabbah *et al*., 1997; Bellioni-Businco *et al*., 1999; Restani *et al*., 1999). People allergic to cows' milk proteins will often have serum IgE to goat or sheep's milk proteins (Besler *et al*., 2002b,c). β-lactoglobulin and caseins have been described as the main cross-reacting allergens. Comparison between milk from the different ruminant species reveals high homologies in both protein composition and structure, particularly for the four caseins (αS1, αS2, β and κ) that are found in cows', sheep's and goat's milk. Moreover, important sequence homologies are observed. Using CMA patient sera by ELISA with purified caseins, Bernard *et al*. (1999) showed that important levels of cross-reactivity occur between bovine, caprine and ovine caseins.

In a study on cows' milk and human α-lactalbumin, proteins showing a high degree of sequence homology demonstrated IgE cross-reactivity by ELISA inhibition (Maynard *et al*., 1999). The authors suggested low clinical

relevance of these cross-reactive IgE. Other reports describe specific allergy to ovine milk with good tolerance to cows' milk (Wüthrich and Johansson, 1995; Umpierrez *et al*., 1999). Businco *et al*. (2000) investigated, by *in vitro* and *in vivo* methods, the allergenicity of mare's milk in a population of 25 children with severe CMA. Only two patients showed positive skin prick tests to mare's milk and one reacted to it in double-blind placebo-controlled food challenge (DBPCFC). Therefore, in some cases, CMA patients may be able to ingest mare's milk.

13.3 Egg

13.3.1 Egg allergy

The prevalence of egg allergy, as studied by skin prick tests, was reported to be about 3.2% in a group of 620 Australian children at risk of atopy (Hill *et al*., 1999), 53% by labial food challenge in a population of 142 food-allergic children (Rancé and Dutau, 1997), and 0.4% in an unselected population of 502 adults (Gislason *et al*., 1999). Cumulative reactive doses were reported as ranging from 0.13 mg (raw whole egg) to 200 mg of proteins (dried whole egg) (Taylor *et al*., 2002). A recent report (Morisset *et al*., 2003) showed that a cumulative reactive dose inferior or equal to 65 mg was observed in 16% of egg allergic patients with 125 oral challenges performed. The lowest reactive dose was observed at less than 2 mg of egg. The conclusion of the study was that the detection tests for egg should ensure a sensitivity of at least 10 ppm taken from minimal quantities as a 95% guarantee for the safety of patients allergic to egg on the basis of consumption of 100 g of food.

13.3.2 Egg allergens

Ovomucoid (Gal d 1), is the dominant allergen in hen's egg. It is a glycoprotein with a molecular weight of 28 kDa and a pI of 4.1 exhibiting trypsin inhibitor activity (see Table 13.2). One study (Bernhisel-Broadbent *et al*., 1994) suggested that the use of commercially purified ovalbumin led to the impression that ovalbumin was the major allergen from egg white, because ovalbumin was contaminated with less than 1% of ovomucoid, resulting in false positive results. The recognition frequencies reported in this study were omucoid 89%, ovalbumin 78% and lysozyme 61% by skin prick test in 18 egg-allergic children. Urisu *et al*. (1997) classified egg white allergen IgE binding prevalence in decreasing order: ovomucoid > ovalbumin >> ovotransferrin and lysozyme by RAST using 72 egg allergic-patients.

Ovomucoid is a highly glycosylated protein comprising 186 amino acids arranged in three tandem domains (Gal d 1.1, 1.2, and 1.3). The three ovomucoid domains were isolated and evaluated with sera from egg-allergic patients to determine B-cell domain specificity, B-cell epitopes and the relative importance

Table 13.2 Egg allergens

	IUIS nomenclature	Relative content	kDa	pl	Sequence database SwissProt	Structure (post-traductional modifications)	Function/ family
Egg white							
Ovomucoid	Gal d 1	11	28	4.1	P01005	glycoprotein	trypsin (serine protease) inhibitor
Ovalbumin	Gal d 2	54	43	4.5	P01012	phosphoglyco-protein	serpin
Ovotransferrin	Gal d 3	12	77.7	6.0	P02789	glycoprotein (15 disulfide bonds)	iron transport
Lysozyme	Gal d 4	3.4	14.3	10.7	P00698	(4 disulfide bonds)	glycosidase bacteriolytic function
Ovomucin		1.5	0.2–8 10^6				
Egg yolk							
α-livetin	Gal d 5		67		P19121	glycoprotein (17 disulfide bonds)	serum albumin

of linear and conformational structures and carbohydrate chains to B-cell epitopes (Cooke and Sampson, 1997). There was significantly more IgE activity against the second ovomucoid domain (median percentage of ovomucoid-specific IgE: Gal d 1.2, 40%; Gal d 1.1, 23%; Gal d 1.3, 26%). Five IgE and seven IgG binding regions were identified and IgE antibodies binding to reduced ovomucoid and IgG binding to oxidized ovomucoid were significantly decreased as compared with that to native ovomucoid (28 and 69%, respectively). The authors showed that conformational B cell epitopes play a significant role in ovomucoid allergenicity and that carbohydrate moieties have a minor effect on allergenicity. In this paper, the authors suggested that persistent egg allergy is related to reactivity to linear sequences and that egg allergy that is outgrown is associated with conformational epitopes.

Ovalbumin (Gal d 2) is a monomeric phosphoglycoprotein with a molecular mass of 43–45 kDa and a pI of 4.5. As noted above, at first it was thought to be the major egg white allergen, but this has been shown subsequently to be less frequently recognized by IgE from egg-allergic patients than ovomucoid. Ovotransferrin (conalbumin, Gal d 3) has a molecular weight of 77 kDa and a pI of 6.0, and has iron-binding properties. Its frequency of recognition in egg-allergic patients was reported in one study to be 22% (Djurtoft *et al*., 1991). Lysozyme (Gal d 4) is a 14.3 kDa protein with a basic pI of 10.7. Lysozyme (1,4-β-N-acetylmuraminidase C) has a bacteriolytic function and hydrolyzes the peptidoglycan polymer of prokaryotic cell walls. The recognition prevalence for lysozyme is reported to range from 6–67% (Djurtoft *et al*., 1991; Yamada *et al*., 1993; Frémont *et al*., 1997).

Specific IgE to egg yolk were first reported by Carrillo Diaz *et al*. (1986). Alpha-livetin (Gal d 5) is the chicken egg yolk protein implicated in 'bird-egg' syndrome. This syndrome occurs in patients in contact with birds and showing respiratory symptoms. This respiratory allergy precedes in all cases the onset of food allergy to eggs. Respiratory symptoms in the presence of certain fowl by sensitization to α-livetin from egg yolk is related to food allergy to egg (de Blay *et al*., 1994; Szepfalusi *et al*., 1994).

13.3.3 Stability of egg allergens

In contrast to milk proteins, egg proteins are very heat-labile. When cooked, egg yolk and white coagulate. Eigenmann (2000) described two cases of anaphylactic reactions after eating raw eggs whereas patients tolerated cooked eggs. Heating egg white for ten minutes at 90 °C significantly decreased RAST for 50% of 16 egg-allergic patients (Anet *et al*., 1985). In a population of 38 subjects with positive response to freeze-dried egg white, 21 had negative challenge responses to heated egg white (Urisu *et al*., 1997). In the same report, ovomucoid was shown to be a heat stable allergen. Oral challenges in 17 (94%) of egg-allergic patients with positive challenges to heated egg white had negative challenges to ovomucoid-depleted egg white.

13.4 Types of detection methods

13.4.1 Diffusion-in-gel methods

Diffusion-in-gel methods are cheap and easily performed but are not very sensitive, especially in processed or heat-treated food products; they are being replaced by immunoenzymic techniques. Langeland (1982) used CRIE and Hoffman (1983) utilized RIE to study egg white allergens. Results showed that ovalbumin and ovomucoid were strong allergens and conalbumin (ovotransferrin) was a less important allergen, while lysozyme was shown to be a weak allergen. Sajdok *et al.* (1990) determined the egg and egg white content in food products using radial immunodiffusion and RIE. Ylitalo *et al.* (1999) detected cows' milk casein in natural rubber latex gloves using RIE. The NMKL (Nordic Committee on Food Analysis) performed a multicenter collaborative study on food allergen detection using double immunodiffusion (Malmheden-Yman *et al.*, 2002). The results showed detection limits of 300 ppm casein in sausages and 30 ppm ovalbumin in pasta. However, technical expertise on the part of the laboratory enhances the possibility of obtaining correct results. The level of detection varies somewhat with antigen and with the titer of antiserum but lies normally at mg levels per 100 g food samples. This level of detection is generally not low enough, as it is usually recommended that methods have detection limits of 1–10 ppm for allergenic residues (Moneret-Vautrin *et al.*, 2003; Morisset *et al.*, 2003). Using radial immunodiffusion, Bertrand-Harb *et al.* (2003) followed the concentrations of β-lactoglobulin and α-lactalbumin during yoghurt fermentation. A RIE method was developed using commercially available anti-casein antiserum (Moen *et al.*, 2003). Quantification of unknown amounts of casein in foodstuffs resulted in a linear correlation between the height of the precipitates and the applied amount of casein. In this study, RIE had a detection limit for casein of 10 ppm and was described as being less expensive than commercial ELISA kits. Nevertheless, RIE is time- and antibody-consuming and requires trained people specialized in this field. Furthermore, ELISA kits are more practical for the food industry than RIE or CRIE.

13.4.2 RAST and EAST inhibition

RAST and EAST (see Chapter 4) are based on the binding of specific IgE to immobilized allergen molecules. RAST or EAST methods have been used for egg or milk allergens (Walsh *et al.*, 1987; Adams *et al.*, 1991; Rugo *et al.*, 1992; Dean *et al.*, 1993; Frémont *et al.*, 1996; Ylitalo *et al.*, 1999). Frémont *et al.* (1996) reported a detection limit of 1 ppm for α-lactalbumin. These techniques using egg-allergic serum are more suitable for clinicians than for the food industry.

13.4.3 Bioactivity of allergens

In order to quantify the allergenic activity of diagnostic allergen extracts, the techniques of RAST or EAST are currently used, as discussed in Chapter 4.

When the biological activity of a diagnostic allergen extract has to be determined, the capacity of the extract to degranulate IgE-sensitized basophils or mast cells reflects the presence of allergenic molecules inducing important cellular changes and finally degranulation (as discussed in Chapter 1). This degranulation is quantified by measuring the mediators (histamine, serotonine) or enzymes (β-hexosaminidase) specifically released by the cells. A choice of cellular systems for measuring such bioactivity of diagnostic allergen extracts is available, and human as well as animal models have been described (May, 1976; Norgaard *et al*., 1992; Prouvost-Danon *et al*., 1994). Nowadays, use of human basophils or mast cells is reserved only for diagnostic or research purposes. Animal mast cells are triggering units allowing the study of allergenic bioactivity, as they may be coated with allergen-specific IgE, after either active or passive sensitization of the cells with the immunoglobulins. In practice, serial ten-fold dilutions of an allergen extract are incubated in the presence of sensitized cells. Bell-shaped curves are obtained when the percentage of total mediator or enzyme release is plotted against the allergen concentration. This indicates that the release observed is the consequence of membrane-bound IgE triggering and not of toxicity on the cells of another component present in the extract. The lowest concentration of the allergen extract inducing the release of a well-defined percentage of the total content of enzyme or mediators represents the bioactivity of the extract.

The newly described transfectants of RBL (rat basophilic leukocytes) cells presenting chimeric receptors fixing human IgE (Marchand *et al*., 2003; Taudou *et al*., 1993) is a technique suitable for the detection of allergenic molecules encountered in an allergic pathology. The cells can be sensitized with a diluted pool of food-allergic sera and afterwards triggered with the allergen extract being studied. The latter techniques may be used for the determination of the bioactivity of food allergens, such as allergenic proteins present in milk or egg.

13.4.4 Dot blot technique

The dot blot technique initially described by Hawkes *et al*. (1982) was first used by Janssen *et al*. (1987) to detect egg and milk proteins in heat-treated meat products. The principle of this technique is to bind the antigen, from a complex mixture supposed to contain it, onto a membrane which is further incubated with a specific antibody enzymatically labeled. Finally, the membrane is incubated with a substrate, the product of which precipitates on the site of enzymic reaction. This precipitation allows the visualization of the presence of the antigen. The method was improved using cyanogen bromide-activated (CNBr-activated) nitrocellulose membranes (Demeulemester *et al*., 1991) which have high binding capacities (Demeulemester *et al*., 1987; Becker, 1989).

13.4.5 Electrophoretic methods, blotting methods, immunodetection

These techniques, based on the separation of samples in an electric field followed by immunodetection using either human allergic sera or animal antibodies, are discussed at length in Chapter 5. They are able to separate and to reveal the proteic components in raw or slightly cooked food products, but their use is not efficient enough for highly cooked and sterilized products because denaturation and aggregation of molecules disturb their separation (King, 1984; Hitchcock and Crimes, 1985; Bonnefoi *et al*., 1986).

Electrophoretic transfer after polyacrylamide gel electrophoresis

The protein unfolding caused by the use of SDS in PAGE may lead to the loss of conformational epitopes with sequential epitopes being preserved; this is the main disadvantage of this technique which was used to study egg and milk antigens and allergens (Quirce *et al*., 1998; Añibarro *et al*., 2000; Kim *et al*., 2002 ; Rupa and Mine, 2003).

Using this technique, Leduc *et al*. (1997) analyzed a few milk- and egg-based ingredients. These ingredients were chosen because they are commonly used in processed foods for their very good protein-binding properties. Results obtained with milk-based ingredients are presented in Fig. 13.1. Silver staining revealed five major proteins in skimmed milk: lactoferrin, bovine serum albumin, caseins, β-lactoglobulin and α-lactalbumin. Bovine serum albumin, β-lactoglobulin and α-lactalbumin are the major constituents of whey, while caseins and lactoferrin compose the other proteins. The polyclonal anti-milk and anti-whey rabbit antisera used in the study had a wide specificity: they recognized all proteins revealed by silver staining, with α-lactalbumin only slightly recognized. These results confirm that the casein fractions can contain

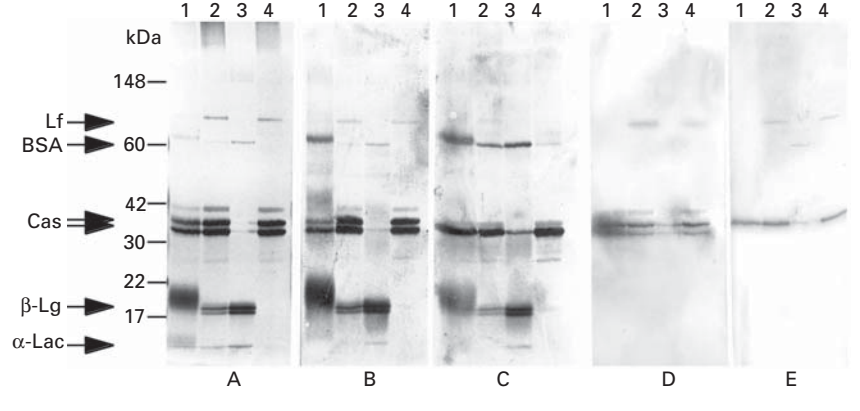

Fig. 13.1 SDS-PAGE of milk products (Leduc *et al.*, 1997). A: silver staining; B: antigens revealed with rabbit antimilk antiseruml C: antigens revealed with rabbit antiwhey antiserum; D: allergen revealed with patient's serum H[15] × M; E: allergen revealed with patient's serum H[17] × M; 1: proteic binder milk by-product; 2: skimmed milk powder; 3: whey; 4: sodium caseinate; Lf: lactoferrin; BSA: bovine serum albumin; Cas: caseins; β-Lg: β-lactoglobulin; α-Lac: α-Lactalbumin.

whey proteins, and that the whey fractions contain caseins, as also previously observed by Janssen *et al*. (1987). The use of two milk-allergic patient sera showed that their IgE recognized allergens in the four milk ingredients studied. For these two sera, major allergens were caseins and, to a lesser extent, lactoferrin.

Blotting transfer after isoelectric focusing in agarose gels

In this method, proteins are separated in agarose gels by isoelectric focusing (IEF) as described by Peltre *et al*. (1982) and are then probed using immunodetection techniques. In contrast to SDS-PAGE, this technique preserves conformational epitopes. Demeulemester *et al*. (1997) used this technique to screen milk and egg white allergens. Briefly, extracts from milk and egg ingredients were focused in agarose gels and then transferred onto CNBr-activated nitrocellulose membranes (Desvaux *et al*., 1989). After incubation of the membranes with milk- or egg-allergic patient sera, IgE-binding was revealed by alkaline phosphatase labeled anti-human IgE and chromogenic substrate. Results showed that IgE recognized caseins in six out of 17 tested sera. β-lactoglobulin and α-lactalbumin, which are not clearly separated during IEF because their pI are very close and because they have scores of isoforms (Holen and Elsayed, 1990), bound IgE from seven sera. With regard to egg white, lysozyme was slightly recognized by five out of 18 sera, ovotransferrin was substantially recognized by IgE in seven sera ovalbumin by five sera and ovomucoid by 11 sera.

A multiple successive immunoprinting after IEF in agarose gel was described by Desvaux *et al*. (1990). A protein extract is focused in an agarose gel, and then subsequently transferred to different CNBr-activated nitrocellulose membranes. Hence, different prints which are copies of the same initial gel are obtained; therefore, they have the same electrophoretic pattern except for molecules present at very low amounts. Leduc *et al*. (1999) performed this technique on raw, heat-pasteurized or heat-sterilized meat paste extracts with or without egg white (see Fig. 13.2). The first print was used to reveal egg white antigens using a rabbit anti-egg white antiserum (see Fig. 13.2a). The second and third prints were each incubated with an egg-allergic serum. Results showed that egg white antigens and allergens can be visualized in raw and pasteurized products using this technique. The IgE of patient 4 recognized ovotransferrin as allergen (see Fig. 13.2b); that of patient 1 recognized ovalbumin and another acidic molecule close to ovalbumin, probably ovomucoid (see Fig. 10.2c). None of the egg white antigens or allergens were detected in heat-sterilized products.

2D-electrophoresis

Two-dimensional (2D)-electrophoresis, initially described by O'Farrell (1975) combines IEF and SDS-PAGE. Holen and Elsayed (1990) used this technique to characterize egg white allergens and showed that in their population of egg-allergic patients, ovalbumin, ovomucoid, ovotransferrin and lysozyme were the major egg white allergens. Brodard *et al*. (1995) used this method

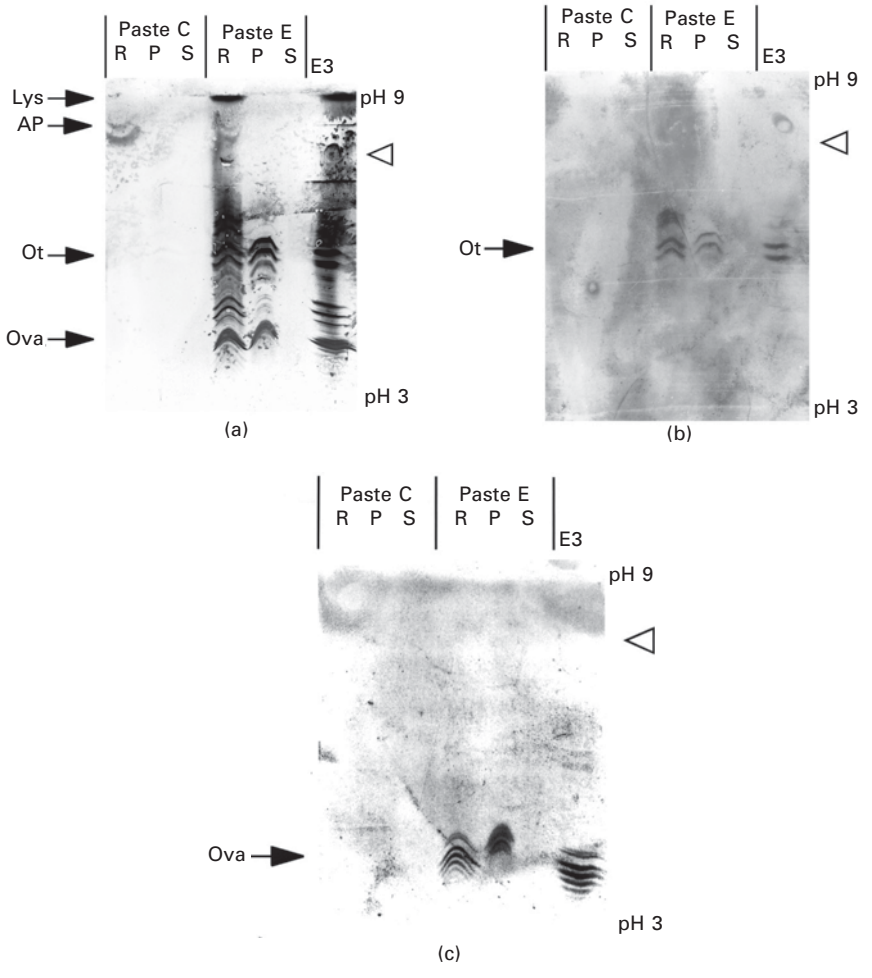

Fig. 13.2 Immunoblots from IEF separation of meat extracts (Leduc *et al.*, 1999). (a) egg white antigens revealed with rabbit antiserum; (b) allergens revealed with human serum H [4] × M; (c) allergens revealed with human serum H [1] × M; paste C: negative control meat product; paste E: meat product containing egg white; R: raw; P: pasteurized; S: sterilized; E3: frozen pasteurized egg white; Lys: lysozyme; AP: alkaline phosphatase; Ot: ovotoransferrin; Ova: ovalbumin; ◁: sample application.

to describe a 2D-map of milk allergens detected by IgE from milk-allergic patients.

13.4.6 ELISA

Many ELISA methods have been described for detection of milk proteins (Gern *et al*., 1991; Mäkinen-Kiljunen and Palosuo, 1992; Mariarger *et al*., 1994; Venien *et al*., 1997; Hefle and Lambrecht, 2004) and egg white proteins

(Breton *et al*., 1988; Rauch *et al*., 1990; Turin and Bonomi, 1994; Demeulemester and Guizard, 1996; Leduc *et al*., 1999; Yeung *et al*., 2000; Hefle *et al*., 2001; Baumgartner *et al*., 2002; Immer *et al*., 2003). Only one ELISA was reported for yolk proteins (Pressi *et al*., 1994). The specific antibodies used in these ELISA methods are IgG; thus, they do not detect the allergenic epitopes, but the antigenic epitopes. Some commercial kits are available. A list of suppliers is available in Table 6.1 in Chapter 6. Currently available ELISA methods are generally based on the detection of caseins and/or β-lactoglobulin, while commercial egg ELISA kits are usually based on the detection of ovalbumin and/or ovomucoid.

Limits of detection in processed food products depend on various parameters, such as fat content, severity of heat processing, muscle origin, state of meat maturation, etc. Hence, detection limits might be different from one product to another. From a theoretical point of view, ELISA methods are quantitative but, for the same reasons as the detection limit, results can be only semi-quantitative or qualitative unless validated appropriately.

In a collaborative study performed by two independent laboratories (Immer *et al*., 2003), 43 food products were assayed using a commercial ELISA kit for egg white detection (see Table 13.3). Of the 43, 32 had labels which mentioned 'egg', 'egg white' or 'egg yolk', egg white was detected in 27 of these 32 products and was not detected in five (505, 512, 515, 539 and 543). Among the 11 products whose labels did not mention egg, egg white was detected in one product (528) at 5 ppm. The discrepancy between egg levels found and the levels expected could be due to a number of reasons: (i) the products do not contain egg; (ii) they contain less egg than mentioned on the labels or less than the detection limits of the ELISA kit; (iii) the egg-based ingredients used or the final products were submitted to high levels of heat or other processing that led to the denaturation of egg molecules and to the lack of detection by the commercial kit.

Hefle and Lambrecht (2004) developed an ELISA for casein determination with a detection limit of less than 0.5 ppm. They analyzed retail non-milk-containing foods such as chocolates, sorbets, fruit drinks and fruit juices and found in many of them undeclared casein from 0.5 to more than 10 000 ppm. They also analyzed products associated with milk-allergic consumer complaints: casein was detected in all of them (5 500–44 500 ppm).

Detection of bovine milk proteins is also useful in quality control to differentiate milk from other species. ELISA methods were published to specifically detect cows' milk in goats' milk (Castro *et al*., 1992) or in ovine milk (Garcia *et al*., 1991; Rodriguez *et al*., 1993) as cows' milk is cheaper than ovine and caprine milk.

13.4.7 DNA methods

As discussed in Chapter 7, polymerase chain reaction (PCR) methods can be used for the detection of food allergens (Holzhauser *et al*., 2000; Dahinden

Table 13.3 Detection of egg white proteins in food products using ELISA (Immer *et al*., 2003). ELISA method used was the Ridascreen® Egg Protein kit (R-Biopharm, Darmstadt, Germany). 501–505: cheese and cheese delicatessens; 506 and 507: rice puddings; 508 and 509: custards; 510 and 511: vegetables purees; 516–520: vacuum-packed meat products; 521–524: fresh pasta; 525–533: dried pasta; 537–541: biscuits; 542 and 543: breads. *: aberrant results. A and B are the laboratories where assays were performed. Some of the foods are typical French foods.

Product No	Designation	Animal species animal proteic binders (label)	Determination of egg white (ppm)	
			A	B
501	Roquefort	Milk (ewe)	< 2	< 2
502	Cheese delicatessens	Milk (ewe, cow)/ egg white	> 5 400	35 000
503	Cheese delicatessens	Milk (cow)/egg white	> 5 400	24 300
504	Cheese delicatessens	Milk (cow)	< 2	< 2
505	Cheese delicatessens	Milk (cow)/egg white	< 2	< 2
506	Rice pudding	Milk/whole egg (7 %)	5.4	4
507	Rice pudding	Milk	< 2	< 2
508	Caramel custard	Milk/whole egg (19 %)	83	64
509	French custard	Milk/whole egg	8.8	6
510	Houmous (chickpea purée)	–	< 2	< 2
511	Aubergine salad	Whole egg	> 540	830
512	Fish rillettes	Tuna/egg white/milk	< 2	*
513	Surimi	Fish/crab/egg white	450	370
514	Shortcrust pastry	–	< 2	< 2
515	Shortcrust pastry	Egg	< 2	< 2
516	Garlic sausage	Pork/egg white	480	440
517	Cocktail sausages	Turkey	< 2	< 2
518	Pike quenelles	Whole egg/pike/milk	91	72
519	Liver pâté	Pork/milk	< 2	< 2
520	Liver mousse	Pork/milk/egg	16	28
521	Gnocchi	–	< 2	< 2
522	Fresh tagliatellas	Whole egg (25 %)	35	21
523	Fresh tagliatellas	Whole egg (14 %)	660	500
524	Fresh tagliatellas	Whole egg (11 %)	3.8	3
525	Nids d'Alsace	Whole egg (30 %)	> 5400	19 200
526	Pâtes d'Alsace	Whole egg (24 %)	> 5400	37 100
527	Crozets de Savoie	Whole egg (15 %)	> 5400	5700
528	Farfalle	–	5.4	5
529	Papillons d'Alsace	Whole egg (30 %)	> 5 400	32 500
530	Farandelles de Savoie	Whole egg (21 %)	> 5 400	25 100
531	Macaroni	Whole egg (13.5 %)	> 5 400	10 300
532	Spaghetti	–	< 2	< 2
533	Spaghetti	Whole egg (19.36 %)	≥ 5 400	4,100
534	Mayonnaise	Whole egg	> 5 400	*
535	Mayonnaise	Egg yolk	1680	850
536	Mayonnaise	Whole egg	> 5 400	8700
537	Palets Saint Michel	Whole egg	82	74
538	Shortbread	Milk/whole egg	190	122
539	Galettes bretonnes	Whole egg (3.7 %), milk	< 2	< 2
540	Biscuits cuiller	Whole egg (32 %), milk	1620	720
541	Biscuits	Whole egg (2 %), milk	380	200
542	Bread	–	< 2	< 2
543	Brioche	Milk/egg	< 2	< 2

et al., 2001; Hird *et al*., 2003). As milk contains DNA from epithelial cells (Lipkin *et al*., 1993), López-Calleja *et al*. (2004) described a PCR method to detect cows' milk in sheep and goat's milk. Eggs contain DNA which can be found in commercial egg white ingredients. DNA methods could therefore be used for detection of milk and egg. But in practice, they are not adapted for detection of egg or milk allergens because the presence of chicken meat and beef, respectively, could give false positive responses.

13.4.8 Biosensors

Surface plasmon resonance (SPR) technology (Biacore™, Biacore AB, Uppsala, Sweden) is discussed in Chapter 9. Allergen detection by SPR immunoassay is based on the measurement of total sample binding to allergen-specific antibodies coupled to the sensor surface. Purified antibodies raised against ovomucoid (egg) and β-lactoglobulin (milk) were covalently bound to a gold-dextran chip (Jonsson and Hellenäs, 2001). Calibration curves established with purified allergens showed a detection limit of approximately 10 ng/mL. No data was given for detection limits in solid food matrices. However, non-specific binding can cause false positive results when allergens are analyzed in untreated samples and total sample binding is used for quantification (Mohammed *et al*., 2001).

13.5 Future trends

Some quick test kits and qualitative methods for milk and egg are on the market now, and some kit companies plan on developing more rapid methods in the form of lateral flow devices (see Chapter 10). Commercial quantitative tests are already on the market but, as quantitation requires more time, these methods are not as 'quick' as the qualitative methods. Use of these techniques by the food industry has allowed the monitoring of egg and milk residues in the manufacturing environment, making immunoassays a useful tool for sanitation assessment and verification/validation. While to date, threshold levels for milk and egg are not known with certainty (see Chapter 1), as far as the appropriate detection limits are concerned, the methods used by kit companies for milk and egg appear to be adequate for allergic consumer protection.

Quantitative tests are unreliable unless appropriately validated, as the precise nature of ingredients used and technological treatments undergone are sometimes hard to determine. The main obstacle to the quantification in some food matrices is low extraction yield; this may be due to the denaturation or aggregation of proteins by processing and to food matrix effects (influence of lipids, Maillard reactions, etc.).

The recent development of array technologies is promising for rapid and multiparametric analysis for allergen detection in food products in the future

(Weinberger *et al*., 2000; Bashir *et al*., 2001; Lin *et al*., 2001; Zlatanova and Mirzabekov, 2001; Talapatra *et al*., 2002; Moreno-Bondi *et al*., 2003) but, at the present time, these are impractical for use in most manufacturing environments.

Quality assurance (ISO, 1999) has become widely used in laboratories. Good practice guidelines, acknowledged or normalized methods, collaborative studies, reference materials and accreditation of analysis by independent organisations are very useful tools for reliable results.

Development of milk and egg with low allergenic properties may be useful (Besler and Mine, 1999). However, they present certain disadvantages: the low allergenic products are quite expensive and do not always protect from possible reactions. Indeed, low allergenic products, prepared to decrease the number of certain allergenic proteins/peptides, may fail to protect people allergic to other proteins/peptides.

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14

Detecting wheat gluten in food

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14.1 Introduction

Although allergy to wheat is not uncommon, especially the occupational aspect of it (baker's asthma), the proteins and peptide motifs involved in IgE-mediated allergy have not been identified in detail. It is known that a broad spectrum of wheat proteins is reactive with sera from wheat-allergic persons. Battais *et al*. (2003) found, by using radioallergosorbent tests (RAST), that 60% of sera from their wheat-allergic subjects had IgE and IgG antibodies against α/β-gliadins and low molecular weight (LMW) glutenin subunits, 55% to γ-gliadins, 48% to ω-gliadins and 26% to high molecular weight (HMW) glutenins. These results were confirmed with immunoblotting, by which it was also shown that 67% of patients have IgE antibodies to the albumin/globulin fraction. Heat processing of wheat dough seems to render these proteins resistant to breakdown. It has been shown by *in vitro* digestion that, while unheated wheat doughs lost their IgE-binding properties during digestion, the IgE-binding potential was retained in heat-processed dough (Simonato *et al*., 2001).

Even although antibodies to whole wheat proteins (including albumins) are commercially available, most of the research to detect wheat proteins is directed towards proteins involved in the pathogenesis of quite another affliction: coeliac disease, a non-IgE-related hypersensitivity in which the toxic principle is the storage protein (gluten) from some cereal species.

Although this disease is different from IgE-mediated allergy, the eliciting proteins are shared to a large extent and, as long as these proteins stick together, testing for one of them will be proof that wheat is present. Because the prevalence of coeliac disease is increasing tremendously, primarily due

to increased awareness and improvements in diagnosis, emphasis is placed on the determination of proteins involved in the pathogenesis of this disease. Wheat-allergic persons may, however, use the same sources of food in complying with a wheat-free diet that coeliac sufferers do.

14.1.1 Coeliac disease

In this introduction, some aspects of coeliac disease, such as history, aetiology, pathology, prevalence, toxic cereals, dietary aspects and developing legislation (Codex, EU), will be covered. The first description of coeliac disease or coeliac sprue was given in 1888 by Samuel Gee. Our modern knowledge concerning the exacerbating factors, the cereal prolamines from wheat, rye and barley and oats, dates back to the years that followed World War II. It was then that the Dutch paediatrician Willem Karel Dicke (1950) confirmed his suspicion about the presumptive toxicity of wheat flour with carefully designed experiments which led ultimately to the conclusion that wheat, rye and barley, and possibly oats were toxic to some of his coeliac patients. Soon afterwards it was discovered that the deleterious factor resided in the protein fraction of the wheat, notably in the alcohol-soluble part, the gliadin.

In 1954 Paully presented the first description of the coeliac lesion, being an atrophy of the villi of the small bowel caused by gluten ingestion, and after that a biopsy of the jejunum, the proximal part of the small bowel, became the hallmark of the diagnosis. Recently, non-invasive diagnostic methods have been developed based on serum parameters like antiendomysial IgA, anti-gliadin IgA and anti-tissue transglutaminase (reviewed by Dieterich *et al*., 2000). These tests have made screening of non-suspect populations feasible, which has revealed that the prevalence of this disease is much higher than was previously thought. A prevalence of 1:300 was found in Italy by Catassi *et al*. (1994) and afterwards similar screening showed that in other European countries, the prevalence was much higher than previously thought. In the US, Fasano *et al*. (2003) reported that 1:133 persons had coeliac disease. Many of these screening-diagnosed persons, however, had no clinical symptoms, although this ‘silent’ disease is believed to become overt later in life and may, if it remains undiagnosed, cause osteopenia and iron deficiency, among other things.

Coeliac disease manifests itself in children with an array of symptoms, among which the most prominent are poor growth and weight loss, diarrhoea and increased fat excretion in the stool (steatorrhoea). This is the ‘classical’ picture. Given the large number of patients who are detected by serological screening without ever presenting with these classical symptoms, this picture of coeliac disease is now subject to change. There is a strong association of coeliac disease with some other diseases, in particular, dermatitis herpetiformis (DH) which has been named by some as ‘coeliac disease of the skin’, even although this particular condition does not improve solely by compliance to a gluten-free diet, but requires further medication.

Coeliac disease is currently considered to be an autoimmune disease and it is believed that it predisposes one to other autoimmune disorders such as type I diabetes, autoimmune thyroid and other endocrine diseases. One of the most serious side effects is osteopenia, while increased risk of malignant diseases, especially intestinal lymphomas derived from mucosal T-cells, reproductive failures, neurological disorders and autoimmune diseases, has also been reported (James and Scott 2001; Pengiran Tengah *et al*., 2002; Vestergaard P, 2003). As there are indications that a gluten-free diet protects coeliac patients against the development of these complications, the importance of compliance to such a diet is stressed. As there is no cure, a lifelong compliance to a gluten-free diet is the only way to alleviate symptoms.

Coeliac disease is believed to have a genetic component because there is a strong association of the disease with the presence of specific HLA class II antigens, predominantly HLA DQ2 and (to a lesser extent) DQ8 (reviewed by Louka and Sollid, 2003). Either of these is needed to develop the disease, although the majority of DQ2 and DQ8 positive persons are coeliac-negative which indicates that more genes are involved. It is believed that the proteins implicated in the disease, the prolamins, bind to the DQ2 or DQ8 molecule, giving rise to T-cell activation and triggering a sequence of events leading to full-blown disease. Questions arose as to how this occurrs, because the prolamines, given their hydrophobic nature, should not bind very strongly to the DQ2/DQ8 peptide binding groove. It was found by Sjöstrom *et al*. (1998) that T-cell reactivity is enhanced by the action of tissue transglutaminase, which deamidates specific glutamine residues of gliadin and glutenin peptides. Extensive research has shown that many sequences (deamidated or not) react with susceptible T-cells, and it has now been found that these reactions are to some extent idiotypic, i.e. different between patients.

A role for the innate immune system has also been hypothesized. The innate immune system consists of a variety of immune responses that constitute the first line of defence against the invasion of foreign substances, e.g. pathogens. Its response to challenge is ahead of the induction of the adaptive immune response, is not antigen-specific and is without memory. Activation at inappropriate sites and/or to an excessive degree can exacerbate tissue damage in several diseases, and a possible role in the first stages of coeliac disease has been suggested (Maiuri *et al*., 2003).

14.1.2 Gluten proteins

The storage protein of wheat, gluten, is composed of monomeric gliadins and polymeric glutenins. Wheat gluten has the unique capacity of forming a highly cohesive, elastic dough which prevents the escape of gas bubbles formed by the yeast cells during leavening. Wheat gluten can be prepared by kneading an amount of wheat flour into dough and washing this dough under running tap water to remove the starch particles until the remaining, chewing-gum-like residue is starch-free. In industry, more sophisticated procedures

are used. Traditionally, cereal proteins have been classified into four types according to their solubility (Osborne, 1907):

- **albumins** – soluble in water or dilute salt solutions;
- **globulins** – insoluble in water, but soluble in dilute salt concentrations and insoluble at high salt concentrations;
- **prolamins** – soluble in aqueous alcohol;
- **glutelins** – soluble in neither water nor dilute salt solutions but soluble in dilute acid or alkali.

An alternative classification to that described above has been proposed based on chemical similarity of reported amino acid sequences of individual components rather than on differential solubility.

A comparison between these nomenclatures is given by Shewry *et al.* (1986). In fact the LMW glutenins can also be considered as aggregated gliadins, because their composition is comparable to the α-gliadin fraction. The difference is that they are present in a polymeric form.

The gliadins and glutenins are characterized by a high content of the amino acids glutamine (Q) and proline (P), ca. 30 and 17 mol%, respectively. They lack nutritionally important amino acids like tryptophan, lysine and methionine which makes their nutritional score rather low. They also lack any enzyme activity and are generally considered as a nitrogen source for the germinating seed. Gliadins are classified into fractions, according to their mobility in an electrophoretic field, as α-, β-, γ- and ω-gliadins: the differences between the first two fractions are small, and they are commonly designated as α/β. A unique property of ω-gliadin is that it does not contain cysteine and is not involved in disulfide exchange.

Which part of the prolamin is toxic?

When the coeliac toxicity of wheat was established, a quest to establish which part of the wheat contained the toxic principle began. Older research confined the toxicity to the prolamin fraction of the wheat protein and not the glutenin portion. More recently, however, T-cell toxicity of glutenin has been described (Van de Wal, *et al.*, 1999). Extensive research has been performed to elucidate the peptide structures that precipitate the disease in susceptible persons. As mentioned before, coeliac-toxic cereals are wheat, rye, barley and their cross-bred varieties, while the toxicity of oats is debated. Oats can be easily contaminated by wheat or barley since the these crops are often grown in close proximity. Quite recently it has also been found that some coeliacs have avenin-reactive mucosal T-cells pointing to intrinsic toxicity of oats. Clinical follow-up of coeliac disease patients eating oats is consequently advisable (Arentz-Hansen *et al.*, 2004).

Buckwheat, maize, rice, sorghum and millet are considered to be non-toxic. Research has been focused on wheat because this is the most abundant species. Clinical testing of gliadin fractions showed that all fractions (α-, β-, γ- and ω-gliadin) were toxic (Ciclitira *et al.*, 1984). Subsequently, in

a series of experiments Wieser *et al.* (1983) isolated gliadin fractions by reversed-phase HPLC and prepared peptides by enzymatic digestion. Further research on isolated peptides showed that those derived from the N-terminal part of the protein (residues 25–55, 1–30, 31–55) were toxic (De Ritis *et al.*, 1988). Much work has since been done with panels of synthetic peptides to narrow the search for the precipitating motif, and many sequences have been found to be toxic (reviewed in Stern *et al.*, 2001). Recently, much emphasis has been placed on T-cell toxicity, and it has been shown that many gliadin and gluten sequences are toxic to T-cell populations of coeliac patients (Koning, 2003).

14.1.3 Regulatory framework for gluten (gliadin) in gluten-free dietary products

A Codex Standard for gluten-free foods was adopted by the Codex Alimentarius Commission in 1976 and published in 1981 as Codex Stan 118, 1981. This standard defines gluten as 'those proteins, commonly found in wheat, triticale, rye, barley or oats to which some persons are intolerant.' In the context of the standard, 'gluten-free' means that the total nitrogen content of the gluten-containing cereal grains used in the product does not exceed 0.05 g per 100 g on a dry matter basis. It is easy to become tangled up by this definition, however, because, although the nitrogen content can be translated into a gluten content by applying the conventional nitrogen conversion factor and correcting for non-gluten protein, the level seems to have been validated only in wheat starch. In wheat starch the aforementioned conversion is not valid because most of the protein is present as 'starch granule protein'. The gluten content of a wheat starch product containing 0.05 g/100 g product is (was) thought to be about equivalent to 200 mg/1000 g and this was formerly supposed to be a safe limit. Nowadays more is known about nitrogen/gluten ratios in wheat starch and it has become clear that the measurement of nitrogen in this matrix can be used only as an approximation of the gluten content. Because most gluten-free products consist of mixtures of non-coeliac-toxic cereals like maize or rice, it is mandatory to measure specifically the gluten content and not the nitrogen content of a product, as the latter would be meaningless in this situation.

The 118–1981 Codex Alimentarius standard is nowadays considered to be outdated, because the threshold is too relaxed for foods which are gluten-free by nature (i.e. maize or rice flours). In addition, the confusing definition of 'gluten-free' needs to be revised. A new standard has been elaborated by the Codex Committee on Nutrition and Foods for Special Dietary Uses (CC NFSDU) but as of this writing this project has not been finalized. A value of 20 mg gluten/kg product has been proposed as a statutory limit for gluten-free food that is 'naturally gluten free', e.g. maize flour and rice flour, and this proposal seems to be universally endorsed. No consensus has yet been reached about food *rendered* gluten free, like wheatstarch,

where the proposed limit of 200 mg/kg has been rejected by coeliac organizations.

14.2 Key requirements for detection and quantization

14.2.1 Accuracy and broad scope

Gluten-free foods will probably be the first type of food where threshold values for the toxic principle will be established. This implies that litigation is likely to occur and, consequently, high standards of reliability are required. It is imperative that methods are highly accurate and sufficiently sensitive to measure well below the levels being proposed as thresholds or already laid down in food regulations. In addition, the method should allow the determination of gluten in a wide range of foods that may have been processed under a variety of conditions (i.e. the method should have a broad scope). In the past, one of the major concerns has been to find a method able to measure gluten in heat-processed food. There are no reports that gluten toxicity is abolished by heat processing and, consequently, the method should be able to measure gluten in food that has been prepared under a wide variety of conditions. Wheat gluten is a key factor in achieving good texture in bakery products. During kneading, leavening and baking of dough, there is an exchange of disulfide bonds which (helped by the action of oxidizing agents) results in insolubilization of the gliadin fraction.

14.2.2 Reference material

Determination of gluten (gliadin) is subject to several constraints, one of which is the reference material used. All the methods published so far are relative methods, and the analysis is conducted with the help of a calibration curve made by measuring known concentrations of a reference substance. It has been shown that the origin and type of gliadin used for calibration has a tremendous impact on the results of the analysis (Van Eckert *et al*., 1998). This fact prompted the European Working Group on Prolamin Analysis and Toxicity (WGPAT – see Section 14.7) to initiate the development and production of a reference material produced from a mixture of the 28 most common European wheat cultivars (Van Eckert *et al*., 2002; 2005, Klein *et al*., 2003). This standard is currently being further evaluated by the EU Institute for Reference Materials and Methods (IRRM) in Belgium, but it is already available from this institute (see Section 14.7).

14.3 Types of detection methods

The vast majority of methods which have been published so far are of the

enzyme-linked immunosorbent assay (ELISA) type (Table 14.1). A key requirement in detecting and quantifying gluten is specificity. The method should detect (coeliac) toxic cereals and produce negative results with cereals considered non-toxic to coeliacs such as rice, maize, sorghum, millet, etc. Of all methods published so far, ELISA methods, which have been developed from the early 1980s on (Windemann *et al*., 1982; Meier *et al*., 1984), form the bulk, due to unrivalled specificity and ease-of-use. In the following, several methods are discussed with the emphasis on those having been made commercially available as kits.

14.3.1 ELISA with antibodies to ω-gliadins

Work of sterling quality has been done by John Skerritt's group at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Australia. They developed the first method of determining gluten to be made available in kit form, and it was also the first method designed to assess the gluten content of heat-processed food (Skerritt *et al*., 1984; Skerritt, 1985; Skerritt and Smith, 1985; Skerritt and Martinuzi, 1986; Skerritt and Hill, 1990, a, b, c).

Events occurring during heat processing of foods are quite complicated. It has been found by Schofield *et al*. (1983) among others that baking quality decreased with heating and was completely destroyed at 75 °C. This was parallelled by a decreased extractability in a sodium dodecyl sulphate (SDS) containing buffer. With gel filtration techniques it was found that predominantly glutenin proteins were destroyed. Extractability of gliadins remained intact until 75 °C, but decreased rapidly when the dough was heated at higher temperatures, and at 100 °C only the ω-gliadins remained soluble, presumably because this fraction did not contain sulfhydryl groups. When a reducing agent such as dithiothreitol (DTT) was added, the extractability could be restored. It is assumed that unfolding of glutenin at elevated temperatures promotes sulphydryl/disulfide exchange not only between glutenin units but also between glutenin and gliadin, and that this exchange becomes fixed upon cooling or heating at temperatures higher than 75 °C. This decrease in extractability was also found by Weegels *et al*. (1994a, b) and confirmed by Rumbo *et al*. (1996, 2001), who found that the effect of heat was almost absent when flour was heated in dry conditions; Wieser (1998) found this also.

The novel aspect of the method developed by Skerritt *et al*. is that it uses a selected monoclonal antibody (mAB) against the most heat-tolerant gliadin fraction, the ω-gliadin. The method is available as a kit and a collaborative trial has been carried out. The method has been approved by the AOAC (Skerritt and Hill, 1991; AOAC, 1995).

Over time it was found that the method had some shortcomings which were difficult to overcome. The first one was the sensitivity. As ω-gliadin is a relatively minor fraction, the method has limited sensitivity compared to

Table 14.1 Overview of immunochemical assays for gluten quantification in food

Year	Number	Reference	Layout	Antibody[1]	Protein	Source	Epitope	Sensitivity[2]	Reactivity[3]	Cross-reactivity[4]	Extraction	Remark
1982	1	Windemann *et al*., 1982*	Sandwich	pAB	A-gliadin	Wheat	Unknown	1 ng/ml (A-gliadin) 10 ng/ml (gliadin)			70% ethanol, 40 °C	
1983	2	Ciclitira and Lennox 1983		pAB	α-gliadin	Wheat	Unknown	1 ng/ml	α-gliadin, no reaction with γ- and ω-gliadin, neither with barley, rye and oats			[5]
1986	4	Troncone *et al*., 1986	Sandwich	pAB	Gliadin	Wheat	Unknown	5 ng/ml		Maize, rice		
1987	5	Freedman *et al*., 1987		pAB/mAB	Gliadin	Wheat	Unknown	15 ng/ml				
1988	6	Friis 1988	Competitive	pAB	Gliadin	Wheat	Unknown	1 ng/ml	Wheat >>> rye >> barley > oats		70% ethanol	
1988	7	Ayob *et al*., 1988		pAB	Gliadin	Wheat	Unknown	30 ng/ml				
1989	8	Mills *et al*., 1989		Capture: IgY, Detection: mAB	Gliadin	Wheat	Unknown					[6]
1990	9	Skerritt *et al*., 1984–1990, a,b,c	Sandwich	mAB	ω-gliadin	Wheat	Unknown	100 ng/ml	Wheat > rye >> barley > oats		40% ethanol	[7]

Table 14.1 *Continued*

Year	Number	Reference	Layout	Antibody[1]	Protein	Source	Epitope	Sensitivity[2]	Reactivity[3]	Cross-reactivity[4]	Extraction	Remark
1994	10	Ellis e*t al*., 1994		pAB/mAB	Gliadin	Wheat	54 amino-acid peptid N-terminal	15 ng/ml	Wheat = rye >> barley >> oats			
1995	11	Chirdo *et al*., 1995	Competitive	pAB	Gliadin	Wheat	Unknown	1 ng/ml	Wheat >> barley > rye >> oats		70% ethanol after pre-extraction of albumins/ globulins by 0.15 M-NaCl	
1995	12	Albrecht and Toth, 1995	Sandwich		Gliadin	Wheat	Unknown	25 ng/ml		Maize		
1998	13	Ellis *et al*., 1998	Sandwich	pAB/mAB	Gliadin	Wheat	A-gliadin 31–49	4 ng/ml	Wheat >> rye > barley = oats		40% ethanol or 50% propane-1-ol with 1% M.E in buffer	
1998	14	Chirdo *et al*.,1998	Sandwich	mAB	Gliadin	Wheat	Unknown	20 ng/ml			70% ethanol after pre-extraction of albumins/ globulins by 0.15 M-NaCl	
1998	15	Chirdo *et al*., 1998	Competitive	mAB	Gliadin	Wheat	Unknown	5 ng/ml				
1998	16	Chirdo *et al*., 1998	Capture	mAB	Gliadin	Wheat	Unknown	1 ng/ml				

Table 14.1 *Continued*

Year	Number	Reference	Layout	Antibody[1]	Protein	Source	Epitope	Sensitivity[2]	Reactivity[3]	Cross-reactivity[4]	Extraction	Remark
1998	17	Sorell *et al*., 1998	Sandwich	mAB 2mAB	Secalin	Rye / wheat	Unknown	3 ng/ml	Rye > wheat = barley >>> oats		Ethanol 60%	
2003	18	Valdez *et al.* 2003	Sandwich	mAB	Secalin	Rye	QXPW/FP	1,5 ng/ml	Barley = rye = wheat >>> oats		Ethanol 60%	[8]
2003	19	Henterich *et al*., 2003	Direct, signal amplified with PCR	mAB	Secalin	Rye	As 18	0,16 ng/ml	As 18		Reducing agent with guanidine hydrochloride	
2004	20	Spaenij-Dekking *et al*., 2004	Competitive	mAB	α-gliadin and γ-gliadin	Wheat	α-59-71, γ142-153 and γ147-159	12 ng/ml	Barley = wheat = rye >>> oats		Ethanol 40%	[9]

* in German
[1] pAB means polyclonal antibody; mAB means monoclonal antibody
[2] sensitivity is given in ng/ml. Actual detection level in food is depending on amount of food extracted
[3] reactivity: the sensitivity with respect to the various prolamins is stated
[4] cross-reactivity: if unspecific, cross-reacting cereal species are stated
[5] radioimmunoassay, Staphylococcus *aureus* used to separate bound and free^{125} iodinated antigen
[6] designed to detect conformation-dependent epitopes in wheat
[7] ringtested in AOAC framework
[8] alternatively an extraction is carrried out in a reducing environment containing guanidine hydrochloride
[9] uses ABs detecting T-cell epitopes

methods using antibodies against a broader spectrum of wheat gliadins. This shortcoming could be alleviated by modifying the reporter system (replacing the initially proposed ABTS substrate by the more sensitive tetramethylbenzidine–TMB). Another option would be to dilute the initial extract less than prescribed, but this would have negative effects on the specificity of the assay.

More serious, however, has been the finding that the ω-gliadin content varied considerably in European wheat varieties. Wieser *et al*. (1994) found that the relative contribution of ω-gliadin to the gliadin pool could vary between 6.2 and 20%, depending on the wheat variety, which means that, related to the ω-gliadin content of the standard, the values could be over- or underestimated by a factor of 2–3.

Recent research by Seilmeier and Wieser (2003) showed that other factors also contributed and that the between-cultivar variation (as determined with the anti-ω kit) was even higher than could be accounted for by ω content as determined by reversed-phase HPLC. It is suggested that frequency or spacing of ω epitopes or non-specific binding of α-gliadin could be the cause of these discrepancies.

Because wheat products in trade are often not present as pure varieties, the effect of variable ω-gliadin/total gliadin ratios might not be as detrimental as it seems at first sight. Using a polyclonal competitive assay, Rumbo *et al*. (2001) found a good correlation between the standard used in the ω-based commercial kit and a gliadin purchased through Sigma Chemical Company (St Louis, MO). The variable ω content was refuted by Skerritt (personal communication) with the argument that in some instances the ω-gliadin in the reference material had been washed out during gliadin preparation. Omega-gliadin is the most soluble gliadin, especially in water at room temperature and above, and is also poorly extracted in 70% ethanol. This view was endorsed by Kasarda (personal comment in Ellis *et al*., 1998).

14.3.2 ELISAs in a competitive format

Principally, ELISAs in a competitive format have the advantage that no multiple or repetitive epitope is needed, in contrast to the majority of sandwich ELISAs. An assay in a competitive format would thus be ideal to determine enzymatically degraded gluten in those situations where such degradation is to be expected, e.g. in malted cereals or ‘solubilized’ wheat protein. Friis (1988) developed a sequentional competitive assay based on a polyclonal, Protein A-purified antiserum against gliadin. The specificity of the antibody was checked by immunoblotting and dot blot. The antibody reacted with α-, β-, and γ- as well as ω-gliadin.

Antibody and sample extract diluted in bovine seralbumin (BSA)/Tween 20 ® (ICI America Inc, Wilmington, DE) mix containing 0.02 mmol DTT was incubated for 16 hours. Polystyrene plates were coated with an optimized amount of gliadin, washed and incubated for 30 minutes with the antibody/

antigen mix for 30 minutes at 20 °C. After washing, horseradish peroxidase (HRP) conjugated swine anti-rabbit IgG, 1:700 diluted in phosphate buffered saline (PBS) containing 0.05% Tween 20 ® and 0.5% BSA was added and the plate was incubated again for 30 minutes at 20 °C. *O*-phenylenediamine (OPD) was used as the peroxidase substrate. The assay had a detection limit of 1 ng/ml. The antibodies, however, showed poor reactivity against rye prolamins and even lower towards barley and oat prolamins. A gliadin-like response was found in several commercial flours, e.g. buckwheat (may have been contaminated) and wheat starch (which always contains residual gluten).

A different, sequentional assay was developed by Chirdo *et al*. (1995) using a polyclonal antibody against gliadin. A pre-incubation in the liquid phase was carried out by mixing standard or sample extracts at appropriate dilutions with antibody dilutions for a predetermined time and temperature (optimal 2 hours at 37 °C). An aliquot of the mixture was subsequently incubated in microtiter wells precoated with an optimized amount of gliadin. Binding of the antibody to gliadin on the wall was visualized by incubating with goat-anti-rabbit IgG HRP conjugate. The optimized assay had a sensitivity of 1 ng/ml which enabled determination of 1 ppm gluten at a sample dilution of 1/50.

It was found that the effect of the extraction solvent (ethanol) was pronounced. Elevated ethanol concentrations in the final incubation mix (0.8–4% when 40% ethanol had been used) resulted in a marked decrease in signal, especially when sample dilutions of 1/50 or 1/10 were used. This could result in an under-estimation of the gliadin content by 2–40%, an outcome which stresses the importance of using high working dilutions. Consequently, a test with high sensitivity allowing for high sample dilutions is preferred in enforcing low statutory gluten threshold values.

The same authors (Chirdo *et al*., 1998) investigated the use of the biotin/streptavidin amplification system using three different mABs developed against commercial gliadin. Three formats were compared: a sequentional competitive, a capture ELISA and a competitive assay using biotin-labelled gliadin. The first had a detection limit of 20 ng gliadin/ml with the most sensitive mAB; the capture format had a sensitivity of 1 ng/ml with a different mAB – the mAB which showed the best performance in the sequentional competitive format gave only poor results. Although there are data suggesting that binding to plastic surfaces is often the cause of structural changes, this seemed not the case here, as all antibodies were equally able to bind biotinylated gliadin. The authors suggest that the cause might have been that the mAB react with epitopes present at a low density. The competitive assay using biotin-labelled gliadin showed a sensitivity of 5 ng/ml. The authors also suggest that the latter type of assay may be used in determining heated or enzymatically degraded prolamin peptides.

14.3.3 ELISAs with mABs and extraction under reduced conditions

Basically, two means are used to circumvent impaired extraction efficiency in products that have been heat processed: the use of an antibody against

heat stable gliadin fractions or the use of reducing agents and/or chaotropic agents to reverse the disulfide interactions that take place during dough kneading and baking.

The latter approach was investigated by Sorell *et al*. (1998) and García *et al*. (2005), and a patent has been applied for this extraction procedure (WO 02/092633). An amount of 0.25 g of a food is extracted with 2.5 ml of a solvent containing 250 mM 2-mercaptoethanol and 2 M guanidine hydrochloride in PBS. The extract is kept at 50 °C for 40 minutes and is then diluted with 7.5 ml 80% ethanol, after which it is vortexed and incubated for one hour at room temperature in a rotary shaker. After centrifugation, the extracts are ready to be used. A panel of antibodies was generated against rye prolamins. Out of this panel, two, R3 and R5, were chosen for the assay in addition to another, 13B4, which has been described elsewhere (Ellis *et al*. 1998). A mixture of R5 and 13B4, which have complementary selectivities (R5 reacting with secalins and hordeins and 13B4 with gliadins), was used to coat the wells. This multiple coating consequently allowed the binding of all three cereal species. A third antibody (R3) was conjugated with horseradish peroxidase and used as a reporter antibody. The assay thus developed was able to detect gliadins, hordeins and secalins at about the same sensitivity. The detection level as reported by the authors was 1.5 ng/ml. No combination of the R-monoclonals was sensitive enough to extend the sensitivity to oats: a separate method was developed to detect this cereal.

A modification of this assay was described by Valdés *et al*. (2003). In this assay (sandwich format) just one monoclonal (the R5) antibody was used for capturing as well as detection. The molecular recognition pattern of this antibody has been investigated by Osman *et al*. (2001) by phage display and pepscan. With both techniques a pentapeptide consensus sequence was found (QXPW/FP, resp. QQPFP). This sequence is one of the amino acid motifs believed to be involved in the pathogenicity of coeliac disease. It is present in a high number of copies in γ- and ω-gliadin, and in a smaller number in α-gliadin and LMW glutenin. The presence of an epitope in LMW glutenin poses problems with respect to correct calibration of the assay because, when comparing to a *gliadin* standard, part of the *glutenin* will be reported as gliadin. In this assay, however, using the same mAB for capture and reporting, this might not lead to substantial errors because multiple epitopes would be needed for detection of glutenin, and LMW glutenin contains only one. In addition, the number of epitopes on γ- and ω-gliadin outweighs the number of epitopes on the LMW glutenin. The authors report a sensitivity of 1.56 ng gliadin/ml corresponding to 1.5 mg/kg in food using a low dilution of 1:25. The reproducibility and repeatability was stated to be 8.7% and the repeatability 7.7% in a small scale investigation.

A collaborative trial was organized in 2002 by the WGPAT (see Section 14.7), the provisional results of which have been published (Immer *et al*., 2003; Immer and Haas-Lauterbach, 2004). Twelve foods were analysed, of which four were spiked with the reference gliadin developed by the WGPAT

in amounts between 35 and 168 mg/kg. The performance of two R5 antibody-based kits from two different manufacturers was compared. Mean recoveries were quite good, ranging from 71–104% with one kit and from 79–111% with the other. The relative standard deviations (RSDs) varied between 22 and 52. Compared to the method as developed by Skerritt, these methods have the advantage that the reactivity shows a better correlation with coeliac toxicity and that they have a broader specificity, allowing the determination of wheat, as well as rye and barley. Given the high abundance of the QQPFP repeat, a strong dependence on ω content of the cultivar might be present.

This 'R5' method has been endorsed by the Codex Alimentarius Committee Methods of Analysis and Sampling (CC MAS) on a temporary basis pending publication of a full statistical report (see report of the twenty-sixth session of the Codex Committee on methods of analysis and sampling, Budapest, Hungary, 4–8 April 2005). A publication of the statistical report of the collaborative trial is in preparation (Méndez *et al.*, 2005, submitted). The method is available as a kit produced by two manufacturers, R-Biopharm (Darmstadt, Germany) and Ingenasa (Madrid, Spain) and through other distributors.

14.3.4 ELISAs with monoclonal antibodies against toxic peptide motifs

Polyclonal antibodies for use in gluten detection methods are regularly prepared by immunizing a rabbit with a gliadin preparation. The antibody can afterwards be purified with standard biochemical techniques (e.g. Protein A-like affinity chromatography) and absorbed to remove unspecific antibodies. The resulting antibody quite often doesn't have the required profile. By producing mABs against whole gliadin, there is a greater possibility of selecting antibodies with the required specificity, but there is no guarantee that this selectivity reflects the toxicity of the distinct cereals.

To overcome this bias, Ellis *et al.* (1998) raised mABs against a synthetic 19-mer of the toxic α-gliadin peptide LGQQQPFPPQQPYPQPQPF (31–49 of α-gliadin) and to point-substituted peptides in which amino acids 31, 33, 36, 38, 39, 41, 42, 44, 46 and 48 were replaced by alanine. A polyclonal anti-unfractionated gliadin antibody was used as a capture antibody in a sandwich ELISA. Gliadin fractions were prepared and standard solutions were made from these fractions as well as from whole gliadin cv. Rektor and cv. Timgalen. The standards were prepared in 40% ethanol and diluted to 100 ug/ml with water. After filtration, the protein content was determined.

The sensitivity of the assay as determined with the prepared prolamins varied from 4 ng/ml (whole gliadin and α-gliadin), 16 ng/ml (β- and γ-gliadin) and 1000 ng/ml for ω-gliadin. Sensitivity against rye, barley and oat prolamin was respectively 500, 1000 and 1000 ng/ml. The assay was tested for ruggedness with respect to several aspects: incubation time, repeatability and reproducibility, and storage stability. The amino acid substitutions revealed that substitution of amino acid Q (33), P (36) and P (38) with alanine reduced

binding substantially, suggesting that the binding was in the region QQQPFP. This region is present in α- and ω-gliadin. The truncated motif is present in avenin (QQQPF) and the more truncated motifs, QQQP and QQPFP, are present in γ-gliadin, hordein and secalins. The reactivity to ω-gliadin is lower than expected given that it is present in this fraction. The authors suggest that this might be due to a lower reactivity of the capture antibody to ω-gliadins.

Wheat starches, gluten-free ingredients and gluten-free products as well as spiked samples were analyzed. The initial extract was made by mixing 50 mg product with 1 ml 40% ethanol for one hour at room temperature, after preliminary defatting twice with four volumes in n-butanol. In a separate experiment, cooked crust and crumb of a loaf prepared from flour spiked with 1% var. Rektor gliadin (spiked before the stage of dough formation) and which had been baked at 230 °C for 10 minutes were analyzed. This loaf was extracted at 60 °C under nitrogen atmosphere for one hour with a reducing buffer containing 50% propane-1-ol, 1% mercaptoethanol, 0.08 mol/l Tris-HCl (pH 7.5) and 2 mol/l urea. In this case, the calibration curve was made up in the reducing buffer diluted 100-fold. Prolamins from coeliac non-toxic rice, maize, millet and sorghum as well as presumably gluten-free ingredients of miscellaneous origin did not cross-react in the assay. The assay could detect gluten in cooked foods, although at reduced sensitivity, the recovery being about 17%. Use of reducing agents was not successful, presumably because they denatured the capture antibodies.

This method might offer good prospects as long as non-reducing conditions are used. The motif QQQPFP is, however, also present in LMW glutenins (which become solubilized under reducing conditions) and this raises the question of what should be taken as a reference material: gliadin or whole gluten. However, at present this issue is not relevant; it appears that the antibodies do not tolerate the reducing agent mercaptoethanol at a concentration of 0.01% and the more efficient extraction is traded off by this phenomenon.

A puzzling phenomenon is the difference between the antibody used in this assay (against QQQPSP) and the R5 used by Sorell *et al*. (1998) and Valdés *et al*. (2003) (against QQPSP) with respect to the difference in reactivity to γ- and ω-gliadin and cereals like rye and barley. What a difference a Q makes! A variety of commercially available gluten-free foods were analyzed and small quantities of gluten were detected in some products.

Of course it would be feasible to design antibody-based assays against other peptides as well. Fraser *et al*. (2003) investigated the toxicity of a sequence containing residues 56–75 of α-gliadin, which proved to be toxic *in vivo*. This peptide would consequently also be a candidate to design an ELISA around. A problem not yet solved is: how many toxic motifs will be found in the future and how should they be weighed in the final result to get a realistic representation of toxicity?

14.3.5 ELISA of native, heat processed and modified gluten after limited hydrolysis

To improve the extraction of gluten, a procedure with a limited hydrolysis was investigated by Denery-Papini *et al.* (2000, 2002). Food or reference material was hydrolyzed with pepsin, which led to about 90% protein extraction in a saline buffer. Monoclonal and polyclonal antibodies were used against repetitive motifs of α-, β- and γ-gliadins. In a competitive-type assay, 35 ppm could be determined with the mAB and 150 ppm with the pAB.

The assay was improved by using anti-γ-gliadin developed against the repetitive domain of γ-gliadins. This domain was isolated and purified with size-exclusion chromatography and reversed-phase HPLC and was found to correspond to a polypeptide of 28 kD. The mAB reacted strongly with ω-gliadins and with α-, β- and γ-gliadins and only weakly with LMW and HMW glutenins. No difference in reaction between native and heated products was found. Detection of hordeins was at same level as gliadins, secalins could be detected at a level ten times lower, and avenins 100 times higher, than gliadin. No reaction occurred with maize and rice. Sensitivity was 0.3 ug/ml, allowing determination at the 150 ppm level.

14.3.6 Other antibody-based methods

As a quantitation of the gliadin is necessary, given the proposed threshold values mentioned in the Codex Alimentarius Standard, immunotechniques which do not produce quantitative results like dot blot, counter-immunoelectrophoresis or immunodiffusion are inadequate. Combining an electrophoretic separation with immunodetection yields a method with very powerful features which can be used conveniently to confirm the results of ELISA tests. The method is very weak with respect to quantitation, as the process of densitometric quantitation of the separated (immunochemically) stained bands in relation to a standard is very complicated. Depending on the electrophoretic method used, the band pattern is often very variable. Acid polyacrylamide gel electrophoresis (PAGE) has been used frequently in cultivar identification. In SDS-PAGE this variation is less, as the separation is based on molecular weight, which is a more conserved attribute, but even this type of electrophoresis often produces a cultivar-dependent band pattern. SDS-PAGE with a general staining like Coomassie Blue has been used extensively in breeding programs, especially because the HMW subunits of glutenin can be solubilized and separated. In SDS-PAGE the sample is prepared by heating in a buffer consisting of buffered SDS with a reducing agent like mercaptoethanol or DTT. Almost all proteins of a food matrix, even when heat-processed, will be solubilized, and consequently, this method offers good potential to determine gluten in heat-processed samples. The variable band pattern, however, quite often precludes an accurate quantitation, although samples may be grouped into classes (e.g. contains between 100

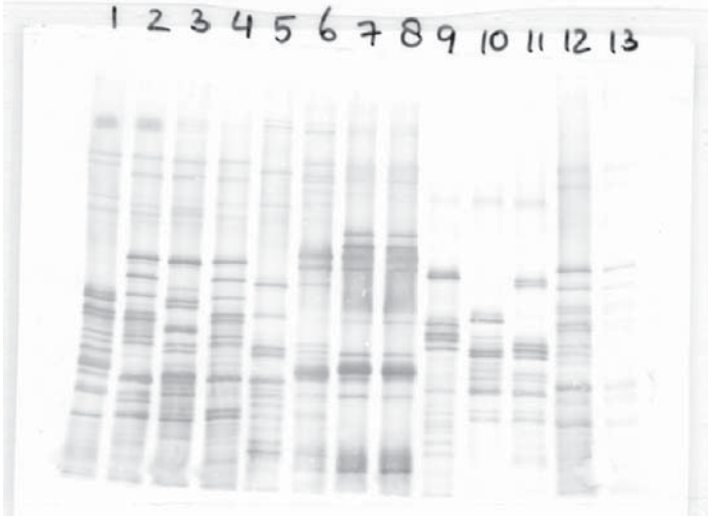

Fig. 14.1 Allelic variance between cereal cultivars. (From left to right): lane 1–5 wheat, 6–8 rye, 9–11 barley, 12, 13 wheat gluten reference. SDS-PAGE (gradient gel and immunochemical staining with a polyclonal anti-wheat gluten antibody).

and 150 ppm gluten). The technique is very efficient at the qualitative level, i.e. it can be used to confirm the presence of gluten in a gluten-free product with a high degree of confidence if the band pattern obtained with the suspected sample matches that of a standard. Due to this cultivar dependence an exact match is, however, very difficult to achieve (see Fig. 14.1).

14.3.7 Comments on antibody-based techniques

The reference standard

There has been much debate about which standard should be used. Van Eckert *et al*. (1998) showed that the results of ELISA methods varied considerably when using different reference standards. This is not unexpected. An antibody reacts with a set of epitopes in the food analyzed. If this set of epitopes is different in term of abundancy and/or affinity of the individual constituting components there will not be a match between the analyte and the reference protein and, consequently, the result is either under- or over-estimated. This mismatch differs from antibody to antibody, causing differences between the various assays. The development of a standard produced from a mixture of cultivars which are important in trade has the advantage of minimizing this bias. However it is impossible to exclude it completely.

Format

The composition of the food is very relevant to the format of the assay. A capture ELISA in sandwich format can be very specific, but it may miss a small size peptide containing just one epitope. Designing antibodies against specific peptide motifs and using these in competitive format might circumvent these shortcomings. Quantitation might be impaired anyway in situations where stoichiometry is disturbed and if there is no prior knowledge about size of the peptides. Ideally (but hardly feasible), quantitation of toxic peptides should be performed with a gliadin standard degraded to the same extent and in the same manner (sites of hydrolytic cleavage and deamidation) as the sample. Another option would be to include a predigestion of the sample to level out differences between them and the reference protein. It remains questionable whether this is feasible. A patent has been applied for using this approach (Denery-Papini *et al*., 2001).

Specificity of antibodies

Increasingly, antibodies are mapped on the molecular level. However, unexpected reactions may occur, dependent on binding on e.g. plastic surfaces (Chirdo *et al*., 1995), temperature (Brett *et al*., 2002) or extraction method. We have repeatedly found that antibodies which behaved quite specifically in a sandwich or competitive assay produced unspecific reactions (a positive response to maize, sorghum, millet and rice) when used in Western blotting. Apparently cross-reacting protein fractions which are not extracted by alcoholic solutions were extracted when using SDS buffer and a reducing environment. Surprisingly, the antibody, which had been prepared with an ethanol-extracted gliadin as immunogen and which had been absorbed to remove all unwanted specificities (and which should thus be specific for gliadin), behaved unspecifically when brought in contact with proteins extracted differently.

Detection of deamidated protein

Wheat gluten is sometimes solubilized by partial hydrolysis. The functional properties of the protein are then modified: the protein becomes less hydrophobic and consequently the functional properties of the protein are changed, which opens the door to wider application of the protein into foods. Although reactivity of the protein with antibodies sometimes remains partially intact, the quantitative aspects are corrupted. Again, quantitation can be achieved only if the appropriate reference material is available. Whether or not modified protein has been added to a food may be detected by performing SDS-PAGE, blotting and immunodetection (Janssen *et al*., 1994). An immunoreactive smear is indicative for the presence of modified gluten; however, the results can be interpreted only in a qualitative way and even then precautions should be taken because severe heating may produce the same effect.

14.4 Non-antibody-based techniques

A number of non-antibody-based techniques have been used in the past. Some of these (nitrogen determination or luminescence) have become obsolete now as their scope is limited and accuracy insufficient.

14.4.1 Polymerase chain reaction

PCR methods are increasingly used in food analysis. They share one significant advantage: the analyte itself is amplified and, principally, extremely low amounts of reporter DNA can be detected (see Chapter 7 for an in-depth discussion of PCR methods). The first PCR method to determine wheat in gluten-free products was described by Allmann *et al*. (1993). The assay consisted of a double PCR: a eukaryote PCR, amplifying a 137 bp fragment of a highly conserved eukaryotic sequence as a control for the condition of the extracted DNA; and a wheat-specific PCR, amplifying a 109 bp segment in the major repeat unit of the intergenic between the 25S and 18S wheat ribosomal RNA genes. This intergenic segment was chosen because it is present in a high number of copies, allowing sensitive detection which would not be possible using a single copy gene. The eukaryotic PCR serves as a control to detect whether false negative results did occur due to DNA degradation. DNA extraction and visualization of the amplified bands were carried out using standard methodology. The assay was specific for wheat: rye produced a very weak signal. The sensitivity of this analysis was 1 pg wheat DNA, which equalled 50 ng wheat. Several commercial products were analyzed with this PCR method as well as with a commercial anti-ω kit as developed by Skerritt. It proved that some thickening agents, such as dextrin, carob starch and guar flour, were refractory to DNA extraction. Other processed, heated or fermented food yielded good-quality DNA. Of all food tested, tapioca, curry and malt extract didn't produce a positive signal with the eukaryotic PCR, while other food gave a positive signal. Comparison of the results obtained with the ELISA kit and the PCR method showed agreement in most cases; in some foods, however (instant soups), they were discordant. There are many inhibitory substances present in DNA extraction reagents used to extract a crude matrix like food which may disturb the amplification. Rossen *et al*. (1992) found that the use of NaOH/SDS inhibited the reaction significantly, while other components, such as detergents, lysozyme, NaOH, alcohols and (not surprisingly) EDTA and EGTA, had a negative effect on amplification. A modified DNA extraction was proposed by Szamos *et al*. (1998).

As mentioned before, the toxicity of oats is debated. Data about its toxicity (especially older data) are often corrupted by a high level of contamination of commercial oat products (e.g. rolled oats) with wheat and/or barley. Using ELISA, it is difficult to detect whether oats are contaminated with wheat, because in most ELISA methods, oat itself shows a low, but distinct reaction.

Detection of a low degree of contamination by ELISA is consequently not possible. Köppel *et al*. (1998) investigated the suitability of the PCR method described above to detect wheat in oat products. With a slightly modified DNA extraction protocol, the method proved to be very sensitive and (at higher contamination levels) a good correlation was found between the results of the ELISA and PCR.

More recently, a quantitative competitive PCR system to detect wheat, barley or rye in gluten-free food was described by Dahinden *et al*. (2001). This assay is based on simultaneous detection of wheat, rye and barley by the amplification of a chloroplast trnL (UAA) intron. Good correlations were found between the PCR and values obtained by ELISA. As stated by the authors, deviations from an expected ratio between PCR signal and an immuno-based response are indicative for wheat starch addition (PCR positive, ELISA negative) or addition of gluten to a food (PCR low or absent, ELISA high) and both cases warrant further investigation.

Real-time PCR using melting curve analysis (LightCycler® FastStart DNA Master^{PLUS} SYBR Green 1 – Roche Diagnostics GmbH, Mannheim, Germany) for identification of wheat, rye, barley and oats was recently described by Sandberg *et al*. (2003). The length of the amplicons varied between 104 and 181 bp and the melting points between 81.2 and 85 °C. DNA was extracted with the Dneasy® tissue kit (Qiagen, Hilden, Germany) according to manufacturer protocol. Target sequences were chosen encoding the ω-gliadin, ω-secalin, hordein and avenin sequences. Though these sequences have a high degree of homology, it proved possible to design species-specific primers by choosing at least one of the primers in the non-coding region of the gene. Wheat species (spring and autumn wheat), durum wheat, spelt and kamut (*triticum polonicum*) were all detected. Several products were analyzed and a good correlation between the PCR assay and an ELISA was found. Of the 17 oat samples, nine were contaminated, some containing two different cereal species as contaminants.

PCR methods offer an attractive alternative to ELISA methods in screening or confirmation. As the technique is completely orthogonal to ELISA, a positive result with both techniques gives almost irrefutable proof that the incriminated cereal species is present. In some matrices it might be difficult to extract DNA of good quality, e.g. thickening agents or emulsifiers. In acid-treated food DNA is often degraded substantially. Thermal treatment of the food poses no problem if short DNA sequences are amplified and consequently the method is ideal to confirm ELISA findings in cooked foods.

14.4.2 Maldi-TOF

A mass-spectrometric method was explored by Camafeita *et al*. (1997, 1998) and Camafeita and Méndez (1998). In this method, the proteins of a food are extracted and measured on a MALDI-TOF mass spectrometry (MS) system.

Mass spectra were recorded in a linear positive mode at 30 kV acceleration voltage and 2 kV linear detector by accumulating 70 spectra of single laser shots under threshold irradiance. Only highly intense, resolved mass signals from 2–3 target spots were considered. The apparatus was calibrated with bovine serum albumin. Patterns of several wheat, barley, rye and oat cultivars were made and were compared to band patterns as revealed by SDS-PAGE and stained with Coomassie Blue. The MALDI-TOF spectra from the four wheat cultivars yielded very similar mass patterns in the 30–40 kD range, which encompasses the molecular weights of α/β- and γ-gliadin. Mass spectra from the other cereal species also showed very consistent patterns, but different from each other, which allowed the unscrambling of mixtures. The authors suggest that this method could be worked out to detect total prolamin from various sources by measuring the mass profile in the 20–45 kD range. No data with respect to non-cereal matrices were presented, however. As the method itself is non-specific, the discrimination between cereals and non-cereals depends completely on differences in mass pattern and on the selectivity of the extraction solvent (in this case 70% ethanol). Compared to SDS-PAGE (separation also based on mass) this method has the advantage of speed. It is open to debate as to whether the intrinsic possibility to measure oats at the same sensitivity as wheat, barley and rye is a substantial advantage. If toxic at all, oats are most probably much less toxic than wheat, rye and barley. The ability to measure wheat, rye and barley contamination in oats by shifting the mass window to higher masses might prove to be useful.

To improve the performance in the maize and rice matrices, the method was modified by changing the extraction procedure (Hernando *et al*., 2003). The extracts, as made in 60% ethanol, were dried and resuspended in 0.5 ml 1M acetic acid and mixed for 15 minutes at room temperature. The work proved that the prolamins from rice and maize remained insoluble (confirmed by taking a mass spectrum of the pellet) while gliadin went into solution (which could be confirmed by preparing a mass spectrum of the supernatant). Sensitivity of the method, however, was not high enough to allow determination of gliadin at the 20 ppm level.

14.5 Selecting a method

The choice of a method strongly depends on the position of the laboratory that is doing the analysis. Whereas, for example, an in-house quality control laboratory would have insight into the formulation of the product analyzed, its ingoing raw materials, additives and the way the food is processed, a laboratory of an official food authority would not have direct access on a regular basis to this information. Having access to these data greatly facilitates the interpretation of the results as a ‘baseline’ response for the food analyzed is known. Laboratories of official food authorities and external private

laboratories doing this type of analysis on an *ad hoc* basis have no knowledge of the baseline response of their test material and would have to discuss whether their results are indeed caused by gluten contamination or are just a false positive result caused by non-specific binding of a quite unrelated substance. Especially in case of trade disputes or litigation, it might be imperative to analyse by two completely unrelated methods. Each method should be validated with respect to trueness (recovery and absence of matrix effects) as well as reproducibility. The latter can be achieved by participating in collaborative trials and proficiency studies as organized, e.g. by any taking part in a proficiency scheme [e.g. the rounds organized by the UK Food Analysis Performance Assessment Scheme (FAPAS)]. A FAPAs round to determine gluten in a dry bread mix was carried out in 2004.

Major flaws in current methodologies are summarized below.

- Methods produce results expressed as 'wheat gluten equivalents', because wheat gluten (either the European reference material certified by IRMM or any other commercially available material) is used in preparing calibration curves. The actual amount of non-wheat gluten (barley, rye, secale, oats, etc.) can relate to this result in a variable and irreproducible way.
- Even if, by using standardized methods, the reproducibility and repeatability might be acceptable, there is no guarantee that the results are true. Only when there is a perfect match between the epitopes of the contaminating cereal (in terms of relative contribution) and the standard will the result be true.

14.6 Future trends

Until now, methods to detect and determine wheat gluten and related proteins from coeliac-toxic cereal species have focused on the determination of the cereal species, without taking into account its toxicity. Presently, much research is being carried out to determine the precipitating factor at the molecular level, i.e. the toxic sequences. Work is also being done to produce non-toxic wheat gluten expressed by yeast cells to be added to non-toxic cereals like maize or rice to improve baking quality. In addition, there is much interest in selecting wheat cultivars which lack the toxic sequences, either by screening of gene databases for attributions naturally lacking these toxic sequences or by removing those sequences by genetic engineering. If this strategy succeeds, it will be a prerequisite for a method that it distinguishes between wheat with, and wheat without, toxic sequences (the same will hold for other cereal species).

It is now generally accepted that coeliac disease is caused by inflammatory T-cell responses to gluten peptides bound to HLA-DQ2 or -DQ8 molecules. There is overwhelming evidence that coeliac disease patients can mount T-cell responses to discrete amino acid sequences found in α- and γ-gliadin and LMW and HMW glutenin.

Assays that would detect the presence or absence of such peptide motifs would thus be accurate indicators of safety of the food for consumption by coeliac disease patients. A novel method to detect such gluten peptides in food has been developed at the Department of Immunohaematology and Blood Transfusion of the Leiden University Medical Centre (LUMC), Leiden, The Netherlands (Spaenij-Dekking *et al*., 2004). Monoclonal antibodies were generated that are specific for T-cell stimulatory epitopes in α-gliadin, γ-gliadin or LMW glutenin molecules. These mABs are used in competition assays in which peptides present in the sample to be measured compete for binding to the peptide-specific mAB with a biotinylated indicator peptide. These assays are highly specific and detect up to 50 ng gluten per ml of the European gliadin standard. This corresponds to detection of 0.5 ppm in extracted gluten preparations. The advantages of the new method are multifold: (i) the assay measures specifically the presence of T-cell stimulatory epitopes known to be involved in the development of CD; (ii) as a consequence of the format, this type of assay requires only one mAB per peptide, allowing the detection of small gluten peptides corresponding to the size of T-cell stimulatory epitopes – this is impossible with the currently available sandwich ELISA systems; (iii) the assay detects gliadin and glutenin molecules simultaneously, allowing a more fair judgement of the potential toxicity of the foods tested for coeliac disease patients. This type of assay offers good possibility to screen novel cereal sources, like tef (*Eragrostis tef*), or other cereals with disputed coeliac toxicity. However, given the almost idiotypic reaction of coeliacs, an antibody mimicking the reactivity of a panel of T-cells might be necessary.

Without any doubt, another trend is the development of rapid screening tests, by which a production lot can be screened rapidly for gluten contamination without requiring access to laboratory facilities. In verifying the performance of hazard analysis of critical control point (HACCP) systems an analysis of the end-product is the ultimate method to check whether all critical points during production are under control. At the time of writing, dipstick assays are commercially available from Tepnel (Deeside, UK), R-Biopharm (Darmstadt, Germany) and Operon (Cuarte de Huerva, Spain) based on the immunochemical capture of coloured microparticles on a blotting medium; however, only limited data about their performance in practice is available yet. Drawbacks for home use include the need to extract the protein from a (most often) solid food, which might be cumbersome and prone to mistakes; however, these methods might have good prospects in HACCP programs.

A biochip is being developed in the framework of an EU funded research project: Quantitation of coeliac disease toxic gluten in foodstuffs using a chip system with integrated extraction, fluidics and biosensoric detection (QLK1-CT-2002-02077) http://www.etseq.urv.es/BBG/dinamic/Cd-chef/. This project, which is coordinated by the Department of Chemical Engineering, Universitat Rovira i Virgili, Tarragona, Spain, includes the development of immuno- and apta-sensor generic platforms with optical/electrochemical/

quartz crystal nanobalance detection. It is aimed at integrating extraction and detection on a disposable microsystem with the time required for total assay being less than 15 minutes at a cost of less than EUR 15, meeting the product design requirements listed both for extraction and for detection. It will be based on either antibodies against the toxic peptide motif or on aptamers (i.e. synthetic RNA/DNA constructs) showing high affinity to the toxic peptide motifs. This project is in its preliminary stages and no performance data are yet available.

14.7 Sources of further information and advice

(Prospective) threshold values for gluten in gluten-free food in national food legislations are increasingly derived from what has been agreed upon in international institutions such as Codex Alimentarius.

Codex Alimentarius is a worldwide organization and its standards are the basis of several national food legislations. Codex standards will have to be implemented in national legislation in order to come into force. Important committees within Codex are: the Codex Committee on Nutrition and Food for Special Dietary Uses (CC NFSDU), the committee on Food Labelling (CC FL) and the committee on methods of analysis and sampling (CC MAS). Current discussions between producers of gluten-free food and consumer organizations have not yet reached consensus about the proposed statutory limit for 'gluten-free'. Of course many producers prefer to produce under relaxed conditions and favor a high limit value while in the eyes of the consumer organizations, a low limit is to be preferred based on the precautionary principle. Ultimately the limit will be a compromise between political (risk management) and scientific (risk analysis) arguments. The reports of the meetings of these committees as well of the commission itself are published on the Codex Alimentarius website: http://www.codexalimentarius.net.

The Working Group on Prolamin Analysis and Toxicity (WGPAT) has brought together a group of scientists of various disciplines, gastroenterologists, cereal/food chemists, pediatricians, with an interest in coeliac disease. The WGPAT has an observer status in Codex Alimentarius. This working group organizes annual meetings, which are appreciated as a platform for multidisciplinary exchange of ideas and may be attended by invitation.

Numerous national coeliac patient organizations exist around the world. If one is interested in issues that are considered important in the coeliac world one should visit one of the national coeliac sites. A list of these is available at www.aoecs.org.

Another item to look at is the recent amendment on the European labelling directive 2000/13/EG (discussed at length in Chapter 21 of this book). This amendment makes the labelling of allergens (and also coeliac-toxic cereals) compulsory. Depending on the outcome of research with respect to the coeliac toxicity of so-called second and third generation products (i.e. products

derived from wheat starch) this amendment will be likely to have dramatic effects on the labelling of many products. Wheat starch-derived products like glucose syrup may be used as a raw material for the production of many food ingredients and additives like polyols, ascorbic acid, acetic acid, etc. In the near future it may thus become mandatory to label food containing these ingredients as derived from wheat unless an exemption is allowed (wheat-based glucose syrups and maltodextrins, glucose syrups based on barley and cereals used in distillates for spirits are (by directive 2005/26/EC) provisionally excluded from this mandatory labelling, because, most probably their gluten content is very low). In the US and other countries, newer labeling laws require, or will soon require, this type of labeling.

Regarding the gliadin standard: the reference material (IRRM-480) developed on the initiative of the WGPAT is available from the IRRM in Geel, Belgium. If one is aiming at producing reproducible results, the use of a worldwide accepted standard is of utmost importance. The IRRM website is http://www.irmm.jrc.be/.

14.8 References

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15

Detecting soy, fish and crustaceans in food

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15.1 Introduction

Detection of peanuts, tree nuts, wheat, milk and egg as members of the 'Big Eight' allergenic foods is described in the preceding chapters. In this chapter we describe the tests available for the remaining allergens: soy, crustaceans and fish. Compared to the other food allergens, detection of the present group of allergens is relatively unexplored, due to several reasons related to these particular foods. Soy is a food stuff that is used in a vast number of applications, undergoing a wide variety of processing steps, with consequences for the detection of allergenic residues. Crustaceans and fish count a large number of species, and for detection purposes it is necessary to choose whether to test for one species, for a subset or for the entire group. Considerations for such choices are based on the allergic patient's characteristics: do they react to one of the species, or even one allergen, or to more species and more allergens? From the food industry perspective, the issue is to detect fish and/or crustacean residue, regardless of the species and the nature of the allergens. The sections below will cover the published and commercially available methods for soy, fish and crustaceans, and will discuss the advantages and disadvantages of the chosen approaches.

15.2 Soy

Soy is a food ingredient with both technofunctionality and biofunctionality. The beneficial effects on health of soy isoflavones have been recently reviewed,[1] and food products containing 25 g of soy per intake may claim to be healthy

(see US Food and Drug Administration (FDA) regulations at www.FDA.gov). Soy is used in a number of applications, as consumer product diversification since the 1980s has required improved technological functionality of ingredients. In terms of authenticity and product adulteration, such as for high-quality meat products,[2] there is a need for methods to detect the presence of soy protein in foodstuffs. Many methods were published in the 1980s, when the use of soy in foods expanded enormously. Test requirements then related to specificity, with respect to other food ingredients, mainly meat, and recovery for processed foods. The sensitivity was of less importance; 0.1 or 1% (w/w) of soy was a suitable detection limit. With food allergies on the increase since the 1990s, it has become increasingly clear that only minute amounts of soy, in particular soy protein, may threaten soy-allergic consumers. Since the minimal provoking doses of food allergens are usually low, in the high μg to mg level, the sensitivity of soy tests is now an important issue. For peanut, another member of the Legumae family, thresholds for subjective reactions of 100[3] and 30 μg[4] have been described. It is expected that the threshold for objective symptoms and more severe reactions will be significantly higher,[5] in the 1–3 mg range, as demonstrated by several case reports on inadvertent intake of peanut-containing foods. There is only limited information on the threshold doses of soy protein for soy-allergic individuals. In fact, a multi-centre study coordinated by the Food Allergy Research and Resource Program at the University of Nebraska (www.farrp.org) to investigate the minimal provoking dose for soy flour has recently commenced. Some case reports show that minor amounts of soy, well below 0.1 and 1% (w/w), can lead to allergic reactions.[6,7] This section aims to review tests that have been described in the literature for the detection of soy, and to discuss advantages and disadvantages of the different methods.

15.2.1 Soy protein solubility and consequences for selecting a detection method

Foods that contain soy proteins are, usually, subjected to some form of heat treatment (e.g. pasteurization, sterilization) during processing to obtain (i) a certain shelf-life, (ii) an inactivation of the anti-nutritional properties, and (iii) certain functional properties. Proteins generally aggregate as a result of heat denaturation and this is also observed for soy proteins. Solubility data on soy proteins are plentiful, but their value in obtaining an overview is frequently limited because of the large variation in heat treatment given, soybean variety used, conditions of determination (pH, ionic strength), solvent used, etc. It is known that these factors all affect solubility.[8] The solubility behavior of the major proteins in soy (glycinin, beta-conglycinin and trypsin inhibitors) was recently reviewed.[9] In general, native soy protein is poorly soluble at neutral pH, while either lowering (< pH 4) or increasing (> pH 8) increases solubility.[8] For heat-treated or otherwise processed soy protein, adjusting the pH will not be sufficient for solubilizing the protein fraction.

Denaturing extraction buffers containing high concentrations of urea or guadinium, or disulphide bridge-reducing agents like beta-mercaptoethanol, detergents like sodium dodecyl sulphate (SDS), or combinations thereof may be necessary to obtain satisfactory solubility levels.[10–16] Recent work showed that at extremely high pH most commonly used soy ingredients are soluble to a large extent.[9] Only some ingredients are soluble at neutral pH, under native conditions. Harsh conditions are required to substantially improve solubility, particularly for processed ingredients (isolates, texturized soy).

Considering the difficulties with soy protein solubility, it could be suggested that the protein fraction may not be the optimal analyte for testing for the presence of soy. DNA-based methods may also be used to detect soy. However, it should be kept in mind that the protein fraction causes allergic responses, and the presence of DNA does not necessarily coincide with that of the protein fraction. This is especially important when processed products, like refined soy oil and lecithin, and fermented products, such as soy sauce, are considered.

15.2.2 Detection of soy proteins

Many different approaches for detection of soy proteins and other soy components in meat have been reported and reviewed.[15,17] Most tests were developed for measuring comparatively larger quantities of soy protein (percents of total weight) in food products in order to detect the use of soy as a protein fortifier/binder. There are only a small number of tests developed for the detection of traces of soy cross-contamination that threaten soy-allergic consumers. These are mainly immunochemical tests based on antibody–antigen interactions, and are usually more sensitive when compared to chemical or biochemical methods. The different techniques used for soy detection are summarized below.

With regard to the safety of foods for soy-allergic consumers, the sensitivity of the detection method is of utmost importance. It is clear that concentrations well below 0.01% (100 ppm) for some allergens can cause allergic reactions, although this has not been proven for soy to date. For peanut, another member of the Legumae family, minimal doses that elicited subjective reactions of 30[4] and 100 μg[3] have been described. This corresponds to 0.3–1 ppm when a serving size of 100 g is considered. For soy, there are no accurate data on minimal provoking doses. Based on information on threshold levels of other food allergens, and taking into account case reports, Taylor *et al.*[18] postulated that a detection limit of 10 ppm is a useful figure. When threshold information for soy becomes available, this number should be reconsidered in light of the new data.

Detection of soy components other than protein; indirect analysis

Since direct protein measurement is sometimes difficult due to solubility issues, indirect methods based on appropriate non-protein markers associated

with soy may be useful. Suitable markers have been chosen for their stability and ease of assay under the processing conditions used. Examples of markers that have been used for assay development include insoluble polysaccharides, oligosaccharides, protein-bound sugars, free amino acids, free peptides, phytate, saponins, sterols and metals.[15] Another approach has used histological techniques. A microscopic examination of calcium oxalate crystals and plant cell components indicate the presence of soy. Staining for cell wall polysaccharides could also be useful in this respect.[15] The detection levels of these methods are too high for quantification of traces of soy for allergenic concerns.

Meyer *et al.*[19] evaluated a DNA-based PCR method for detecting soy residue in food products, and achieved a detection limit of 70 ppm. More recent public discussion on genetically modified organisms (GMO) and foods originating from such crops led to the development of assays for GMO soy, with a reasonable sensitivity (for GMO concerns; 0.9%, EU legislation, for more information see http://europa.eu.int/comm/food/fs/gmo/gmo_ongoinit_en.html). Methods based on DNA, most often polymerase chain reaction (PCR) tests, are applied to distinguish between wild-type and GMO soy. As a test control, a gene present in wild-type soy was detected as well. Van Duijn *et al.*,[20] for example, measured the soy lectin gene as a positive control. This test has also been used to detect soy in processed food with a reported detection limit of 0.01% (100 ppm).

The sensitivities of the described indirect methods are between 0.01% and 1% (100–10 000 pm). These are appropriate for detecting soy added as a functional ingredient, but not for detecting traces originating from cross-contamination. Besides this practical disadvantage, there is a theoretical concern. In attempting to protect soy-allergic consumers, the allergic component, i.e. protein, should be measured rather than a marker. This is of key importance because industrial soy processing results in a variety of soy protein products that may have lost other (marker) components during processing.

Antibody-based tests

Enzyme-linked immunosorbent assay (ELISA) is a powerful analysis tool for detection of specific proteins. It has the advantage of simultaneously testing a larger series of samples at a high level of sensitivity. A large number of investigators have used ELISA methods for the detection of soy protein in meat products. Immunochemical methods are generally limited to the qualitative screening of raw or mildly processed products, since protein denaturation often alters the antigen–antibody interaction.[15] This has been studied by different authors[15,21–32] and the most important results are discussed below.

Antibodies against soy can be raised in laboratory animals, usually rabbits, mice, goats, sheep or donkeys, as described in Chapter 3. Individual animals may react differently to immunization protocols, and it is difficult to predict which species is the best host for raising antibodies against soy. In addition,

there is another issue that influences the success of raising antibodies against soy. Soybean protein is a common substance in animal food, so the antigenic responses obtained after immunization are not expected to be high. This may lead to antibodies with low affinity and, consequently, ELISAs constructed with such antibodies have poor sensitivities. This may be overcome by using animals kept on soy-free diets.[24] Aiming for a sensitive method to detect soy protein, it seems to be logical to choose glycinin or β-conglycinin as antigenic protein since these are the major soy proteins and they are abundantly present in soy. However, using a single-allergen method creates the risk of false negative test results in the case that the analyte is not present or does not react while other allergens may still be present.

ELISAs for soy glycinin

Ravenstein and Driedonks[11] raised antibodies against the SDS-denatured acidic polypeptides of glycinin in rabbits. An ELISA constructed with these antibodies had a reported detection limit of 0.5% (5000 ppm). Applying the antibodies in an immunoblotting experiment decreased the detection limit enormously, but this was not considered relevant since soy is usually used in concentrations at 1% and higher. Meisel[27] immunized chickens with SDS-denatured glycinin and obtained specific IgY antibodies from egg yolk. With these antibodies, an ELISA with a sensitive range between 0.5 and 256 μg/ml for dilutions of soy protein isolate was constructed. The detection limit was 0.5 μg/ml, corresponding with 0.1% soy protein spiked in a food matrix. IgY antibodies used in immunoblotting even allowed the detection of the acidic polypeptide of glycinin at nanogram levels.[27] Plumb *et al*.[28] raised antibodies against native glycinin. They found that heating glycinin at pH 7.6 caused the immunoreactivity to decrease to around 50% of the original value but it increased sharply above 92 °C. These results are in agreement with Demonte *et al*.[30] but differ from the results of Iwabuchi and Yamauchi,[22] who found that the antigenic activity disappeared after heating. Huang *et al*.[31] found that the epitope identified by Plumb *et al*.[28] corresponded to residues 86–104 of the acidic polypeptides of glycinin A1aB1b and lies on the C-terminus of the proteolytic intermediate known as glycinin-T. The epitope seems to be continuous in nature. Iwabuchi and Yamauchi[22] studied the effect of ionic strength (I) on thermal denaturation of soybean glycinin. Up to I = 0.7, no effects of ionic strength were found. A reduction in immunogenicity also occurred when glycinin was taken to pH 2.0 and pH 11.0 and exposed to high temperatures.[21]

ELISAs for soy beta-conglycinin

Plumb *et al*.[29] raised antibodies that were specific for the native α and α′ subunits of β-conglycinin. β-conglycinin immunoreactivity was increased as the protein was heated, reaching a maximum at the denaturation temperature of 65 °C. This phenomenon is unusual as most thermally denatured proteins have low immunoreactivity when probed with antibodies raised against native

protein. This was also found to occur at pH 7.6 at different ionic strengths.[21,33] The epitope of the antibody used by these researchers corresponds to the residues 78–84 in the acidic extension present in the α′ subunit of β-conglycinin,[29] and seems to be continuous in nature.[31] A linear epitope, in contrast to a conformational epitope, is expected to be more heat stable and may become more exposed after denaturation. This may explain the increased detectability of heated β-conglycinin.

ELISAs for soy trypsin inhibitors

Brandon *et al.*[23] worked with antibodies raised against native and heat denatured soybean Kunitz trypsin inhibitor.[23] Antibodies raised against the denatured inhibitor were specific for the denatured conformation of the protein. In contrast, the native inhibitor elicited antibodies that selectively recognized determinants in both native and heat-treated proteins. Barkholt *et al*.[25] raised an antibody against Kunitz-type soybean trypsin inhibitor that worked equally well against native and denatured protein. Because Kunitz-type soybean trypsin inhibitor is an anti-nutritional factor, soybeans lacking this protein were bred.[26] At present, there are no commercial cultivars lacking the Kunitz-type soybean trypsin inhibitor, but these may become available in the future. From that perspective, using the Kunitz-type soybean trypsin inhibitor as a marker for residual soy protein is not a good choice. Brandon *et al*.[24] used antibodies that bind the Bowman-Birk protease inhibitor. They identified an epitope that was destroyed by heat and developed an ELISA for specific recognition of native Bowman-Birk inhibitor in the presence of denatured forms. None of these tests were optimized for sensitivity.

ELISAs for total soy protein

Several methods use antibodies raised not against one particular protein but against the whole soy protein fraction. For example Janssen *et al*.[34] detected soy proteins in meat products up to 0.1% by gel electrophoresis followed by blotting and dot blot. All major soy fractions were recognized by the antibody. Hitchcock *et al*.[15] raised antibodies against soy protein which had been 'renatured' from a hot urea solution by removing or diluting the denaturant. This method could recognize heat-treated soy fractions as well as native ones. Some specialized products, however, gave little or no response, presumably because the proteins added were hydrolyzed (it should be noted that hydrolyzed soy proteins can retain allergenicity). It was found that the method of Hitchcock *et al.*[15] could also be used with commercially available anti-soy antibodies[35] and a commercially available soy protein assay kit (Cortecs, presently Tepnel Biosystems, Deeside, UK) was developed. The antibody that was used is not specified in the kit insert. This test is meant for quantitation of soy protein levels between 1 and 10% of total weight of raw or processed mixed meat products. The relatively high detection limit is partially due to the fact that the extracted samples need to be diluted in order to prepare an environment that is compatible with ELISA. In this case the

required dilution factor was 20. Recently a method with a high sensitivity (around 1 ppm) was described.[9] This method uses an easy extraction protocol, a one-step extraction at pH 12. Table 15.1 shows that this extraction has high recoveries for a variety of different soy ingredients as compared to extraction under native condition or extraction with urea and reducing agents. A mixture of soy protein isolate, soy protein concentrate and texturized soy protein was extracted at pH 12, and the extract used for raising antibodies in goats and rabbits. A classical protocol for immunization was used and, prior to that, the animals were kept on a soy-free diet for at least three months. An inhibition

Table 15.1 Extractability of processed soy ingredients using three different methods

Sample description	Type	Extraction recovery (% related to product specification)		
		TRIS pH 8.2	Urea/DTT	pH 12
Soy 200/90 (own supply) light toasted	Meal	39	52	67
Central Soya Centex 4220 Texturized Soy Protein	Flour2	46	45	
Central Soya Pro Fam 981	Isolate	39	43	60
ADM Pro Fam 891	Isolate	33	46	51
ADM Pro Fam 781	Isolate	28	36	37
Central Soya Pro Fam 646	Isolate	28	46	66
ADM Arcon S	Concentrate	34	47	64
ADM Arcon SM 066-405	Concentrate	41	50	67
ADM Arcon F	Concentrate	3	48	52
Central Soya Response 4400 Textured	Concentrate	5	35	52
Central Soya Procon 2000	Concentrate	4	48	59
Central Soya Promine DS Functional	Concentrate	3	45	42
ADM Arcon T	Flakes	ND	ND	ND
Mean (± SD)		**20 (± 15)**	**45 (± 5)**	**55 (± 10)**

Protein concentration measured by Bradford method.[36]

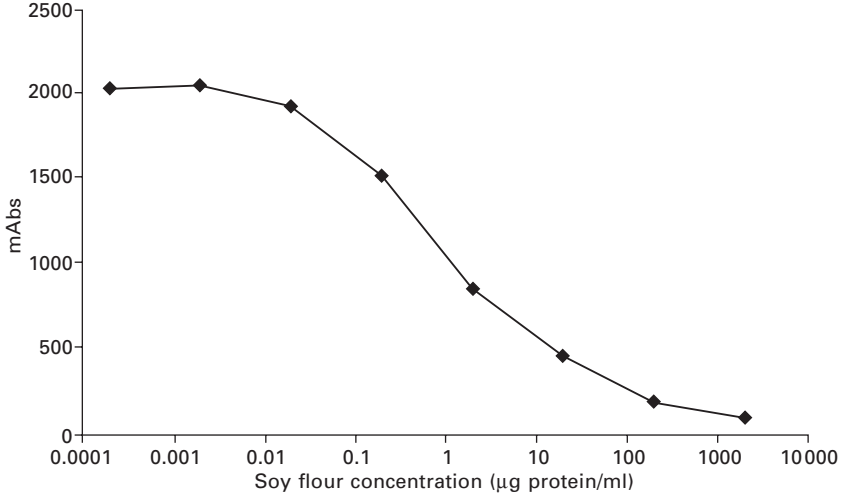

Fig. 15.1 Standard curve for soy protein ELISA. (ELISA plates were coated with pH 12-extracted soy and incubated with mixtures of rabbit antibody against pH 12-extracted soy with calibrator (pH 12-extract of lightly toasted soy flour).

ELISA using rabbit antibodies was constructed and the detection limit is 0.02 µg/ml (Figure 15.1) which corresponds to 0.4 ppm for solid food samples. Table 15.2 shows that the recovery of this test is high (mean 73%) and that the variation between individual samples is only moderate (35%). These characteristics are promising as they allow soy detection with sensitivity

Table 15.2 Recovery of soybean detection

Type of soy	Percent measured soy protein[1]	Listed on product specification sheet	Recovery (%)
Meal	Used as standard	–	–
Texturized Flour	35	54	65
Isolate	87	90	97
Isolate	79	90	88
Isolate	27	90	30
Isolate	123	90	136
Concentrate	61	72	86
Concentrate	83	70	119
Concentrate	25	69	37
Concentrate	39	65	60
Concentrate	35	68	52
Concentrate	20	68	29
Flakes	36	Not specified	–
Mean (± SD)	–	–	**73 (± 35)**

[1]Protein concentration measured by Bradford method[36]

Table 15.3 Commercially available methods to detect soy

Analyte	Type of method	Kit manufacturer	Sensitivity	Web site
Soy protein	Inhibition ELISA	Tepnel	5000 ppm	www.tepnel.com
Soy trypsin inhibitor	Sandwich ELISA	Elisa Systems	1 ppm	www.elisasystems.net
Soy lectin gene	Real-time PCR	Congen	10 ppm	www.congen.de
Soy protein	Sandwich ELISA	Neogen (under development)	1–5 ppm	www.neogen.com

relevant for soy-allergic patients. Assay validation is now in progress and commercialization is expected in 2005 (Neogen Corporation, www.neogen.com).

Available methods for soy detection

Since the 1990s several tests to detect soy in food have become commercially available. Most of these are ELISAs, but more recently DNA-based methods have been launched. Table 15.3 gives an overview of the available methods together with their most important characteristics. The ELISA for total soy protein from Tepnel is meant for detecting large additions of soy protein in, for example, meat products (p 278). The relatively high detection limit is not suitable for allergen detection. Recently, ELISA Systems Ltd launched a new soy protein screening assay. The detection limit is 1 ppm, and it is therefore well suited to allergen detection. The kit is suitable for screening the presence of soy protein and is based on the detection of Kunitz-type soybean trypsin inhibitor. It is not applicable for samples that have been subjected to extensive hydrolysis, fermentation and heat treatment (ELISA Systems, personal communication). As mentioned above, if commercially available cultivars lacking the Kunitz trypsin inhibitor were to become available, this type of method would no longer be useful.

15.3 Crustaceans

Shrimp is the most commonly consumed crustacean shellfish and causes severe allergic reactions, but other members of the Crustacean family such as lobster, crab and crawfish can also elicit severe reactions.[37,38,39,40] Estimates of the number of shrimp-allergic individuals range from 0.6–2.8% of the population. In the US, allergy to crustacean shellfish is more prevalent than peanut allergy.

15.3.1 Crustacean allergens

The major allergen of shrimp is tropomyosin, a muscle protein[41] which is readily isolated from the boiling water and meat of cooked shrimp. Monoclonal antibodies directed against this 36 kD shrimp allergen reacted to a 36 kD protein in crayfish, crab and lobster extracts[41]. The allergen nomenclature for this protein is Pen a 1. The molecular weight of various crustacean tropomyosins has been reported as 34–39 kD; this protein is the target of some of the detection methods that will be discussed next. At least 80% of shrimp-allergic subjects have IgE-binding to tropomyosin[42], making it a major allergen. Another shrimp allergen (Pen m 2) was identified as having extensive similarity to crustacean arginine kinase and to possess arginine kinase activity[41]. Yu *et al.*[42] made specific polyclonal antibodies to Pen m 2 and also developed a competitive ELISA using shrimp-allergic patient sera to show that Pen m 2 was a cross-reactive crustacean allergen. In addition, a yet-unidentified and unnamed allergen of 16.5 kD has been reported to cause IgE-binding in a significant majority of shrimp-allergic subjects in one study[42]. Many other minor IgE-binding proteins have been described in several reports, but their significance is unknown.

15.3.2 Cross-contamination and cross-reacting allergens

The seafood processing, soup and frozen snack and meal industries have the most interest in detecting the undeclared presence of shrimp residues in other foods. Of concern is the transfer of shrimp allergens through frying oil in restaurants and in battering operations in the food industry, and also in the manufacture of shrimp-containing flavors and other ingredients on shared lines with non-shrimp ingredients. There are also significant reactions reported as a consequence of occupational exposure to aerosolized crustacean allergens.[44,45] In some cases, cross-reactivity was found using IgE immunoassays between shrimp tropomyosin and non-crustacean shellfish (in a restaurant seafood handler and also in seafood processing workers), even though these shellfish are of different phyla.[46,47]

15.3.3 Detecting crustacean allergens

There are few methods that have been described for the detection of crustacean residues. ELISA or radioallergosorbent testing (RAST) utilizing sera from shrimp-allergic patients have been used for research purposes and to investigate and characterize crustacean allergens, but only two methods have been reported to date to quantitate shrimp residues. Jeoung *et al.*[48] made monoclonal antibodies to shrimp tropomyosin and developed a sandwich ELISA for the purpose of quantitating the protein in commercial skin test extracts. In these clinical extracts, the ELISA could detect 4–125 ng tropomyosin/ml. In a method designed for detecting shrimp in foods, Ben Rejeb *et al.*[49] described an ELISA made with antibodies against shrimp tropomyosin that possessed

a detection limit of approximately 2.5 ppm. However, up to now, this method has been described only in the abstract and without many details, but it was apparently used to determine the presence of tropomyosin in some food matrices. There is one crustacean ELISA method on the market currently, made by ELISA Systems Pty Ltd, Windsor, Queensland, Australia (http://www.elisas.com.au). The claimed limit of detection of this sandwich tropomyosin assay is 0.05 mg/kg (0.05 ppm). Although the assay is positioned as a screening assay for tropomyosin, the reactivity of tropomyosin from crustaceans other than shrimp is not shown. While some efforts to raise specific polyclonal or monoclonal antibodies have resulted in antibodies that have high specificity for the species of interest,[48,49] one group reported producing a monoclonal antibody against abalone tropomyosin that had surprisingly high reactivity to chicken and crustacean tropomyosins.[50] Therefore, any anti-tropomyosin antibody/detection method should be checked against various types of tropomyosins for possible cross-reactivity issues. In particular, due to their being in the same phyla, anti-crustacean tropomyosins should be checked for cross-reactivity with insect tropomyosins. Sometimes, storage mites contaminate food ingredients such as breading materials, and this has the potential to lead to cross-reactions from the mite tropomyosin. Tropomyosins from different species/phyla do share highly conserved regions but, interestingly, crustacean-allergic subjects do not react to chicken or other non-seafood tropomyosin, which means that the IgE-binding sites are somewhat specific.

There is also one report in which direct ELISA and dot-immunoblotting was used to detect crab residues in heated and sterilized surimi-based products.[51] Polyclonal anti-lobster arginine kinase antibody was utilized and resulted in an ELISA with a detection limit of 10–25 g of crab per kg of surimi-based product; however, this detection limit is too high for allergen detection requirements. Work on detection systems for other crustacea besides those mentioned above has not been reported to date.

15.4 Fish

Fish allergy is relatively common, especially in geographic regions where fish is an important part of the diet. Fatal reactions have occurred as a result of allergic reactions to fish.[39,52] Numerous studies have attempted to estimate the prevalence of fish allergy, usually ranging from 0.3–0.5%.[38,53,54] Although the exact prevalence of fish allergy is not well established, it is listed among the most common of all food allergens.[55] Fish can also cause occupational reactions. While asthma is the most prominent symptom, anaphylaxis has been reported, and physical contact with seafood can also elicit symptoms in allergic individuals.[56,57]

15.4.1 Fish allergens

The best-characterized fish allergen is the major allergen of codfish, named Gad c 1, which belongs to a group of muscle tissue proteins known as parvalbumins[58]. Parvalbumins are responsible for mediating the concentration of calcium in muscle. Gad c 1 is a member of the parvalbumin family found in muscle tissue of amphibians, fish and other animals. Parvalbumins are found in high amounts in lower vertebrates and in smaller amounts in higher vertebrates.[59] Gad c 1 is an acidic protein of 12 kD which is stable to heating, extremes of pH and mild proteolysis.[60] All codfish-allergic individuals react to Gad c 1.[61,62] Another codfish allergen was identified, in an *in vitro* study with cod-allergic patient sera, to have a molecular size of 41 kD, in addition to six other bands at 13, 21, 27, 47 and 58 kD. The 13 kD band was a major allergen for all subjects in the study, and the 13 and 41 kD bands were recognized by anti-parvalbumin monoclonal antibodies, as were bands at 28 and 49 kD. Although the 41 kD protein was dissimilar to Gad c 1, cod-allergic sera demonstrated IgE binding to this protein.[63] In another study investigating IgE binding in sera of fish-allergic patients, pre-rigor cod muscle was found to contain IgE-binding bands of 12, 22, 30, 45, 60, 67, 104 and 130 kD.[64] A major IgE-binding band for all sera was the 12 kD band; the 30 and 67 kD bands also possessed good IgE-binding activity. It was interesting to note that when the cod had been dead for several days, new IgE-binding bands of 18, 41 and 80 kD appeared, and the relative content of IgE-binding proteins was increased. The action of naturally occurring proteases could have cleaved the allergen and opened up sections of the peptides making them more accessible to IgE-binding during this period. Anti-codfish parvalbumin monoclonal antibody was also found to bind to all IgE-binding bands identified in this study, indicating that there was shared similar structures in all peptides.

While many fish-allergic patients are allergic to multiple fish species, monospecificity has been reported to tuna and cod[65], and swordfish.[66] In these studies, subjects with multiple allergies to fish species showed IgE binding to proteins at 12–13 kD bands, while the monospecifically-sensitive subjects showed IgE-binding to unique bands at 40 kD in tuna and 25 kD in swordfish.

15.4.2 Pan-allergens among fish species

The existence of structurally-related parvalbumins in divergent fish species may offer an explanation of cross-reactivity to fish species in certain allergic individuals. Hansen *et al.*[62] noted that in adults with clinical sensitivity to cod, reactions were reported to mackerel, herring and plaice, and *in vitro* results showed IgE-binding to a single band in the 11–14 kD region of these fish extracts. An IgE-binding protein from salmon, named Sal s 1, was identified by *in vitro* analyses, and was shown to be salmon parvalbumin.[67] In one study, almost 50% of cod-allergic patients had clinical sensitivity to salmon[68].

Sal s 1 is described as having two bands, at 12 and 14 kD, and therefore has at least two isotypic variants. The allergens from other fish species are also parvalbumins including horse mackerel (Tra j 1) and bigeye tuna (Thu o 1).[69–71] While tuna-allergic individuals have been noted to have IgE-binding to Gad c 1, one study noted that raw tuna extracts seemed to lack IgE-binding bands in the parvalbumin size range that were present in extracts of catfish, cod and snapper.[65] However, Park *et al.*[72] described IgE-binding to tuna proteins at 12–13 kD using sera from fish-allergic persons and IgE-binding bands at 12–13 kD in mackerel, pollock and cod. Also, unique binding to a band at 19 kD from saury and 37 kD from tuna were observed. In one study, canned tuna did not present problems for five fish-allergic children;[73] canning may decrease the allergenicity of cod. However, amino acid sequencing has shown that the 12–13 kD band from tuna does not have the same sequence as does cod parvalbumin; this may explain why tuna did not cross-react extensively with other species in this study.

Bernhisel-Broadbent *et al.*[74] found that in 11 subjects demonstrating *in vitro* allergy to fish, positive oral challenge only occurred to one species in seven subjects, to two species in one subject, and to three in two others. This observation has been also seen in other studies.[62,75,76] However, some studies have found that subjects clinically react to multiple fish.[65,77] A recent survey found that the rate of reactions to multiple fish among those with fish allergy was 67%.[39]

15.4.3 Detection of fish allergens

Because of the complexity of fish allergy, attempts to develop detection systems for fish residues have been lagging behind efforts for other allergens. Several commercial monoclonal antibodies against different invertebrate parvalbumins are available; a few methods have been described for detection of fish parvalbumins, and most use monoclonal antibodies. In one recent study,[78] a rapid method using a surface plasmon resonance biosensor was developed that could detect parvalbumins from carp, sardine and skipjack tuna (katsuonut) in five minutes. This work built upon previous accomplishments by this group in developing monoclonal antibody (against bluefin tuna parvalbumin) that was cross-reactive to all vertebrate parvalbumins.[79] No detection limit in food for the biosensor technique was reported, but the authors felt the method would be useful in detecting undeclared fish residues in foods and that the biosensor chip has the advantages over ELISA methods of regeneration and continuous use. However, it is unknown whether this method will detect many different types of fish.

15.5 Future trends

For soy, some tests with good sensitivity have been described, and it is

expected that more sensitive ELISAs will soon be commercially available. Together with DNA-based methods, tools are available to test for the presence of soy residues in a wide variety of ingredients and food matrices in a sensitive way. There is, however, a strong need for soy reference materials in order to enable quantification and comparison of test results between different laboratories. In addition, highly-processed soy products will still be difficult to analyze, especially when the processing history of the ingredients is not known.

More research in the area of detection of fish and also crustacea residues in foods needs to be conducted to provide a variety of methods that are sufficiently easy and relatively inexpensive for use by the food industry in protecting seafood-allergic consumers. A main challenge for detection of crustacea and fish is related to specificity. Are all relevant species within the large family of crustaceans and fish recognized to the same extent? An additional issue concerns proteins that are potent allergens in crustacea/fish while their homologues in other species are harmless; appropriate assessment of cross-reactivity issues will be important in this respect.

The 'Big Eight' allergenic foods are the most relevant for the food industry to measure as they represent the foods that sensitive consumers are most often allergic to. This chapter deals with three of them while the others were discussed in earlier chapters. The list of allergenic foods in the EU was recently extended with adoption of legislation on food allergen labelling. The added allergens are celery, mustard and sesame. For these allergenic foods no commercial tests are available. A polymerase chain reaction (PCR) for celery has been described, and for mustard a polyclonal antibody-based ELISA was recently developed (Chapter 12). Future challenges for these foods are numerous: for some of the allergenic foods, methods need to be prepared from scratch by raising antibodies and constructing ELISAs. Other allergens are further along and opportunities exist for commercialization. For all of them, even including the allergenic foods dealt with earlier, reference materials are required, and methods that are available now need to be validated in interlaboratory studies. All in all, for this latter group of allergenic foods, much effort is still required in order to have the tools needed to protect affected consumers.

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Part IV

Issues in allergen detection methods

16

Allergen quality assurance for hypoallergenic formula

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16.1 Introduction

The development and production of hypoallergenic infant formulas (HIF) present some unique food allergen control challenges. Almost all infants using HIF have already developed strong allergies to cows' milk and soy proteins. Some of these infants will react violently to extremely small amounts of allergen.[1] Formulas made for this population represent a severe test of allergen content control in manufactured foods.

HIF are usually made using extensively hydrolyzed proteins selected based on their almost completely diminished immunologic reactivities.[2] Current HIF use enzymatically hydrolyzed cow milk casein or whey proteins. Manufacturing hydrolysates for HIF is a challenging biochemical task. As the amount of hydrolysis increases, the functional properties, organoleptic characteristics, and biochemical stability of the hydrolysate deteriorate. During hydrolysate development these factors must be balanced while achieving the required reduction in immunologic reactivity. Once a satisfactory hydrolysis procedure is set, it must be validated and controlled, and each hydrolysate ingredient batch must be tested against strict allergen content specifications.

HIF are often produced in facilities that also manufacture other products containing intact or partially hydrolyzed cows' milk and/or soy protein allergens, often using at least some shared equipment. In this environment, HIF allergen quality assurance requires careful control of ingredients, effective and consistent equipment cleaning procedures, processing and packaging controls, and valid testing methods to verify product identity and quality. These methods and procedures must be executed in an appropriately documented total quality environment that includes allergen awareness training for plant personnel.

Procedures and training must be targeted to the needs of the specific manufacturing process. Quality systems used to successfully manufacture HIF may be applied, where appropriate, to aid in allergen control in more general food production settings.

This chapter will present strategies and technology used to successfully produce formula intended for the most severely food-allergic populations. Section 16.2 will define key terms and discuss some important assumptions that simplify the study of the immunology and immunochemistry of food allergens. Relationships between clinical allergen reactivity thresholds and analytical method's sensitivity requirements for HIF will also be addressed. Section 16.3 will provide a description of allergen quantitation methods used to test HIF. Enzyme-linked immunosorbent assay (ELISA) methods are the most valuable, and strengths and weaknesses of various ELISA procedures will be discussed. Section 16.4 will present examples of how the analytical tools can be applied to validate allergen-cleaning protocols and verify product quality. Section 16.5 will summarize the current state of allergen quality control technology, identify unresolved issues and uncertainties, and attempt to integrate the analytical capabilities with operational procedures and plant worker training to develop a comprehensive allergen management quality system.

16.2 Key terms and clinical and analytical performance targets

16.2.1 Definitions and characteristics

'Hypoallergenic' literally means 'less allergenic'. This description has no specific quantitative limits and can accurately represent comparisons between many cooked and raw foods, processed versus unprocessed foods, or hydrolyzed and unhydrolyzed proteins. Management of food allergy in infants requires a more rigorous definition that relates to the clinical performance of hypoallergenic formulas. American and European Pediatric Societies and regulatory agencies now agree that 'clinically hypoallergenic' formulas must not cause allergic symptoms when fed to 90% of infants allergic to the base protein used to manufacture the formula. This definition also requires that sufficient clinical research using double-blinded, placebo-controlled food challenges (DBPCFC) must be done to have at least a 95% statistical confidence in establishing the > 90% negative allergy reaction frequency.[3,4] It is important to note that some clinical allergic reactivity should be expected for HIF (up to 10%) and that these products are not 'non-allergenic'.

Protein hydrolysates used in HIF are selected for their minimal allergenic reactivity. Successful development of these products requires an understanding of the structure/function and dose/response relationships between food allergens and the immune system. Research in this area is complicated by the highly

variable clinical manifestations of food allergy and the inability to comprehensively identify all food allergen epitopes. This makes it difficult to study food allergens directly. However, it is possible to predict the allergenic potential of modified protein systems using much more standardized animal models of immunogenicity (all allergens are immunogens) and immunochemical assessments of antigen content (allergens and immunogens must be antigens). Data demonstrating very low formula immunogenicity in hyperimmunized animals and very low antigen content by sensitive immunochemical methods are important prerequisites for clinical challenge studies in allergic patients.[2] DBPCFC in specific food-allergic patients are still required to establish the true hypoallergenic clinical performance of HIF.[5]

16.2.2 Identifying hypoallergenic ingredients

The first step in HIF development is to identify a suitable hypoallergenic protein hydrolysate ingredient. This involves an immunochemical assessment of the degree of reduction in protein antigenic reactivity following hydrolysis. An important question is, 'How much reduction in immunologic reactivity is sufficient to achieve hypoallergenic clinical performance?' This question is analogous to the key allergen question in the general food industry, 'How much unlabeled food allergen cross-contamination is too much?' Both questions are addressed by exploring clinical data describing allergic reaction threshold doses. Data from HIF clinical studies are not comprehensive but indicate that 200 μg of milk allergen / feeding might be well in the 'hypoallergenic range' with actual formula reactivity rates of 2–3% of milk-allergic infants.[6] However, there are isolated cases where less than 2 μg of allergen protein has created strong reactions in the most highly allergic individuals.[1]

The allergen content data described here were obtained using sensitive inhibition-format ELISA (discussed in Section 16.3 and Chapter 6). ELISA results can readily differentiate clinically hypoallergenic products (and hydrolysates) from more allergenically active products based on partially hydrolyzed proteins (Table 16.1). As a general rule for formulas based on cows' milk protein hydrolysates, hypoallergenic clinical performance can be achieved at residual allergen concentrations below 15 μg/mL, with the most effective formulas containing less than 1 μg allergen/mL. (Testing is performed at ready-to-feed concentrations on liquid formula or reconstituted powdered formula, serving size is approximately 250 mL.) Therefore, to be useful in quality assurance testing of hypoallergenic formula, analytical methods to measure the casein and whey allergen components must have quantitative sensitivity limits in the 100 ng/mL range. This sensitivity limit also seems adequate for general food industry applications. Key complicating issues for adapting these methods to other products are variability in serving size and the need to test non-liquid products. The most important challenge in setting allergen content limits is the difficulty in obtaining clinical data from blinded,

Table 16.1 Antigen concentration of hydrolysate infant formulas[1]

Formula	Casein	Whey	Total	Reactions[2]
Alimentum (H)	0.14	0.20	0.34	3
Good Start	4.40	520	524	45
Nutramigen (H)	0.19	0.43	0.62	2
Pregestimil (H)	0.19	NA	NA	NA
Profylac (H)	0.50	13.5	14.0	4

[1]By inhibition format ELISA based on rabbit antisera to bovine casein or whey, reported as µg immunologically active antigen/mL, at ready-to-feed formula concentration (approximately 15–18 gm protein/L)
[2]Clinical reaction rate (%) in milk-allergic infants (from reference 6)
H = hypoallergenic clinical performance

controlled, progressive allergen challenges in highly allergic patients. Some of the most sensitive patients are also at the greatest risk during challenges. Current data also indicate that sensitivity limits are not the same for all food allergens (Figure 16.1).[7] Dose–response data have been reported for peanut,[8] hazelnut,[9] egg,[10] milk,[11] and soy.[11] Interestingly, recent data from soy-allergic infants and children suggest a substantial difference in reaction thresholds between cows' milk and soy allergens.[11] If confirmed, this might ease the sensitivity requirements of soy allergen quantitation methods.

When selecting method sensitivity performance targets for product allergen control applications, the most (clinically) meaningful data are derived by measuring the allergen content in a single serving of the food product. There is no agreed allergen content limit for food products at this time.[12] The best performing hypoallergenic formulas contain less than 200 µg of immunologically active antigen per 250 mL serving. This could be considered an upper limit for acceptable serving doses of other strong food allergens.

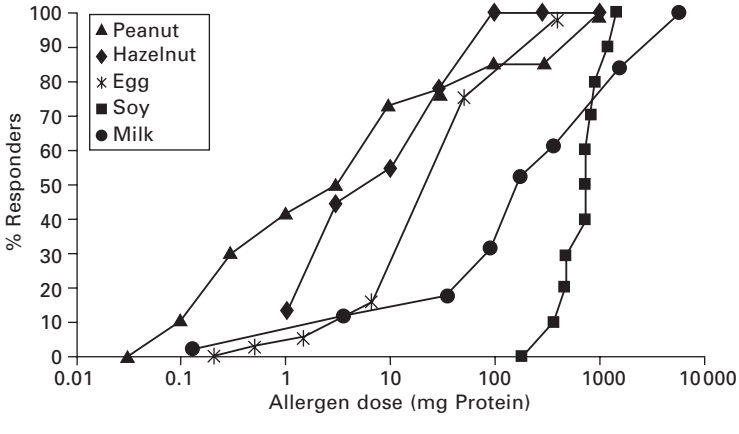

Fig. 16.1 Food allergen reaction thresholds (by DBPCFC). Ingested allergen dose (mg protein) vs % allergic responses in challenged patients. Adapted from reference 7.

Method sensitivity requirements for a specific food product can then be estimated by dividing this amount by the product serving size. These results should be modified based on more extensive information on the acceptable dosage limits for specific allergens as this information becomes available. Finally, HIF allergen doses are delivered in liquid form, while most general food allergens are solids. Liquid allergen doses may be more immediately active than solid allergens. If so, liquid allergen reactivity thresholds may be lower than the same allergen ingested in solid form.

16.2.3 Hypoallergenic formula production controls

Once the hypoallergenic hydrolysate has been selected and the analytical methods are in place, commercial production of HIF presents its own challenges. HIF must be free of allergen contamination. HIF allergen quality assurance programs begin with ingredient qualification and include manufacturing equipment cleaning validation, manufacturing process control, and finished product release testing to meet very low antigen content specifications. The ELISA methods used during HIF product development are also useful in quality assurance. The analytical testing goal is to assure HIF products are free of 'immunologically significant' levels of contaminating antigens. How much antigen is 'immunologically significant' in the context of preventing allergic symptoms in already-sensitized patients? As discussed above, there is no agreed standard. Manufacturing HIFs that successfully achieve hypoallergenic clinical performance provides an interesting model for food allergen control. Analysis of this process illustrates methods to avoid contamination, confirm production equipment cleaning, and judge product quality.

16.3 Analytical methods

16.3.1 Method applications

Methods for food allergen quantitation have three main applications in hypoallergenic formula manufacturing. First, quantitation of residual immunologically active allergen fragments in hydrolysate ingredients. Second, detecting and quantitating potential protein contaminates from other non-protein ingredients, shared processing equipment, and the manufacturing environment. This includes validating and monitoring production equipment and environmental cleaning procedures. Finally, allergen content of the finished formula must be measured as a fitness for use specification. Each application has its own requirements and technical challenges. In addition to allergen quantitation and detection of contamination, it is also sometimes important to have rapid and/or inexpensive methods to identify hypoallergenic ingredients and formula with non-immunologic tests.

Measuring residual allergen content in the hydrolysate ingredient is complicated because the analytical method must accurately measure fragmented

antigen that may contain only one or two antibody-binding epitopes. Even these small fragments can trigger allergic reactions in some patients. This makes assay formats based on antigen capture or 'sandwich' approaches unusable. Measuring hydrolysate allergen content is made easier because these ingredients are usually highly soluble powders that can be dissolved at relatively high concentrations to achieve desired method sensitivity. The assay may also be carried out in buffer systems that are optimized for method performance. Data are best reported in units of μg immunologically active antigen/g total protein. Hypoallergenic hydrolysates usually range from 20–100 μg immunologically active antigen/g protein. Assuming that these ingredients are tested at a concentration of 10 mg protein/mL, the ELISA method should be sensitive in the range of < 200 ng/mL.

Testing for contaminants from non-protein ingredients, carry-over from production equipment, and the manufacturing environment can be accomplished using sandwich-type assays since the contaminants will usually be intact proteins from other ingredients and products. These capture assays are highly sensitive and are generally less time-consuming. Capture assays also operate with less interference from sample matrix anomalies and can be validated for use with a number of liquid and solid product forms. Currently, a number of commercial ELISA kits are offered to measure allergenic food residues (Neogen, Lansing, MI; ELISA Systems Ltd, Brisbane, Australia; R-Biopharm, Darmstadt, Germany; Tepnel Life Sciences PLC, Deeside, UK).

Samples for these tests include product contact surfaces and environmental samples collected by swabbing techniques. The swabbing methods are similar to those used to collect samples for microbiological testing. A standard surface area is swabbed, then the swab is placed in a set volume of solvent (usually a neutral buffer with dilute detergent) and the dissolved allergen is measured. A second sample type is rinse water following a clean-in-place (CIP) cycle used to clean closed liquid processing systems. Care must be exercised in collecting these samples. The samples must be a representative rinse water sample and not a diluted cleaning fluid. Chemicals in cleaning fluids in many cases will interfere with the assay immunochemistry. The preferred protocol is to perform the CIP cycle, then flush the system with a volume of clean water similar to the normal volume of liquid product that is used to flush the system prior to initiating product collection during normal production. Sensitivity requirements for assays used in this application are difficult to assess since there is usually not a clear relationship between antigen levels found on product contact surfaces or in rinse water samples and antigen content of the initial amounts of formula product produced at start-up. This will be discussed in more detail in Section 16.4.

Measuring residual allergen in finished hypoallergenic products has immunochemical and sensitivity requirements similar to hydrolysate characterization methods. Capture format assays are not useful. The tests are also made more difficult by interference from matrix effects associated with the emulsified formula. Similar problems in non-hypoallergenic food

applications can be largely overcome by using capture format assays. Non-hypoallergenic foods are not routinely tested for allergen content. However, selectively applying these tests to the first product samples made after an equipment cleaning trial is a useful way to help interpret data from the trial and to better understand relationships between equipment cleaning trial results and product allergen content (see Section 16.4).

16.3.2 ELISA methods for hypoallergenic formula

Currently the most widely used analytical method for allergen quantitation is the ELISA. Appropriate ELISAs can be used to measure fragmented antigens (characterize hydrolysates and HIF) and intact antigens (detect HIF contamination and monitor equipment/environmental cleaning). Successful ELISA development requires knowledge of specific food allergen immunochemistry. There are a number of complicating issues: food allergens are complex mixtures of immunologically active proteins; specific allergen reactivity will be affected differently by various food processing methods, including hydrolysis; the allergen epitope recognition pattern will vary widely among allergic patients and may also be different from the antigen epitope specificity of the anti-food protein antisera used in the assay (usually animal-derived); allergens are not measured directly, antigen content (measured with IgG antibodies) is used as a surrogate; individual allergens are also complex with many antibody recognition sites (epitopes) per allergen; data are reported as 'immunologically active (parent protein system)' based on standards and control samples; there are no 'certified' food allergen standards so the potency, specificity, and stability of assay standards and control samples must be individually established and be internally consistent from batch to batch of standard.

Food antigen ELISA methods use either direct (sandwich) or inhibition formats and are easily sensitive to 10 ng antigen / mL, with typical accuracies of ± 5–20%. These methods are covered in detail in Chapter 6 so the discussion here will be limited to elements of ELISA validation for testing hypoallergenic formula. Quality elements for ELISA methods used to test hypoallergenic formula include: potency, specificity, and stability of the anti-parent protein antisera used as the key reagent in the test; solubility, consistency, and stability of the selected standards and control samples; test sample integrity; and optimization of operating conditions including validation of electronic data collection, calculation, and reporting systems.

16.3.3 ELISA quality elements

Anti-food allergen antibody potency

ELISA quality starts with selection or generation of the anti-food protein antisera. This antibody is the most important reagent, determining specificity and sensitivity of the test. Polyclonal antisera generated in hyperimmunized

laboratory animals seem to be the best choice. These antisera can be obtained in relatively large amounts using standardized food allergen samples as immunogens. This yields reproducible potency and specificity, especially when more than 10 animals are immunized and the processed and qualified antisera are pooled. Animal antisera generated in this way are preferable to food allergen-specific human IgE pools because they can be reproducibly generated, have a broader and more consistent specificity profile, and are cheaper and more widely available. The disadvantage of using animal antisera is that allergen epitopes recognized by allergic humans are not necessarily the same as immunogen epitopes recognized by hyperimmunized animals. Food allergy in humans tends to have limited and individually variable reactivity patterns to the complex mixture of proteins contained in a food allergen. Establishing a pool of human IgE antisera that reacts strongly with all proteins in a selected food allergen (and only those proteins) has not been feasible to this point. Use of IgG antibodies from animal antisera means that the ELISA is technically measuring food antigens, not food allergens. However, while antigens may or may not be allergens, all allergens must be antigens. Using animal antisera to measure food antigens may overestimate food allergen content, but this theoretical error is on the side of product safety. Operationally, the use of animal antisera has not been a problem in interpreting food allergen information in the hypoallergenic formula industry for more than 15 years.

Having selected hyperimmunized animals as the source of antibody for food antigen analysis, interest then turns to optimizing antibody potency and specificity. Both are controlled by the hyperimmunization protocol.[9] Animal immunizations should be given at relatively high antigen doses (5 mg protein/immunization in 4 mL of immunogen), in a potent oil-based adjuvant (Complete Freund's Adjuvant, CFA) processed mechanically to form a stable emulsion (two volumes CFA: one volume antigen solution). Animals are given a booster immunization at three weeks (5 mg protein, one volume CFA: one volume antigen solution) with additional boosters at four to six week intervals (substituting Incomplete Freund's Adjuvant for CFA). Antisera collection is started at four weeks and can continue for several months. Typically, at least 10 animals are immunized. This immunization protocol assures antisera potency and can generate antibody titers greater than 10 million.[13] Antigen-specific antibody titers are obtained from individual animals at all time points and evaluated for sample pooling. After the desired serum pool is made and mixed thoroughly, the pool is aliquoted into cryo storage vials and stored at < -70 °C. Antibody activity stability under these conditions is > 10 years. Upon thawing, the cryo vial contents are realiquoted into smaller freezer tubes that are used as working sources. These are stored frozen (~ –20 °C) in a non-frost-free freezer and are stable for at least 12 months.

Anti-food allergen antibody specificity

With antisera potency established by hyperimmunization, specificity must be determined. Hyperimmune antisera specificity will largely be controlled

by the purity of the immunogen used for hyperimmunization. A second factor is the animal's diet. While the hyperimmunization protocol will be sufficiently vigorous to break antigen-specific oral tolerance, it is still advisable to restrict the food antigens of interest from the animal's diet, through the previous generation if possible, or at least from birth.

Since all food allergens are complex protein mixtures, it is important to establish that the antisera will detect (bind to) each antigen contained in the mixture. This can be accomplished qualitatively using immunoblotting or immunoelectrophoresis, and quantitatively using protein-specific ELISAs. Quantitative specificity analysis requires the availability of purified individual protein components of the food allergen. In the quantitative tests purified individual antigens are coated onto ELISA plates and the antiserum antibody titer (reciprocal antiserum dilution generating a specified ELISA signal) to each allergen component is established. These titers indicate the utility of the antisera and can be used to quantitatively compare its relative specificity (Table 16.2, column 3: ELISA titer). The titer testing profile used to establish specificity should also be used to show equivalence of new antibody preparations to specificity ranges of previous antisera pools. If antisera specificity does not meet acceptance criteria, the antibody must be regenerated, usually using an immunogen supplemented with antigens that gave sub-potent responses in the original immunization.

When using an inhibition format ELISA, antisera specificity is only half of the assay specificity equation. The protein content and purity of the plate coating antigen are other important determiners of overall assay specificity. Note, in Table 16.2, for the bovine casein and soy ELISAs, high antibody titers to the desired antigen and low concentrations (absence) of other proteins in the coating antigens combine to give these assays very high specificities.

Table 16.2 Relative antisera and assay specificity (from ELISA data)

Antibody to	Antigen	ELISA titer	Coat[1]	% Reactivity[2]
Bov. casein	Bov. casein	1 930 000	99.7	100
	Bov. β-lactoglobulin	9470	0.29	0
	Bov. α-lactalbumin	9500	0.01	0
	Bov. serum albumin	16 200	0	0
Bov. whey	Bov. whey	6 670 000	70	89.0
	Bov. casein	1 930 000	30	11.0
Soy protein	Soluble soy protein	318 000	100	100
	Bov. casein	6400	0	0
	Bov. β-lactoglobulin	543	0	0
	Bov. α-lactalbumin	272	0	0
	Bov. serum albumin	149	0	0

[1]Individual protein concentration in plate coating antigen (g/100 g)

[2]Assay specific reactivity (%) $= \dfrac{(\text{antigen titer}) \times (\text{antigen [coat]})}{\Sigma[(\text{antigen titer}) \times (\text{antigen [coat]})]} \times 100\ \%$

For the whey ELISA, specificity is lower (89%). This is an unavoidable consequence of the nature of commodity whey protein concentrates. Approximately 70% of the protein in these ingredients is made up of the expected whey proteins, β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulin. However, the remaining 30% of protein is κ-casein macropeptide and proteose peptone, both soluble casein fragments generated during the cheese manufacturing process. Therefore, common food ingredient whey protein concentrates will contain casein antigens and immunochemical methods to detect 'whey' will also usually have some level of casein reactivity.

In some cases, selecting the optimal coating antigen can reduce the complexity of ELISA analysis. By incorporating acceptable protein ingredients used in formula production as sources of coating antigens and antigen standards it is possible to achieve close alignment between the assay's performance with standards and unknown samples. This is appropriate since contamination, if present, will be derived from the protein ingredients used in the production facility. The success of this approach relies on the lot-to-lot consistency of composition and character of the commodity ingredients. These usually vary more than defined biochemical standards. However, careful comparative characterization of commodity protein ingredient lots has allowed this strategy.

Another consideration in measuring complex food antigens is that single protein components in purified form should not be used as coating antigens or antigen standards to assess total protein antigen content or residual antigen in protein hydrolysates. For example, the four major bovine whey proteins have different heat and enzymatic digestion stabilities.[14] Removal of one specificity by heat treatment or enzymatic digestion does not guarantee the same level of removal of the other whey protein antigens.

Judging ELISA method specificity requirements should be done with the manufacturing facility's ingredient inventory in mind. Assay specificity can be custom selected for each location. Key questions are, 'What allergen-containing ingredients are present in the facility?' and 'Is the cleaning efficiency of each allergen the same?' (answer to the latter question is usually 'no' or 'not sure'). Example: if a manufacturer used cows' milk protein as an ingredient, this could come in the form of condensed skimmed milk, non-fat dried milk, caseinates, or whey protein concentrates. Each contains 'milk allergens'. However, these ingredients have different impacts on processing equipment cleaning requirements since whey proteins are sometimes more difficult to clean from equipment than caseinates. If the plant ingredient inventory shows both whey protein concentrates and caseinates, then separate assays for casein and whey may give a clearer picture of processing equipment cleaning validation trials. Specificity will then be judged separately to establish that each antiserum adequately recognizes the different protein components of casein or whey antigens contained in the ingredients.

Specificity of capture format ELISAs is determined by the specificities of the capture antibody and the detecting antibody, plus the composition and character of the 'sandwich' antigen. Specificities of the two antibodies must

be determined independently if a different antibody preparation is used for each. At least one of the antibodies should be polyclonal, but several combinations of monoclonal and polyclonal antigen-specific antibodies can be successfully employed. Use of appropriate protein commodity ingredients as antigen standards in capture format ELISAs can also increase the value of these ELISA data in assessing food allergen control strategies.

Establishing food antigen standards and control samples

Antigen standard preparations are used to establish the accuracy of ELISA antigen quantitation methods while the precision of the tests and their stability over time are monitored using antigen control samples. An antigen standard is simply a stable antigen solution of known concentration. However, preparing antigen standards for ELISA methods is more complicated than simply dissolving food proteins in appropriate buffers and determining protein concentration. The objective of the assay is to measure the amount of immunologic reactivity of the sample, its antigen content. This is not always the same as protein content, especially for commodity food proteins that have been exposed to heat treatment and other food processing stresses. Processing treatments tend to decrease the amount of immunologic reactivity for a set amount of food protein. For some commodity ingredients there can be more than an 80% lot-to-lot difference in immunologic reactivity per unit protein. It is therefore important to select antigen standards that are as undenatured (immunologically active) as possible. Unfortunately, at this time there are no commercially available 'gold standard' food antigen preparations. Therefore, careful selection of the best materials available and complete characterization of each lot of these assay standards is important. Key quality assessment targets for standards and control samples are purity, compositional completeness of protein elements, high relative immunological reactivity, consistency, and solubility.

Once selected and characterized, the stability of the standard must be established. Stability of common powdered food antigens stored at –70 °C is excellent (> 10 years), while reconstituted standards are usually stable when stored as concentrated solutions (1–3 mg protein/mL) at –20 °C in non-frost-free freezers for 12–24 months. Assay control samples are usually made from different batches of the same materials used for assay standards. Control samples are stored under the same conditions as standards and will demonstrate similar stabilities.

Sample integrity

Sample integrity is critical in food allergen testing, especially with the high sensitivity of the test methods. Sample contamination must be avoided. Special care must be used in sample collection where contaminated collection equipment and poorly cleaned equipment sampling ports can cause problems. Another concern is that very low but significant levels of antigen can be lost from sample solutions if the antigenic proteins adsorb to the surface of the

sample container. For rinse water samples this can be avoided by using EPA (US Environmental Protection Agency)-approved glass vials designed for water testing. Place 5 mL of a 10X concentrated buffer containing Tween® 20 (ICI Americas Inc, Wilmington, DE) detergent into the vial, then add 45 mL of the rinse water sample. The resulting solution is a 10% dilution of the sample that is properly buffered while any protein is kept in solution by the dilute detergent. Sample integrity issues should be resolved on a case-specific basis during method validation.

Optimizing assay operating conditions

A wide variety of elements within an ELISA can (must) be optimized to achieve the desired accuracy, precision, sensitivity range, and ruggedness. Assay conditions can also be changed to achieve some time and cost savings goals. There is not a single answer to the question 'How to best perform an ELISA?' Each situation must be addressed based on its unique needs. Evaluation of these needs should be a key element in prospective assay validation planning. Unfortunately, unlike testing hypoallergenic formula, the analytical targets for allergen testing of general foods to control allergen contamination have not been defined by extensive clinical research, and are therefore poorly understood. Without a clear target for 'How much is too much?' it is impossible to know with certainty the meaning of food allergen test results, and also impossible to set firm analytical performance requirements. On the other hand, when applied in an integrated fashion, by testing environmental and equipment cleaning samples and linking these results to antigen testing on first-produced product, it is possible to identify environmental and equipment locations that show much higher than normal antigen levels. These 'hot spots' can then be lowered or eliminated by environmental or equipment cleaning modifications. This is a major goal of food allergen testing of shared processing equipment.

Data handling

The final element of analytical quality is data manipulation and reporting. As in all cases, the automated data collection and calculation hardware and software must be justified and validated. Standard curves will be used and the fit parameters for standard curve acceptance must be established. Acceptable control sample performance parameters will be established and a data quality acceptance tree can be constructed (Figure 16.2). Consistent data reporting in meaningful units of measurement is also important. Since agreed food allergen standards do not exist, antigen content data will not be traceable to officially designated standard materials. The chosen assay standards must therefore be internally consistent to establish data quality. Linking data collected using internal standards with the clinical performance of formula in DBPCFC trials establishes the utility of the assay data as a product quality control tool. Optimal reporting units for hypoallergenic formula quality assurance testing are situational: for hydrolyzed protein ingredients and hypoallergenic formula,

1. Standard curve fit performance:

   Is the correlation coefficient (R^2) of the standard curve fit > 0.98?

   Yes: Go to step 2.

   No: Discard the data, repeat the test.

2. Control sample performance:

   Are results for the control sample(s) within acceptable limits?

   Yes: Sample data are acceptable.

   No: Discard the data, repeat the test.

Fig. 16.2 ELISA data quality acceptance decision tree.

μg immunologically active parent protein/g total protein (multiplied by g protein/serving = total antigen dose/serving, the key indicator of clinical reactivity); for rinse water samples, μg immunologically active parent protein/mL of sample; for swab samples, μg immunologically active parent protein/mL (assumes a standard swab solvent volume [usually 5.0 mL] which can be converted to μg immunologically active parent protein/m^2, based on the area of the equipment surface that was swabbed).

16.3.4 Non-immunologic testing

Hypoallergenic formulas must undergo all normal infant formula nutrient testing and microbiological/sterility testing to demonstrate quality. These formula and their unique ingredients must also be properly identified. The specificity that makes food antigen immunochemical assays so valuable can also limit their utility. Biochemical methods have been developed for use in these situations.

Biochemical characterization of hypoallergenic hydrolysates

Hypoallergenic protein hydrolysates cannot be identified and clearly differentiated by ELISA from intact proteins from sources other than the hydrolysate parent protein. For example, a hypoallergenic casein hydrolysate and intact soy proteins both will give very low to negative results in a milk protein or casein ELISA test. Low/negative ELISA data must be interpreted as 'no/low milk antigen', but should not be interpreted 'hypoallergenic hydrolysate' since intact soy proteins, protein-free carbohydrates, and a number of other ingredients will also be devoid of milk allergens and yield negative ELISA results. A separate identification method is required. As hydrolyzed protein, the hypoallergenic hydrolysate will have a characteristically high amino nitrogen to total nitrogen ratio (AN/TN). Hypoallergenic hydrolysates are easily identified by a combination of high AN/TN (matching set specifications) plus a very low (also to specification) antigen content by ELISA. Both tests are needed to establish the hypoallergenic ingredient's identity.

Biochemical identification of hypoallergenic formula
Most current hypoallergenic formulas are based on hydrolyzed cows' milk casein or whey proteins. These formulas must meet rigorous casein and/or whey antigen content lot release specifications. However, as above, other formulae based on non-cows' milk proteins will also show negative results in casein and whey protein ELISA methods. Polyacrylamide gel electrophoresis (PAGE), when visualized using sensitive silver staining, will show strong banding patterns individually characteristic of non-hypoallergenic formula. PAGE analysis of hypoallergenic formula does not yield staining.[15] Most non-hypoallergenic formulas manufactured in our facilities contain significant amounts of intact milk proteins. The presence of these formulas as contaminants will be easily detected using a casein ELISA. Soy-based formula is not detected by casein ELISA, but is easily detected with PAGE. Using both methods, from a food allergen content perspective, it can be shown that a formula sample contains 'very low casein antigen, according to hypoallergenic formula specification' and that it is 'not intact soy-based formula'; therefore, 'positive identification as hypoallergenic formula'.

16.4 Applications

16.4.1 Ingredient identification

Quality assurance for hypoallergenic formula begins with selection of protein hydrolysates with the capacity to achieve hypoallergenic clinical performance. Whether these ingredients are self-manufactured or purchased from a vendor, a careful analysis of the hydrolysate manufacturing process is required. The goal of this effort is to reproducibly manufacture a hydrolysate with an extremely consistent and well-controlled degree of hydrolysis that is free of contaminants. Hypoallergenic utility of the hydrolysate is best predicted using animal models of immunogenicity[1, 13] and established in blinded food challenge clinical studies with food-allergic patients. Before this research is started, it is critical that the process for manufacturing the hydrolysate be consistent and fixed. This is not a straightforward task. Variables include the nature and consistency of the intact protein substrate, the consistency and composition of the enzyme and/or chemical systems used for hydrolysis, the reproducibility and control of the hydrolysis process (time, temperature profiles, concentrations of reactants over time, pH and ionic character control), microbiological control, reproducibility of post-hydrolysis purification, drying conditions, packaging, storage conditions, and transportation. In this process the ability to measure very low levels of all allergenic protein systems present in the hydrolysate manufacturing facility is important. The inhibition format ELISA is clearly the best method available for this purpose. The end result of this research is a rigorously defined and validated set of ingredients, procedures, and specifications, along with a quality system that will ensure

their execution, which produces a consistent hydrolysate with very low residual antigen content that is free of contamination.

The use of single protein-dedicated or 'functionally dedicated' facilities for hypoallergenic hydrolysate production is most helpful and allows monitoring of only one protein allergen source to establish adequate cleaning. If additional proteins must be present in the production environment, additional allergen testing will be necessary. Environmental controls deserve special attention when configuring these facilities. Special care must be taken to control allergen-containing dust if the protein substrate or any of the hydrolysis components are powdered products (as is most often the case). Creative air handling and filtration can be of great value in controlling airborne allergens. Protein dust may be vacuumed or washed clean but should never be blown with compressed air. The analytical goal is to perform environmental testing to identify and eliminate allergen hot spots, then correlate the cleaned environment with production of hydrolysate batches that are free of contamination. Controlled repetition of the cleaning process with appropriate ELISA monitoring of antigen content of the environment and hydrolysate produced after cleaning will establish validity of the cleaning process for both environment and production equipment. These data will also validate the allergen integrity of the production processes and their controls.

16.4.2 Ingredient receipt, testing, and storage

Obviously, care must be taken to avoid ingredient contamination during transportation and at receipt. Usually the ingredients are powdered products in a container that may not be airtight. This allows the generation of some dust that, if not controlled, can lead to significant levels of contamination. Strategies to minimize dust risk include air movement and filtration optimization, a firm policy for spill cleanup, and sequestering ingredients for hypoallergenic products away from other protein ingredients. It is important to note that all ingredients used in the hypoallergenic formula require this treatment.

Ingredient acceptance testing is carried out to establish the quality of the ingredients. Hydrolysates will be tested against biochemical, microbiological, and antigen content specifications (by inhibition format ELISA). Non-hydrolysate ingredients will receive normal characterization testing, but special care must be taken to ensure that these ingredients are free of protein. Both are then stored under conditions that will ensure stability and prevent contamination.

16.4.3 Allergen awareness in the manufacturing environment

A number of strategies can be employed to ensure that hypoallergenic products are free of contaminating allergens. All these start with food allergen awareness training for production managers and operators. The goal of this training is

to raise awareness of the serious nature of food allergies in all who contribute to formula quality. It is vital to gain commitment from operators, analysts, and managers to proactive implementation of food allergen safety programs. Training should foster continuous improvement in procedures and equipment to ensure the food allergy safety of products. There is no substitute for informed, committed, and empowered employees in food allergen control.

Generally, food allergen awareness training should include the what, why, and how of food allergen control. This will begin with a description of food allergies, what causes the allergic reactions, and the serious consequences including death that can result from uncontrolled allergic reactions to food. The regulatory environment should also be addressed, describing the increased emphasis on food allergen regulation and FDA inspection, and the growing food allergen product recall pattern. The potentially life-threatening consequences for consumers should be re-emphasized and product liability risks should be covered. Training should raise awareness in the context of the regulations and establish personal commitment to proactively managing food allergens in the manufacturing environment. With that accomplished, training on allergen management can begin. This will include training on plant-specific allergen control procedures for ingredient receipt, acceptance testing and storage, equipment and environment cleaning procedures, allergen testing and data interpretation, allergen control in formulation and processing, and the importance of labeling information and packaging. Finally, the quality systems that ensure compliance with food allergen management procedures should be described and the trainees should be invited to suggest further improvements as opportunities arise.

Training for operators and production supervisors is usually most meaningful when it is custom designed for individual production environments. Training for middle and upper managers should be more global and take advantage of combined information from a variety of food industries. The University of Nebraska's Food Allergy Research and Resource Program (Lincoln, NE, USA; www.farrp.org) provides an excellent short course on food allergen management in industry and is recommended for this group.

16.4.4 Production environment and equipment cleaning

Production environment and equipment cleaning will involve dry cleaning, wet washing, and clean-in-place approaches. In all cases the objective is to reproducibly remove allergens using controlled, documented cleaning procedures that have been demonstrated to be effective by repeated testing (validated). Use of sensitive antigen detection methods is critical to success here. As mentioned earlier, the capture format ELISA is probably the most rugged and useful method in this application and a number of commercial ELISA kits can be used effectively. An important element of using commercial kits is to validate kit performance in detecting the specific antigens present in the facility prior to initiating cleaning trials.

Advice on allergen cleaning procedures will be equipment-specific and is beyond the scope of this chapter. However, there are some common principles that deserve mention. First, contamination can be 'systematic' or 'spot'. Systematic contamination can be detected, causes identified, and procedural corrections made to avoid the problem. Spot contamination is usually from environmental sources and is much more difficult to address. Spot contamination appears randomly and is especially hard to detect in powdered or solid products where homogeneity of the formula is not assured. Second, the relationship between equipment/environment allergen test result and level of product allergen contamination is almost always unknown. Just as there is no firm answer to the clinical question, 'how much is too much?', there is no firm answer when the same question is asked of cleaning/manufacturing data. This is especially challenging in interpreting environmental test results. It is possible to produce uncontaminated products (sometimes) in the presence of relatively high levels of antigen in the production environment. However, inability to interpret the data in absolute terms should not be used to justify not performing the testing.

Environmental testing should be conducted as a risk management exercise designed to lower risks by identifying antigen hot spots and modifying cleaning procedures to minimize environmental antigen content. While the antigen results have limited absolute meaning, less is better, and 'undetectable' is an assurance of very low risk. Finally, antigen results from swabbing food contact surfaces and from rinse waters of production equipment will tend to be most closely related to formula contamination potential. These tests will provide the most valuable information in identifying hot spots and establishing validated cleaning systems. In this effort, special care should be given to processing equipment that heats liquid products during manufacturing since 'burn on' of food allergens is a common cleaning problem. 'Dead spots' in liquid processing systems (valves, sampling ports, joints, etc.) where turbulent flow is not maintained during cleaning also represent special cleaning challenges and should be included in the sampling plan.

Antigen content data from environmental and equipment cleaning trials should be analyzed for correlation with antigen testing of products made following the various cleaning efforts. A future goal is to better understand the relationship between the more abstract data from cleaning trials and clinically-relevant data on product antigen content.

A theoretical example of trials designed to measure and optimize allergen cleaning is shown in Table 16.3. Trial 1 represents the starting point before efforts to optimize allergen cleaning. After identifying hot spots on the dump room wall and air duct, environmental cleaning procedures are changed to assure cleaning of these areas. For trial 2 the CIP cycle that cleans the surge tank, homogenizer 4, and the heat exchange loop including the valve, is modified by increasing circulation time. This improves surge tank and homogenizer antigen results to undetectable levels, but there is still antigen in the heat exchange loop. Also in this trial, vacuum cleaning the can filler

Table 16.3 Antigen cleaning optimization (example)

Sample	ELISA data (ng antigen/mL or ng antigen/100 cm^2			
	Type	Trial 1	Trial 2	Trial 3
Dump room wall	Swab	> 3000	< 10	< 10
Blend tank 7	Rinse water	< 10	< 10	< 10
Surge tank 1	Rinse water	350	< 10	< 10
Homogenizer 4	Rinse water	30	< 10	< 10
Heat exchange loop	Rinse water	1250	175	50
Heat exchange valve	Swab	> 3000	1850	30[1]
Air duct 8	Swab	> 3000	< 10	50
Can filler 3	Swab	465	375	< 10[2]
Can sealer 3	Swab	< 10	< 10	< 10

[1]After disassembly and hand cleaning
[2]After warm water rinse

has reduced but not eliminated antigen. In trial 3, modifying the CIP cleaning fluids has effectively cleaned the heat exchanger while acceptable cleaning of the valve requires disassembly and hand cleaning. The can filler is also effectively cleaned using a warm water rinse. This scenario, while theoretical, is based on actual cleaning trial data and is presented to illustrate data interpretation. Once the cleaning processes are optimized they should be fully documented and replicated to yield an allergen cleaning validation package and standard operating procedures (SOPs) for allergen cleaning.

16.4.5 Manufacturing controls

All of the normal quality assurance procedures and methods used for non-hypoallergenic formula are also employed to assure the quality of hypoallergenic formula. These are extensive but will not be discussed here. Instead, the additional controls and testing required to manufacture hypoallergenic formula will be detailed. Once hypoallergenic ingredient testing methods have been developed and validated, ingredient specifications set, allergen cleaning procedures validated and SOPs written, the next step is to put in place procedures to control manufacturing to eliminate the risk of releasing contaminated formula.

The best way to manufacture hypoallergenic formulas is to use facilities that are physically separated and completely dedicated for that purpose. If only hypoallergenic ingredients enter the facility and only hypoallergenic formula are produced by the facility, there are no normal circumstances whereby the formula can become contaminated. Because hypoallergenic formulas tend to be small-volume products, use of completely separate production facilities is not usually economically feasible. However, it is highly advantageous to analyze production facilities to determine what equipment can be dedicated for use only for hypoallergenic formula. Dump stations, batching tanks, transfer pumps and lines, heat exchangers, filling

lines, sampling and cleanup tools might all be considered for dedication. Some disadvantages of equipment dedication in a mixed product manufacturing setting are maintaining operator training to use only the right equipment and keeping accurate records of compliance. Clearly, dedicated equipment in a mixed manufacturing environment does not ease the requirements to execute validated equipment and environmental cleaning.

Assuming a mixed manufacturing or partially dedicated facility, the first step in manufacturing control is to optimize product production scheduling. This is a simple but powerful tool that takes advantage of a known hierarchy in product allergenic reactivity: hypoallergenic products are least allergenically active and are made first following the required cleaning, soy-based products have relatively low allergenic reactivity[7] and are made next, cows' milk-based products are most allergenic and are manufactured last in the cycle. It is also helpful to schedule longer run times, producing a number of formula batches and thereby minimizing the costs and down time associated with cleaning between product change-overs.

In-process testing of the formula for antigen content is not required but may be useful, especially when starting new production lines or incorporating new production equipment. The advantage of in-process testing is that it allows savings of sterilization and packaging costs of contaminated batches that must be discarded, and it provides information useful in investigating possible sources of contamination.

16.4.6 Finished formula release testing

In addition to the normal nutritional and microbiological release testing performed on all formula, hypoallergenic formula must be tested to confirm their identity and low antigen content. This is accomplished using an inhibition format antigen ELISA as discussed in Section 16.3. Formula antigen content specifications are tied to the antigen content of the formula batches used in the clinical food challenge studies that established the hypoallergenic clinical performance of the formula. If non-cows' milk based formulas are manufactured in the same facility, a PAGE test (described in Section 16.3.4) is used to identify the formula by showing that it does not contain intact non-milk proteins that are not detected by the casein ELISA.

16.5 Summary and future trends

Successful food allergen control in sophisticated food manufacturing facilities has been practised to very exacting standards in the production of HIF for an extended period of time. Experience gained with HIF can be used in the general food industry. Many of the allergen management techniques directly apply. These include: allergen control guidelines and inspections for suppliers to ensure ingredient integrity; ingredient inventory control to provide allergen

segregation during storage; dust control and air handling optimization to minimize contamination; production scheduling to segregate allergens; and, most important, employee allergen awareness training to obtain broad commitment to food allergen management. Information gained from equipment and environmental cleaning research is also useful, and it is likely that new research programs in this area will provide additional valuable data on food allergen cleaning efficiencies.

The quixotic solution to food allergen contamination is to build individual manufacturing plants for each food allergen. While this is not realistic, it is important to remember that segregation of allergens is the best strategy for food allergen control. It should also be noted that, although most food manufacturing plants are not new, they are often undergoing change. Substantial progressive improvements in food allergen management can be achieved if allergen management becomes a design goal in ongoing plant renovations. Operator creativity is also a powerful force in identifying allergen management improvements. This enhances the value of allergen awareness training and fosters ownership of product allergen quality among those closest to production.

Most of the specialized and expensive immunology tools used in manufacturing HIF do not seem to have a place in daily mainstream food manufacturing. It should not be necessary to establish immunochemistry laboratories in all food manufacturing facilities. Instead, the procedures for sampling and data analysis used to control HIF can be referenced and coupled with commercially available food antigen ELISA kits that are used with plant-specific food allergen ingredients to investigate food antigens in the production environment and processing equipment. In a relatively short time environmental and equipment antigen-cleaning procedures can be developed and validated. If needed, some of this work can be accomplished using consultants and contract laboratories. Once food allergen management practices are validated and in place, maintaining control becomes an exercise in recurrent training and taking advantage of equipment changes over time to enhance allergen segregation.

A number of food allergen management issues are currently without solution. The most basic unresolved question is ‘How much is too much?’ or, more directly, ‘What is an actionable limit?’ Without a clear answer to this question food allergen management programs are left to the uncertainties of clinical reaction thresholds. If a product causes a clinical reaction, the allergen level was too much. However, field reports of allergic reactions are not controlled clinical trials and tend to represent unusual cases with an ‘n’ of one. How will the balance between food cost contributed by extraordinary allergen control methods and allergic reaction frequency be determined? Is this issue scientific or political? When and by whom will it be resolved? While these questions are being debated it is important to maintain focus on efforts to improve allergen management and lower risks using cost-effective measures.

Another key weakness is in the analytical area. A number of useful food allergen ELISA kits are on the market, but the list of available assays does

not include many key allergens, and there is a general lack of standardization and validation. There are no widely accepted standards for allergens, antigens, or antisera. Also lacking are standard assay protocols, sample collection and preparation procedures, data calculation and reporting tools, and assay validation data for food quality assurance applications. Substantial progress is being made to fill these gaps and new options, better understanding, and new partners will change the future.

A postscript regarding allergen quality assurance of 'non-allergenic' 'elemental' diets: Recall that by definition, a 'clinically hypoallergenic' formula can have an allergic reaction failure rate of up to 10%. Patients who react to hypoallergenic formula must use diets that are devoid of immunological reactivity. The protein components of these formulas are based on purified amino acids and the products must be completely free from food protein contamination. Amino acid-based diets were initially developed to manage digestive difficulties. In these applications small amounts of intact protein contamination did not have a significant negative metabolic effect on the diet's performance. However, when the diets are used to control extremely severe food allergies, additional allergen quality assurance requirements must be observed. HIF testing technology can be used with some slight modifications: first, product contamination will only be with intact proteins from the production environment, therefore commercially available capture-format ELISAs can be used; second, testing will need to include all protein sources present in the manufacturing facility; finally, while amino acid-based diets can be shown to be clinically hypoallergenic, there are (can be) no testable clinical performance standards for 'nonallergenic' formulas. Operationally, the modified quality assurance programs have enabled us to successfully manage the allergen quality of amino acid-based diets used in highly allergic patients for more than six years.

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17

Common issues in detecting allergenic residues on equipment and in processed foods

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17.1 Introduction

Food allergy has been long recognised as a clinical phenomenon, with numerous reports in the 20th century medical literature.[1,2,3] However, while it was known that patients could suffer extremely severe and sometimes fatal reactions following ingestion of minute amounts of the offending food, food allergy was perceived as a problem for the individual sufferers. Since the late 1980s, however, this perception has changed, and food allergy is now recognised as an important public health problem. A major factor in this increased concern is probably the rise in the prevalence of atopic disease,[4] of which it can be considered a manifestation. The prevalence and incidence of food allergy and the number of severe reactions[5] may be increasing, although the lack of sound baseline epidemiological data precludes firm conclusions. The new perception of food allergy has been accompanied by the recognition that the solution to the problem lies with collaboration between all the stakeholders, including patients and those who look after them, clinicians, public authorities and the food industry.

The ultimate aim for all stakeholders is to avoid food allergy sufferers reacting to the allergens to which they are sensitised. This can be achieved in two ways. One is to ensure accurate allergen declaration through labelling, the other is to ensure that where a specific allergen is not declared, the product does not contain it in an amount that would pose a risk and food allergy sufferers can assume it is safe for them. Both these requirements can only be fulfilled by detailed knowledge of the composition of products. Food manufacturing processes are extremely complex. This complexity derives from several factors including material sourcing, processing, efficient use of

equipment and other resources, and product formulation. Managing allergen risks requires an integrated approach, which takes into account all these factors throughout the supply chain, from ingredient suppliers through to retailers, and ultimately the consumer.

Total avoidance of cross-contact and therefore absence of specific allergens from products where they are not part of the formulation is often not practicable. Such circumstances require an analysis of the risk arising from residual allergen, and subsequently a quantitative risk assessment. Although knowledge of minimum provoking doses for many allergens is inadequate, knowing how much allergen is present in a product is a key element in this assessment, and the subsequent management of the allergen risk. Allergen detection methods can play an important role at several points in the analysis of this risk. These include the initial analysis phase, in which the current risk is determined, and the validation of specific risk management procedures, such as line cleaning. Upstream of the food manufacturer, analytical methods can be used as part of the supplier audit process. Detection of allergen also plays a role in investigations of incidents and compliance with process standards. This chapter will discuss the role of allergen detection methods, but will not address details of individual methods, except where these could have a bearing on the specific use of such methods.

17.2 Food allergy and product safety

Before considering the role of detection methods in ensuring the safety of food-allergic consumers, it is useful to take a broader view of food allergy as a safety issue. A first consideration with food allergy is that the nature of the hazard differs from that of other toxicants. Indeed, schemes for the classification of adverse effects of food usually distinguish it from toxic reactions, which potentially affect anyone who eats the food.[6] Specifically, food allergy only affects a defined section of the population, and food allergens present no risk to non-allergic persons, irrespective of the level of intake. However, while risk assessment in food allergy must therefore focus on the specific population at risk, it is still valuable to analyse it through the accepted framework of hazard identification, hazard characterisation, exposure assessment and risk characterisation.

The first step, hazard identification, is encompassed in the definition of the problem, namely food allergy. The hazard under consideration is any reaction to a food mediated by the immune system, although for practical purposes it extends only to those responses in which antibodies of the IgE class are implicated. Intrinsic in the accepted definition of allergy is the concept of clinical reactivity. It thus excludes situations where people are only sensitised, as revealed by skin prick testing or measurement of specific IgE, but do not otherwise react to contact with the food.

Risk characterisation consists of establishing the relationship between the dose of a material and the response it produces. In conventional toxicology, this is usually achieved by experiments in suitable animal species. These provide information about dose–response relationships and result in the definition of a no observed adverse effect level (NOAEL) from which a safe dose for man is calculated. Hazard characterisation in food allergy differs from this situation, firstly because animal experimentation is irrelevant, and secondly because it can be viewed at both an individual level and a population one. The population dimension is probably the most relevant from the public health point of view, and consequently for the food manufacturer, while the individual dimension is most relevant to the clinician advising a patient on management of the condition. For ethical reasons it is extremely difficult to obtain information about individual responsiveness to different doses of allergen. Full characterisation, up to the point of the most severe reaction, can only occur inadvertently, where the dose increment used proves to be too large. Characterisation of the population response to food allergens is, however, feasible and ethical, in the context of helping patients manage their condition. This is achieved through studies using double-blind placebo-controlled food challenges (DBPCFC) with increasing doses of allergen up to the lowest dose producing an objective response. Such experiments provide information on the frequency of response to particular doses in the population tested. However, such studies have a number of limitations as tools to identify a precise NOAEL. Firstly, because of their logistics, they can only be performed in relatively small numbers of allergic individuals, which limits their statistical power. Typically 29 or 58 individuals would be challenged, and it can be shown that statistically, such numbers give 95% confidence that fewer than 10 or 5% respectively of the population from which they are drawn would react to the dose identified as the NOAEL.[7] A further limitation is that individuals who have experienced a severe reaction to the allergen of interest are excluded from the studies by some clinicians. A modelling approach to overcome such problems has recently been proposed.[8] Although this approach is proving promising, much validation work is still required before it can be used in risk management.

Once the hazard has been characterised, and a NOAEL defined, exposure assessment is required. For individuals with a food allergy, the risk arises from acute ingestion of often quite small amounts of allergen, rather than ingestion over a period of time. The usual measure to consider for purposes of risk assessment is the amount of allergen that could be present in a portion of food. An important unresolved issue exists, however, with respect to the period over which the intake of allergen should be summed, as well as the possible effect on the provoking dose of exposure to small amounts, unable by themselves to stimulate an allergic reaction. Indeed some publications have attributed increased reactivity to exposure to such doses via routes other than the oral one.[9]

17.3 Management of food allergy risks

17.3.1 Aims of food allergen management

The first aim of food allergen management must clearly be to protect food-allergic consumers, while not limiting their food choices unnecessarily. This implies a risk assessment, as described above, as the alternative would be to use precautionary labelling for almost all products in some instances. Indeed the role of detection methods for allergenic residues is predicated upon a risk-based approach to management of food allergens in food manufacturing. If allergens were declared under all circumstances, irrespective of the risk they pose, there would be no need for methods to detect residual allergen. The allergic consumer would simply be informed of the presence of particular allergens, and left to manage the risk individually. However, this approach is viewed by allergic consumers as an abdication of responsibility, and is much disliked.[10,11] Furthermore, rather than safeguarding allergic consumers, it can actually place them at increased risk by leading them to erroneous conclusions about the safety of products. From the manufacturer's point of view, managing allergens on a risk basis means effectively taking a view as to what proportion of the allergic population it is feasible to protect, based on knowledge of NOAEL for individual allergens, coupled with an assessment of achievable residual allergen content for particular products. Defining the aims of allergen management is important not only in setting process control objectives, but also in providing a basis for clear communication with stakeholders, such as allergic consumers and health practitioners who they consult for advice. For instance, decisions must be made about whether the policy aims to avoid all reactions in allergic individuals or just severe ones. The implications of achieving this aim must also be evaluated in a wider socio-economic context. For instance, more thorough cleaning procedures may result in undesirable environmental consequences, or the introduction of water into dry systems may introduce a microbiological hazard. The range of responsiveness of allergic patients is extremely wide, and some react to extremely low doses. Protection of such consumers may only be achieved by advising them not to consume manufactured foods. If so what does that mean for assays?

17.3.2 Integrated approach

Current approaches to the management of the allergen risk in the food industry recognise that it has to be integrated into the whole product life-cycle, from its design right through to the point at which the consumer eats it. It is within that integrated approach that the role of allergen detection methods must fit. Major food manufacturers have devised specific corporate policies for the handling of allergens, supplemented by guidelines which provide practical advice to individual manufacturing units. These methods ensure that a high minimum standard exists for the handling of allergens throughout the company.

For instance, Unilever has a policy for dealing with allergens which states that it shall declare the presence in its products of any allergen which is a common cause of allergic reactions. At a minimum, any allergen required by local regulations will be declared. However, beyond that, the allergenic risk from foods not commonly known to be allergenic may be assessed if clinical or epidemiological data indicate the need. If then classed as a common cause of allergic reactions, this food component would be declared on labels and included in hazard analysis of critical control points (HACCP) plans. Unilever also undertakes to inform any consumer on request about the presence of uncommon allergens in specific products.

Allergen management guidelines need to ensure that allergens are correctly and intelligibly declared in products, but also to make sure that allergen is not present inadvertently at levels likely to cause adverse effects on health. Such guidelines specifically need to address all stages in the product life-cycle, from its design, through the sourcing of ingredients, to manufacture, labelling and distribution. Specifically, they need to deal with:

- **Innovation.** Is the use of the allergenic ingredient necessary for the functionality of the product or could an equivalent non-allergenic ingredient serve as well?
- **Supply chain.** Control of allergens in the supply chain requires a close relationship between suppliers and manufacturers so that the needs and requirements of the latter can be met. Typically, the starting point of the supplier assessment will be a questionnaire about allergens handled and precautions in place to avoid cross-contact, including the existence of a HACCP plan. This is backed up by periodic audits of the suppliers' facilities. Additionally, suppliers are required to seek agreement to any change in the formulation of the ingredient they supply.
- **Manufacturing protocols.** Main considerations are the inclusion of common allergens in HACCP plans, production scheduling to minimise cross-contact, validated cleaning procedures and clear labelling and separation of specific allergenic ingredients within the factory. Procedures need to cover rework, where sound product is not packaged but 'recycled'. Staff training to understand the importance of allergen control procedures is vital and improves support for what can be additional procedures in the production process. Finally, the same degree of attention is needed whether the company's own manufacturing facility is concerned or that of co-packers.
- **Packaging, promotion and advertising.** Packaging carries the label and therefore the allergen information. Care is required to ensure that information remains with the product until it reaches the consumer. Other considerations include warnings if the formulation has changed to include an allergenic ingredient previously not present.
- **Retailers.** Generally, the manufacturer's allergen information will be sufficient. However, situations such as in-store promotions require care to ensure that the consumer is fully informed. Sound product, which fails to meet all standards for general sale, may be repackaged and sold in specialised

outlets or even in a different market. The manufacturer needs to ensure that appropriate allergen information is retained and is available to the ultimate consumer.

- **Food professionals.** Most allergic reactions to foods occur outside the home, in conditions where the product is often not labelled and even when asked, food professionals fail to provide correct information. Where pre-prepared food is provided to that sector, the manufacturer has a responsibility to ensure that accurate allergen information is provided and conveyed to the consumer.

17.3.3 Role of allergen detection in the integrated approach

Consideration of the various phases of the product life-cycle reveals a number of points where the detection of allergenic residues has a role. These will include all the points at which there is uncertainty about the presence of allergens or their levels. By definition, detection only applies to allergens which could be present inadvertently and will therefore apply to the manufacturing stages and any upstream of those stages. Detection of allergenic residues will have a relatively limited role at the innovation stage, although it may be useful in making a choice between suppliers of an ingredient. As discussed, assessing the risk arising from inadvertent allergen presence begins with ingredient suppliers, and clearly measurement of residual allergen levels can provide valuable information.

As mentioned earlier, the use of detection methodology in allergen risk management must be guided by the objectives of the policy. Total elimination of the allergen risk, in the sense of a guarantee that no allergic individual may be affected, irrespective of their sensitivity or the severity of reactions they experience, is rarely, if at all, possible unless specific allergens are excluded from manufacturing facilities. A key element in deciding what needs to be achieved in order to afford a specified level of protection to the allergic population is the minimum dose which provokes a reaction in such individuals (threshold). Data on such doses are unfortunately still scarce, and subject to much debate,[9] even in the case of the most common food allergens. They can be difficult to use confidently in management of allergen risks, particularly since the uncertainties surrounding their derivation are difficult to quantify. Recently attempts have been made to investigate the distribution of such doses in the population, and to estimate by mathematical modelling below what level in food a residual allergenic protein must be kept in order to protect a specified proportion of the allergic population.[8] As work progresses on defining such levels, they will provide more effective ways of monitoring the success of risk management measures. Methods for the detection of residues will thus increase in importance in roles such as confirming that products and ingredients meet set specifications, and the validation of risk measures such as cleaning. They will also provide, of course, the basis for assessment of compliance by authorities.

17.4 Role of allergen detection and other considerations

17.4.1 Why do we need detection methods?

Methods for the detection of allergenic residues can be deployed for a variety of uses. In industry, these will include what is effectively the exposure assessment part of risk assessment. Typical activities would be assessment of the extent of cross-contact at different points, as part of a HACCP study, and subsequently validation of the measures put in place to control the extent of low level continuous cross-contact. Extending up the supply chain, such methods could also be used to confirm suppliers' statements about their ingredients, as part of the audit of their processes, while downstream, product analysis could be envisaged where incidents have occurred, or there is a suspicion that allergenic residues may exceed specification. Similarly, confirmation that residues are present and in what amount would be an important starting point for investigation of incidents. However, industry is not the only potential user of detection methods. Public authorities need to provide evidence to support compliance activities, and demonstration of the presence of residual allergen in products which are not supposed to contain them can form a strong part of such evidence. Allergic consumers may also be potential users of such methods, although none are currently suitable for this type of application.

Different users are likely to require different methods with different characteristics, with respect to detection limits, quantitation, robustness and ease-of-use. Risk assessment activities imply quantitative evaluations and require methods which measure accurately and reliably the residues of interest, even in complex matrices. (In HACCP studies, although desirable, it is probably not essential for the method to be easy to use.) In contrast, enforcement authorities will only in practice be interested in quantitation if the relevant regulations specify an action level. If no level is specified, it would presumably be sufficient for a method to have an adequate detection limit and to be known not to produce false positives. For potential allergic users, a key requirement is no false negatives, as well as an adequate limit of detection.

17.4.2 What should assays for allergenic residues detect?

The allergenic activity of a food usually depends on a range of proteins, and it has been shown many times that the pattern of response of allergic people to the different proteins can differ considerably.[12] It has also been shown more recently both in allergic patients[13] and in experimental animals[14] that the overall response to an allergenic food is a summation of the responses to the individual proteins. The implication of those observations is that immunoassays for food allergens should essentially be considered as means of quantitating the relevant protein(s), rather than measuring allergenic activity in the food, which will differ for each allergic patient. Another implication is that quantitation of single allergenic proteins may be valuable if one is

trying to monitor the effect of processing on such proteins, but may give highly misleading results if used in an assay intended for other purposes, such as the estimation of the extent of cross-contact or establishing whether a product contains more allergenic material than a set limit. The key consideration with respect to assay development should therefore be the purpose of the assay. The allergenic protein, although the obvious candidate, may not always be the optimal choice. However, detecting the protein is probably the most common approach and it is therefore appropriate to discuss the options available.

In developing an assay based on protein detection, two main choices of methodology exist: monoclonal and polyclonal antibody technology. Both have advantages and drawbacks. As monoclonal antibodies recognise single epitopes on proteins, this technology usually results in a highly specific assay, with relatively low incidence of cross-reactivity, even with closely related proteins, provided the antibodies have been correctly screened. Theoretically there is also an endless supply of antibody with exactly the same performance characteristics as the original antibody. However, the narrow specificity of the monoclonal antibody can also be its Achilles' heel, as detection of the protein of interest will only take place if the antibody binding site remains intact and accessible in the various food matrices in which the protein may be present. Polyclonal antibody technology relies on the production of a range of antibodies, either to a single protein of interest, or to all the proteins within the food, depending on the preparation which is used. This provides a detection system which is less likely to fail completely to identify the presence of proteins of interest, although quantitation may remain problematical if processing alters the relative proportions of the different immunochemically active proteins in a food. However, the main drawbacks of polyclonal technology lie in the need for extensive purification procedures that may need to be applied to the protein(s) of interest, as well as to the resulting antiserum to ensure specificity and absence of unwanted cross-reactivity, together with the need to develop procedures to ensure batch to batch reproducibility.

Once the technology itself has been selected, there remain several possibilities in developing immunoassays based on protein. As discussed, monoclonal technology results in a highly specific detection system, but it can nevertheless be broadened by using a combination of detection antibodies against different epitopes of the same protein, and/or different proteins. However, beyond a few proteins, this becomes complex to optimise. Polyclonal technology leaves open the choice of the material against which the antiserum can be raised. Thus it can be as general as a protein extract of the whole food, or as specific as a highly purified protein.

However, detection of the protein, or proteins, may not be necessary or even be the method of choice in all instances where detection of allergenic residues is sought. Instead, a marker molecule, for which a robust and sensitive analytical method exists and which is always found in a known ratio to the

proteins, can be used. An example would be lactose in milk which could be used as a tracer for estimating the amount of milk protein left on a line by cross-contact. Similarly, a marker compound could be used in supplier audits. However, only measurement of the protein could help an allergic individual decide whether a particular product is safe to eat. Similarly, compliance activities by food safety authorities are likely to be required to demonstrate directly the presence of the offending allergen, rather than a marker material.

The reverse transcriptase polymerase chain reaction (RT-PCR) assay has recently become the subject of considerable interest, and is described elsewhere in this book. It takes advantage of amplification of any relevant (species) DNA present in the food of interest to the point where it can be detected. It can also be used in a mode which can be considered semi-quantitative. However, in relation to allergens, it relies on an implicit assumption that the presence of DNA in a processed food denotes the presence of (allergenic) protein, a contention that needs experimental justification.

17.4.3 Limit of detection

The limit of detection of any assay is an important parameter, but it needs to be considered together with the other parameters that make up the assay. Again, the purpose of the assay should dictate this factor, as it does the others. For most purposes, such as monitoring the effectiveness of allergen control measures or verifying compliance with set limits, it would be reasonable for the detection limit to be such that the assay could detect allergenic material in an amount in a portion of food that was close to the lowest amount shown to provoke some reaction under controlled clinical conditions. This, of course, begs the question as to what those levels are, and to what extent any uncertainty in determining minimum provoking doses should also be allowed for. Minimum provoking doses vary considerably between individuals[15,16] and Bindslev-Jensen *et al*.[8] have recently described their cumulative frequency versus log-normal dose in the population of allergic patients as a sigmoidal log-normal plot. The implication of this distribution is that there is a small proportion of individuals who will respond to very small amounts of allergen. Assays could be developed to detect such small amounts but, even leaving aside possible technical issues of signal-to-noise ratio, it is questionable whether such sensitivity is actually required, except for forensic purposes. A recent suggestion, based on clinical findings[17] proposed that measures to minimise the inadvertent presence of allergen in industrial food manufacture should aim to protect 95% of the allergic population. On that basis, the lowest levels that residual allergen should not exceed were of the order of 5 ppm protein, a requirement which most current commercially available assays meet, assuming minimal losses during extraction. It should also be borne in mind that the concept of a valid lower detection limit is only meaningful in the context of an assay developed to detect a representative range of proteins in a food product. Assays developed to detect a single

protein could be very difficult to interpret in these circumstances, for reasons already discussed.

17.4.4 Characteristics of the ideal allergen detection assay

Having considered various parameters of assays for the detection of allergenic residues, it is appropriate to examine what an ideal assay might look like. However, simply in formulating such a question, one is inevitably drawn to question whether there can be a single 'ideal assay' equally suitable for all purposes. The 'ideal assay' concept will therefore be examined in some of the different contexts in which it might be used.

Assays for monitoring effectiveness of allergen risk management measures

A typical application of such an assay would be to assess effectively different line cleaning measures, for instance. A primary requirement would be that it is sensitive enough to detect relevant levels of allergen and that it possesses adequate accuracy, providing a true measure of the analyte. A high degree of precision is not as important as accuracy, however. High specificity is also a requirement, as food products will often contain a number of proteins from different sources. Detection of irrelevant proteins could lead to inappropriate decisions with regard to allergen management, e.g. implementing more stringent measures than necessary or defensive labelling, if the analysis suggests the problem cannot be overcome. However, in addition to the appropriate technical parameters, the assay also needs to be designed for those who will be the primary users. In the factory environment, these will not necessarily be people with specialised laboratory training. The assay design must therefore be sufficiently robust for use by non-laboratory personnel, and formats such as calibrated test strips, for instance, are worth investigating. Similarly, experience of the development of in-home diagnostic assays used for clinical monitoring may hold valuable lessons.

Assays for measuring residual allergen in finished products

Although measuring residual allergen in finished products is not a common application of assays, there are occasions when it is necessary. These may be, for instance, where a process failure leads to the conclusion that there is a high probability of residual allergen being present at a level likely to endanger allergic individuals, or where there have been reports of reactions to the product. While this type of application requires of an assay many of the same properties as described for monitoring assays, the assay is likely to be performed by laboratory personnel, rather than in a factory, and therefore 'user-friendliness', while always desirable, is of a lower priority.

Assays for investigating compliance

Where limits have been set on the residual allergen amounts in food products, the assay must meet more stringent requirements in some respects than in the

previous instances. Sensitivity and specificity are key elements as before, while precision is more important than for other assays, given the potential legal implications of the test detecting allergen. In particular, the assay should have a low incidence of false positives.

Assays for measuring single allergenic proteins
Many assays have been developed for single allergenic proteins. While these can sometimes serve for the purposes described above, they have many shortcomings in this regard, as discussed earlier. However, they could have a role in evaluating the effects of processing, for instance, on the protein of interest. Such assays will essentially be highly specific, and will probably also be sensitive. However, they will be used almost exclusively in the laboratory, and therefore simplicity of operation will not be a primary design consideration.

17.4.5 Common limitations

Most of the assays discussed above have a number of limitations, some of which are inherent in the methodology, while others result from particular combinations of methodology and the substrate in which the analyte is sought. The most significant limitations relate to extraction of the analyte from the food for analysis, interference by other components of the matrix which cannot be readily be separated and changes in the analyte itself which reduce the ability of the method to detect it.

Variability in extracting the analyte from the food
Most common methods, including many of those for total protein analysis (e.g. Bradford,[18] BCA[19]) and ELISA methods, operate in an aqueous environment and require extraction of the protein prior to analysis. The efficiency of this extraction will depend on the solubility of the protein(s) of interest in the aqueous buffer used for extraction. Many foods and food products include lipids as part of their formulation, and many proteins, including allergens, are associated in the food or product with the lipid component. In experiments to measure the total residual protein content of edible oils, we consistently recovered only 50% by extraction into phosphate-buffered saline, based on a comparison with the content measured by excited nitrogen analysis, which does not require extraction. This effect can be difficult to detect. In the example quoted, recovery of protein spiked into oils was virtually quantitative, presumably because of differences in their physicochemical properties, compared with the proteins remaining in oils after refining. In a different context, Keck-Gassenmeier *et al.*[20] found very low recoveries (2–3%) of peanut protein added to chocolate products, but were able to improve this to near-quantitative recovery by the addition of fish gelatine to the extraction buffer. These experiences indicate the need for a thorough knowledge of the physicochemical characteristics of both the matrix and the protein(s) of interest in order to obtain reliable results.

Matrix interference

As well as interfering with the recovery of the analyte(s) of interest, the food matrix, or some of its components, may actually interfere with the subsequent assay, if those components are co-extracted in sufficient amounts. For instance, we have found that on occasions, solutions with very high sugar content (although within the range used in several foods) reduced considerably the recovery of β-lactoglobulin (unpublished results). Other materials commonly used in foods, such as colours, could obviously also interfere with the performance of assays based on colorimetric endpoints, depending on their fate during extraction.

Changes to proteins due to processing

Food processing probably poses the greatest challenges with respect to allergen detection, particularly for the most common type of assay, namely immunoassays. Processing can alter either the allergenicity of a protein, or its ability to be detected in the food matrix, either because of changes in immunoreactivity or in the interactions between the protein and the matrix, or indeed both. Thus, fermentation of milk with certain strains of Lactobacilli reduces the IgE-binding capacity of the product compared to native milk, suggesting a reduction in its ability to provoke reactions. Under these circumstances detection of lower amounts of milk protein would be a true reflection of a reduction in hazard to the allergic individual. A similar situation occurs in the case of the apple allergen, which is known to be heat-labile[21]. However, assays can also significantly underestimate the content of heat-treated milk proteins, as a result of what must be assumed to be altered recognition of the protein analyte, since a total protein assay yielded total recovery (unpublished results). Similar findings were reported by Koch *et al*.[22] for roasted peanut proteins. Clinical data on reactivity to heated milk and peanut proteins suggests that under those circumstances the apparent reduced protein content does not reflect a reduction in hazard to the allergic patient. These examples illustrate the need for a thorough understanding of the pitfalls of an assay before it is used to generate data which will be used in risk assessment.

17.5 Future trends

The need for detection of allergenic residues has now been established as the importance of food allergy as a public health problem has become acknowledged. Several current trends are likely to influence the development and application of allergen detection. One is the developing legal framework, which will ultimately lead to defined action levels. Another is the determination of NOAELs for many of the main allergenic foods. A third may be the need for ways of monitoring particular foods as they are modified to reduce their allergenicity. Finally, while it not currently feasible, pressure from allergic

patients and their support groups for the means of monitoring foods for the presence of cross-contact allergens may lead to development of some rapid assays. The likely influence of each of these trends will be examined separately.

The legal framework with respect to food allergens is developing fast, with Switzerland, Japan, Australia/New Zealand, the United States and the European Union bringing in legislation specifying which allergenic foods must be declared. The lists are usually based on the list of allergens in the Codex General Standard on Labelling, but extend it to cover allergens of regional importance such as celery and mustard in the European Union. This legislation can be anticipated to drive food manufacturers to use test kits much more extensively to demonstrate for legal purposes that their allergen risk management procedures are effective. Although allergen testing has not proved to be the primary mechanism of enforcement, in some legislatures where it has a longer history such as the USA, enforcement authorities will undoubtedly seek to use them to support other evidence. Except for Switzerland, current legislation does not address the issue of allergen presence through cross-contact, and action levels have not yet been set. However, as allergen test kits become used to a greater extent, pressure is likely to grow, particularly from manufacturers, for defined action levels, below which the presence of the allergen would not constitute an infringement of the law. If action levels are not set by the agencies or the legislators, they will likely be defined by case law, which is probably not an ideal mechanism for this type of issue.

Determination of NOAELs and their use will provide manufacturers with defined targets for their allergen management policies, in terms of what amounts constitute a risk to what proportion of food allergy sufferers. They will also provide manufacturers with information for improved control of allergen hazards. Such control will, however, require that they know what level of allergenic residues is present in their products. Measurement of allergenic residues at appropriate points during the manufacturing process will be one way to obtain this information and could therefore increase considerably from its current relatively limited use.

Monitoring the allergenicity of certain foods or food products is another area where detection of allergenic residues could play an increasing role, as manufacturers seek to provide foods with reduced allergenicity. However, this area is probably likely to have a lower impact than the previous two, as looking for residual allergenicity by protein quantification is only one of several steps in defining reduced allergenicity.

Food allergy significantly impairs quality of life for sufferers.[23,24] Greater control over their condition by food allergy sufferers would undoubtedly help restore some of this quality. Demand for means to do so could spur an extension of the measurement of allergenic residues to this totally new area. This prospect is probably still quite distant, inasmuch as it requires methods which are simple to use and robust. A critical question will be the extent of the test manufacturer's legal liability in the event of an allergen not being detected and producing a reaction in a sufferer.

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18

Factors affecting the effectiveness of allergen detection

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18.1 Introduction

More than 40 years have passed since the first publication of the radioimmunoassay technique by Yalow and Berson.[1] Despite pessimistic prophecies about its efficacy, it has survived, developed, and recently been augmented by other diagnostic tests to become a powerful tool to investigate very small amounts of substances in the nanogram to picogram range. Developed originally to analyze hormones, the method is nowadays used for a wide range of applications, for example, in the area of clinical diagnosis, food and feed diagnostics and to test for environmental pollutants.

The effectiveness of any immunoassay depends directly on the quality of the antigen used as a target and the quality of the antibody used for capture and detection but in addition to this, the performance of the assay itself is important. Allergens are proteins, and therefore immunoassays are appropriate tools to measure them. Often more than one allergenic protein exists in a complex matrix amongst many other proteins. The nature of food can vary over an extremely wide range and sample preparations are often very difficult to work with. Many food products are affected by heating, which can alter the allergenic and non-allergenic proteins. So there are two components which can influence the assay performance; one comes from the extraction of the allergen and the other from the assays themselves.

18.2 Factors affecting the determination of allergenic residues

18.2.1 Factors resulting from the assay

Before the implementation of an immunoassay, whether it is an in-house method or a commercial kit, it is necessary to become familiar with the basic principle of the test. Therefore, the protocol, (or instructions/kit inserts if a commercial test) have to be read thoroughly. As discussed in Chapter 6, there are two main types of enzyme-linked immunosorbent assay (ELISA) design: competitive and sandwich ELISAs. In the case of competitive (inhibition) ELISAs, the more allergenic residue in the sample, the fewer antibodies are bound and the less color is developed (indirect ratio). In the case of a sandwich-type ELISA the amount of developed color is proportional to the amount of allergenic residue in the sample (direct ratio). The two types of curves represented by these two assays are illustrated in Fig. 18.1 for egg protein and beta-lactoglobulin.

Two kinds of errors can be found in immunoassays: one is systematic (which is manifested in the form of bias) and the second is random error (which is reflected in poor reproducibility or 'imprecision'). Bias and imprecision are two statistical parameters which describe an assay. Systematic errors leading to bias are often difficult to perceive. It implies that the 'true' value of a sample is known. The assessment and minimization of random errors of measurement, which lead to imprecision and to reduced accuracy of quantitative measurement, will now be discussed. Important parameters are:

- **performance** – the precision (intra-/interassay) reproducibility/recovery, sensitivity and reliability;

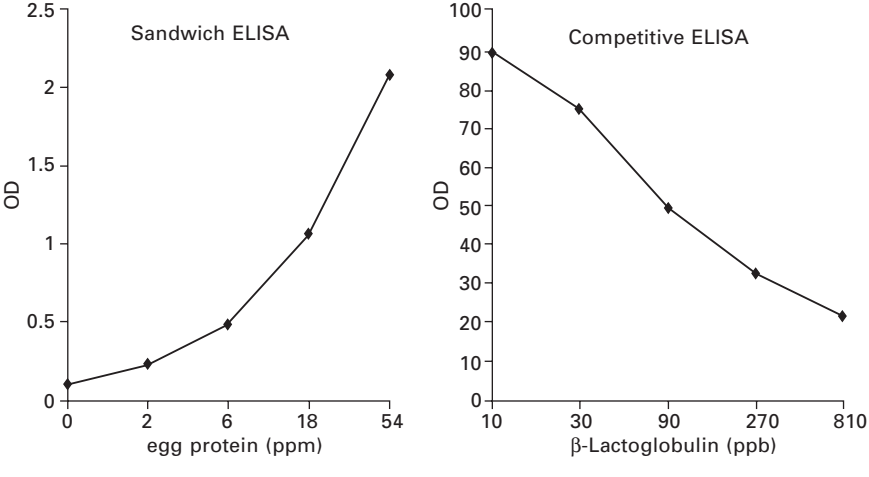

Fig. 18.1 RIDASCREEN® Egg ELISA (sandwich) and RIDASCREEN® ß-Lactoglobulin ELISA (competitive assay).

- **convenience** – the analysis time from sample preparation to final results, the environment, the quality of equipment (pipettes and instruments); and
- **skill** – of technical staff.

Test performance

Because it is desirable that results should be available as fast as possible, the tendency is to shorten the assay performance time more and more. Speed, however, comes at a price. Antigen–antibody reactions are subject to the laws of chemical equilibrium. If the reaction is interrupted before the steady state is reached, the assay becomes subject to greater variability. Usually it is required that assays should not take longer than a few minutes, at most one to three hours. To obtain consistent results in commercial kits, the manufacturer-recommended temperature and incubation times have to be strictly adhered to. During each moment of incubation, the result is changing. Times and temperatures are optimized in such a way that the recovery of the analyte should be nearly 100%. The manufacturer has to check the robustness of the assay concerning variation of time and temperature (in most cases a variation of about 5% is tolerated) to guarantee the correctness of results within certain limits.

It is necessary to understand the basic principle of the type of test being used. In competitive assays, the reaction is initiated by adding the antibody to the wells, which allows for slow and deliberate pipetting of samples and standards. In the sandwich-type format, the reaction is more immediate, and pipetting must take place without delay, since the capture antibody on the plate binds the allergenic residues on initial contact. In the latter type of assay, an analyst has a little bit more time if a reaction step covers about 30 minutes or more. But if the assay time takes only a few minutes (5–10) the technician has a challenge to pipette quickly. Furthermore, for commercial kits, it is clear that more assay wells can be processed if the assay takes a longer time. Hence, it is important that the same velocity of pipetting is done throughout the entire test procedure.

It is clear that the maximum number of possible samples which may be investigated depends on the reaction time as well. An assay with a total of five minutes incubation time will be designed with only a few standards, with the standards and samples running as single determinations and the number of samples not greater than 10 so that the technician is able to pipette them within a minute. Otherwise the delay between the first and the last pipetting is too long. If an incubation time of about 30 minutes or more is included, it is possible to prepare half a plate or more, provided that the pipetting is finished in less than five minutes.

For commercial kits, all of the included test components have to be handled carefully according to the manufacturer instructions. Before using, kit components should reach room temperature and they need to be mixed thoroughly without creating foam within the solutions.

One of the most important points for either in-house methods or commercial

kits is the avoidance of contamination. Two types of contamination are possible: bacterial contamination (washing buffer, sample dilution buffer as well as standards or conjugates) and contamination due to the allergenic residue itself. If, for example, the conjugate is delivered as a concentrate, and it is going to be used more than once for dilution, it is essential that bacterial contamination is avoided. On the other hand, it is also necessary to make sure that the medium (buffer or water) which is used for diluting the concentrate is not contaminated bacterially or by the analyte itself. This is also a consideration for in-house methods where buffers and conjugates may be used for several days or by several technicians for other assays also. Another example would be that if the conjugate concentrate is diluted with the same buffer in which the sample is to be diluted, a sufficient amount of buffer for diluting the conjugate should be set aside separately before the buffer is used for sample preparation. Otherwise, a contamination of sample dilution buffer by the analyte can occur and may lead to false positive results or uninterpretable standard curves.

Absolutely clean containers should be used for the preparation of washing buffer, as well as sample dilution buffer. Allergenic residues sometimes adsorb strongly onto certain surfaces, which can result in contamination of solutions. Such contamination can also lead to uninterpretable results (high absorbances over the whole standard curve without differentiation, see Table 18.1).

Dust containing allergenic residues present in the air or on laboratory equipment can contaminate solutions too. In these cases, the assay should be carried out in rooms separated from the sample preparation room. The analyst should ensure that there is no contamination of laboratory equipment (tables, pipettes, tubes) as well as hands (wash hands, wear gloves) before starting the assay. The microtiterplate should be covered during each reaction step to avoid contamination. In methods with very low detection limits, there can be problems if dust and hand contamination are not controlled. An example of contamination of components of a commercial kit by a customer's laboratory is shown in Table 18.2. The first run had results in accordance with the standard curve which was done in the kit manufacturer's quality assurance laboratory. But the second run shows very high absorbance in the lower range of the standard curve, a typical sign of contamination.

Table 18.1 RIDASCREEN ® FAST Gliadin ELISA absorbances: there was wheat flour contamination in the cap of the sample dilution buffer bottle in which the buffer concentrate was diluted, and afterwards this buffer was used for diluting the conjugate-concentrate

Standards (ng/ml)	Contaminated sample dilution buffer, used for diluting the conjugate	Fresh prepared buffer, clean lab, clean equipment
0	3.067	0.075
10	3.067	0.594
20	3.240	1.118
40	3.064	1.931
80	2.882	2.651

Table 18.2 RIDASCREEN ® Gliadin ELISA absorbances: contamination of the second test run in a customer's lab

Standards (ng/ml)	Fresh opened kit	Second run	Curve of the quality assurance lab
0	0.040	0.685	0.056
5	0.145	0.767	0.196
10	0.303	0.790	0.440
20	0.612	0.888	0.746
40	1.123	0.938	1.235
80	1.815	1.703	1.943

For both commercial methods and in-house methods, the washing of the ELISA plate between the reaction steps counts as an additional factor which may influence the results. It is important to use the right washing buffer, the right amount of buffer and the right number of washing steps. It is also important to guarantee that no cross-contamination from well to well occurs during washing. It is better to use more washing steps than too few. The plate should be inverted strongly against very absorbent paper two or three times after each washing step. It is necessary to ensure that the wells are clearly empty between the washing steps and before each new reagent addition begins, but the wells should not be allowed to dry. Improper washing often leads to a higher background level, which results in higher absorbance values in the blank control and, in both types of ELISA formats, gives rise to a poorer differentiation between the lower standards. Often, a high coefficient of variation (CV) indicates improper washing. Table 18.3 shows high absorbance at the low standards in a sandwich-type commercial method. The very high CV values highlighted are due to incomplete washing.

Technicians should make sure that pipettes are calibrated and take care to pipet without splashing into neighbouring wells. In addition, the use of a calibrated ELISA reader is absolutely necessary and should be maintained. Moreover, some chromogens/substrate solutions should be kept in the dark and for these, of course, the color reaction should be run in the dark as well. Parallelism between standard and analyte is essential to producing an accurate

Table 18.3 RIDASCREEN® Gliadin ELISA: very high OD in the lower area of curve connected with high CV due to incomplete washing

Standards (ng/ml)	Standard Curve	Coefficient of Variation (CV %)
0	0.639	**13.1**
5	0.988	**20.1**
10	1.247	1.5
20	1.832	1.0
40	2.126	6.2
80	2.682	7.1

concentration estimate of an analyte in a sample using an immunoassay. Unfortunately, pure standards or defined standards are seldom available in the field of allergenic residue determination. For commercial assays, each includes its own standard. For in-house methods, investigators make their own standards, too. The degree to which the standard is adapted to reality is a quality parameter. Often proteins are altered during food processing. How can the standards be adapted to all of these possibilities? This issue will be discussed later in Chapter 19.

What does correct pipetting imply?
It is well-known that proteins often adsorb onto surfaces, so pipette tips should be rinsed several times in standard or sample before pipetting can begin. Standards can be pipetted from zero to the highest amount with one tip if the tip is rinsed several times in each standard before pipetting into the wells. If more than one dilution from a sample must be pipetted, the technician should start with the lowest concentration (the highest dilution) and go to the most concentrated, using the same tip rinsing technique as with the standards. For each sample a new tip should be used. Spreading from well to well during pipetting should be avoided since it gives rise to bubbles.

After pipetting, the plate should be covered and gently shaken (either manually or with a microplate shaker). For commercial kits, during the incubation steps, the plate should only be shaken if the manufacturer recommends this step; otherwise the optical density (OD) may become too high for photometric measurements. In connection with this, the incubation temperature is important. Most commercial assays are developed at what is considered room temperature in the US and middle Europe (22–24 °C). Usually higher temperatures lead to higher OD values. Most modern ELISA readers can measure OD ranges between 4.0 and 6.0, but sometimes old photometers are not capable of reading more than OD 2.5. In hotter climates, the incubation period for color development may be shortened, if this is in agreement with the manufacturer. For in-house methods, developers/users can adapt their conditions to their laboratory temperatures by changing incubation times or other parameters. ELISA readers read high ODs in a less accurate fashion than low ODs, and this may result in unreliable results. In addition, the standard curve can often end up sigmoidal. For commercial assays, sigmoidal curves are not ideal, but for in-house methods sigmoidal curves can elicit good results if the developer/user uses the linear portion of the curve. Samples which fall outside linear portions of curves should be repeated by using a further dilution step to yield an OD which does fall on the appropriate part of the curve. In the same way, extrapolation below the first and above the highest standard of curve should be avoided, as these lead to inaccurate values, which may be misleading.

Test characteristics
Whether using a commercial ELISA kit or an in-house method, the ELISA

must be robust and reliably employ a procedure that ideally has a minimum number of manipulations.

For ELISA methods:

- For commercial kits, the standard curve should be nearly identical to the one documented by the manufacturer. Most manufacturers deliver a quality control data sheet for comparison.
- For in-house methods, internal quality control methods should be used to make sure that standard curves run similarly on different days, with different technicians, etc.
- Sandwich ELISAs should start with very low OD at the zero standard, and maximal OD should be similar to that reported by the manufacturer; in the case of an in-house method, again, internal quality control should be done to ensure that the assay is running identically from day to day.
- Competitive ELISA curves should begin in a steep manner between the first and second standard to guarantee accurate determination of samples in the low range of the curve.
- Benchmarks of 80%, 50%, and 20% values can be used to characterize the curve as well. These are the points of intersection of the curve at 80/50/20% binding of the analyte and can be used to describe the reproducibility of the standard curve. For commercial kits, most manufacturers indicate the 50% level in the kit insert, which is that concentration reached at 50% binding of the analyte.
- It is advisable to establish quality control data, which should indicate the 80/50/20% levels (or whatever benchmark in-house methods users define) from run to run as well as the maximum and minimum OD to evaluate the method performance. Over time the analyst has the possibility to compare the data from run to run and this will make it easier to judge a run that experienced problems.
- Of additional importance is the CV of samples and standards, which is calculated by dividing the standard deviation by the mean of replicate results and multiplying by 100. A CV of greater than 10% within the assay can indicates insufficient washing or pipetting. The CV calculated within one run refers to the intra-assay reproducibility, while the CV calculated between runs refers to the inter-assay reproducibility. Both terms help to evaluate the assay performance.
- To evaluate the accuracy of test runs, known negative and positive controls should be included within each run. The results can be included additionally in the quality control data. This also helps to promptly recognize outlying data.
- To control the reproducibility of a run, the suggestion would be to run one to three recovery samples with known contents of analyte (one negative and two positive). Intra-assay and inter-assay reproducibilities as well as the recoveries (measured mean value of the sample divided by the theoretical value multiplied by 100) can then be calculated.

All of these parameters help to identify outlying data and enhance the accuracy and reliability of the results obtained.

18.2.2 Factors resulting from the extraction method

The reliability of an ELISA method depends not only on the assay itself but also on the development of sample extraction procedures. Extraction procedures ideally should give a complete extraction of an analyte from all foods containing it. Specific difficulties in determining the presence of allergenic residues in foods can be related to the complexity of ways in which the proteins are presented in foods. The allergenic residue should be measurable in any food matrix, or the limits of application have to be defined. Food matrices are very complex and range from liquids and pastes to solids and powders. Allergenic residues can be found in raw materials or in processed food. The components of food may have been processed by a variety of heat treatments or extractive procedures. The food may have been stored raw, frozen or even pasteurized or sterilized at over 100 °C. The sensitivity and selectivity of the immunoassay must be combined with a selective extraction procedure. Much work is necessary to ensure the robustness of extraction. For commercial kits, the duration of the extraction method should ideally not be longer then the incubation time of the kit, but for in-house methods the user has a lot more flexibility in this regard. For commercial methods, the extraction should be rapid and simple and acceptable by the user. For both commercial and in-house methods, the extraction must be validated. Validation for allergenic residue methods can include spiking experiments or use of 'manufactured' standards, such as peanut-in-chocolate (discussed in Section 18.3.3).

In commercial kits, the manufacturer describes in the kit instructions the matrices for which the assay is validated, and also which extraction procedures are recommended for different matrices. The buffer used as extraction buffer is one of the most important elements of successful immunoassays for food analysis, to provide the efficient extraction of the allergenic proteins from the sample into a liquid phase and to minimize background effects due to non-specific binding. Sometimes special extraction buffers or extraction procedures for complicated matrices are used. If a matrix of interest is not mentioned in the commercial kit instructions, the manufacturer can often help with special supplements for special matrices or with developing a new extraction procedure.

In most cases, samples are not homogeneous. Therefore it is recommended to collect a solid sample from different areas of the food of interest. A greater amount (100–200 g) of a solid sample should be collected, which then has to be ground and homogenized to a fine powder or homogeneous mixture.

The samples are then weighed. Instruments and containers used have to be clean to avoid traces of the analyte (especially peanut which adsorbs very effectively or dusty materials, such as cereal flours in the case of gliadin and milk powders), which can lead to cross-contamination. If a lot of samples must be weighed, the spatula should be properly cleaned after each sample (especially peanut samples or gliadin-containing samples) or disposable spatulas can be used to avoid cross-contamination. If the concentrations of an analyte in a series of samples are known, weighing should be performed starting from the

smallest to the largest concentration. Dust should be avoided, especially when weighing flours (for example to determine gliadin). Sample preparation and implementation of assay should be carried out in different rooms and the ELISA procedure should then take place away from the weighing area.

The extraction buffer should be applied, if using a commercial kit, strictly following the manufacturer's instructions. Ice cream should be thawed and homogenized before weighing. Meat, sausages and canned products have to be finely ground or minced. It can be helpful to work with deeply frozen samples (–20–80 °C). Often the allergen extraction needs special additives to extract the allergen more completely. Fish gelatin, skim milk powder or other proteins can be helpful in extraction, especially from chocolate.

Chocolate is one of the most difficult matrices. This matrix should be heated to approximately 30–40 °C, and afterwards well homogenized and divided into portions (for repeated analysis) by weighing. It is convenient to heat the extraction buffer also in order to produce completely homogenized samples. Before each repeating analysis can begin, the chocolate must be melted and homogenized again. All of the different types of matrices have to be mixed very well after adding the extraction medium to homogenize the solution or suspension.

In the case of commercial ELISAs, extraction time and temperature are optimized by the manufacturer. The former should be as short as possible. Often an additional centrifugation step is needed to get clear supernatant solutions. If further fine particles in the extract are still discovered, a filtration step should be carried out afterwards. It is important to get a solution as clear as possible to minimize background effects. Fat-containing samples, such as meat products, ice cream or chocolate, can be centrifuged in the cold. The fat layer is then situated on the top and can be discarded easily with a laboratory spoon or spatula.

In addition, it can be helpful to freeze such solid samples at –20 or –80 °C (before homogenizing) which contain a high amount of fat or cocoa (for example chocolate bars, cream-containing bakery goods). Alternatively, defatting with acetone can be performed before the extraction (acetone dried powder), but this process can modify the allergenic protein itself. Food matrices can be composed of a lot of different substances, and sometimes one or more of these may disturb the extraction procedure. We distinguish between two types of problem:

- substances which disturb the extraction of an analyte and
- substances which disturb the reaction between the antibody and the analyte due to cross-reactivities or masking processes.

In some cases accompanying substances themselves disturb the determination of the allergen. For example, chestnut can decrease the determination of gliadin considerably. A high content of cocoa can hinder the extraction of allergenic residues because the tannins from cocoa can mask the allergens. Therefore additives should be used which can displace the tannins from the

allergens. Such additives, as mentioned above, should be added in relatively high concentration (10 –> 25%).

In addition, the samples themselves are of interest. We distinguish between raw materials (nuts, cereals, seeds, flours) and processed food products (bakery goods, meat products, canned products). Processing of food in most cases leads to partial denaturation of proteins (hazelnut, egg white and gliadin are especially sensitive to heating), which can affect the ability of animal antiserum to recognize them; however, they can retain their allergenicity after processing. The extent to which processing can be recognized depends on the antibody; different commercial kits use different antibodies. Nuts are used mostly roasted, but can also be used in a dried, non-heat-treated state. If roasted, they can be processed at different times and temperatures. An assay should be able to detect both raw and roasted material. The correct result also depends on what protein standards are used in the methods. Unfortunately, to date internationaly approved standards for the different kinds of nuts do not exist, but Chapter 19 deals with reference matertials in detail.

The detection of nut residues depends on the extent of roasting (peanut, hazelnut, almond) and, for egg white proteins or milk proteins, depends on the extent of heating processes (for example pasteurizing or sterilizing of canned products). Peanut proteins are changed during the roasting process only after prolonged times (> 30 minutes) or high temperature (> 140 °C), methods which are not used in normal roasting of peanuts for consumption. Hazelnut proteins are much more sensitive to roasting and can denature much earlier. Some of the egg white allergenic proteins (e.g. ovalbumin) denature during the process of pasteurizing or sterilizing whereas others (ovomucoid) are stable. Prolamins from wheat, rye or barley can change during bakery processes. A specialized extraction method is therefore recommended to extract heat-processed prolamins.[2] This procedure is also useful if processed soy milk is evaluated for gliadin, as these types of samples can give false positive results if done without a special extraction additive (cocktail). All of these examples should show how important it can be to know a great deal about the samples being tested. The more knowledge the analysts have, the better they can judge the result.

The quality of the antibody used in ELISA methods is most important. The main question is whether the antibody is able to recognize heat-modified proteins from allergenic foods in the same way as the raw form. Often, in commercial kits, the manufacturer indicates which kind of proteins the antibody can detect. In many kits the antibody recognizes denatured proteins but not completely, while occasionally only a qualitative result can be obtained.

In many cases, cross-reactivities may be encountered. It is important to know how specific the antibody is. A wide range of food ingredients which can occur in food products should be investigated (cereals, nuts, legumes, seeds, food additives, spices, processing aids, etc.). With most commercial kits for the detection of allergenic residues, antibodies are very specific for the analyte of interest, but it could be the case that antibodies raised against

peanut or hazelnut could also detect walnut, or antibodies against almond could detect hazelnut also. Very often roasted nuts show much higher cross-reactivity than raw. When using a commercial kit, the insert should be read thoroughly to see what types of substances cross-react in the method, or one should call the manufacturer and inquire. Substances which have similar species structures in relation to the analyte proteins to be determined can also be recognized by antibody (e.g. peas in the case of peanut, apricots in the case of almond, sunflower seeds in the case of hazelnut). Again, check the insert of the kit or contact the manufacturer regarding this.

Special problems: prolamins

Gluten is the term for a protein mixture found in wheat cereals which contains glutelins and prolamins (for example gliadins) in approximately equal quantities. (Chapter 14 covers gluten detection methods in detail). One exception is wheat starches.[3] Their ratio of prolamins/gluten depends on the washing process used (ratios exist between 1.6 and 2.6). Prolamins are ethanol-extractable protein fractions of wheat (gliadins), rye (secalins) and barley (hordeins) which are responsible for coeliac disease. Currently, the Codex Alimentarius definition for 'gluten-free' means 'that the total amount of the gluten from wheat, rye, barley and oat in the products or those crossed species in food or ingredients is not more than 200 ppm (mg/kg) on the dry substance basis'[4]. But today a lower cut-off (10 ppm gliadin/20 ppm gluten) is in discussion. Most of the manufacturers of 'gluten-free' food apply this lower limit even now. Therefore, very sensitive and specific immunoassays are necessary to find gluten contamination of these products or to find hidden, undeclared prolamins. In the past the dominant method for gluten analysis were Skerritt and Hill's monoclonal antibodies.[5] However, due to its higher sensitivity and specificity the R5-mAB based ELISA[6] is now recommended and was endorsed as 'Method One' by Codex Alimentarius in 2005.[7] On the other hand, special extraction procedures are necessary to extract heat-treated prolamins. A new gliadin material has been purified which aims to make gluten analysis safer and more efficient,[8] and based on it the Institute for Reference Materials and Measurements (IRMM) has issued a gliadin standard. Thanks to this, a new level of quality has been achieved in the field of gliadin analysis with assays capable of detecting 1.5 ppm gliadin. The new, lower sensitivity of the assays means that, when handling gliadin-containing samples in the laboratory, there are some issues needing greater care than previously, for example minimizing airborne contamination, etc. One consideration which is not often discussed is that equipment should be tested for contamination by swabbing from time to time.

18.3 Troubleshooting

This part of chapter is dedicated to troubleshooting in case something goes wrong with an assay.

18.3.1 The standard curve does not match the manufacturer's

The recommendation would be to look at the concentration of the standard curve which represents 50% binding (50% dose). If the concentration values differ +/– 20% from the 50% value, the assay should be repeated. The preparation of the components as well as the incubation time and temperature should be checked. Be sure that there is no contamination of conjugate, sample dilution buffer or washing buffer. In addition, control of the calibration of pipettes and of the photometer as well as the quality of substrate and chromogen is necessary.

If the curve of a sandwich ELISA starts with an OD at the zero standard point (0 ppm) much higher than expected from the manufacturer's kit insert, or if the CV of the curve and/or samples is over 10%, the washing process should be evaluated. It is important to have a clear blank value at standard point 1 and the second standard should be far enough from zero, otherwise false positive results can occur.

If the analyst finds nearly the same OD for two neighbouring standards, perhaps one of them is contaminated with the analyte, but also be sure that a single standard is not pipetted twice. If the OD is less than the OD_{max} expected, check the dilution of components and the photometer, and be sure that the reagents do not show deterioration (cloudy or fluffy solution). The chromogen and substrate should also be tested, and there exists a simple method to do this. If conjugate and chromogen/substrate are pipetted together into a test tube, the color reaction should start immediately, otherwise one of the components has deteriorated. However, two standards with the same OD could also mean a stability issue with the standards and, if using a commercial kit, the manufacturer should be contacted if the problem recurs.

If the OD_{max} of a sandwich ELISA standard curve is too high, the incubation time and temperature should be checked, and this should also be done if the ODs are too low. Also, check the washing procedure, as incomplete washing can lead to an increase in non-specific binding. It can be important, especially for analytes which are present in a very low concentration, to minimize issues by good performance of the assay. If the conjugate has to be diluted, make sure the proper dilution was made.

If a standard curve shows nearly the same, very high OD over the whole curve, it is an unmistakable sign of contamination. All components of the assay should be prepared fresh in new, absolutely clean containers, with new water (pure and sterilized water may be bought from a pharmacy). The laboratory should be cleaned before starting the next assay. Sometimes (for example, prolamin contamination) an ethanolic solution should be used for cleaning. In some cases, water purification systems can have components that contain milk residues, so this should be considered if running casein or whey ELISAs. Components of a test kit from different lots or different manufacturers should not be mixed, because the components of each lot and kit are adjusted to each other.

18.3.2 Bad accuracy of known samples

The extraction procedure should be checked for the correct extraction buffer and additive and the right ratio of sample to buffer. Is it necessary to use an extraction additive? Does the analyst have to test a heat-processed sample? Could it be that the sample is modified significantly enough through processing such that the antibody cannot detect the protein residues appropriately?

The measured OD for the samples should be found in the middle and steepest part of the curve. If the OD is in that part of standard curve which is not linear, the sample determination should be repeated with a further dilution. The analyst should not extrapolate below the first or over the last standard of the curve. Extrapolations are not precise enough and are usually not guaranteed by commercial test kit manufacturers. If the analyst has in addition used a high dilution of the sample, the extrapolated mistake will be multiplied. If the evidence shows that the extraction process was less than perfect, check the procedure and repeat the extraction.

Check the CV for these samples and the run of the standard curve. If reagents of a commercial kit have to be diluted, check whether the dilution was in the right order and check the washing steps. Eventually, the measurement of samples or even the extraction procedure of the samples should be repeated if results continue to be poor.

18.3.3 Bad recovery of spiked samples

The analyst should be sure that the spiking of samples was done as exactly as possible. The ELISA method can only be run with correctly spiked samples. For spiking experiments two possibilities exist:

- weigh the required amount of a blank sample and then add the spiking analyte solution or solid to the flask; or
- produce a higher concentration of the analyte in the desired blank matrix and then gradually dilute it with the blank matrix until the desired concentration is reached. This method can lower the error made by weighing. Stir it very well over a long period of time (0.5–1 hour) at each step to be sure that the step is homogeneous. Some matrices take more than four hours of mixing to achieve homogeneity.

The best way to approach this depends on the matrix itself and the desired concentration of the analyte. Spiking experiments with extracts of the analyte provide a correct measure of the ability of the extraction buffer to do its job, because the extraction procedure is then used twice (first for the analyte and afterwards again for the analyte within the matrix), and gives important data on possible matrix interferences.

If matrices are to be spiked with a known content of protein/food, one should be sure that the matrix which will be used is free of the analyte. For example, some types of European chocolate can contain hidden hazelnuts. Therefore, it can be necessary to produce chocolate in-house for spiking

experiments. Peanut can cause cross-contamination quite easily due to its tendency to be adsorbed onto vessels and spatulas, so precautions should be taken.

Gliadin contamination can occur by airborne means, if flours or a powder of a product are used. Solid matrices like cereals or cookies should be ground to a fine powder before starting the spiking experiments, in a room separate from the assay procedure laboratory in certain cases. If it is certain that the matrix is free of the analyte, spiking can begin. Concentrations of interest rank in the µg/g (ppm) range. The procedure cannot be carried out with sufficient precision by weighing on a balance, because micrograms must be weighed into a large amount of the blank matrix (for example 100 µg/10 g sample). Therefore it is better to start with a higher concentration of analyte (1.0–0.5% of analyte) and mix it down by repeating dilutions with the blank matrix until the desired concentration is reached. After mixing and strong homogenizing, the sample is further diluted step by step with the blank matrix, for example from 1.0 to 0.1%, from 0.1% to 0.01% and then further down to 0.001%. Appropriate mixing and homogenizing at each step is imperative. This is the best method to get the correct concentrations in the case of spiking solid food matrices. In the case of chocolate, the matrix should be heated at 30–40°C while stirring during spiking.

For spiking experiments of dry solid matrices (cereals, cookies) it may be better to use a suspension of the analyte (for example hazelnut, peanut or almond) for spiking, which allows weighing of a higher amount of the analyte (depending on the amount in the suspension) in a homogeneous solution. Otherwise pockets or clumps of analyte may result.

18.3.4 Poor precision (reproducibility and variation (CV))

Intra-assay problems

In a test, the OD has a very high variance between duplicates. The analyst should test the components for bacterial contamination (cloudy and flaky solutions). Inspection and evaluation of the pipetting procedure should be done. Did the pipette tips get rinsed several times before pipetting? The washing process should be checked again. Incomplete washing and inadequate evacuation of wells after washing can influence the zero standard and the CV, and therefore the detection limit of the assay as well as the precision of the sample determination. Also, incomplete mixing of reagents in the microwells can cause this problem. Ensure that the components are mixed by gently rocking the plate after adding the reagents or, in some cases, a microplate shaker can be employed. Check background effects with an extracted blank sample or with wells into which only conjugate and the reagents for color reaction are pipetted.

Inter-assay problems
In a test, the ODs between runs of the same lot or different lots show a large range of variance. Pipetting errors or reagent deterioration could be the reason. Further, the stability of sample extracts should be tested. Also, the reproducibility of the extraction itself should be inspected by a reference sample (known concentration) by repeating the extraction. The homogeneity of the sample should be examined. The analyst should increase the sample size and should repeat the extraction again.

18.3.5 Inadequate color development

If all of the wells show the same OD, one of the reagents could have deteriorated, the washing buffer could be contaminated, the reagents could be mixed incompletely or the conjugate or standards could be contaminated. If there is no color development in the wells, possibly the conjugate or color reagent are missing. The analyst should check the color development by mixing color reagents and conjugate. Color development should start immediately, otherwise it is likely that one of the components has deteriorated.

18.4 Future trends

18.4.1 Fast assays

The goal in the future will be to achieve shorter immunoassays which are nevertheless able to guarantee the detection limit that is demanded for allergenic residue detection. Concentration limits of allergens in food should be defined according to appearance of clinical reaction in food-allergic individuals (see Chapter 2 for a discussion of thresholds).

In the commercial kit market, more rapid assays, which possess, in general, incubation times of 3×10 minutes, are already available for some of the most important allergenic residues. Incubation times will be further decreased in relation to a low detection limit, which can be reached, assuming that the quality of antibodies is high enough. Also, extraction procedures will become faster, easier, and more effective at getting complete recovery. One day internationally-approved reference materials for all kinds of food allergens will exist.

18.4.2 Dipsticks

Dipstick formats have been developed for allergenic residues – the antibodies or antigens are immobilized to a microporous surface on a strip. The assay can be easily performed by transferring the stick into a sample after extraction from food. For a complete discussion of dipsticks/lateral flow devices, see Chapter 10 of this book.

There are a few commercially available dipsticks which allow determination of allergenic residues in the range of a reasonable cut-off point to get a yes/no answer. A decision within 5–10 minutes is possible. Two types of design are available:

- determination of a single allergen; or
- determination of a group of allergens (e.g. tree nuts) on one dipstick.

For example, a very sensitive dipstick is now available for detection of gliadin in food (R-Biopharm AG, Darmstadt, Germany), based on the monoclonal antibody R5[6] which is able to recognize prolamins from wheat, rye and barley at 100% and fails to recognize prolamins from maize and rice. The antibody recognizes a toxic peptide, which is repeatedly found in gliadins and glutenins, and is also able to recognize heat-denaturated gluten provided a special extraction procedure is conducted.[2] In a basic design common to all lateral flow devices, the test principle is based on an immunological capture of colored microparticles (which are bound to the monoclonal antibodies against gliadin) that migrate through a membrane, in which also the monoclonal antibody against gliadin has been immobilized. The sample flows through the porous membrane. If there are gliadins in the sample, the colored microparticles are bound and the complex moves across to the zone of immobilized gliadin capture antibodies, where it is immobilized. A red colored line will appear if the sample contains gliadin.

This assay works quickly (within five minutes a visible result is obtained) and is easy to perform. The use of the stick is very safe because, additionally, a blue safety control band appears if the stick works correctly. This gliadin dipstick is able to detect less than 10 ppm gliadin in a sample. Further dipsticks are under development by various companies for various allergenic foods. Two manufacturers have had dipsticks for peanut on the market for the past year. It is expected that commercial dipsticks for milk, egg, almond and hazelnut will be developed in the next few years.

Dipsticks are helpful in evaluating incoming raw material quickly, to check finished goods and to inspect the production process and equipment for allergenic residues. Often the production of allergen-containing and non-allergen-containing food occurs on the same line or on parallel lines. It is very important to assess the production process to guarantee that cross-contamination of allergenic residue is avoided or to indicate if the processing line is free of residues. The dipstick can be used directly by swabbing processing/packaging equipment. Usually, an area of 10×10 cm^2 is swabbed. Then the stick is dipped into a buffer solution and the migration process begins. In the case of the R-Biopharm AG gliadin test, as little as 1.5 µg/100 cm^2 can be detected. Dipsticks are also very useful for analyzing rinse waters from clean-in-place systems.

18.5 Summary

The success of any immunoassay depends directly on the main components (antigens, antibodies), but the performance of the assay is also important in obtaining a reliable result.

Two kinds of errors can be found – a systematic (in the form of bias) and a random error (reflected in poor reproducibility or 'imprecision'). Bias and imprecision are two statistical parameters which describe each assay. Some suggestions to minimize random errors of an assay are made in this chapter. The performance of a test influences the precision (intra-/interassay), reproducibility, recovery, sensitivity and reliability of the system. It is neccessary to understand the basic principle of the assay type one is using. The assay conditions and the handling on the one hand and the analyte extraction procedure on the other are important parameters influencing the result. Before starting, the user should be clear about the assay format, the time requirements, the number of possible samples with one run, and the standardizing/calibration of the assay. Further, the careful handling of test components and mistakes that can occur (adsorption, contamination, degradation) and the factors influencing the procedure (time, temperature, washing steps, starting point of the reaction) are covered. Today's immunoassays are very sensitive. It is clear that care must be taken to avoid cross-contamination in the laboratory and some examples are given concerning possible origins of contamination. The evaluation of results should come from a standard curve which fits the measured values in the best way; often a cubic spline method is needed. Extrapolations above and below the standard range of the curve should not be made. Hints are given for an internal quality control regime, which should be introduced into each laboratory that deals with immunoassays, to secure the results.

The sensitivity and selectivity of the immunoassay has to be combined with a selective and effective extraction procedure of the analyte. Factors of interest are the incubation time and temperature during the extraction, but most important is the suitability of the extraction buffer. Optimization is absolutely neccessary. Tips are given on how to spike different kinds of matrices effectively to control the assay and the extraction procedure. Finally, recommendations are made concerning troubleshooting in the case of problems in obtaining good analytical results.

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19

Reference materials and method validation in allergen detection

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19.1 Introduction

Reliable detection and quantification methods for food allergens are necessary in order to improve consumer protection and to ensure compliance with food labelling regulations. However, the analysis of allergenic components in food products can be very difficult, because their chemical identity is often not sufficiently known and it is suggested that they are frequently only present in trace amounts. Another question yet to be answered is the required detection power and selectivity of corresponding analytical methods, as scientifically sound threshold levels have not yet been established, e.g. by human oral challenge studies. Threshold levels for specific allergic reactions determined until now by double-blind placebo-controlled food challenges (DBPCFC) range between less than 1 mg and more than 1 g of allergenic protein depending on the food concerned and the selected individuals (Taylor *et al*., 2002). There seems to be a general assumption that the detection limits for different food products need to be around 10 ppm [mg allergen (protein)/kg food] or lower, depending on the particular food (Koppelman *et al*., 1996, Poms and Anklam, 2004). But one should keep in mind that mass is usually not an appropriate quantification and measurement parameter for analytes with varying chemical/biological activity such as proteins.

Currently, there are several analytical approaches for the detection of potential allergens in food products (reviewed in Poms *et al*., 2004). The methods employed are targeting either the allergen (protein) itself or a marker that indicates the presence of the offending food. At present, detecting the allergen *per se* is not always feasible, as the crucial chemical properties may not be well characterized and understood or the detection limits of the methods

used are insufficient. Typically, specific proteins or DNA fragments are targeted as markers for the presence of potentially allergenic food products or ingredients. Protein-based methods usually involve immunochemical detection protocols such as the radio-allergosorbent test (RAST), enzyme-allergosorbent test (EAST), enzyme-linked immunosorbent assay (ELISA) and immunoblotting (discussed in other chapters of this book), whereas only the ELISA technique is presently used in routine food analysis. Methods operating on the DNA level are usually based on an amplification of a specific DNA fragment by the polymerase chain reaction (PCR) (see Chapter 7). Currently the employment of DNA-based methods for the detection of allergens in food products is a matter of controversy, since proteins are the causative agents in allergy and PCR results cannot be linked to any allergen/protein content. However, the choice of method is still mainly dependent on the food concerned (availability of specific antibodies/DNA-primers and the achievable detection limit) and on the effects of food processing during production. Therefore, protein-based and DNA-based methods each have their own characteristic merits and drawbacks concerning their applicability in the detection and quantification of allergens in various food products (Poms and Anklam, 2004).

19.2 Quality assurance for the analysis of allergens

Whatever approach is used for the analysis of allergen-containing food components, it must be assured that the measurement results are reliable and relevant for the assessment of the allergic potential of the investigated food. Therefore the whole, so-called analytical process (Fig. 19.1) has to be properly

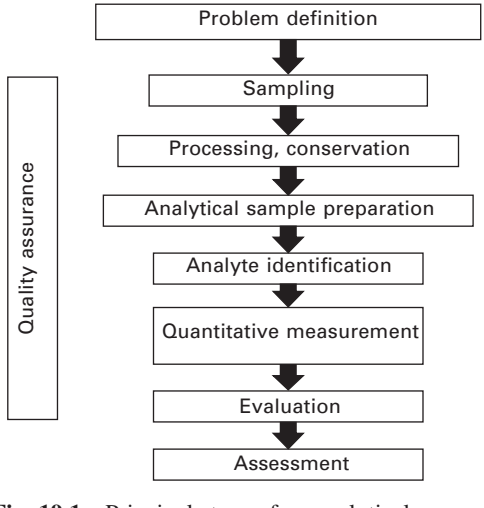

Fig. 19.1 Principal steps of an analytical process.

designed. For instance, technologies used in food processing such as roasting and extrusion can have a significant influence on the availability (solubility) of specific proteins for allergic reactions. This must be taken into account when antibodies are raised for immunochemical techniques.

Especially during the 1990s, general concepts and tools have been developed for assuring the reliability and comparability of measurement results in quantitative (bio)chemical analysis. It seems to be generally accepted now that analytical quality assurance (AQA) is based on three main components, namely the systematic use of validated methods, reference materials and proficiency testing. Consequently, the general prerequisite for the implementation of any AQA system is the availability of problem-adjusted combinations of reference methods and reference materials.

19.3 Towards validated methods for allergen determination

The urgent need for method standardization in this field has been recognized by the international analytical community and has recently led to the establishment of a new working group (WG 12) in the technical committee on food horizontal methods (TC 275) of the European Committee for Standardisation (CEN). American and European institutions e.g. the AOAC International Research Institute (AOAC-RI) the European Commission's Joint Research Centre (EC-JRC) the German Institute for Standardization (DIN) Health Canada, and others) have taken the initiative to validate the analytical performance of several test kits for various food allergens.

However, recent studies showed that quantitative results obtained by the application of different test methods varied significantly (Hurst *et al*., 2002; Koch *et al*., 2003; Poms and Anklam 2004). These findings underline the fact that the detection and particularly the quantitative determination of allergenic residues in food products can be impaired by: (i) interactions with compounds from the food matrix (e.g., polyphenols and tannins); (ii) reduced solubility and reactivity of heat-denatured proteins; (iii) differences in antibody affinity/recognition of allergenic proteins from different species and geographical origin (Hurst *et al.*, 2002; Hischenhuber, 2002; Keck-Gassenmeier *et al*., 1999). A recent inter-laboratory validation study for peanut allergen detection methods involving more than 30 international laboratories concluded that quantitative results varied strongly with differences of up to 300% (Poms *et al.*, 2005).

Only a very few inter-laboratory validation studies for allergen detection methods have been completed to date, and even their status and conclusions suffer from the lack of international guidelines for validation of test kits and of corresponding reference materials.

19.4 Characteristics and use of reference materials

In recent years the harmonization of terminology with respect to reference materials and the understanding of underlying scientific principles has considerably improved in the various analytical communities. Nevertheless, there still exists some confusion in corresponding communications. One should keep in mind that ISO Guide 30 (ISO, 1992) and the current VIM (BIPM *et al.*, 1993) list the following definitions with respect to reference materials:

- **Reference material (RM)**. Material or substance one or more of whose property values are sufficiently homogeneous and well-established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.
- **Certified reference material (CRM)**. Reference material accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realization of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence.

According to these definitions, CRMs form a subgroup of RMs, namely those RMs that possess additional characteristics, namely a certificate and traceable assigned values with an uncertainty statement. It should be noted that traceability is defined in this context as a property of the result of a measurement or the value of a standard, whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties (BIPM *et al.*, 1993).

There are many different terms in use for the other subgroup, the non-certified RMs, with a tendency to call them quality control materials (QCM) (Emons *et al.*, 2004). Certified and non-certified reference materials are used for method development and validation, calibration, statistical quality control within a laboratory (mainly for establishing control charts) and to assess the performance of laboratories by third parties, especially by proficiency testing schemes. All these applications allow detection of pitfalls of the respective analytical methodologies and/or their implementation, thus increasing comparability of measurements between laboratories and improving confidence in their analytical data.

Any use of a material must be preceded by a definition of its characteristics, as they determine whether a material is ‘fit for the purpose’. The most important characteristics of materials for analytical quality control are minimum sample size to assure in-sample homogeneity, between-bottle homogeneity, stability during transport, stability during storage, commutability and, if necessary, the assignment of traceable property values with an uncertainty statement and the presence of a certificate. In this course, certification of a reference material is only part of an integrated process (Fig. 19.2) comprising

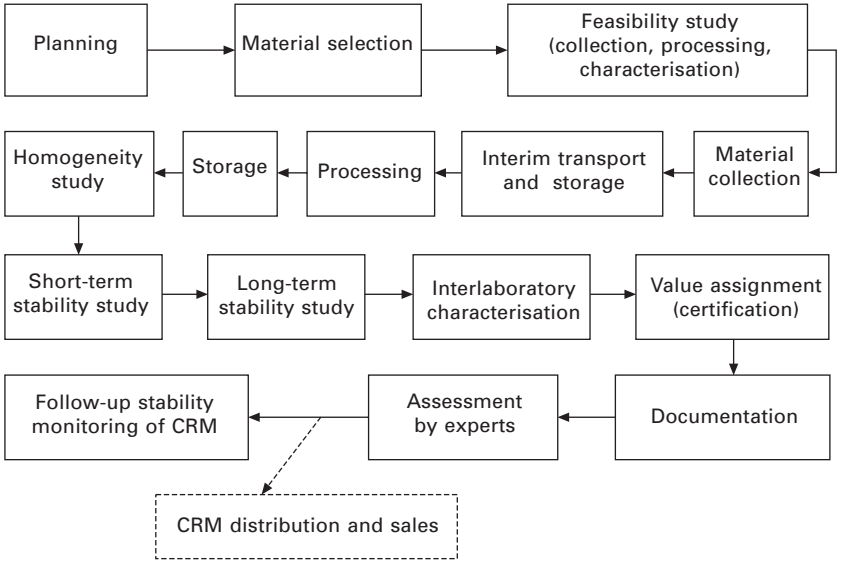

Fig. 19.2 Process of CRM development and production at IRMM.

correct preparation, homogeneity and stability demonstration, and accurate material analysis that leads to a certified value together with its total uncertainty.

The term 'commutability' originates from clinical analysis and describes the similarity of the analytical response obtained for a given material to the response obtained from routine samples (ISO, 2003). This term covers all 'matrix effects' and all effects that arise from the processing of the candidate reference material. For example, processing such as fine grinding can facilitate the extraction of an analyte from a material. Analysis of such a RM would then not allow a correct assessment of the extraction efficiency of a method with respect to routine samples.

Most of the parameters required for method validation and for the estimation of measurement uncertainty can be derived without assigned values. But for the assessment of the trueness – and consequently accuracy – of a method, assigned values with a stated uncertainty which are traceable to the same reference as the analytical results of the method used are required. CRMs provide exactly this traceable assigned value. Such a certified value is also required if the material is to be used for calibration. In addition, a minimum sample size is defined beforehand for CRMs, enabling analysts to know without testing of their own whether the material is homogeneous enough for method development. An additional advantage is the ensured stability of the materials. All these advantages must be balanced against the potential mismatch between the particular CRM and the routine sample.

Disadvantages of using CRMs for certain purposes result from the compromises which have to be accepted because of additional material

manipulations to achieve the necessary homogeneity and stability for a CRM. It has to be decided by the user whether the resulting deviation from the real sample to be analyzed can be accepted within the QA process.

Though usually overlooked by technical staff, legal issues can play an important role in the discussion about CRMs and QCMs. The situation is well established for CRMs: the CRM producer guarantees the accuracy of the certified property value, including homogeneity and stability for each unit (bottle) of material. Therefore, a CRM producer who negligently brings insufficiently characterized materials on the market could be held liable for any costs arising. For example, if a laboratory validates a method using a falsely certified CRM, the producer is at least morally responsible for wrong assessments on real samples based on this validation.

19.5 Towards reference materials for allergens

The availability of reference materials for allergens in food would offer the potential to harmonize, standardize and calibrate corresponding methods and to achieve international traceability, i.e. worldwide comparability, of such measurement results. Furthermore, RMs could offer the basis for further development and production of antibodies and detection systems. Several food matrix CRMs are available from producers of reference materials (e.g. National Institute of Standards and Technology, NIST, from the USA, Institute for Reference Materials and Measurements, IRMM, from the European Commission) that may also contain allergens, such as milk powder, egg powder and peanut butter; however, these materials are certified for components other than allergens. But so far no material for food allergens which fulfils the quality criteria described above for CRMs or QCMs has become commercially available. Moreover, it is important to have real food matrix materials (not only spiked with extracts of allergenic food) available. Obviously the development and production of such reference materials for the analysis of food allergens face several challenges.

19.5.1 The analyte

Firstly, the analyte for food allergen determination is in many cases not clearly defined. Typically, a number of key proteins and epitopes are known and described, which cause corresponding reactions. In addition, hypersensitive patients react to one or more different epitopes and, furthermore, the severity of reaction may vary between different epitopes of the same protein. Thus the analyte may be a certain protein or a variety of different proteins specific for a particular species. Moreover, the spatial structure of these allergenic proteins may vary, thus exhibiting different *in vivo* and *in vitro* reactivity. Consequently, measurement results of food samples can be influenced by intrinsic factors (i.e. biological variability such as protein content and food

composition due to geographical and seasonal variability) and extrinsic factors, such as food processing history and different protein denaturation; but also the time of harvest and the duration of storage may affect the characteristics of food allergens, particularly for plant allergens. Additionally, the state of the extracted analyte also often depends on the extraction method used.

19.5.2 The method

Secondly, there is no appropriate reference method available to date to which procedures and results are traceable. The currently employed methods in allergen determination involve either an immunochemical detection or an indirect species-specific DNA analysis. Protein-based immunochemical methods are typically very specific for the food commodity concerned or for a specific allergenic protein; however, the detected analyte is often not well-defined (binding sites of employed antibodies are mostly unknown and usually numerous different binding sites are targeted). On the other hand, results from DNA-based methods cannot be linked to protein/allergen quantities in a certain food. Promising developments are aiming at specific peptide mapping employing high-performance liquid chromatography (HPLC) and mass spectrometry (Shefcheck and Musser, 2004). Current problems associated with these techniques are the lack of sensitivity and specificity. Moreover, the matrix of the foodstuffs analyzed may have a strong effect on the reliability of the method used, which has to be taken into account.

19.5.3 The material

Thirdly, a reference material for a specific food allergen or a particular allergenic food component needs to fulfill certain quality criteria depending on its intended use as outlined above. Even non-certified RMs have to be sufficiently homogeneous, depending on the required sample size of the method, and stable with respect to the intended time-frame from preparation to final application. Such parameters have to be checked before an RM can be made available and used. Therefore, the target properties of 'allergen materials' must be defined in the beginning, and appropriate analytical methods have to be available for their control. In general, certification of a candidate reference material will only be possible for a well-defined property based on either molecular structure of the analyte or a method-defined parameter.

19.6 Future trends

Several attempts are ongoing to break the 'vicious circle' concerning the interrelation between lack of methods and materials for allergen analysis. For instance, the IRMM in co-operation with Health Canada and the Prolamin Working Group of Codex Alimentarius has developed a candidate reference

material 'gliadin from European wheat' to support quality assurance for gluten determination. The material is in the process of characterization and its certification is envisaged for the end of 2005. In addition, IRMM has recently developed processing techniques for the homogenization of peanuts. This should allow the preparation of a 'reference matrix' for peanut analysis in the near future. It is foreseen that defined powders will be made available originating from several peanut varieties which have undergone different food processing. Further the International Association for Cereal Science and Technology will make testing materials available that incorporate these well-defined powders in various food products (e.g. in dark chocolate, cookies, breakfast cereal flakes). In addition, the US Food and Drug Administration and the Food Allergy Research and Resource Program at the University of Nebraska have similar efforts ongoing in the area of manufactured standards. All those activities should pave the way into the future of appropriate quality assurance tools in allergen analysis.

19.7 Sources of further information and advice

- Codex Alimentarius
  http://www.codexalimentarius.net/web/index_en.jsp
- IRMM (Institute for Reference Materials and Measurements)
  http://www.irmm.jrc.be/
- Health Canada
  http://www.hc-sc.gc.ca/
- ICC (International Association for Cereal Science and Technology)
  http://www.icc.or.at/
- FDA (US Food and Drug Administration)
  http://www.fda.gov/
- FARRP (Food Allergen Research and Resource Program) at the University of Nebraska http://www.farrp.org

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20

US regulation of undeclared allergens in food products

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20.1 Introduction

A company selling food products in the US is vulnerable to potential regulatory and product liability when the food product causes injury due to the presence of an undeclared allergen. There is potential regulatory liability because the US Food and Drug Administration (FDA) has long taken the position that foods should be recalled when they are found to contain an undeclared major allergen. In addition, the Food Allergen Labeling and Consumer Protection Act, signed into law in August 2004, deems a food product misbranded if the label fails to declare a major allergen by a common English name such as 'milk,' 'egg,' or 'peanut.' Once a company discovers that one of its products contains an undeclared major allergen, either through its own due diligence, in response to a consumer complaint, or in response to an FDA inquiry, the company generally will recall the offending product. Given the severity of reaction that can occur when a food-allergic consumer is exposed to a major allergen, companies generally must issue press releases announcing the recall. A company can easily spend hundreds of thousands of dollars recalling products with an undeclared allergen.

The sale of a food product containing an undeclared allergen also presents potential product liability. Americans have long been known for their fascination with lawsuits and their propensity to resolve disputes through the court system. The media carries numerous stories of consumers who have been harmed from food and are seeking huge damages from restaurants or food manufacturers. Indeed, customers have sued food restaurants for serving hot coffee that caused a serious burn and for serving food that allegedly contributed to obesity. There have also been hundreds of cases that have been brought

against the food industry for selling foods that contained pathogens such as *Salmonella* or *E. coli* that have caused consumer injury. Numerous cases have also been filed against the food industry for selling a food that contained an undeclared major allergen. While the vast majority of cases are settled out of court, the few reported cases available indicate that the potential liability for selling a food with an undeclared allergen can range from a few thousand to over a million dollars.

The food industry has invested considerable time and resources since the 1990s in attempting to reduce the presence of undeclared allergens in the food supply. Other chapters in this publication focus on the various ways in which undeclared allergens can become integrated into the food supply and the manufacturing controls that can be implemented to reduce them. Indeed, many companies are now using allergen test kits to monitor for the presence of undeclared allergens that could be incorporated into foods by use of shared equipment. These test kits can be incorporated effectively into a company's allergen control practices by identifying the presence of an undeclared allergen that otherwise is not visible to the naked eye. Companies must realize that the results from the test kit may one day be available to regulators. If the company becomes involved in a product liability action resulting from an alleged injury due to an undeclared allergen, the allergen test results would also need to be made available to lawyers that are bringing the action against the company. Although a powerful tool for incorporation into an allergen control program, companies must give careful consideration to how they will incorporate test kits into their allergen control program.

20.2 Regulatory liability

20.2.1 Misbranding provisions

The Federal Food, Drug, and Cosmetic Act (FFDCA) prohibits the introduction into interstate commerce of foods products that are misbranded.[1] The FFDCA deems a food misbranded unless its label bears an ingredient statement identifying each ingredient that has been added to the product by an appropriately descriptive common or usual name.[2] Spices, flavors, and colors that do not have to be certified are exempt from the ingredient labeling requirements and may be declared by generic terms such as 'spice,' 'flavor,' 'natural flavor,' 'artificial flavor,' or 'artificial color,' as appropriate.[3]

The FDA regulations exempt incidental additives from the ingredient labeling requirement.[4] To be eligible for regulation as an incidental additive, and therefore be exempt from the ingredient labeling requirements, a substance must be present at an insignificant level and have no technical or functional effect in the food.[5] Examples of incidental additives include substances that become incorporated into a food because they are present in a sub-ingredient (such as carriers for flavors and colors), processing aids (such as enzymes

that are used during the manufacturing process but then later removed), and substances migrating to food from equipment and packaging materials.

The Food Allergen Labeling and Consumer Protection Act establishes new requirements for the labeling of major allergens. The President signed the law in August 2004 and its provisions become effective for products labeled after January 1, 2006.[6] At the time of this article, FDA has not yet provided any guidance on how it intends to interpret this new law. The new law amends the FFDCA in two significant ways. First, it defines the term 'major food allergen' and second, it deems a food misbranded unless the major food allergens are declared by a 'common English name' that is easily recognizable by consumers. In large part, the legislation is limited to what are commonly called the 'Big 8 allergens.' 'Major food allergen' is defined as 'milk, egg, fish (e.g. bass, flounder, or cod), crustacean shellfish (e.g. crab, lobster, or shrimp), tree nuts (e.g. almonds, pecans, or walnuts), wheat, peanuts, and soybeans' and food ingredients that contain proteins derived from the major food allergens.[7] These proteins nevertheless may be excluded from this definition if they fall under one of two exceptions.

First, highly refined oils, and ingredients derived from these oils, are not a 'major food allergen.[8] These "highly refined oils" are refined, bleached, deodorized oils. In its report, the Committee notes that the legislation would not change the common or usual name of these highly refined oils. Thus, oils, such as peanut oil, would still be labeled as such in ingredient statements.

Second, a manufacturer may obtain an exemption for ingredients derived from major allergens by submitting a premarket notification or petition demonstrating that such ingredient should not be considered a major allergen.[9] The premarket notification procedure would be available when (i) scientific evidence establishes that the food ingredient does not contain allergenic protein or (ii) FDA has made a determination under a Section 409 premarket approval or premarket notification program that the ingredient does not cause an allergic response that poses a risk to human health.[10] The Committee explains that the Generally Recognized as Safe (GRAS) notification process is not included as part of this exception.

It is difficult to predict how the agency will implement the allergen law, particularly when it comes to its application to processing aids and other incidental additives that are derived from major allergens. Ingredients such as soy lecithin, fish gelatin, and wheat starch have been used for decades as processing aids and have been exempt from ingredient labeling requirements. While these ingredients may have detectable levels of protein, they are frequently used at very low levels in finished foods. In many instances the level of protein in the finished food contributed by the use of an ingredient such as soy lecithin can be well below 1 part per million.

It would be reasonable for FDA to take the position that allergenic proteins present at *de minimis*, or very low levels, essentially are not present in finished foods and should not be subject to the allergen labeling requirements. Indeed, the courts have applied a *de minimis* concept with regard to the

regulation of food additives that may be present at very low levels in foods.[11] At the time of this article, FDA has not offered guidance on whether it would apply a *de minimis* analysis and exempt from the allergen labeling requirements those food ingredients derived from major allergens that contribute insignificant levels of allergenic protein in finished foods.

Moreover, even if FDA does agree that a *de minimis* analysis is appropriate, it remains unclear what level of allergenic protein will be considered a *de minimis* level. The absence of an established threshold below which allergens will not cause an allergic reaction complicates the agency's ability to establish a *de minimis* level. There are factors, however, that would support the establishment of 10 parts per million or a similar level as the *de minimis* level. This is the level identified by some experts as being unlikely to trigger an allergenic reaction in most food-allergic consumers. It also is the level that has been used informally by many in the food industry for years in determining whether an allergen should be declared. In addition, 10 parts per million is reasonably close to the one to five parts per million analytical level found in some of the commercially available allergen test kits.

The *de minimis* concept not only is important when determining whether a food ingredient should be subject to the allergen labeling requirements, but also could apply when determining if an ingredient is eligible for the notification or petition process. The notification process is available in those instances when a manufacturer can demonstrate that an ingredient derived from a major allergen, although containing protein, does not contain an allergenic protein. The law does not comment, however, on what is meant by 'containing allergenic protein.' The law does not comment on whether the agency should consider the limit of detection of the most sensitive analytical method or the intended use of the ingredient and the level of allergenic protein that would theoretically be present in the finished food through such use.

It presumably would be reasonable for the agency to focus on the manner in which the ingredient is used in the finished food and the theoretical levels of allergenic proteins that would be present through such use. In instances when the ingredient would contribute a *de minimis* level of allergenic protein under its intended conditions of use, it would be reasonable for the agency to take the position that the ingredient does not contain allergenic protein and is eligible for the notification process. If the agency instead focuses on whether the ingredient contains a detectable level of allergenic protein, there would be very few ingredients that would be eligible for the notification program. Under such an interpretation, an ingredient such as soy lecithin that may contain less than 50 parts per million of total protein would be ineligible for the notification process.

A petition process is established for those ingredients derived from major food allergens that are ineligible for the notification program.[12] An exemption would be obtained by submitting a petition with data demonstrating that the ingredient does not cause an allergic response that poses a risk to human health. The petitioner would bear the burden of proof. The petition would be

deemed denied after 180 days unless the petitioner and FDA mutually agree to an extension. FDA is required to post to a public site the notifications and petitions that have been received and any responses or objections thereto.

Once within the definition of 'major food allergen,' ingredients would be subject to the provisions of the law regardless of whether they had previously been treated as 'incidental additives.' If the agency is unwilling to apply a *de minimis* analysis or find some other legal means to interpret the allergen law flexibly so it is possible to obtain an exemption for processing aids that contribute insignificant levels of allergenic proteins in finished foods, there will be many food products that will be subject to the allergen labeling requirements. Indeed, products containing soy lecithin, fish gelatin, wheat starch, and other common processing aids would suddenly be subject to allergen labeling. The food-allergic consumer in many instances has been consuming these products without incident and will suddenly be presented with a label disclosing the presence of the major allergen in the food.

In addition to defining major food allergen, the new law amended Section 403 of the FFDCA to provide three new misbranding provisions. The first provides FDA the authority to require by regulation the appropriate labeling of any spice, flavoring, coloring, or incidental additive that includes, as a constituent, a food allergen that is not one of the major food allergens. The second requires colors, flavors, and incidental additives that contain major allergens to be subject to the labeling requirements for major allergens. The third requires the eight major food allergens to be labeled on foods that are not raw agricultural products.

Manufacturers will have the following two options for identifying the eight major food allergens on the food label:[13]

1. By placing the statement 'Contains_____' with the blank filled in with the name of each major food allergen, at the end of, or immediately adjacent to, the ingredient statement; or
2. By using a parenthetical following the name of the ingredient that identifies the name of the major allergen, such as 'casein (milk).' The parenthetical would not be required when the ingredient name uses the name of the major food allergen (such as 'milk' or 'soy protein') or when the name of the major food allergen appears elsewhere in the ingredient statement (unless the other listing is for a food ingredient that the Secretary has determined does not cause an allergenic response).

Regardless of the option selected, the major food allergens would need to be identified by the name of the food source from which the major allergen is derived, such as milk, egg, wheat, peanut, or soybean. The specific type of tree nut (e.g., almonds, pecans, or walnuts), crustacean shellfish (e.g., crab, lobster, or shrimp) or fish (e.g., bass, flounder, or cod) would also need to be identified.

The agency has for several years had an allergen labeling policy that applies to the same 'major allergens' that are covered by the allergen labeling

law.[14] The Allergen Labeling Policy is of lesser significance today because it has been superseded by the Food Allergen Labeling and Consumer Protection Act. It, nonetheless, is appropriate to review the policy because it remains in effect until the allergen legislation becomes effective in January 2006.

The FDA Allergen Policy applies to the labeling of ingredients that are added directly to the food and to ingredients that may otherwise be eligible for an ingredient labeling exemption. FDA states:

> Ingredients Added Directly to Foods: Products which contain an allergenic ingredient by design must comply with 21 U.S.C. 343(i)(2). Where substances that are, bear, or contain allergens are added as ingredients or sub-ingredients (including rework), the Federal Food, Drug, and Cosmetic Act (the Act) requires a complete listing of the food ingredients (section 403(i)(2); 21 U.S.C. 343(i)(2); 21 C.F.R.101.4) unless a labeling exemption applies.
>
> Exemptions from Ingredient Labeling: Section 403(i)(2) of the Act provides that spices, flavors, and certain colors used in a food may be declared collectively without naming each one. In some instances, these ingredients contain sub-components that are allergens. FDA's regulations (21 CFR 101.100(a)(3)), provide that incidental additives, such as processing aids, which are present in a food at insignificant levels and that do not have a technical or functional effect in the finished food are exempt from ingredient declaration. Some manufacturers have asserted to FDA that some allergens that are used as processing aids qualify for this exemption. FDA, however, has never considered food allergens eligible for this exemption. Evidence indicates that some food allergens can cause serious reactions in sensitive individuals upon ingestion of very small amounts; therefore, the presence of an allergen must be declared in accordance with 21 CFR 101.4. The exemption under 21 CFR 101.100(a)(3) does not apply to allergenic ingredients.[15]

FDA has clarified that it considers products that do not comply with this policy misbranded. FDA reasons 'the article was misbranded when introduced into and while in interstate commerce and is misbranded while held for sale after shipment in interstate commerce, within the meaning of the section 403(i)(2) of the Act, in that it is fabricated from two or more ingredients, and its label fails to bear the common or usual name of each such ingredient, namely (specify the undeclared allergenic ingredient).'[16]

Since 1996, FDA has stated 'processing aids that contain allergenic ingredients must be declared in accordance with 21 CFR 101.4(a)(1).'[17] Under this FDA policy, a substance that may be present at very low levels and have no technical or functional effect on the food would be subject to ingredient labeling if it contains a protein from a major allergen.

20.2.2 Adulteration provisions

The FFDCA also prohibits the introduction into interstate commerce of foods products that are adulterated.[18] A food is deemed adulterated for many reasons, including if it has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health.'[19]

The FDA Allergen Policy addresses those allergens that may become inadvertently incorporated into food during the manufacturing process. FDA recognizes that allergens may be unintentionally added to food 'as a result of practices such as improper rework addition, product carry-over due to use of common equipment and production sequencing, or the presence of an allergenic product above exposed product lines.'[20] FDA notes 'such practices with respect to allergenic substances may be insanitary conditions that may render the food injurious to health and adulterate the product under section 402(a)(4) of the Act [21 U.S.C. 342(a)(4)].'[21] FDA states such a product is 'adulterated when introduced into and while in interstate commerce and is adulterated while held for sale after shipment in interstate commerce, within the meaning of the section 402(a)(4) of the Act, in that it has been prepared, packed and held under insanitary conditions whereby it may have been rendered injurious to health.'[22]

The food industry routinely uses the same equipment to manufacture different products in much the same way that consumers will use the same mixers, cake pans, cookie trays and utensils when baking cookies and cakes. Given the ability of allergens to produce an allergic reaction when present at very low levels – and certainly levels below what can be seen visibly – allergenic proteins can adhere to mixers, utensils, and baking pans, and other food contact surfaces and be released in subsequently manufactured products. The unintentional contamination can occur in the food manufacturing facility, in restaurant kitchens, and in homes.

The courts have never been asked to address whether FDA can invoke section 402(a)(4) (21 U.S.C. § 342(a)(9)) to require the labeling of allergenic substances that may become incorporated into foods through inadvertent cross-contact during the food production process. Manufacturers that fail to implement practices to control the inadvertent cross-contact of major allergens face potential regulatory action. In general, the food industry has implemented numerous practices and procedures to reduce the likelihood that allergens will be introduced through inadvertent cross-contact. There may be some instances, however, when cross-contact cannot be avoided even when following state-of-the-art manufacturing and cleaning practices. In instances when cross-contact cannot be avoided when companies are following good manufacturing practices (GMPs), FDA will allow companies to use precautionary labeling such as 'may contain _____' with the blank filled in with the name of the allergen that may be present in the food through inadvertent cross-contact. FDA has made it clear that it will allow 'may contain' statements provided manufacturers are not using them in lieu of GMPs.

20.2.3 Product recalls

A company that has introduced into interstate commerce a food product that contains an undeclared allergen will be subject to potential FDA regulatory action. Although the agency does not have the legal authority to mandate product recalls, the agency does have the legal authority to seek judicial intervention through an injunction against the company or a seizure of the products.[23] The Bioterrorism Statute promulgated in June 2002 expanded FDA's authority to include administrative detentions as well.[24] FDA can also generate tremendous publicity in the event that the company refuses to recall a product that contains an undeclared allergen. Given the powerful enforcement tools and the power of the press available to the agency, it would be unprecedented for a company to refuse to recall a food product when *requested* to do so by the agency.

Under FDA's administrative procedure regulations, a recall is defined as 'a firm's removal or correction of a marketed product that the Food and Drug Administration considers to be in violation of the laws it administers and against which the agency would initiate legal action, e.g., seizure.'[25] There are three classifications of recalls, Class I, Class II, Class III, with Class I being the most serious. A Class I recall is defined as 'a situation in which there is a reasonable probability that the use of, or exposure to, a violative product will cause serious adverse health consequences or death.'[26] Almost all recalls involving major food allergens, with the exception of wheat, will result in a Class I recall. Undeclared wheat generally will result in a Class II recall because, although wheat can trigger an anaphylactic reaction, it has not been reported to cause death, as has been reported with exposure to the other major allergens.

During a Class I recall, FDA ordinarily will require the company to issue a press release notifying the consumers about the recall. These press releases, when picked up and broadcast by the media, are an effective tool in notifying the food-allergic consumer that he or she needs to return the product. The publicity generated by a recall can adversely impact the company's image and the cost of recovering foods from the marketplace can easily exceed $ 100 000, depending on the quantity of food introduced into commerce and the breadth of the distribution. The publicity generated by recalls also notifies lawyers that there may be a harmed consumer or consumers that could serve as the basis of a legal challenge against the company.

20.3 Legal grounds for product liability actions

The presence of undeclared allergens in the food supply will present potential product liability. It is difficult to quantify the potential product liability risk, however, because there are relatively few reported cases. Many companies will settle potential product liability actions involving undeclared allergens before cases are filed. These settlements will be done quietly and generally

will include as a condition of settlement a clause prohibiting the injured consumer from disclosing the terms of the settlement.

Of the few cases that are filed, many are settled before the trial and once again the terms of the settlement generally prohibit disclosure of the terms. These confidentiality clauses make it difficult to fully assess the damages that a company may incur. The difficulty is further exacerbated by the nature of product liability laws which generally are brought under state law. The liability, therefore, potentially may vary from state to state, depending on the product liability laws of the jurisdiction.

Although state product liability laws differ, there are similarities in product liability actions. Under most state laws, a consumer will be able to sue a company to recover damages for injuries allegedly caused by food allergens, which, when consumed, have caused severe or life-threatening adverse reactions. These plaintiffs sue under a product liability theory, which refers to a variety of legal claims, including negligence, breach of warranty, and failure to warn. These claims, which are not mutually exclusive, are used by plaintiffs who are seeking damages for injuries suffered because of alleged 'defects' in the goods they have purchased.

20.3.1 Overview of product liability cases and negligence

An entity that markets a product in the US faces potential product liability exposure to the extent that the product causes harm to consumers. Generally, the aggrieved party must bear the burden of proof in demonstrating that the product was 'defective' and that the defect caused the individual's injuries. There are numerous causes of action under which a plaintiff can bring a product liability action, including negligence, breach of express and implied warranty or misrepresentations. In negligence actions, an aggrieved party would need to demonstrate that the company marketing a product failed to meet the standard of care when making and marketing the defective product.[27] In an action alleging breach of express or implied warranty, the aggrieved party alleges that he or she had an express or implied contract with the manufacturer of the allegedly defective product and that the company breached the terms of that contract.

The traditional theories of product liability would present potential hurdles in the instance of undeclared allergens in the food supply. In many instances, companies could allege that they were in fact following standard industry practice and that the injury resulted from no fault of the company. For example, a chocolate manufacturer could claim that it should not be held negligent for selling a chocolate bar that contained trace levels of peanuts in an instance when the company implemented state-of-the-art allergen controls, had data demonstrating that its allergen controls were effective, that it adhered to the allergen controls and that due to an unforeseeable circumstance, a trace level of peanut found its way into the product. Under these circumstances, it could be difficult to hold the food company liable under a theory of negligence or breach of warranty.

20.3.2 Strict liability

The rules changed in the 1950s, and for the first time the courts recognized that a company could and should be held strictly liable for harm caused by its product even in the absence of negligence on the company's part.[28] The theory of strict liability is applied to many industries, including the food industry. Under a theory of strict liability, a plaintiff essentially has to prove one of three key elements (i) there was a mistake in the manufacturing process, (ii) the manufacturer formulated or designed the product improperly, or (iii) the manufacturer placed the product into commerce without adequate warnings. The element that likely will be involved in most allergen cases is the third element regarding the company's failure to provide adequate warnings about the potential danger.

Perhaps most troubling for the food industry is that liability can attach in the absence of any negligence. There are numerous theories of 'strict liability' that can be used by plaintiffs to recover damages even when the defendant is found to have exercised due care in the conduct of his or her business. In most strict liability actions, the issue is not whether the food manufacturer acted negligently, but whether the manufacturer provided its customers with sufficient warnings about the product. For example, in the prior example regarding the chocolate company, although that manufacturer could not be held liable under a theory of negligence, the company could be held liable under a theory of strict liability.

In product liability actions against the food industry, the issue is generally whether the company had a responsibility to provide an adequate warning and, if so, whether the company provided an adequate warning. The Restatement Second of Torts, a treatise that many judges use when determining whether liability should attach in certain instances, specifically addresses the warning requirements for food allergens. The Restatement provides

> In order to prevent the product from being unreasonably dangerous, the seller may be required to give directions or warning, on the container, as to its use. The seller may reasonably assume that those with common allergies, as for example to eggs or strawberries, will be aware of them, and he is not required to warn against them. Where, however, the product contains an ingredient to which a substantial number of the population are allergic, and the ingredient is one whose danger is not generally known, or if known is one which the consumer would reasonably not expect to find in the product, the seller is required to give warning against it, if he has knowledge, or by the application of reasonable, developed human skill and foresight should have knowledge, of the presence of the ingredient and the danger.[29]

The Restatement makes it clear that there will be circumstances in which the seller of foods should provide a warning that the product contains an allergen. It is important to note that the restatement applies to all sellers of

foods and, as such, imposes potential liability on manufacturers of packaged foods as well as to restaurants and others that provide food directly to consumers. A warning is not required under the Restatement when the presence of the allergen is obvious. For example, a restaurant that serves a chef salad with large chunks of egg does not need to provide a warning that the entrée contains eggs because the food-allergic consumer would see the eggs and be aware of their presence.

A warning arises when the presence of an allergen is not obvious to the consumer. First, the Restatement requires a substantial portion of the population to be allergic to the ingredient. It is safe to assume that the Restatement would apply to warnings for the major allergens. It is less clear, however, whether the Restatement would apply to warnings for those foods which are known to be allergenic to only a very limited number of individuals. Unfortunately, there is no guidance in the Restatement or in the case law whether a warning would be required for allergenic ingredients to which very few are allergic. A seller reasonably could argue that a warning is not required to the extent that a consumer has an allergy that affects only a very small number of individuals. There can be no assurance, however, that the courts would agree and the issue ultimately may need to be treated as a factual issue that must be decided by a judge or jury.

If there are a substantial number of people in the population that are allergic to the ingredient, a warning would be required when the consumer reasonably would not expect to find the allergen in the product and the seller has knowledge, or by the application of reasonably developed human skill and foresight, should have knowledge of the presence of the ingredient and the danger. A warning would be required, for example, in instances when a manufacturer develops a new ingredient that contains an allergenic component. For example, if a company develops a new fat substitute from peanut protein, the marketer of this product should warn the peanut-allergic consumer of the presence of the peanut protein. This could be accomplished through the product name (e.g., Acme Peanut Derived Fat Substitute) or through a warning statement on the food label (e.g., Attention Peanut Allergic Community: Acme Fat Substitute is Made from Peanuts), or through an ingredient statement disclosing the presence of peanuts in the product (e.g., Acme Fat Substitute (contains peanuts)).

A warning may also be required to inform a consumer of the presence of an allergen in a food that would not ordinarily be expected to be in such food. For example, assume that a restaurant adds peanut butter to its chili. The restaurant should consider providing notice to consumers even if peanut butter is the 'secret ingredient,' because the consumer would not ordinarily expect to find peanut butter in a chili. In addition, a warning such as 'may contain peanuts' could be required when an allergen becomes a component of food through inadvertent cross-contact.

As of the time of this article, there are few cases that provide any guidance on when a warning should be required. In one case, a plaintiff sued McDonald's

claiming that the restaurant had a duty to warn of the presence of carageenan in its McLean hamburger.[30] McDonald's filed a summary judgment motion requesting that the case be dismissed, in part, because the company made available to consumers upon request brochures that identified the carageenan in the product. The trial court granted the motion without issuing a decision. The plaintiff appealed and the appellate court reversed the trial court decision finding issues of 'material fact upon which reasonable minds could differ.'

While acknowledging that McDonald's listed 'carageenan' as an ingredient in a flier that it made available to consumers, the appellate court noted that the flier 'does not inform the reader that carageenan is derived from seaweed, nor that persons who are allergic to seaweed may experience an adverse reaction to that ingredient.'[31] The court also noted that the plaintiff purchased the sandwich at a drive-through window and there was no evidence as to whether she received a flyer or that it was offered to her at that time. Under the applicable Ohio statute, a jury reasonably could find, for instance, that a manufacturer should have known of a risk to even a small number of consumers. The statute asks

> whether a manufacturer exercising reasonable care would warn of the risk in light of both the *likelihood* and the seriousness of the potential harm. Within this framework, whether the plaintiff's harm was unusual or not would be a factor in calculating whether a manufacturer exercised reasonable care in its decision not to warn. The incidence of the kind of harm at issue in this case is only one factor a jury would consider in finding a duty to warn.[32]

McDonald's also claimed that there was no evidence indicating that the carageenan in its hamburger caused the plaintiff's reaction. The appellate court rejected this line of argument noting that the plaintiff had alleged that the product caused the reaction and that she supplemented this evidence with a note from a treating physician identifying the hamburger as the cause of her reactions. Because the plaintiff offered evidence 'tending to prove causation, the element remains a question for the jury.'[33] Noting that the plaintiff had raised genuine issues of material fact, the appellate court held that it would be up to a jury to decide whether McDonald's should be liable for the plaintiff's harm.

The McDonald's case is recognizably troubling because McDonald's did in fact identify the presence of carageenan as an ingredient in flyers made available to consumers and because carageenan is not known or recognized as an allergen. McDonald's likely reached an out-of-court settlement after losing the appeal. Had the case gone to trial, McDonald's then could have presented evidence establishing that (i) its flyer constituted adequate warning and (ii) that its hamburger did not cause the plaintiff's injury. It would have been up to the judge or jury, however, to decide these issues of fact.

20.3.3 Damages

The failure to provide adequate warnings to allergens exposes the food and restaurant industries to potential product liability lawsuits. Plaintiffs that have suffered injury from consuming the allergen may decide to file lawsuits seeking damages against the food manufacturer or restaurant. Damage awards can include both compensatory damages and punitive damages. Compensatory damages are intended to compensate the consumer for injuries, which may include medical expenses and the amorphous 'pain and suffering.'

A food company or restaurant can also be liable for punitive damages if its failure to warn is judged to be the result of what is known as 'actual malice' or a 'reckless disregard' for the safety of customers. Although the definitions of 'actual malice' and 'reckless disregard' vary by jurisdiction, the general concept describes a situation where the defendant was aware of the dangers posed by his conduct but went ahead anyway. For example, punitive damages might be imposed where a server had serious doubts about the truth of his answer when he told a customer a dish did not contain a particular ingredient, but he chose to ignore those doubts, thereby endangering the customer. Punitive damages also could be awarded, for example, if a company becomes aware that it failed to include eggs in the ingredient statement, but fails to recall the product. Although no cases could be found when a company has been held to pay punitive damages, these are the types of activities that in some jurisdictions may expose a company to a punitive damages award. Importantly, punitive damages could end up being many times the amount of actual damages sustained.

Other damages that should not be overlooked are the adverse publicity, the lost business that may result from a lawsuit, and the legal fees associated with defending the action. It is the potential for adverse publicity that likely is responsible for the large number of cases that are settled out of court. With regard to lost business, in some instances the loss of business has caused companies to go out of business.

20.3.4 Summary of product liability actions

There have been numerous product liability actions in the US resulting from the presence of undeclared allergens in foods. Many of the actions are brought against restaurants because, in many instances, the waiting staff have failed to provide accurate information about the allergens in the food. Other actions have been brought against packaged food manufacturers for marketing products with labels that do not identify all of the allergens. The tables in the appendices track many, although not all, of the product liability actions that have been filed. Appendix I summarizes cases that have been filed involving known allergens. Appendix II summarizes cases involving ingredients that are not known allergens, but nonetheless have been alleged to cause an adverse reaction. A review of the cases reveals that damages can range from zero to well over one million dollars.

20.3.5 Food and restaurant industry responsibility and due diligence

Many of the actions brought against restaurants are the result of misinformation that has been communicated by the waiting staff to the customers. All restaurants should educate their employees about the serious adverse reactions that can result if an individual with a food allergy is exposed to an allergen. The staff must realize that allergic reactions can be severe and in many instances produce life-threatening adverse reactions. If the waiting staff has a better understanding of the severity of the reaction, they be more inclined to take seriously customer comments about allergies to food ingredients.

Restaurants and food service providers should also designate at least one person, preferably a manager, as a 'point person' to respond to customer questions about food allergies. This person should be well-informed of the ingredients that are used to prepare the foods and of the severe reactions that can occur when the food-allergic consumer is exposed to an allergen. The waiting staff must be instructed to direct all questions about food allergies or intolerances to the point person. The point person must be instructed to provide accurate information about the ingredients in the food, even if it means disclosing a secret ingredient.

There are also numerous steps that food companies can take to reduce their risk of liability. First, companies should make certain that the ingredient statement accurately reflects the composition of the product. Companies should also become familiar with the requirements of the Food Allergen Labeling and Consumer Protection Act and with FDA policy on the labeling of allergens. Companies should develop an allergen control policy that identifies the manufacturing, procurement, processing, packaging, labeling, and other issues that will be implemented to address allergens. In instances when inadvertent cross-contact with allergens cannot be controlled through GMPs, companies should use a label statement such as 'may contain ___' with the blank filled in with the major allergen that may be present in the food. This type of warning should provide the food-allergic consumer with adequate notice of the potential for the food to contain an allergen that needs to be avoided.

Regular audits of the labels are advisable to ensure that all ingredients are declared. This can be a daunting task because many food products have labels with 10, 20, 30, or even more ingredients. The finished food manufacturer needs to review carefully the ingredients that it adds to the product as well as the ingredient decks on products that it purchases from outside vendors. This audit should be performed whenever a change is made to the formulation of the product or when new labels are designed.

Companies should also conduct an internal audit of the manufacturing process. The allergenic ingredients in the manufacturing process must be identified and the process should be carefully analyzed to identify any instances where cross-contamination could occur. If the same machinery is used to manufacture products with and without allergenic ingredients, there is a potential for allergen residues to end up in subsequently manufactured product.

There also is a potential for cross-contamination when product lines cross, unless they are covered. In the event that cross-contamination can be avoided by good manufacturing practices, the manufacturer should institute procedures to address the issue. If not, the manufacturer should consider labeling that will inform the consumer of the presence of the allergen, such as 'may contain peanuts.'

Manufacturers should consider whether they will incorporate allergen test kits into their allergen control program. Before using the test kit, however, it is imperative that the company develop a firm policy on how the test kits will be used. This policy should address, for example, whether the test kits will be used to test finished product, products not intended for commercial sale, or the manufacturers' cleaning operation. If the manufacturer tests finished product, it would be advisable to establish procedures that identify when the product will be tested and specify that the product should be retained until the test results are received. The manufacturer should also consider the level of detection of the test kits. While it may be tempting to use the most sensitive method of analysis, there may be little value in using a method that can detect allergenic proteins below one to five parts per million, a level that may very well be well beyond the threshold for a safety concern.

In the event that manufacturers test finished product, find detectable levels of allergenic protein, and allow the products to enter commerce, the analytical results could be made available to regulators or plaintiff lawyers in any product liability action resulting from any consumer harm from the consumption of the product. Although it is difficult to imagine that any reputable company would release products under this factual situation, if a company did find itself in litigation it could be facing not only compensatory but potential punitive damages as well.

Conducting an analysis on the finished product is the best way to ascertain whether a company has adequate cleaning procedures to ensure that allergens do not end up in subsequently manufactured foods. Identification of the allergen in the finished product, however, would preclude the company from introducing that product into interstate commerce. To minimize the adverse consequences of a positive test, companies may want to scale back their batch sizes so that the consequences of a positive test can be minimized. Alternatively, companies could test product that is not intended for introduction into interstate commerce. Other companies may limit allergen testing to cleaning materials (such as push through materials in dry cleaning operations) or swabbing of food contact surfaces.

It could be difficult to protect the confidentiality of the test results by having the allergen test results conducted by an in-house or outside lawyer. While most states will maintain confidentiality of materials developed by lawyers in anticipation of litigation, including analytical results, few jurisdictions may be inclined to extend the protection in an instance when the lawyer routinely analyzes the product and the product is released after the lawyer receives results from the analysis. It could, therefore, be difficult

to maintain the confidentiality of the test kit results by having the analysis performed by an in-house or outside lawyer. A company, therefore, should assume that the allergen analytical results would need to be made available to a plaintiff that is suing the company for injuries resulting from the alleged presence of undeclared allergens in the food.

Allergen test kits provide the industry with a useful tool in controlling the presence of undeclared allergens. Careful thought and consideration, however, must be given to how the company will incorporate the test kits into its overall allergen management program.

20.4 Future trends

The issues presented by allergens in foods are continuing to receive a great deal of attention in the US by regulators and legislators. This issue received the attention of the US Congress and legislation has been passed that requires the declaration of major allergens on food labels by common English names. The legislation directs the FDA to prepare numerous reports on major food allergens. By February 2006, the FDA is required to submit reports that: (i) analyze the ways in which foods are unintentionally contaminated with major food allergens (cross-contact) and estimate how common these practices are in the food industry; (ii) advise whether good manufacturing practices or other methods can be used to reduce or eliminate cross-contact of foods with the major food allergens; (iii) describe the various types of advisory labeling, the conditions of manufacturing that are associated with the various types of advisory labeling, and the extent to which advisory labels are being used on food products; (iv) determine how consumers with food allergies would prefer that information about the risk of cross-contact be communicated on food labels; (v) state the number of inspections of food manufacturing and processing facilities conducted in the previous two years and discuss the findings of these inspections; and (vi) assess the extent to which the Secretary and the food industry have effectively addressed cross-contact issues.

The legislation also requires the FDA, within two years, to issue a proposed rule that would define and permit the use of ‘gluten-free’ on food labels. The reports that must be prepared by the FDA and the mandate to establish gluten labeling will ensure that the FDA will continue to focus its resources on allergen labeling issues.

The FDA will also continue to conduct allergen inspections of the food industry and will take regulatory action when appropriate. Manufacturers that have introduced foods containing undeclared allergens will also continue to face potential recalls.

In the event that consumers are injured by an undeclared allergen in the food, the industry should anticipate potential product liability. One of the unintended consequences of the recent media attention on the legislative and regulatory allergen initiatives is that it increases the awareness in the plaintiff’s

bar of the potential for a new and potentially lucrative cause of action to be brought against the food industry. Because test kits are available to the general public, savvy plaintiff's lawyers could begin testing products for the presence of undeclared allergens and then attempt to bring class action lawsuits against companies marketing products with undeclared allergens. The number of product liability actions brought against the food industry for undeclared allergens may very well increase, unless the industry is able to implement procedures to reduce the release into commerce of products with undeclared allergens.

20.5 Conclusion

The presence of undeclared allergens in the food supply is proving to be an issue that subjects the food industry to potential product liability. Restaurants can reduce their potential liability by educating their waiting staff of the importance of providing consumers with accurate information about products. Restaurants also should consider ways in which they can inform the food-allergic community of the presence of allergens in products where they would not ordinarily be expected.

Manufacturers of finished food can also take numerous steps to reduce the potential regulatory and product liability. The labeling of products with ingredient statements that identify allergens is the best way to minimize product liability. Although there are no magic formulas that will guarantee immunity from liability for an allergic reaction or sensitivity to food, there are numerous steps that can be taken to minimize potential liability.

20.6 Sources of further information and advice

Additional information on the regulatory and legal developments on food allergens can be found on FDA's website at http://vm.cfsan.fda.gov/list.html.

20.7 References

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4. 21 U.S.C. § 101.100(a)(3).
5. 21 C.F.R. § 101.100(a)(3).
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7. 21 U.S.C. § 321(gg); FFDCA § 201(gg).
8. 21 U.S.C. § 321(gg)(2)(A); FFDCA § 201(gg)(2)(A).

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18. 21 U.S.C. § 331(a); FFDCA § 301(a).
19. 21. U.S.C. § 342(a)(4); FFDCA § 402(a)(4).
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23. 21 U.S.C. § 332 (injunctions), § 334 (seizures); FFDCA §§ 302, 304.
24. 21 U.S.C. § 334(h); FFDCA § 304(h).
25. 21 C.F.R. § 7.3(g).
26. 21 C.F.R. § 7.3(g).
27. *Larsen v. General Motors Corp.*, 391 F.2d 495 (8th Cir. 1968).
28. See, e.g., *Greenman v. Yuba Power Products, Inc.* 377 P.2d 897 (Cal. 1962).
29. Comment j to Section 402 of *2 Restatement of the Law 2d, Tort* (1965).
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31. *McDonald's*, 655 N.E. at 443.
32. *McDonald's* 655 N.E. at 444 (*emphasis original).*
33. *McDonald's* 655 N.E. at 444 (*emphasis original).*

Appendix I: Summary of cases involving known allergens

Style of case	Damages	Comments
Thompson vs. East Pacific Enterprises, Inc. *et al*.[1]	-0- Case dismissed	Plaintiff went into anaphylactic shock, suffered respiratory arrest and heart attack. Dish did not contain peanuts; trace amounts of peanut product were present in food as result of cross-contamination. Plaintiff did not inform restaurant of her allergy to peanut products.
Abbhi vs. AMI *et al.*[2]	Not reported	Nine year old child died from anaphylactic shock after eating pastry.
Knight vs. Just Born, Inc. *et al*.[3]	Not reported	Defendants asserted idiosyncratic allergic response defense.
Mitchell vs. Eddie's Place on Chagrin, Inc.[4]	$10 000	Allergic reaction caused anaphylaxis requiring epinephrine shot, resulting in scarring at injection site on leg and emotional distress.
Marshall Randall vs. Trans Pacific Stores, Ltd.[5]	$57 137	Plaintiff regularly bought cookies at store, was told no nuts in cookie. Store substituted new cookie with nuts; had told employee to change label but employee had not done so. Allergic reaction resulted in anaphylactic reaction and angioedema with respiratory failure and acidosis; was unconscious and close to death upon arrival in emergency room and admitted to intensive care unit.
Vosko vs. Wing Hong, Inc.[6]	$8096	Restaurant mistakenly put shrimp, instead of chicken, in egg foo yung. After eating meal, plaintiff took Benadryl and went to ER with symptoms of anaphylactic shock. Was given adrenalin, observed for two hours and released.
Dobeus vs. The Chef's Place, Inc.[7]	$500	Allergic reaction resulted in asthmatic episodes and hospitalization.
Plaintiff vs. Trattoria Spago[8]	$1 000 000	Allergic reaction resulted in stroke with permanent brain lesion and inability to return to work. Had informed waitress of allergy. Restaurant denied that dessert contained nuts.
Bocon vs. Pizzeria Uno, Inc.[9]	-0- Verdict for defense	Plaintiff informed waitress of dairy allergy; after starting to eat, plaintiff developed nausea, diarrhea and vomiting, then went to emergency room with irritable bowel syndrome (IBS). Plaintiff had pre-existing IBS.
McGowan vs. Public House, Inc.[10]	$3500	Plaintiff suffered allergic reaction and emotional distress; was told cookie contained no nuts.
Krueger vs. Camaraderie Restaurant, Inc.[11]	$5000	Allergic reaction caused pain and swelling in face and increased heart rate. Plaintiff alleged that waitress stated that dessert was nut-free.

Appendix I: Continued

Style of case	Damages	Comments
Castiello vs. WLH Management Corp.[12]	$172 500	Anaphylactic attack resulting in permanent asthmatic condition.
Balison vs. JB's Restaurant, Inc.[13]	$12 287	Allergic reaction resulting in anaphylactic shock. Disputed whether plaintiff asked about cooking methods or defendant made representations regarding same.
Ficken vs. Edy's Grand Ice Cream, Inc.[14]	$2654	Allergic reaction resulted in sleeplessness, vomiting, and headaches.
MacLeod vs. Univ. of Mass. Health Services[15]	$350	Student suffered psychological trauma following allergic reaction to mayonnaise on sandwich. $500 award reduced by $150 for contributory negligence for not checking food before eating.
Yost vs. Chapter Eleven Restaurant[16]	-0- Verdict for defense	Allergic reaction resulted in permanent asthmatic condition.
O'Brien vs. Moishs Addison Bakery[17]	$100 000	Plaintiff suffered acute respiratory arrest, anaphylactic shock and temporary coma. Fiancée had been told by sales clerk that there were walnuts, but not peanuts, in brownies.

[1]Unpublished, 2003 WL 352914, Washington Ct of Appeals No. 49924-6-I (Feb. 18, 2003). (Appeal from trial court grant of summary judgment for defendants on all counts; decision affirmed.)
[2]1997 WL 325580, Conn. Superior Court, No. CV-96032195-S (June 3, 1997). (Motion to strike non-products liability claims granted.)
[3]2000 WL 924624, US District Court, Oregon (Mar. 28, 2000). (Cross motions for summary judgment. Summary judgment denied as to manufacturing defect and negligence claims. Summary judgment granted as to failure to warn claim because inconsistent with manufacturing defect claim.)
[4]Settlement, Cuyahoga County, Ohio, Court of Common Pleas, No. 396582 (Oct. 17, 2000).
[5]Unpublished Jury verdict, San Francisco County, Cal., No. 995772 (Sept. 10, 1999).
[6]Unpublished, Jury verdict, Oakland County, Michigan Circuit Court.
[7]Unpublished Settlement, Lake County, Illinois Circuit Court, No. 96-AR-2078 (Nov. 1997).
[8]Unpublished Settlement, LA County Superior Court, No. BC-105-476 (April 1996).
[9]Unpublished Jury verdict, Norfolk County, Mass. Superior Court, No. 94-494 (Sept. 25, 1995).
[10]Unpublished Settlement, Worcester County, Mass. Superior Court, No. 93-0252-A (Feb. 1995).
[11]Unpublished Settlement, Eau Claire County, Wis. Circuit Court, No. N/A (Feb. 1994).
[12]Unpublished Settlement, York County, Maine Superior Court, No. CV-92-610 (Dec. 1993).
[13]Unpublished Settlement, Kootenai County, Idaho District Court, No. 88757 (March 1993).
[14]Unpublished Verdict, Jackson County, Mo. Circuit Court, No. CV-90-22738 (Sept. 1992).
[15]Unpublished Verdict, Hampshire County, Mass. Superior Court, No. 87-173 (Feb. 1990).
[16]Unpublished Verdict, Spokane County, Wash. Superior Court, No. 85-2-01946-1 (Oct. 1988).
[17]Unpublished Settlement, Philadelphia County, Penn., No. 1996-12-918 (1999).

Appendix II: Summary of cases involving ingredients that are not allergens

Style of case	Damages	Comments
Livingston vs. Marie Callendar's Inc.[1]	Not reported	Plaintiff suffered MSG Symptom Complex including respiratory arrest, hypoxia, cardiac arrest and brain damage after eating soup containing MSG. Waitress had told plaintiff that soup did not contain MSG.
Brown vs. McDonald's Corp.[2]	Not reported	Carageenan is seaweed-derived product to which people allergic to seafood may have an allergic reaction. After eating hamburger plaintiff developed rash, tight chest blue lips and hives, required five-hour hospital stay.
Trapnell vs. Sysco Food Services, Inc. *et al.*[3]	Not reported	Chronic asthmatic died after eating food containing sulfites. Decedent had been told that no sulfites had been used in the food.
McPike vs. Enciso's Cocina Mejicana, Inc.[4]	-0- Verdict for defense	Ten year old asthmatic girl died after allergic reaction to sulfite in guacamole.
Caravia vs. Sbarros Licensing Inc.[5]	$1 500 000	Female died after asthma attack caused by sulfites in salad. Jury found defendant 60% liable and decedent 40% negligent.

[1]85 Cal.Rptr.2d 528, Cal. Court of Appeal, 2d District, No. B-115078, June 3, 1999. (Appeal from trial court dismissal of strict liability claim; Reversed and remanded.)
[2]655 N.E.2d 440, Ohio App. 1995, No. 94-CA-005904. (Appeal from trial court grant of summary judgment for defendants; affirmed in part and reversed in part; Case remanded to trial court.)
[3]850 S.W.2d 529, Tex. App. 1992, 890 S.W.2d 796, Tex. 1994. (Appeal from trial court grant of summary judgment. Reversed by Court of Appeals; reversal affirmed by Texas Supreme Court. Case remanded.)
[4]762 P.2d 315, Oregon Ct. Appeals 1988. (Appeal from jury verdict for defendants; affirmed.)
[5]Unpublished Jury verdict, Broward County, Fla. Circuit Court, No. 86-31404 (Sept. 1990).

21

EU regulation of undeclared allergens in food products

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21.1 Introduction

The European Union promotes the free movement of goods between Member States through the EC Treaty, which prohibits quantitative restrictions on imports between them (Article 28). This principle of free movement was affirmed in the *Cassis de Dijon* case.[1]

In the late 1990s, the BSE crisis led to major changes in the regulatory regime for foodstuffs. The White Paper on Food Safety, presented by the Commission, stated that 'assuring that the EU has the highest standards of food safety is a key policy priority for the Commission,' and that 'a radical new approach is proposed.' The White Paper recommended the establishment of an independent European Food Authority to address the need to guarantee a high level of food safety. A range of other measures aimed at improving and standardising legislation covering all aspects of food products, from 'farm to table', is needed to support this recommendation.

The supporting measures proposed by the Commission are presented in the Annex, with an indication of priority and likely timing. The 'Action Plan on Food Safety' (Action Number 3) proposes a General Food Law Directive. The objectives are to establish food safety as the primary objective of EU food law and to lay down the common principles underlying food legislation, in particular the scientific basis for legislation, responsibility of producers and suppliers, traceability along the food chain, efficient controls and effective enforcement. Action Number 16 concerns a proposal to amend the existing directive on the labelling, presentation and advertising of foodstuffs. The objectives of this action are to ensure that the components of compound ingredients forming less than 25% of the final product are indicated, and to

lay down a list of allergenic substances. The objectives of Action Number 3 were laid down in 2002 in Regulation 178/2002/EC,[2] and those of Number 16 in 2000, in Directive 2000/13/EC.[3] Recently, this directive has been amended by Directive 2003/89/EC,[4] which introduces the obligation to indicate a number of allergen ingredients on the label.

Regulation 258/97/EEC[5] concerns the placement of novel foods or novel food ingredients on the market within the Community. Foods and ingredients are novel if they have not *hitherto been used for human consumption to a significant degree within the Community*. Those responsible for placing novel foods on the market must submit a request to the Member State where the product is to be introduced.

The European Commission also has a number of legal instruments concerned with compensation for victims of defective products, including the Product Liability Directive, Directive 85/374/EEC,[6] and the General Product Safety Directive, Directive 92/59/EEC.[7] The Product Liability Directive was amended by Directive 1999/34/EC[8] to include primary agricultural products. The General Product Safety Directive was repealed by Directive 2001/95/EC.[9] On the basis of these instruments, producers are obliged to place only safe (food) products on the market, and are liable for damage caused by any defective (food) products.

21.2 Food legislation concerning the labelling of ingredients

Directive 2000/13/EC[10] addresses the labelling, presentation and advertising of foodstuffs, and has superseded the older Directive, 79/112/EEC.[11] The major elements of the new directive are:

- 'the need to inform and to protect the consumer';
- 'to enact Community rules of a general nature applicable horizontally to all foodstuffs put on the market'; and
- to promote the use of 'detailed labelling, in particular giving the exact nature and characteristics of the product, which enables the consumer to make his choice in full knowledge of the facts'.

The most important requirements of this Directive are discussed below.

21.2.1 Definitions

Labelling shall mean any words, particulars, trade marks, brand name, pictorial matter or symbol relating to a foodstuff and placed on any packaging, document, notice, label, ring or collar accompanying or referring to such foodstuff. Pre-packaged foodstuffs are those where the contents cannot be altered without opening or changing the packaging.

21.2.2 Misleading information

One of the most important requirements is that the labelling and methods used must not mislead the purchaser. Foodstuffs should not be labelled, presented or displayed in a way that suggests they possess properties or special characteristics that are either not present, or are in fact typical for all similar foodstuffs.

21.2.3 Information required on the label

The following particulars are compulsory on the labels of foodstuffs:

(1) the name under which the product is sold;
(2) the list of ingredients;
(3) the quantity of certain ingredients or categories of ingredients;
(4) in the case of pre-packaged foodstuffs, the net quantity;
(5) the date of minimum durability or, in the case of foodstuffs that are highly perishable (microbiologically), the 'use by' date;
(6) any special storage conditions or conditions of use;
(7) the name or business name and address of the manufacturer or packager, or of a seller established within the Community;
(8) particulars of the place of origin or provenance where failure to give such particulars might mislead the consumer to a material degree as to the true origin or provenance of the foodstuff;
(9) instructions for use when it would be impossible to make appropriate use of the foodstuff in the absence of such instructions; and
(10) the actual alcoholic strength by volume for beverages containing more than 1.2% by volume of alcohol.

21.2.4 Name

The name under which a foodstuff is sold shall be the name provided for in the Community provisions applicable to it or, where there are no Community provisions, the name provided for in the laws, regulations and administrative provisions applicable in the Member State in which the product is sold. Failing this, the name or description of the foodstuff and, if necessary, its use must be clear enough to let the purchaser know its true nature and distinguish it from other products.

21.2.5 List of ingredients

An ingredient is any substance, including additives, used in the manufacture or preparation of a foodstuff and still present in the finished product, even if in an altered form. If an ingredient of the foodstuff is itself the product of several ingredients, the latter shall be regarded as ingredients of the foodstuff in question. Additives that serve no technological function in the finished product are not ingredients, nor are solvents, media or other processing aids.

In general, the list of ingredients shall include all the ingredients of the foodstuff, in descending order of weight, as recorded at the time of their use in the manufacture of the foodstuff. Exceptions include added water and volatile products, ingredients used in concentrated or dehydrated form, concentrated or dehydrated foods, mixtures of spices or herbs, and ingredients constituting less than 2% of the finished product (may be listed in a different order after the other ingredients). Ingredients may be listed by their specific name, or by category name where applicable (see Annex I of the Directive). Additives that belong to one of the categories listed in Annex II of the Directive must be designated by the name of that category, followed by their specific name or EC number. Flavourings shall be designated in accordance with the rules laid down in Annex III of the Directive. There are some cases where ingredients do not need to be listed, for instance single-ingredient foodstuffs such as fresh fruit and vegetables, carbonated water, fermentation vinegars, cheese, butter, fermented milk and cream.

21.2.6 Quantitative ingredients declaration (QUID) regulation

The QUID regulation specifies the circumstances under which the quantity of an ingredient or category of ingredients used in the manufacture or preparation of a foodstuff must be stated. The quantity indicated, expressed as a percentage, must correspond to the quantity of the ingredient or ingredients at the time of its/their use, and must appear either in or immediately next to the name under which the foodstuff is sold, or in the list of ingredients in connection with the ingredient or category of ingredients in question.

21.2.7 Net quantity

The net quantity of pre-packaged foodstuffs must be expressed in units of volume in the case of liquids and in units of mass in the case of other products, using the litre, centilitre, millilitre, kilogram or gram, as appropriate. Additional regulations concern other aspects of labelling net quantity, such as the weight of pre-packaged items that are sold in combination, foodstuffs that are normally sold by number, and use of drained net weight for foodstuffs normally sold in a liquid medium.

21.2.8 Date of minimum durability

The date of minimum durability of a foodstuff shall be the date until which the foodstuff retains its specific properties when properly stored. The date shall be preceded by the words: ‘Best before …’ when the date includes an indication of the day, and ‘Best before end …’ in other cases. Either the date itself or a reference to where the date is given on the labelling shall accompany these words. If need be, these particulars shall be followed by a description of the storage conditions which must be observed if the product is to keep for

the specified period. The date of minimum durability does not apply to all foodstuffs. In the case of highly perishable foodstuffs, which are likely to constitute an immediate danger to human health after a short period, the date of minimum durability is replaced by the 'use by' date.

21.2.9 Instructions for use

Instructions for use of a foodstuff shall be indicated so that it is used appropriately.

21.2.10 Foodstuffs without pre-packaging

The Member State itself shall adopt detailed rules concerning the labelling of foodstuffs offered for sale to the ultimate consumer or to mass caterers without pre-packaging, or where foodstuffs are packaged on the sales premises at the consumer's request or pre-packaged for direct sale.

21.2.11 Language

The information on the label should be written in a language easily understood by the consumer, unless the consumer is informed by other means. Member States may specify the language(s) used to label products marketed within their own territory, in accordance with the rules of the Treaty.

21.3 Food legislation concerning the labelling of allergens

Directive 2003/89/EC[12] amends Directive 2000/13/EC[13] concerning the information requirements for foodstuffs, and is designed to achieve a high level of health protection for consumers and to guarantee their right to information through accurate labelling of ingredients. The amendment includes the requirement to label all the ingredients, additives, processing aids and other substances that may cause allergic or intolerance reactions in consumers.

21.3.1 Considerations

Allergic or intolerance reactions constitute a danger to the health of the consumer. The Scientific Committee on Food has stated that food allergies affect the lives of many people, and reactions range from very mild to potentially fatal. Common food allergens include cows' milk, fruits, legumes, especially peanuts and soybeans, eggs, crustaceans, tree nuts, fish, vegetables (celery and other foods of the Umbelliferae family), wheat and other cereals. The most common food allergens are found in a wide variety of processed foods,

so labelling rules are necessary to ensure that consumers suffering from food allergies receive appropriate information.

A list of allergenic substances has been drafted, and includes those foodstuffs, ingredients and other substances recognised as causing hypersensitivity. The list of allergens may be revised if new allergens are identified, or if technological developments permit the removal of allergenicity in ingredients and other substances, in order to avoid unnecessary obligations on labelling.

21.3.2 List of allergenic ingredients

Annex IIIa shows a list of allergenic substances that must be indicated in the list of ingredients, and includes:

- cereals containing gluten (i.e. wheat, rye, barley, oats, spelt, kamut or their hybridised strains) and products thereof;
- crustaceans and products thereof;
- eggs and products thereof;
- fish and products thereof;
- peanuts and products thereof;
- soybeans and products thereof;
- milk and products thereof (including lactose);
- nuts, i.e. almond (*Amygdalus communis* L.), hazelnut (*Corylus avellana*), walnut (*Juglans regia*), cashew (*Anacardium occidentale*), pecan nut (*Carya illinoesnis (Wangenh.) K. Koch*), Brazil nut (*Bertholletia excelsa*), pistachio nut (*Pistacia vera*), macadamia nut and Queensland nut (*Macadamia ternifolia*) and products thereof;
- celery and products thereof;
- mustard and products thereof;
- sesame seeds and products thereof; and
- sulphur dioxide and sulphites at concentrations of more than 10 mg/kg or 10 mg/litre expressed as SO_2.

The words 'allergenic ingredient(s)' and 'allergenic substance(s)' mean an allergenic substance(s) named in Annex IIIa, and include the substance itself and also the products thereof. There are other foodstuffs that can cause adverse reactions, but on the basis of this Directive, the presence of such an ingredient or product thereof in a foodstuff does not have to be indicated on the label. However, the Directive does indicate that it is important to be able to revise the list of ingredients rapidly, when necessary by including or deleting certain ingredients or substances. Recently the Directive 2005/26/EC[14] has established a list of food ingredients or substances which are provisionally excluded from Annex IIIa of Directive 2000/13/EC (see Table 21.1). These ingredients or substances will be excluded from this annex until 25 November 2007 and so do not have to be indicated on the label.

Table 21.1 List of food ingredients and substances provisionally excluded from Annex IIIa of Directive 2000/13/EC

Ingredients	Products thereof provisionally excluded
Cereals containing gluten	Wheat-based glucose syrups including dextrose[1] Wheat-based maltodextrins[1] Glucose syrups based on barley Cereals used in distillates for spirits
Eggs	Lysozyme (produced from egg) used in wine Albumin (produced from egg) used as fining agent in wine and cider
Fish	Fish gelatine used as carrier for vitamins and flavours Fish gelatine or isinglass used as fining agent in beer, cider and wine
Soybean	Fully refined soybean oil and fat (1) Natural mixed tocopherols (E306), natural D-alpha tocopherol, natural D-alpha tocopherol acetate, natural D-alpha tocopherol succinate from soybean sources Vegetable oils derived phytosterols and phytosterol esters from soybean sources Plant stanol ester produced from vegetable oil sterols from soybean sources
Milk	Whey used in distillates for spirits Lactitol Milk (casein) products used as fining agents in cider and wines
Nuts	Nuts used in distillates for spirits Nuts (almonds, walnuts) used (as flavour) in spirits
Celery	Celery leaf and seed oil Celery seed oleoresin
Mustard	Mustard oil Mustard seed oil Mustard seed oleoresin

[1]And products thereof, in so far as the process that they have undergone is not likely to increase the level of allergenicity assessed by the European Food Safety Authority for the relevant product from which they originated

21.3.3 Labelling of allergenic ingredients of beverages containing more than 1.2% by volume of alcohol

Any ingredient listed in Annex IIIa shall be indicated on the labelling where it is present in the beverage. This indication shall comprise the word 'contains' followed by the name of the ingredient(s) concerned. However, an indication is not necessary when the ingredient is already included under its specific name in the list of ingredients or in the name under which the beverage is sold. When necessary the EU can adopt detailed rules for the presentation of the indication.

21.3.4 Labelling of allergenic ingredients in other foodstuffs

Any ingredient listed in Annex IIIa or originating from an ingredient listed in Annex IIIa that is used in production of a foodstuff and still present in the

finished product, even in altered form, must be indicated on the label with a clear reference to the name of the ingredient. This means that any allergenic ingredient used in production and present in the foodstuff has to be indicated on the label even if a list of ingredients is not needed. This requirement also applies to the ingredients of a compound ingredient, food additives, a component of a food additive, flavourings, a component of a flavouring, processing aids, and carriers, solvents or media for additives, flavours or processing aids. Indication is not required if the name under which the foodstuff is sold clearly refers to the ingredient concerned.

21.3.5 Method of labelling an allergenic ingredient

The directive states that 'the ingredient shall be indicated on the label with a clear reference to the name of this ingredient,' and that 'the indication shall not be required if the name under which the foodstuff is sold clearly refers to the ingredient concerned.' This open formulation for labelling allows some leeway in deciding whether an indication is needed, the text of the indication itself and positioning on the label. In practice, it can lead to differences regarding the labelling of allergenic substances, and it is crucial that the considerations described above are taken into account when drawing up the indication. It is also important to assess the knowledge of consumers, because, for example, there are consumers who do not know that yoghurt is made of (cows') milk.

21.3.6 Indication and labelling of an allergenic ingredient

If a list of ingredients is not needed for a particular foodstuff, the allergenic ingredient should be labelled as 'Contains' followed by the name of the allergenic substance. If a list of ingredients is required, then the presence of allergenic ingredient(s) can be stated in the list of ingredients. The allergenic substance should be stated after the ingredient concerned, preceded by the word 'contains', and followed by the name of the allergenic substance, e.g. Ingredients: yoghurt, contains milk, and so on. A second possibility is to indicate the presence of an allergen in a sentence under the list of ingredients, e.g. 'This foodstuff contains the allergenic ingredients' followed by the name of the allergenic substance, or 'Contains' followed by the name of the allergenic substance. In these cases, it is advisable that the allergenic substance(s) in the list of ingredients and in the sentence under the list are joined, e.g. by a character or figure between brackets in front of or after the allergenic ingredient concerned.

This Directive has to be implemented in accordance with the associated national laws of each Member State, and, under the Directive, it is possible for a Member State to define another system of indication and/or wording for labelling allergenic substances in foodstuffs. For example the Netherlands describes coercively the labelling of allergenic ingredient(s) in the list.

21.3.7 Guidance documents

Since publication of the Directive, organisations in the food industry have set up guidelines for the practical application of the new labelling rules with respect to ingredients and allergens. These guidelines do not have legal status.

In the Netherlands, the 'Centraal Bureau Levensmiddelenhandel' and 'Federatie Nederlandse Levensmiddelen Industrie (FNLI) have drawn up 'Wijziging van de etiketteringvoorschriften ingevolge de publicatie van RICHTLIJN 2003/89/EG VAN HET EUROPEES PARLEMENT EN DE RAAD van 10 November 2003 tot wijziging van Richtlijn 2000/13/EG met betrekking tot de vermelding van de ingrediënten van levensmiddelen; Een Handleiding'.

The Confederation of the Food and Drink Industries of the EU (CIAA) developed the 'Final Guidance Document on the practical application of Directive 2003/89/EC on the ingredient and allergen labelling'.

21.3.8 Limits of detection, standards

The directive does not include any limit of detection or standard. If an allergenic ingredient has been used in the preparation of a foodstuff, it should be labelled. If it is not used but is present, then it does not have to be labelled.

21.4 Legislation concerning general product safety (Directive 2001/95/EC)

Directive 2001/95/EC[15] superseded Directive 92/59/EEC[16] pertaining to the safety of products and ensuring that products placed on the market are safe. The most important requirements of this Directive are discussed below.

21.4.1 Considerations

Where Member States have different legislation on product safety, this not only affects the levels of protection provided to consumers, but also tends to create barriers to trade and distortion of competition within the European Community market. The Community aims to protect the health and safety of consumers, and Community-wide legislation introducing general product safety requirements and containing provisions on the general obligations of producers and distributors should contribute to that aim. Because it is very difficult to adopt Community-wide legislation for every product, there is a need for a broad-based, legislative framework that establishes general safety requirements for any product.

21.4.2 Product definitions

A product is defined as any product, including the provision of a service, which is intended for consumers or likely, under reasonably foreseeable conditions, to be used by consumers even if not intended for them, and is supplied or made available, whether for consideration or not, in the course of a commercial activity, and whether new, used or reconditioned.

A 'safe product' is defined as any product which, under normal or reasonably foreseeable conditions of use including duration and, where applicable, putting into service, installation and maintenance requirements, does not present any risk or only the minimum risks compatible with the product's use, considered to be acceptable and consistent with a high level of protection for the safety and health of persons.

Important aspects of this definition for foodstuffs are:

- the characteristics of the product, including its composition, packaging, instructions for assembly and, where applicable, for installation and maintenance;
- the presentation of the product, the labelling, any warnings and instructions for its use and disposal and any other indication or information regarding the product; and
- the categories of consumers at risk when using the product, in particular children and the elderly.

A 'dangerous product' means any product, which does not meet the definition of 'safe product'. The feasibility of obtaining higher levels of safety or the availability of other products presenting a lesser degree of risk shall not constitute grounds for considering a product to be 'dangerous'.

21.4.3 General safety requirement, conformity assessment criteria and European standards

Producers shall be obliged to place only safe products on the market. A product shall be deemed safe, when, in the absence of specific Community provisions governing the safety of the product in question, it conforms to the specific rules under national law of the Member State in whose territory the product is marketed. A product shall be presumed safe as far as the risks and risk categories covered by relevant national standards are concerned when it conforms to voluntary national standards in place of European standards.

21.4.4 Other obligations of producers and distributors

Within the limits of their respective activities, producers shall provide consumers with information that enables them to assess the risks inherent in a product throughout the normal or reasonably foreseeable period of its use, and precautions against those risks. This obligation applies if such risks are not immediately obvious without adequate warnings.

21.4.5 Product recall

A recall means any measure aimed at achieving the return of a dangerous product that has already been supplied or made available to consumers by the producer or distributor. The producer is obliged to monitor the products made, and producers and distributors are obliged to recall products from the market, and consumers, if they present risks. Member States shall ensure that producers and distributors comply with their obligations under the directive in such a way that products placed on the market are safe. Governments can oblige producers and distributors to carry out a product recall.

21.4.6 Guide to corrective action including recalls

Intertek Research and Testing Centre published 'Product safety in Europe, A guide to corrective action including recalls' on behalf of the UK Consumers Association. This is a voluntary guide to carrying out corrective actions regarding product safety, and covers all types of corrective action aimed at removing a safety risk arising from a non-food product placed on the market. It also gives information for corrective actions concerning foodstuffs if they pose a risk. The text is available on the Internet (see Section 21.9).

21.5 Legislation concerning food safety (Regulation (EC) No. 2002/178/EC)[17]

This regulation provides the basis for assuring a high level of protection of human health and consumer interests in relation to food. The regulation lays down the general principles and requirements of food law, establishes the European Food Safety Authority and defines procedures in matters of food safety.

21.5.1 Considerations

The free movement of safe and wholesome food can be achieved only if food and feed safety requirements do not differ significantly from Member State to Member State. Experience has shown that measures are needed to guarantee that unsafe food is not placed on the market, and that systems exist to identify and respond to food safety problems. To ensure the safety of food, all aspects of the food production chain must be considered, from and including primary production and the production of animal feed, up to and including sale or supply of food to the consumer, because each element may have a potential impact on food safety. Community policies aim to ensure a high level of protection of human life and health, and Community food policies form a common basis for measures governing food and feed taken by individual Member States and at Community level.

21.5.2 Definition of food

Food or foodstuff means any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be, ingested by humans. Food includes drink, chewing gum and any substance, intentionally incorporated into the food during its manufacture, preparation or treatment. It includes water. Food shall not include feed, live animals unless they are prepared for placing on the market for human consumption, plants prior to harvesting, medicinal products, cosmetics, tobacco and tobacco products, narcotic or psychotropic substances, residues and contaminants.

21.5.3 Food safety requirements

Unsafe foodstuffs must not be placed on the market. Foodstuffs are deemed unsafe if they are considered to be injurious to health or unfit for human consumption. In determining whether any food is unsafe, normal conditions of use of the food by the consumer and at each stage of production, processing and distribution, and all information provided to the consumer should be taken into account. In determining whether any food is injurious to health, the probable immediate and/or short-term and/or long-term effects of that food on the health of a person consuming it (and subsequent generations), the probable cumulative toxic effects, and the particular health sensitivities of a specific category of consumers should be taken into account.

21.5.4 Responsibilities of food and feed business operators

Food and feed business operators at all stages of production, processing and distribution within the businesses under their control shall ensure that foods or feeds satisfy the requirements of food law that are relevant to their activities and shall verify that such requirements are met.

21.5.5 Traceability

Food and any other substance intended to be, or expected to be, incorporated into a food shall be traceable through all stages of production, processing and distribution. Traceability is of great importance for product liability. In the case a defective product, the plaintiff can make each producer in the chain liable, from raw material to end-product. Producers therefore need to have a traceability system in place that enables them to check whether the cause of a defective product lies in their own factory, is due to a defective raw material, or arose later with a user in the chain. In the latter two cases the producer can take recourse against the supplier or user. Food which is placed on the market or is likely to be placed on the market in the Community shall be adequately labelled or identified to facilitate its traceability, through relevant documentation or information.

21.5.6 Responsibilities of food business operators for non-compliant foodstuffs

If a food business operator has reason to believe that a food which they have imported, produced, processed, manufactured or distributed is not in compliance with food safety requirements, then they must immediately initiate procedures to withdraw the food, whether from other food business operators or from the consumers themselves. Food business operators are also obliged to inform and collaborate with the relevant authorities when a food has been recalled, and provide reasons for withdrawal to consumers, where applicable. Food business operators that are not producers also have obligations in the withdrawal of unsafe foods, including passing on information necessary to trace a food, and co-operating in the actions taken by producers, processors, manufacturers and/or the competent authorities. Food business operators shall not prevent or discourage any person from co-operating, in accordance with national law and legal practice, with the competent authorities, where this may prevent, reduce or eliminate a risk arising from a food.

21.5.7 Liability

The provisions stated in Chapter 2 of the Regulation (EC) No 2002/178/EC concerning General Requirements of Food Law, Food Safety Requirements, Responsibilities and Traceability, shall be without prejudice to Directive 85/374/EEC[18] concerning liability for defective products. This means that the rules concerning Food Safety Requirements, Responsibilities and Traceability, and those concerning defective products, exist side by side.

21.6 Legislation concerning product liability

The liability for defective products in the European Community is laid down in Directive 85/374/EEC[19] and is based on strict liability for the producer, 'liability without fault'. Each Member State also has national rules concerning liability, which are generally based on fault and can be used if the case meets conditions laid down in the national rules.

21.6.1 Considerations

Different laws among Member States concerning the liability of the producer for damage caused by a defective product may distort competition and affect the free movement of goods. National laws also result in differing levels of consumer protection against damage to health or property caused by a defective product. Standardisation of laws across the Community is not and cannot be total, but this Directive lays down the principle of strict liability for the producer. Even in the absence of fault, the producer may be held responsible for damage caused by a defective product that the producer has put on the market.

21.6.2 Optional possibilities for Member States

The Directive contains three optional possibilities for Member States. Firstly, that in some cases a producer shall not be liable if he proves that the state of scientific and technical knowledge at the time when the product was put into circulation was such that the existence of the defect could not be discovered. Secondly, that a Member State might limit total liability for damage, so that damage resulting from death or personal injury and caused by identical items with the same defect can be limited to an amount not less than 70 million ECU (Euro 70 million). Thirdly, that a Member State might legislate that the term 'product' also includes agricultural products and game. The Member States Greece, France, Luxembourg, Finland and Sweden used this option, but it has now been abrogated by the implementation of Directive 1999/34/EC,[20] which was aimed at restoring consumer confidence in the safety of agricultural products following the BSE crisis.

21.6.3 Requirements for liability

Article 1 lays down the requirements for a producer to be liable for a defective product. The four requirements are:

- the person claimed is the producer;
- there is damage;
- the product is defective; and
- there is a causal relationship between the defect and the damage.

The four requirements, opportunities for defence, and the limitation or expiry period are discussed below.

21.6.4 Producer

On the basis of Article 3, 'Producer' means the manufacturer of a finished product, the producer of any raw material or the manufacturer of a component part and any person who, by putting his name, trademark or other distinguishing feature on the product presents himself as its producer. In addition, any person who imports a product into the Community for sale, hire, lease or any form of distribution in the course of his business shall be deemed to be a producer within the meaning of the Directive and shall be responsible as a producer. The producers are liable jointly and severally, and the victim of damage may make a claim against any of these persons for compensation, whether they are the actual producer or not. If the producer of the product cannot be identified, each supplier of the product shall be treated as its producer unless he informs the injured person, within a reasonable time, of the identity of the producer or of the person who supplied him with the product.

21.6.5 Damage

The scope of the damage to be compensated is restricted according to Article 9, to include damage caused by death or by personal injuries, and damage to, or destruction of, any item of property other than the defective product itself, with a lower threshold of 500 ECU (Euro 500). The item of property should be of a type ordinarily intended for private use or consumption. This directive does not cover damage to professional property, where national laws concerning liability are applicable. National laws are also needed for damage that is not covered by the scope of product liability, such as the lower threshold of Euro 500. As described above, damage resulting from death or personal injury and caused by identical items with the same defect can be limited to an amount not less than 70 million ECU (Euro 70 million).

21.6.6 Defective product

A product is defined in Article 2 and described in Section 21.4.2. For the purpose of this Directive, 'product' also means all movables even if incorporated into another movable or into an immovable, and foodstuffs are products in the sentence of this directive.

Article 6 shows the requirements for a defective product. A product is defective when it does not provide the safety which a person is entitled to expect, taking all circumstances into account, including:

- the presentation of the product;
- the use to which it could reasonably be expected that the product would be put; and
- the time when the product was put into circulation.

The presentation of the product includes the way the product is advertised, as well as the information concerning the product on the label, and instructions and directions for use. All these have to point out the dangers and risks of using the product, and the consumer may expect that there are no other dangers and risks than those indicated.

The producer has to take into account intended and reasonably expected use of the product, and allow that, in practice, a user will not take all safety and precautionary measures. The safety standards in force at the time when the product was put into circulation are deciding factors in qualifying the product as defective or not.

The production chain comprises the stages a product passes through on its route from raw material to finished consumer product. Typically a product could go from the raw material producer to a processor to be combined with other raw materials into a semi-product before passing to a manufacturer who might combine several semi-products into an end product for sale and delivery to the consumer. It is generally accepted that each time a raw material, semi-product or end product moves along the chain it has been 'put into circulation'. Hence the same commodity can be put into circulation several

times during the production and distribution process. It is only when the commodity is in circulation (and if it is defective) that the producer will be liable. During storage and processing the product is deemed not to be in circulation and, based on the legislation concerning product liability, the producer cannot be liable. However, producer liability is possible based on other legislation such as, for example, labour laws. Processing aids are not deemed to have been put into circulation if they are used for the preparation of foodstuffs, but only if they have been passed along the chain.

The expectations of the consumer play an important part in determining whether a product is defective or not. Hence other circumstances under which a product is considered defective relate to the nature of the product (for instance, a medicine has a different nature to a foodstuff), the advantages and the usefulness of the product, the chance or frequency of appearance of the danger, the appearance of the harmful additional workings, and the knowableness of the danger. A product is not considered defective if there is a mere lack of conformity to specifications, stemming from regulations or from a contract, and the safety of the product is not at stake.

The cause of a product defect can be reduced to faults in production, where there are three kinds of faults.

- **Design defects**. This kind of fault leads to categorical defects, and therefore to a series of defective products. The fault only can be removed by a new or changed design.
- **Manufacturing faults**. This kind of fault occurs during production, and results in one or more products which deviate from other products made according to the same specifications. Producers have a duty to check products against specifications and ensure that defective products are not put into circulation.
- **Inadequate instructions or warnings**. If the instructions or warnings included in the advertisement of the product, on the label, in the instructions and on the directions for use do not give any or enough information about the safe use of the product, then they are inadequate.

The appearance of one of these faults does not mean that the product is defective, unless the safety of the product is at stake.

Products may become unsafe after they are put into circulation, which is not covered by this Directive. If a product becomes defective after being put into circulation, the claim for damages must comply with national rules based on fault. The producer has a duty of care as laid down in Directive 2001/95/EC[21], and must monitor products accordingly, warning consumers if the product, or its use, becomes unsafe.

21.6.7 Causal relationship

The plaintiff has to prove the damage, that the product is defective, and the causal relationship between the defect and the damage. In practice, this is

very difficult for the plaintiff to prove, and the Directive does not allow a conversion of the burden of proof. But it is within the scope of the Directive for a judge to charge the producer with contradicting the thesis of the plaintiff, if the plaintiff has provided a sufficient part of the proof.

21.6.8 Means of defence

There are a number of means for disclaiming liability, although all are very difficult to fulfil in practice. Article 7 lays down that a producer shall not be liable if they prove the existence of one or more of the following circumstances.

- The producer charged did not put the product into circulation, e.g. if the product was stolen from the factory site, or the product has been counterfeited.
- It is probable that the defect which caused the damage did not exist at the time when the product was put into circulation, or that this defect came into being afterwards. Wear of a product does not make it a defective product.
- The product was neither manufactured by the producer for sale or any form of distribution for economic purpose, nor manufactured or distributed by the producer in the course of business. Both conditions must be fulfilled for a successful appeal.
- The defect is due to compliance with mandatory regulations issued by the public authorities. In this case, the government has prescribed the composition or labelling, or certain conditions needed for permissibility and approval. In the case of a successful appeal, the product is still a defective product but the producer is not liable for it.
- The state of scientific and technical knowledge at the time when the product was put into circulation was not such as to enable the existence of the defect to be discovered. As described earlier, each Member State is permitted to disallow this means of defence. In practice, an appeal under this provision is not likely to be successful. The producer has a duty to research the objective state of scientific and technological knowledge at the time when the product was put into circulation.
- In the case of a manufacturer of a component, that the defect is attributable to the design of the product into which the component has been fitted, or to the instructions given by the manufacturer of the product.

21.6.9 Limitation period

Article 10 states that a limitation period of three years shall apply to proceedings for the recovery of damages as provided for in this Directive. The limitation period shall begin to run from the day on which the plaintiff became aware, or should reasonably have become aware, of the damage, the defect and the identity of the producer. These conditions are cumulative. The laws of the

Member States concerning suspension and interruption are unhindered by these provisions.

21.6.10 Period of expiry

The period of expiry is laid down in Article 11, 'the rights conferred upon the injured person pursuant to this Directive shall be extinguished upon the expiry of a certain period.' This period is ten years from the date on which the producer put the actual product which caused the damage into circulation, unless the injured person has in the meantime instituted proceedings against the producer.

21.6.11 Product recall

Rules concerning product recall are laid down in Directive 2001/95/EC,[22] which has repealed Directive 92/59/EEC.[23] Regulation 178/2002/EC [24] also shows rules concerning recalls

21.7 Key issues in labelling of allergens, undeclared allergens, food safety and product liability

21.7.1 Possibilities leading to liability under amended Directive 2000/13/EC

There are a number of possibilities that can lead to a producer being liable for a defective product when it contains one or more allergenic ingredients. Those that come under the scope of amended Directive 2000/13/EC[25] include:

- an allergenic ingredient is listed in Annex IIIa of the amended Directive 2000/13/EC[26] and has been consciously used in production and is still present in a foodstuff but has not been indicated on the label.
- an allergenic ingredient is listed in Annex IIIa of the amended Directive 2000/13/EC[27] and has been used in production and is still present in a foodstuff, and has been indicated on the label.

These situations are discussed further within the framework of Directive 2001/95/EC,[28] Regulation 178/2002/EC,[29] Directive 85/374/EEC,[30] Directive 2000/13/EC[31] and Directive 2003/89/EC[32].

Directive 2001/95/EC[33]

Product definitions

Foodstuffs with one or more allergenic ingredients are products for the purpose of this directive, under the definitions listed in Section 21.4.2. A foodstuff containing one or more allergenic ingredients which are not indicated on the label is not a safe product under the definitions listed in Section 21.4.2.

Important considerations for foodstuffs containing one or more allergenic ingredients are:

- the characteristics of the product, including its composition, packaging, instructions for assembly and, where applicable, for installation and maintenance;
- the presentation of the product, the labelling, any warnings and instructions for its use and disposal and any other indication or information regarding the product;
- the categories of consumers at risk when using the product, in particular children and the elderly.

A foodstuff which contains one or more allergenic ingredients that are not indicated on the label will be considered a dangerous product.

General safety requirement, conformity assessment criteria and European standards
There are specific Community provisions governing product safety for foodstuffs containing one or more allergenic ingredients (Section 21.4.3). A foodstuff containing allergenic ingredients which are not indicated on the label is not a safe food and may not be placed on the market.

Other obligations of producers and obligations of distributors
Other obligations (Section 21.4.4) apply to foodstuffs containing allergenic ingredients because they present a risk to some consumers.

Product recall
Producers of foodstuffs have an obligation to monitor their products and to recall products from the market if they present risks (Section 21.4.5). If an allergenic ingredient is not indicated on the label of a foodstuff, its presence forms a risk for some consumers and hence the product is dangerous. Once the producer is aware of this risk, the foodstuff must be withdrawn from the market. Producers and distributors are also obliged to recall dangerous products from consumers. In some cases, other appropriate actions may include adequately and effectively warning consumers. Governments can oblige producers and distributors to recall a product, from the market and/or consumers.

Regulation 178/2002/EC[34]
Definition of food
Foodstuffs with one or more allergenic ingredients belong to food under the definition of food for this regulation (Section 21.5.2).

Food safety requirements
Under the definition of unsafe foods (Section 21.5.3), foodstuffs with allergenic ingredients that are not indicated on the label are unsafe for a specific category of consumers and should not be placed on the market.

Responsibilities of food and feed business operators
Producers of foodstuffs must ensure that the foodstuffs do not contain allergenic ingredients if these are not indicated on the label (Section 21.5.4).

Traceability
Producers of all foodstuffs, with and without allergenic ingredients, indicated on the label or not, must meet traceability requirements as listed in Section 21.5.5

Responsibilities of food business operators for non-compliant foodstuffs
Producers of foodstuffs with allergenic ingredients which are not indicated have the ultimate responsibility for placing safe foods on the market (Section 21.5.6). They are obliged to withdraw such foodstuffs as soon as possible after they become aware of requirements to indicate the allergenic ingredients.

Directive 85/374/EEC[35]
Requirements for liability
The producer shall be liable for damage caused by a defect in his product under the requirements described in Section 21.6.3.

Producer
The definition 'Producer' as described in Section 21.6.4 applies to the producer of a foodstuff containing an allergenic ingredient that is not indicated on the label.

Product
Foodstuffs with and without allergenic ingredients are products in the sentence of this directive (Section 21.6.6).

Damage
A plaintiff can claim damage as described in Section 21.6.5.

Defective product
A foodstuff containing an allergenic ingredient is a defective product if the allergenic ingredient is not indicated on the label, because the allergenic ingredient presents a risk to some consumers (Section 21.6.6). A product containing an allergenic ingredient may also be defective if there are faults during production, such as defects in the design of the product and the label. If the instructions or warning concerning the indication on the label do not give enough or any information about one or more allergenic ingredients, then the warnings or instructions are inadequate.

Causal relationship
The plaintiff has to prove the damage, that the product is defective, and the causal relationship between the defect and the damage (Section 21.6.7). The

proof of the damage and the defect will be relatively straightforward. The causal relationship will be more difficult to prove, because it will be possible that the plaintiff will have consumed other foodstuffs containing the same allergenic ingredients.

Means of defence
There are a number of means of defence for a producer to prove that he is not liable (Section 21.6.8). But, in the case of foodstuffs containing allergenic ingredients, the defect which caused the damage will have existed at the time when the product was put into circulation. In addition, it will be clear that the product was manufactured for sale and distribution by the producer, that the defect is not due to compliance with mandatory regulations issued by the public authorities, that the state of scientific and technical knowledge at the time when the product was put into circulation made it possible to discover the existence of the defect, and that the producer is not the manufacturer of a component. The defect is therefore attributable to the design of the product made by the producer.

Limitation period
The limitation period of three years applies in the case of foodstuffs containing allergens (Section 21.6.9).

Period of expiry
The 10-year period of expiry applies in the case of foodstuffs containing allergens (Section 21.6.10).

Directive 2000/13/EC[36] *and Directive 2003/89/EC*[37]
Definitions
The indication of the presence of an allergenic ingredient in a foodstuff comes under the requirements for labelling (Section 21.2.1).

Misleading information
Omitting to indicate the presence of allergenic ingredients could be qualified as misleading because it concerns the characteristics of the foodstuff (Section 21.2.2).

Information on the label
The following particulars on the labelling of foodstuffs concern allergenic ingredients (Section 21.2.3):

- the name under which the product is sold;
- the list of ingredients; and
- instructions for use when it would be impossible to make appropriate use of the foodstuff in the absence of such instructions.

An allergenic ingredient must be indicated in order to comply with the requirements.

Name of the foodstuff
A foodstuff may be sold under a name which clearly refers to the allergenic ingredient. If this is the case, further indication is not needed (Section 21.2.4).

List of ingredients
Ingredients must be indicated under the requirements to list ingredients (Section 21.2.5). In addition to this, allergic ingredients which are listed in the Annex to Directive 2003/89/EC must be indicated in a prescribed manner on the label (Sections 21.2.5 and 21.3.2).

Labelling of allergenic ingredients of beverages containing more than 1.2% by volume of alcohol
If a foodstuff is a beverage and one or more allergenic ingredients are not indicated, then the labelling does not meet the requirements (Section 21.3.3).

Labelling of allergenic ingredients of other foodstuffs
Any allergenic ingredient used in production and present in the foodstuff must be indicated on the label even if a list of ingredients is not needed (Section 21.3.4). The lack of an indication does not meet the requirements. If the name under which the foodstuff is sold clearly refers to the ingredient concerned, the indication is not required.

Conditions for labelling an allergenic ingredient
The Directive has open specifications for indication, stating that 'the ingredient shall be indicated on the label with a clear reference to the name of this ingredient', and that 'the indication shall not be required if the name under which the foodstuff is sold clearly refers to the ingredient concerned' (Section 21.3.5). But if a required indication is missing, then the label does not meet the requirements.

Researching the need to indicate an allergenic ingredient
The producer is expected to have researched the need to indicate any allergenic ingredients in their products (Section 21.3.2). If the producer has judged incorrectly that an indication is not needed, whereas in fact it is required, then the labelling will not meet the requirements.

QUID regulation
The QUID regulation is relevant if the quantity of an allergenic ingredient is such that it must be stated (Section 21.2.6).

Net quantity
The requirements concerning the net quantity are not relevant for this subject (Section 21.2.7)

Date of minimum durability
The requirements concerning the date of minimum durability are not relevant for this subject (Section 21.2.8).

Instructions for use
The presence of one or more allergenic ingredients listed in Annex IIIa has to be indicated under instructions for use requirements (Sections 21.2.5 and 21.3.2).

Language of the information on the label
Language requirements for labels apply to foodstuffs containing allergenic ingredients (Section 21.2.11).

21.7.2 Other possibilities for liability

There are other possibilities for liability. If there is no legal requirement to indicate the allergenic ingredient, then the producer may indicate the presence of an allergenic ingredient by the indication 'contains' or 'may contain'. Examples include:

- an allergenic ingredient is not (yet) listed in Annex IIIa of the amended Directive 2000/13/EC,[38] has been consciously used in production and is still present in a foodstuff. This ingredient has not been indicated on the label because listing it is not mandatory.
- an allergenic ingredient, which may or may not be listed in Annex IIIa of the amended Directive 2000/13/EC,[39] has not been used in production of a foodstuff, but it is present because of cross-contamination. It has not been indicated on the label, but it is known or one could reasonably expect that this contamination could occur.
- an allergenic ingredient, which may or may not be listed in Annex IIIa of the amended Directive 2000/13/EC,[40] has not been used in production of a foodstuff, but it is present because of cross-contamination. It has not been indicated on the label and one could not reasonably expect that this contamination should occur.

In these instances, the claim of the plaintiff has to be based on national rules based on fault, and the plaintiff has to prove the fault. The circumstances have to be taken into account, such as the prevailing knowledge about the danger of allergenic ingredients in general, and the specific knowledge regarding the particular allergenic ingredient concerned. An increasing awareness of the dangers of allergenic ingredients increases the responsibility of the producer.

21.8 Future trends

21.8.1 Labelling in general

Additional information will be required on the label, e.g. information about nutritional facts. Currently, including nutritional information is voluntary, but if a producer makes a claim such as 'light', then supporting information must be provided on the label. The type and content of nutritional and health claims will be regulated in the near future.

21.8.2 Labelling of allergens

At present there are 12 allergens that must be labelled according Directive 2003/89/EC. However, there are other ingredients used in foods that can cause allergies or intolerances in consumers which do not come under mandatory labelling requirements. In order to protect consumers, it is anticipated that additional allergens will be listed in the future. The opposite also applies, in that ingredients which have been scientifically established not to cause adverse reactions will be deleted from the list of allergens.

21.8.3 General product safety

Public authorities can oblige producers (and distributors) to carry out a product recall, from the market and from consumers where necessary, on the basis of Directive 92/59/EEC[41]. An extension of this obligation may be to authorise consumers to obtain injunctions, in order to have dangerous products be withdrawn from the market.

21.8.4 Product liability

The supplier of foods may be assimilated with the producer where product liability is concerned. At present, the liability of a supplier can only be established based on fault under national law. The strict liability regime may also be extended to cover damages to professional products.

21.9 Sources of further information and advice

21.9.1 Additional information on regulatory and legal developments

Additional information on the regulatory and legal developments on labelling of foods, labelling of allergens, product liability and product safety can be found at the following web-sites:

- CBL
  Centraal Bureau Levensmiddelenhandel
  http://www.cbl.nl/

- CIAA
  Confédération des Industries Agro-Alimentaires de l'EU
  http://www.ciaa.be/
- European Community
  http://europa.eu.int/eur-lex/nl/index.html
- FNLI
  Federatie Nederlandse Levensmiddelen Industrie
  http://www.fnli.nl/
- TNO Quality of Life
  http://www.voeding.tno.nl/
- VAI
  Nederlandse Voedingsmiddelen Industrie
  http://www.vai-voeding.nl/
- **Europe**
  — Labelling
  http://europa.eu.int/comm/food/food/labellingnutrition/foodlabelling/index_en.htm
  — Consumer affairs/product safety/product liability
  http://europa.eu.int/comm/consumers/index_en.htm
  — Public health
  http://europa.eu.int/comm/health/horiz_publications_en.htm
  — European Food Safety Authority (EFSA)
  http://www.efsa.eu.int/index_en.html

21.9.2 Tabling of the main legislation of the European Union concerning establishing community, product liability, labelling and product safety

- EC Treaty[42]
  Treaty establishing the European Community.
- Directive 79/112/EEC[43]
  Council Directive 79/112/EEC of 18 December 1978 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. This directive has been repealed by Directive 2000/13/EC,[44] so it is no longer in force.
- Directive 85/374/EEC[45]
  Council Directive of 25 July 1985 on the approximation of the laws, regulations and administrative provisions of the Member States concerning liability for defective products (85/374/EEC).
- Directive 92/59/EEC[46]
  Council Directive 92/59/EEC of 29 June 1992 on general product safety. This directive has been repealed by Directive 2001/95/EC,[47] so it is no longer in force.
- Directive 1999/34/EC[48]
  Directive 1999/34/EC of the European Parliament and of the council of

10 May 1999 amending Council Directive 85/374/EEC on the approximation of the laws, regulations and administrative provisions of the Member States concerning liability for defective products.

- Directive 2000/13/EC[49]
  Directive 2000/13/EC of the European Parliament and the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs.
- Directive 2001/95/EC[50]
  Directive 2001/95/EC of the European Parliament and of the Council of 3 December 2001 on general product safety.
- Regulation 178/2002/EC, General Food Law or GFL[51]
  Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.
- Directive 2003/89/EC[52]
  Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs,
- White Paper food safety
  White Paper on Food Safety, COM (1999) 719 final, Brussels, 12.1.2000.

21.10 References

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3. *OJEC* 2000 L109/29
4. *OJEC* 2003 L308/15
5. *OJEC* 1997 L043/1
6. *OJEC* 1985 L210/29
7. *OJEC* 1992 L228/24
8. *OJEC* 1999 L141/20
9. *OJEC* 2001 L011/4
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Conclusions

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22.1 Recent literature and trends

While this book was in production, several new articles were published concerning allergen detection methods. Some methods for other allergenic foods were developed as were several novel experimental approaches for detection of allergenic residues or markers of allergenic residue. The majority of the new publications dealt with validation trials of commercially available test kits and the optimizing recoveries for these tests. We believe that all these developments are important and we hereunder summarize the main conclusions of the papers published in 2004–05 on allergen detection methods.

Several methods have been developed for allergenic foods that could previously not be detected. A sandwich enzyme-linked immunosorbent assay (ELISA) for lupin (Holden *et al*., 2005) utilizing polyclonal antibodies was reported to have a 1 ppm detection limit, with high specificity for lupin and only minor cross-reactivities for other legumes. Recoveries in spiking studies were 60–115% in various food matrices, but model foods were not utilized in this study. Additionally some polymerase chain reaction (PCR) methods for some allergens not previously described were published. A PCR method for mackerel contamination was developed by Aranishi and Okimoto (2004). Another one for crustacean shellfish was described by Brzezinski (2005), but it had a reported detection limit for shrimp of <0.1%, which is not sensitive enough for allergen concerns. Also, a buckwheat PCR method was described by Hirao *et al*. (2005) and had a detection limit of 1 ppm (w/w) of buckwheat DNA spiked in wheat DNA. Before these newly developed methods can be applied on a routine basis in the food industry, the methods should be validated in terms of precision, recovery, sensitivity and specificity, etc.

Such validation studies have been undertaken recently for some allergen detection kits and several publications became available in 2005:

- A validation trial designed by the U.S. Food and Drug Administration (Park *et al*., 2005) utilized three laboratories in a performance validation of three commercial peanut ELISA kits at 0 and 5 ppm to be used for screening purposes using peanut butter spiked into breakfast cereal, cookies, ice cream, and milk chocolate spiked at 0 and 5 ppm peanut. They found when two validated kits are used in parallel, the probability of detecting 5 ppm is >95% peanut protein in four different food matrixes.
- Another interlaboratory trial of five commercial ELISA kits for peanut in biscuits (cookies) and dark chocolate model foods containing peanut at 0–10 ppm was published by Poms *et al*. (2005). They found that, in general, these five quantitative kits performed better in the 5–10 ppm range than at lower ranges of 2–2.5 ppm, but in some cases, 2 ppm was below the official detection limit of the kit, so the authors were pushing the technology in some cases beyond what the kit was designed to do. Impressively, 34 laboratories participated in this trial, and results were obtained from 12–14 laboratories for each commercial kit. For the biscuits (cookies), peanut flour was mixed and then baked in household-size portions and equipment. For dark chocolate, peanut-free chocolate was melted at 60 °C and then peanut flour was mixed in. Average recoveries varied with all types of test kits and matrices, and both the type of kit and the matrix seemed to be important for recovery.
- More work has been performed on interlaboratory validations for allergen detection methods. Akiyama *et al*. (2005) described two interlaboratory trials on peanut and buckwheat ELISAs: extracts of biscuit, sauce, chocolate and butter spiked with peanut standard protein at a level of 5–20 ng/mL were found to be reproducible in the study. Mean recoveries of the peanut standard protein from the food extracts were over 40% using two ELISA methods. The detection limits of both ELISA methods were 2–2.5 ng/mL in sample solutions. Inter-laboratory evaluation studies were conducted for the ELISA methods for buckwheat. Extracts of snacks, buns and udon noodles spiked with buckwheat protein at a level of 5–20 ng/mL as sample solutions were analyzed in replicate at 10 laboratories. Mean recoveries of the buckwheat standard protein from the food extracts were over 40% in the two ELISA methods. The detection limits of both ELISA buckwheat methods were 1 ng/mL.

With the data of the validation studies available, we believe that the detection and quantification of peanut residue in food products, as desired by the food industry and food regulators, is possible for the majority of food products. One should, however, always be aware of specific situations that may disturb proper analysis. Recovery from the food matrix may be the most important issue here, and some food matrices are particularly notorious. Chocolate, for example, is a difficult matrix to extract peanut residue from. Akkerdaas *et al*.

(2004) designed a protocol to improve the extraction of hazelnut allergens from dark chocolate. This assay took advantage of the protein-solubilizing properties of pepsin, a proteolytic enzyme. Pepsin-digested hazelnut was used to make polyclonal antibodies. The reported detection limit was 1 ppm and good recovery (97%) was obtained even from a normally difficult dark chocolate matrix. The study employed model foods manufactured to contain different amounts of hazelnut. The requirement to digest the food matrix for each individual sample with pepsin makes it impractical to use in a food production setting in most cases, however.

In addition to new methods and validation studies of commercial test kits, we observed several trends in the detection of allergenic residues. Stephan *et al*. (2004) found that the use of celery PCR and a peanut ELISA, together with more inexpensive total protein measurement techniques, to detect protein traces can be a fast and cost-effective method for monitoring the effectiveness of wet cleaning procedures. Regulatory agencies and some food companies could make use of multiple allergen detection in one assay; one paper by Ben Rejeb *et al*. (2005) described a competitive multi-assay for peanut, hazelnut, almond, cashew and Brazil nuts in a single run. The assay was adapted for screening purposes applied to chocolate samples. The limit of detection was reported to be 1 ppm. Another interesting approach was to use a lipid transfer protein (LTP), a highly conserved protein present in plants, to design a pan-allergen ELISA (Zuidmeer *et al*., 2005); a single sandwich ELISA method can be used to detect LTP from apple, cherry, nectarine, and hazelnut. However, this might not be practical for food companies that are only interested in one of these allergenic substances (example hazelnut) in the presence of other LTP-containing ingredients in the food matrix (example apple). Nevertheless, such approaches can be further explored for allergens from sources that are closely related such as fish or crustaceans.

There may be novel methods in the future for the quick and reliable assessment of the content of allergenic residue in food, either based on immunochemical methods, DNA techniques, or based on an entirely new methodology. One unconventional new approach to peanut PCR was described by Sforza *et al*. (2005): circular dichroism on directly stained peanut DNA amplified by PCR. The detection limit was very low, reported to be a few picomoles. However, both the low detection limit and the technical aspects of the method do not make it very practical for the food industry to use. We should be aware, though, of emerging technologies that are complementary to or may replace conventional methods in the future.

22.2 Relating detection limits to clinically relevant doses

Some state and national regulatory agencies have used allergen detection methods to investigate consumer complaints from food-allergic consumers. The availability of methods for allergenic residue detection will make

investigation of consumer complaints and gathering of data easier for risk assessment for all of these stakeholders, but the reliability of some analytical results may be questioned until methods are validated and collaboratively studied. In exquisitely allergic individuals, the threshold dose for elicitation of such reactions is often considered to be zero. However, some food-allergic patients report that they can tolerate small quantities of allergenic food. Studies covered in the various chapters in this book include some that have used allergen detection methods to discover that some packaged food products contain undeclared allergen residues at levels that could be potentially hazardous to allergic individuals. These findings suggest that residues do exist in retail food products, with no report of allergic reactions. This could be because either the eliciting doses are higher than the levels present or perhaps that food-allergic individuals do not buy certain products that they feel are vulnerable to contamination with undeclared allergenic food residues. Some uncertainty is likely to remain regarding whether the threshold dose has been determined for the most sensitive individuals. Clinicians will never be completely certain that the most sensitive individual has been identified and tested. However, an analogy can be drawn to the infant formula industry where hypoallergenic infant formula based upon extensively hydrolyzed casein is safe for the vast majority of milk-allergic infants. Yet, hypoallergenic infant formula is known to elicit adverse reactions in a very small number of milk-allergic infants. This analogy illustrates the difficulty in developing threshold doses for all allergic consumers. Instead, the goal should be to identify threshold doses that protect the vast majority of allergic consumers. Allergic individuals can (and probably do) ingest foods, on occasion, containing lower amounts of their offending food without any adverse reactions.

Threshold doses are currently reported to be in the low mg range, with subjective (not observable) symptoms reported in some studies of peanut to be 100 ug. As discussed in this book, detection limits of current commercial methods (1–2.5 ppm, or 1–2.5 mg/kg) for residues of allergenic foods appear to be in the ideal range to detect potentially hazardous residues of undeclared allergens in foods. Some noteworthy publications over the years have suggested that detection methods be 5–10 ppm, and that advice remains fairly sound now that more evidence exists on threshold doses. The development of analytical procedures that are more sensitive than the current ones does not seem to be justified clinically. The ideal range of sensitivity of detection methods for allergenic residues should be driven by knowledge of minimal eliciting dose levels. Good manufacturing practices in the food industry and regulatory limits imposed by governmental agencies should also be based on minimal eliciting doses. The goal should be to protect allergic consumers while allowing them the greatest variety of foods in their diets rather than the detection of ever-vanishing levels of allergenic food residues that have no adverse health consequences to even the most sensitive consumers. Use of low detection limit methods may actually contribute to unjustified proliferation of ‘may contain’-type labeling and therefore decrease the quality of life for food-

allergic consumers. An increase in 'may contain'-type labeling may also contribute to food-allergic consumers being driven to exhibit risky behavior (especially teenagers, risk-takers by nature), and make dangerous choices to disregard this type of labeling.

22.3 Reference materials, extraction and recovery

Short-term needs in the allergen detection area include the critical need for reference materials and validation trials, and also the development of assays for some commonly allergenic foods for which none exist. Any method used for the detection of allergens in foods needs to be validated for its sensitivity, specificity, and detectability in the food matrix of interest to guarantee its applicability. In many studies, no information on the allergenic source material used to spike or even to make model foods is provided. Even in validation trials that have already been published, little or no detail is given on the nature of the material used to make the spiked or model (manufactured to contain specific amounts of allergen) matrices. It is therefore difficult to make a true comparison between one kit and another. Currently, efforts to generate such reference materials are in progress at various centers. The use of standard reference materials is necessary to make the results of methods comparable and to be able to attain reproducible interlaboratory results. The development of quantitative allergenic methods can still be challenging due to the lack of suitable standard reference materials.

In addition, simple 'spiking', i.e. putting a peanut extract into a food extract, or dry milk into a dry food powder, is no longer considered appropriate in assessing the performance of allergen detection methods, except when investigating possible matrix interference purposes. The true extraction efficiency of a method and the true recovery from a food matrix can only be ascertained with the use of model foods. The development and production of such reference materials for the analysis of food allergens face several challenges, and is it not practical to make model foods for every matrix. While efforts to make model foods should be applauded, making them in a home kitchen or on a small scale is of limited use, as they do not mimic true processing conditions or practical situations. Making model foods according to pilot plant or true industrial conditions is not necessarily an easy task, but some research groups are doing work in this area, and these materials will be key in the evaluation of allergen detection methods.

22.4 Developing realistic and practical detection methods

Before 1998, no commercial methods were available for the detection of allergenic residues in foods or on food processing equipment. The methodology

for detection of allergenic residues in foods improved with the advent of ELISA and PCR methods. As new methods have been developed and commercialized, the food industry has increasingly used them. However, there is a lack of easy-to-use methods for some of the most commonly allergic foods (for example crustaceans and fish). For the majority of users (food companies), useful methods will include rapid, simple (easy-to-use) kits, with on-(production) line monitoring. Rapid ELISAs and lateral flow devices/dipsticks will, therefore, continue to be the most-used format in the food industry for some time. Complicated, long extraction protocols and lengthy methods are not workable. Recombinant antibodies are starting to be used in developing ELISA methods for phytotoxin analysis, and the ability to engineer specificity of antibodies may offer unique opportunities in the future in allergenic residue detection. PCR does not seem to be practical for analysis on food production lines, whereas lateral flow devices/dipsticks are favored because of easier and faster use. However, other techniques such as PCR can be very useful for regulatory agencies, companies which possess the appropriate equipment and space, or through contract laboratories in providing additional information about a sample. In addition, newer methods such as surface plasmon resonance (SPR) on automated platforms can provide rapid, efficient analysis of large quantities of samples without direct supervision, and might therefore be feasible in some production settings. Other technologies may become more feasible in the future. An example is microfluidic separation where it is anticipated that separation devices may eventually be handheld. The more recent developments in assay technologies are promising for rapid and multiple analysis for allergen detection in food in the future. However, at the present time, these techniques are impractical for use by the food industry. The food industry's need for multiplex methodologies remains to be fully met.

22.5 Summary

The unintended presence of allergenic residue in foods is, and will be in the future, an issue that affects the food allergic consumer, and thereby the food industry. Data on threshold levels of an allergen to elicit an allergic reaction in food-allergic patients are available for a selected number of allergens, and based on these threshold levels, a sensible detection limit for food allergen detection assays can be set. We propose that this detection limit should be between 1 and 10 ppm, providing that the recovery of the analytical procedure is at least 50% for the food products where the test is applied, and preferably higher. Since the early 1990s many methods have been published to detect allergenic residue in food and food ingredients. Some assays have been validated in multi-laboratory trials (peanut) and have been shown to be useful for assessing the amount of allergenic residue in foods. For some allergens

other than peanut such validation studies are in progress, and for other allergens, methods need to optimized before validation studies can be considered.

Trace contamination can arise from practices in the food industry, restaurants, and in homes and schools. Food-service employees and food processors alike must be very vigilant about the use of shared utensils and equipment. Most of the deaths from inadvertent exposure to allergenic foods have occurred in food-service situations (restaurants, schools, homes). However, in many countries, food-service establishments do not have specific regulations about handling or labeling allergens. The increased demand among the general consuming public for a wide variety of food products, and the ever-increasing consumption of food prepared outside the home, can assist in producing reactions to hidden food allergens. While the majority of reactions occur after ingestion of a food that is prepared in a food-service environment rather than packaged food products, the food processing industry has been subjected to increasing scrutiny of its allergen controls. The resulting impact has been remarkable: the food industry has made significant investment, effort and improvements in allergen control in recent years. Food processors often use shared equipment and facilities for the production of processed foods with a variety of formulations. This is often out of necessity rather than just fueled by economic reasons. Due to various limitations and practices in food processing, foods may occasionally contain trace residues of other foods that may not be declared on the ingredient label. The food industry began investing a significant amount of time, money, and effort to attain better allergen control in manufacturing facilities in the late 1980s. Since the late 1990s, the commercialization of some test methods for the detection of some allergenic foods has allowed food manufacturers to use these tests to create important data to assess and design allergen control strategies and protocols.

Debate and discussion about the effects of processing on allergenicity and allergenic residue detection in foods will be ongoing. In addressing new labeling legislation in the EU and the US, analytical methods are being used to assess allergenicity of some ingredients. However, analysis of some allergen-derived ingredients can pose serious challenges. Problematic ingredients include oils and lecithin due to their oily nature, and also hydrolyzed proteins and residues of allergenic growth substrates in gums, starter cultures, enzymes, etc. Standard ELISAs cannot be used in the case of hydrolyzed/proteolyzed/fermented ingredients and PCR cannot be used to assess how allergenic the ingredient is. Methods involving IgE obtained from individuals allergic to the particular food are the only way to determine allergenicity of these types of ingredients with the exception of oral challenge studies in allergic individuals.

Although there is much work to do to deal with the issue of detecting allergenic residues in food, we believe that the enormous output of many investigators and test kit manufacturers, and the use of test kits by the food industry, has already succeeded in providing better protection for the food-allergic consumer. This is a solid basis for the further development of tools for the detection of allergenic residues.

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