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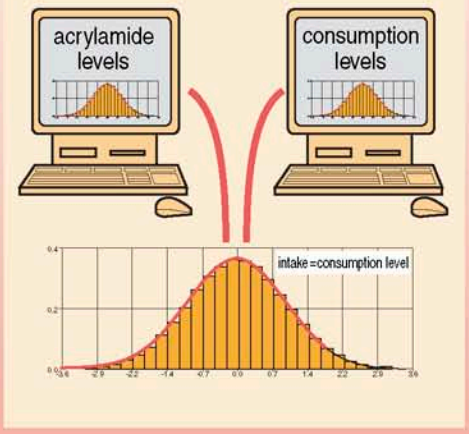

Acrylamide and other hazardous compounds in heat-treated foods

Edited by K. Skog and J. Alexander

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CRC Press
Boca Raton Boston New York Washington, DC

WOODHEAD PUBLISHING LIMITED
Cambridge England

Published by Woodhead Publishing Limited, Abington Hall, Abington,
Cambridge CB1 6AH, England
www.woodheadpublishing.com

Published in North America by CRC Press LLC, 6000 Broken Sound Parkway, NW,
Suite 300, Boca Raton, FL 33487, USA

First published 2006, Woodhead Publishing Limited and CRC Press LLC

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library.

Library of Congress Cataloging in Publication Data
A catalog record for this book is available from the Library of Congress.

Woodhead Publishing Limited ISBN-13: 978-1-84569-011-3 (book)
Woodhead Publishing Limited ISBN-10: 1-84569-011-7 (book)
Woodhead Publishing Limited ISBN-13: 978-1-84569-201-8 (e-book)
Woodhead Publishing Limited ISBN-10: 1-84569-201-2 (e-book)
CRC Press ISBN-13: 978-0-8493-9096-8
CRC Press ISBN-10: 0-8493-9096-6
CRC Press order number: WP9096

The publishers' policy is to use permanent paper from mills that operate a sustainable forestry policy, and which has been manufactured from pulp which is processed using acid-free and elementary chlorine-free practices. Furthermore, the publishers ensure that the text paper and cover board used have met acceptable environmental accreditation standards.

Project managed by Macfarlane Production Services, Dunstable, Bedfordshire, England
(e-mail: macfarl@aol.com)
Typeset by Godiva Publishing Services Ltd, Coventry, West Midlands, England
Printed by TJ International Limited, Padstow, Cornwall, England

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Foreword

Interest in the effects of diet on health has increased in the Western world, and much effort has been devoted to increasing knowledge both of the relationship between food and health and the effects of toxic compounds in our diet. Science involves hard and sometimes frustrating work, but through science long-lasting friendships between people from different scientific disciplines and different parts of the world may be established. With our main interests in food processing and chemistry and food toxicology (Kerstin is the expert in food processing and chemistry and Jan in toxicology), we met almost twenty years ago at a Nordic meeting on cooked food mutagens. We have worked together in two EU-funded projects, 'Heterocyclic amines – role in human health' and 'HEATOX – heat-induced food toxicants, identification, characterisation and risk minimisation'. It is a great pleasure for us to be editors of this book and we would like to thank the scientists with whom we have collaborated in various projects or met at conferences and workshops for their contributions.

Introduction

Kerstin Skog and Jan Alexander

Everybody has a relation to food and our dietary habits are frequently discussed in the media and between people. The human diet provides us with energy and several essential nutrients. It is a complex mixture of compounds, including both protective and harmful components, which may interact with each other or with other food constituents. The cooking process aims at making the food appetising and more easily digestible. In addition, cooking ensures microbiological safety and is important for inactivation of toxic lectins and removal of some toxic compounds such as cyanogenic glycosides. Cooking or heat processing adds aroma and flavour to the food and provides variation in the diet. During heating, several physical and chemical changes take place, such as the change of size or shape, melting of fat, starch gelatinisation, protein denaturation and water evaporation. Heating also causes free amino acids and sugars to react via the Maillard reaction and form a plethora of chemical compounds.

Many Maillard reaction products are important for the sensory properties such as colour, flavour and aroma of the heated products. But some of these compounds may not be beneficial or may even be toxic to humans. Among these are acrylamide and heterocyclic amines (HCAs), which are the main focus of this book. HCAs have been known for many years, whereas the presence of acrylamide, traditionally known as an industrial chemical, in heat-treated food rich in carbohydrates was announced by Swedish researchers just a few years ago. Since then much research has been initiated on its formation and toxicity. Acrylamide is mainly found in coffee, bread and fried potato products. Acrylamide is classified as a probable human carcinogen by the International Agency for Research on Cancer (IARC). Also HCAs may play a role in the aetiology of cancer and the IARC has classified several of these compounds as possible or probable human carcinogens. HCAs are present in meat, fish and

other proteinaceous foods cooked well done. One of the HCAs, PhIP, which occurs at the highest concentrations in cooked food, induces tumours in the prostate gland, breast and large intestine of experimental animals. These are the cancers associated with a so-called Western diet. Even epidemiological studies lend some support to the hypothesis that HCAs might represent a risk for humans consuming large quantities of well-done meat. The IARC recommends a reduced intake of HCAs.

Since compounds, being both carcinogenic and genotoxic, theoretically may represent a risk even at very low exposures, the formation of such compounds during heat processing of food has been the focus of interest. The majority of these compounds, however, need enzymatic bio-activation into reactive metabolites capable of reacting with DNA leading to mutations and cancer. This process occurs in competition with enzymatic detoxication processes both involving a multitude of metabolising enzymes.

One strategy of risk reduction is to reduce human exposure to food-borne acrylamide and HCAs by decreasing their formation in foods. Modification of cooking practices, while still creating tasty meals or products and maintaining microbial safety and acceptable shelf life, or removing meat from our diet to avoid HCAs would not be a good solution, since meat is a nutritious food. Changing cooking practice and food composition to reduce HCAs and acrylamide could potentially lead to an increase of unknown compounds with potential harmful health effects. However, this is not easy to address scientifically. The identification of potential carcinogens from complex food matrices is not a trivial task. It has taken three decades of research by many research groups to isolate, characterise and explore the adverse effects of heterocyclic amines. Thanks to an extremely sensitive bacterial assay containing the right bio-activating enzymes it was possible to find very low levels of heterocyclic amines in cooked meat. The bio-assay directed isolation of these compounds was complicated and time consuming. The same system would, however, not pick up acrylamide since acrylamide is bio-activated by enzymes not present in that system. It is therefore a great challenge to reveal whether hazardous compounds are also present among the thousands of compounds formed during heat processing of food. Obviously, it is not possible to devise a biological screening system to solve this. Other strategies must be applied, such as chemical modelling of compound formation combined with experimentation and screening of the compounds by structure activity analysis coming up with compounds containing structural alerts. The resulting compounds should then be subjected to testing in biological systems. Lots of progress has been achieved in the construction of biological test systems, such as bacteria and cells, which contain human bio-activation enzymes being more relevant for assessing potential risks for humans.

The trend of eating ready-made food products is increasing and consumers who have to rely on industrially prepared foods have the right to demand safe and nutritious food. The choice of cooking/heating method may be used to decrease the formation of heat-induced toxic compounds. There is also a need

for accurate advice on home cooking practices from our food safety authorities. Until more is known about the health risks of heat-induced food toxicants, it is practical to reduce exposure to them. By learning more about reaction mechanisms and conditions in favour of their formation, it may be possible to find strategies to prevent or markedly reduce their presence in our diet. Such precautions are motivated from both food quality and food safety aspects.

This book covers several of these aspects: mechanisms of the formation of hazardous compounds during food processing, ways to reduce the formation, molecular modelling in the identification of toxic compounds, human exposure, biomarkers in humans, toxicological aspects, and risks to humans following exposure from food.

Note: given the lack of a standard nomenclature, the terms heterocyclic amines and heterocyclic aromatic amines, and the abbreviations HCA and HAA, are both used in this book to refer to this class of compounds.

Part I

Formation and analysis of hazardous compounds in heat-treated foods

1

The Maillard reaction and its role in the formation of acrylamide and other potentially hazardous compounds in foods

D. S. Mottram, M. Y. Low and J. S. Elmore, The University of Reading, UK

1.1 Introduction

The Maillard reaction has been recognised for over 60 years as a major route to flavour and browning in cooked foods (Kawamura, 1983). This extremely complex reaction between amino compounds (principally amino acids) and reducing sugars has been the subject of much research by food scientists seeking to identify compounds that provide the flavour and colour characteristics of heated foods (see reviews by Hodge, 1967; Hurrell, 1982; Mauron, 1981; Mottram, 1994; Nursten, 1980, 2005). The reaction has implications in other areas of the food industry, including the deterioration of food during processing and storage (owing to the loss of essential amino acids and other nutrients) and the protective effect of the antioxidant properties of some Maillard reaction products (Nursten, 2005). In recent years the physiological significance of the reaction has been recognised in relation to *in vivo* glycation of proteins and the link to diabetic complications, cardiovascular and other diseases (Ledl and Schleicher, 1990; Nursten, 2005). The possibility of mutagenic compounds being formed in the Maillard reaction has also been recognised for many years and this was given particular attention in the 1980s when carcinogenic heterocyclic aromatic amines were isolated from well-grilled or charred steaks and were shown to derive from Maillard reactions involving amino acids, reducing sugars and creatinine (Negishi *et al.*, 1984). However, in April 2002 the discovery by Tareke *et al.* (2002) of acrylamide (2-propenamide) at concentrations as high as

5 mg/kg in a range of fried and oven-cooked foods, resulted in one of the biggest issues in food science for many years. There was major interest world-wide because acrylamide has been classified as a probable human carcinogen (IARC, 1994) and is also a known human neurotoxin (Friedman, 2003).

It was recognised that acrylamide is formed by the thermal reaction of natural food components and it is only formed in foods cooked at high temperatures, when moisture levels become low. In considering possible mechanisms by which acrylamide may be formed, the only sources of nitrogen for the amide group, which are common to all foods, are proteins and amino acids. This implicated the Maillard reaction, since the amino acids provide the nitrogen for many of the aroma and colour compounds found in baked and roasted foods. Although triacylglycerols were initially suggested as possible precursors for acrylamide, the close similarity in structure between the amino acid asparagine and acrylamide led to the hypothesis that this amino acid was the principal precursor of acrylamide (Mottram *et al.*, 2002). The role of the Maillard reaction involving asparagine as the route by which acrylamide was formed during heating was demonstrated by a number of research groups (Becalski *et al.*, 2003; Mottram *et al.*, 2002; Stadler *et al.*, 2002; Zyzak *et al.*, 2003).

1.2 The chemistry of the Maillard reaction

The formation of colour through the interaction of amino acids with glucose was first recognised by L.C. Maillard in 1912 (Maillard, 1912). However, it was Hodge in 1953 who first attempted to draw up a scheme to explain the essential steps in the complex reaction (Hodge, 1953). It is noteworthy that some 50 years later the Hodge scheme still provides the basis for our understanding of the reaction.

1.2.1 Stages in the Maillard reaction

The scheme devised by Hodge divides the Maillard reaction into three stages. The reaction is initiated by the condensation of the carbonyl group of a reducing sugar with an amino compound (Fig. 1.1) producing a Schiff base. This cyclises to an *N*-substituted aldosylamine if the sugar is an aldosugar. Acid-catalysed

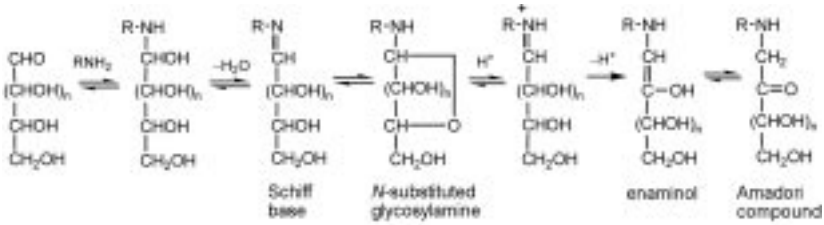

Fig. 1.1 Initial stage of the Maillard reaction.

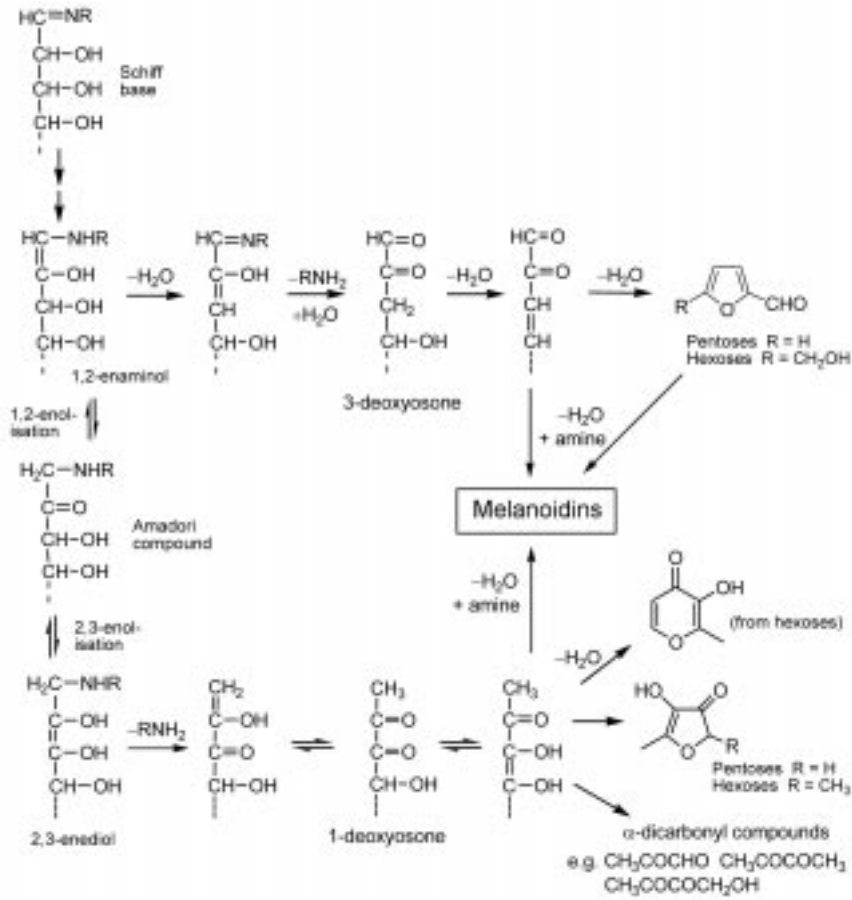

Fig. 1.2 Decomposition of the Amadori compound in the intermediate stages of the Maillard reaction.

rearrangement gives a 1,2-enaminol, which is in equilibrium with its keto tautomer, an *N*-substituted 1-amino-2-deoxyketose, known as an Amadori rearrangement product. Ketosugars, such as fructose, form the Heyns rearrangement product by related pathways.

The Amadori and Heyns rearrangement products are unstable above ambient temperature. They have various keto-enol tautomers, which undergo enolisation, deamination, dehydration, and fragmentation steps giving rise to a collection of sugar dehydration and fragmentation products, containing one or more carbonyl groups, as well as furfurals, furanones and pyranones (Fig. 1.2). In this intermediate stage of the Maillard reaction the amino acid also undergoes deamination and decarboxylation through Strecker degradation (Section 1.2.2). The aldehydes, furfurals and other carbonyls produced at this stage may contribute to flavour characteristics associated with the Maillard reaction.

The products of the initial and intermediate stages of the Maillard reaction are colourless or pale yellow and Hodge attributed colour formation to the final stage of the reaction, where condensation between carbonyls (especially aldehydes) and amines occurs to give high molecular mass, coloured products known as melanoidins. These have been shown to contain heterocyclic ring systems, such as pyrroles, pyridines, pyrazines and imidazoles, but their detailed structures are unknown. The final stage of the reaction is of great importance for flavour formation when carbonyl compounds react with each other, as well as with amino compounds and amino acid degradation products, such as hydrogen sulphide and ammonia. It is these interactions that lead to the formation of flavour compounds, including important heterocyclics, such as pyrazines, pyrroles, furans, oxazoles, thiazoles and thiophenes.

1.2.2 Strecker degradation

An important reaction associated with the Maillard reaction is the Strecker degradation of amino acids (Schonberg *et al.*, 1948; Strecker, 1862). While a large part of the Maillard reaction focuses on the degradation of sugar, initiated or catalysed by amino compounds, Strecker degradation, on the other hand, can be seen as the degradation of α-amino acids initiated by carbonyl compounds. It is usually considered as the reaction between an amino acid and an α-dicarbonyl compound in which the amino acid is decarboxylated and deaminated, yielding an aldehyde, containing one fewer carbon atoms than the original acid (termed a Strecker aldehyde), and an α-aminoketone (Fig. 1.3). However, the reaction need not be restricted to dicarbonyls. Any active carbonyl group which can form a Schiff base with the amino group of an amino acid should, under appropriate conditions, promote the decarboxylation and deamination of an amino acid. Thus, α-hydroxycarbonyls and deoxyosones, formed as Maillard intermediates,

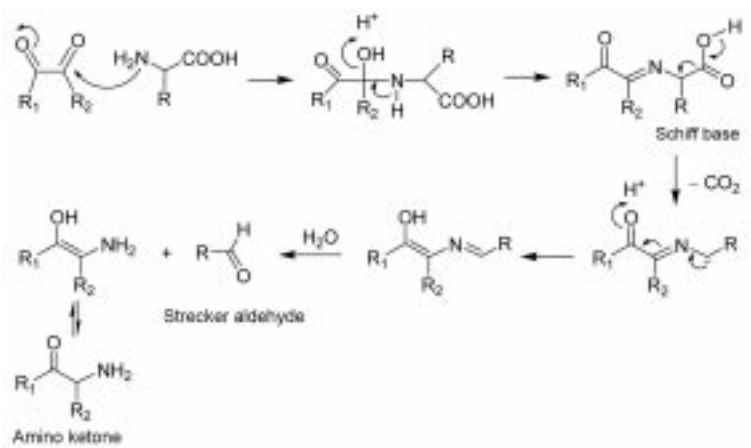

Fig. 1.3 Strecker degradation of amino acids.

as well as dicarbonyls, can act as Strecker reagents and produce Strecker aldehydes. Similarly, it is possible for the Schiff base, formed between a reducing sugar and an amino acid in the initial step of the Maillard reaction, to break down, via a Strecker type reaction, to yield a Strecker aldehyde. Other carbonyl compounds found in foods which could act as Strecker reagents include 2-enals, 2,4-decadienals, and dehydroascorbic acid.

Strecker degradation is very important in flavour generation, as it provides routes by which nitrogen and sulphur can be introduced into heterocyclic compounds in the final stage of the Maillard reaction. The α-aminoketones are key precursors for heterocyclic compounds, such as pyrazines, oxazoles and thiazoles. In the case of alkylpyrazines, the most direct and important route for their formation is thought to be via self-condensation of α-aminoketones, or condensation with other aminoketones (Vernin and Parkanyi, 1982). If the amino acid is cysteine, Strecker degradation can lead to the production of hydrogen sulphide, ammonia and acetaldehyde, while methionine will yield methanethiol. These compounds, together with carbonyl compounds produced in the Maillard reaction, provide intermediates for reactions giving rise to important aroma compounds, including sulphur-containing compounds such as thiophenes, thiazoles, trithiolanes, thianes, thienothiophenes and furanthiols and disulfides (Mottram and Mottram, 2002).

1.3 Acrylamide and the Maillard reaction

Shortly after acrylamide was first reported in carbohydrate-rich heated foods (Tareke *et al.*, 2002), the thermal degradation of free asparagine in the presence of sugars in the Maillard reaction was proposed as the major route for acrylamide formation (Mottram *et al.*, 2002; Stadler *et al.*, 2002). Labelling experiments confirmed that the carbon skeleton of acrylamide and the nitrogen of the amide group derived from asparagine (Zyzak *et al.*, 2003). Suppressing the Maillard reaction would be expected to reduce the levels of acrylamide. However, the Maillard reaction is responsible for the generation of desirable flavours and colours in food, and is indispensable for ensuring the organoleptic quality expected by consumers. An understanding of the relationship between flavour generation and acrylamide production, both mechanistic and kinetic, is required to be able to develop a strategy to minimise acrylamide without adverse effects on the flavour of foods. Being a by-product of the Maillard reaction, acrylamide levels are affected by the same factors that influence flavour and colour formation during heating. These include reactant concentrations (i.e., the reducing sugar and free amino acid content of food), time-temperature conditions during processing, moisture levels, pH, and the presence of additives. Reactant levels are influenced not only by the type of food but also by cultivar, soil conditions, harvesting times, and storage conditions of the raw food.

Proposals for means to lower acrylamide levels in food include using raw products with low sugar or asparagine content (Amrein *et al.*, 2003; Becalski *et*

al., 2004; Biedermann-Brem *et al.*, 2003; Grob *et al.*, 2003; Haase *et al.*, 2003), reducing cooking times and temperatures (Amrein *et al.*, 2004; Surdyk *et al.*, 2004; Taubert *et al.*, 2004), and lowering the pH (Amrein *et al.*, 2004; Jung *et al.*, 2003; Rydberg *et al.*, 2003), all of which are fairly straightforward solutions, based on prior knowledge of the factors affecting the Maillard reaction. While it is indeed possible to reduce acrylamide levels in food, the solutions presented were mostly food-specific and involved a combination of measures that were determined empirically. It has been recognised that for the development of a holistic strategy, which can be adapted for different foods and processing conditions and can be implemented successfully in industry, a fundamental understanding of the reaction mechanisms and kinetics behind acrylamide and flavour formation is necessary.

1.3.1 Mechanisms of acrylamide formation from asparagine in the Maillard reaction

In the initial work demonstrating the role of asparagine and the Maillard reaction in acrylamide formation, Mottram *et al.* (2002) heated asparagine and other amino acids with glucose or 2,3-butanedione at temperatures between 120 and 185 ºC. This confirmed the importance of asparagine, although small quantities of acrylamide were also formed from methionine. Both glucose and 2,3-butanedione were effective and it was suggested that Strecker degradation was a possible mechanistic route to acrylamide. However, it seemed unlikely that the Strecker aldehyde of asparagine (3-oxopropanamide) was an intermediate, as such a route would necessitate reduction of the aldehyde to an alcohol, followed by dehydration. Stadler *et al.* (2004) showed that pyrolysis of 3-hydroxypropanamide (the Strecker alcohol from asparagine) at 180 ºC, yielded only very small amounts of acrylamide compared with a glucose asparagine model system, confirming that a pathway to acrylamide from a Strecker aldehyde was most unlikely. Nevertheless, the initial stages of Strecker degradation with the Schiff base formation and decarboxylation to an imine are feasible steps in the route to acrylamide. In parallel work, Stadler *et al.* (2002) heated 20 amino acids with glucose and found that only asparagine gave significant quantities of acrylamide. Different sugars (glucose, fructose, galactose, lactose, sucrose) gave similar quantities of acrylamide. Using ^{15}N-amide-labelled asparagine, they were able to demonstrate the incorporation of the ^{15}N into acrylamide. They also showed that pyrolysis of *N*-glycosides of asparagine, at 185 ºC, readily yielded acrylamide.

Zyzak *et al.* (2003) provided further conclusive evidence that asparagine is the amino acid precursor for acrylamide. Stable isotope substitution studies, using a potato model system, showed that all three acrylamide carbon atoms originate from asparagine, and that the acrylamide nitrogen is derived specifically from the amide nitrogen of asparagine. These researchers were also able to show the presence of 3-aminopropanamide (a proposed precursor of acrylamide) in heated glucose–asparagine model systems using LC-MS. Addi-

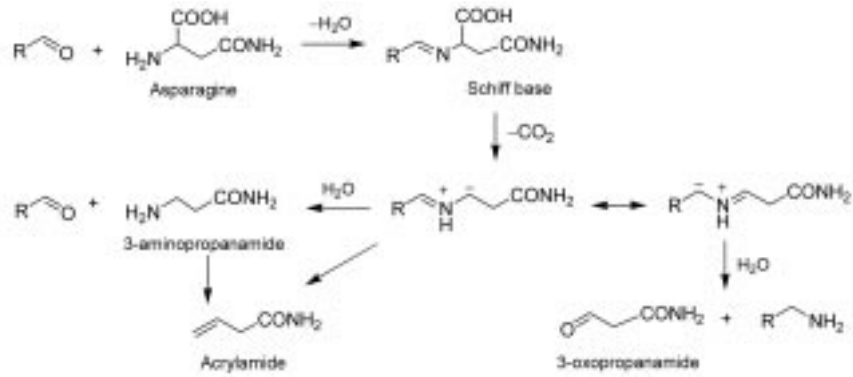

Fig. 1.4 Scheme for the formation of acrylamide from asparagine and a carbonyl compound. Derived from Wedzicha *et al.* (2005) and Zyzak *et al.* (2003).

tionally, the chromatogram contained a peak which had MS ions corresponding either to the decarboxylated Schiff base of glucose and asparagine or to the decarboxylated Amadori compound. From these observations, a mechanism for acrylamide formation was proposed which, like previous suggestions, involves the formation of a Schiff base from the reaction of a carbonyl compound with asparagine, followed by decarboxylation in a Strecker-type reaction to give an unstable intermediate (Fig. 1.4). Hydrolysis of this decarboxylated Schiff base gives 3-aminopropanamide which, on elimination of ammonia, yields acrylamide. The ready thermal degradation of 3-aminopropanamide to acrylamide under aqueous or low water conditions at temperatures between 100 and 180 ºC confirmed that it is a very effective precursor of acrylamide (Granvogl *et al.*, 2004). Alternatively the decarboxylated Schiff base could form acrylamide via elimination of an imine.

The general reaction scheme for acrylamide formation shown in Fig. 1.4 encompasses the essential steps, although the detailed mechanism of each step may depend on the species involved (i.e. the nature of the carbonyl compound) and on conditions of temperature, water content and pH. This has been studied in some detail (Schieberle *et al.*, 2005; Stadler *et al.*, 2004; Yaylayan and Stadler, 2005; Yaylayan *et al.*, 2003). The effectiveness of different carbohydrate moieties in forming acrylamide has been investigated by a number of research groups. Zyzak *et al.* (2003) investigated the ability of different carbonyls to generate acrylamide in a potato snack model system and found that a variety of carbohydrate sources could generate acrylamide from asparagine, including glucose, 2-deoxyglucose, ribose, glyceraldehydes, glyoxal and decanal. Schieberle *et al.* (2005) heated equimolar mixtures of asparagine and five different monosaccharides and two disaccharides at 170 ºC for 30 min. in closed glass vials in the presence of 10% water. Yields of acrylamide ranged from 0.8 to 1.3 mol-%, with glucose the most effective (Table 1.1). It is interesting to note that sucrose, a non-reducing sugar, produced almost as much acrylamide as some of the reducing sugars. This suggests that thermal processing of foods,

Table 1.1 Acrylamide (mmol/mol asparagines) formed by heating asparagines with different sugars (0.1 mmol each mixed with 3 g silica gel, 10% water, 170 ºC, 30 min.). Adapted from Schieberle *et al.* (2005)

Carbohydrate	Acrylamide	Carbohydrate	Acrylamide
Glucose	9.6	Arabinose	6.7
Fructose	8.9	Erythrose	6.4
Lactose	8.1	Sucrose	5.7
Ribose	7.6		

sucrose may undergo hydrolysis to glucose and fructose. Stadler *et al.* (2002) also noted that glucose, fructose, galactose, lactose and sucrose all gave comparable yields of acrylamide when heated with asparagine at 180 ºC. Other carbonyls have been evaluated for their effectiveness in producing acrylamide. Stadler and co-workers (Blank *et al.*, 2005; Stadler *et al.*, 2004) compared dicarbonyls and hydroxycarbonyls with glucose using model systems containing equimolar quantities of carbonyl and asparagine heated, in the presence of water, in sealed tubes at 180 ºC for 5 min. The highest yields of acrylamide were for hydroxycarbonyls, followed by glucose, with α-dicarbonyls, such as butanedione, giving yields ten-fold lower than glucose (Table 1.2). Schieberle *et al.* (2005) also found α-dicarbonyls, such as glyoxal and methylglyoxal, to be less effective than hydroxyacetone or glucose in producing acrylamide.

It is generally recognised that the key step in the mechanism for acrylamide formation, from the Maillard reaction between glucose and asapargine, is the decarboxylation of the Schiff base. Stadler *et al.* (2002) first proposed that the *N*-glycoside of asparagine was the direct precursor of acrylamide and subsequently demonstrated that the potassium salt of *N*-(D-glucos-1-yl)-L-asparagine was over 20 times more effective at producing acrylamide than the corresponding Amadori compound (*N*-(deoxy-D-fructos-1-yl)-L-asparagine) (Stadler *et al.*, 2004). This, along with the observed efficiency of α-hydroxycarbonyls in producing acrylamide with asparagine, indicates that a Schiff base with an hydroxyl group in the β-position to the nitrogen atom, such as would arise from an α-hydroxy carbonyl or a reducing sugar, is more favourable to degradation to

Table 1.2 Acrylamide (mmol/mol asparagines) formed by heating asparagines with different carbonyls (0.2 mmol each, 20 μl water, 180 ºC, 5 min.). Adapted from Blank *et al.* (2005)

Carbonyl compound	Acrylamide	Carbonyl compound	Acrylamide
2,3-Butanedione (diacetyl)	0.26	1-Butanal	0.01
2-Oxopropanal (methylglyoxal)	0.52	1-Hydroxyacetone (acetol)	3.97
Ethanedial (glyoxal)	0.38	2-Hydroxy-1-butanal	15.8
Oxoacetic acid (glyoxylic acid)	0.08	Glucose	2.22

acrylamide than a Schiff base with a β-carbonyl group, arising from an α-dicarbonyl compound.

In order to explain the significance of the β-hydroxyl group, it may be necessary to consider an alternative route to the classical Strecker degradation for the decarboxylation of the Schiff base. Yaylayan *et al.* (2003) proposed a mechanism in which the Schiff base undergoes an intramolecular rearrangement to give an unstable oxazolidine-5-one derivative (Fig. 1.5, pathway Ia). This readily decarboxylates to an intermediary azomethine ylide which is resonance stabilised. Alternatively, this azomethine ylide may be formed by the decarboxylation of the zwitterionic form of the Schiff base (pathway Ib) (Grigg *et al.*, 1988; Stadler *et al.*, 2004). A similar carbonyl-assisted decarboxylation of sarcosine (*N*-methylglycine) was first reported by Rizzi (1970). The azomethine ylide can readily undergo irreversible 1,2-prototropic shift yielding the imines 1 and 2 (pathways IIa and IIb). Since the 1,2-prototropy is irreversible, the two imines cannot be interconverted. Imine 1 (a decarboxylated Schiff base) can hydrolyse to 3-aminopropanamide and yield acrylamide by the elimination of ammonia. Alternatively, tautomerism of imine 1 (which requires the β-hydroxyl

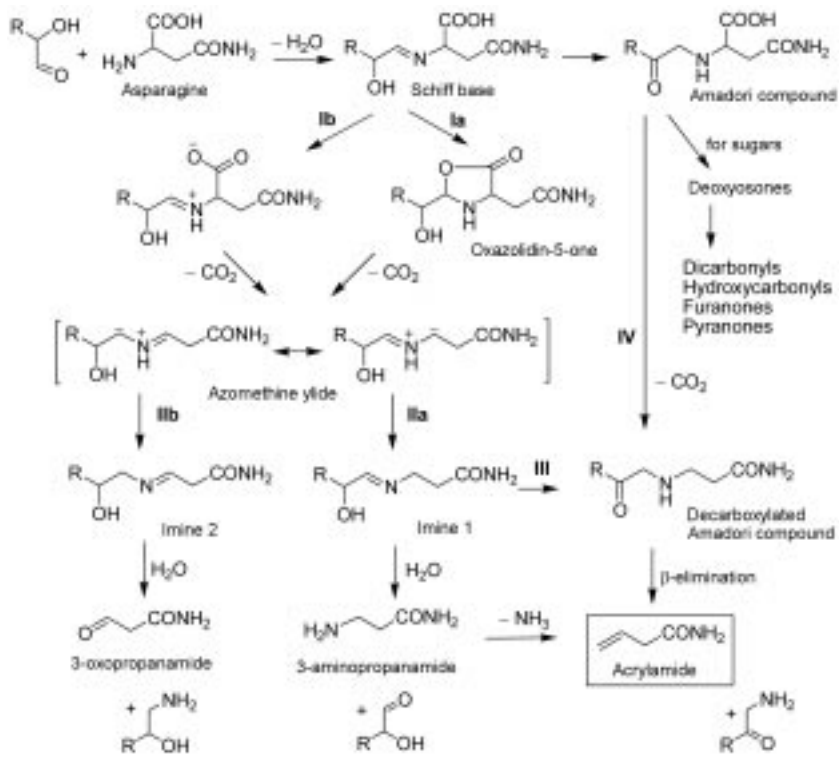

Fig. 1.5 Proposed mechanism for the formation of acrylamide from the reaction of asparagine with an amino carbonyl or a reducing sugar. Derived from Stadler *et al.* (2004) and Yaylayan *et al.* (2003).

group) would lead to a decarboxylated Amadori compound (pathway III) which would yield acrylamide, along with an amino ketone, via a β-elimination reaction (Yaylayan *et al.*, 2003). Imine 2 cannot produce acrylamide, but hydrolysis will result in the Strecker aldehyde of asparagine, 3-oxopropanamide. Formation of acrylamide from the Amadori compound (pathway IV) is not energetically favoured compared with the azomethine ylide route (Yaylayan *et al.*, 2003). However, the Amadori compound will be an important pathway to other Maillard reaction products, including carbonyls and colour and flavour compounds.

The nature of the carbonyl compound is important in determining the extent to which the different pathways occur and consequently the yields of acrylamide. A hydroxyl group in the β-position to the nitrogen atom of the Schiff base will favour the rearrangement of imine 1 to the decarboxylated Amadori compound (pathway III). Indeed with a carbonyl group in this position such a rearrangement cannot occur. Thus, this additional pathway for acrylamide formation, available only to Schiff bases formed from α-hydroxycarbonyls, may explain why hydroxyacetone, an α-hydroxycarbonyl, formed almost eightfold more acrylamide than methylglyoxal, the corresponding α-dicarbonyl, when heated with asparagine (Table 1.2). For α-dicarbonyls the mechanism for the decarboxylation of the Schiff base is similar to that for α-hydroxycarbonyls and reducing sugars (Fig. 1.6) but imine 1 cannot form the secondary amino compound (decarboxylated Amadori compound) and direct β-elimination (Fig. 1.6, pathway V) will be much less favoured. Thus the main route to acrylamide will be via 3-aminopropanamide. Furthermore, the presence of a carbonyl group in the azomethine ylide will have a tendency to delocalise the negative charge on the carbon atom adjacent to the carbonyl and this may cause the azomethine ylide to form preferentially imine 2. This imine hydrolyses to the Strecker aldehyde, 3-oxopropanamide, which cannot form acrylamide. Thus, overall acrylamide formation is less favoured from the reaction of asparagine with an α-dicarbonyl than an α-hydroxycarbonyl.

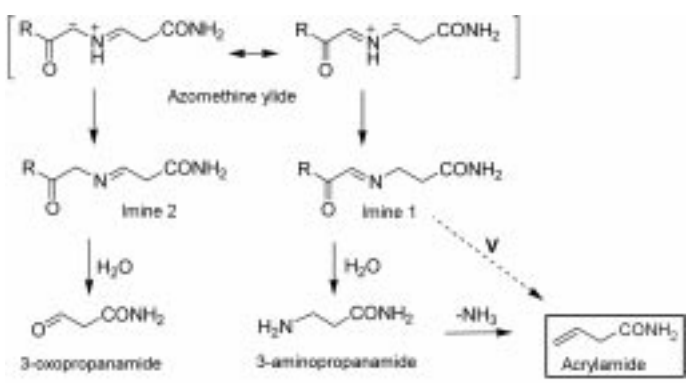

Fig. 1.6 Intermediates formed in the reaction of asparagine with a dicarbonyl compound and their degradation to acrylamide and a Strecker aldehyde.

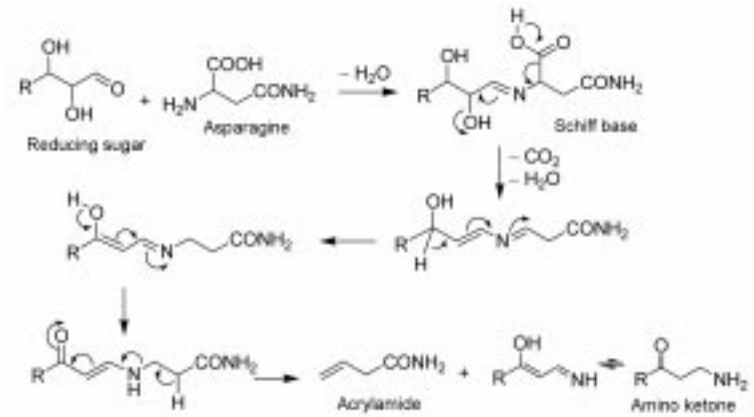

Fig. 1.7 Possible pathway for the formation of acrylamide from asparagine and a reducing sugar via a classical Strecker degradation of the Schiff base. Adapted from Blank *et al.* (2005).

Blank *et al.* (2005) have suggested that in reaction systems involving reducing sugars the polyhydroxy system may contribute to the degradation of the Schiff base and have proposed a scheme employing classical Strecker degradation together with the release of water (Fig. 1.7). The driving force for the reaction is the hydroxyl group in the γ-position to the nitrogen atom of the Schiff base. However, none of the intermediates has yet been isolated to confirm the hypothesis.

The schemes shown in Figs 1.4, 1.5 and 1.6 postulate the formation of the Strecker aldehyde of asparagine (3-oxopropanamide) as an alternative route for the breakdown of the Schiff base (classical Strecker degradation). Attempts to identify 3-oxopropanamide in heated mixtures of fructose and asparagine, using on-line monitoring with proton transfer ionisation mass spectrometry, were unsuccessful (Blank *et al.*, 2005), probably because the compound is too unstable to survive the severe conditions needed to produce acrylamide.

The mechanisms discussed in this section have been used to explain acrylamide formation in model systems containing asparagine and acrylamide. However, real foods are more complex and contain a number of free amino acids and sugars (Elmore *et al.*, 2005), all of which will participate in the Maillard reaction. Thus, the cooking food will contain a pool of reactive carbonyl compounds, which will be available to form Schiff bases with asparagine, as well as other amino acids. As a consequence, acrylamide formation in real foods may proceed through a number of different intermediates.

Studies on the kinetics of the Maillard reaction in model systems and foods have clearly demonstrated the relationship between time and temperature of heating and acrylamide formation, and the requirement of low moisture levels for maximum acrylamide production (Elmore *et al.*, 2005; Knol *et al.*, 2005; Wedzicha *et al.*, 2005). Prolonged heating results in a loss of acrylamide and it has been reported that the apparent loss in heated potato and cereal products

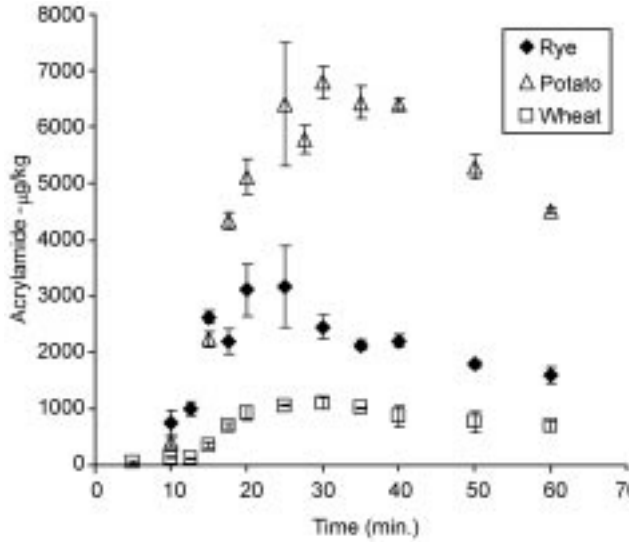

Fig. 1.8 Effect of cooking time at 180 ºC on acrylamide concentrations in rye, potato and wheat cakes (error bars represent standard deviations of three replicates). Adapted from Elmore *et al.* (2005).

occurs when all the free amino acid has reacted. Typical curves for acrylamide against cooking time are shown in Fig. 1.8. Similar losses of acrylamide have been reported by other researchers. The heating conditions needed to bring about a decrease in acrylamide are more severe than those normally encountered in processed foods, with the exception of roasted coffee, where high roasting temperatures generally result in products that have decreased levels of acrylamide compared with the maximum attainable under mild heating conditions (Taeymans *et al.*, 2004).

The loss of acrylamide could be due to evaporation or polymerisation of acrylamide. However, a much more likely explanation is that acrylamide reacts with other food components. Acrylamide contains an electrophilic α,β-unsaturated system that reacts, via a Michael addition, with nucleophilic groups such as amino or thiol groups, which may be present in free amino acids or as peptides and proteins such as the sulfhydryl group of cysteine, ϵ-amino group of lysine, or N-terminal amino group of proteins. The Michael reaction may be reversible which could, in certain circumstances, lead to the release of bound acrylamide.

1.3.2 Other possible routes to acrylamide

Acrylic acid is a possible intermediate to acrylamide, by reaction with a source of ammonia. There are a number of possible reactions by which acrylic acid could be formed in heated foods, although it is not generally recognised as a processing-derived compound. A possible pathway is the degradation of aspartic

acid, in the Maillard reaction, by analogous routes to those reported for formation of acrylamide from asparagine. When aspartic acid was heated with glucose or fructose, acrylic acid was formed in yields comparable with those of acrylamide from asparagine reacted under similar conditions (Stadler *et al.*, 2003). However, only trace quantities of acrylamide were formed when acrylic acid was heated with glucose (Mottram *et al.*, 2002), suggesting that a source of ammonia was limiting.

Acrylic acid may also arise form the oxidation of acrolein, which can be formed in the thermal degradation of lipids, either from the oxidation of fatty acids or from the glycerol moiety (Umano and Shibamoto, 1987). Other sources of acrolein may be amino acids (Alarcon, 1976). It has been shown that heating methionine with glucose in the absence of water at 185 ºC yielded acrylamide (Mottram *et al.*, 2002) and it was postulated that the pathway was via acrolein and ammonia, both of which are believed to be products from the Strecker degradation of methionine (MacLeod and Seyyedain-Ardebili, 1981). In pathways involving acrylic acid a source of nitrogen is required. This is likely to be from the deamination of amino acids; glutamine, asparagine and cysteine have been shown to release ammonia readily when heated at 180 ºC (Sohn and Ho, 1995).

The possible role of lipids in acrylamide formation has been the subject of some debate, with the acrolein route as one possible pathway (Yasuhara *et al.*, 2003). As discussed above, when fats and oils are heated at high temperature, acrolein can be formed, from the degradation of lipid, and its oxidation to acrylic acid followed by reaction with a source of ammonia could yield acrylamide. Alternatively, lipid oxidation products such as 2-alkenals and 2,4-decadienals could provide a carbonyl source for reaction with asparagine in Strecker type reactions. Ehling *et al.* (2005) showed that acrylamide was formed when fats and oils were heated with asparagine in model systems and that fish oils, with higher polyunsaturated fatty acid content, gave tenfold higher amounts of acrylamide than beef fat or corn oil. However, in models based on a potato matrix the addition of cooking oils did not significantly increase acrylamide (Biedermann *et al.*, 2002; Taeymans *et al.*, 2004), although other researchers reported an increase of acrylamide in potatoes when oil was added during heating (Tareke *et al.*, 2002). Overall these results suggest that lipid, *per se*, is not an important precursor for acrylamide compared with the main acrylamide–sugar route, although it may play a subsidiary role as well as providing a good medium for heat transfer.

1.4 The formation of other potentially toxic compounds in the Maillard reaction

The Maillard reaction produces a very large number of volatile compounds. We have examined 35 papers that have identified thermally derived volatile compounds in simple Maillard reaction systems comprising an amino acid and a

sugar. Over 550 different compounds have been identified. The majority were heterocyclic and included furans, pyrazines, pyrroles, pyridines, imidazoles, thiophenes, thiazoles, and oxazoles. Over 330 of the compounds found in the reaction systems have also been reported in the volatiles of cooked foods, where many contribute to the flavour characteristics of the food. In addition, there are numerous non-volatile compounds formed in the Maillard reaction, including melanoidins, which are largely uncharacterised. A number of these volatile compounds, as well as some amino acid reaction systems, have been tested for mutagenicity using the Ames test and some showed evidence of mutagenic activity (Lee and Shibamoto, 2002) but none of the compounds has been classified as a possible carcinogen. Furthermore, most of these compounds are present at extremely low levels in foods (typically 1 g/kg or lower) and it will be extremely difficult to determine any link between the consumption of cooked foods containing these compounds and human cancer.

There is, however, one group of non-volatile compounds that are highly mutagenic and have been found in cooked meat and fish products that have been subject to temperatures high enough to cause charring, such as occurs in grilling and barbecuing (Friedman and Cuq, 1988; Jagerstad *et al.*, 1998; Lee and Shibamoto, 2002). These compounds have been classified as heterocyclic aromatic amines (HAA). Over 20 compounds of this class have been identified (Jagerstad *et al.*, 1998) although they are found only at the low g/kg level. They can be divided into two groups: the amino-imidazo-azaarenes, which contain an aminoimidazole fused to a quinoline, quinoxaline, or pyridine ring system, and the aminocarbolines, which comprise a pyrrole ring fused to a phenyl ring and a pyridine (Fig. 1.9).

The aminoimidazoquinolines and the aminoimidazoquinoxalines, often referred to collectively as IQ-type HAAs, are believed to be formed as Maillard products from the reaction of pyrazines or pyridines, an aldehyde, such as acetaldehyde or formaldehyde, and creatinine (Fig. 1.10). Pyrazines are secondary products of the Strecker degradation of amino acids by carbonyl compounds, formed from the condensation of two amino carbonyls (see Fig. 1.3). Similarly, pyridines can be produced from the reaction of amines with deoxyosones (Belitz *et al.*, 2004). Creatinine is derived from creatine, which is an essential component of all muscle, and this provides the link between IQ-type HAAs and well-cooked or charred meat. PhIP, an aminoimidazopyridine found in cooked meat, is believed to derive from the reaction of creatinine with phenylacetaldehyde, the Strecker aldehyde of phenylalanine (Zochling and Murkovic, 2002). It was proposed that the reaction involved an aldol addition reaction between phenylacetaldehyde and creatinine followed by dehydration to give an aldolcondenation product, which was suggested to be an important imtermediate for the formation of condensation of PhIP (Fig. 1.11). The last step to form the aminoimidazopyridine ring system was not fully explained although a source of nitrogen was clearly required. IQ-type HAAs are highly mutagenic and several (MeIq, MeIQx, PhIP) have been classified as possible human carcinogens and one (IQ) a probable carcinogen by the IARC (Nursten, 2005).

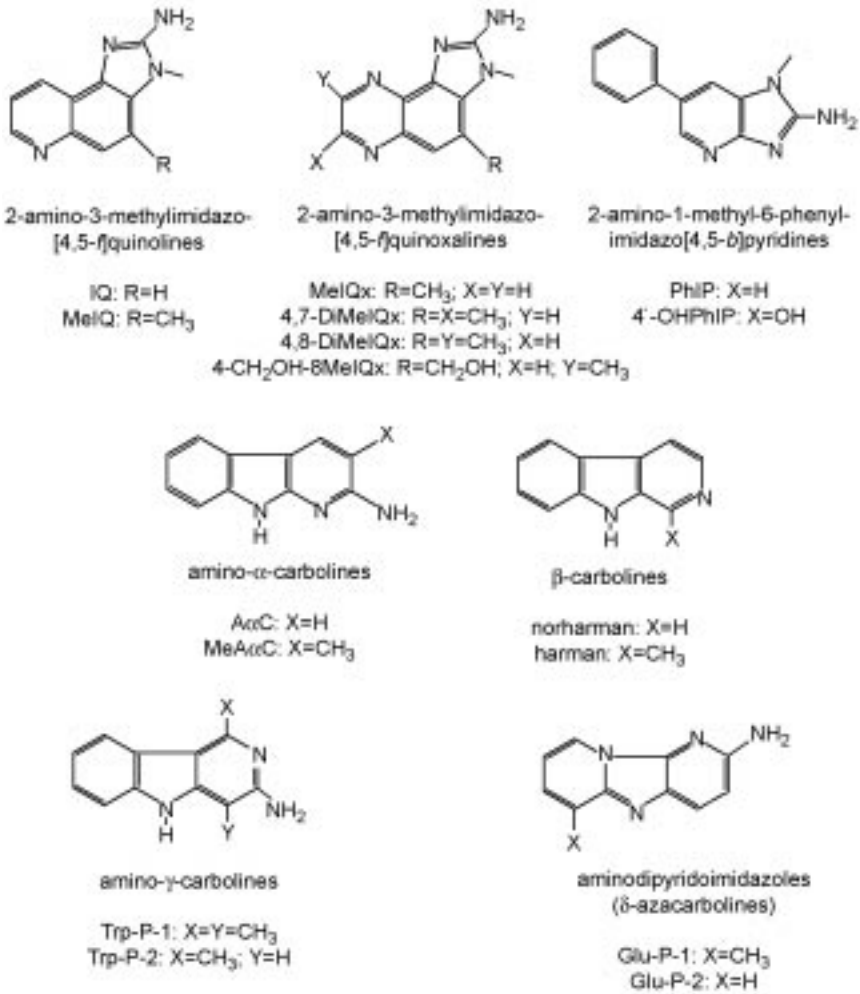

Fig. 1.9 Structures of some heterocyclic aromatic amines found in cooked foods.

The nonIQ-type HAAs do not involve creatinine in their formation and result from the high-temperature treatment of amino acids, such as tryptophan or glutamic acid. Carbolines are formed by the pyrolysis of tryptophan, either free or protein bound (Friedman and Cuq, 1988). The β-carbolines, norharman and harman, are formed much more readily than the α- or γ-carbolines. In the case of the free amino acid the β-carbolines may be formed from the reaction of tryptophan with formaldehyde or acetaldehyde (Fig. 1.12), while with bound tryptophan an internal Schiff base is formed with the carbonyl of the amide group (Friedman and Cuq, 1988). Pyrolysis of glutamic acid yields related HAAs, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-amino-dipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2). Although the nonIQ-type HAAs

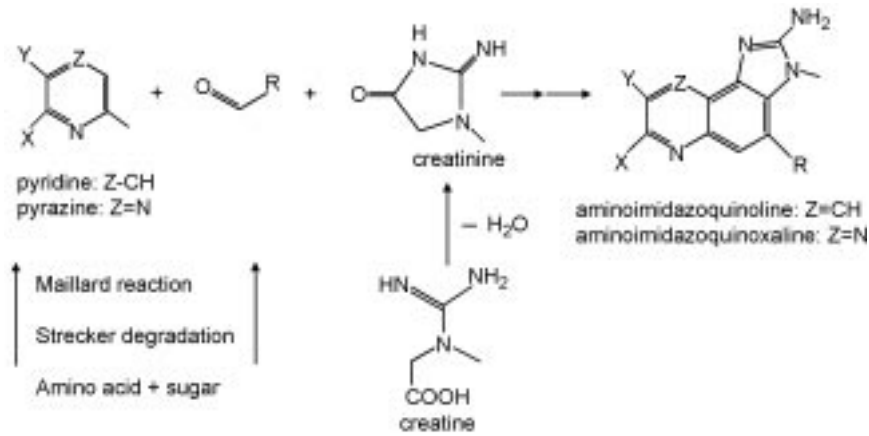

Fig. 1.10 Formation of aminoimidazo-quinolines and -quinoxalines from Maillard reactions involving creatinine. Adapted from Jagerstad *et al.* (1998).

have not been reported as frequently as the IQ-type HAAs, they have been found in various meat, chicken and fish products heated at high temperatures although only at the low g/kg level (Jagerstad *et al.*, 1998; Nursten, 2005).

1.5 Conclusions

The Maillard reaction between amino acids and reducing sugars is essential for the formation of the characteristic flavour and colour of most thermally processed foods. This very complex reaction produces a wide range of volatile compounds, contributing to the flavour of the food, and non-volatile melanoidins, which are responsible for colour. It was recognised almost 30 years ago that high temperature cooking of meat, fish and chicken, under the grill or on the

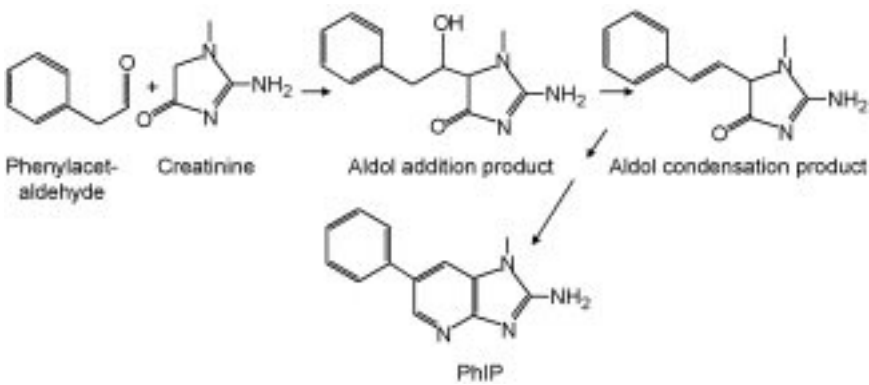

Fig. 1.11 Proposed pathway for the formation of PhIP, an imidazopyridine, from creatinine and phenyl acetaldehyde. Adapted from Zochling and Murkovic (2002).

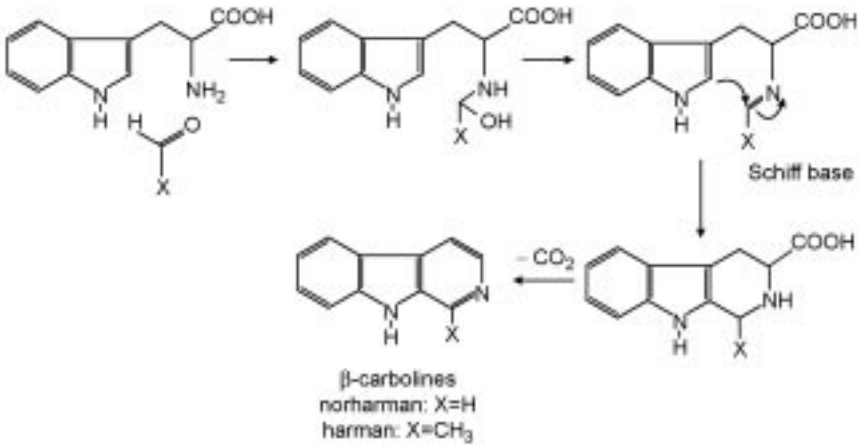

Fig. 1.12 Formation of β-carbolines in thermal degradation of tryptophan in presence of formaldehyde or acetaldehyde. Adapted from Friedman and Cuq (1998).

barbeque, could lead to the formation of highly mutagenic heterocyclic aromatic amines at low g/kg levels. Their formation has been associated with the Maillard reaction. More recently acrylamide, which has been classified as a probable carcinogen, has been reported in fried and baked potato and cereal products at much higher levels (up to 10 mg/kg). The Maillard reaction, involving the amino acid asparagine, has been conclusively shown to be responsible for the formation of acrylamide. Thus the Maillard reaction gives rise both to undesirable compounds and highly desirable compounds during the cooking of food. The challenge to food scientists and technologists is to find a means of maintaining the desirable flavour and colour while minimising the production of potentially harmful compounds, such as acrylamide and heterocyclic aromatic amines.

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2

The formation of acrylamide in cereal products and coffee

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2.1 Introduction

After the joint announcement by the Swedish National Food Authority and the University of Stockholm of the finding of relatively high levels of acrylamide in a wide range of heated foodstuffs, global research efforts were launched to understand how this undesired process chemical is formed in foods. Several pathways that may lead to the formation of acrylamide have been described in the literature, and first concrete evidence of the key reactants in food was provided by researchers from industry, academia, and food control laboratories working in Switzerland (Stadler *et al.*, 2002), United Kingdom (Mottram *et al.*, 2002), Germany (Weisshaar and Gutsche, 2002), Canada (Becalski *et al.*, 2002) and the USA (Sanders *et al.*, 2002). These studies have independently shown that the amino acid asparagine furnishes the backbone of the acrylamide molecule, and that the well-known Maillard reaction plays a major role in its formation.

Compared to potato-based products, only few studies have been published to date on the formation and mitigation strategies of acrylamide in cereal-based foods and coffee. The Confederation of the European Food and Drink Industries (CIAA) has contributed to the knowledge on acrylamide formation in these product categories through two peer-reviewed articles, and a recent Status Report (CIAA, 2004). In fact, the CIAA has, since the acrylamide issue, established a Technical Expert Group which recently presented a 'Toolbox' approach to identify common interventions and measures throughout the food supply chain. An important goal of the 'Toolbox' is a collection of key guiding principles of good practice for all stakeholders, i.e., consumers, manufacturers, retailers, caterers, etc.

This chapter summarizes the knowledge acquired to date in cereal-based products and coffee related to the formation of acrylamide, taking a view at the

different stages of food production, including agronomical aspects, recipes, technologies, and where relevant final preparation by the consumer.

2.2 Formation and possible mitigation strategies

2.2.1 Mechanistic pathways based on asparagine

A first insight into a more detailed mechanism of formation of acrylamide was provided by Zyzak *et al.* (2003) and Yaylayan *et al.* (2003). Both groups have confirmed the early proposal by Stadler *et al.* (2002) of the importance of the initial glycoconjugate of asparagine and reducing sugar or a reactive carbonyl compound, i.e. the Schiff base or *N*-glycoside. A key finding by Yaylayan and coworkers is a feasible route to the decarboxylated Schiff base and subsequently decarboxylated Amadori product. A final and rate limiting step is the cleavage of the carbon-nitrogen covalent bond to produce acrylamide (see Fig. 2.1).

A similar mechanism was proposed by Zyzak *et al.* (2003). They provided concrete evidence for the decarboxylated species (decarboxylated Amadori or Schiff intermediate of asparagine) by mass spectrometry using model systems. Furthermore, in this scheme, acrylamide may be formed directly by elimination of the early decarboxylated Schiff intermediate (azomethine ylide). Alternatively, acrylamide could also be formed indirectly from the decarboxylated Schiff (hydrolysis) or Amadori (elimination) intermediates via 3-aminopropionamide, that can further degrade (elimination of ammonia) at elevated temperatures to furnish acrylamide.

No direct evidence has been provided to date for the involvement of the decarboxylated Amadori compound in the formation of acrylamide (Route A, Fig. 2.1). A recent study has, however, demonstrated that the β-elimination reaction proceeds at higher temperatures, rapidly releasing the corresponding vinylogous compound (Stadler *et al.*, 2004). The Strecker aldehyde of asparagine may also represent a transient intermediate (route B, Fig. 2.1). The imine II releases 3-oxopropionamide that upon reduction and subsequent loss of water (promoted at higher temperatures) affords acrylamide (Stadler *et al.*, 2004; Wedzicha *et al.*, 2005).

A further important aspect is that the sugar type may affect the yield of acrylamide. Initial work indicates that *keto* sugars such as fructose seem more efficient than *aldehydo* sugars (e.g. glucose), generating acrylamide at relatively lower temperature (Stadler *et al.*, 2002). A mechanistic explanation is the ability of the fructose Schiff intermediate to stabilize the azomethine ylide intermediate through H-bonding (Yaylayan and Stadler, 2005). An alternative possibility is the higher reactivity of fructose due to its lower melting point and thus greater molecular mobility, thereby favoring faster reaction with the amine to form the Schiff base (Vuataz *et al.*, 2004).

The formation of acrylamide and related vinylogous compounds takes place in an essentially ‘dry’ environment. In aqueous systems, the (carboxylated) Amadori compounds are considered the first stable intermediates formed in the

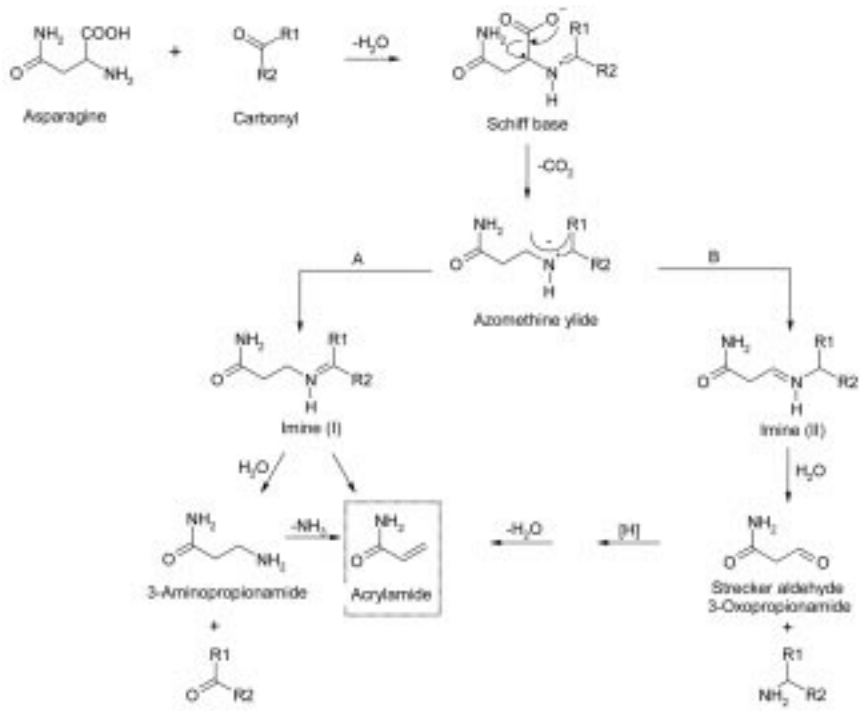

Fig. 2.1 Proposed mechanism for the formation of acrylamide (adapted from Stadler *et al.*, 2004, and Wedzicha *et al.*, 2005).

Maillard cascade and that lead to 1- and 3-deoxysones, and that further decompose to form important color and flavor compounds. As mentioned above, a dry or low moisture state with adequate thermal input enables the decarboxylation of the *N*-glycoside, opening the route to acrylamide. It is important to note that the yield of acrylamide in model systems (food and pure reactants) is low, i.e., typically less than 1% of the asparagine is converted to acrylamide (Stadler *et al.*, 2002; Surdyk *et al.*, 2004; Biedermann and Grob, 2003; Becalski *et al.*, 2003).

Overall, reaction yields in foods are difficult to predict, as acrylamide is also rapidly degraded at temperatures above 170 ºC (Stadler *et al.*, 2002; Mottram *et al.*, 2002). Studies using stable isotope-labeled compounds may provide a basis for kinetic models in the different foods and enable better estimation of final amounts under the different cooking regimes.

2.2.2 Cereal products

Cereal-based foods are part of the staple diet and encompass an extremely diverse range of products, and those of importance in relation to acrylamide exposure include bread, bakery items (cakes, biscuits, morning goods, etc.), and

breakfast cereals. Compared to potato products, relatively fewer data have been published related to formation and mitigation measures, mainly attributable to the large complexity of this category and the plethora of individual formulations and technologies used in the food industry (Taeymans *et al.*, 2004; CIAA, 2004). A recent survey of bakery products sold in the UK showed highest amounts in dry biscuit-type products and not necessarily in those that have strong Maillard flavours (Sadd and Hamlet, 2005).

Bread (including crispbread)

The levels of acrylamide found in bread are relatively low, but due to the high consumption may become a relevant source of exposure to acrylamide. Typical levels reported in different studies reside in the range of 15 to 90 μg/kg (based on whole loaves) (Ahn *et al.*, 2002; Surdyk *et al.*, 2004; Becalski *et al.*, 2003; Tareke *et al.*, 2002; Sadd and Hamlet, 2005), but may become significantly higher in white bread that is toasted (Ahn *et al.*, 2002). Therefore, it is important to consider the different definitions, diverse formulations, and usage patterns of bread in different countries, as these may differ substantially and consequently lead to different amounts in the products as consumed.

Raw materials

It is well established that the amount of reducing sugars is more important than free asparagine for the formation of acrylamide in potato-based products (Amrein *et al.*, 2003). However, in cereal foods, including bread, the main determinant of acrylamide formation is free asparagine. This has been corroborated by spiking bread dough with free asparagine, that has led to a subsequent increase in acrylamide concentration after baking (Surdyk *et al.*, 2004). Further evidence of the importance of asparagine was furnished by adding the enzyme asparaginase to the model doughs, that afforded a significant reduction of acrylamide (Weisshaar, 2004). The addition of fructose or glucose had no such effect in cereal model systems (Surdyk *et al.*, 2004; Bråthen and Knutsen, 2005). Overall, the studies conducted so far indicate that the amount of asparagine in the raw materials and ingredients, in particular cereal flour, is a key determinant of the formation of acrylamide during baking.

The concentrations of free asparagine have been studied in different commercial milling fractions of wheat and rye (Fredriksson *et al.*, 2004). Whole grain flours showed higher amounts of asparagine (for wheat and rye 0.5 g/kg and 1.1 g/kg, respectively) versus sifted flours. Of the wheat fractions, wheat germ was reported to have the highest level of asparagine (4.9 g/kg). An earlier study by Springer and coworkers (2003) confirmed the impact of milling degree and the relatively higher levels of asparagine in rye as compared to wheat and in whole grain versus flour fractions. In fact, the concentration of free asparagine in cereal grains varies widely and typical ranges are shown in Table 2.1.

However, agronomical factors (e.g. crop variety, climatic conditions fertilizer regimes) may significantly impact the amount of free asparagine in cereal crops (Taeymans *et al.*, 2004) and consequently the concentration of acrylamide in the

Table 2.1 Overview of the amounts of free asparagines in different cereal grains and fractions

Commodity	Fraction	Free asparagine (g/kg)[a]	Reference
Wheat	Germ	55.5–57.4	Fredriksson *et al.* (2004)
	Bran	1.12	Nestlé Research Centre[b]
	Bran	1.48	Fredriksson *et al.* (2004)
	Whole wheat flour	0.17	Elmore *et al.* (2005)
	Flour	0.18–0.19	Nestlé Research Centre[b]
	Flour	0.14–0.17	Fredriksson *et al.* (2004)
	Flour	0.15–0.4	Noti *et al.* (2003)
Oats	Bran	0.71	Nestlé Research Centre[b]
	Flour	0.5	Nestlé Research Centre[b]
Rye	Whole grain	1.07	Fredriksson *et al.* (2004)
	Flour	0.53–0.68	Fredriksson *et al.* (2004)
	Flour	0.63	Elmore *et al.* (2005)
	Flour	0.26	Nestlé Research Centre[b]
Maize	Flour	0.596–1.07	Wang *et al.* (2001)
	Semolina	0.22	Nestlé Research Centre[b]
	Corn starch	<0.01	Biederman and Grob (2003)
Rice	Germ	0.23	Friedman (2003)
	Bran	0.28	Friedman (2003)
	Flour	0.07	Nestlé Research Centre[b]

[a] On a fresh or dry weight basis.
[b] Personal communication, Drs E. Campos and T. Benet.

final product. Regional differences may account for levels that vary by > fivefold, and today the scientific data that may explain this variability is lacking. Long-term studies are warranted to assess the full potential of measures at the agronomic level, considering the overall effect on crop yield, nutritional status, and the economic impact.

Product composition

The composition and ingredients of bread may contribute substantially to the acrylamide levels. Major differences have been reported in fermented versus non-fermented bread and crispbread (CIAA, 2004). During fermentation, the amino acid pool is rapidly depleted and provides a source of nitrogen for growth, and yeast preferentially assimilates asparagine. As shown in model dough, the fermentation time is an important parameter to consider. Longer fermentation times in dough mixtures comprising sifted wheat flours and whole grain can decrease acrylamide by on average 87% (Fredriksson *et al.*, 2004). A reduction of free Asn down to < 0.1 g/kg equates to approx. 4 μg/kg of acrylamide in a whole grain wheat bread. An overview of the proposed measures in the bread category are shown in Table 2.2 (non-exhaustive list).

Table 2.2 Selected measures identified that may afford a reduction of acrylamide in bread products

Product	Study (impact of)	Major finding	Reference
Crispbread	Time/temp. profiles	Decreasing average longitudinal oven baking time affords reduction of acrylamide.	Taeymans *et al.* (2004)
	Temp./ moisture	Up to 50% reduction of acrylamide by optimization of inlet/outlet temp. in the oven and adjustment of recipes.	Springer *et al.* (2003)
Bread			
Rye	Milling degree	Relationship between milling degree and free asparagine, highest potential of acrylamide formation in rye meal and whole rye flour.	Springer *et al.* (2003)
Wheat (yeast leavened)	Baking temperature	Increase of acrylamide with increasing temp. No reduction even at high baking temp.; 99% of the acrylamide formed in the crust.	Surdyk *et al.* (2004)
	Addition of free asparagine	Increase in acrylamide concentration.	
	Addition of fructose	No change in acrylamide content.	
	Color	Acrylamide formation related to color.	
White	Toasting	Increase in acrylamide amount with toasting time and color; kinetic model established.	CIAA (2004)
Fermented rye and wheat	Fermentation (yeast and sourdough)	Up to 87% reduction in acrylamide amount upon extended fermentation times. Yeast utilizes available free asparagine within two hours incubation. Only marginal effects in terms of reduction of acrylamide in sourdough fermented bread.	Fredriksson *et al.* (2004)
Bread and flatbread (rye and wheat)	Glycine	Significant reduction of acrylamide after addition of glycine to the dough, e.g., 81 mmol/kg flour reduces acrylamide by up to 73% in bread crusts.	Bråthen *et al.* (2005)
Bread and flatbread (rye and wheat)	Time and temp.	Linear correlation of acrylamide formation with time/temp.	Bråthen and Knutsen (2005)

Studies on sourdough have, however, not shown such promising results, with only a small difference in free asparagine versus only yeast fermentation. The effects of lactic acid bacteria are most probably attributable to lower pH of the dough and consequently less Maillard chemistry taking place. On the other hand, the proteolytic activity of bacteria may also contribute to increasing the amount of available asparagine (Fredriksson *et al.*, 2004). Therefore, one possible option to reduce acrylamide is to extend the time of yeast fermentation and use as an endpoint of acrylamide formation the fermentation time.

The addition of competing amino acids such as glycine has been suggested (Rydberg *et al.*, 2003; Amrein *et al.*, 2004). Bråthen and coworkers (2005) reported a significant reduction of acrylamide concentration in flat breads (whole meal rye flour) and breads (white wheat flour), 73% and 96%, respectively, after adding 6.08 g of glycine per kg of flour. In the Maillard reaction, glycine could compete with available asparagine for the sugar/carbonyl source. Alternatively, acrylamide may also react with the nucleophilic amino group of glycine through Michael addition.

The employment of the enzyme asparaginase to reduce free asparagine is a possible future option. In fermented breads, asparaginase could be introduced as a selective measure. However, depending on the enzyme characteristics, this approach may imply longer incubation times. So far, only experimental work in the laboratory has been conducted as the enzyme is not available on a commercial scale as a food additive or processing aid. Prior to industrial usage, questions related to the regulatory approval, labeling, and safety will need to be addressed.

Process management

The thermal input and final moisture content are decisive factors in the formation of acrylamide. Pilot-scale studies assessing the effect of time and temperature profiles during baking have been established for white bread (CIAA, 2004) and wheat leavened bread (Surdyk *et al.*, 2004). Both studies show that acrylamide is formed predominantly in the outermost crust layer (i.e. hottest and driest zone), and increases in the bread crust with baking time and temperature, contributing up to 99% of the total acrylamide in the whole loaves. Thus, consistently lower concentrations of acrylamide are found in the bread crumb (Sadd and Hamlet, 2005). In a dry cereal system (water removed prior to baking by lyophilization), acrylamide passes through a maximum at around 200 ºC, and then declines due to a faster rate of loss (evaporation or removal through protein interaction) (Bråthen and Knutsen, 2005). One explanation for the different behavior of acrylamide in a dehydrated model is that in the bread system, which contains residual water, the effective product temperature is lowered due to evaporation of water off the surface. This is supported by earlier work in test tube models that illustrate the reduction of acrylamide after prolonged heating (Becalski *et al.*, 2003; Stadler *et al.*, 2002; Tareke *et al.*, 2002).

In most cases, acrylamide is also well correlated to the color of the bread, representing in this category an important end-point of acrylamide formation

(Surdyk *et al.*, 2004, Stadler and Scholz, 2004). However, exceptions to this rule have been reported, for example when ingredients such as ammonium bicarbonate are added to the recipe (Sadd and Hamlet, 2005). The CIAA has recently presented modeling work to predict acrylamide formation over time in toasted white bread, and have shown that formation follows well defined Arrhenius kinetics (CIAA, 2004). Therefore, toasting bread to a golden yellow colour provides slices with acrylamide concentrations that are relatively low, and one could envisage a colour scale on the food product package as practical guidance to consumers to avoid overheating. In addition, the formation of other undesired processing chemicals such as 3-monochloropropane-diol (3-MCPD) is also linked to the degree of toast (Breitling-Utzmann *et al.*, 2005).

Crispbread can be broadly categorized into fermented and non-fermented products. Fermented products tend on average to contain lower amounts of acrylamide compared to non-fermented cripsbreads. The addition of rework (< 5%) has been studied in crispbread manufacture, and has been shown not to have a negative impact on acrylamide formation in the finished product. However, manufacturers will need to study the effect of rework on a case-by-case basis for each individual product (CIAA, 2004).

In non-fermented crispbread manufacture, an average acrylamide reduction of 75% in 2004 versus figures initially published by the UK Food Standards Agency in 2002 has been achieved (Taeymans *et al.*, 2004; CIAA, 2004). The key parameter that was adjusted to accomplish this was optimization of the thermal input during the oven baking process, i.e., temperatures at the beginning of the sequence were initially set higher, and toward the end of the toast lowered by roughly 30 ºC compared to the standard baking profile. This measure has an impact on the line efficiency due to the extended overall time of the bake (CIAA, 2004).

Bakery wares

In bakery items, concentrations of acrylamide have been reported > 1000 μg/kg. Based on data extracted from the EU monitoring database (www.irmm.jrc.be/ffu/acrylamidemonitoringdatabase_statusJune04.xls), the 75%ile lies below 400 μg/kg for the category of fine bakery wares, and below 650 μg/kg for gingerbread that is listed separately (Stadler and Scholtz, 2004). Studies conducted in the UK on acrylamide formation in cereal products have reported lower amounts in cakes. This may be related to a relatively lower free asparagine concentration in cake flours, i.e., about one-third of those found in bread flours, as cake flours contain a lower proportion of the outer parts of the wheat grain. In addition, the lower oven temperatures encountered during cake baking may also contribute to a lower amount of acrylamide in cakes (Sadd and Hamlet, 2005).

This category of foods is very diverse, and thus represents a far higher level of variability and complexity in terms of recipes and processing technologies as compared, for example, to potato-based products and bread. Consequently, a major focus of the mitigation in this food category is at the processing stage,

where changes to recipes and optimization of baking conditions may afford products with relatively lower acrylamide amounts, taking into consideration the important organoleptic properties of the finished products.

Raw materials

As in the case of bread, trials conducted on a range of biscuits showed a linear relationship between the amount of free asparagine in the flour and acrylamide formation in the final product (CIAA, 2004). However, as mentioned on page 27, the concentration range of free asparagine in cereal flours varies widely (Taeymans *et al.*, 2004). Added to this will be the variability caused by seasonal changes and other agronomic factors not yet adequately studied (fertilization regime, harvest time, etc.).

Product composition

Recipes and ingredients play a major role in the formation of acrylamide. Different ingredients may have varying amounts of the precursors (sugars, asparagine) that can participate in the Maillard reaction. Sugar is a key ingredient in the biscuit recipe, and a proportional increase of reducing sugar leads to more acrylamide being formed in the biscuit product (Haase *et al.*, 2003; Taeymans *et al.*, 2005). Replacing invert sugar syrup with sucrose, a non-reducing sugar, has led to a marginal improvement with regard to acrylamide in a sweet biscuit used as an ingredient, albeit affecting also the color of the biscuit (Graf *et al.*, 2005). However, sucrose can be considered the main sugar used by the baking industry and therefore in most cases already represents an 'ideal' condition (CIAA, 2004).

Bench-scale and pilot-scale trials by academic and industry research groups have identified certain measures at the recipe stage that may represent future opportunities in relatively lowering the acrylamide concentration in bakery products (e.g. crackers, biscuits). One of the most studied interventions is the replacement of the baking agent – ammonium bicarbonate – by the corresponding sodium salt (Amrein *et al.*, 2004, Vass *et al.*, 2004, Graf *et al.*, 2005). Ammonium apparently catalyses the breakdown of sugars to afford more reactive carbonyl by-products, that can condense with free asparagine to furnish the early Maillard intermediate in the reaction cascade (Stadler *et al.*, 2004; Yaylayan and Stadler, 2005). Sodium bicarbonate has recently been shown to contribute significantly to the elimination of acrylamide in a biscuit cracker model (Levine and Smith, 2005), indicating two favorable but independent effects of the substitution of ammonium bicarbonate with sodium bicarbonate. Thus, this approach has afforded measurable reductions of up to 70% versus a standard product. It must, however, also be remembered that in most cases in biscuit manufacture a mixture of both ammonium and sodium bicarbonate are employed (Sadd and Hamlet, 2005), and full replacement may have an impact on the textural properties of the final product. More importantly, complete substitution may lead to an increase in dietary sodium levels should such replacements be conducted systematically.

A further option proposed at the recipe stage is the employment of citric acid, reported to afford an approximate fourfold reduction of acrylamide in gingerbread, depending on the dose (Amrein *et al.*, 2004). Citric acid lowers the pH of the system and thereby affects the overall Maillard reaction. In an industrially produced sweet biscuit, the addition of 244 g/100 kg has achieved a lowering of the acrylamide content by one third. Similarly, lactic acid was shown to reduce acrylamide in a model biscuit (plain flour matrix) on average by > 30% (Taeymans *et al.*, 2004). Any measures that will impact the Maillard reaction and consequently browning/flavor generation will, however, also have an impact on the sensorial properties of the product.

In model biscuits, certain spices such as ginger and cardamon may increase the amount of acrylamide, whereas nutmeg may afford a relative reduction (Taeymans *et al.*, 2005). An additional variable is the mixing time of the dough, that may reverse the effect of sugars or pH in the system (Taeymans *et al.*, 2005). Thus, the interpretation of certain observations in biscuits is not straightforward, and again emphasizes the need for individual studies for each recipe/technology/product combination.

Process management

As previously highlighted, bakery wares represent an extremely heterogeneous product category, where multiple variants must be considered and will need to be studied at the process management level, e.g. temperature, time, moisture (Stadler and Scholtz, 2004; Haase *et al.*, 2003). Clearly, modeling experiments as already presented by certain research groups to predict formation/elimination will be helpful in optimizing formulation and process conditions (Sadd and Hamlet, 2005; Elmore *et al.*, 2005).

In a biscuit model system, acrylamide could be reduced by approximately 20% by changing the baking profile (time/temp regime), in that higher temperatures are employed at the early stages of baking and lower temperatures in the end zone. Analogous to the measures described for cripsbread, this implies longer baking times and consequently reduced line efficiencies (CIAA, 2004). A further parameter to target is end-product moisture that typically resides at ~2% in a commercial biscuit. The results of several pilot-scale studies have demonstrated that moisture is inversely proportional to acrylamide formation (Taeymans *et al.*, 2004). However, moisture is in most cases closely correlated to the surface color of the biscuit, and will also impact the organoleptic properties (texture, taste) and shelf-life of the product.

A promising route that may in the future achieve substantial reductions in acrylamide is the use of the enzyme asparaginase, that converts free asparagine by removal of the amide group to aspartic acid. However, for the enzyme to act it must reach the substrate. Furthermore, additional process steps may become necessary, e.g. to ‘inactivate’ the enzyme.

Patents related to the use of asparaginase have been filed by several companies (e.g., Frito Lay, Procter & Gamble, DSM, Novozymes), but experiments conducted so far have been restricted to laboratory and bench-scale trials

(Amrein *et al.*, 2004; Zyzak *et al.*, 2003; JIFSAN II). Therefore, this research avenue can be considered medium term, i.e., 2–5 years before a commercial product becomes available.

Breakfast cereals

Similar to biscuit and bakery items, breakfast cereals comprise a large and diverse category of foods made from a range of cereal grains by a variety of distinct processes, leading to many different forms of flake, puffs, extrudates and biscuit-like pieces. Sugar is in most cases an important ingredient and can be added either prior to toasting or after toasting as a coating. Data extracted from the EU Joint Research Centre database (www.irmm.jrc.be/ffu/acrylamidemonitoringdatabase_statusJune04.xls) shows that typical amounts of acrylamide in breakfast cereals range from 5–846 μg/kg ($n = 162$, 75th percentile = 152.5 μg/kg).

Raw materials

The most promising means to reduce acrylamide content in breakfast cereals is by reduction of the free asparagine content in the crops from which they are made. The key agronomic factors that can significantly influence the chemical composition and performance of a crop are the variety of the grain, the crop year, and fertilizer regimes (CIAA, 2004).

The crop year has a significant effect on asparagine and hence acrylamide formation in cereal grains. A fluctuation of up to 40% (based on acrylamide concentration) was observed and attributed to seasonal variation, viz., climatic conditions (CIAA, 2004). Data for a selection of European wheat varieties grown mainly in the UK in 2002 were published in Taeymans *et al.* (2004). The 31 different varieties (total of 45 samples) revealed a range of free asparagine from 0.074–0.66 g/kg. Within the same cereal variety, a twofold variabiality in the values was not uncommon. Wheat grown over a range of nitrogen fertilizer regimes showed a correlation of the content of asparagine to protein ($r^2 = 0.553$, $n = 38$). The same study (CIAA, 2004) reported an inverse relationship of sucrose to protein, and no association of reducing sugars with protein.

Product composition

Asparagine is the key determinant of acrylamide formation. Thus, some cereal grains may yield more acrylamide due to higher amounts of asparagine than others within a common process (Fig. 2.2). However, the choice of the grain defines the product and thus ‘dilution’ with grains that harbor relatively less asparagine, such as rice, will lead to substantial changes of the product (CIAA, 2004).

Process management

Acrylamide is mainly formed during the toasting step, in particular when the moisture content falls below 5% and the color and flavor begin to form. Within the variety of processes the products made by extrusion puffing are at the low end of the scale with regard to acrylamide concentration (typically <100 μg/kg).

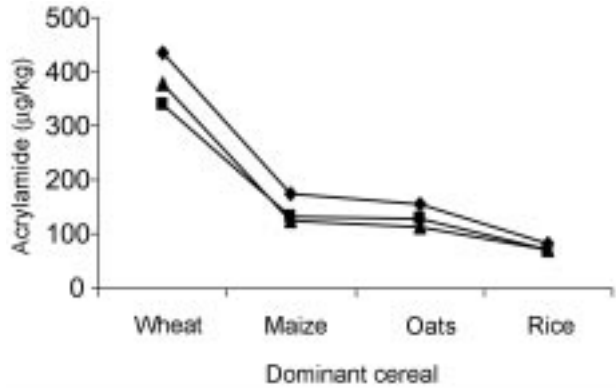

Fig. 2.2 Acrylamide content (three independent experiments) for a cereal made by a common process from dough in which the dominant cereal was varied (adapted from CIAA, 2004).

The extrusion cooker gelatinizes starch but develops little Maillard color or flavors, the water in the cereal being flashed off at the end of the extruder with little or no toasting. Much of the flavor of such products is derived from the coating applied after puffing or from ingredients included in the recipe. For most other processes there is a distinct toasting step with development of flavor and color and a tendency for acrylamide to exceed 100 μg/kg (CIAA, 2004).

Studies on the distribution of acrylamide in a toasted wheat cereal have shown that the temperature threshold required to initiate formation of acrylamide is apparently not as high as observed in model test tube experiments (i.e. > 120 ºC). The temperature measured in the centre of the biscuit cereal was well below 100 ºC but still revealed about half the amount of acrylamide measured on the surface (Taeymans *et al.*, 2004). This implies the formation of a precursor during the cooking step that decomposes at relatively low temperatures during the toast. All breakfast cereal processes aim to gelatinize starch quickly by bringing the raw cereal to a high temperature very quickly. The processes essentially do not offer scope for washing or use of enzymes such as asparaginase to reduce asparagine content.

2.3 Coffee

Coffee is not consumed as such, but prepared by the addition of hot water and subsequent filtration. Acrylamide is a polar molecule and efficiently extracted with hot water. Hence, the brewing process most probably allows full extraction of the acrylamide present in ground coffee to the brew. A survey of acrylamide in roast and ground coffees and instant coffees in the USA has revealed levels ranging from 45–374 μg/kg (n = 31, powder basis) and 169–539 μg/kg (n=*12*, powder basis), respectively (Andrzejewski *et al*., 2004). These numbers compare well with those for roast and ground coffee in the JRC database, i.e. median =

264 μg/kg, $n = 102$ (Dybing *et al.*, 2005) and in the scientific literature (Granby and Fagt, 2004).

The calculation of the acrylamide content per cup is an important term of exposure levels. For example, assuming an acrylamide content of 250 μg/kg in the powder, and a brew strength of 50 g/l, then one liter of coffee (6–7 cups) will result in an acrylamide uptake of 12.5 μg (considering total extraction of the acrylamide from the powder).

Raw materials

Coffee beans of the varieties Robusta and Arabica have been reported to harbor relatively low concentrations of free asparagine, typically in the range 0.3–0.9 g/kg (Stadler and Scholz, 2004). Robusta coffees contain – on average – slightly higher amounts of asparagine than do Arabica beans (CIAA, 2004). However, the relatively narrow range does not provide much opportunity for control by selecting/blending green beans with lower asparagine levels. Importantly, the blend contributes substantially to the sensorial properties of the final product. In addition, it is has been shown in model systems that at high temperatures above 220 ºC, i.e. as typically encountered during roasting, other marginal pathways may become important, such as the amino dehydroxylation of acrylic acid to furnish acrylamide (Stadler *et al.*, 2003; Yaylayan and Stadler, 2005; Yasuhara *et al.*, 2003).

Carbohydrates are present in green coffee beans at far higher concentrations than free asparagine. Sucrose ranges from 30–100 g/kg, and is far more abundant than the reducing sugars glucose or fructose (typically < 1 g/kg). Sugars decompose rapidly during the roasting process and contribute to the formation of advanced Maillard reaction products such as the melanoidins, but show no relationship to acrylamide formation.

Product composition

Roast coffee and soluble coffee are based 100% on pure green coffee, without addition of any extraneous material or other ingredients. Consequently, there are no possible mitigation options at the recipe stage, as any additives would forfeit the claim of pure coffee. Nevertheless, the possible application of asparaginase has been suggested in a patent filed by Procter & Gamble, but the efficacy can be expected to be marginal, mainly due to the difficulty of the enzyme solution penetrating the bean and reaching the substrate.

Process management

Coffee is typically roasted at temperatures in the range of 220–250 ºC, and the roasting time and speed of roast have an important impact on the sensorial properties (aroma/taste). These are carefully fine-tuned to achieve a characteristic profile leading to a clear identity of roasted coffee. Coffee beans are thus subjected to relatively higher temperatures than other foods, and as mentioned above one can expect more than one chemical pathway leading to acrylamide.

Experiments conducted by the food industry at the pilot scale and in a factory

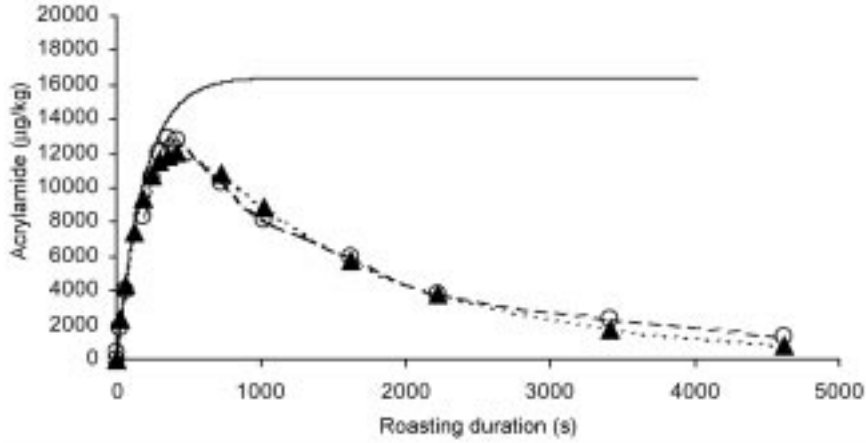

Fig. 2.3 Kinetic model to predict the formation of acrylamide in coffee during roasting (230 ºC) over time. Solid line = predicted amount taking into account no loss of acrylamide during the roasting process; open circles = predicted amount formed; shaded triangles = experimentally measured formation/loss over roasting time. Data kindly provided by Drs Remy Liardon and Viviane Theullerat, Nestlé Product Technology Centre, Orbe, Switzerland.

setting have shown that acrylamide is degraded/eliminated during roasting. The profile of acrylamide formation during the roasting cycle reflects this effect very clearly (Taeymans *et al.*, 2004). In coffee, acrylamide is formed at the beginning of the roasting step, reaching > 7 mg/kg, and then declining steeply toward the end of the roasting cycle due to higher rates of elimination. Kinetic models and spiking experiments with isotope labeled acrylamide have revealed that > 95% of acrylamide is lost during roasting (Fig. 2.3), either due to evaporation (b.p. 193 ºC) or degradation/polymerization. Increasing the degree of roast at constant roasting time leads to darker coffees and subsequently a slight decrease in acrylamide concentration.

Varying the total roast times (e.g., 1.5–8 minutes) by modifying the roasting temperatures does not change the maximum amount of acrylamide formed during the process. Longer roasting times may afford a minor reduction of acrylamide towards the end of the roasting cycle but with a major impact on the sensory properties of the product (CIAA, 2004). However, darker roasting as a potential option to reduce acrylamide could generate other undesirable compounds and negatively impact the taste/aroma of the product. Consequently, no practical solutions are today at hand that would reduce acrylamide levels and concomitantly retain the quality characteristics of coffee, since the roasting step cannot be fundamentally changed.

Stability of acrylamide in coffee

Two research groups have reported that acrylamide is not stable in commercial coffee stored in its original container (Andrzejewski *et al.*, 2004; Delatour *et al.*,

2004). Losses of 40–60% have been recorded in roast and ground coffees stored at room temperature over a period of 6–12 months (Delatour *et al.*, 2004). Acrylamide seems stable in coffee after brewing over a period of five hours (Andrzejewski *et al.*, 2004). Investigations are currently under way to understand the underlying mechanisms and possibly devise mitigation strategies based on this phenomenon.

2.4 Conclusion

Significant progress has been made in the past 2–3 years in understanding how acrylamide is formed in foods, and this knowledge has been shared rapidly and widely through different channels on a global scale. In the cereal product category, a number of options and tools have been identified that can be assessed. However, no common solutions are available and each manufacturer will need to identify the most promising tools based on the particular processes and product characteristics. Any intervention studies, however, should always be coupled to the sensory properties of the final product. Researchers working on approaches to reduce acrylamide often fail to consider this aspect. In this context, it is important to maintain the identity of a branded product, and any changes, however subtle in quality, may consequently impact consumer acceptability.

Coffee and certain cereal-based foods pose considerable challenges in devising adequate and practical approaches to reduce acrylamide, based in part on the multi-composition and/or diverse technologies that are employed. In the case of coffee, an additional uncertainty is that at the high temperatures of roasting, other marginal pathways of formation of acrylamide may become important. Despite intensive efforts on behalf of the food industry, no evident measures have been identified to date that could lead to a relevant reduction without impacting the important organoleptic properties and safety aspects of the final product.

Any efforts to reduce acrylamide intake via the diet must be concerted. Based on data presented at FDA/CFSAN (JIFSAN II), a theoretically complete removal of acrylamide, for example in coffee, would reduce the average exposure from 0.43 g/kg bw/d to 0.40 g/kg bw/d, i.e. 7% reduction. In the 90th percentile group, this reduction equates to 4.3%. Therefore, meaningful reduction can be achieved only by addressing all pertinent food groups and sources, i.e. home cooking/food service outlets and industrial processed food, concomitantly.

Major progress in reducing acrylamide has been achieved in some food categories such as, for example, potato crisps, French fries, and crisp bread. It is imperative that the food industry maintains its momentum and engagement in tackling the acrylamide issue, sharing across sectors in an open and transparent manner. Essentially, the short-term measures that have been identified and implemented are mainly at the recipe and processing levels. New technologies

and processes beyond those used in normal practice may provide future opportunities (e.g., steam roasting, asparaginase). In the case of cereal-based foods, further progress will need to address the raw material composition and thus entails long-term agronomical research. In general, any mitigation measures should be considered within a broader frame taking into account added risks or reduced benefits (nutritional). Currently the procedure by which such an analysis could be conducted remains to be established.

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3

The formation of acrylamide in potato products

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3.1 Introduction

The potato is the world's fourth major staple food crop, with a total estimated production of 328,865,936 metric tons in 2004 (FAOSTAT, 2004). Consumer demand encompasses fresh potatoes, and fried and roasted potato products. The economic significance of fried and roasted potato products, which exceeds that of fresh potatoes in several countries, is due to the Maillard reaction, which occurs during processing and has significant taste benefits. Along with staple products such as French fries and potato crisps, potato-processing companies have diversified their offerings by adding a number of semi-finished and finished products. The presence of acrylamide in fried and roasted potato products is a cause for major concern, and several projects have already begun to address this problem. Fundamental and applied research into acrylamide has explored the issues of product quality and consumer health.

Boiling potato tubers initiates several biochemical processes. Among others, the starch granules gelatinise, most of the organic nitrogen (in the form of protein) coagulates, the cell wall structure changes, and volatile components that are responsible for the typical potato flavour are generated (Mutti and Grosch, 1999). Further heating initiates the Maillard reaction, which is driven by free amino acids and carbonyl groups from reducing sugars (Roe *et al.*, 1990). As a result of the Maillard reaction, additional flavour components and dark-coloured pigments are formed (Roe *et al.*, 1990; Whitfield, 1992).

Acrylamide in fried and roasted potato products is formed via several chemical reactions, e.g. lipid degradation via acrolein (Yasuhara *et al.*, 2003), and enzymatic modification of the amino acid asparagine (Granvogel *et al.*,

2004; Zyzak *et al.*, 2003). The principal reaction involves the free amino acid asparagine and carbonyl groups, which are derived from carbohydrates and especially from reducing sugars (e.g., glucose, fructose) (Mottram *et al.*, 2002; Stadler *et al.*, 2002; Yaylayan *et al.*, 2003). Thus the reaction pathway is linked with the early Maillard reaction, including decarboxylation of the Schiff base, rearrangement to Amadori products, and finally a β-elimination reaction to release acrylamide (Yaylayan *et al.*, 2003). Biedermann *et al.* (2002a) have shown that the final acrylamide concentration in food is a result of concurrent formation and elimination reactions. Model studies with different food matrices (Amrein *et al.*, 2003; Becalski *et al.*, 2003; Biedermann *et al.*, 2002a,b) have demonstrated that specific factors determine the extent of acrylamide formation in different food categories.

The overall reaction efficiency is low in relation to the concentrations of the precursors (Stadler *et al.*, 2004). Potatoes are known to have a relatively high level of free asparagine (Brierley *et al.*, 1997; Mack and Schjoerring, 2002). The concentration of the second precursor, carbonyl groups from reducing sugars, is much lower but varies widely, depending on several factors to be discussed below. Consequently, acrylamide formation in potatoes is limited by the concentration of reducing sugars, and hence these must be controlled in the raw material.

3.2 Acrylamide and the raw material

Consistent quality of the raw material is critical in potato processing, because a high variability between batches will lead to large variations in the quality of the final products. Potato tubers (*Solanum tuberosum* ssp. *tuberosum* L.) are vegetative parts of the plant, grow underground, and are therefore influenced by environmental factors, and the wide range in composition that can result is shown in Table 3.1.

Asparagine (Surdyk *et al.*, 2004) is the most important amino acid in acrylamide formation, and was found to comprise 39% of total free amino acids (Gerendas *et al.*, 2004). In a set of nine cultivars, grown at two locations in 2003,

Table 3.1 Composition of potato tubers (average values, according to Lisinska and Leszczynski, 1989)

Composition	% fresh weight
Starch	8–29.4
Protein	0.69–4.63
Organic acids	0.4–1.0
Minerals	0.44–1.87
Crude fibre	0.17–3.48
Reducing sugars	0–5.0
Lipids	0.02–0.2

the concentration of free asparagine (ASN) ranged between 58.7 and 143 mmol ASN/kg dry matter (DM). The corresponding values of reducing sugars (RS) were between 3.64 and 13.8 mmol RS/kg DM (Haase *et al.*, 2004). Sucrose can be used as an indicator for the reducing sugar potential with regard to enzymatic cleavage and thermal breakdown. It also indicates maturity, an important tool for post-harvest physiology (Sowokinos and Preston, 1988).

Reducing sugars, defined as the sum of glucose and fructose and regardless of other reducing sugars, have been studied for some time because colour formation in heated potato products that undergo the Maillard reaction is closely connected with reducing sugars (see Kumar *et al.* (2004) for a recent review). In a stepwise regression analysis, around 90% of the colour variation could be explained by reducing sugars (Roe *et al.*, 1990), while free asparagine was not correlated with Maillard-derived colour (Rodriguez-Saona and Wrolstad, 1997).

Continuous monitoring of sugar concentration in tubers during storage is important for determining when critical concentrations are reached. Different strategies have been developed by the industry to control sugar levels, using laboratory techniques or rapid test procedures. As well as the concentration of reducing sugars, glucose and sucrose levels are also used as criteria for accepting or refusing a batch of potatoes. The acrylamide issue has raised awareness of this approach among the different stakeholders.

3.2.1 The cultivar

Potato breeding companies have introduced many potato genotypes to address the large range of specific requirements, from the very basic, such as the length of the growing season, to complex internal quality tools that relate to the final use of the potatoes, e.g., fresh consumption, dehydrated or fried food, starch isolation, ethanol production, or animal feeding. About 4000 cultivars are listed in the *World Catalogue of Potato Varieties* (Hils and Pieterse, 2005), but most of these cultivars result in fairly poor sensory and nutritional quality after processing. Breeders have therefore started breeding programmes aimed at enhancing final product quality. The number of useful cultivars is considerably restricted because the genotypes determine the mean concentration, expected range, and storage behaviour of reducing sugars. In the German list of potato varieties, one section contains cultivars with processing potential (Bundessortenamt, 2005). Seventy-one out of 209 cultivars are suitable for frying processes, but only 15 cultivars are used for more extensive operations (Haase, 2005). Unfortunately, it has been shown that a quantitative relationship between crisp colour and specific compositional or environmental factors determined for one cultivar cannot be used to predict the colour of crisps made from other cultivars (Loiselle *et al.*, 1990). Therefore, individual test procedures have been developed to estimate the processing facilities of each cultivar. Most breeding companies also organise field trials, to evaluate the best cultivars for different local conditions.

Crisp colour is correlated to sugars, but a quantitative trait loci (QTL)

Table 3.2 Cultivar-dependent sucrose and reducing sugar level of four cultivars, grown over seven years at three locations; combined harvest and after storage data (Haase, 2005)

Cultivar	n	Sucrose (mg/100 g FW)		Reducing sugars (mg/100 g FW)	
		Average	Std dev.	Average	Std dev.
Erntestolz	41	494[a]	275	97.1[a]	67.1
Panda	42	331[b]	119	82.5[ab]	75.8
Sempra	42	325[b]	111	40.4[b]	25.8
Saturna	42	278[b]	93.9	65.4[c]	49.7

[a,b] A significant difference (LSD-test; $p < 0.05$) between two samples exists if their codes have no letter in common.

analysis to identify genetic factors that contribute to crisp colour suggested that additive effects contribute a significant portion of the variation in crisp colour (Douches and Freyre, 1994). The overall correlation in an experiment with four German crisp cultivars, grown over seven years at three locations, was weak. At harvest time, sucrose accounted for 3% of crisp colour and reducing sugars for only 29%. After storage at 8 ºC for five months, the values were 23% and 38%, respectively (Haase, 2005). Table 3.2 shows cultivar-dependent differences in sucrose and reducing sugars from that experiment. Both sucrose and reducing sugars were different between cultivars. Absolute sucrose level was lowest for Saturna and highest in Erntestolz, reducing sugars were lowest in Sempra and highest in Erntestolz.

3.2.2 Growing conditions

The vegetative character of the potato tuber has a direct impact on the final food quality, so agronomic factors are important in relation to how the potatoes will be processed.

Temperature

The potato crop has few requirements with regard to climate, but low temperatures (Grob *et al.*, 2003), especially during the final growing stage, will result in unacceptably high sugar levels in the tubers (Ezekiel *et al.*, 1999). Furthermore, high temperatures (above 25 ºC) also result in elevated sugar levels, because increased respiration has a negative effect on the rate of starch biosynthesis (Krause and Marschner, 1984), which is followed by a decline in 3-phosphoglycerate (Möller, 2004) and hence inhibition of ADP-glucose pyrophosphorylase and starch synthesis (Geigenberger *et al.*, 1998). Sucrose and reducing sugars were found to behave differently at higher temperatures, with sucrose increasing but reducing sugars decreasing, independently of sunshine intensity (Kolbe, 1996). Consequently, the level of reducing sugars will be lowest at moderate temperatures, between 15 and 25 ºC.

Fertilisation

Anorganic and organic fertilisation strategies influence the composition of potato tubers as a whole and the content of acrylamide precursors in particular, but data are sometimes inconclusive and conflicting because several promoting and opposing mechanisms are involved. Specific cultivars respond differently to the level of nutrients in the soil and their availability over time, and this may also affect any response to additional nutrient dressing. Weather conditions during the growing season influence the biochemical reactions in both soil and plant, along with the nutrient levels in the root space. As a result, many field trials have only a limited expressiveness and cannot be generalised.

A balanced nutrient supply will result in high yields with acceptable internal qualities. Any shift moves the balance and the final quality drops. Specific interactions with the nutrient offering have been identified for most cultivars. Amrein *et al.* (2003) investigated ten potato cultivars under different farming systems (organic, integrated, conventional) for their response to nitrogen supply (50–200 kg N/ha). The acrylamide formation potential was correlated to the content of reducing sugars, and was up to 2000 g acrylamide per kg potato (determined according to Biedermann *et al.* (2002a)). No significant relationship was found with the farming system or the nitrogen regime. Adding nitrogen typically increases yield when applied in a redundant way, but also leads to an accumulation of amino acids, and amides in particular (e.g., asparagine) (Marschner, 1995). A pot experiment under low potassium with the cultivar Agria confirmed this (Gerendas *et al.*, 2004). In the same experiment, the concentration of reducing sugars increased, particularly at higher and intermediate doses of nitrogen.

Potato production for the crisp industry is sometimes linked with specific potassium strategies, because this nutrient is known to influence dry matter and reducing sugar content. In a pot experiment (cultivar Agria), sugars were reduced by increasing potassium supply (Gerendas *et al.*, 2004), and this was attributed to osmotic homeostasis (Marschner, 1995).

Nutrient interactions between nitrogen, potassium and phosphorus inside tubers were investigated by Kolbe (1996), and demonstrated different reactions for reducing sugars and sucrose. Increasing nitrogen levels were accompanied by a drop in reducing sugars, and potassium or phosphorus variations had little effect. On the other hand, sucrose increased at higher levels of phosphorus, and a high nitrogen level was necessary to reduce sucrose concentration. No interaction was detected between nitrogen and potassium.

Growing location

Soil type and local atmospheric conditions are expected to influence tuber size, distribution and internal composition, and therefore processing quality, but a study of three growing locations used for crisp cultivars indicated only non-significant differences among the average values (Table 3.3). Processing companies contract potato production to specific areas, avoiding areas that are known to produce potatoes of inferior quality, and also refrain from mixing potato batches from different sites, which could also reduce the overall quality.

Table 3.3 Location-dependent sucrose and reducing sugar levels of four cultivars, grown over seven years at three locations; combined harvest and after storage data (Haase, 2005)

Location	n	Sucrose (mg/100 g FW)		Reducing sugars (mg/100 g FW)	
		Average	Std dev.	Average	Std dev.
A	56	335[a]	198	75.3[a]	69.3
B	55	384[a]	161	57.9[a]	36.3
C	56	350[a]	189	80.2[a]	69.8

[a] A significant difference (LSD-test; $p < 0.05$) between two samples exists if their codes have no letter in common.

Maturity

The storage potential of potatoes is largely determined by the maturity of the tubers at lifting, which is influenced by growth conditions and date of harvest (Pritchard and Adam, 1992). Potatoes are physically mature when vines have senesced and tubers have developed a degree of skin set. However, tubers may be physically mature without being chemically mature (i.e. low sugar concentration). To predict final tuber quality, a model was designed that describes the accumulation of reducing sugars in potato tubers as a function of both time and temperature (Hertog *et al.*, 1997).

The level of sucrose in crisp potatoes should be as low as possible, to minimise the accumulation of reducing sugars during long-term storage. Sowokinos and Preston (1988) propose an upper level of 150 mg/100 g FW at harvest time.

3.2.3 Post-harvest facilities

Lifting potatoes and post-harvest treatments induce cultivar-specific stress reactions. Cell wall damage induced by mechanical action can be directly linked to increased respiration rates and sugar levels (Pisarczyk, 1982). Generally, stored potatoes experience a physiological drift that affects their composition, including reduction in turgor and remobilisation of many components through respiration and sprouting, e.g. starch. Different cultivars differ in their ability to form reducing sugars in storage, possibly because of invertase activity and/or compartmentalisation of sucrose (Sowokinos *et al.*, 1989). Along with genetic control, the environment also has an influence. Unusually warm periods during growth result in reduced dormancy, possibly even resulting in a break in dormancy before lifting.

The impact of the high variability of the growing season is shown in the content of sucrose and reducing sugars in a set of four cultivars grown at three locations (Fig. 3.1). Increases in sugar concentration between spring and autumn values depend on the growing season. A given value at harvest time did not

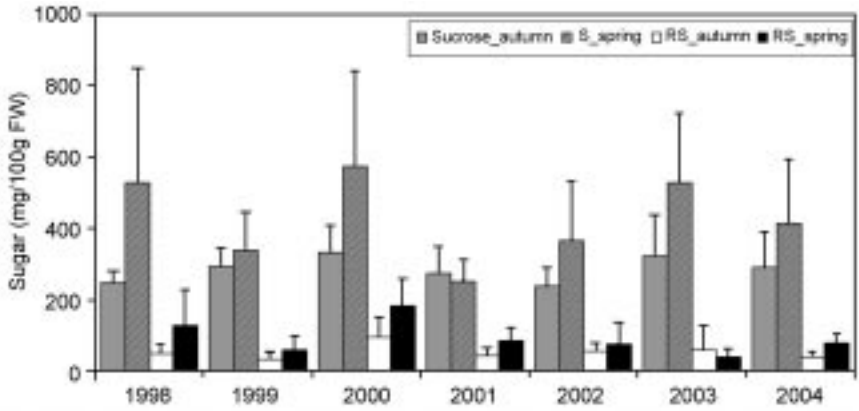

Fig. 3.1 Average sucrose and reducing sugars concentration of four crisp cultivars from 1998 to 2004 (samples from three locations, each analysed in autumn and in spring; vertical bars indicate the standard deviation of values) (Haase, 2005).

mean that the increase during storage could be predicted. In field trials, Kolbe *et al.* (1995) indicated a positive correlation between harvest and long-term storage for sucrose levels, but not for reducing sugars.

During dormancy, sprouting is completely inhibited, even under ideal conditions for sprouting (Reust, 1984). Sprouting itself is associated with the remobilisation of high molecular carbohydrates (mainly starch). The onset and development of sprouts is accompanied by a serial of biochemical reactions, including an increase in reducing sugars. To prolong dormancy, tubers can be stored at temperatures below 8–10 ºC, but that regime leads directly to 'cold sweetening' (Coffin *et al.*, 1987), an enrichment of low molecular carbohydrates along the respiration pathway. Potato tubers for fresh consumption are regularly stored under such conditions, and typically taste sweet in spring, indicating a high sugar level. These potatoes are not suitable for fried products, even in the domestic sector.

Potatoes for processing are stored at 8–10 ºC, and anti-sprouting chemicals (natural or synthetic) are applied to prevent sprouting (Kleinkopf *et al.*, 2003). The primary method for controlling sprouting in storage is still post-harvest application of isopropyl *N*-(3chlorophenyl) carbamate (chlorpropham, or CIPC). CIPC inhibits sprout development by interfering with cell division. Among the different natural substances available, carvone (monoterpene from caraway seed) has become popular, and is licensed in several countries. Carvone treatments were found to have no negative effect on the sensory quality or fry colour of the potatoes (French fries) in comparison with CIPC (Hartmans *et al.*, 1995).

During cold-induced sweetening in stored potatoes, starch degradation occurs primarily through the action of starch phosphorylase. Reducing sugars may accumulate through various enzymatic reactions (Sowokinos, 1990) along the biochemical pathway of respiration. Three key loci are involved in the process of sweetening and reconditioning and one or more genes may be common to

Table 3.4 Sucrose and reducing sugar content in five processing cultivars, stored at 8 and 4 ºC, respectively (2002 data, average of samples from three growing locations) (Haase, 2005)

Cultivar	Criterion	Harvest	8 ºC-Store	4 ºC-Store
			(mg/100 g FW)	
Agria	Sucrose	144 ± 17	174 ± 70	367 ± 133
	Red. sug.	54 ± 2	45 ± 7	225 ± 62
Bintje	Sucrose	139 ± 25	397 ± 119	272 ± 109
	Red. sug.	133 ± 39	168 ± 50	281 ± 69
Panda*	Sucrose	249 ± 50	376 ± 79	389 ± 169
	Red. sug.	57 ± 26	40 ± 19	103 ± 39
Saturna	Sucrose	210 ± 52	229 ± 45	290 ± 85
	Red. sug.	69 ± 11	62 ± 30	159 ± 69
Sempra*	Sucrose	254 ± 69	282 ± 90	473 ± 275
	Red. sug.	24 ± 5	31 ± 12	78 ± 54

* Cultivar, suitable for cold storage.

both processes. Alleles A and B for the enzyme Uridine-5-diphosphoglucose pyrophosphorylase (UGPase) were present in different ratios in cold-resistant and cold-susceptible cultivars. Cold resistance to sweetening was found in a specific UgpA:UgpB ratio favouring the UgpA allele, while cold-sensitive cultivars exhibited predominance for the UgpB allele (Thill and Peloquin, 1994).

Potato breeders have tried to overcome the cold sweetening effect, so that tubers can be stored at low temperatures without sugar accumulation and without any anti-sprouting treatment. The list of German potato cultivars includes six cultivars with this characteristic (Bundessortenamt, 2005). These cultivars (e.g., cv. Panda) showed a disproportional increase in sucrose concentration when stored at 4 ºC, whereas the content of reducing sugars remained low compared with typical processing cultivars (Agria, Bintje, Saturna) (Geigenberger *et al.*, 1998) (Table 3.4). The genetic background of cold sweetening is under investigation and the first GMO lines are being tested (Lynch *et al.*, 2003; Sowokinos, 2002).

A rise in sugar levels during the first storage period can be reversed, in a process known as reconditioning. Increasing storage temperature to about 15 ºC for several days allows the accumulated sugar to be respirated. In contrast, senescent sweetening of tubers is characterised by an irreversible reaction, which generally occurs after prolonged storage. Senescent sweetening is influenced by a number of other factors besides storage temperature, including storage period, genotype, growth history, and soil and environmental conditions at the time of lifting (Isherwood, 1976; Williams and Cobb, 1992).

Storage atmosphere

During storage, the tubers release carbon dioxide through respiration, and an increase in carbon dioxide levels can be observed in modern storage facilities

that have impermeable walls and low ventilation (Veerman, 1998). Cultivar and season-dependent rises in reducing sugars and sucrose have been observed under such storage conditions (Mazza and Siemens, 1990), therefore a change in storage atmosphere could be beneficial. A significantly lower oxygen level in the storage atmosphere would decrease the level of reducing sugars in the products, even at low storage temperatures (Harkett, 1971), because a very low oxygen concentration inhibits the cold-induced enzymes, such as invertase, alternative oxidase and one form of amylase (Zhou and Solomos, 1998).

3.3 Acrylamide and potato processing

Consumers are attracted by products that reduce food preparation time. Often these products are frozen, for easier handling and longer shelf-life. Potato products range from peeled potatoes, which still have to be cooked, to finished products. Acrylamide formation occurs in both industrial cooking and domestic preparation. In the latter case, the amount of acrylamide formed depends on the intensity of heat, and may contribute a substantial part of the overall daily acrylamide intake. Industrially finished products are controlled by national food authorities, who use processing guidelines and other regulations to ensure reduced acrylamide content in food. The dynamic minimisation concept in Germany aims at a gradual reduction of acrylamide content, agreed between the authorities and the German food industry. Acrylamide data collected from official food surveillance laboratories are evaluated for the different food groups. If acrylamide contents are above a specific signal value, food control authorities will contact the food producers concerned and they will enter into the minimisation dialogue to check whether ingredients or processes should be changed to minimise acrylamide contents, and identify which changes are necessary (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit – BVL, 2004).

Reducing the potential for acrylamide formation depends on two things, reducing the precursors in the raw materials, and optimising heat treatment to minimise acrylamide formation. Both these factors must be considered carefully, because the overall organoleptic and nutritional quality of the products must be maintained.

3.3.1 Semi-finished products

The potato industry has developed a number of par-fried potato products, either chilled or frozen. They are characterised by acrylamide formation during final preparation, whether in restaurants, by catering services, or in the domestic situation. Actual human exposure to acrylamide is highly variable because cooks and consumers have different preferences regarding discoloration and have developed individual preparation techniques to meet their specific requirements. In addition, professional and domestic appliances have widely varying heat

control facilities, and cooking or re-heating instructions may not always be followed accurately. Despite these uncertainties, a number of influences on final acrylamide potential have been identified for industrial preparation.

Size

The surface to volume ratio is particularly important because acrylamide formation typically occurs in the outside layer of the products. Croquettes and related products were found to have a low acrylamide potential, whereas potato fritters and Swiss *Rösti* were found with a broad range of acrylamide values, ranging from 15 to 2779 μg/kg food (European Commission Joint Research Centre, 2004). A direct link with the composition of the raw material was demonstrated in an experiment with Swiss *Rösti* (Hebeisen *et al.*, 2005).

Both home-made and industrially par-fried French fries are cut with different geometries. Coarse-cut strips (14 × 14 mm) with a surface to volume ratio (SVR) of 3.3 cm^{-1} resulted in significantly lower ($P < 0.05$) amounts of acrylamide than fine-cut strips (8 × 8 mm) with a SVR of 5.4 cm^{-1}, using a constant frying time (Matthäus *et al.*, 2004).

Reducing acrylamide precursors

A strategy to produce French fries with a very low acrylamide level was designed by Grob *et al.* (2003), based on lowering the concentration of reducing sugars. After careful selection of the potato tubers (cultivar × storage conditions), fine strips from the outer part of the tubers were removed, because they reach the optimum frying temperature earlier than the bulk, rapidly turn brown and contain high levels of acrylamide. This is accentuated because the sugar content in the peripheral layers of the potato is particularly high (Grob *et al.*, 2003). The cut potatoes were immersed in water for several minutes to reduce the amount of precursors in the surface layer. Finally, the fryer was loaded with no more than 100 g potato per litre of oil.

To improve the golden-yellow colour of the finished product, French fries are sometimes dipped in glucose or sugar solution before par frying. Analyses have shown that this produces a concentration-dependent effect on acrylamide formation. The acrylamide content increased with finishing temperature at all glucose concentrations. At glucose contents above 0.5%, acrylamide concentrations increased more substantially (Taeymans *et al.*, 2004). The use of sugar dips should therefore be reconsidered.

pH value of the soaking or blanching medium

Immersing potatoes in organic acids (e.g., citric acid) did not reduce the content of glucose and asparagine in slices when compared with a control, but acrylamide formation was reduced by almost 70% for slices fried at 150 °C. Higher frying temperatures did not show this (Pedreschi *et al.*, 2004). Jung *et al.* (2003) described a pH drop of about 1.0 and 1.3 units in an experiment with French fries, when using a concentration of about 10 g/litre and 20 g/litre citric acid, respectively. Acrylamide formation was inhibited up to 80% even at higher

frying temperatures. The reduction of acrylamide formation seemed to be due to both the lowering of pH and leaching of precursors. The colour was not affected, but a slightly sour taste and harder texture were described with the 20 g/litre citric acid dipping, indicating an upper critical level of additives.

Temperature

An experiment with cooking temperatures for French fries indicated that the final acrylamide concentration was strongly related to the temperature at which the par-fried material was finished, and not related to the par-frying temperature (Taeymans *et al.*, 2004). The content of acrylamide increased with both temperature and frying time. While the amount of acrylamide was relatively low at temperatures between 150 and 175 ºC, there was a dramatic increase when the temperature reached 180–190 ºC (Matthäus *et al.*, 2004). The increase of acrylamide formation at constant temperature followed a linear function, with greater slope at higher temperatures. Varying temperature at constant frying time resulted in an exponential shift of the acrylamide concentration in the product, with disproportionate acrylamide concentrations at higher temperatures (Matthäus *et al.*, 2004; Taeymans *et al.*, 2004) (Fig. 3.2).

Low frying temperatures are linked with negative quality effects, such as higher fat uptake and poorer texture (Taeymans *et al.*, 2004). It is also important to monitor the final moisture content of the French fries, so that they are not too dry (lower than 38%) or too soft and wet (higher than 45%), otherwise they will be rejected by the consumers. In the experiment described above, both increasing temperature and increasing frying time led to a lower final moisture

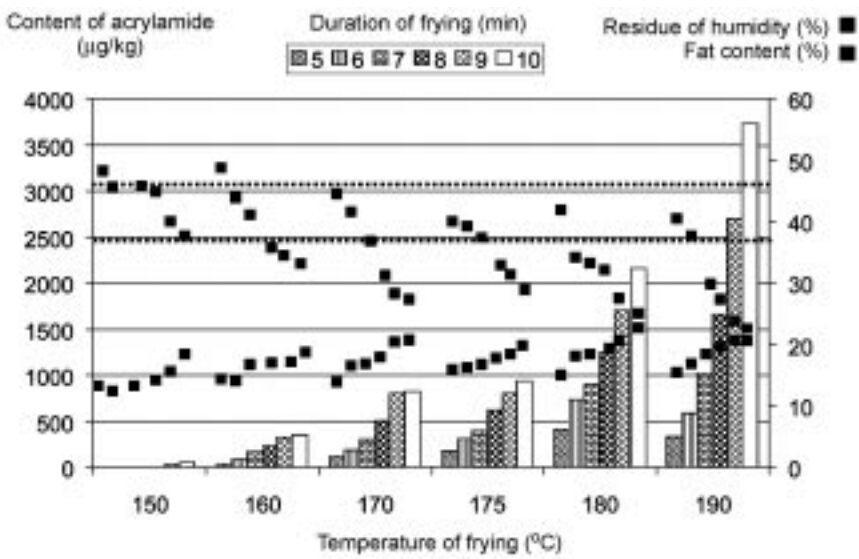

Fig. 3.2 Finishing frying of par-fried French fries and its relationship between acrylamide, final moisture and fat content (Matthäus *et al.*, 2004).

content in the product. While frying at 150 ºC resulted in an acceptable moisture level, several other samples prepared at higher temperatures became too dry. Since acrylamide formation in the product follows the Maillard reaction, high processing temperatures promote the formation of acrylamide due to the fast reduction of water content in the outer layers of the product, thus these French fries contained higher amounts of acrylamide.

Frying medium

The influence of several edible oils on acrylamide formation in French fries was compared by Matthäus *et al.* (2004), but no significant differences could be observed. Gertz and Klostermann (2002) detected higher acrylamide levels in French fries fried with Palmolein compared with rapeseed or sunflower oil. The oil used for finish frying on a commercial level frequently involves additives, to reduce surface tension. As a result, frying may take place at a slightly reduced temperature without prolonging the duration. Grob *et al.* (2003) compared French fries of similar quality, and found that while acrylamide contents might be marginally lower, the differences remained at the limit of being significant.

The effect of oil quality on acrylamide concentration was tested by analysing the oligomer triglycerides. Heat degradation forms glycerol, which reacts with acrolein, a potential source of acrylamide (Yasuhara *et al.*, 2003), but despite using progressively older oil, there was no noticeable increase in acrylamide (Matthäus *et al.*, 2004). The influence of dimethylpolysiloxane (DMPS) (silicone oil, E900) on acrylamide formation is controversial. DMPS is permitted as an antifoaming agent in frying fats and oils. It acts by excluding foam-forming substances from the interface between the liquid and gaseous phase, by forming an entire interfacial film or by increasing the interfacial tension of water. Gertz and Klostermann (2002) found a higher level of acrylamide in French fries prepared with DMPS, whereas Matthäus *et al.* (2004) did not.

Modified cooking advice

Providing consumers or end-users in restaurants and catering with modified preparation instructions can reduce acrylamide formation without affecting product quality (aid Infodienst Verbraucherschutz Ernährung Landwirtschaft, 2002):

- maximum finish frying temperature of 175 ºC (formerly 180 ºC or above)
- do not overcook – finished product should be ‘golden yellow’ (formerly ‘golden brown’)
- do not prepare more than 100 g potatoes per litre of oil
- lower final oven temperature for ‘baked’ French fries to 200 ºC (180–190 ºC in fan ovens).

3.3.2 Industrially finished products

The market for industrially finished products is dominated by potato crisps (US ‘chips’), products made either from cut or sliced potatoes or from a potato dough

(flakes or granules) and other ingredients, e.g. cereal products. Potato crisps made from cut potatoes have been popular for over 150 years. Crisps directly reflect the composition of the raw material (see Chapter 2), whereas for other products it may be possible to reduce the amount of acrylamide precursors, e.g. by substituting some critical ingredients. The potato flakes or granules used in crisps may be high in reducing sugars, due to the typical processing methods for dehydrated potatoes and wet reconstitution.

Reducing acrylamide precursors

Reducing the concentrations of acrylamide precursors will reduce the acrylamide formation potential of the finished product. Soaking sliced tubers in distilled water for 90 minutes reduced sugar content by about 30%. The resulting decrease in acrylamide formation was directly related to the frying temperature (20–38% reduction at frying temperatures of 150, 170 and 190 ºC) (Pedreschi *et al.*, 2004). Blanching the sliced potatoes was more effective and reduced glucose by 76% and asparagine by 68% (Pedreschi *et al.*, 2004). This resulted in a very significant reduction in acrylamide formation (Haase *et al.*, 2003), but unfortunately that reduction was accompanied by a loss of textural quality, based upon changes within cell wall components, especially pectin and esterification of pectin (Haase, 2001). Immersing the potato slices in organic acids (e.g., citric acid) did not significantly reduce glucose and asparagine content, but acrylamide formation was reduced by almost 70% against a control for slices fried at 150 ºC. Surprisingly, this effect was not present at higher frying temperatures (Pedreschi *et al.*, 2004).

Heat treatment

Frying time must be adjusted if the frying temperature is changed, in order to keep the final moisture content below 3% and thus preserve crispiness. The acrylamide concentration of the crisps followed a non-linear progression line (Fig. 3.3) with a correlation coefficient of $r = 0.92$ against frying temperature. Low frying temperatures resulted in moderate acrylamide concentrations despite the longer frying time, whereas temperatures above 170 ºC led to a sharp increase. As the temperature decreased from 180 to 165 ºC, the acrylamide content in potato crisps fell by 51% during traditional frying (Granda *et al.*, 2004). The colour intensity of the product (expressed as a*-value <red-green>) followed the frying temperature with $r = 0.96$ (Fig. 3.3). A calculation of the temperature load (temperature $\times$ time) indicated a non-linear decline at increasing frying temperature, inverse to the acrylamide increase (Table 3.5).

With regard to temperature-dependent acrylamide formation, a negative temperature shift during frying was tested, which heightened the absolute temperature load. After an initial frying temperature of 170 ºC over 90 seconds, frying was finished at a lower temperature (Table 3.6). The temperature gradient indicates that frying time had to be adapted in order to get a comparable product with a suitably low moisture content. Colour and acrylamide content decreased with decreased temperature loads, whereas fat content increased as temperature

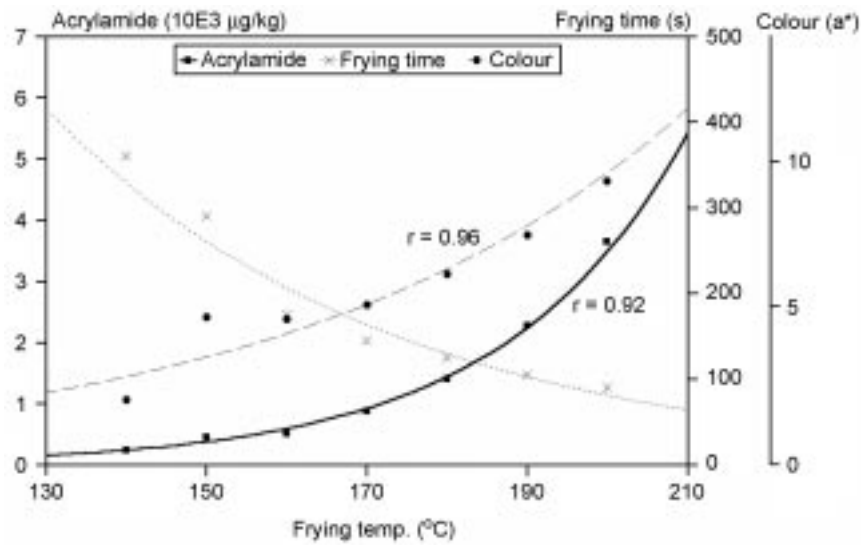

Fig. 3.3 Acrylamide concentration and colour (a* < red-green) of potato crisps and corresponding frying time in relation to frying temperature (Haase *et al.*, 2004).

Table 3.5 Temperature load (temperature × time; $T \times t$ [°Cs]) of potato crisps in relation to the frying regime (Hasse, 2005)

No.	Temperature (°C)	Temperature load (°Cs)	Acrylamide (μg/kg)
1	140	50,400	240
2	150	43,500	461
3	160	28,000	517
4	170	24,650	879
5	180	22,500	1410
6	190	19,950	2280
7	200	18,000	3650

Table 3.6 Quality profile of potato crisps produced with different temperature profiles (Hasse, 2005)

No.	Temp1 (°C)	Time (s)	Temp2 (°C)	Temperature load (°Cs)	Colour (a*)	Fat (%)	Acrylamide (μg/kg)
1	170	140	–	23,800	5.69	42.3	2990
2	170	90	160	34,500	6.98	51.7	1960
3	170	90	150	40,050	5.87	59.8	1290
4	170	90	140	42,600	5.05	59.2	968
5	170	90	130	44,550	3.45	58.4	318

load decreased, indicating the need for further optimisation tools, e.g. de-fatting techniques. Otherwise, the fat uptake would become extremely high and the snack would be soft. Reducing air pressure to achieve a lower boiling point could provide a solution. Several experiments have indicated a significant reduction in acrylamide formation, e.g. by 63%, under vacuum frying at lower temperatures, from 140 to 125 ºC (Granda *et al.*, 2004). The organoleptic quality of the vacuum fried crisps was not significantly different to normal frying for texture and flavour characteristics (mouth feeling, flavour), whereas colour (b-value) was significantly different between frying methods (Granda *et al.*, 2004).

The inverse technique with a pressurised frying system is used in some special cases to reduce frying time (especially fabricated crisps), but it must be carefully assessed to determine if the anticipated reduction in temperature load will break the inverse relationship with acrylamide (see above).

3.4 Conclusion

Research into potato food products has shown several starting points for a systematic and permanent reduction in acrylamide formation, but modifications of a single factor often carry the risk of quality loss in the finished product. Therefore, a combination of several approaches is needed to reduce acrylamide levels while preserving overall food quality.

As a consequence of the considerable variation in amounts of acrylamide precursors in potato tubers, the concrete acrylamide potential differs enormously. As well as cultivar-specific conditions, there are several other factors that influence the content of free asparagine and reducing sugars. Estimating or predicting the expected acrylamide level during processing is difficult or almost impossible, even in a specified product. Therefore, minimising acrylamide levels requires new or adjusted strategies in potato production. Breeding new cultivars with low sugar levels seems to be the easiest solution, but other quality criteria, such as organoleptic, growing or processing attributes, must also be met if the cultivar is to be acceptable to processors and consumers.

Acrylamide potential can be influenced by the effects of storage conditions on the production of reducing sugars in the tubers. Greater and more frequent quality control will make it possible to modify storage parameters (e.g., temperature, humidity, carbon dioxide concentration) as necessary.

The processing methods are ultimately responsible for the formation of acrylamide, with high temperatures enhancing acrylamide levels. However, lowering the processing temperature below a certain level will reduce the quality of the finished product due to increased fat uptake and an adverse effect on texture. Thus a reduced temperature with a prolonged heating time should be combined with other treatments. Vacuum frying is a useful technique, but typical quality criteria must be observed to determine the best frying parameters.

Blanching cut potatoes reduces the amount of acrylamide precursors, but this technique may also have some negative results. A high leaching intensity

negatively impacts on food quality. Implementing or prolonging the blanching step requires new or modified blanching units, which may require major modifications to existing plants. An enzymatic reduction of asparagine in cut potatoes and in potato dough by asparaginase is under investigation, but its effectiveness is not yet established because it adds to the overall processing time.

Changing the design of frying units to reverse the flow direction of the heated oil may alter the thermal load, which will reduce acrylamide levels in finished products. Semi-finished products should be accompanied by precise preparation advice, and consumers need to be educated so that the required change in overall thermal load of the potato food product is achieved.

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4

Mechanism for the formation of PhIP in foods

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4.1 Introduction

Heterocyclic aromatic amines (HAs) are substances with a high mutagenic and carcinogenic potential (Sugimura, 1997). They occur in heated meat and fish and it has been suggested that the Maillard reaction is involved in their formation. At the beginning of last century, Maillard proposed the browning reaction to account for the brown pigments and polymers produced from the reaction of the amino group of an amino acid and the carbonyl group of a sugar. The chemistry underlying the Maillard reaction is very complex. It encompasses not one reaction pathway but a whole network of various reactions. The original comprehensive reaction scheme of Hodge (Hodge, 1953) has been improved continuously since that time. At some stages of the browning reaction, e.g., pyrazines (Hwang *et al.*, 1994), quinoxalines (Morita *et al.*, 1981), and pyrido[3,4-d]imidazoles (Baltes and Gi, 1995), are formed that are involved in the formation of HAs. The formation of mutagens during the Maillard reaction was shown by the groups of Spingarn (Spingarn and Garvie, 1979), Shibamoto (Shibamoto *et al.*, 1981), and Wei (Wei *et al.*, 1981).

The formation of the HAs can be studied in chemical model systems (Jägerstad *et al.*, 1998). The advantage of the model system is that complex side reactions are reduced and reactions from other constituents of meat that are not involved in the formation of the HAs are excluded. Additionally, some of the HAs were first identified in the model systems and later found in heated meat. The first published model systems where mutagenic compounds were identified were pyrolysis reactions of amino acids and proteins. Other food constituents did not form mutagenic substances during pyrolysis (Nagao *et al.*, 1977b; Sugimura *et al.*, 1977). The substances identified were the same as found in the charred

parts of roasted or grilled meat and fish (Nagao *et al.*, 1977a). Using a reflux model system, Jägerstad *et al.* (1983) verified that IQ and IQx compounds were formed from the precursors creatine, amino acids and sugar. This review will give an overview of the investigative work on formation of PhIP using model systems.

4.2 Formation of PhIP

PhIP was first identified by the group of Felton (Felton *et al.*, 1986). The molecular structure and carbon numbering of PhIP are shown in Fig. 4.1. Using model systems Shioya showed that phenylalanine, creatinine and glucose were probable precursors of PhIP (Shioya *et al.*, 1987). By dry heating of ^{13}C-labelled phenylalanine and creatinine it has been convincingly demonstrated that phenylalanine and creatinine are precursors of PhIP (Felton *et al.*, 1986). PhIP may also be produced from creatine heated together with leucine, isoleucine and tyrosine (Johansson *et al.*, 1996; Övervik *et al.*, 1989). Accordingly, glucose seems not to be a necessary precursor using dry heating conditions (Skog *et al.*, 1998). However, glucose was found to have a considerable influence, either enhancing or inhibiting depending on its concentration, on the yield of PhIP produced from phenylalanine and creatine in a liquid model system (Skog and Jägerstad, 1990) and also during dry heating (Felton *et al.*, 1986). Manabe reported that a tetrose (erythrose) is the most active in the formation of PhIP, when phenylalanine and creatinine dissolved in water are heated at temperatures of 37 and 60 ºC (Manabe *et al.*, 1992). The other carbohydrates namely arabinose, ribose, glucose and galactose are not as active. This group found PhIP in heated mixtures of creatinine, phenylalanine and aldehydes (Manabe *et al.*, 1992), as well as in mixtures of phenylalanine, creatinine and nucleic acids (Manabe *et al.*, 1993). The 4′-hydroxy derivative of PhIP was found in an analogous reaction using tyrosine instead of phenylalanine (Wakabayashi *et al.*, 1995). Skog and Jägerstad investigated in detail the influence of glucose added to the model system. They showed that the addition of glucose to a mixture of phenylalanine and creatinine increased the formation of PhIP. However, higher than equimolar amounts of glucose lead to a decrease of the formed PhIP (Skog and Jägerstad 1991; Skog *et al.*, 1992).

Further experiments were carried out to identify the intermediates of PhIP formation. One possible intermediate is phenylacetaldehyde which is a Strecker degradation product of phenylalanine and is also formed by thermal degradation at

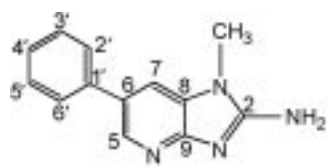

Fig. 4.1 Molecular structure and used carbon numbering of PhIP.

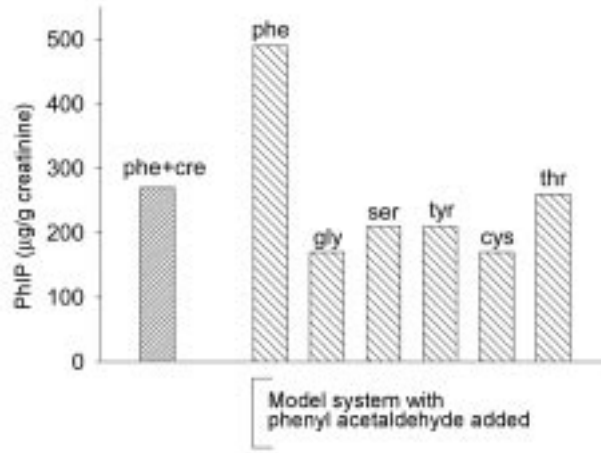

Fig. 4.2 Comparison of the formation of PhIP using a model system with phenylalanine and creatinine (phe + cre) with phenylacetaldehyde and creatinine in a first reaction and amino acids added (phe, gly, ser, tyr, cys, thr) ten minutes after the start of the reaction (Zöchling and Murkovic, 2002).

temperatures at which PhIP is usually formed. Testing a mixture of phenylacetaldehyde and creatinine with an additional nitrogen source (e.g., glycine) PhIP was formed as well (Fig. 4.2) (Zöchling and Murkovic, 2002). Other thermal degradation products that were identified by GC-MS were also tested for their ability to form PhIP. Typical degradation products occurring at higher concentrations comprised styrene, phenylethylamin, phenylethanol and phenylacetic acid. From these only phenylethylamin was capable of forming PhIP. Phenylethylamin, which is formed in a decarboxylation reaction, occurs at rather high concentrations and probably contributes more to the formation of PhIP than phenylacetaldehyde. In an experiment comparing phenylacetaldehyde and phenylethylamine in the reaction with phenylacetaldehyde the yield of PhIP was ten times higher compared to phenylethylamine (Zöchling and Murkovic, 2002).

In order to find the positions of incorporation of the phenylalanine framework into PhIP, studies using specifically labelled phenylalanine with ^{13}C in positions C-1, C-2, C-3, were undertaken (Murkovic *et al.*, 1999). The products of these reactions were isolated and their ^{13}C-spectra measured, showing a largely increased carbon-signal for those carbons with specific labelling. For this a thorough investigation of the NMR spectrum of PhIP using INEPT (insensitive nuclei enhanced by polarisation transfer) and selective excitation was necessary. The resulting spectrum of PhIP was as follows: 28.45 (Me-C), 112.06 (C-7), 129.19 (1s, C-2', C-6'), 128.23 (1s, C-4'), 126.6 (1s, C-6), 126.94 (1s, C-3', C-5'), 139.68 (1s, C-5), 156.7 (1s, C-2), 158.36 (1s, C-9), 139.44 (1s, C-1', C-8) (Fig. 4.1; Murkovic *et al.*, 1999).

Labelling at C-1 of phenylalanine showed no incorporation of ^{13}C. This was probably due to the very fast decarboxylation of the intermediate product and

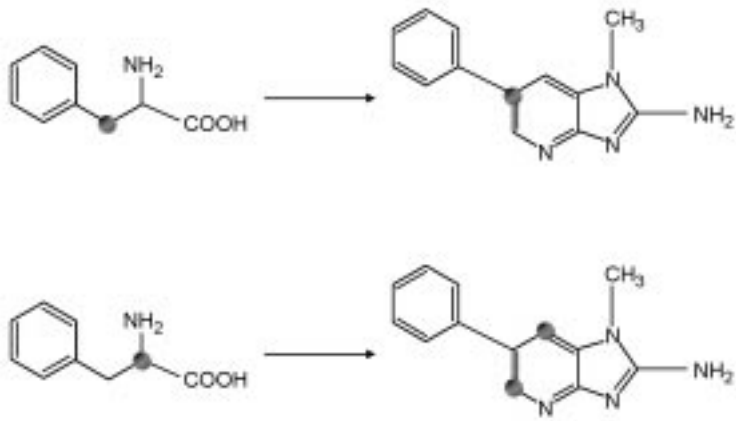

Fig. 4.3 Experiments using ^{13}C-labelled phenylalanine (Murkovic *et al.*, 1999).

loss of $^{13}CO_2$. Labelling the C-2 of the amino acid, signals were found at 139.68 and 113.06. These increased signals could be identified as C-5 and C-7 in PhIP suggesting two different possibilities for the formation of the six-membered pyridine ring. When phenylalanine was labelled at C-3 only one increased signal was found at 126.60 which was suggested to be a result of incorporation of the labelled carbon into C-6 of PhIP (Fig. 4.3, Murkovic *et al.*, 1999).

Using LC-ESI-MS for determination of the molecular weight of the reaction products that were formed in the model reaction with labelled phenylalanine in both reactions only one single labelled PhIP was obtained. In the reaction with ^{13}C-3-phenylalanine the molecular mass increased by one as expected and in the reaction with ^{13}C-2-phenylalanine the molecular mass increased by two.

Similar experiments with ^{15}N-labelled phenylalanine were carried out to identify the origin of the nitrogen. MS experiments showed – after correcting for the ^{13}C-satellites – that 10% of the formed PhIP had no ^{15}N incorporated. 77% of the PhIP had a mass that was increased by one and 13% had a mass increased by two. These data show that the amino group of the amino acid is not the only source of nitrogen for the formation of PhIP. Since the only unlabelled nitrogen occurs in creatinine, it is possible that this is introduced into the pyridine ring. The incorporation of two nitrogen atoms could be interpreted as one is put into the pyridine ring and the other one is an exchange with the amino group of creatinine.

To further investigate the mechanism of PhIP formation postulated intermediates were synthesised. These intermediates were the aldol addition product (2-amino-1-methyl-5-(1′-hydroxy-2′-phenylethyl)-imidazol-4-one) and the aldol condensation product (2-amino-1-methyl-5-(2′-phenylethyliden)-imidazol-4-one or 2-amino-1-methyl-5-(2′-phenylethenyl)-imidazol-4-one) (Fig. 4.4). Both of these substances (Fig. 4.5) were able to form PhIP. Using the synthesised standards the aldol condensation product was identified in heated meat and in a heated model system in which the precursors phenylalanine and creatinine were

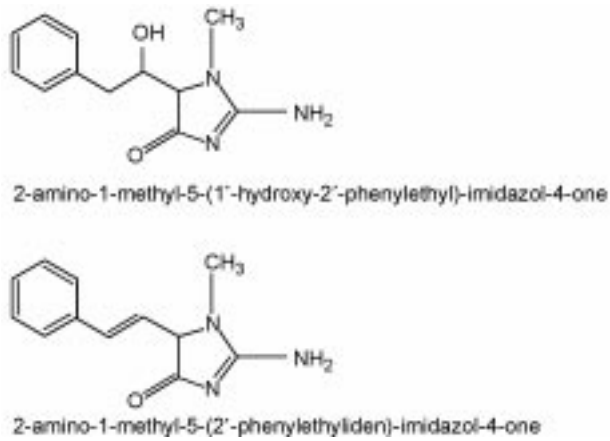

Fig. 4.4 Postulated intermediates of PhIP formation.

heated dissolved in diethylene glycol. At 200 ºC the aldol condensation product reached the highest concentration after 10–20 minutes in the model system (Zöchling and Murkovic, 2002).

The results discussed here are collated in Fig. 4.5. The suggested pathway is as follows: phenylalanine is decomposed to phenylacetaldehyde which reacts with creatinine to form the aldol addition product (A). At the high temperatures occurring during this reaction this intermediate is not stable and eliminates water

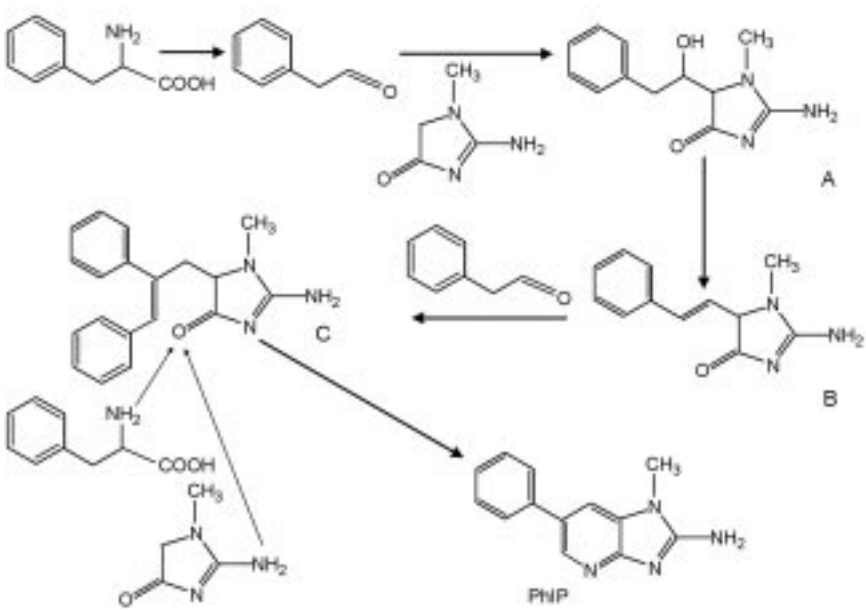

Fig. 4.5 Formation of PhIP with identified intermediate reaction products (adapted from Murkovic *et al.*, 1999; Zöchling and Murkovic, 2002).

forming the aldol condensation product (B). At this stage phenylacetaldehyde could form a second aldol condensation product (C). Then the nitrogen is introduced either from phenylalanine or from creatinine. From these not yet identified intermediates PhIP is formed.

4.3 Conclusions

The formation of heterocyclic amines in foods can be simulated with model reactions with different stages of complexity. The simplest model system is a mixture of amino acids with creatinine and carbohydrates. In some cases model reactions like the one simulating the formation of PhIP even the carbohydrates are not needed as reaction partners. The main advantage of these simple model systems is that the HAs are formed at chemically and physically defined conditions that are highly reproducible. This enables the study of parameters that influence the formation like temperature, molar ratio of reaction partners or inhibitors. In the model system limitations due to heat and mass transfer are also eliminated. The evaluation of the mechanism of formation of PhIP which included the identification of intermediate reaction products gives a detailed picture showing that not a single pathway is followed but several different reactions occur in parallel that lead to PhIP.

On the basis of the results of the model experiments and the detailed knowledge of the chemical background strategies can be developed to minimise the formation of this carcinogenic compound in heated foods. From the chemical point of view it is important that the molar ratio of amino acid/creatinine/carbohydrate should be changed to an unfavourable composition. It was shown by Olsson (Olsson *et al.*, 2002) that an increased level of glucose in the meat (due to genetic variations of the animals) decreased the heterocyclic amine formation. The effect of antioxidants influencing the chemical reactions is – at least in the case of PhIP – equivocal. In model systems the addition of antioxidant plant extracts resulted in an increase of the content of PhIP (Zöchling *et al.*, 2002). Using the same plants as spices for the preparation of meat a clear inhibiting effect was shown (Murkovic *et al.*, 1998). Moreover, the use of the heat stable antioxidant TBHQ in a complex model system (homogenised meat) showed no significant effect on the formation of PhIP in the meat models originating from chicken, beef, pork, or turkey meat (Messner and Murkovic, 2004). It is difficult to draw conclusions from these inconsistent results and much more detailed experiments have to be carried out.

It seems that the only realisable possibility to reduce the PhIP content – besides the increased presence of glucose – is to change the temperature/time regime of cooking. Lowering the temperature on the surface of the meat and reducing the time of heating significantly reduces the amount of PhIP present in the food. However, this has to be optimised with respect to microbiological safety as well as visual and sensory quality and acceptability of the heated meat.

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5

Latest developments in the analysis of heterocyclic amines in cooked foods

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5.1 Introduction

The first reports on mutagenic activity in cooked foods appeared in the late 1970s, when Japanese scientists demonstrated that the charred surface of grilled or broiled meat and fish and the tar resulting from pyrolysis of some aminoacids were highly mutagenic in the Ames bacterial system.[1,2] Some of the compounds responsible for this were later identified as heterocyclic amines (HCAs), which are isolated from pyrolysed proteins and amino acids and from a high variety of protein-rich foods, such as meat or fish. To date, more than twenty-five heterocyclic amines have been isolated from food samples. The structures of these compounds are given in Fig. 5.1.

The amounts of HCAs in a variety of foods cooked with different procedures and under different conditions need to be determined in order to develop studies on their risk to human health and to assess procedures to prevent their formation during cooking. Until now, considerable effort has been devoted to developing and improving analytical methods for the reliable determination of HCAs in a variety of food samples and to establishing a standard methodology for their analysis. Determination of HCAs in foods is hindered by several factors, such as the complexity of the food matrices to be analysed and the very low concentration levels of the mutagens. Cooked foods are a complex heterogeneous mixture of a lot of chemical substances, which makes it difficult to quantify individual chemical compounds accurately. In practice, the first problem to overcome is the extraction, isolation and pre-concentration of analytes present in the sample at part-per-billion (ng/g) levels. Then, highly efficient separation techniques, such as gas chromatography, liquid chromatography or capillary electrophoresis, are required to separate the compounds. In addition, selective

and sensitive detection systems are needed to identify and quantify the mutagens generated.

This chapter gives a general picture of the status of the analysis of heterocyclic amines and discusses the most important contributions and advances of the last five years. The literature prior to 1999 is not included, because it was reviewed in two papers published in the *Journal of Chromatography* in 2000 by Pais *et al.*[3] and Toribio *et al.*,[4] which give a complete overview of the analytical techniques, sample treatment and clean-up procedures used in HCA analysis up to then. Our aim is to report the latest developments in the analysis of HCAs in food matrices, based on the literature available, and to suggest the methods to achieve the best results. In the first section of the chapter, we discuss the most common sample pre-treatment procedures and clean-up and pre-concentration techniques and we lay down a few guidelines on the best approach to analysis. In the second section, the separation methods currently used for the determination of HCAs in food samples are reviewed, with special attention paid to liquid chromatography-mass spectrometry which is thought nowadays to be the best technique for identifying and quantifying these mutagens at the low levels found in food samples. Comments on gas chromatography-mass spectrometry and on

Key to Fig. 5.1 on pages 70–71

2-amino-3-methylimidazo[4,5-f]quinoline, **IQ**;
2-amino-3,4-dimethylimidazo[4,5-f]quinoline, **MeIQ**;
2-amino-3-methylimidazo[4,5-f]quinoxaline, **IQx**;
2-amino-3,4-dimethylimidazo[4,5-f]quinoxaline, **4-MeIQx**;
2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline, **8-MeIQx**;
2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline, **4,8-DiMeIQx**;
2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline, **7,8-DiMeIQx**;
2-amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline, **TriMeIQx**;
2-amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-f]quinoxaline, **4-CH_2OH-8-MeIQx**;
2-amino-1,7,9-trimethylimidazo[4,5-g]quinoxaline, **7,9-DiMeIgQx**;
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, **PhIP**;
2-amino-1,6-dimethylimidazo[4,5-b]pyridine, **DMIP**;
2-amino-1,5,6-trimethylimidazo[4,5-b]pyridine, **TMIP**;
2-amino-1-methyl-6-[4-hydroxyphenil]imidazo[4,5-b]pyridine, **4′-OH-PhIP**;
2-amino-1,6-dimethylfuro[3,2,e]imidazo[4,5,b]pyridine, **IFP**;
2-amino-9H-pyrido[2,3-b]indole, **AαC**;
2-amino-3-methyl-9H-pyrido[2,3-b]indole, **MeAαC**;
1-methyl-9H-pyrido[4,3-b]indole, **H**;
9H-pyrido[4,3-b]indole, **NH**;
3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole, **Trp-P-1**;
3-amino-1-methyl-5H-pyrido[4,3-b]indole, **Trp-P-2**;
2-amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole, **Glu-P-1**;
2-aminodipyrido[1,2-a:3′,2′-d]imidazole, **Glu-P-2**;
2-amino-5-phenylpyridine, **Phe-P-1**;
4-amino-6-methyl-1H-2,5,10,10b-tetraazafluoranthene, **Orn-P-1**;
4-amino-1,6-dimethyl-2-methylamino-1H,6H-pyrrolo[3,4-f]benzimidazole-5,7-dione, **Cre-P-1**;
3,4-cyclopentenopyrido[3,2-a]carbazole, **Lys-P-1**

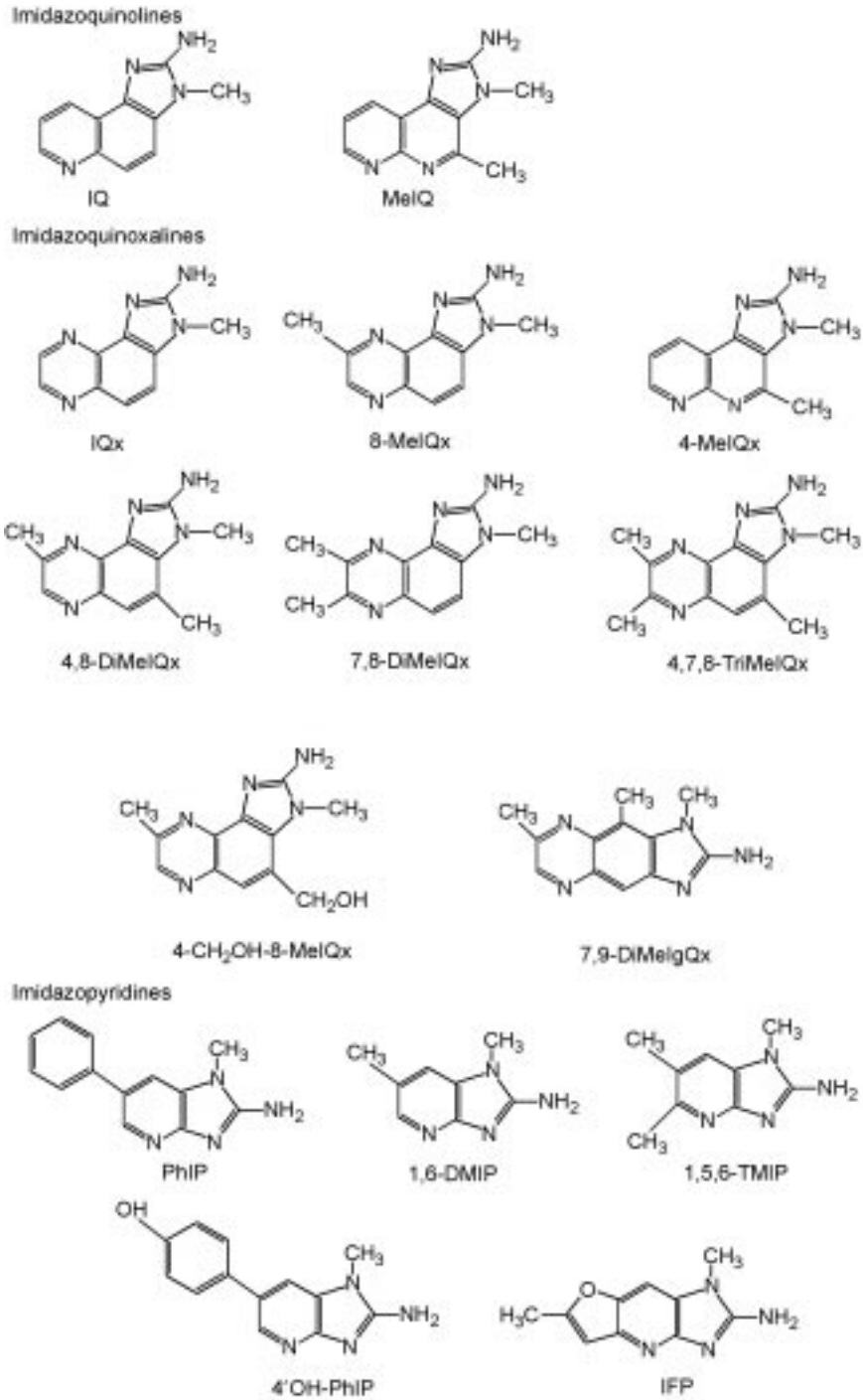

Fig. 5.1 HCAs abbreviations.

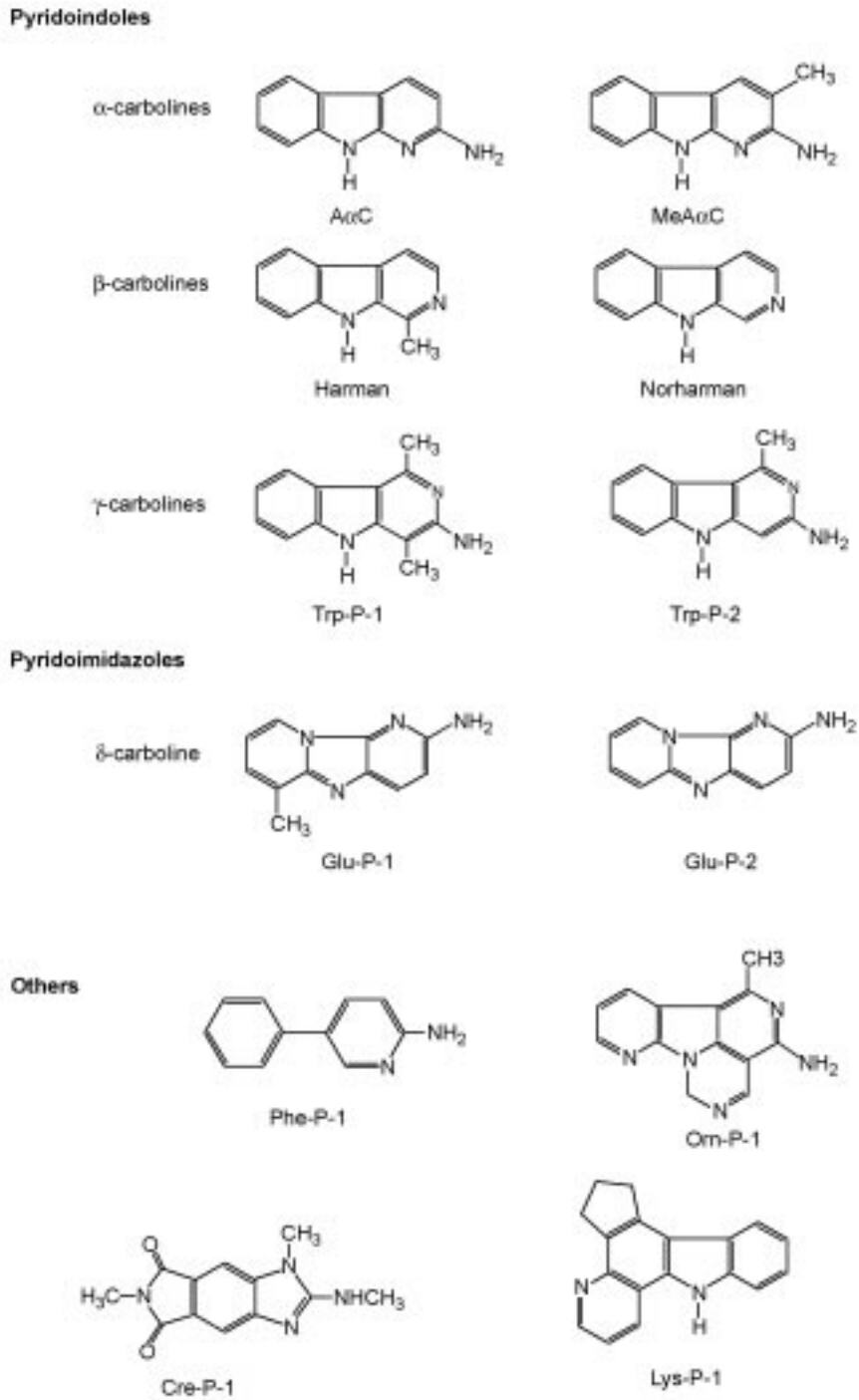

Fig. 5.1 continued

liquid chromatography with other detection systems are also included. A section devoted to the techniques used for identifying new mutagens and quantitation strategies is also included.

5.2 Extraction of HCAs and sample preparation

The extraction and purification of analytes is probably one of the most important steps in the analysis of heterocyclic amines. The origin of the sample to be analysed and the analytical technique selected for the determination greatly influence the degree of purification required and, therefore, the complexity of the sample preparation process. Moreover, heterocyclic amines are analysed in a wide variety of samples such as proteinaceous foods, model systems, beverages, cigarette smoke, cooking fumes, rainwater, incineration ash and biological matrices including plasma, urine, faeces or bile. The sample preparation procedures always require consideration of the composition of the sample matrix, which entails laborious approaches including several steps to obtain extracts clean enough for analytical purposes. In consequence, there is no standard procedure for HCA analysis regardless of the kind of sample. It is also difficult to compare heterocyclic amines found in the same kind of samples, such as cooked foods, since varying food composition and cooking conditions affect values.

Depending on the objective of the analysis, i.e. determination of mutagenic activity or quantification of mutagenic analytes, several strategies for sample preparation have been adopted. Toribio *et al.*[4] reviewed the analytical work published up to 2000 on the separation procedures, sorbents and solvents used. When the analysis concerns mutagenic activity in foods, and the main aim is to isolate and characterise the compounds responsible for this mutagenicity, extensive sample fractionation is required. In general, analytical protocols start by describing an initial homogenisation step, mainly carried out by adding hydrochloric acid to the sample. Other solvents used are acetone, water, methanol and a water/acetonitrile mixture.

When total mutagenic activity is measured, the procedures are very simple. They are mainly based on successive liquid-liquid extractions at different pH after protein precipitation. However, when the objective is the mutagenicity or the characterisation of a single compound, intensive fractionation is required. Thus, highly laborious procedures, which require large amounts of starting material (10–100 kg) to obtain enough mass for the analyses, have been developed. After the initial homogenisation, further purification is carried out by acid-base partition, liquid chromatography using different sorbents or combinations of these two methods. Final purification is attained by means of one or more HPLC steps, which also provide the analytes isolated in different fractions, whose genotoxicity is tested with the Ames/*Salmonella* test. The isolated mutagens are then characterised by more selective methods, such as UV and fluorescence spectrophotometry, high-resolution MS or NMR.

Once a mutagenic compound has been identified and standard solutions are available, analytical-scale purification procedures and chromatographic methods for the accurate quantification of this analyte are used. Sample preparation procedures before the identification and quantification of mutagenic amines also contain several steps. As mentioned above, the first is a dissolution step, in which the sample is homogenised and dispersed by various solvents. In most cases, the solvents used are organic, such as methanol, acetone, ethyl acetate or hydroalcoholic mixtures; or aqueous, like hydrochloric acid, water or sodium hydroxide. In all the procedures, except when hydrochloric acid is used, sodium hydroxide is the solvent of choice, and mild heating is sometimes proposed. In all cases, the sample treatment after the dissolution step involves separation techniques such as centrifugation or filtration after protein precipitation. Further purification is carried out by one or various separation procedures, including liquid-liquid extraction, column liquid chromatography, and solid-phase extraction. In addition, a pre-concentration stage is required to achieve low detection limits. This is commonly performed by evaporating the final extract to dryness and re-dissolving the residue in a small volume of the appropriate solvent for the determination procedure.

5.2.1 Liquid-liquid extraction

Liquid-liquid extraction is the separation method preferred by most of the authors for the first step in the isolation of the analytes from the food matrix. In Tables 5.1 and 5.2, analytical studies published since the 1999 review are described. In most of the procedures summarised in these tables, after homogenisation of the sample, elimination of the solids and change of the solvent if necessary, an acid-base partition is performed. The acidic solution obtained is directly extracted with an organic solvent, which may be dichloromethane, diethyl ether or ethyl acetate, in order to remove acidic or neutral interferences. If the solution obtained is basic, the analytes are directly extracted in their neutral form with dichloromethane or ethyl acetate. If the sample is homogenised in an organic solvent, the analytes are extracted with HCl. In most cases, further purification is achieved by consecutive acid-base partition processes or by combining this technique with extraction using sorbents. This will be discussed in the following sections.

Liquid-liquid extraction can also be achieved by using inert solid materials such as diatomaceous earth, a sand-like porous material commercially available in several forms such as Kieselguhr, Diatomaceous earth®NT or Hydromatrix. These materials can be added to the liquid in the batch mode or, more frequently, as a support in a chromatographic column. Tables 5.1 and 5.2 show a large number of examples of this method. The procedure that uses diatomaceous earth is generally referred to as liquid-liquid extraction. In some cases, this procedure is coupled on-line with several solid-phase extraction steps and thus becomes a tandem extraction process, as described in Section 5.2.3.

Table 5.1 Liquid chromatography using conventional detection systems

Compounds	Sample	Sample treatment	Chromatographic conditions	Detection/ quantitation mode	Ref.
A) IQ: MeIQ, IQx, MeIQx, 4,8-, 7,8-DiMeIQx B) PhIP, AαC, MeAαC, harman, norharman, Trp-P-1, Trp-P-2	Meat juice	Solution in 8 M urea, 2 M NaOH.: LLE (diatomaceous earth) with DCM. SPE: PRS, washing with 0.01 M HCl and MeOH/0.1M HCl (6:4), elution with 0.5 M $AcONH_4$ pH 8; C_{18}, elution with MeOH/NH_3 (9:1). Evaporation, reconstitution in MeOH. Additional step in heated (225 ºC) samples: CBA.	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 10 mM TEA-acetic acid pH 3.2/10 mM TEA-acetic acid pH 3.6/ACN, grad., 1 mL/min	A) UV(DAD) B) Fluorescence External calibration	65
IQ: MeIQ, IQx, MeIQx, 4,8-, 7,8-DiMeIQx, PhIP, AαC, MeAαC, harman, norharman, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2	Model system	SPE: PRS, washing with 0.01 M HCl, elution with: – nonpolar amines: MeOH/0.1 M HCl (4:6) – polar amines: 0.5 M NH_4AcO pH 8. Nonpolar amines: Neutralise with NH_3, dilute with H_2O to <20% MeOH; C_{18}, elution with MeOH/NH_3 (9:1). Polar amines: C_{18}, elution with MeOH/NH_3 (9:1). Nonpolar and polar combined extracts, evaporation and reconstitution with MeOH.	TSKgel ODS 80 TM (5 μm, 250 × 4.6) 0.05 M $AcONH_4$ pH 3.6/ACN, grad., 1 mL/min	UV Internal standard added to the extract	66
PhIP	Model system	Solution in 0.1 M HCl. LLE (diatomaceous earth) with DCM at pH<2 (discard organic phase). Aqueous phase at pH 9, LLE with DCM. Organic phase, evaporation and reconstitution with MeOH. SPE: *blue cotton*, elution with MeOH/NH_3 (50:1). Evaporation and reconstitution with MeOH.	LiChrospher 60 RP-Select B (5 μm, 250 × 4.6 mm) MeOH/ACN/acetic acid/H_2O (15:25:2:58) pH 5.1, 1 mL/min	UV(DAD) Fluorescence External calibration	35
MeIQx, 4,8-DiMeIQx, PhIP, IFP	Several meats	Solution in 1 M NaOH. LLE (diatomaceous earth) with DCM/toluene (95:5). SPE: PRS, washing with 0.1 M HCl and MeOH/0.1 M HCl (4:6), elution with 0.5 M $AcONH_4$ pH 8; C_{18}, elution with MeOH/NH_3 (9:1). Evaporation, reconstitution in MeOH	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 10 mM TEA-formic acid pH 3.6/ACN, grad., 1 mL/min	UV(DAD) Fluorescence External calibration, recovery correction	25

IQ: MeIQ, IQx, MeIQx , 4-MeIQx, 4,8-DiMeIQx, DMIP, PhIP, AαC, MeAαC, harman, norharman, TMIP, IFP, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2	Several meats, meat drippings	Homogenisation in 1 M NaOH. LLE (diatomaceous earth) with DCM/toluene (95:5) or EtAc. Following as[66] without combining extracts, and polar extract from heated models are treated with additional SPE step: SCX.	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 10 mM TEA-formic acid pH 3.6/ACN, grad., 1 mL/min	UV(DAD) Fluorescence External calibration, recovery correction	18
A) IQ, MeIQ, IQx, MeIQx, 4,8-, 7,8-DiMeIQx B) PhIP	Process flavours, bouillon concentrates, pan residues	Homogenisation in 1 M NaOH. LLE (diatomaceous earth) with DCM/toluene (95:5). Following as[66] without combining extracts. Additional clean-up steps for very complex matrices: TSK gel[8] or SCX[67] or CBA (for both polar and nonpolar extracts separately) rinsing with 0.01 M $AcONH_4$ pH 6, elution with MeOH/NH_3 (4:1).	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 10 mM FTEA pH 3.2/10mM TEA-formic acid pH 3.5/ACN, grad., 1 mL/min	A) UV(DAD) B) Fluorescence External calibration, recovery correction	19
IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP, AαC, : harman, norharman, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2	Lyophilised meat extract	Solution in 1 M NaOH. LLE (diatomaceous earth) with DCM. SPE: PRS, washing with MeOH/H_2O (4:6) and H_2O, elution with 0.5 M $AcONH_4$ pH 8; C_{18}, elution with MeOH/NH_3 (9:1). Evaporation, reconstitution in MeOH.	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 10 mM TEA-formic acid pH 3.3/ACN, grad., 1 mL/min	UV(DAD) External calibration, internal standard added to the extract	48
A) IQ, MeIQ, MeIQx, 4,8-DiMeIQx B) PhIP	Beef patties	Homogenisation in 1 M NaOH. LLE (diatomaceous earth) with DCM. Following as[66] without combining extracts.	TSKgel ODS 80 TM (5 μm, 4.6 × 250 mm) 10 mM TEA-formic acid pH 3.2/ACN, grad., 1 mL/min	A) UV(DAD) B) Fluorescence Standard addition	68
IQ, MeIQx,4,8-, 7,8-DiMeIQx	Soup cubes	Extraction with 1 M HCl, alkalisation with 6 M NaOH. LLE (diatomaceous earth) with DCM/toluene (10:1). SPE: PRS, washing with 0.1 M HCl and H_2O, elution with MeOH/NH_3 (9:1). Evaporation, reconstitution in mobile phase.	Luna phenyl-hexil (2 μm 250 × 2.1 mm) 30 mM trichloroacetic acid pH 2.5/THF (96:4), 0.23 mL/min	Electrochemical (coulometric array) Standard addition	26

Table 5.1 Continued

Compounds	Sample	Sample treatment	Chromatographic conditions	Detection/ quantitation mode	Ref.
MeIQx, 4,8-DiMeIQx, DMIP, TMIP, PhIP, IFP	Model system, meat	Homogenisation in 1 M NaOH. LLE (diatomaceous earth) with EtAc. Following as[66] for polar amines.	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 0.01 M TEA-formic acid pH 3.2/ACN, grad., 1 mL/min	UV(DAD) Fluorescence External calibration, recovery correction	46
A) IQx, MeIQx, 4,8-DiMeIQx, B) PhIP, AαC, MeAαC, harman, norharman, Trp-P-1, Trp-P-2	Meat juice model system	Same as[68] but LLE (diatomaceous earth) step with DCM/toluene (95:5). Most of samples are treated with an additional SPE step with CBA.[19]	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 10 mM TEA-acetic acid pH 3.2/10 mM AcTEA pH 3,6/ ACN, grad., 1 mL/min	A) UV(DAD) B) Fluorescence External calibration, recovery correction	27
IQ, MeIQ, MeIQx, 4,8-, 7,8-DiMeIQx, PhIP, MeAαC, harman, norharman	Spiked beef extract	Extraction in 1 M NaOH added with MeOH. LLE (LiChrolut EN), previous washing with methanolic NaOH (MeOH/NaOH, 55:45) and ethanolic hexane (EtOH/hexane, 20:80) and hexane, then elution with EtOH/DCM, 10:90: Evaporation, reconstitution in mobile phase.	A) Zorbax SB-Phenyl (5 μm, 250 × 4.6 mm) B) LiChrospher RP18e (5 μm, 125 × 4.6 mm) 0.01 M TEA-formic acid pH 3/ ACN, grad., 1 mL/min	UV(DAD) External calibration, recovery correction	11
IQ, MeIQ, IQx, MeIQx, 4,8-, 7,8-DiMeIQx, PhIP, DMIP, harman, norharman, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, TMIP, IFP	Meat boullion, fried pork	Same as[68] but LLE (diatomaceous earth) step with DCM /EtAc mixtures. For PhIP: elution from C_{18} with MeOH/NH_3 (4:1).	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 10 mM TEA-acetic acid pH 3.6/ACN, grad., 1 mL/min	UV(DAD) Fluorescence External calibration	69
A) IQx, MeIQx, 4,8-DiMeIQx B) PhIP, AáC, Trp-P-1, Trp-P-2	Several foods	Same as[68]	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 10 mM TEA-formic acid pH 3.6/ACN, grad., 1 mL/min	A) UV(DAD) B) Fluorescence Not indicated	70

IQ, MeIQ, MeIQx, 4,8-DiMeIQx , PhIP	Pork	Solution in 1 M NaOH. LLE (diatomaceous earth) with DCM. SPE: PRS, washing with 1 M HCl and H_2O, elution with 0.5 M $AcONH_4$ pH 8; C_{18}, elution with MeOH/NH_3 (9:1). Evaporation, reconstitution in ACN.	A) SynChropak RP-8 (5 μm, 250 × 4.6 mm) B) TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) A) TEA formic acid pH 3.2/ ACN, grad., 1 mL/min B) TEA-formic acid pH 3.3/ ACN, grad., 1 mL/min	UV(DAD) External calibration	58
AαC, MeAαC, harman, norharman	Fish fibre	Same as[66].	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 0.05 M $AcONH_4$ pH 3.6/ACN, grad., 1 mL/min	UV(DAD) Fluorescence External calibration	71
IQ, MeIQ, MeIQx, 4,8-, 7,8-DiMeIQx, PhIP	Several foods	Homogenisation in 0.5 M HCl and LLE with DCM (discard organic phase). Alkalisation the aqueous phase, LLE (diatomaceous earth) with DCM. SPE: PRS, elution with MeOH/NH_3 (98:2). HPLC, Nucleosil 5 SA using $AcONH_4$ (pH 4.5 and 6)/ACN (60:40) solutions in gradient mode.	LiChrospher 60 rp-select B (5 μm, 250 × 4.6 mm) A) 0.01 M TEA-formic acid pH 3.3/ACN, grad., 1 mL/min B) 0.05 M $AcONH_4$ pH 4.7/ MeOH, grad., 1 mL/min	UV External calibration, recovery correction	47
IQ, MeIQ, IQx, MeIQx, 4,8-, 7,8-DiMeIQx, PhIP, AαC, MeAαC, harman, norharman, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2.	Marinated foods	Same as[68].	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 50 mM $AcONH_4$ pH 3.6/ACN, grad., 1 mL/min	UV(DAD) External calibration, recovery correction	40
IQ, MeIQ, IQx, MeIQx, 4,8-, 7,8-DiMeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2	Chicken	Same as[68] but LLE (diatomaceous earth) step with EtAc.	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 10 mM TEA-formic acid pH 3.2/10 mM TEA-formic acid pH 3.5/ACN, grad., 1 mL/min	UV(DAD) Fluorescence External calibration, recovery correction	21
PhIP	Model system	Solution in 0.1 M HCl. SPE: Oasis MCX, washing with 0.1 M HCl, MeOH and MeOH(40%)/NH_3 (95:5), elution with MeOH/NH_3 (95:5). Evaporation and reconstitution with MeOH.	LiChroCART Superspher 60RP-select B (5m, 125 × 2.1mm) MeOH/ACN/$AcONH_4$ pH 5/ H_2O, grad., 0,4 mL/min	Fluorescence External calibration	32

Table 5.1 Continued

Compounds	Sample	Sample treatment	Chromatographic conditions	Detection/ quantitation mode	Ref.
MeIQx, 4,8-DiMeIQx, PhIP, harman	Pig meat	Same as[68], washing PRS with 0.01 M HCl, MeOH/ 0.1 M HCl (40:60) and H_2O.	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 10 mM TEA-acetic acid pH 3.6/ACN, gradient, 1 mL/min	UV (DAD) External calibration, recovery correction	72
MeIQx, 4,8-DiMeIQx, PhIP, IFP	Model system	Same as[21].	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 0.01 M TEA-phosphoric acid pH 3.2/ACN, gradient, 1 mL/ min	UV(DAD) Fluorescence External calibration	73
MeIQx, 4,8-DiMeIQx, PhIP, IFP	Pet foods	Same as[21].	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) 0.01 M TEA- phosphoric acid pH 3.2/ACN, gradient, 1 mL/ min	UV(DAD) Fluorescence External calibration, recovery correction	44
MeIQx, PhIP	Beef hamburgers, chicken	Same as[68].	TSKgel ODS 80 TM (5 ×m, 250 × 4.6 mm) 10 mM TEA-acetic acid pH 3.6/ACN, gradient, 1 mL/min	UV(DAD) Fluorescence External calibration, recovery correction	74
A) DMIP, IQ, MeIQx, 4,8-DiMeIQx, B) PhIP, harman, norharman	Chicken	Same as[68] but using 0.01 M HCl, MeOH/0.1 M HCl (60:40) to elute nonpolar amines from PRS. Most of samples are treated with an additional SPE step with CBA.	C_8 Symmetry (5 μm,150 × 2.1 mm) 50 mM acetic acid-ammonium acetate A) pH 5.25/ACN 90:10 B) pH 6.0/ACN 70:30	Electrochemical Standard addition	31

Table 5.2 Liquid chromatography coupled with mass spectrometry

Compounds	Sample	Sample treatment	Chromatographic conditions	Interface/ analyser	Acquisition and quantitation modes	Ref.
IQ, MeIQx, 4,8-, 7,8-DiMeIQx, PhIP	Beef, bacon	Homogenisation in 1 M NaOH. LLE (diatomaceous earth) with DCM. SPE: PRS, washing with 0.01 M HCl, elution with: – nonpolar amines: MeOH/0.1 M HCl (4:6) – polar amines: 0.5 M NH_4AcO pH 8. Nonpolar amines: Neutralise with NH_3, dilute with H_2O to <20% MeOH; C_{18}, elution with MeOH/NH_3 (9:1). Polar amines: C_{18}, elution with MeOH/NH_3 (9:1). Final extracts: evaporation and reconstitution with MeOH/H_2O (1:1).	Vydac C18 (5 μm, 250 × 2.1 mm); 25 mM $AcONH_4$ pH 8.5/ ACN, grad., 0.2 mL/min	APCI QqQ	MRM, product ion scan, precursor ion scan, neutral loss; labelled standards	57
MeIQx, 4,8-DiMeIQx, DMIP, TMIP, PhIP, IFP	Model system	Homogenisation in 1 M NaOH. LLE (diatomaceous earth) with EtAc. SPE: PRS, washing with 0.01 M HCl and MeOH/0.1M HCl (4:6), elution with 0.5 M $AcONH_4$ pH 8; C_{18}, elution with MeOH/NH_3 (9:1). Evaporation, reconstitution in mobile phase.	ODS-A (250 × 3 mm) Acetic acid /MeOH/ ACN, grad., 200 μL/min	ESI IT	Product ion scan –	46
IQ, MeIQ, MeIQx, 4,8-, 7,8-DiMeIQx, PhIP, AαC, MeAαC, harman, norharman, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2	Lyophilised meat extract	Homogenisation in 1 M NaOH. LLE (diatomaceous earth) with DCM. SPE: PRS, washing with MeOH/H_2O (4:6) and H_2O, elution with 0.5 M $AcONH_4$ pH 8.5; C_{18}, elution with MeOH/NH_3 (9:1). Evaporation, reconstitution in MeOH.	TSKgel ODS 80 TM (5 μm, 4.6 × 250 mm) $HCOONH_4$ pH 3.25 / $HCOONH_4$ pH 3.7 / ACN, grad., 1 mL/min	APCI IT	Full scan; standard addition IS in extract	20,75
IQ, MeIQ, IQx, MeIQx, 4,8-, 7,8-DiMeIQx, DMIP, PhIP, IFP AαC, Me AαC, Trp-P-1, Trp-P-2	Chicken	Homogenisation in 1 M NaOH. LLE (diatomaceous earth) with EtAc; evaporation, reconstitution in 1 M NaOH. SPE: Blue Chitin, washing with H_2O, elution with MeOH/NH_3 (9:1). Evaporation, reconstitution in MeOH.	Zorbax SB-C8 (5 μm, 150 × 4.6 mm) Acetic acid pH 3.5/ ACN, grad., 1 mL/min	ESI IT	SIM External calibration, recovery correction	13

Table 5.2 Continued

Compounds	Sample	Sample treatment	Chromatographic conditions	Interface/ analyser	Acquisition and quantitation modes	Ref.
IQ, MeIQ, MeIQx, 4,8-, 7,8-DiMeIQx, PhIP, AαC, MeAαC, harman, norharman, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2	Lyophilised meat extract	Same as[75].	TSKgel ODS 80 TM (5 μm, 250 × 4.6 mm) $HCOONH_4$ pH 3.25/ $HCOONH_4$ pH 3.7/ ACN, grad., 1 mL/min	APCI IT	Product ion scan; standard addition IS in extract	37
IQ, MeIQ, 7,8-DiMeIQx, , PhIP Trp-P-1, Trp-P-2	Beefburgers, chicken	Homogenisation in 0.1 M HCl. LLE with DCM (discard organic phase). Aqueous phase is made basic with 10 M NaOH, LLE with DCM. Organic phase, LLE (back-extraction) with 0.2 M HCl. Aqueous phase is made alkaline with 10 M NaOH, LLE with DCM, evaporation and reconstitution with MeOH/0.1 M HCl, (20:80). SPE: PRS, washing with 0.1 M HCl, MeOH/0.1 M HCl, (20:80) and H_2O, elution with ACN/0.5 M $AcONH_4$ pH 8.5 (4:6); partial evaporation, C_{18}, elution with MeOH/NH_3 (9:1) and MeOH. Evaporation, reconstitution in mobile phase.	TSKgel Super ODS (2 μm, 100 × 4.6 mm) HCOOH; $AcONH_4$/ACN, grad.	ESI QqQ	SIM, SRM External calibration recovery correction (labelled standards)	64
DMIP, IQ, MeIQx, MeIQ, 4,8-DiMeIQx, PhIP, AαC, MeAαC harman, norharman, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2,	Beef, pork, chicken, lamb, sausages	Same as[57] but using 0.01 M HCl, MeOH/0.1 M HCl (6:4) and H_2O to elute nonpolar amines. Final extracts are reconstituted in MeOH.	Symmetry C_8 (5 μm, 150 × 2.1 mm) Acetic acid/ $AcONH_4$ pH: 4.5/ACN, grad., 0.3 mL/min	ESI QqQ	SRM Standard addition IS in extract	51
MeIQx, 4,8-DiMeIQx, PhIP	Beefburgers	Same as[51].	Zorbax SB-C8 (5 μm, 150 × 4.6 mm) Acetic acid pH:3.5/ ACN, grad.	ESI IT	SIM External calibration, recovery correction	76

MeIQx, 4,8-DiMeIQx, PhIP, norharman	Beefburgers	Same as[76] but LLE (diatomaceous earth) step with EtAc.	Zorbax SB-C8 (5 μm, 150 × 4.6 mm) Acetic acid pH:3.5/ACN, grad.	ESI IT	SIM External calibration, recovery correction	77
IQ, MeIQx, MeIQ, 4,8-, 7,8-DiMeIQx, PhIP, harman, norharman,Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2	Hamburgers, kebab, meat rolls	Same as[77].	Zorbax SB-C8 (5 μm, 150 × 4.6 mm) Acetic acid pH 3.5/ ACN, grad.	ESI IT	SIM External calibration, recovery correction	78
DMIP, IQ, MeIQx, MeIQ, 4,8-DiMeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2	Model system	Homogenisation in 1 M NaOH. LLE (diatomaceous earth) with EtAc. SPE: Oasis MCX, washing with 0.1 M HCl and MeOH, elution with MeOH/NH_3 (19:1). Evaporation, reconstitution in MeOH.	Semi Micro ODS-80 TS (5 μm, 250 × 2 mm) Acetic acid pH 5.5/ MeOH/ACN, grad., 0.3 mL/min	ESI IT	SIM Standard addition IS in extract	33
DMIP, IQ, MeIQx, 4,8-, 7,8-DiMeIQx, PhIP, MeAαC, harman, norharman	Model system	Same as[75] but reconstituting in MeOH/mobile phase (1:1).	Symmetry C_8 (5 μm, 150 × 2.1 mm) Acetic acid/ $AcONH_4$ pH 4.5/ACN, grad., 0.3 mL/ min	ESI IT	Product ion scan Standard addition IS in extract	79
DMIP, IQ, MeIQx, MeIQ, 4,8-DiMeIQx, PhIP, AαC, MeAαC harman, norharman, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2	Lyophilised meat extract	Same as[51]. Final extracts are reconstituted in MeOH/30 mM $AcONH_4$ pH 4.5 (1:1).	Symmetry C_8 (5 μm, 150 × 2.1 mm) Acetic acid/ $AcONH_4$ pH 4.5/ACN, grad., 0.3 mL/ min	ESI Q, QqQ, IT	SIM, SRM, product ion scan Standard addition IS in extract	50
DMIP, IQ, MeIQx, MeIQ, 4,8-DiMeIQx, PhIP, AαC, MeAαC harman, norharman, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2	Lyophilised meat extract	Same as[50].	Symmetry C_8 (5 μm, 150 × 2.1 mm) Acetic acid/ $AcONH_4$ pH 4.5/ACN, grad., 0.3 mL/ min	ESI Q-TOF, QqQ	Product ion spectra, SRM, product ion scan, neutral loss Standard addition IS in extract	38

Table 5.2 Continued

Compounds	Sample	Sample treatment	Chromatographic conditions	Interface/ analyser	Acquisition and quantitation modes	Ref.
IQ, IQx, 4,8-, 7,8-, 1-iso-8-MeIQx, 4,8-,7,8- DiMeIQx, 7,9-DiMeIgQx, PhIP, AαC, MeAαC	Beef	Homogenisation in 1 M NaOH. LLE (diatomaceous earth) with DCM/toluene (95:5). SPE: Oasis MCX, washing with 0.04 M HCl in MeOH (40%, v/v), MeOH and 2% NH_3 containing 15% MeOH; elution with 5% NH_3 in MeOH. Evaporation, reconstitution in mobile phase.	Aquasil C_{18} (3 μm, 150 × 1 mm) HCOOH/ ACN, grad., 50 L/min	ESI Q-TOF, QqQ	Product ion spectra, SRM, labelled standards	28
IQ, IQ[4,5-b], IQx, 8-MeIQx, 4,8-DiMeIQx, , PhIP, 7,9-DiMeIgQx, AαC, MeAαC	Beef, chicken	Same as[28].	Aquasil C_{18} (3 μm, 150 × 1 mm) HCOOH/ ACN, grad., 50 L/min	ESI QqQ	SRM, product ion scan, precursor ion scan, labelled standards	34

5.2.2 Column liquid chromatography

Liquid chromatography is based on a physical separation process that involves a stationary phase and a liquid mobile phase. Both the liquid solution containing the analytes and the stationary phase placed in an adequate recipient, usually a column, are brought into contact. In general, the stationary phase interacts with the analytes to allow their selective separation. Liquid chromatography can be used as a semi-preparative technique to collect fractions with the different compounds. For example, a reversed-phase LC can isolate the fraction corresponding to the analytes before quantitative analysis by LC-UV or LC-Fluorescence. In other cases, preparative LC can be used as a fractionation step, by means of an open column filled with sorbents such as Sephasorb HP. Another chromatographic technique used for the separation of the analytes in a preparative step is thin-layer chromatography, which has been used, for instance, in the isolation of IQ from ground beef.[5]

When the objective is not the fractionation but the purification of the analytes, liquid chromatography can be used as a clean-up step. In an ideal case, the relevant compounds are completely retained on the surface of the solid, interferences are eliminated by washing of the sorbent, and finally the analytes are eluted using the most appropriate solvent. In most cases, this process is performed using open columns, but sometimes the two phases are mixed in a batch with mechanical stirring, and separated by filtration after distribution equilibria are achieved. As Toribio *et al*. described,[4] adsorption in resins was one of the first chromatographic mechanisms used to purify HCAs. Amberlite XAD-2, a non-ionic polymeric adsorbent based on polystyrene, was the most popular. After the corresponding treatment, the aqueous phase is neutralised and passed through the sorbent in order to concentrate the relatively non-polar chemicals. The analytes are then eluted with acetone, combined in some cases with methanol.

Based on the observation that HCAs have a planar structure and form complexes by means of hydrophobic interactions with compounds with analogue structures, Hayatsu *et al*. developed a specific sorbent named blue-cotton, a cellulose cotton bearing covalently linked copper phthalocyanine trisulphonate.[6] This material adsorbs HCAs in aqueous solution very efficiently by means of hydrophobic interactions between the copper-phthalocyanine nucleus and the aromatic substances. Afterwards, the mutagenic amines can be eluted easily with methanol-ammonia solution. The first applications developed for the analysis of HCAs in food matrices introduced blue cotton directly into the aqueous solution, and the material was filtered before the elution. However, this sorbent can be placed in preparative columns for the same treatment. Some modified procedures using the copper-phthalocyanine complex over several support materials were recently reviewed by Skog.[7] For example, a product called CPC Sephasorb, which consists of copper phthalocyanine bound to the dextran polymer Sephasorb HP, was developed and successfully applied to the analysis of meat extracts by Gross.[8] Furthermore, the use of other support material such as rayon instead of cotton improved HCA extraction, making Blue Rayon a

more efficient adsorbent than Blue Cotton.[9–11] By using poly-N-acetyl-glucosamine (chitin) as the support material, the blue pigment content is increased and thus higher extraction efficiency is obtained. Blue chitin columns were developed especially for extracting planar polycyclic compounds such as HCAs and have been successfully applied to the analysis of food samples.[12,13]

Other sorbents have also been used. For example, Sephasorb HP, which fractionates by size exclusion and gel adsorption, was used by Gross to obtain different fractions by means of a preparative LC column.[14] After an initial LLE step using diatomaceous earth as a solid support and dichloromethane as extraction solvent, a glass column filled with Sephasorb HP provided very clean fractions of HCAs using a medium-pressure (60 bar) LC system. Other authors used silica gel to extract HCAs from foods. After a liquid-liquid extraction treatment, the neutralised analytes are extracted from the ethyl acetate solution by means of a column filled with the sorbent. The analytes are then eluted by ethyl acetate/MeOH or ethyl acetate/MeOH/NH_3.[4]

The use of specific sorbents containing monoclonal antibodies (MAbs) for the purification of heterocyclic amines provides simple and rapid sample preparation. However, specific antibodies are needed for each compound, and the synthesis of MAbs is highly complex. In consequence, MAbs are not commercially available, which hinders the use of this method and has meant that very few papers on it have been published. Among those that have used this approach is the article by Turesky *et al*. on the analysis of IQ and MeIQx in beef.[15]

5.2.3 Solid-phase extraction

Solid-phase extraction (SPE) provides a quick alternative to classical adsorption columns. Analytes are extracted by disposable commercial cartridges, which normally contain from 100 mg to 500 mg of a solid sorbent as stationary phase. In SPE, the analytes from the sample extract interact more with the solid phase than the unwanted matrix components. As mentioned in the column liquid chromatography section, in an ideal case the compounds studied are retained on the surface of the solid, while interferences are eliminated by washing the column. Finally, the analytes are eluted by using a different solvent. Most SPE procedures can be used on a microanalytical scale and also integrated on-line with the separation analytical technique. Moreover, analytical sensitivity and selectivity can be optimised by using several sorbents and eluents and, in some cases, by coupling of different sorbents in tandem. Thus, most of the sample preparation procedures for the analysis of HCAs use SPE, which gives extracts that are sufficiently purified to prevent interferences, and a high throughput analysis. As seen in Tables 5.1 and 5.2, most analyses of proteinaceous foods were by means of solid-phase extraction. The method mainly used was that described by Gross and Grüter,[16] with various modifications.[17-21]

Most of these procedures use on-line coupling of the liquid-liquid extraction, with diatomaceous earth as solid support and several SPE steps allowing the development of tandem extraction procedures. Thus, many of the applications

described in Tables 5.1 and 5.2 concern techniques which can be integrated into these tandem extraction procedures. Solid phase micro-extraction (SPME) has also been suggested for the analysis of HCAs in food samples.[9-11] For instance, recently Cardenes *et al.* described the use of carbowax-templated resins as fibre coatings for the analysis of spiked meat extracts by coupling with HPLC, although low recovery factors were obtained for several polar and non-polar amines.[22]

Tandem extraction strategies

As mentioned above, when liquid-liquid extraction using diatomaceous earth and solid-phase extraction are coupled, the result is a time-saving and high-throughput analysis, because few sample transfer and evaporation steps are required during the work-up. This is beneficial not only for sample handling, but also ensures high analyte recovery. In addition, the high number of commercial stationary phases and the possible optimisation of each step by changing the working solvents greatly enhance both the selectivity and the reliability of these tandem methods. Therefore, they can be regarded as standard procedures, although their suitability for the chromatographic analysis of a given sample depends on both the selectivity of the detection technique and the sample matrix.

Some examples of the methods based on LLE/SPE tandem extraction proposed in the literature are summarised in Tables 5.1 and 5.2. These tables describe the sample treatments used for the extraction and purification of HCAs in food analysis published from 1999 till now. All treatments begin by homogenising the sample in HCl or NaOH aqueous solutions, with subsequent solvent extraction using in most cases a solid support of diatomaceous earth. Finally, an additional step of solid-phase extraction is carried out. In this case, different sorbents alone or in combination are used. The extraction tandem using PRS and C_{18} is one of the most common combinations. The final elution using adequate solvents can provide one or two purified extracts containing non-polar and polar amines, combined or separated, respectively.

In tandem extraction procedures, diatomaceous earth is coupled to the SPE by placing of the solid material in an empty preparative column, which is also commercially available. The use of diatomaceous earth as solid support for liquid-liquid extraction is recommended for the sample preparation of aqueous samples, and can be used within the pH range 1–13. When this material is mixed with the sample, which has been previously homogenised in sodium hydroxide solution, the aqueous phase is distributed in the form of a thin film over the chemically inert matrix. Subsequently, HCAs are eluted using organic solvents that are non-miscible with water, which means this process could be considered a liquid-liquid extraction. When diatomaceous earth is used for this process, lipophilic substances are extracted from the aqueous into the organic phase, and macromolecules such as proteins and carbohydrates remain adsorbed on the inert material. Unlike classic liquid-liquid extractions, this method has the advantage of avoiding emulsions, the process is faster and less solvent is required. The organic solvents most commonly used are DCM and ethyl acetate. In addition, some authors observed that elution from diatomaceous earth is

improved by adding 5–10% toluene or phenol to dichloromethane.[23,24] This improvement was used in several studies.[18,19,25-28]

Hitherto, the most popular tandem method is the one proposed by Gross in 1990. This consists of combining diatomaceous earth with propylsulphonate silica (PRS method). The method is the basis for a number of procedures that give a single purified extract containing HCAs.[4] In this protocol, the sample homogenised with 1 M sodium hydroxide is loaded in a diatomaceous earth column, which is coupled to a PRS cartridge. The analytes are then transferred from the diatomaceous earth to the cationic exchanger sorbent by passing dichloromethane (DCM), DCM with additives or ethyl acetate through the tandem. To activate the ion exchange process, 0.01 M hydrochloric acid is passed by the PRS sorbent, and the cartridge is then washed with a methanol/hydrochloric acid (MeOH/0.1 M HCl, 6:4) solution. The analytes are then eluted with ammonium acetate pH 8 and retained in a C_{18} cartridge, to achieve a pre-concentration prior to chromatography. The diagram of this method is shown in Fig. 5.2. It is important to underline that, with this procedure, most of the less polar amines (i.e. carbolines) are missed in the final extract. In spite of this drawback, a slightly modified version of this method has recently been applied for the analysis of polar compounds in several meat dishes prepared following traditional Polish recipes.[29]

Several studies have sought to improve the potential of the Gross method for analysing the highest number of amines. The main modifications of the original method are the increase in hydrochloric acid concentration from 0.01 M to 0.1 M, in order to raise ionic activation, or the change in the MeOH/H_2O ratio from (6:4) to (4:6), to minimise the elution of some of the analytes during the washing step. Some methods include both modifications. To avoid the losses of the analytes during the step in PRS of washing with hydrochloric acid, acidic preconditioning before the sample treatment has been suggested. Despite all these modifications, the PRS extraction method shows its limitations when highly complex samples, such as process flavours, bouillon concentrates or pan residues, are analysed by UV or electrochemical detection. Therefore, additional clean-up steps are needed to purify these samples more efficiently, to improve chromatographic efficiency and to obtain detection sensitivities similar to those obtained with heated meat products. Some examples shown in Tables 5.1 and 5.2 include an additional purification step,[30,31] an alternative replacement of the PRS+C_{18} tandem by only one sorbent such as MCX,[32-34] or copper phthalocyanine trisulphonate-derived sorbents such as Blue cotton[35] or Blue Chitin,[13] which give sufficiently good purification for analysis.

As stated above, the acidic washing of the PRS sorbent results not only in the activation of the ionic exchange, but also in the losses of the less polar analytes (PhIP, carbolines). Therefore, another tandem was developed to allow the recovery of all HCAs: this is known as the Gross-Grüter method (Fig. 5.2).[16] These authors tested the effect of the ratio MeOH/HCl on the washing solution and found that the best compromise between selectivity and recovery was obtained when 0.1 M hydrochloric acid containing 40–50% of methanol was

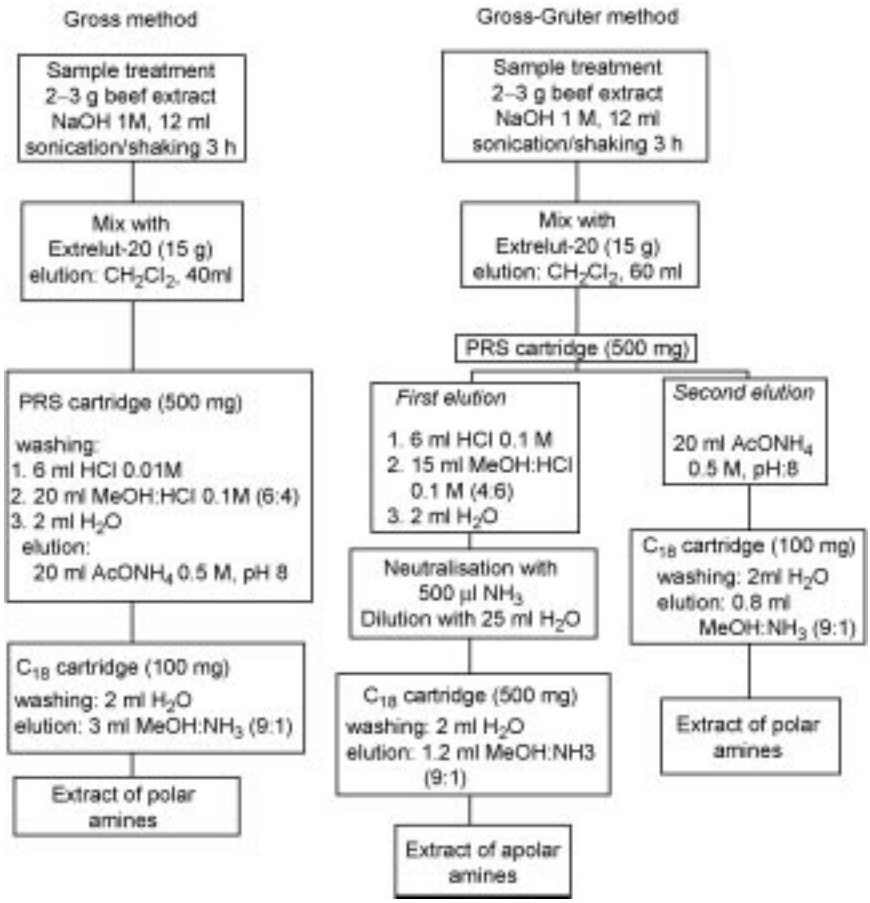

Fig. 5.2 Original tandem extraction procedures.

used, achieving selective desorption of the less polar amines. As shown in Tables 5.1 and 5.2, in most of the analyses of HCAs in several thermal processed meals, the sample treatment is based on this tandem extraction system, which allowed the independent analysis of both polar and non-polar HCAs. In some cases, the two final extracts that the Gross-Grüter-based procedures provide are combined for simultaneous analysis of the whole group of amines, which is less time-consuming.

Galceran *et al.*[17] studied the influence of the concentration of HCl in the rinsing step, and better results were obtained when 0.01 M HCl and MeOH : 0.1 M HCl (6:4) were used in the first elution of PRS sorbent. The increase of MeOH in the solution allows the collection of PhIP in the less polar extract. The ratio MeOH/HCl is also changed and, in some cases, the modification implies the use of ammonium acetate at pH 8.5. This modified method has been recommended for the analysis of a potential reference material in a recent inter-laboratory

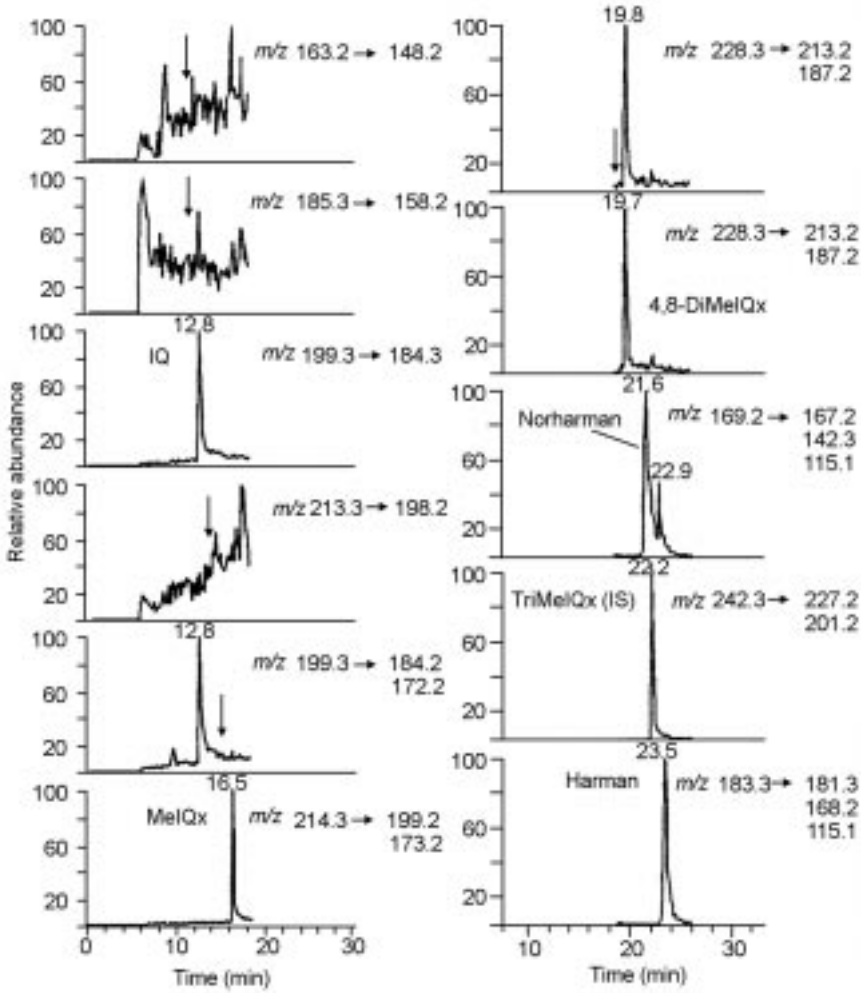

Fig. 5.3 LC–APCI-MS/MS chromatogram obtained for a meat extract purified with clean-up procedure based on the Gross method, but modified by Toribio *et al.* to prevent losses of non-polar amines. The arrows indicate where other non-detected HCAs such as DMIP, Glu-P-2, MeIQ, Glu-P-1, 7,8-DiMeIQx and Trp-P-2 would be expected (reprinted from ref. 37 with permission).

exercise.[36] Other changes are aimed at the analysis of more complex samples, and include an additional clean-up step. In some cases, this additional step uses a Fractogel TSK CM column, a weak cation-exchange gel. The use of a polymeric sorbent avoids the irreversible adsorption produced when silica gel is used. In other cases, Bond Elut SCX or Isolute CBA, which is a weak cation exchanger consisting of propylcarboxylic acid, is used for the additional clean-up step. Other possible modifications are not only the recombination of the final extracts

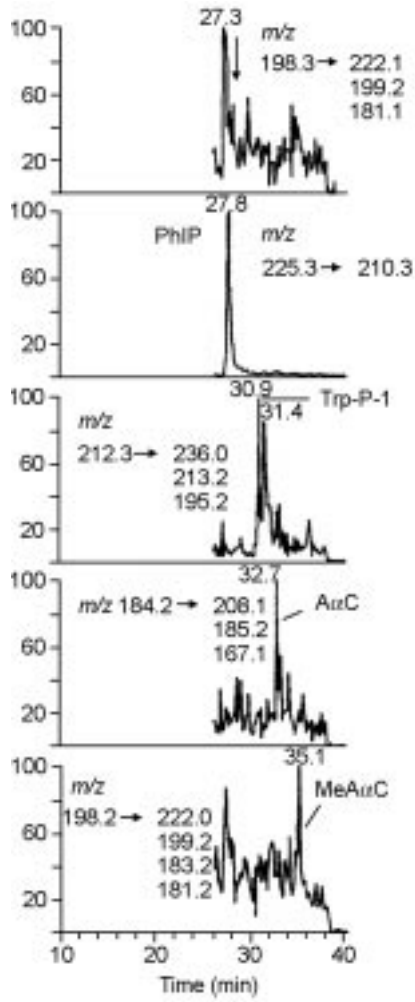

Fig. 5.3 Continued

obtained, but the replacement of the LLE using diatomaceous earth by a classic LLE process.

Finally, the tandem extraction procedures can also be classified in two groups according to the number of extracts yielded, i.e. single-extract or two-extract methods. In general, a compromise between high recovery and clean-up efficiency must be achieved in the selection of the most appropriate procedure. Actually, the choice of some of these methods for a particular analysis will depend on the matrix sample and must also take into account both the required sensitivity and the detection technique. For example, Fig. 5.3 shows the chromatogram obtained with a clean-up procedure based on the Gross method, but modified by Toribio *et al.* to prevent losses of non-polar amines.[37] In this

case, the method provides a single purified extract and, even in the analysis of complex matrices such as a meat extract, a final purified extract clean enough for analytical purposes is obtained. The clean-up strategies described in the most recent publications can also be integrated as tandem extraction procedures. Thus, several authors determined both non-polar and polar amines in a lyophilised meat extract[38,39] and a fried chicken breast, using a modified version of the Gross-Grüter method,[17] which can be integrated as a two-extract procedure. In addition, the analysis of several fried ground beef and chicken breast samples using a single-extract procedure based on the use of MCX sorbent has recently been described.[34] This sorbent exhibits a mixed-mode mechanism (ion exchange and C_{18}) and has some advantages over those used in the method originally developed by Gross-Grüter. This method enables the sample to be cleaned up in one step and all the HCAs to be extracted in only one fraction.

5.3 Chromatographic analysis

Chromatography has been involved in the analysis of HCAs since the very beginning of the identification of these compounds in cooked food matrices. At that time, preparative liquid chromatography was used in combination with the Ames test to measure mutagenic activity of fractions obtained from cooked foods. Even now, these methods are used by some authors to study the effect of several treatments on the formation of HCA mutagenicity in cooked foods.[40-42] In addition, liquid chromatography can be used to isolate HCAs from various matrices and to assist purification. The compounds obtained were characterised by their ultraviolet (UV) or fluorescent spectra, proton nuclear magnetic resonance (^{1}H NMR) and mass spectrometry (MS). However, the most important use of chromatography in the study of HCAs is related to the determination of the concentration levels of this family of compounds in proteinaceous foods such as meat or fish processed by different cooking practices, meat extracts, beef flavours and other matrices including wine and beer.

Both chromatographic techniques, liquid chromatography (LC) and gas chromatography (GC), have been used to determine HCAs in food samples. GC is mainly coupled with mass spectrometry, but several detector systems such as UV, fluorescence or electrochemical detection are used with LC. Nevertheless, in recent years the enormous potential advantage of LC-MS over selective detection, on-line identification and good detection limits has led to its being the technique of choice for the analysis of these compounds. Capillary electrophoresis (CE) has also been put forward by some authors as a way of determining HCAs in some cooked foods, as reported in the review by Pais *et al.*[3] CE takes advantage of high-resolution separations at a minimal cost in terms of staff time and reagent use. However, CE lacks the sensitivity of liquid chromatography and its coupling to mass spectrometry is not a straightforward task. Even so, an attempt to perform on-line CE pre-concentration and coupling to nanospray mass spectrometry has been recently reported.[43] These limitations are

probably sufficient explanation of why no information on the use of CE to analyse HCAs in foods has appeared recently in the literature.

5.3.1 Liquid chromatography

Most of the analytical procedures for HCA determination in the literature are based on LC with UV detection, although the poor specificity of this detection system requires exhaustive clean-up processes that often lead to lower analyte recovery and so less accuracy. Table 5.1 summarises the compounds studied, the samples analysed and the chromatographic conditions proposed in the literature published from 1999 till now that use liquid chromatography coupled with conventional detection systems. Most of the papers studied the formation of HCAs in model systems or the effect of different cooking practices on the generation of HCAs. In UV detection, ultraviolet absorbance spectra using diode array devices help in peak confirmation and this reason, along with the low price and widespread use of this detector in most analytical laboratories, explains its general application to HA analysis (Table 5.1).

As an example, Fig. 5.4 shows the chromatogram obtained for a puppy food,[44] with identification of MeIQx and PhIP using UV spectra. However,

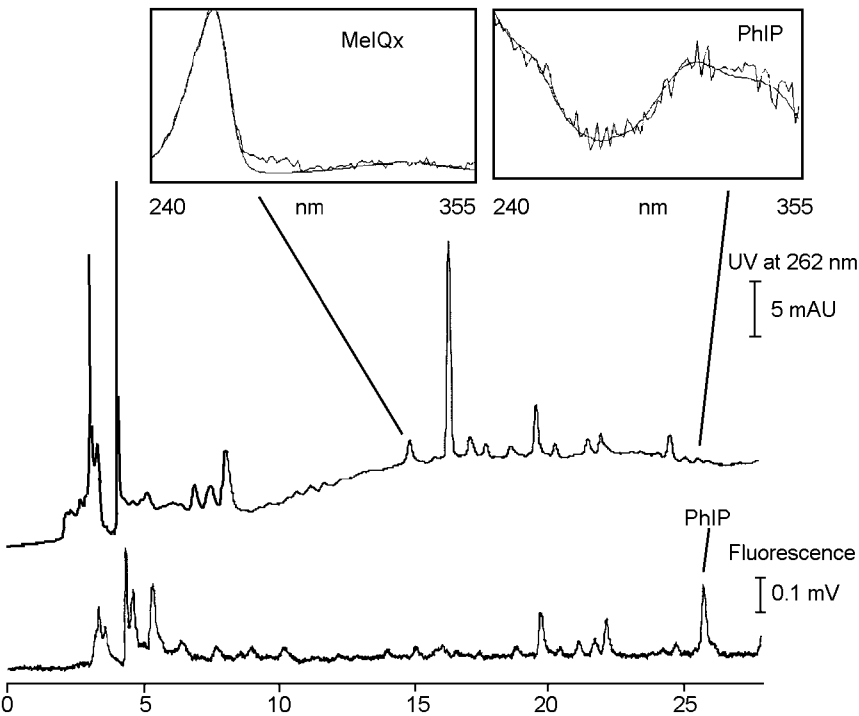

Fig. 5.4 LC chromatograms and UV absorbance spectra obtained for a puppy food sample (reprinted from ref. 44 with permission).

interfering compounds frequently co-elute in the chromatogram with the analytes, decreasing sensitivity and making quantification difficult. In fact, limits of detection in UV are relatively high, between 0.5 and 1.3 ng injected for standard solutions. Table 5.3 shows detection limits found in our laboratory for several HCAs, using UV (diode array), fluorescence and electrochemical detection for standard solutions and a lyophilised meat extract.[45] Due to the fluorescent properties of some HCAs and the resulting higher selectivity and sensitivity, this last detection system is frequently used simultaneously with UV (Table 5.1), as happens in the example in Fig. 5.4, in which a better signal for PhIP is obtained with fluorescence detection. Very low limits of detection (0.02–0.2 pg injected for standard solutions) are obtained with fluorescence, although in the solvents commonly used in reversed-phase liquid chromatography (water, methanol or ACN), the IQ-type compounds are not fluorescent and so reduce the applicability of this technique.

In contrast, as all HCAs are electrochemically active, electrochemical detection giving high selectivity and sensitivity can be used for their analysis. Detection limits, between 5 and 80 pg for standard solutions, are not as low as those obtained with fluorescence detection (Table 5.1), but a larger number of HCAs including IQ-type compounds give good responses. Conventional electrochemical detectors cannot help in the confirmation of the chromatographic peaks, but coulometric array detection systems have been proposed[26] for this purpose.

Sample matrix and sample treatment greatly influence chromatographic interference and detection limits. For instance, as cooking temperature is a major

Table 5.3 Detection limits in LC coupled to conventional detectors

Compound	Standard solution pg injected			Lyophilised meat extract ng/g		
	UV	EC	FD	UV	EC	FD
DMIP	1100	16	–	n.d.*	15	–
Glu-P-2	800	79	0.22	–	15	0.6
IQ	300	40	–	140	8	–
MeIQx	350	44	–	650	8	–
MeIQ	550	65	–	n.d.*	30	–
Glu-P-1	850	55	0.09	–	15	0.5
7,8-DiMeIQx	500	7	–	–	2	–
4,8-DiMeIQx	500	41	–	n.d.*	30	–
Norharman	650	25	0.09	–	2	0.2
Harman	450	25	0.03	–	4	0.2
Trp-P-2	400	5	0.03	n.d.*	5	0.5
Trp-P-1	450	54	0.04	n.d.*	n.d.*	0.5
PhIP	550	11	0.18	n.d.*	5	0.2
AαC	400	22	0.12	240	6	1
MeAαC	600	33	0.16	n.d.*	n.d.*	0.4

* Not determined because of interferences.

factor affecting detection limits in food samples, at high cooking temperatures chromatograms became more complex and the detection limits of HCAs increased. Therefore, detection limits in cooked food samples depend not only on the characteristics of the detector, but also on the capacity of the clean-up procedure to reduce interference. LODs found in the literature for UV detection embrace a very wide range, probably due to the diverse samples and cooking practices used by different authors, but also to the method used to evaluate detection limits. Most publications give no information about LOD calculation procedure whereas in others LODs were calculated by spiking the sample with increasing amounts of standard solution. In general, most values for cooked foods lie between 0.05 and 10 ng/g, depending on the presence or absence of co-extracted interferences.[11,44,46,47] The values increase up to 50 ng/g in flavours[3] or > 100 ng/g in lyophilised meat extracts (Table 5.3), due to the complexity of these samples, which leads to a higher number of interfering co-eluting compounds in the chromatogram. In such cases, additional clean-up steps are recommended, even though this causes a decrease in the recoveries and higher standard deviations.[19,48]

Detection limits of HCAs in cooked food samples using fluorescence or electrochemical detection are better than those with UV. As an example, Table 5.3 gives LOD values obtained with UV, fluorescence and electrochemical detection. To evaluate these LODs, a lyophilised meat extract that did not contain detectable amounts of HCAs[45] was spiked at very low concentration levels. Data of Table 5.3 show that LODs with fluorescence detection are generally more than ten times lower than with electrochemical detection, although only a small group of HCAs has intrinsic fluorescence. Interference frequently prevents the use of electrochemical detection for food sample analysis. For instance, in Table 5.3, the LODs for some of the HCAs could not be evaluated due to the coelution of interfering compounds. Moreover, in an inter-laboratory study of several lyophilised meat extracts, electrochemical detection could only be used to analyse a sample with high concentrations (75 ng/g).[36] Nevertheless, matrices less complex than beef extracts could be analysed by electrochemical detection, such as soup cubes[26] or chicken.[31]

In chromatographic separation, reversed-phase stationary phases such as C_8 and C_{18} on silica-based particles of 5 μm are the most widely used. Of these, the TSKgel ODS column is the most common and phosphate, acetate or formate buffers at low pH (3–4) with acetonitrile as organic modifier are the most popular mobile phases for the analysis of HCAs by liquid chromatography. To obtain narrow and symmetrical peaks, triethylamine is generally added to the mobile phase and gradient elution is performed with UV or fluorescence detection (Table 5.1), which gives enough resolution for all the eluted compounds. In electrochemical detection, gradient elution is difficult to use because of major distortions in the base line. Therefore, isocratic elution is used instead, although frequently all the compounds cannot be eluted in just one chromatogram and two mobile phases with different amounts of organic solvent are needed.

5.3.2 Liquid chromatography-mass spectrometry

The coupling of mass spectrometry and liquid chromatography combines the capacity and versatility of this separation technique with the high selectivity and sensitivity of mass spectrometry. One of the most important benefits of MS over other detection techniques is that it can confirm analyte identity, by giving both molecular mass and structural information. Moreover, MS/MS experiments performed with triple quadrupole analysers and ion traps improve selectivity. For these reasons, in recent years, most of the papers dealing with the analysis of HCAs in food samples reported the use of this kind of coupling. A thorough review on the application of LC-MS to HCA analysis was published by Pais *et al.* in 2000.[3] In this chapter the improvements and new applications of this technique from this date to the present are discussed. Table 5.2 summarises the samples and compounds analysed and the experimental conditions, and refers to the original literature.

Silica-based reversed-phase columns (C_8 and C_{18}) with particle diameters between 2 and 5 m are commonly used in LC-MS analysis of HCAs. As columns with small internal diameters, 2 or 1 mm, improve sensitivity when an electrospray ionisation source is applied, their use has increased in recent years. The behaviour of several reversed-phase columns for the separation of 16 HCAs was evaluated in a recent study.[49] The authors indicate that, for peak symmetry and number of theoretical plates, the traditional TSK Gel column ODS-80TS gives the best performance and, in addition, takes less equilibration time. However, good results have also been obtained in several laboratories with other columns such as Symmetry C_8 and Aquasil C_{18} (Table 5.2). As mobile phases need to be volatile to be compatible with MS systems, acetic and formic ammonium salts are mostly used as buffers and ACN as organic modifier for gradient elution.

Over the years, a variety of ionisation techniques have been used to identify, confirm and analyse HCAs in cooked foods and model systems. The main advantages of MS are its sensitivity and selectivity, which detect and quantify HCAs at low levels without the addition of purification schemes to prevent interference. The first LC-MS analyses of HCAs were performed with thermospray (TSP) as ionisation source and the spectra obtained showed mainly the protonated molecular ions of the compounds. At that time, in most applications the coupling was only used for confirmation, and quantification was mostly performed by fluorescence or UV detection.[3] However, since the mid-1990s when atmospheric pressure ionisation sources (API) became more popular, the use of TSP has been replaced by electrospray (ESI) and atmospheric pressure chemical ionisation (APCI). Both sources are used for HCA analysis, although ESI is more common (Table 5.2).

In both cases, HCAs provide the protonated molecular ion $[M+H]^+$ as base peak, but the responses seem to be higher when ESI is used. However, it is difficult to compare the LODs found in the literature for standards and food matrices obtained with the two ionisation techniques, since LODs depend on instrument design and experimental conditions such as mobile phase, type of column, acquisition parameters and sample matrix. Nevertheless, generally

better detection limits are obtained with ESI. For instance, LOD values 2–6 times lower were obtained with ESI[50] than with APCI,[37] using the same lyophilised meat extract spiked at very low levels and the same clean-up procedure. Similar results were obtained with other analysers and working modes, such as MS (single ion monitoring) and MS/MS (product ion scan and selected ion monitoring). Thus, for HCAs, ESI provides the best ionisation efficiency, regardless of the instrument employed. This is probably why in recent years most authors used this ionisation source (Table 5.2).

Nevertheless, it is known that ionisation in the ESI source improves with a high amount of organic solvent in the mobile phase because of the decrease in electrospray droplet surface tension. This can cause a problem in some instruments when the analysis of those HCAs eluted at short retention times must be determined, since low percentages of organic solvent in the mobile phase are needed to obtain adequate chromatographic separation. In these cases, the post-column addition of organic solvent enhances ionic evaporation and improves the signal. For example, three- to fourfold signal improvement was obtained for HCAs by the post-column addition of 0.1% formic acid in acetonitrile (100 l/min) when an on-axis ESI source was used.[50]

Of MS analysers, quadrupoles and those that permit tandem mass spectrometry (MS/MS), mainly triple quadrupoles (QqQ) and ion traps (IT), remain the most commonly used. Although tandem mass spectrometry with hybrid instruments such as a quadrupole-time-of-flight (Q-TOF) can be a good alternative because of their great accuracy in mass determination, these instruments have been used very little in HCA analysis (Table 5.2). The easier operating performance, better robustness in routine analysis and the relatively low cost of both quadrupole and IT analysers, compared with Q-TOF instruments, explain their widespread use. Although single quadrupole instruments do not allow confirmation of the quantified analytes, they have sometimes been used, as in the study by Messner *et al.*[33] on the formation of HCAs in a fresh meat model system. These authors used selected ion monitoring (SIM) to increase sensitivity and reduce interference by monitoring only one or several masses at a time. This acquisition mode, selecting the *m/z* corresponding to the protonated molecular ion $[M+H]^+$, has also been used by other authors (Table 5.2) with ion trap analysers, mainly to study the effect of several additives or cooking procedures in the formation of HCAs. However, in ion traps, working with SIM mode does not represent any significant advantage, since the acquisition time needed for each mass is similar to that needed to obtain the spectrum in full scan.

For high selectivity and sensitivity when analysing HCAs in complex matrices, many laboratories use tandem mass spectrometry with both triple quadrupoles and ion traps. As triple quadrupole analysers display the highest sensitivity when working in selected reaction monitoring (SRM), this is the acquisition mode most frequently used. For example, Fig. 5.5 shows the chromatogram obtained for a griddled chicken sample using a QqQ instrument and SRM.[51] When performing tandem mass spectrometry, ion traps are

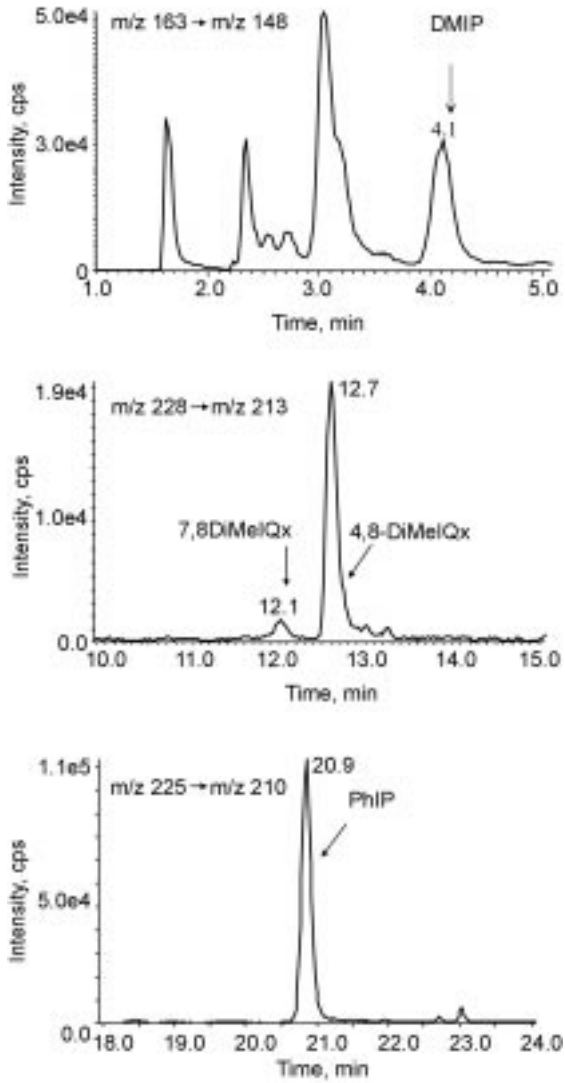

Fig. 5.5 LC chromatogram obtained for a griddled chicken sample using a QqQ instrument in SRM mode (reprinted from ref. 51 with permission).

generally less sensitive than triple quadrupole analysers, but they have the advantage of working on product ion scan without losses in sensitivity, which allows product ion spectra to be obtained even near the detection limit. This helps in identification and confirmation purposes. Figure 5.6 shows the LC-ESI-MS/MS chromatogram of a lyophilised meat extract spiked at very low levels (~2 ng/g) of IQ and PhIP where the high quality of the spectra obtained can be seen.

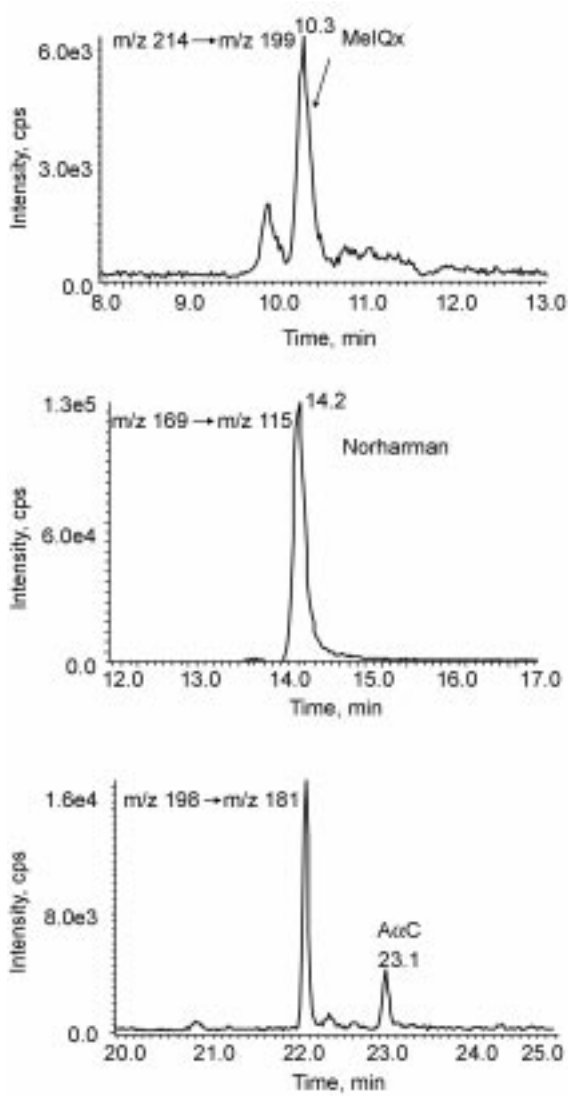

Fig. 5.5 Continued

Comparison of the responses of different mass analysers from data found in the literature is not easy. Most of the publications do not include values of limits of detection and/or quantification, and different food samples, clean-up procedures, chromatographic conditions and MS instruments are used. Nevertheless, in general, LODs down to 0.02–0.05 ng/g are found when using SRM and triple quadrupole instruments, whereas these values increased to 0.1–3 ng/g with ion traps. Table 5.4 gives the LODs obtained for HCAs with several different acquisition modes (SIM, product ion scan, SRM and single MS), four MS

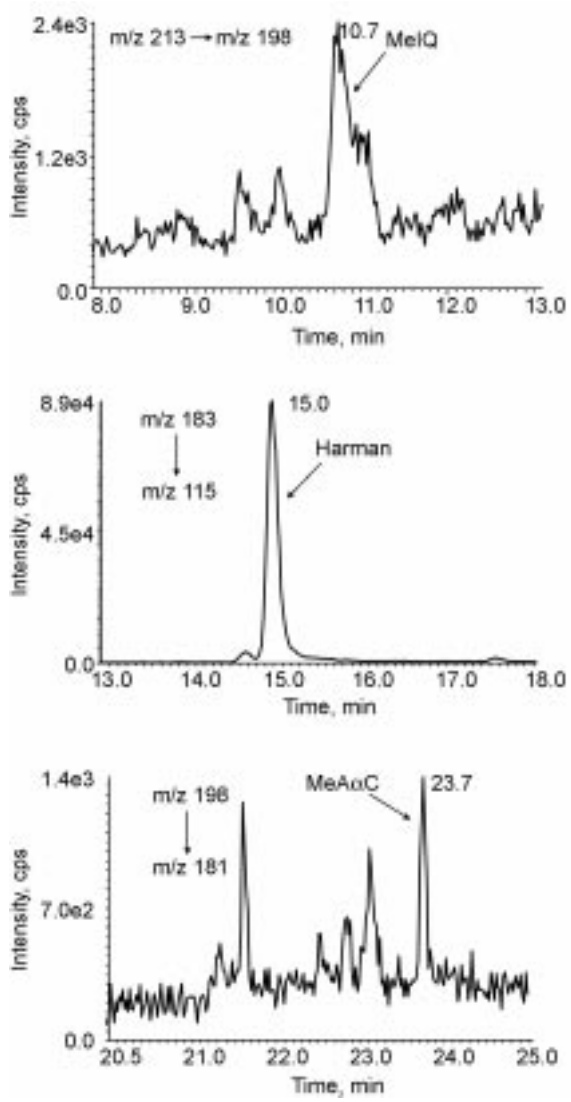

Fig. 5.5 Continued

analysers and ESI as ionisation source. These LODs were calculated by spiking a lyophilised meat extract with low amounts of HAs and performing the same extraction and clean-up procedure.[38,50]

The best LODs were obtained for the triple quadrupole instrument working with SRM acquisition mode, while higher values, similar in the two cases, were obtained for the ion trap instrument working in product ion scan mode and for the Q-TOF working in single MS mode. However, the high accuracy of the TOF analyser allows unambiguous identification of the compounds and

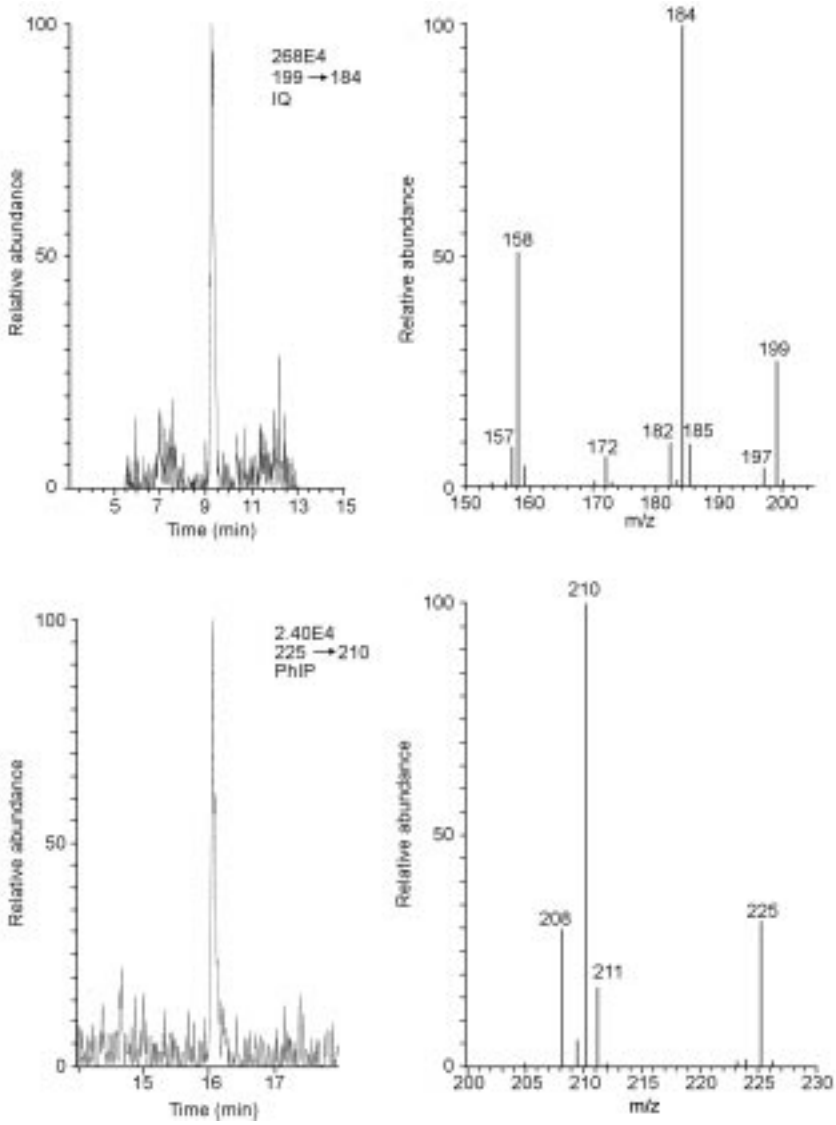

Fig. 5.6 LC-ESI-MS/MS chromatogram and mass spectra obtained for a lyophilised meat extract spiked at 2 ng/g of IQ and PhIP.

ion traps provide spectral information that helps in identification when complex samples are analysed. Moreover, it is important to realise that detection limits not only depend on the analyser but also on the design of the ionisation source. In consequence, instruments that provide higher electrospray efficiency, such as the QqQ and the Q-TOF used to obtain data in Table 5.4, provide higher sensitivity. In fact, LODs found recently in our working group with a latest-generation ion trap, a linear trap, which has

Table 5.4 Detection limits in a lyophilised meat extract (pg injected and ng/g) using ESI and several MS analysers and acquisition modes

HCAs	Ion trap				Quadrupole		Triple quadrupole				Q-TOF	
	Full scan		Product ion scan		SIM		SIM		SRM		MS mode	
	pg inj.	ng/g	pg inj.	ng/g	pg inj.	ng/g	pg inj.	ng/g	pg inj.	ng/g	pg inj.	ng/g
DMIP	563	8.4	179	3.6	25	0.5	18	0.4	3	0.1	23	1.4
Glu-P-2	253	5.1	94	1.9	29	0.6	40	0.8	5	0.1	18	1.1
IQ	180	3.6	30	0.6	25	0.5	37	0.7	2	0.04	31	1.9
MeIQx	400	8.0	147	2.9	85	1.7	56	1.1	4	0.1	9	0.5
MeIQ	68	1.4	37	0.7	61	1.2	21	0.4	2	0.04	31	1.9
Glu-P-1	213	4.3	69	1.4	14	0.3	32	0.6	5	0.1	14	0.8
7,8-DiMeIQx	136	2.7	54	1.1	7	0.1	25	0.5	3	0.1	7	0.4
4,8-DiMeIQx	141	2.8	59	1.2	9	0.2	26	0.5	3	0.1	9	0.5
Norharman	214	4.3	14	0.3	18	0.4	10	0.2	2	0.04	30	1.8
Harman	385	7.7	32	0.6	32	0.6	21	0.4	4	0.1	19	1.1
Trp-P-2	225	4.5	28	0.6	43	0.9	9	0.2	1	0.02	8	0.5
Trp-P-1	168	3.4	6	0.1	17	0.3	2	0.04	1	0.02	4	0.2
PhIP	450	9.0	63	1.3	41	0.8	18	0.4	0.5	0.01	10	0.6
AαC	341	6.8	86	1.7	25	0.5	11	0.2	1	0.02	49	1.9
MeAαC	352	7.0	83	1.7	27	0.5	10	0.2	1	0.02	16	1.0

an optimised ion source, were two to ten times lower than those obtained in SRM with the QqQ instrument.

An important characteristic of ion traps is the possibility of undertaking multiple-stage fragmentation $(MS)^n$, which is very useful for studying the fragmentation pathways of compounds. Knowledge of the characteristic fragmentation pathways for the different families of HCAs, aminoamidazoazaarenes and carbolines, facilitates the screening of unknown mutagens belonging to these families. Since the early days of HCA study with MS, several authors have proposed tentative fragment assignments using different MS techniques such as direct introduction and electron ionisation with high-resolution mass spectrometry,[52] LC-ESI-MS/MS with QqQ instruments[53] or LC-MS and in-source fragmentation in quadrupole instruments.[54,55] In these studies it was found that imidazoquinolines and imidazoquinoxalines have a similar fragmentation pattern, with the loss of the methyl group as the main fragment, although quinoxalines also showed the cleavage of the aminoimidazol group. For carbolines, the loss of amino and methyl groups was the most important fragmentation, though HCN was eliminated too.

More recently, Toribio *et al.*[56] used the MS^n approach with an ion trap and labelled compounds (^{13}C and D_3) in a thorough study of the fragmentation of HCAs in order to establish the fragmentation pathways of these compounds. In this study, MS^2, MS^3 and in some cases MS^4 were used to propose assignments of the fragments via genealogical relationships, to confirm the fragments previously proposed by other authors and to identify new ones. As an example of the kind of information that can be obtained by using multiple-step fragmentation in an ion trap, Fig. 5.7 gives the tentative fragmentation pathway for MeIQ, 4,8-DiMeIQx and TriMeIQx. In this context, it is important to mention that sometimes, owing to the low resolution of the ion trap, fragment ions can be assigned to more than one elemental composition, giving as a result several possible fragmentation pathways. In these cases, the accurate mass measurement provided by the Q-TOF instruments enables wrong assignments to be discarded. This approach was used by Barceló-Barrachina *et al.*[38] to confirm some HCA fragmentations and to explain differences found between spectra obtained from different instruments. For instance, Fig. 5.8 shows the MS/MS spectrum obtained with a Q-TOF instrument in which the corresponding mass assignments are given. The high resolution of the instrument allowed two fragments to be distinguished at *m/z* 172, one from a direct loss of $\cdot CN_2H_2$ and the other from the consecutive losses of $\cdot CH_3$ and $\cdot HCN$ due to multiple collisions occurring in the Q-TOF. The high accurate mass capacity of this analyser was used by other authors[28] to identify new compounds in meat. For example, Fig. 5.9 shows the LC-ESI-Q-TOF-MS chromatogram of a grilled meat sample, in which the mutagen 8-MeIQx and its putative isomer 1-Iso-MeIQx were found.

Turesky *et al.*[34,57] used triple quadrupole analysers and several scan modes to identify and confirm the presence of unknown HCAs in food samples. For the screening of new HCAs, Guy *et al.*[57] applied three different scan acquisition

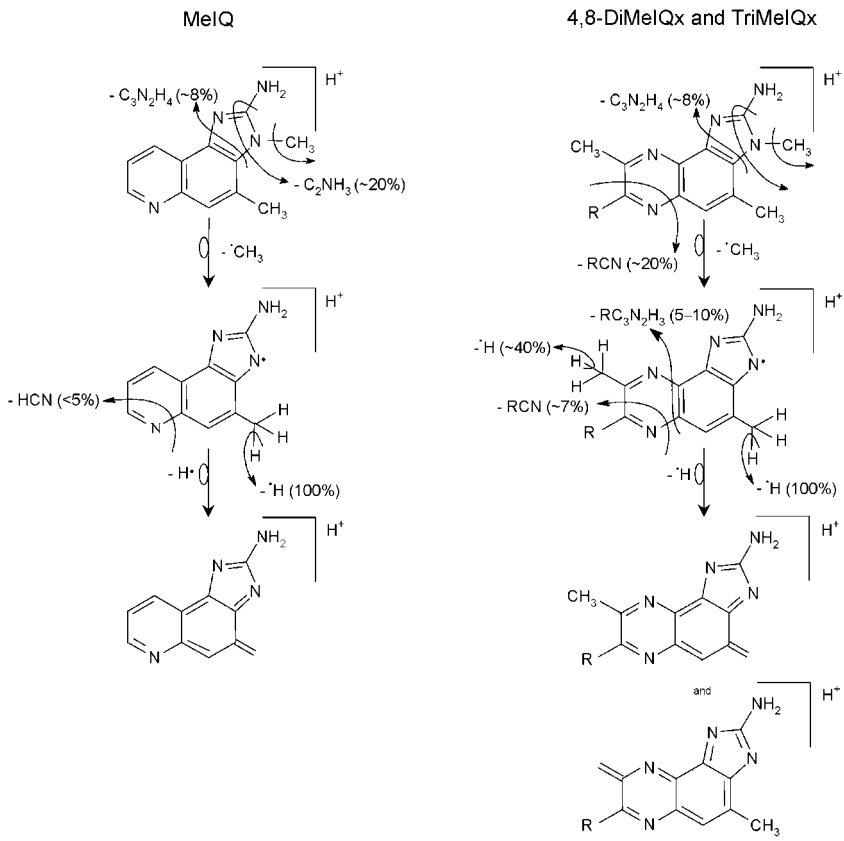

Fig. 5.7 Tentative fragmentation pathway for MeIQ, 4,8-DiMeIQx and TriMeIQx by LC-APCI-MSn(IT).

modes, neutral loss, precursor ion and product ion. With this approach, the authors confirmed the presence of 7,9-DiMeIgQx in a meat extract. In a recent paper, Tureski *et al*.[34] used a similar approach to identify and quantify IQ [4,5-b] in several foods. Moreover, six other compounds, probably isomers of IQx, 8-MeIQx and DiMeIQx, were also detected. In this study, the authors used the highly sensitive SRM scan mode to detect and quantify known HCAs. To identify the analytes, they selected several characteristic fragment ions for each compound, taking advantage of their knowledge of the corresponding fragmentation patterns. In addition, the authors used the product ion scan mode to obtain information about unknown eluted compounds and to suggest plausible structures.

In general, the spectra obtained using different analysers are quite similar and no important differences were detected.[38,50] Nevertheless, for carbolines the spectra on ion trap instruments show ions at *m/z* values higher than the protonated molecular ion, which are due to ion-molecule association reactions

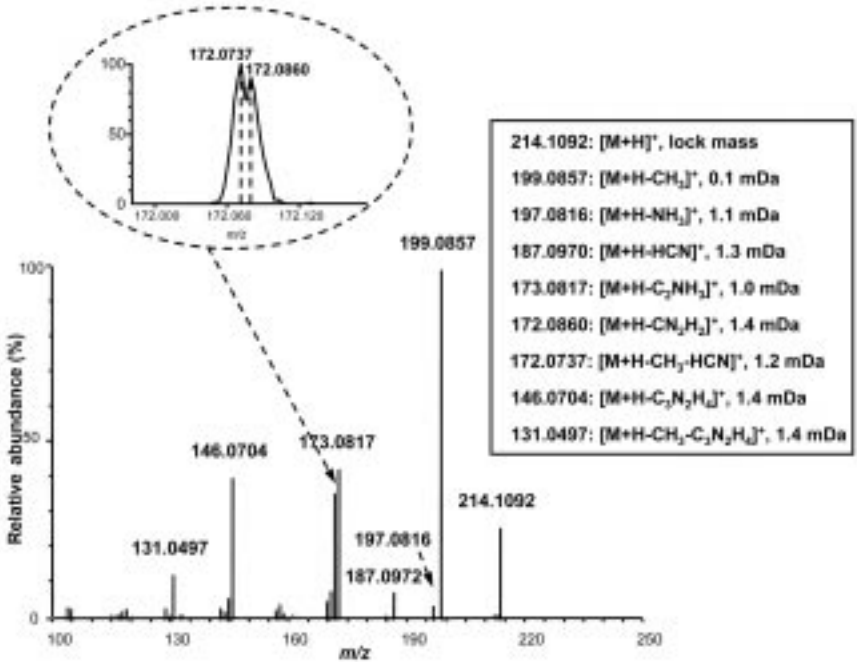

Fig. 5.8 MS/MS spectrum of MeIQx obtained with a Q-TOF instrument showing the assignments of each fragment with its corresponding mass accuracy (reprinted from ref. 38 with permission).

occurring inside the trap. These reactions take place between the product ion $[M+\mathrm{H}-\mathrm{NH_3}]^+$ of carbolines and solvent molecules present in the ion trap, such as water, methanol or ACN. In some cases, such as for α-carbolines, the resulting ions are very abundant and correspond to the base peak. Figure 5.10 shows the spectra obtained for MeAαC using several analysers, a QqQ, a classic IT, a linear trap and a Q-TOF, in which the spectrum obtained with the classic IT shows the ions at *m/z* 199 and *m/z* 222. These ions that correspond to adducts with H_2O and ACN[56] do not appear in the spectra obtained with the triple quadrupole and with the Q-TOF. Their presence was much lower when the new ion trap, the linear trap, was used because the new design of this instrument reduces the entrance of the solvents in the trap. The abundance of adduct product ions is highly dependent on small changes in the experimental conditions. Therefore, to achieve reproducible results, these ions must be taken into account in the quantification of HCAs by MS/MS.

5.3.3 Gas chromatography-mass spectrometry

Gas chromatography coupled with mass spectrometry (GC-MS) has been the most common procedure used to identify new mutagens in cooked food samples and model systems, because of the advantages of the high separation efficiency

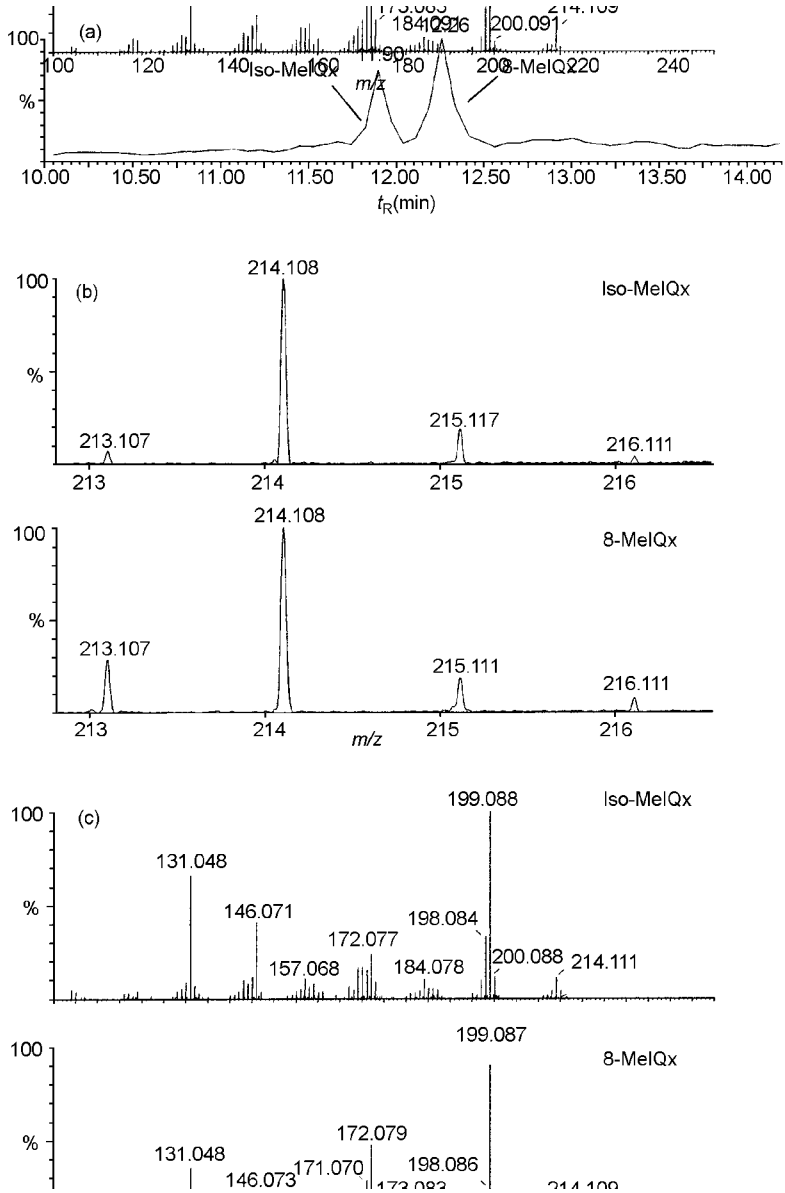

Fig. 5.9 On-line LC-ESI-Q-TOF/MS chromatogram of (A) 8-MeIQx and putative 8-MeIQx isomer in grilled meat, (B) limited full scan spectra, and (C) product ion spectra of [M + H]+. The instrument was recalibrated, postacquisition, using the spectrum of synthetic 8-MeIQx (m/z 214.109), and data are smoothed and centred (reprinted from ref. 28 with permission).

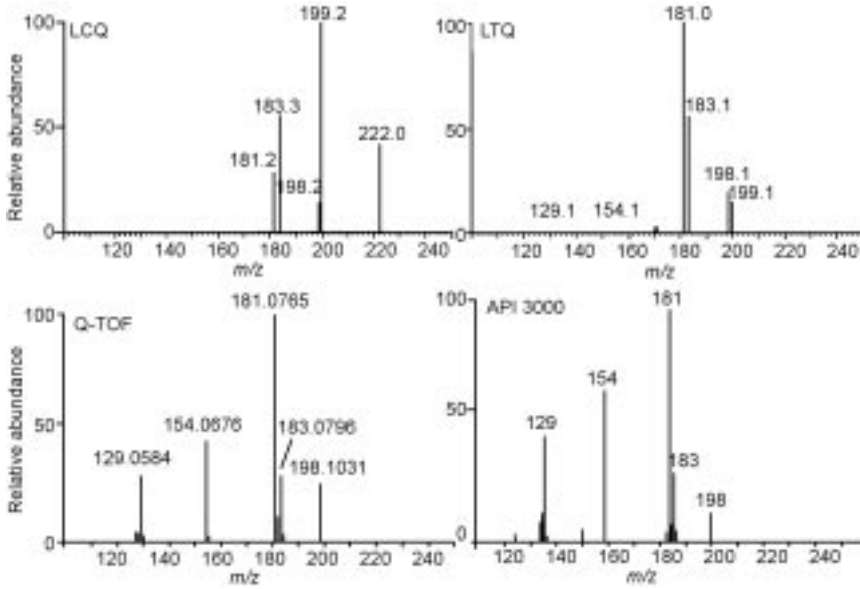

Fig. 5.10 Comparison of the MS/MS spectra obtained for MeAαC using several analysers.

provided by GC capillary columns and of the high sensitivity and specificity of mass spectrometry. Even nowadays, when LC-MS is becoming the technique of choice for this kind of analysis, GC-MS is sometimes used to confirm the identity of HCAs. This is reported, for instance, in the papers on the presence of these mutagens in several meat dishes.[29,58] However, HCAs are polar and non-volatile compounds, which makes it difficult to analyse them by gas chromatography. Although there are some references in the literature to the analysis of some HCAs, mainly the less polar ones, Trp-P-1, Trp-P-2, AαC, Me AαC, harman and norharman, by GC without derivatisation, generally a derivatisation step is performed. For the analysis of HCAs without GC-MS derivatisation, a semi-polar stationary phase (50% methyl, 50% phenyl polysiloxane) is used[19] but even so there is strong retention of HCAs in the column. To improve the characteristics of these compounds, by increasing volatility and reducing polarity to prevent strong adsorption in the GC system, derivatisation of the amine group is recommended.

Most of the derivatisation methods developed for the analysis of HCAs by GC-MS were reviewed in a paper published by Pais and Knize.[3] Only those analyses of HCAs in foods that appeared in the literature later than the publication of this review are discussed here. The first alkylation reagent proposed for the derivatisation of the primary amino group of HCAs was 3,5-bis-trifluoromethylbenzoil bromide, but the poor chromatographic behaviour of the derivatives meant they could not be used for quantitative analysis. Later, this reagent was replaced by 3,5-bis-trifluoromethylbenzyl bromide, which gives

alkyl derivatives with good chromatographic properties. This procedure has been frequently used,[59,60] but not all HCAs can be efficiently derivatised: mono- or di-derivatives depending on the compounds are obtained and detection limits are not as good as with LC-ESI-MS/MS.[60] Acylation products using acid anhydrides such as heptafluorobutiric and pentafluoropropionic have also been tried out as HCA derivatives. However, most of these yield derivatives with poor chromatographic properties. To improve peak shape, mainly for heptafluorobutiric derivatives, an additional methylation step with diazomethane or dimethylformamide dimethylacetal is needed. The reaction with pentafluoropropionic acid anhydride was recently used to confirm the identities of HCAs found in several foods analysed by LC-UV.[29,58]

Another approach to the determination of HCAs by GC is the preparation of the N-dimethylaminoalkylene derivatives by alkylation using N,N-dimethylformamide dialkyl acetal. Two different reagents have been suggested, N,N-dimethylformamide dimethylacetal[61] and N,N-dimethylformamide di-*tert*-butylacetal.[39] One of the advantages of this method is that, unlike other procedures, it enables a large number of HCAs to be derivatised. This approach has been used for the analysis of these mutagens in several food samples by GC with nitrogen-phosphorous detection (NPD)[61] and also in a meat extract with mass spectrometry.[39] In this latter case, the molecular ion $[M]^+$ was found to be the base peak for most of the compounds and was used for quantification, while the fragment $[M\text{-}56]^+$ was proposed for confirmation. Recently, a method based on the silylation of the amino group[62] was suggested. In this case, HCAs were transformed to their *tert*-butyldimethylsilyl derivatives in a one-step reaction with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide. Silyl derivatives for most of the HCAs were obtained, except Glu-P-1, Glu-P-2 and harman that gave incomplete derivatisation. Although characterisation of the compounds by MS is easy, since an intense ion $[M\text{-}57]^+$ caused by the loss of the *tert*-butyl group appears in the spectra and very good detection limits, 0.02 to 0.35 ng/g, are obtained, the stability of the silylated derivatives is limited. Thus, the procedure is difficult to be used in routine analysis. In fact, the authors indicate that the derivatised samples must be injected in the same working day.

Electron ionisation (EI) is the most common ionisation mode used to analyse HCAs by GC-MS, as it provides excellent fragmentation patterns with the loss of the derivatising group that allows the characterisation of the compounds. Electron capture/negative ionisation (EC/NI) with ammonia as reaction gas has been also used for the analysis of the 3,5-bis-trifluoromethylbenzyl derivatives of some HCAs in meat samples.[59] As the sensitivity of EC/NI for compounds containing fluor atoms is high, in general, better detection limits than with EI are obtained. Quadrupole instruments working in SIM are the mass analysers mostly used by the researchers that used GC-MS for HCA analysis or confirmation purposes. Only one researcher[39] used an ion trap to evaluate dimethylformamide dialkyl acetal derivatisation for the analysis of HCAs in a meat extract.

5.4 Identification and quantification methods

The analysis of HCAs in model systems and cooked foods to study the formation and presence of HCAs in these kinds of samples is currently directed to the determination of known mutagens, although in some cases the objective is the identification of new compounds. In the first case, both quantification and confirmation of target analytes are required. When LC with UV-DAD detection is used, the identities of the compounds are confirmed by comparison of their retention times and ultraviolet spectral shapes with those of reference standards, although the information about the nature of the analyte that is obtained from the UV spectra is rather limited. In some cases, co-chromatography by adding standards to the samples is applied. Then, the enhancement of peak area or height helps in identification. The tolerance of the relative retention time, i.e., the retention time of the analyte to that of the internal standard, is $\pm 0.5\%$ for GC and $\pm 2.5\%$ for LC.[63] In addition, those compounds that are fluorogenic have often been confirmed by the presence of fluorescence peaks corresponding to UV peaks that have the correct spectral shape and retention time. Even so, the analytes are not fully identified and, in fact, two different columns and/or two chromatographic conditions are needed, so as to have enough data to confirm the presence of the compounds in a real sample.

The advantage of using mass spectrometry coupled with liquid chromatography in this context is that it allows the unequivocal identification of the analyte if a spectrum with enough fragmentation is recorded. However, it should be mentioned that, in most of the applications of MS to the analysis of HCAs, SIM or MS/MS monitoring, only one transition is used, although current legislation requires more than one transition for each target compound to confirm the presence of an analyte in a given sample. Indeed, the EU Council Directive on analytical methods and the interpretation of results[63] requires a minimum of three points for correct confirmation. In low-resolution MS, the monitoring of two transitions from the same precursor ion allows four points to be obtained, and thus is enough for confirmation.

For quantification, several approaches such as external calibration, internal standard and standard addition have been used. When analysing HCAs, matrix effects must be taken into account, particularly when studying complex samples such as foods. In this context, external calibration seems to be less suitable for quantification, although it has been frequently used by several authors (Tables 5.1 and 5.2). In fact, most of the papers on LC-UV analysis of HCAs used this quantification method without adding any internal standard. Internal standards are currently added to the final extract before the injection in the chromatographic system in order to control the final volume obtained in the clean-up and pre-concentration steps and the injected volume in GC and CE. Though various compounds have been put forward as internal standards, TriMeIQx is the most commonly used. The addition of an internal standard at the beginning of the clean-up procedure is also recommended, in order to control the extraction and purification steps and sometimes to correct the results for incomplete recoveries.

Nevertheless, different classes of compounds are extracted with varying efficiencies, which makes use of just one standard for all analytes difficult. Moreover, extraction efficiency is highly dependent on sample matrix and significant differences in recoveries, from 15–30% to 60–90%, have often been obtained[25,51] when analysing different meat matrices, even when using the same extraction and clean-up procedure. Therefore, to calculate the values needed to correct the results for incomplete recoveries, samples of each meat type spiked with all the compounds to be determined must be analysed in parallel with the calculation of the respective recoveries.

Matrix effects are important not only when UV detection is used, but also with MS. In this latter case, ion suppression is the main problem, especially when working with LC-MS with electrospray sources. The presence of co-eluting compounds in the chromatogram causes as much as a 30–65% decrease in response.[34] This causes a considerable increase in detection limits, for instance, LODs found in a meat extract when using LC-ESI-MS and various analysers were 2 to >100 times higher than those obtained for standards.[50] One possible way of overcoming matrix effects is to perform quantitation by standard addition, since this permits correction of differences in recoveries for both analytes and matrices. However, this method is not appropriate when a large number of different samples are analysed, because multiple extractions and determinations have to be performed for each sample after it is spiked at several levels. In addition, it seems that the nature of the matrix is not the only factor responsible for low recoveries, since frequently on analysis of a large number of samples, in addition to finding different recoveries for different kinds of samples, a small number of incidences of low recoveries occur[18,51] for each type of sample, different meat or different cooking procedure. This is probably related to the robustness of the method, which affects its reliability during normal usage. The relatively high number of clean-up steps needed to extract and pre-concentrate HCAs from food samples means that the analytical procedure is very unlikely to remain unaffected by small changes.

To solve the above problems of quantitative analysis, the best option is isotopic dilution. This approach allows correction of non-complete recoveries in the sample treatment and changes in the instrument performance such as signal suppression in LC-MS detection, since both isotopically labelled and native compounds have very similar ionisation response and extraction and chromatographic behaviour. Moreover, quantification by isotope dilution reduces the number of samples to be analysed, since correction for recovery is made by using the labelled compounds as surrogates. Figure 5.11 shows the chromatogram obtained for a meat-based bouillon sample containing low amounts of HCAs, to which deuterated standards for IQ, MeIQx, 4,8- and 7,8-DiMeIQx and PhIP were added.[57] The main drawback with this approach is that isotopically labelled standards are commercially available only for a very small number of HCAs. Some authors have used these standards to correct the results obtained by external calibration; the recoveries obtained with labelled standards were taken as representative of the behaviour of various families of HCAs.[34,64] However,

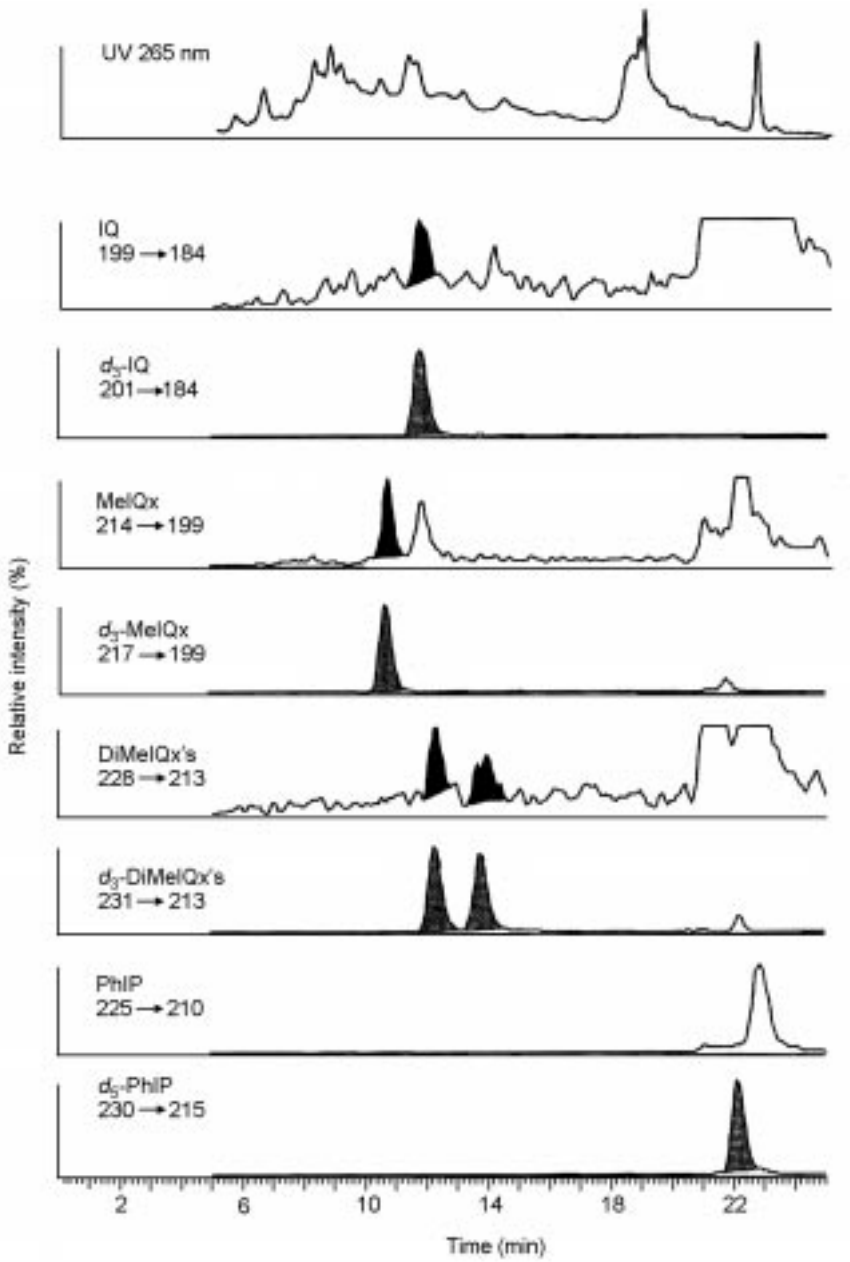

Fig. 5.11 LC-APCI-MS/MS chromatogram obtained for a meat-based bouillon sample containing < 1 ng/g of total HCAs monitored in SRM acquisition mode. The UV trace reported on top has been recorded with a wavelength of 265 nm and corresponds to a meat extract sample contaminated with high levels of total HCAs (>10 mg/kg) (reprinted from ref. 57 with permission).

only rough values for the HCAs could be obtained, since different recoveries were found for different compounds and because ion suppression due to matrix interferences is not corrected by this approach. Therefore, the recommended method for those compounds for which labelled standards are not available is standard addition.

5.5 Conclusions

The analysis of HCAs in cooked foods has been an active area of research during the last 15 years. To date the methods most commonly used for the analysis of these mutagens are those based on tandem extraction procedures, coupling on-line several clean-up steps, and liquid chromatography-mass spectrometry (LC-MS) for separation, identification and determination.

The extraction and clean-up procedures are mainly based on the Gross-Grüter method, that allows obtaining two extracts, one containing the polar HCAs and the other containing the less-polar ones. It can be considered as the reference method since its suitability has been demonstrated in several interlaboratory studies among laboratories experienced in the analysis of HCAs in food samples. However, this method is time consuming and requires high purity of the extracts which forces in some cases the application of additional clean-up steps. In this context, the improvement of the extraction and clean-up methods using new extraction techniques such as pressurised liquid extraction (PLE), on-line clean-up procedures and selective sorbents such as imprinted polymers or immunosorbents is still an outstanding matter. Although some specific sorbents have been prepared, they are adequate for only a small number of compounds and are used only in the analysis of body fluids probably because food samples are more complex.

Liquid chromatography coupled with mass spectrometry has become a powerful tool in the quality control of foods products and the safeguarding of human health and it is also the technique of choice for the analysis of HCAs in foods. Both ionisation techniques ESI and APCI, have been used for the analysis of HCAs but ESI provides the best detection limits for these compounds. Ion-trap and triple quadrupole analysers have been frequently used in this field but the choice of one or the other depends on the characteristics of the analysis to be performed. Triple quadrupole instruments working in SRM generally provide lower detection limits and better repeatability than ion-traps and are the instruments recommended for quantification of target compounds in food samples. In contrast, ion-traps are at present the best approach for identification of unknown compounds taking advantage of their capability of performing MS^n.

The new instruments recently developed such as the linear trap, with important improvements in sensitivity and repeatability, will be likely to allow in the near future the analysis of unknown mutagens at very low concentration levels. Other analysers, mainly the Q-TOF, also have their place in this field because of their capability of high mass accuracy that can help in the unambiguous identification of the compounds in the food samples.

Several quantification methods such as external calibration, internal standard and standard addition have been often used, but undoubtedly isotopic dilution is the best option for accurate determination. It allows accurate results to be obtained and overcomes matrix effects and potential changes on the recoveries with an important reduction of the analysis time. Nevertheless, actually only few isotopically labelled food mutagens are commercially accessible. This means that isotopically labelled standards are necessary at least for the compounds most frequently found and those showing the highest genotoxic activity in cooked foods. Moreover, to obtain reliable results and to establish a consistent risk assessment, further studies are required on the occurrence, biovailability, genotoxicity and carcinogenicity of new compounds in cooked foods.

Finally, to generate data on the concentration of HCAs in cooked foods, validated methods under statistical control must be used. To validate the new analytical methods and to ensure the accuracy of those currently used, efforts must be directed to perform inter-laboratory exercises using different food matrices, and certified reference materials whenever possible. Nevertheless, no reference materials are actually available and their preparation is one of the actions to be promoted in the near future.

5.6 References

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6

Analysis for acrylamide in foods

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6.1 The analytical task

We need to analyse foods for acrylamide for several reasons. These include: to support estimates of consumer exposure via the diet, to judge the effectiveness of control measures undertaken by food producers and government agencies, and to allow kinetic and mechanistic models to be formulated to understand the basic science underlying acrylamide formation. It is useful to first consider the nature of the foods affected by acrylamide formation along with the levels of interest. These define the challenge that the analytical chemist must meet.

6.1.1 The affected foods

Acrylamide is formed when foods rich in carbohydrates are subjected to high temperatures during cooking or other thermal processing (Hellenas *et al.*, 2005). Asparagine is the only important precursor that has been identified so far, reacting with reducing sugars in the Maillard reactions that also form colour and flavour (Mottram *et al.*, 2002; Stadler *et al.*, 2002). Foods containing free asparagine along with reducing sugars are therefore particularly prone to form acrylamide. As would be anticipated given the link with Maillard reactions, acrylamide formation seems to be favoured by low moisture conditions such as exist during the end stages of baking, frying, toasting and grilling these foods.

Cereal products and potato products are particularly affected and, for example, the affected foods constitute about 40% of the calorific value of a typical Western diet. The eight major dietary food groups are (in no particular order); French fries, oven-baked chips (US = 'fries'), potato crisps ('chips'), breakfast cereals, biscuits ('cookies'), coffee, bread (especially toasted bread),

and pies and cakes. Other foods of particular interest, because they are products with a high potential for acrylamide formation, are gingerbread, almonds and Christmas cakes. Other foods of particular interest because they are high-consumption foods for some consumers include chicory and other coffee substitutes, teething biscuits, baby rusks and other baby foods.

6.1.2 The detection levels needed

The concentrations of acrylamide in the affected foods cover a wide range. For some unique, highly contaminated products, such as overcooked chips or crisps, levels can exceed 3,000 μg/kg. On the other hand, for high-consumption items of major dietary importance such as coffee, infant formula and baby foods, measurement of acrylamide concentrations down to 10 μg/kg or lower is desirable. Similarly, methods with good sensitivity have to be used when estimating human exposure to acrylamide by analysis of total diet samples or duplicate diet samples, where the acrylamide concentration is diluted by combining all sorts of different food types into composite samples.

6.2 Physical and chemical properties of acrylamide

Along with the nature of the foodstuffs to be tested, the physical and chemical properties of acrylamide (Habermann, 1991; Cyanamid, 1969) have to be considered because they too influence the choice of analytical approaches. Acrylamide (also known as 2-propenamide) is a white crystalline solid. It has a molecular weight of 71 Daltons, a melting point of 84.5 $\pm$ 0.3 ºC and a high boiling point (136 ºC at 3.3 kPa). Acrylamide is freely soluble in water, lower alcohols such as methanol and ethanol, and in other polar organic solvents such as acetonitrile, ethyl acetate and acetone. It is virtually insoluble in non-polar organic solvents such as heptane and carbon tetrachloride. As is evident from its structure (CH_2=CH–CO–NH_2) the limited conjugation involving π-electrons means that acrylamide lacks a strong chromophore for UV (ultraviolet) detection and does not fluoresce. This solubility behaviour dictates the extraction strategy used for analysis of foods and the low molecular weight and low volatility of acrylamide have consequences for the analytical measurement techniques used.

6.3 Sampling requirements

6.3.1 Taking a representative sample

Acrylamide formation is a largely surface phenomenon where the conditions of high temperature and low moisture pertain during cooking or thermally processing foods. The affected foods are mostly solids. Acrylamide is not distributed homogeneously throughout these foods but it is concentrated at the surface. There can be significant differences between individual food pieces – such as

potato crisps or French-fries. There can also be differences in acrylamide content within individual items since, for example, the edges and tips of fried potato products brown more than the rest of the item as does the crust of a bread loaf compared to its inner crumb. So to get a true result, the whole portion or serving of food as eaten should be homogenised thoroughly before a specimen is taken for extraction and analysis. Acrylamide is freely water soluble and virtually insoluble in oil and fat phases, so if there is any tendency for foods to separate this should be avoided. This said, most of the affected foods can be homogenised quickly and effectively.

An important data gap is that acrylamide levels are very dependent on the exact cooking conditions used and the time/temperature profiles operating. Relative to surveys of commercially processed foods, there have been few studies of acrylamide formation during home cooking. It is therefore not clear how representative, or not, are the few studies that report home- or lab-based cooking tests.

6.3.2 Stability in foods prior to analysis

Acrylamide is a difunctional monomer containing a reactive electron-deficient double bond and an amide group, and it undergoes reactions typical of those two individual functionalities (Friedman, 2003). It exhibits both weak acidic and weak basic properties. Because they are in conjugation, the electron withdrawing carboxamide group activates the double bond, although the activation is not as great as by a carbonyl or acid group in conjugation. Consequently, acrylamide reacts with nucleophilic reagents in a reaction similar to the Michael addition reaction to α,β-unsaturated systems. The importance of this route of acrylamide 'destruction' in heated foods, and indeed if the reaction is reversible, has not been elucidated yet.

$$\text{R–X–H} + \text{CH}_2\text{=CH–CO–NH}_2 \longrightarrow \text{R–X–CH}_2\text{–CH}_2\text{–CO–NH}_2 \qquad (6.1)$$

Acrylamide levels have been reported to decline slowly in some food products during long-term storage. One example is ground coffee for which a 40 to 65% decline was found after six months' storage at room temperature. In contrast, there was no significant decline of acrylamide levels in a coffee sample stored at −40 ºC (Andrzejewski *et al.*, 2004). When the level of acrylamide in coffee was measured when freshly brewed and also after standing heated for up to five hours, no significant change in the concentration was found. In general, however, for home-cooked, take-away and restaurant meals that are freshly cooked and then consumed hot, there seems to have been no systematic study reported on the short-term stability of acrylamide. The current view is that these products can be allowed to cool and then be analysed later, but this should be verified.

Given that acrylamide seems to be rather stable in the large majority of the affected foods, any discrepancy between the date of analysis of retail samples compared to the 'normal' date of consumption by the consumer is not expected to be a major source of error in estimates of intake via the diet.

6.4 Extraction procedures

A schematic of the main steps in analysis of foods for acrylamide is shown as Fig. 6.1 (Wenzl *et al.*, 2003; Castle and Eriksson, 2005). The purpose of sample extraction is to remove acrylamide from the food matrix into a liquid solvent suitable for subsequent instrumental analysis.

6.4.1 Addition of internal standard

Nearly all published methods use internal standards added at the start of the procedure. Both deuterium-labelled ($^{2}H_3$-acrylamide) and carbon-labelled ($^{13}C_3$-acrylamide) standards are used widely. The triple-label in each takes the internal standard well away from the mass ions used to measure acrylamide itself in methods that employ mass spectrometry. In most published methods, the internal standard is added to the specimen of food and a period of incorporation is allowed before commencing extraction. It is then assumed that the internal standard behaves in the same way as the 'native' acrylamide. This is usually checked by determining the recovery of acrylamide spiked into the matrix.

6.4.2 Extraction using water

The high water-solubility of acrylamide means that extraction of foods using plain water is effective. Water extraction is most usually conducted at room temperature with a mass ratio of about one part sample plus ten parts water (Rosén and Hellenäs, 2002; Tareke *et al.*, 2002). Acrylamide is neither strongly acid nor basic and so adjustment of pH is not required. The sample needs to be finely divided to ensure efficient extraction. Extraction seems to be rather rapid and just a few minutes agitation of a dispersed sample in cold water is effective.

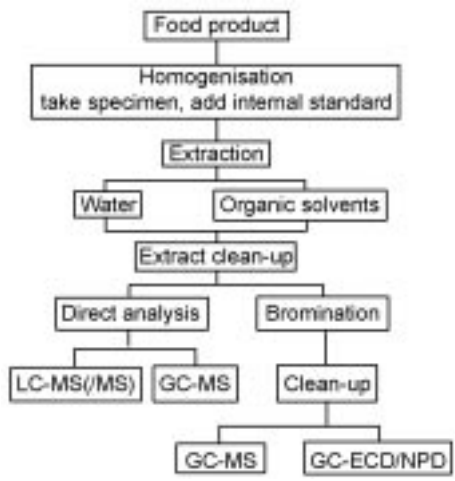

Fig. 6.1 General schematic for the main techniques used for acrylamide analysis.

Cereal and potato products are naturally hydrophilic and extract well. Extraction using hot water does not seem to give any higher recovery (Owen *et al.*, 2005). However, fatty matrices such as chocolate or peanut butter benefit from using hot water to promote dispersion and effective extraction. Alternatively, room temperature extraction using a mixture of water and an immiscible organic solvent such as dichloroethane to break up and remove the fat phase (Zyzak *et al.*, 2003; Eberhart *et al.*, 2005) is very effective. Other organic solvents, including hexane, have been used by others for defatting the sample prior to water extraction.

6.4.3 Extraction using organic solvents

Accelerated solvent extraction with acetonitrile has been used as an alternative to water extraction for analysis of acrylamide in potato products and crisp breads (Cavalli *et al.*, 2003). Other polar solvents, including methanol, propanol, acetonitrile, and ethanol/dichloromethane mixture, have been employed to extract acrylamide from foods (Owen *et al.*, 2005). These alternative solvents are used only because the subsequent GC-MS analysis (gas chromatography coupled to mass spectrometry) of underivatised acrylamide (see later) tolerates water only poorly if at all. Pre-swelling with water is necessary to ensure efficient extraction of some foods (Biedermann *et al.*, 2002).

6.4.4 Efficiency of extraction – trueness of results

When the same materials are analysed by laboratories using different extraction solvents and extraction conditions, such as in proficiency exercises, there is a general consensus of results with no evidence that one combination of solvent/conditions gives rise to higher results than other combinations. Some laboratories have used enzymatic treatment of food samples during water extraction but found that treatment with amylase or protease does not liberate any additional acrylamide in the foods tested.

In marked contrast, whilst Eriksson and Karlsson (2006) also found no significant effect of enzymatic treatment, they did observe a very marked increase in the amount of acrylamide extracted from certain samples when extracted at alkaline pH. For example, for one type of Swedish whole grain bread the acrylamide extracted was around 200 μg/kg in the pH range 2–8 but rose steeply from pH 9–10 to give a fivefold increase to 1000 μg/kg when the water used for extraction was at pH 12 and above. The authors interpreted this as possibly due to a pH-induced relaxation of the food matrix making acrylamide more accessible and so giving higher extraction efficiency. This would be a kinetic phenomenon and it seems an unlikely explanation given the absence of increased acrylamide extraction using the exhaustive (both time-wise and solvent-wise) studies mentioned above. It should be borne in mind that acrylamide formation in heated foods sits within a very complex series of reactions (Stadler and Scholz, 2004) that do not drive through to completion but,

rather, are placed in suspense when cooking is stopped. Perhaps a more plausible explanation then is that the alkaline pH forms additional acrylamide from degradation of other substances sitting either up-stream (i.e. from precursors of acrylamide) or downstream (i.e. reversion of reaction products from acrylamide) in the whole maze of reaction pathways.

Notwithstanding the mechanistic explanation, this important work has prompted extra studies to test its relevance with respect to the bioavailability of acrylamide. It has to be noted in this context that food does not experience alkaline pH of 9 and above during digestion and metabolism. For the time being, therefore, the preponderance of evidence from different extraction studies indicates that extraction of acrylamide from food samples is complete when using the normal analytical procedures (JIFSAN, 2004). After the writing of this chapter, others have demonstrated that the increased yield of acrylamide at alkaline extraction pH is an artefact (see Chapters 1 and 2).

6.5 Determination by GC-MS after bromination of acrylamide

Virtually all acrylamide tests use either GC-MS or LC-MS (liquid chromatography coupled to mass spectrometry) approaches. GC-MS has a much longer history of use and so it will be discussed first although probably the majority of high-throughput commercial testing labs use LC-MS nowadays.

6.5.1 Background to the approach

Analysis for acrylamide using bromination and GC determination was established well before acrylamide was discovered in heated foods, because of the need to test drinking water, discharge waters, and crops for acrylamide (Bologna *et al.*, 1999; Castle *et al.*, 1991; Habermann 1991). Bromination affords an analyte that is much easier to analyse at trace levels than acrylamide itself. The product of bromination is 2,3-dibromopropionamide which can be back-extracted from the aqueous bromination solution into a solvent such as ethyl acetate. Bromination is an addition reaction and if a labelled internal standard is used, the isotope label (^{2}H or ^{13}C) is retained.

$$Br_2 + CH_2{=}CH{-}CO{-}NH_2 \longrightarrow Br{-}CH_2{-}CHBr{-}CO{-}NH_2 \qquad (6.2)$$

In some methods the first-formed 2,3-dibromopropionamide is then dehydrobrominated to form the more stable 2-bromopropenamide, by treatment with triethylamine before GC-MS analysis. In this case, one of the three deuterium labels is lost if $^{2}H_3$-acrylamide is used as the internal standard, but quantification via the monobromo derivative (now double-labelled) is still successful (Nemoto *et al.*, 2002).

$$Br{-}C^{2}H_2{-}C^{2}HBr{-}CO{-}NH_2 \longrightarrow Br{-}C^{2}H{=}C^{2}H{-}CO{-}NH_2 + {}^{2}HBr \qquad (6.3)$$

6.5.2 Confidence in the identification and quantification aspects

It is possible to determine acrylamide by bromination followed by GC analysis using an alkali flame-ionisation detector or using an electron capture detector. However, virtually all laboratories make use of the extra selectivity and confidence offered by mass spectrometry coupled with the facility this offers to use an isotopically labelled internal standard. The Br-GC-MS method has a high intrinsic level of specificity originating from three attributes: (i) water extraction, bromination to make a less polar derivative, and then back-extraction into ethyl acetate, eliminates many potential interferences; (ii) capillary GC is a high-resolution procedure that can deliver a pure acrylamide peak into the MS; (iii) brominating the acrylamide gives a higher molecular weight analyte which, especially with the characteristic $^{79}Br/^{81}Br$ isotope pair, gives several MS ions to monitor and confirm the identity of the analyte.

6.5.3 Trueness of the Br-GC-MS analysis

There was an initial concern that the harsh conditions used in the bromination procedure, with strong acid and potentially oxidising conditions with elemental bromine, might give rise to acrylamide formation from precursors as an artifact of the method. These concerns have added resonance now that the influence of alkaline pH on extraction (see above) has been reported. However, the concerns were dispelled early on when parallel analysis of sample extracts using bromination-GC-MS and underivatised LC-MS gave broad agreement for a range of food types (Ahn *et al.*, 2002; Ono *et al.*, 2003). Similarly, in check-sample exercises there has been no evidence of any bias from Br-GC-MS methods compared with other methods of test used (Owen *et al.*, 2005; Klaffke *et al.*, 2005; Wenzl and Anklam, 2005).

6.5.4 Performance of the Br-GC-MS methods

In a typical procedure (Ahn *et al.*, 2002) homogenised food is extracted by shaking with water in a 1:10 ratio. A portion of the filtered extract is brominated overnight at ca. 5 ºC and the derivative extracted into ethyl acetate. The organic extract is dried over sodium sulfate and then evaporated to a small volume. Analysis on a normal bench-top GC-MS instrument gave a limit of quantification of less than 10 μg/kg. At this level, the brominated acrylamide gave a good response in all 4 m/z channels monitored. In this procedure, 1 ml of final analysis solution corresponded to 1 g of food, i.e. no effective concentration.

It is possible to increase the effective concentration for GC-MS analysis by using a procedure described by Ono *et al.* (2003) in which a final analysis solution of 25 μl corresponds to 0.28 g food, or an 11-fold concentration. The limit of detection (LoD) and limit of quantitation (LoQ) was said to be 1 μg/kg and 3 μg/kg respectively. Other clean-up steps have been described, using Carrez solutions I & II, clean-up of the brominated derivative using Florisil, dehydrobromination using triethylamine and then GC-MS (Pittet *et al.*, 2004).

The concentration factor was 34 and the LoD and LoQ were quoted to be 2 μg/kg and 5 μg/kg. Clean-up of the brominated food extract on silica (Castle *et al.*, 1991; Castle, 1993), on Florisil (Nemoto *et al.*, 2002, Pittet *et al.*, 2004) and by size exclusion chromatography (Tareke *et al.*, 2000) have also been employed to achieve lowered detection limits. An LoQ of 5 to 10 μg/kg is more than adequate for testing most individual food items although improved sensitivity is desirable when testing composite diet samples.

6.5.5 Bromination GC-MS – conclusions

It can be concluded that methods using water extraction, bromination of acrylamide followed by GC-MS, are well developed and have been demonstrated to be accurate in check-sample exercises. Limits of quantification down to 5 μg/kg are well within the reach of the average laboratory equipped with a standard benchtop GC-MS instrument. The current state of the art using readily available equipment, is represented by the bromination GC-MS procedure applied to the analysis of 20 different food groups that make up the UK total diet study, measuring down to 1–5 μg/kg levels for each food group (Food Standards Agency, 2005).

6.6 Determination by GC-MS with no derivatisation

Acrylamide is not a natural candidate for direct GC-MS analysis for several reasons. Firstly, the polar solvents (and preferably water) that are required to effect a good extraction of acrylamide from foods are not well suited for preconcentration and injection onto a capillary column. Secondly, acrylamide is very polar and has a low volatility compared to its molecular weight, and so selection of the column phase is critical. Lastly, such a small molecule with a molecular weight of 71 Daltons does not give a very convincing or unique mass spectrum. Nevertheless, a number of laboratories have persisted with the difficult task of developing a direct GC method for acrylamide (Biedermann *et al.*, 2002), largely because it offers higher sample throughput (avoiding the time-consuming bromination step) and it reduces the use of corrosive and hazardous chemicals. Given the limited solubility of acrylamide in most organic solvents, a polar solvent such as methanol, propanol or butanone is needed for efficient extraction from the food sample. Water is still required, however, since swelling of many food samples with water is necessary otherwise the recovery is very poor. Fatty samples require defatting, normally by extraction with hexane. Due to the high polarity of acrylamide, a polar column such as Carbowax is used and on-column injection is preferable.

Extract clean-up for underivatised-GC-MS is more demanding than for the bromination-GC-MS procedure because of the possibility that precursors of acrylamide may be extracted and so extra acrylamide may be formed as an artifact during the GC analysis. It has been noted by others that extraction of acrylamide precursors from foods can lead to extra acrylamide formation as an

artifact during subsequent heating (e.g., Soxhlet extraction) and this may occur also in the hot injection port of a gas chromatogram. In proficiency check-sample exercises a bias of the results obtained by GC-MS without derivatisation has been noted (Owen *et al.*, 2005; Klaffke *et al.*, 2005; Wenzl *et al.*, 2005). Some laboratories using the direct GC-MS approach reported high results whilst others consistently reported satisfactory results. This suggests that whereas the direct GC-MS approach can be successful, extra care is necessary – especially in extract clean-up. The current state of the art for commercial analysis using an underivatised GC-MS approach is represented by the procedure of Hoenicke *et al.* (2004). Their GC-MS analysis and associated extraction protocol enabled analysis of difficult matrices like cocoa, soluble coffee, molasses and malt, measuring down to 5 μg/kg.

6.7 Determination by LC-MS

LC-MS analysis has the advantage that the LC technique in reversed-phase mode is directly compatible with an aqueous solvent that is best suited for extraction of acrylamide from foods. However, the high polarity and the low molecular weight of acrylamide meant that considerable method development was needed to establish useful LC separation and MS quantitation (Rosén and Hellenäs, 2002; Tareke *et al.*, 2002).

6.7.1 Extract clean-up requirements for LC-MS

As stated before, the best extraction solvent for acrylamide in foods is water and this extract is directly compatible with reversed-phase LC using an aqueous mobile phase with a small amount of organic modifier. However, some prior clean-up of the aqueous extract is required. Clean-up for LC-MS methods has three approaches that are often used in combination. First, to use SPE (solid phase extraction) with complementary stationary phases. Secondly, to use chemical deproteination with the classical Carrez I and II reagents. Thirdly, to remove unwanted co-extractives by physical methods of freeze-thaw precipitation or membrane filtration.

Different kinds of SPE cartridges have been used, including graphitised carbon, ion-exchange resins, and mixed mode materials. In most cases, the choice of any particular SPE cartridge has been made largely by trial and error until the problem (generally a specific interference or a non-specific suppression of ionisation) has been solved. In many cases, the SPE clean-up step has been combined with a molecular size cut-off filter (3 to 5 kDa) to remove larger molecules that would otherwise give problems in the analysis.

6.7.2 Chromatographic performance in LC-MS

The chromatographic resolving power of LC columns is much lower than for GC columns and so column choice is critical for a successful analysis. This is

especially true because acrylamide is so water soluble and it is a challenge to get useful retention on most reversed-phase LC columns. As with the SPE clean-up, a number of different stationary-phase chemistries have been used to get separation of acrylamide from other co-extractives. These include graphitic carbon, octadecyl-modified silica (ODS), other modified silicas and ion-exchange resins – some with a supplementary size-exclusion mode too. The column type used most frequently is graphitic carbon, eluted with water and a small amount of organic modifier.

6.7.3 Detection by LC-MS

Triple-quadrupole mass spectrometers for LC-MS/MS are quite expensive but single-stage instruments are not usually sensitive enough to conduct acrylamide analysis on water extracts of foods unless a degree of pre-concentration is used. Even so, most modern LC-MS/MS instruments perform relatively poorly below ca. 100 Daltons unless tuned specifically for low mass/charge ratio ions; acrylamide has a molecular weight of just 71 Da. Most LC-MS/MS methods use electrospray ionisation (ESI) in the positive ion mode.

6.7.4 Confidence in LC-MS identification of acrylamide

LC-MS/MS identification of acrylamide rests on the chromatographic retention time and on the presence and relative abundance of characteristic ions (JIFSAN, 2004). The main ions observed for acrylamide are m/z 72 (protonated molecular ion), 55 (loss of amino) and 27 (subsequent loss of CO). Three SRM (selected reaction monitoring) traces may be recorded, although the acquisition of just two SRM traces fulfils the criteria required in the Commission Decision 2002/657/EC (Riediker and Stadler, 2003). Another criterion that has been used is to examine the full mass spectrum obtained. So, for example, the ion m/z 55 was used for quantification and for identification the spectra should be identical for the sample and for the standard at 10 eV and 20 eV collision energy (Tareke *et al.*, 2002).

6.7.5 Quantitative aspects of LC-MS analysis

In many reports it is not clear exactly how the reported LoD and LoQ values were derived. Some laboratories have derived the values from simple standards and others from real sample extracts. It is also frequently unclear if the LoD and LoQ values cited are for the main qualifying ion only or if they take proper account of the need to record and measure the qualification ions so that the relative abundance ratios can be checked. For acrylamide the qualification ions are often much weaker (less abundant) than the main quantification ion, depending on the instrument and the conditions used. Most laboratories still attain about the same performance as in the first reports (Rosén and Hellenäs, 2002; Tareke *et al.*, 2002), independent of which LC-MS method they have used. LoD values are typically 3 to 20 μg/kg, LoQ values are 10 to 50 μg/kg.

6.7.6 LC-MS methods – conclusions

Most survey data for acrylamide have been obtained using LC-MS/MS analysis. The technique has proved to be well correlated with GC-MS measurements and is accurate. There is one relative weakness that LC-MS methods share with GC-MS methods. The precision of measurements, within a lab but especially between laboratories, is satisfactory but is not good. For estimates of intake using large databases of concentration data, accuracy (trueness) of reported values is the major concern and precision is relatively unimportant. On the other hand, when the result for a single food sample has to be interpreted, for example, comparing the result against a target value (legal or in-house QC) then if the method is imprecise this can hinder interpretation of the test result.

Three recent papers represent the current state of the art of the LC-MS approach. In the aforementioned paper (Hoenicke *et al.*, 2004), HPLC-MS/MS and an associated extraction protocol was used for high sample throughput with straightforward food types (potato chips, French fries, cereals, bread, and roasted coffee). This allowed the analysis of up to 60 samples per person/day measuring down to 30 μg/kg. GC-MS was reserved for more difficult matrices. Similarly, another dual-track procedure for simple matrices and for complex matrices has been published (Eberhart *et al.*, 2005) using simple and inexpensive LC-MS instrumentation with good performance characteristics and applicable to a wide range of food products. Lastly, as an example of non-routine work, LC-MS was applied to the main categories of Swedish baby food products, i.e., breast milk substitute (infant formula), gruel, porridge and canned baby food (Fohgelberg *et al.*, 2005). The LoQ was 0.5 μg/kg for liquids and 2 μg/kg for other foods, although at these levels the weaker MS fragment ions may not meet the signal : noise criteria or the relative abundance criteria for analyte confirmation.

6.8 Other instrumental methods

Although existing GC-MS and LC-MS methods are perfectly adequate, several labs have tried different instrumental approaches. These have tended to be research projects into method development *per se* and little routine data have been generated outside niche areas. Some limited examples are covered here.

6.8.1 Determination by LC-UV

LC-UV exhibits rather poor sensitivity and selectivity because acrylamide possesses only a poor UV chromophore which is a weak and rather uncharacteristic absorber. Nevertheless, LC-UV has found application, especially in the testing of food samples prepared in the laboratory to simulate home cooking or industrial cooking and so for which blank (uncooked) samples are readily available to help guard against interferences in the analysis. Thus, LC-UV has been used to test mainly potato products and instant noodles for which

acrylamide levels can be rather high and so sensitivity is not a major issue. To compensate for the lack of selective detection, column switching techniques have been used to get better separation (Terada and Tamura, 2003) but this then complicates the procedure. For French fries and others foods, LC-UV at low wavelengths gave broadly the same results as LC-MS (Cavalli *et al.*, 2003; Peng *et al.*, 2003). Again with UV detection, micro-emulsion electrokinetic chromatography on uncoated silica capillaries had a detection limit of 0.7 μg/ml (700 μg/kg) and day-to-day precision around 12%. This was demonstrated by analysing samples of home-made French fries (Bermudo *et al.*, 2004).

LC-UV has also been used as the determination step after bromination (similar derivatisation procedure as used for GC) for acrylamide in aqueous samples and in sugar using a column switching technique (Brown and Rhead, 1979). Perhaps of greater potential scope for application is the derivatisation of acrylamide using mercaptobenzoic acid followed by LC-MS analysis (Jezussek and Schieberle, 2003). If this derivatisation reaction (eqn 6.1) could be made reliable with an LC-UV analysis in place of LC-MS, then using the added benzoyl chromophore could provide a simplified LC-UV method suitable for quality control.

As a last example, in order to be able to use a rapid headspace solid-phase microextraction method, Lagalante and Felter (2004) overcame the limited volatility of acrylamide by derivatisation to form N,O-bis(trimethylsilyl)-acrylamide. The detection limit claimed was 0.9 μg/kg and three commercial cereals were tested using the method.

6.9 Prospects for rapid tests

All of the methods reported above require specialist lab instruments and trained staff and they are not suitable for deployment into food processing plants for on-line or at-line tests. That said, since no major country has established regulatory limits on acrylamide in foodstuffs, there is less impetus for a rapid control measure that would be used particularly for routine in-house testing of batches. Several research groups have undertaken developmental work to try to raise antibodies to acrylamide with the objective of deploying a test method in ELISA (Enzyme-Linked Immunosorbent Assay), dip-stick or lateral-flow formats. No publications have reported success. It seems likely that the reactivity of acrylamide allied to its small size has so far defeated attempts to raise useful antibodies. Derivatisation of acrylamide to increase its size and make it more immunogenic could be used, but this would be a less attractive approach since it loses the simplicity of a rapid direct test

6.10 Conclusions

By the end of 2002, a few months following the discovery and reporting of acrylamide formation in heated foods, our basic capability to measure

concentrations of acrylamide in the main foods affected was established using GC-MS and LC-MS procedures. The large number of papers published since then have provided further refinement, especially to deal with difficult matrices, but our core capabilities remain essentially the same. There are both GC-MS and LC-MS(MS) techniques which fulfil the requirements for today's acrylamide analysis, both for 'routine' and for 'difficult' food matrices. The performance of the methods is sufficient with respect to their scope, detection limits and accuracy. The precision of the methods could be improved and the establishment of validated test methods and certified reference materials would assist in this.

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7

A molecular modelling approach to predict the toxicity of compounds generated during heat treatment of foods

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7.1 Introduction to molecular modelling

There has been a strong move in recent years to search for non-animal alternatives to testing chemical toxicity in vertebrates. The main drivers behind this move have been the need for chemical risk assessment in the absence of test data, cost/time implications of testing large numbers of chemicals on animals, and strong ethical reasons. For example, the 3Rs principle (Reducing, Refining and Replacing the use of animals in laboratory procedures), which is a policy adopted by many EU Member States, provides a strong basis for the development and use of non-animal alternatives in toxicity testing. The most important among the few available alternatives, e.g. *in vitro* and read-across techniques, is the molecular modelling approach that is based on either structure activity relationships (SAR), or quantitative structure activity relationships (QSAR). The *in silico* predictive models based on (Q)SAR approach are already in use for the assessment of physicochemical properties, environmental behaviour, and biological activity of a wide range of organic compounds. Over the years, (Q)SARs have become much more reliable and accepted tools for predicting complex biological phenomena, including specific toxicity endpoints (Price and Watkins, 2003; Cronin and Livingstone, 2004). The use of (Q)SARs in chemical risk assessments is encouraged by the ECB (European Chemicals Bureau), ECVAM (European Centre for the Validation of Alternative Methods), OECD (Organisation for Economic Co-operation and Development), and the US EPA (Environment Protection Agency). Under the proposed EU REACH (Registration,

Evaluation and Authorisation of CHemicals) regulations, (Q)SARs have been recognised as potential tools for use in the assessment of chemical properties and toxicity hazards.

The discovery of acrylamide in foods (Friedman and Mottram, 2005) has led to a further search for other potential toxicants that may be produced during heat treatment of foods. A database of compounds that may be produced in foods as a result of Maillard reaction (560 entries) or lipid oxidation (over 180 entries), has recently been compiled under the EU HEATOX project (www.heatox.org). There has been, however, a need for carrying out an initial assessment of any potential toxicities associated with these compounds. The study reported here assessed 247 of the compounds, with the aim to determine the usefulness of (Q)SAR approach in predicting the toxicity of compounds for which there is little or no existing experimental data available.

7.1.1 Prediction of chemical toxicity by molecular modelling

Linear and non-linear approaches

The basic paradigm behind the development of (Q)SARs is that biological activity of members of a series of compounds is proportional to one or more physicochemical properties of the molecules. Thus biological activity $= fn$ ($\text{property}_1 + \text{property}_2 + \text{property}_n$). The concept that biological activity of a series of related chemicals can be related mathematically to one or more of physicochemical properties emerged in the 1960s. Early pioneers such as Hansch and Leo (1979) built upon the underlying concept using such principles as substituent parameters (properties that change in a predictable way when the subsistent of a common core molecule is changed). It became widely accepted that three types of property could influence biological activity. These were; steric (size and shape), electronic (resulting from the partial charges on atoms), and hydrophobic (determining the partition of compounds between aqueous and organic phases). Because of the limitations of computational methods, early calculations were based on a series of look-up tables and manual regression analysis to relate biological activity to chemical property(ies). Largely because of these limitations, the (Q)SAR paradigm became associated with linear mathematics. Even though it was realised that most properties would have an optimum value (a second order relationship), most populations of sample compounds would not straddle the optimum and would thus yield to simple first-order linear solutions (Fig. 7.1).

With such simplistic properties and computational methods, it gradually became clear that (Q)SAR methods worked well only in certain well-defined situations. The set of compounds in the dataset had to be closely related and preferably congeneric, varying only at a single substituent. The study set had to have the same mode of action and the biological data had to be assessed close to the point of action, for example receptor binding or enzymic assays. Although many attempts were made to apply (Q)SAR studies to more complex situations, they rarely succeeded.

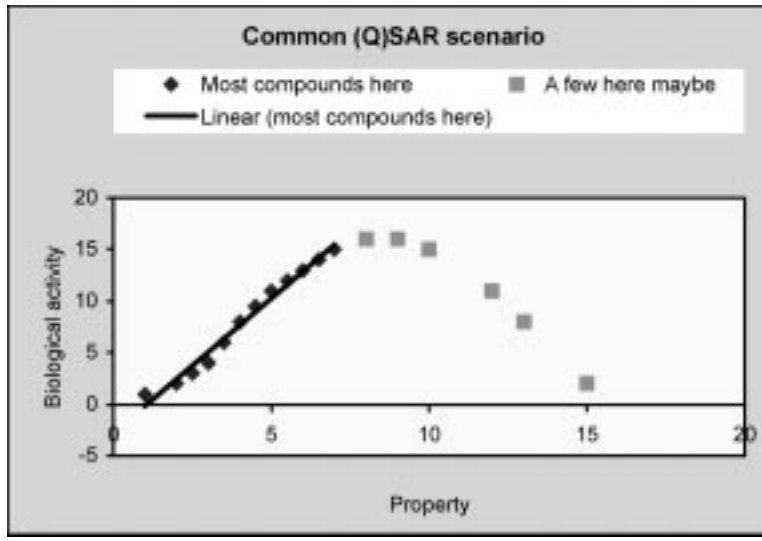

Fig. 7.1 This shows a situation often encountered in (Q)SAR studies. Most compounds fall on the linear part of the response and thus the entire response is erroneously assumed to be linear.

(Q)SAR models for relatively simple sets of molecular data are usually still based on linear statistical methods. However, the usefulness of linear algorithms in finding multi-dimensional relational patterns in complex sets of data is limited. Linear models are also often hard to generalise across different chemical classes or test species. This is where non-linear algorithms and soft-computing techniques play a very useful role in deciphering the relational patterns in complex datasets. Powerful data-mining techniques, such as those based on an artificial neural network approach have also become available in recent years (Agrafiotis *et al.*, 2002; Kaiser, 2003; Pintore *et al.*, 2003). With rapid developments in computing power, and data-mining techniques, it has become possible to build virtual models of chemical compounds and to estimate a wide range of physicochemical properties that hitherto had been very difficult, or even impossible, to determine experimentally.

At the same time, the development of non-linear mathematics, such as fuzzy systems and probabilistic methods, has enabled non-linear methods to be readily applied to large and imprecise data sets. This shift in the (Q)SAR paradigm has now opened the possibility that (Q)SARs may provide a reliable alternative to animal testing for the assessment of safety of chemicals to human health and the environment. The development of (Q)SARs, however, needs a combination of expertise in computational chemistry (to understand the physicochemical nature of chemicals and to develop models), biochemistry (to understand interaction of chemicals with biological systems), toxicology (to understand quality and meaning of test data) and statistics (to analyse complex sets of data, and to establish significance of results).

Neural networks and artificial intelligence approaches
The realisation that real life (Q)SAR problems are usually multidimensional and non-linear, has led to a search for mathematical methods to describe such models. The strictures of polynomial non-linear regression have been discarded in favour of methods that excel at dealing with the inherent uncertainty or 'noise' in biological data. Such methods include fuzzy logic, genetic algorithms, and the many 'flavours' of neural networks (e.g., Neurosolutions: *www.nd.com/*). The latter, because of their 'biological' inspiration, have usually been defined as a set of connected nonlinear elements. However, neural networks are essentially another family of parameterised nonlinear functions, which like polynomials, wavelets, Fourier series, radial basis functions, and splines are nonlinear approximators. In the context of (Q)SAR modelling, these nonlinear functions are intended to approximate the regression function of the predicted property, i.e. the expectation value of the latter (viewed as a random variable), conditional to the set of variables of the model (i.e. the descriptors of the molecules in a (Q)SAR). The use of genetic algorithms (GA) allows a further optimisation of virtually any parameter in a neural network to produce the lowest error (Niculescu, 2003). For example, the number of hidden units, the learning rates, and the input selection can all be optimised to improve the network performance. This has also led to the development of hybrid models that can combine genetic algorithm (GA), principle component analysis (PCA) and fuzzy methodology with neural networks (Neagu, 2002; Neagu *et al.*, 2004).

7.2 Development of a (Q)SAR model

It is beyond the scope of this chapter to review the variety of approaches that can be used to develop a (Q)SAR model or the enormous number of models and other software tools that have already been developed to predict a vast array of physicochemical properties (e.g., hydrophobicity, sorption, vapour pressure, solubility), environmental behaviour (e.g., persistence, photolysis, aqueous hydrolysis, biodegradation) and biological effects (e.g., ecotoxicity, acute and chronic toxicity, mutagenicity, carcinogenicity). A brief overview of (Q)SAR modelling is provided below only as a guideline, and interested readers are advised to obtain further information from relevant articles, reviews and books (e.g., Cronin and Livingstone, 2004).

The chemical structure of an organic compound provides a wealth of embedded information that can be deciphered by calculating a range of physicochemical properties (descriptors) using specialist software. These descriptors can then be modelled against a specific (experimentally determined) biological property/activity associated with a group of compounds to find a relationship between the structural aspects and biological activity of the compounds. Once tested and validated, a (Q)SAR model can be used, within its domain of applicability, as a tool to predict the activity of other compounds.

7.2.1 Training a model

A typical (Q)SAR modelling approach requires a 'training' set of compounds for which experimentally measured biological property/activity values are available. The first step of modelling thus involves selection of a set of compounds that can be used to train a model. Ideally, the compounds in a training set belong to the same chemical class, or closely related classes, with a similar mode of action. Chemical structures (2D and 3D) of each compound are assembled *in silico*, and optimised using specialist software, such as ChemX (Chemical Design Ltd, Oxford Molecular Group, UK), CaChe (FQS Poland: www.cachesoftware.com/), and CORINA (www2.chemie.uni-erlangen.de/software/corina/index.html). Compared to 2D structures, the optimisation of 3D structures requires much a greater effort to obtain a molecular configuration that resembles its 'real life' form. This is because the use of wrong configuration has a drastic negative effect on the quality of the final model. However, because it is not straightforward to determine the 'real life' conformation, usually the lowest energy conformation of a chemical structure is used in modelling. Where available, comparison of an optimised 3D structure is carried out with its crystal structure to add confidence to the selection of a final conformation for modelling. A number of other factors are also considered, for example whether a compound can exist in different interchangeable tautomeric forms, and whether the right geometrical or stereo-isomeric form of the compound has been selected for modelling.

In rare cases, experimentally measured physicochemical property data (descriptors) may be available for a set of compounds. In a large majority of cases, however, optimised chemical structures are used to calculate a range of descriptors. Indeed, up to several thousand chemical descriptors can be calculated for each compound using a suite of specialist software (e.g., Dragon (www.disat.unimib.it/chm/) and Codessa (www.semichem.com/codessa/default.php)). These descriptors represent a range of physicochemical properties of a compound, such as constitutional, hydrophobic, steric, electronic (quantum mechanical), electrotopographical, topological, functional groups, molecular fragments, etc. Generally, the descriptors based on molecular orbitals are regarded as carrying inherently accurate information, whereas other descriptors may be based on more empirical descriptions.

7.2.2 Biological activity data

One of the main and essential requirements for (Q)SAR model building is the availability of experimentally obtained good-quality data on biological activity of compounds in a training set. It should be stressed that the reliability of (Q)SAR predictions are largely dependent on the quality of test data used in building the model, although other factors may also have varying degrees of effect on the quality of (Q)SARs; for example, the chemical information contained in the descriptors, the statistical algorithms used, and the extent to which a model has been tested and validated. The use of too few compounds in a

training set generally leads to a poor model. However, scarcity of good quality toxicity data often limits the use of sufficient number of compounds that is needed to rigorously train and test a (Q)SAR model. It is preferable to use data obtained by studies that have been carried out under accepted guidelines for toxicity tests (such as OECD or US-EPA); however, data obtained from studies under GLP (Good Laboratory Practice) are usually sufficiently reliable for the purposes of (Q)SAR modelling.

7.2.3 Feature selection (data reduction)

The use of thousands of calculated descriptors also raises the possibility of finding a false correlation between the parameters that describe a chemical structure and its biological activity. Different methods are, therefore, used for data reduction to select only the appropriate parameters for use in modelling. For example, unnecessary parameters can be removed by using a cross-correlation matrix, through stepwise regression, genetic algorithm, K-nearest neighbour method (to classify a new object on the basis of attributes and training samples), principal components analysis or a variety of other statistical methods.

7.2.4 Statistical basis for structure-activity relationships

A statistical algorithm is used to find the relationship between a chemical structure (represented in the form of calculated descriptors) and biological activity. Depending on the complexity of the datasets, a method based on linear (such as multiple linear regression) or non-linear (such as principal component analysis and partial least squares) algorithm may be used. These can be further aided by feature selection using a genetic algorithm (GA) approach, cluster analysis, probabilistic methods, neural networks, or a combination of different methods. Machine learning is another computational technique, which incorporates data reduction, statistical correlation and data handling in a single package (e.g. WEKA: www.cs.waikato.ac.nz/%7Eml/weka, TANAGRA: http://eric.univ-lyon2.fr/~ricco/tanagra/index.html, and YALE: http://yale.cs.uni-dortmund.de/).

7.2.5 Model testing and validating

A separate set of compounds, for which data on experimentally determined properties is available, is used to rigorously test model predictions. A number of statistical indicators (such as R^2 and Q^2) are used to provide an indication of the reliability of model performance and predictions. Other external datasets may be used for further validation of the models. This step provides a real test for the robustness and reliability of a model, and also determines the domain of its applicability.

7.3 The use of *in silico* models as a predictive tool in chemical risk assessment

The use of validated (Q)SARs has been allowed under certain provisions of the current EU regulations, where information may be generated by means other than animal tests, in particular by (Q)SARs (Cronin, 2004). For example, a confirmatory test may be waived if results can be derived from a validated (Q)SAR, provided that the results are adequate for the purpose of classification, labelling and risk assessment, and that an adequate and reliable documentation of the applied method has been provided. (Q)SARs may also be used to derive physicochemical properties data, to identify substances for which a test is not possible, and to support the clustering and grouping of chemicals into categories.

For regulatory use, (Q)SARs need to be relevant to specific regulatory endpoints, to have been developed using a transparent methodology and an unambiguous algorithm, and tested and validated as suggested by the so-called Setubal Principles (OECD, 2004). For a number of reasons, that include scarcity of good quality data and duly validated models, the use of (Q)SARs for regulatory purposes has so far been limited, and is based mainly on precautionary principles. A number of agencies around the world have, however, either tested commercial (Q)SAR systems to assess suitability for regulatory use, such as Danish-EPA (Environment Protection Agency), US-NTP (National Toxicology Program) and Environment Canada, or have developed or used (Q)SAR based methodologies for the assessment of chemical properties and toxicity hazards, such as US-EPA, US-ATSDR (Agency for Toxic Substances and Disease Registry), US-FDA (Food and Drug Administration), NIOSH (National Institute for Occupational Safety and Health), Danish-EPA, and BgVV (the German Federal Institute for Health Protection of Consumers and Veterinary Medicine) (Cronin, 2004).

7.4 Prediction of chemical toxicity by expert systems

The progress in molecular modelling has also led to the development of expert systems that combine the predictive power of a large number of individual models, or are based on rules extracted from existing knowledge about the properties and behaviour of chemical compounds. In this study, two expert systems were used to assess the potential toxicity of compounds that may be generated during heat treatment of foods. These were TOxicity Prediction by Komputer-Assisted Technology (TOPKAT), which is a quantitative toxicity expert system based on a number of validated (Q)SARs, and Deductive Estimation of Risk from Existing Knowledge (DEREK), which is a rule-based expert system based on the SAR approach.

7.4.1 TOPKAT (www.accelrys.com/products/topkat/)

TOPKAT version 6.2, developed by Accelrys Inc. USA, contains a range of robust, and cross-validated (Q)SARs that are based on multivariate statistical

relationships between experimentally derived toxicity data and chemical descriptors. The program uses linear SMILES (simplified molecular input line entry system; http://www.daylight.com/dayhtml/doc/theory/theory.smiles.html) notation as an input to build a chemical structure from which it calculates chemical descriptors and predicts different toxicity endpoints using the multivariate statistical relationships. For many endpoints, the program also computes a probability value. A probability below 0.3 indicates a negative result, between 0.3 and 0.7 an indeterminate zone, and above 0.7 a positive prediction for the endpoint. Table 7.1 shows a selection of the toxicity prediction modules that are available in TOPKAT program.

Rodent carcinogenicity can also be assessed according to sex and species (rat and mouse), or according to either the NTP or the US FDA datasets. The program is also able to determine whether a compound of interest falls within the optimum prediction space of the model/relationship. The optimum

Table 7.1 A selection of toxicity prediction modules that are available in TOPKAT

TOPKAT module	Description
Weight of evidence Carcinogenicity call (v. 5.1)	This quantitative structure–toxicity relationship (QSTR) model scores the chemical using the US FDA Center for Drug Evaluation weight-of-evidence protocol, which scores the chemical as a carcinogen if it is a multiple-site carcinogen in at least one sex/species combination, or it is a single-site carcinogen in at least two sex/species combinations.
Ames mutagenicity (v. 3.1)	The mutagenicity QSTR model was developed from compounds assayed according to the US EPA Gene Tox protocol (a chemical is tested against five strains of *Salmonella typhimurium*, using the Histidine Reversion Assay).
Rat oral LD_{50} (v. 3.1)	The rat oral LD_{50} module comprises 19 statistically significant and cross-validated QSTR models derived from experimental LD_{50} values for approximately 4000 compounds.
Chronic LOAEL (v. 3.1)	The rat chronic lowest observed adverse effect level (LOAEL) module comprises five statistically significant and cross-validated QSTR models derived from experimental values for 393 compounds. All data used were for oral rat chronic studies of at least one year's duration.
Skin irritation (v. 6.1)	The skin irritation module is a discriminant model consisting of 13 submodels of various structural categories. The models compute the probability of a compound being a skin irritant.
Skin sensitisation NEG v SENS (v. 6.1)	QSTR models derived from 335 uniform guinea pig maximisation tests. The models compute the probability of a compound being a skin sensitiser or non-sensitiser.

prediction space is a distinct multivariate descriptor space in which the model is applicable. Predictions are reported only if they fall within the optimum prediction space and all validation criteria are satisfied, or if they fall outside the optimum prediction space but within a permissible range of the program. Results are not reported for end-points where the program identifies that a prediction may be unreliable because either the prediction was outside the optimum prediction space and outside the permissible range of the model/sub-model or, a structural fragment from the query compound was not represented in the training set of the model/sub-model.

7.4.2 DEREK for Windows (www.lhasalimited.org/index.php)

DEREK for Windows version 8.0.1, developed by Lhasa Ltd, UK, works by matching structural entities in a query structure with predetermined structural alerts that are known to be associated with different toxicity endpoints (termed as toxicophores). A structural alert is the set of structural features in a molecule that makes a toxicologist suspect that the substance may show a particular toxic effect. This is similar to the definition of a toxicophore (a structural feature believed to be responsible for its toxic effect) or a pharmacophore (a structural feature believed to be responsible for a useful pharmacological effect), but alerts and toxicophores are not always identical. An alert may include information about additional features that increase or decrease the effectiveness of a toxicophore, such as hydrophobicity. In total, the program uses 482 structural alerts associated with different toxicity endpoints.

DEREK predicts the following toxicity endpoints for species that include bacteria (*Salmonella typhimurium*), guinea pig, hamster, human, mammal, mouse, primate, rat and rodent:

- carcinogenicity, such as photocarcinogenicity
- irritation, such as irritation of the eye and the gastrointestinal tract
- miscellaneous endpoints, such as anaphylaxis and anticholinesterase activity
- genotoxicity, such as mutagenicity and chromosome damage
- respiratory sensitisation, such as occupational asthma
- skin sensitisation, such as photoallergenicity
- thyroid toxicity.

DEREK also provides an indication of the likelihood of each predicted adverse effect using the terminology shown in Table 7.2.

7.5 The use of the (Q)SAR approach to identify potential toxicants in heat treated foods

7.5.1 Prediction of toxicity by TOPKAT

The study involved assessment of 247 compounds that have been reported to be produced during heat treatment of different foods, to generate a priority list of

Table 7.2 Terminology used in the outputs provided by DEREK for each endpoint

Terminology	Description
Certain	There is proof that the proposition is true
Probable	There is at least one strong argument that the proposition is true and there are no arguments against it
Plausible	The weight of evidence supports the proposition
Equivocal	There is an equal weight of evidence for and against the proposition
Doubted	The weight of evidence opposes the proposition
Improbable	There is at least one strong argument that the proposition is false and there are no arguments that it is true
Impossible	There is proof that the proposition is false
Open	There is no evidence that supports or opposes the proposition
Contradicted	There is proof both that the proposition is true and that it is false

compounds on the basis of predicted toxicity. The main aim of this study was to enable identification of the most toxic compounds so that they could be tested by standard laboratory procedures. For this purpose, the compounds were assessed by two toxicity expert systems, TOPKAT and DEREK, and toxicity predictions were used to prioritise them according to toxicity hazards. Furthermore, the reliability of the results was assessed by comparing the predicted toxicities of selected compounds with the published data in different online toxicity databases.

The compounds included in the study belong to a number of different chemical classes: pyrazines, thiophenes, thiazoles, pyrroles, furans, pyridines, oxazoles, as well as miscellaneous S-containing, N-containing, and O-containing compounds. Some of the 2D chemical structures were downloaded using the online program ChemIDPlus (National Library of Medicine: http://chem.sis.nlm.nih.gov/chemidplus), whilst others were built using the following specialist software:

- ChemX Version 2000.1. (Chemical Design Ltd, Oxford Molecular Group, UK)
- CaChe Version 6.1 (FQS Poland: www.cachesoftware.com/)
- MDL-ISIS/Draw Version 2.5 (www.mdli.com/).

The chemical structures were saved in MDL Mol file format for use as inputs to DEREK, and converted to SMILES codes using the chemistry tool kit (ChemTK-Lite: http://sageinformatics.com/) for use as inputs to TOPKAT.

The results from TOPKAT assessment were used to prioritise compounds on the basis of predicted toxicities. For this purpose, we adopted a simplified scoring system. For the endpoints where TOPKAT predictions were reported as a probability, the results were assigned a score of 1 for positive (probability > 0.7), 2 for indeterminate (probability 0.3 to 0.7), and 3 for negative (probability < 0.3). In some cases, the compounds were outside the prediction space of the models (OPS) and as such a prediction was not reliable. Such results were scored

as not available (n/a). The results were then used to order compounds according to the highest predicted toxicity hazards using the following ranking system:

1. The compounds of most concern were considered to be those that were predicted to be either carcinogenic or mutagenic. Therefore, all of the compounds were first ranked for those that were predicted to be both carcinogenic and mutagenic.
2. These were followed by compounds that were predicted to be either carcinogenic or mutagenic.
3. These were followed by compounds that were predicted to be negative for both carcinogenicity and mutagenicity, or for which a valid prediction was not obtained.

Within each of the above three categories, compounds were further ranked in the order of increasing rat oral LD_{50}. The results of other endpoints, such as chronic LOAEL, skin irritation score, and skin sensitisation score were not considered in prioritising the compounds.

The results of TOPKAT analysis are shown in Table 7.3. Out of the 247 compounds studied, the program predicted:

- 17 compounds to be both carcinogenic and mutagenic
- 134 compounds to be positive for either carcinogenicity or mutagenicity (and indeterminate or negative for the other)
- 91 compounds to be either indeterminate or negative for both carcinogenicity or mutagenicity
- 5 compounds were outside the model prediction space, and results were not available either on carcinogenicity or mutagenicity.

7.5.2 Comparison of results with other available information

The 17 compounds that were predicted to be both carcinogenic and mutagenic by TOPKAT, and another 17 compounds that were predicted to be neither carcinogenic nor mutagenic, were further evaluated using the toxicity expert software DEREK. The predictions obtained by DEREK analysis, where reasoning showed that the likelihood of a toxicophore was at least plausible, are shown in Table 7.4.

The TOPKAT predictions were further compared with any published information on the toxicity of these selected compounds using the online program ChemIDPlus. This program is linked to a database of over 379,000 records of which over 177,000 include chemical structures, and provides hazard information by searching the number of online databases linked to TOXNET (http://toxnet.nlm.nih.gov). The results of the search (Table 7.4) showed that partial information on carcinogenicity and mutagenicity was available for only some of the compounds. However, this still provided some evidence for either carcinogenicity and/or mutagenicity of seven out of the 17 compounds (2-butenal; Benzofuran, Quinoxaline; 2,3-butanedione; 2,4-pentanedione; 5-(hydroxymethyl)-2-furfural; and 2,3-dihydro-3,5-dihydroxy- 6-methyl-4H-pyran-4-one) that were

Table 7.3 A priority list of potential food toxicants on the basis of predicted toxicity by the expert system TOPKAT. For carcinogenicity, mutagenicity, skin irritation or skin sensitisation, a score of 1 = positive, 2 = indeterminate, 3 = negative

Compound name	CAS number	Chemical class	Carcinogenicity score	Mutagenicity score	Rat oral LD50 (mg/kg)	Chronic LOAEL (mg/kg)	Skin irritation score	Skin sensitisation score
3-methyl-3-buten-2-one	814-78-8	Misc O	1	1	149	364	3	1
3-penten-2-one	625-33-2	Misc O	1	1	246	29	3	3
2-butenal	4170-30-3	Misc O	1	1	301	85	1	1
3-Aminopyridine	462-08-8	Pyridine	1	1	310	29	3	1
Benzoxazole	273-53-0	Oxazole	1	1	333	26	3	3
Benzofuran	271-89-6	Furan	1	1	399	26	3	3
Quinoxaline	91-19-0	Pyrazine	1	1	469	56	3	3
2,3-butanedione	431-03-8	Misc O	1	1	836	518	1	2
2,4-pentanedione	123-54-6	Misc O	1	1	967	545	2	3
4,5-dihydro-2-methyl-3(2H)-furanone	3188-00-9	Furan	1	1	1200	209	3	1
2,3-dimethyl-2-cyclopenten-1-one	1121-05-7	Misc O	1	1	1400	21	2	1
2-methylquinoxaline	7251-61-8	Pyrazine	1	1	1500	59	3	3
3-hydroxy-2-methyltetrahydrofuran	29848-44-0	Furan	1	1	2100	39	1	1
3-methyl-2H-1-benzopyran-2-one	2445-82-1	Misc O	1	1	2400	62	3	3
5-(hydroxymethyl)-2-furfural	67-47-0	Furan	1	1	2900	56	1	1
1-(acetyloxy)-2-propanone	592-20-1	Misc O	1	1	4300	235	3	3
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	28564-83-2	Misc O	1	1	10000	42	1	1
2-acetyl-1-ethylpyrrole	39741-41-8	Pyrrole	2	1	146	157*	1	3

Table 7.3 Continued

Compound name	CAS number	Chemical class	Carcinogenicity score	Mutagenicity score	Rat oral LD50 (mg/kg)	Chronic LOAEL (mg/kg)	Skin irritation score	Skin sensitisation score
2-cyanofuran	617-90-3	Furan	2	1	400	14	1	1
Vinylpyrazine	4177-16-6	Pyrazine	1	2	403	50	1	1
2-Amino-3-methylpyridine	1603-40-3	Pyridine	1	2	735	31	3	1
2-formylthiophene	98-03-3	Thiophene	1	2	1100	55	3	1
Furfural	98-01-1	Furan	1	2	1100	23	1	1
1-(propionyloxy)-2-propanone	72845-79-5	Misc O	1	2	2400	273	3	2
1-(acetyloxy)-3-butanone	10150-87-5	Misc O	1	2	5200	281	3	3
2-hydroxy-3-methyl-2-cyclopenten-1-one	80-71-7	Misc O	1	2	7500	154	1	1
5-methyl-6,7-dihydro-5H-cyclopentapyrazine	23747-48-0	Pyrazine	1	3	86	4100*	N/a	N/a
1-ethyl-2,5-dimethyl-1H-pyrrole	5044-19-9	Pyrrole	N/a	1	157	55	1	3
2-acetyl-1-methylpyrrole	932-16-1	Pyrrole	3	1	162	146	3	3
benzonitrile	100-47-0	Misc N	1	3	250	51	3	1
1-(5-methyl-2-furyl)-1,2-propanedione	1197-20-2	Furan	1	3	251	66	1	1
2-acetyl-2-thiazoline	29926-41-8	Thiazole	N/a	1	267	9	3	3
acetonitrile	75-05-8	Misc N	1	3	268	6	1	1
2-methyl-2-thiazoline	2346-00-1	Thiazole	N/a	1	319	19	1	3
2,3-dihydrothiophene	1120-59-8	Thiophene	3	1	391	153	1	N/a
1H-pyrrolo[2,3-b]pyridine	271-63-6	Pyridine	1	3	420	210	3	3
1H-pyrrolo[2,3-c]pyridine	271-29-4	Pyridine	1	3	422	210	N/a	3
2-vinylfuran	1487-18-9	Furan	1	3	425	23	1	1
1-methyl-1H-pyrrole-2-carboxaldehyde	1192-58-1	Pyrrole	1	N/a	472	51	1	3

2-Amino-6-methylpyridine	1824-81-3	Pyridine	1	3	497	31	3	1
2-acetyl-5-methylfuran	1193-79-9	Furan	1	3	512	151	1	1
thieno(3,2b)thiophene	251-41-2	Thiophene	1	3	577	65	N/a	N/a
2-methylpyridine	109-06-8	Pyridine	1	3	609	46	3	2
thieno(2,3b)thiophene	250-84-0	Thiophene	1	3	619	65	N/a	N/a
3-methylpyridine	108-99-6	Pyridine	1	3	661	46	1	2
methylpyrazine	109-08-0	Pyrazine	1	3	673	46	N/a	2
2-acetylfuran	1192-62-7	Furan	1	3	681	59	2	3
2-acetylthiophene	88-15-3	Thiophene	1	3	686	159	3	1
5-ethyl-2-methylpyridine	104-90-5	Pyridine	1	3	693	54	1	3
2-ethyl-5-methylpyrazine	13360-64-0	Pyrazine	1	3	701	55	1	3
1H-pyrrole	109-97-7	Pyrrole	1	3	704	143	3	3
2-ethyl-6-methylpyrazine	13925-03-6	Pyrazine	1	3	716	55	1	3
4-methylpyridine	108-89-4	Pyridine	1	3	738	46	1	3
2-methylfuran	534-22-5	Furan	1	3	742	21	1	2
2-acetyl-3-methylthiophene	13679-72-6	Thiophene	1	3	778	170	3	1
1-(5-methyl-2-furyl)-1-propanone	10599-69-6	Furan	1	3	783	74	1	2
Thiazole	288-47-1	Thiazole	1	3	783	46	N/a	1
2-ethylpyridine	100-71-0	Pyridine	1	3	794	50	1	3
Pyridine	110-86-1	Pyridine	1	3	802	40	3	3
4-ethyl-2-methylpyridine	536-88-9	Pyridine	1	3	810	54	1	3
3-propionylpyridine	1570-48-5	Pyridine	1	3	837	70	2	3
ethanethioic acid S-methyl ester	1534-08-3	Sulfur	1	3	839	409	1	3
ethylpyrazine	13925-00-3	Pyrazine	1	3	849	51	1	3
thiophene	110-02-1	Thiophene	1	3	882	45	N/a	1
3-methylthiophene	616-44-4	Thiophene	1	3	892	50	N/a	1
2-ethylfuran	3208-16-0	Furan	1	3	897	23	1	3
3-ethylpyridine	536-78-7	Pyridine	1	3	909	50	N/a	3
4-methylthiazole	693-95-8	Thiazole	1	3	912	51	N/a	1
2-(methylthio)furan	13129-38-9	Sulfur	1	3	964	28	1	N/a

Table 7.3 Continued

Compound name	CAS number	Chemical class	Carcinogenicity score	Mutagenicity score	Rat oral LD50 (mg/kg)	Chronic LOAEL (mg/kg)	Skin irritation score	Skin sensitisation score
5-methyl-2-furfural	620-02-0	Furan	1	3	984	51	1	1
3-acetyl-2,4-dimethylpyrrole	2386-25-6	Pyrrole	1	3	1000	614*	1	3
1-(2-thienyl)-1-propanone	13679-75-9	Thiophene	1	3	1000	79	3	N/a
1-(2-furyl)-1-propanone	3194-15-8	Furan	1	3	1100	35	1	3
2,5-dimethyl-3-ethylpyrazine	13360-65-1	Pyrazine	1	3	1100	58	1	3
3,5-dimethyl-2-ethylpyrazine	13925-07-0	Pyrazine	1	3	1100	58	1	3
3-butylpyridine	539-32-2	Pyridine	1	3	1100	58	N/a	1
2-ethylthiophene	872-55-9	Thiophene	1	3	1100	55	1	N/a
2-acetyl-3-methylpyrazine	23787-80-6	Pyrazine	1	3	1300	156	3	3
methylmercaptan	74-93-1	Sulfur	1	3	1300	175	3	3
2-(methoxymethyl)furan	13679-46-4	Furan	1	3	1400	2	1	3
N-(2′-phenylethyl)-acetamide	877-95-2	Misc N	1	3	1400	30	3	1
2-methyl-1H-pyrrole	636-41-9	Pyrrole	1	3	1500	165	1	3
2-methylthiazole	3581-87-1	Thiazole	1	3	1500	51	N/a	1
2-formyl-3-methylthiophene	5834-16-2	Thiophene	1	3	1500	59	3	1
(Z and E)-1-(2-furyl)-1-buten-3-one	623-15-4	Furan	1	3	1600	98	N/a	3
4-Methylimidazole	822-36-6	Misc N	1	3	1600	167	N/a	3
5-methyl-2(1H)-pyridinone	1003-68-5	Pyridine	1	3	1600	203	1	2
3-pentanol	584-02-1	Misc O	1	3	1700	467	3	3
1-(2-thiazolyl)-1-propanone	43039-98-1	Thiazole	1	3	1800	81	3	N/a
2-furanmethanol	98-00-0	Furan	1	3	1900	24	3	1

5-methyl-2-furanmethanol	3857-25-8	Furan	1	3	1900	52	1	1
1-hydroxy-2-propanone	116-09-6	Misc O	1	3	1900	329	1	3
acetic acid	64-19-7	Misc O	1	3	2000	120	3	3
2-methyl-5-propylpyrazine	29461-03-8	Pyrazine	1	3	2000	58	1	1
2-ethyl-1H-pyrrole	1551-06-0	Pyrrole	1	3	2100	185	1	3
1H-pyrrole-2-carboxaldehyde	1003-29-8	Pyrrole	1	3	2100	185	1	1
2-ethylthiazole	15679-09-1	Thiazole	1	3	2100	55	N/a	1
propylpyrazine	18138-03-9	Pyrazine	1	3	2400	55	2	1
3-thiophenecarboxylic acid	88-13-1	Thiophene	1	3	2400	84	N/a	1
2-ethyl-3-methylpyrazine	15707-23-0	Pyrazine	1	3	2500	55	1	3
2-thiophenecarboxylic acid	527-72-0	Thiophene	1	3	2600	95	3	1
3-pentanone	96-22-0	Misc O	1	3	2700	562	3	3
Dimethyl trisulfide	3658-80-8	Sulfur	1	3	2900	610*	N/a	N/a
Dimethyl disulfide	624-92-0	Sulfur	1	3	3100	721*	3	N/a
2-hexanone	591-78-6	Misc O	1	3	3300	470	3	3
4-propylpyridine	1122-81-2	Pyridine	1	3	3300	54	N/a	2
Dimethyl tetrasulfide	5756-24-1	Sulfur	1	3	3400	953*	N/a	N/a
methyl 2-furoate	611-13-2	Furan	1	3	3600	31	1	3
1-(2-furfuryl)-1H-pyrrole	1438-94-4	Pyrrole	1	3	5100	30	N/a	1
4-(methylthio)-2-butanone	34047-39-7	Sulfur	1	3	6600	496	1	N/a
5-(2-hydroxyethyl)-4-methylthiazole	137-00-8	Thiazole	1	3	9700	64	N/a	1
1-methyl-1H-pyrrole	96-54-8	Pyrrole	1	3	>10000	41	3	3
2-furanmethanethiol	98-02-2	Sulfur	1	3	4600*	28	3	1
acetaldehyde (tetramer)	108-62-3	Misc O	1	N/a	665	618	N/a	N/a
Pyrazine	290-37-9	Pyrazine	1	N/a	925	41	N/a	3
2-methyl-3-furanthiol	28588-74-1	Sulfur	1	N/a	1100	28	1	1
1,2-ethanedithiol	540-63-6	Sulfur	1	N/a	1400	350	N/a	N/a
4-thio-2-butanone	34619-12-0	Sulfur	1	N/a	1600	269	3	3
tetrahydrofuran	109-99-9	Furan	3	1	558	128	1	1
2,3,4,5-tetramethyl-2-cyclopenten-1-one	54458-61-6	Misc O	3	1	789	22	3	1

Table 7.3 Continued

Compound name	CAS number	Chemical class	Carcinogenicity score	Mutagenicity score	Rat oral LD50 (mg/kg)	Chronic LOAEL (mg/kg)	Skin irritation score	Skin sensitisation score
1-ethylpyrrole-2,5-dione	128-53-0	Pyrrole	3	1	995	9	3	1
3-thiopropionic acid	107-96-0	Sulfur	3	1	998	116*	3	3
4-methyl-2,3-pentanedione	7493-58-5	Misc O	3	1	1100	401	1	1
tetrahydro-5-methyl-2-furanmethanol	6126-49-4	Furan	3	1	1700	79	1	1
2,4-hexanedione	3002-24-2	Misc O	3	1	1700	416	3	2
3-methyl-1,2-cyclopentanedione	765-70-8	Misc O	3	1	1700	85	3	1
2,3-pentanedione	600-14-6	Misc O	3	1	1900	365	1	1
4,5-dihydro-5-methyl-2(3H)-furanone (valerolactone)	108-29-2	Furan	3	1	2000	256	3	3
2-hydroxycyclohexanone	533-60-8	Misc O	3	1	2000	15*	3	3
4,5-dihydro-2(3H)-furanone (butyrolactone)	96-48-0	Furan	3	1	2700	142	3	3
2(5H)-furanone	497-23-4	Furan	3	1	2700	135	2	3
5-methyl-2(3H)-furanone	591-12-8	Furan	3	1	2900	51	1	2
3,5-dimethyl-2-hydroxy-2-cyclopenten-1-one	21834-98-0	Misc O	3	1	3300	238	1	1
2,4-(3H,5H)-furandione	4971-56-6	Furan	3	1	4700	26	1	3
2(3H)-furanone	20825-71-2	Furan	3	1	4800	136	3	3
3-butene-1,2-diol	497-06-3	Misc O	3	1	5100	111	3	3
5-methyl-2(5H)-thiophenone	7210-64-2	Thiophene	3	1	>10,000	75	3	1
4,5-dihydro-5-methyl-4-thio-3(2H)-furanone	56078-99-0	Sulfur	3	1	>10,000*	288	3	3
2,5-dimethyl-4-hydroxy-3(2H)-furanone	3658-77-3	Furan	3	1	>10000	172	1	N/a

2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone	27538-10-9	Furan	3	1	>10000	601*	N/a	N/a
3-hydroxy-4,5-dimethyl-2(5H)-furanone	28664-35-9	Furan	3	1	>10000	163	N/a	1
3,4-hexanedione	4437-51-8	Misc O	3	1	1700*	687	N/a	1
2-methylthiazolidine	24050-16-6	Thiazole	3	1	2300*	66	3	3
2-cyanopyrrole	4513-94-4	Pyrrole	3	1	679*	111	1	1
2,4,6-trimethyl-1,3,5-trithiane (thioacetaldehyde trimer)	2765-04-0	Sulfur	N/a	1	596	471	N/a	N/a
2-methylthieno (3,2b)thiophene	13393-75-4	Thiophene	N/a	1	2300	69	N/a	N/a
2,5-dimethyl-3(2H)-furanone	14400-67-0	Furan	N/a	1	3000	66	1	1
2,4,5-trimethyl-3-thiazoline	60633-24-1	Thiazole	N/a	1	6100	84	N/a	1
2-ethyl-2-thiazoline	16982-46-0	Thiazole	N/a	1	144*	25	1	3
2-propyl-2-thiazoline	23185-09-3	Thiazole	N/a	1	155*	24	1	N/a
2,4-dimethyl-3-thiazoline	60755-05-7	Thiazole	N/a	1	5200*	71	3	3
2-propenenitrile	107-13-1	Misc N	2	3	68	2	1	1
propanenitrile	107-12-0	Misc N	2	3	242	6*	3	1
3-methyl-2-butanone	563-80-4	Misc O	2	3	525	62	1	3
N-(2′-phenylethyl)formamide	23069-99-0	Misc N	2	3	1000	64	2	1
2-butanone	78-93-3	Misc O	2	3	1600	381	3	3
2-pentanone	107-87-9	Misc O	2	3	2600	424	3	3
2,3-diethyl-5-methylpyrazine	18138-04-0	Pyrazine	2	3	3000	61	1	N/a
4,5-dihydro-2(3H)-thiophenone	1003-10-7	Thiophene	3	2	443	168	3	3
3-hydroxy-4-hexanone	4984-85-4	Misc O	3	2	1400	311	N/a	3
1-(2-furyl)-2-hydroxyethanone	17678-19-2	Furan	3	2	2300	15	3	3
3-hydroxy-2-butanone	513-86-0	Misc O	3	2	2600	315	1	3
2-methylphenol	95-48-7	Misc O	3	3	290	73	1	3
1-(2-furyl)-1,2-propanedione	1438-92-2	Furan	3	3	319	31	1	3
2-acetylpyridine	1122-62-9	Pyridine	3	3	513	146	3	3
2(1H)-pyridinone	142-08-5	Pyridine	3	3	573	63	3	3

Table 7.3 Continued

Compound name	CAS number	Chemical class	Carcinogenicity score	Mutagenicity score	Rat oral LD50 (mg/kg)	Chronic LOAEL (mg/kg)	Skin irritation score	Skin sensitisation score
2-Hydroxypyridine	72762-00-6	Pyridine	3	3	573	63	3	3
3-thio-2-pentanone	67633-97-0	Sulfur	3	3	597	301	N/a	3
acetylpyrazine	22047-25-2	Pyrazine	3	3	631	146	3	3
3-acetylthiophene	1468-83-3	Thiophene	3	3	652	154	N/a	1
pyrrole-2,5-dione	541-59-3	Pyrrole	3	3	773	49	3	1
4-hydroxyacetophenone	99-93-4	Misc O	3	3	817	1400	1	3
Phenol	108-95-2	Misc O	3	3	866	75	1	3
pyrrolidine-2,5-dione	123-56-8	Pyrrole	3	3	887	51	N/a	1
2-acetylpyrrole	1072-83-9	Pyrrole	3	3	995	538*	3	3
2-pyrrolidone	616-45-5	Pyrrole	3	3	1000	48	3	3
3-methylpyrrole-2,5-dione	1072-87-3	Pyrrole	3	3	1100	17	2	1
2-cyclopenten-1-one	930-30-3	Misc O	3	3	1200	142	3	3
3-thio-2-butanone	40789-98-8	Sulfur	3	3	1200	373	N/a	3
cyclopentanone	120-92-3	Misc O	3	3	1300	150	2	3
propanoic acid	79-09-4	Misc O	3	3	1500	174	1	3
cyclohexanone	108-94-1	Misc O	3	3	1600	232	2	3
3-(methylthio)-2-butanone	53475-15-3	Sulfur	3	3	1700	39*	1	3
1-(2-furyl)-3-butanone	699-17-2	Furan	3	3	1900	83	1	1
acetophenone	98-86-2	Misc O	3	3	2100	863	3	3
3-hydroxy-2-pentanone	3142-66-3	Misc O	3	3	2400	260	2	3
2-phenyl-1-ethanol	60-12-8	Misc O	3	3	2400	617	3	1
2,6-diethylpyrazine	13067-27-1	Pyrazine	3	3	2400	58	1	N/a
2-thiopropionic acid	79-42-5	Sulfur	3	3	2400	1800*	1	3
2,5-diethylpyrazine	13238-84-1	Pyrazine	3	3	2500	58	1	N/a
tetrahydro-4H-thiopyran-4-one	1072-72-6	Sulfur	3	3	3700	268	3	3
methional	3268-49-3	Sulfur	3	3	3800	269	1	N/a
1,2-butanediol	584-03-2	Misc O	3	3	4800	324	3	3

Furfuryl ethanoate	623-17-6	Furan	3	3	5300	6*	1	3
4,5-dihydro-3(2H)-thiophenone	1003-04-9	Thiophene	3	3	5700	175	3	3
1-(methylthio)-2-butanone	13678-58-5	Sulfur	3	3	6900	496	3	3
4,5-dihydro-2-methyl-3(2H)-thiophenone	13679-85-1	Thiophene	3	3	7400	243	3	3
1,2-dithiolane	557-22-2	Sulfur	3	N/a	528	188*	N/a	N/a
ethylmercaptan	75-08-1	Sulfur	3	N/a	699	226	3	1
2-thioethanol	75-08-1	Sulfur	3	N/a	699	226	3	1
1,2,4-trithiolane	289-16-7	Sulfur	3	N/a	3100*	220*	N/a	N/a
2-methyl-2-butenal	1115-11-3	Misc O	N/a	3	265	29	N/a	1
2-methyl-5-vinylpyrazine	13925-08-1	Pyrazine	N/a	3	288	54	1	1
2,5-dimethyl-2,5-cyclohexadien-1,4-dione	137-18-8	Misc O	N/a	3	295	0.38*	N/a	N/a
2-methyl-2-cyclopenten-1-one	1120-73-6	Misc O	N/a	3	415	58	1	1
2-acetyl-5-methylthiophene	13679-74-8	Thiophene	N/a	3	447	170	3	1
2,5-dimethylpyridine	589-93-5	Pyridine	N/a	3	496	50	3	3
2,5-dimethylthiazole	4175-66-0	Thiazole	N/a	3	571	55	3	1
2,5-dimethylthiophene	638-02-8	Thiophene	N/a	3	589	55	1	N/a
2,4-dimethylthiazole	541-58-2	Thiazole	N/a	3	603	55	N/a	1
2,4-dimethylthiophene	638-00-6	Thiophene	N/a	3	605	55	N/a	N/a
2,4-dimethylfuran	3710-43-8	Furan	N/a	3	625	23	1	3
2,5-dimethylfuran	625-86-5	Furan	N/a	3	653	47	1	3
2,6-dimethylpyrazine	108-50-9	Pyrazine	N/a	3	691	51	3	3
2-ethyl-5-methylfuran	1703-52-2	Furan	N/a	3	692	51	1	3
3,5-dimethylpyridine	591-22-0	Pyridine	N/a	3	700	50	3	N/a
2,5-dimethylpyrazine	123-32-0	Pyrazine	N/a	3	708	51	3	3
2-methylthiophene	554-14-3	Thiophene	N/a	3	779	50	3	1
5-methylthiazole	3581-89-3	Thiazole	N/a	3	860	51	3	1
2-formyl-5-methylthiophene	13679-70-4	Thiophene	N/a	3	871	59	1	1
4-methyl-5-vinylthiazole	1759-28-0	Thiazole	N/a	3	967	58	N/a	1
2,3-dimethylthiophene	632-16-6	Thiophene	N/a	3	1000	55	N/a	N/a
2,6-dimethyl-3-ethylpyridine	23580-52-1	Pyridine	N/a	3	1100	58	1	3
2,4-Dimethylimidazole	930-62-1	Misc N	N/a	3	1200	187	3	3

Table 7.3 Continued

Compound name	CAS number	Chemical class	Carcinogenicity score	Mutagenicity score	Rat oral LD50 (mg/kg)	Chronic LOAEL (mg/kg)	Skin irritation score	Skin sensitisation score
2,4-dimethyl-1H-pyrrole	625-82-1	Pyrrole	N/a	3	1300	185	1	3
2,4,5-trimethylthiazole	13623-11-5	Thiazole	N/a	3	1300	59	3	N/a
3,4-dimethylthiophene	632-15-5	Thiophene	N/a	3	1400	55	N/a	N/a
2,4,5-trimethyloxazole	20662-84-4	Oxazole	N/a	3	1600	52	3	N/a
tetramethylpyrazine	1124-11-4	Pyrazine	N/a	3	1700	58	N/a	N/a
2-ethyl-4,5-dimethyloxazole	53833-30-0	Oxazole	N/a	3	1800	56	1	3
2,3,4,5-tetramethyl-1H-pyrrole	1003-90-3	Pyrrole	N/a	3	1800	219	N/a	N/a
4,5-dimethylthiazole	3581-91-7	Thiazole	N/a	3	1900	55	N/a	1
4-ethyl-2,5-dimethyloxazole	30408-61-8	Oxazole	N/a	3	2100	56	1	3
5-ethyl-2,4-dimethylthiazole	38205-61-7	Thiazole	N/a	3	2100	63	1	N/a
2,3-dimethylpyrazine	5910-89-4	Pyrazine	N/a	3	2400	51	N/a	3
2-(1-thioethyl)thiophene	94089-02-8	Thiophene	N/a	3	2600	121	N/a	N/a
5-ethyl-4-methylthiazole	31883-01-9	Thiazole	N/a	3	3300	59	N/a	1
4-ethyl-5-methylthiazole	52414-91-2	Thiazole	N/a	3	3300	59	N/a	1
3-methyl-2-cyclopenten-1-one	2758-18-1	Misc O	N/a	3	4200	54	1	1
4-ethyl-2-methyl-5-propylthiazole	41981-75-3	Thiazole	N/a	3	>10,000	69	1	1
2-methyl-3-(methyldithio)furan	65505-17-1	Sulfur	N/a	3	1300*	35*	1	N/a
bis(2-furylmethyl) disulfide	4437-20-1	Sulfur	N/a	3	2000*	20*	N/a	N/a
bis(2-methylbutyl)amine	27094-65-1	Misc N	N/a	N/a	653	2*	1	1
trimethylpyrazine	14667-55-1	Pyrazine	N/a	N/a	800	55	3	N/a
2,5-dimethyl-3-furanthiol	55764-23-3	Sulfur	N/a	N/a	926	60	1	1
3,5-dimethyl-1,2,4-trithiolane (E and Z)	23654-92-4	Sulfur	N/a	N/a	1100	328*	N/a	N/a
2-methyl-3-thiophenethiol	2527-76-6	Thiophene	N/a	N/a	1100	64	N/a	1

N/a Not available.
* Outside the prediction space of the program.

Table 7.4 Comparison of toxicity predictions by TOPKAT with other available information

Compound name, CAS number, (chemical class)	Predicted toxicity (TOPKAT)	Predicted toxicity (DEREK)	ChemIDPlus toxicity information
3-methyl-3-buten-2-one CAS No. 814-78-8, (Misc O)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 149 mg/kg	Chromosome damage, skin sensitiser	Highly toxic by oral, percutaneous and respiratory routes. Irritant. Animal carcinogenicity studies are limited, but are generally negative. Agents in this group generally have little mutagenic activity (HSDB), Negative in AMES mutagenicity studies (CCRIS). Rat oral LD_{50} = 180 mg/kg
3-penten-2-one CAS No. 625-33-2, (Misc O)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 246 mg/kg	Chromosome damage, skin sensitiser	No information available on carcinogenicity or mutagenicity. Rat oral LD_{50} = 3200 mg/kg
2-butenal CAS No. 4170-30-3 (Misc O)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 301 mg/kg	Mutagenic, irritant, skin sensitiser	Possible human carcinogen (HSDB), genotoxic, mutagenic, and carcinogenic (e.g. Fernandes *et al.*, 2005; Budiawan, 2001), Positive in AMES mutagenicity studies (CCRIS). Rat oral LD_{50} = 80 mg/kg
3-Aminopyridine CAS No. 462-08-8 (Pyridine)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 310 mg/kg	Carcinogenic, skin sensitiser	No information available on carcinogenicity or mutagenicity. Irritant (eyes and skin). Quail oral LD_{50} = 178 mg/kg
Benzoxazole CAS No. 273-53-0 (Oxazole)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 333 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity. Mouse oral LD_{50} = 750 mg/kg

Table 7.4 Continued

Compound name, CAS number, (chemical class)	Predicted toxicity (TOPKAT)	Predicted toxicity (DEREK)	ChemIDPlus toxicity information
Benzofuran CAS No. 271-89-6 (Furan)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 399 mg/kg	Nothing to report	Postive carcinogenicity studies; negative AMES mutagenicity, but positive mutagenicity result for mouse lymphoma (CCRIS), possibly carcinogenic in humans (HSDB), carcinogenic in female rats (Robbianno *et al.*, 2004).
Quinoxaline CAS No. 91-19-0 (Pyrazine)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 469 mg/kg	Nothing to report	One positive AMES mutagenicity test result (CCRIC), Negative results sperm morphology (GENE-TOX), Reported as being mutagenic (Sutherland *et al.*, 1996; Bashir *et al.*, 1990). No information on available carcinogenicity.
2,3-butanedione CAS No. 431-03-8 (Misc O)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 836 mg/kg	Skin sensitiser	Positive for several AMES mutagenicity studies (CCRIS), probably has irritant and CNS depressant action (HSDB). No information available on carcinogenicity. Rat oral LD_{50} = 1580 mg/kg
2,4-pentanedione CAS No. 123-54-6 (Misc O)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 967 mg/kg	Chromosome damage, skin sensitiser	One positive AMES mutagenicity test result (CCRIS), Irritant (HSDB). Rat oral LD_{50} = 55 mg/kg
4,5-dihydro-2-methyl-3(2H)-furanone CAS No. 3188-00-9 (Furan)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 1200 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity. Mouse oral LD_{50} = 1860 mg/kg
2,3-dimethyl-2-cyclopenten-1-one CAS No. 1121-05-7 (Misc O)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 1400 mg/kg	Chromosome damage	No information available on carcinogenicity or mutagenicity.

2-methylquinoxaline CAS No. 7251-61-8 (Pyrazine)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 1500 mg/kg	Nothing to report	Negative for several mutagenicity studies (CCRIS), no information available on carcinogenicity.
3-hydroxy-2-methyltetrahydrofuran CAS No. 29848-44-0 (Furan)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 2100 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity. Mouse LD_{50} (iv) 4360 mg/kg
3-methyl-2H-1-benzopyran-2-one CAS No. 2445-82-1, (Misc O)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 2400 mg/kg	Skin sensitiser	Chromosomal effects in plants (GENE-TOX), reported to be Hepatoxic in rats (Toxline). No information available on carcinogenicity or mutagenicity. Mouse LD_{50} (sc) 316 mg/kg
5-(hydroxymethyl)-2-furfural CAS No. 67-47-0 (Furan)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 2900 mg/kg	Nothing to report	Negative for two mouse carcinogenicity studies, but positive for some mutagenicity studies (CCRIS), found to possess cytotoxic, genotoxic and tumorigenic activities, but the mechanisms of its toxic actions remain unclear (Surh *et al.*, 1994). Shows cytotoxicity and mutagenicity at high concentrations (Janzowski *et al.*, 2000). Rat oral LD50 = 2500 mg/kg
1-(acetyloxy)-2-propanone CAS No. 592-20-1 (Misc O)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 4300 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity.
2,3-dihydro-3,5-dihydroxy- 6-methyl-4H-pyran-4-one CAS No. 28564-83-2 (Misc O)	Carcinogenicity = positive Mutagenicity = positive Rat oral LD_{50} = 10,000 mg/kg	Chromosome damage, skin sensitiser	Positive for some mutagenicity studies (CCRIS), the compound generated active oxygen species to cause DNA strand breaking and mutagenesis (Hiramoto *et al.*, 1997).
2,6-diethylpyrazine CAS No. 13067-27-1 (Pyrazine)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 2400 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity.

Table 7.4 Continued

Compound name, CAS number, (chemical class)	Predicted toxicity (TOPKAT)	Predicted toxicity (DEREK)	ChemIDPlus toxicity information
acetylpyrazine CAS No. 22047-25-2 (Pyrazine)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 631 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity.
4,5-dihydro-3(2*H*)-thiophenone CAS No. 1003-04-9 (Thiophene)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 5700 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity.
3-acetylthiophene CAS No. 1468-83-3 (Thiophene)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 652 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity.
Methional CAS No. 3268-49-3 (Sulfur)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 3800 mg/kg	Mutagenic, skin sensitiser, genotoxic	Negative for AMES mutagemicity tests, but positive for two mouse lymphona studies (CCRIS). Rat oral LD50 = 700 mg/kg
1-(methylthio)-2-butanone CAS No. 13678-58-5 (Sulfur)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 6900 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity.
3-thio-2-pentanone CAS No. 67633-97-0 (Sulfur)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 597 mg/kg	Skin sensitiser	No information available on carcinogenicity or mutagenicity.
Pyrrolidine-2,5-dione CAS No. 123-56-8 (Pyrrole)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 887 mg/kg	Nothing to report	Rat oral LD_{50} = 14000 mg/kg
3-methylpyrrole-2,5-dione CAS No. 1072-87-3 (Pyrrole)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 995 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity.

1-(2-furyl)-1,2-propanedione CAS No. 1438-92-2 (Furan)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 5100 mg/kg	Skin sensitiser	No information available on carcinogenicity or mutagenicity.
furfuryl ethanoate CAS No. 623-17-6 (Furan)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 5300 mg/kg	Nothing to report	Positive for three of nine AMES mutagenicity tests (CCRIS).
2-acetylpyridine CAS No. 1122-62-9 (Pyridine)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 513 mg/kg	Nothing to report	Negative for AMES mutagenicity tests, but positive for two mouse lymphona studies (CCRIS). Rat oral LD_{50} = 2280 mg/kg
2(1*H*)-pyridinone CAS No. 142-08-5 (Pyridine)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 573 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity.
Cyclopentanone CAS No. 120-92-3 (Misc O)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 1300 mg/kg	Nothing to report	No information available on carcinogenicity or mutagenicity. Rat inhalation LC_{50} = 19500 mg/kg
4-hydroxyacetophenone CAS No. 99-93-4 (Misc O)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 817 mg/kg	Skin sensitiser	No information available on carcinogenicity or mutagenicity. Mouse oral LD_{50} = 1500 mg/kg
2-phenyl-1-ethanol CAS No. 60-12-8 (Misc O)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 2400 mg/kg	Nothing to report	PEA was not mutagenic in the Ames test or in an *Escherichia coli* DNA-polymerase-deficient assay, system (Anonymous, 1990). Rat oral LD_{50} = 1790 mg/kg
Acetophenone CAS No. 98-86-2 (Misc O)	Carcinogenicity = negative Mutagenicity = negative Rat oral LD_{50} = 2100 mg/kg	Nothing to report	Negative for all AMES mutagenicity tests (CCRIS), negative for prokaryotes – other genotoxic effects (GENETOX), not classified as carcinogen (due to lack of any animal or human data) (HSDB). Rat oral LD_{50} = 815 mg/kg

The online databases HSDB, CCRIS and GENETOX are accessible through TOXNET (http://toxnet.nlm.nih.gov).

predicted to be both carcinogenic and mutagenic by TOPKAT. This comparison also indicated that predictions for either carcinogenicity or mutagenicity of two of the compounds (3-methyl-3-buten-2-one, and 2-methylquinoxaline) were probably false positives. For the same 17 compounds, DEREK predicted it to be plausible that one compound will exhibit mutagenic activity, one compound carcinogenic activity, and five compounds cause chromosome damage.

For the 17 compounds that were predicted to be neither carcinogenic nor mutagenic, there was evidence to back up predictions for two compounds, whilst data were not available for 12 compounds. The comparison showed that predictions for either carcinogenicity or mutagenicity of three of the compounds (2-acetylpyridine, furfuryl ethanoate, Methional) were probably false negatives. For the same 17 compounds, DEREK predicted it to be plausible that one compound will exhibit mutagenic activity. DEREK also predicted it to be plausible that one of the three TOPKAT false negative compounds (Methional) will exhibit mutagenic and genotoxic activity, but yielded no alert for the other two false negative compounds.

Despite the indication that predictions for a small proportion of compounds (two to three out of each category of 17 compounds) may not be accurate, the comparison still provided a very good degree of certainty in the combined toxicity prediction by TOPKAT and DEREK. This further indicated that the reliability of toxicity predictions by expert systems can be enhanced by the use of a combination of approaches, because a single program alone may not be adequate for accurately predicting a variety of toxicity endpoints for such a wide range of chemical classes.

7.6 Conclusions

The use of the (Q)SAR approach to identify potential toxicants among the compounds that are generated during heat treatment of foods has demonstrated that the methodology can be successfully used in situations where there is little or no prior toxicity information available. The comparison of predicted toxicities with available published information has also shown that, despite a few discrepancies, the reliability of the predictions by the two expert systems used in this study is excellent for the purpose of ranking compounds to identify those that would need testing on a priority basis. Because the (Q)SAR approach is based on well defined statistical algorithms, it eliminates the involvement of any guesswork or random selection of compounds, which could lead to errors through human bias. The priority list generated by this study thus provides a useful basis on which compounds may be targeted for toxicity testing through standard laboratory procedures. For situations that require an even greater accuracy of toxicity predictions, for example a regulatory dossier, it would be feasible to develop and use specific (Q)SAR models for each class of the chemicals separately, and/or to use more than one expert system and cross-validate the predicted toxicity values by a read-across approach.

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Part II

Health risks of acrylamide and other hazardous compounds in heat-treated foods

8

Biomonitoring of acrylamide

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8.1 Introduction

The terms 'biomonitoring' and 'molecular dosimetry' generally refer to the measurement of a biomarker to assess the health risk associated with exposure to a chemical. A biomarker is a measured chemical or biological endpoint in samples from humans (or other studied organisms). Different biomarkers may reflect the successive events from exposure to biological effects via concentration and dose (integrated concentration) in the body for exposure to a genotoxic compound (Fig. 8.1).

Biomarkers are sometimes divided into categories, such as biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility, etc. (Aitio 1999; Bond *et al.* 1992; Watson *et al.* 2004).

In this chapter different biomarkers for acrylamide (AA) exposure and methods for their measurement will be reviewed, as well as results obtained on biomarkers in experimental studies. This will continue with an overview of results obtained in human biomonitoring studies and a discussion of the usefulness of different biomarkers in studies of exposure and as a basis in risk assessment.

Our focus is on chemical biomarkers, mainly because of the specificity of chemical analysis. In contrast, biological biomarkers, e.g. cytogenetic changes such as micronuclei, chromosomal aberrations or sister chromatid exchange, are non-specific to a particular chemical but respond to various genotoxic factors in the environment. The chemical biomarkers that have been considered for biomonitoring of exposure to AA include free AA or its metabolite glycidamide (GA) in body fluids, products excreted in the urine, and reaction products (adducts) with DNA or the protein haemoglobin (Hb) in erythrocytes.

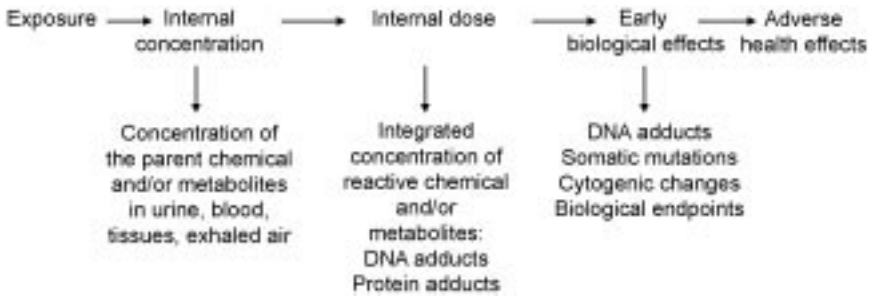

Fig. 8.1 Principal scheme of biomarkers that could be applied for measurement of parameters/events in the process from exposure to a genotoxic compound to the development of adverse health effects.

For the application of a biomarker it is important to know the time window during which a biomarker reflects the exposure. Both AA and GA are reactive compounds with half-lives in humans of about 4.6 h for AA and 1.9 h for GA (Calleman 1996). This implies that the compounds do not accumulate in the tissues and that their concentrations give a short-term measure of the exposure. Similarly, the major part of the urinary mercapturic acids of AA and GA are rapidly excreted with a half-life of about 3.5 hours (Boettcher *et al.* 2005a) and, therefore, timing of analysis after exposure is critical as illustrated in Fig. 8.2.

The major adduct with DNA formed from exposure to AA has a half-life of approximately four days (see Section 8.3.4). For chemically stable adducts with Hb the life span is equal to that of the erythrocytes – about 120 days in humans. Thus, in humans the time period after exposures covered by adduct measurement is about a week for DNA adducts, and a few months for Hb adducts (Fig. 8.2). The adducts accumulate during continuous exposure to reach a steady-state level. The steady-state level of DNA adducts, assuming a half-life of four days, would correspond to about six times the adduct increment from one day of exposure. The steady-state level of Hb adducts corresponds to 60 times the daily increment (Fig. 8.3). In addition to the lifetimes of the various biomarkers, their relative abundance, as well as the sensitivity of the analytical methods available for quantification, are determining their utility in human biomonitoring studies.

8.2 Metabolism and reactivity

8.2.1 Metabolism

The metabolism of AA in the body follows two major pathways; (i) conjugation with glutathione (GSH) and subsequent formation of mercapturic acids and (ii) oxygenation of the double bond in the transformation to the epoxide GA (Fig. 8.4). The formation of GA is shown to be catalysed by the CYP 450 enzyme 2E1 in mice (Ghanayem *et al.* 2005; Sumner *et al.* 1999) and it is reasonable to

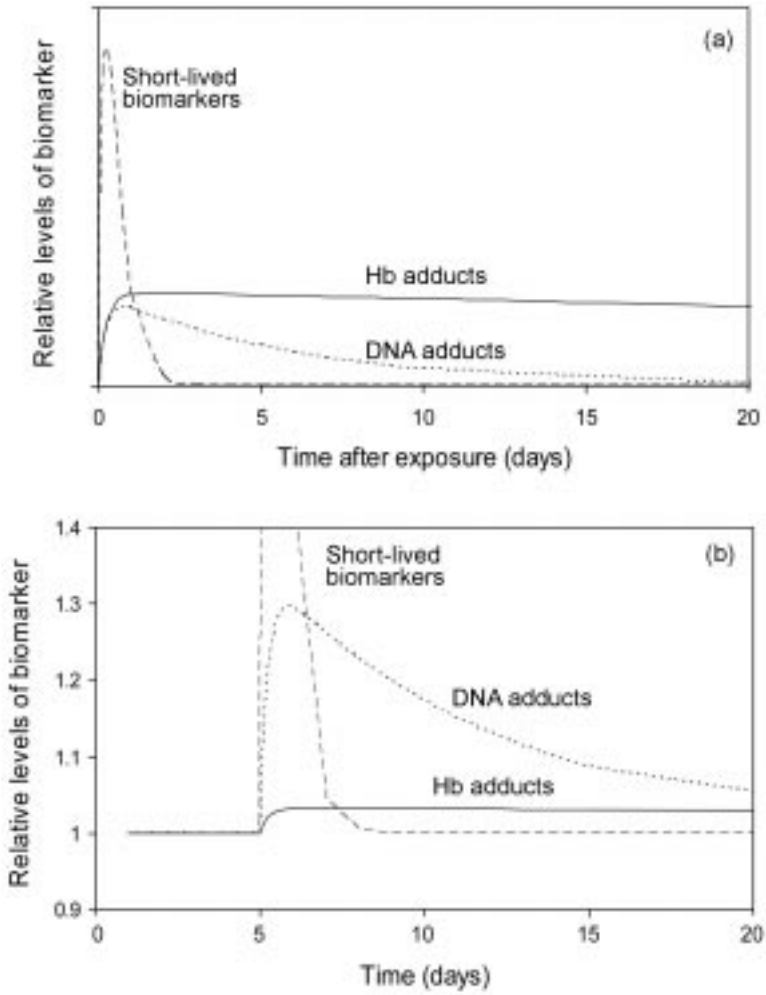

Fig. 8.2 Monitoring time ranges for short-lived biomarkers (e.g., urinary metabolites), stable haemoglobin (Hb) adducts and DNA adducts (assuming a half-life of about four days). Case (a) illustrates a single exposure which is high compared to the background exposure (linear scale); Case (b) illustrates a single exposure (at day five) corresponding to two days of background exposure (normalised values). Scheme of principle.

assume that the same enzyme is also responsible for this metabolism in humans. The hydrolysis of GA to glyceramide may be catalysed by epoxide hydrolase (EH) and the conjugation with GSH by glutathione transferases (GST). However, *in vitro* studies have indicated that neither GST nor EH influences the detoxification rates of AA or GA in human blood, and that these detoxification pathways are largely chemical reactions without enzymatic catalysis (Paulsson *et al.* 2005).

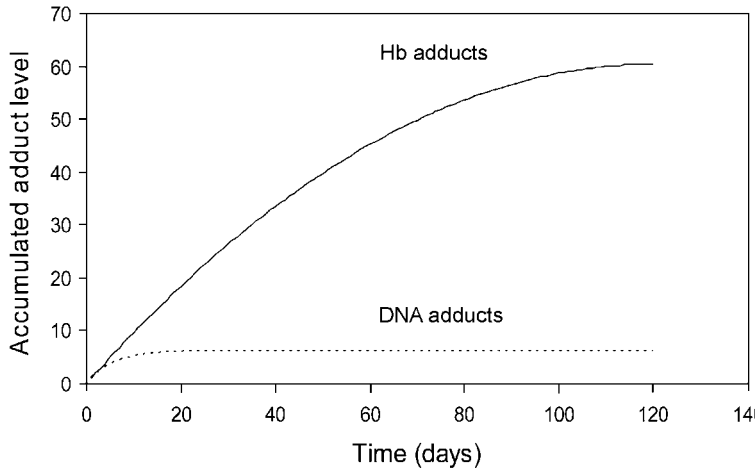

Fig. 8.3 Accumulation of haemoglobin (Hb) adducts and DNA adducts, respectively, during continuous exposure (exposure starts at day 0). The Hb adducts are assumed to be stable with a life span of 120 days in humans (equal to that of the erythrocytes) and the half-life of the DNA adducts is assumed to be four days.

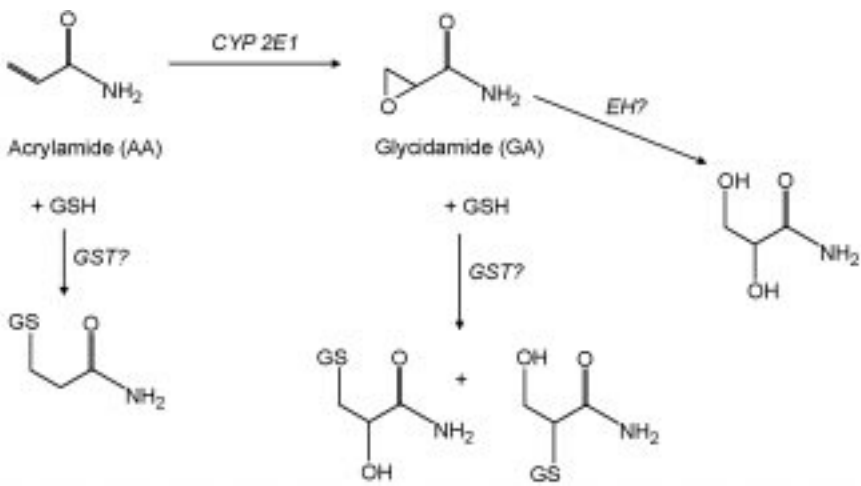

Fig. 8.4 Detoxification pathways of AA and formation of GA, with indication of possible enzymes involved.

8.2.2 Reaction with nucleophiles

AA and its metabolite GA are electrophilically reactive substances that bind to nucleophilic sites, for example in biomacromolecules such as proteins and DNA, and form adducts. The reactivity of nucleophilic atoms generally decreases in the order S > N > O. However, the reactivity patterns of AA and GA are quite different. Being an α,β-unsaturated carbonyl compound, AA reacts through an

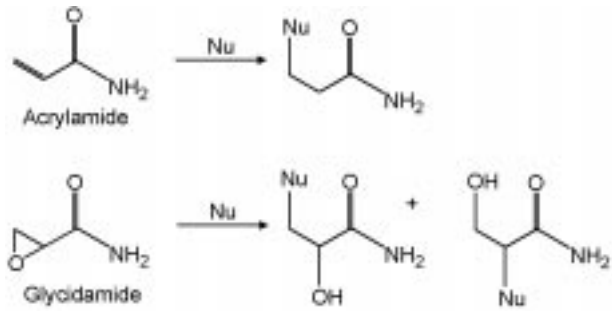

Fig. 8.5 Scheme over AA and GA reactions with an arbitrary nucleophile (Nu), showing the formation of 2-carbamoylethyl adducts from AA, and the formation of 2-carbamoyl-2-hydroxyethyl or 1-carbamoyl-2-hydroxyethyl adducts from GA.

addition mechanism (Michael type) with a strong propensity for reactions with SH-containing compounds (Friedman 2003). AA has a much lower reaction rate towards the nucleophilically reactive nitrogens in the DNA bases than its metabolite GA. The epoxide GA reacts via nucleophilic substitution with a reactivity similar to that of other simple epoxides, for example ethylene oxide (Silvari *et al.* 2005). GA shows a spectrum of adducts with nitrogen atoms in DNA (see Section 8.3.4). Presumably, GA forms adducts also with oxygen atoms in the bases and in the phosphate groups (Johansson *et al.* 2005; Silvari *et al.* 2005). Depending on which carbon the nucleophile attacks, two isomeric forms are formed (Fig. 8.5).

The reactivity of a nucleophilic site in biomacromolecules is also dependent on its pK_a – the base in an acid base equilibrium being by far the most reactive species. This influences the rate of adduct formation and choice of biomarker. Thus, because of the low pK_a (about 7) of the N-terminal amino group in Hb, this site has a comparatively high reactivity and could be favourable for adduct monitoring (see Section 8.3.3). Furthermore, adduct formation is influenced by the tertiary structure of the biomacromolecule. For instance, a certain cysteine in rat Hb ($Cys^{\beta 125}$) has an about 100 times higher reactivity than cysteines in human Hb, and therefore cysteine adducts are abundant in the rat, also in the case of AA (see Section 8.3.3), (reviewed in Törnqvist *et al.* (2002)).

8.3 Chemical biomarkers, methods and experimental results

8.3.1 Acrylamide and glycidamide in body fluids

Determination of free AA and GA in body fluids has mainly been used to study the rate of formation of GA from AA, and rates of elimination of the two compounds in experimental animals. In early studies ^{14}C-labelled AA was used, and AA and GA were quantified by radioactivity measurements (Miller *et al.* 1982). Today sensitive and specific high throughput chemical analytical methods are available for the two compounds. Twaddle *et al.* have described a method based on liquid chromatography (LC) with electrospray tandem mass

spectrometry ((ES)/MS/MS) that allows quantification of concentrations down to 10 nM AA and 100 nM GA in samples of serum – the detection limit being approximately three times lower (Twaddle *et al.* 2004b). ^{13}C-Substituted AA and GA, respectively, were used as internal standards and the samples were purified by solid phase extraction prior to the LC-MS/MS analysis.

The animal experiments have shown that AA is rapidly absorbed from the site of administration and distributed to the tissues. Furthermore, the experiments have shown that the extent of formation of GA is dependent on administered dose, dose rate and route of administration and differs between species. There is also a variation in the half-lives of AA and GA obtained in these studies depending on the exposures (Barber *et al.* 2001; Doerge *et al.* 2005b; Twaddle *et al.* 2004b). At low dietary exposure (0.1 mg/kg) the half-lives in mice could be estimated to 1.2 h (males) and 3.0 h (females) for AA, and to 2.6 h (males) and 3.7 h (females) for GA (Doerge *et al.* 2005b). The results from the measurements of AA and GA in serum show that the ratio of the internal dose (AUC; area under the concentration curve) of GA to the internal dose of AA is two to three times larger in mice than in rats when compared at relatively high exposure doses (Barber *et al.* 2001; Twaddle *et al.* 2004b). This is in agreement with results from Hb and DNA adduct measurements (see Sections 8.3.3 and 8.3.4).

Serum concentrations of AA and GA have been measured in rats administered daily doses of 1 mg AA per kg body weight (bw) in the drinking water (Doerge *et al.* 2005a). The serum concentrations did not vary significantly between the various time points studied (0–28 d). The average concentrations of AA and GA were about 0.6 μM. These data may be used for a tentative estimate of the concentrations that may occur in humans at current exposure levels/intake, estimated to be about 0.5 μg AA/kg bw/day (see Section 8.5.2). A linear extrapolation to this intake from the experiments in rats would give a serum concentration of about 0.3 nM.

If analysis of AA in serum or other body fluids is to be used in biomonitoring of low levels of AA exposure, there is a need for further improvement of the sensitivity of the methods for analysis. There are sensitive methods developed for the analysis of the AA in food, which may as well be used for the analysis of AA in body fluids. Particularly methods exploring the reactivity of AA to form derivatives for analysis may be advantageous. A method where a tripeptide was used for trapping of AA, followed by gas chromatography tandem mass spectrometry (GC-MS/MS) analysis of a derivative in the same way as in the analysis of Hb adducts with N-termini (cf. Section 8.3.3), achieved high sensitivity in the analysis of AA in coffee (limit of detection < 0.4 nM) (Licea Pérez *et al.* 2003). There are today only a few studies reporting measurements of free AA or GA in human exposure situations. Those are discussed in Section 8.4.1.

8.3.2 Urinary metabolites

Determination of AA-derived metabolites in the urine may become an important tool for surveillance of AA exposure; the advantages being that the sampling is

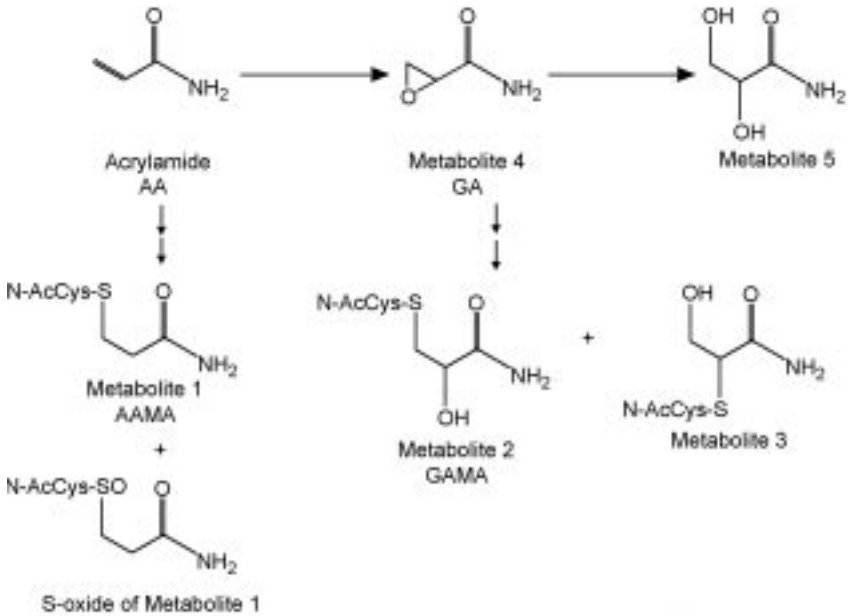

Fig. 8.6 Structures of the different urinary metabolites from AA identified in rodents and humans.
Metabolite 1 = *N*-acetyl-*S*-(3-amino-3-oxopropyl)cysteine (AAMA) and its *S*-oxide.
Metabolite 2 = *N*-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA).
Metabolite 3 = *N*-acetyl-*S*-(1-carbamoyl-2-hydroxyethyl)cysteine.
Metabolite 4 = glycidamide (GA).
Metabolite 5 = 2,3-dihydroxypropionamide (glyceramide).

non-invasive and that sensitive and specific methods that require a minimum of handling of the samples are available. A majority of studies on AA-related urinary metabolites have concerned identification of the various products formed, and evaluation of differences between species in the pattern of products, as well as evaluation of the impact of different routes of exposure. In these studies ^{13}C-NMR spectroscopy has been a useful approach which allows the identification and quantification of metabolites directly in the urine following exposure to ^{13}C-substituted AA (Sumner *et al.* 1992). Structures of the different urinary metabolites found in rodents and humans are shown in Fig. 8.6.

The ratio of products formed directly from AA to those derived from GA is of interest because it reflects the flux through the two major metabolic pathways – direct reactions of AA and formation of GA, respectively. About 50% of the administered compound was recovered over 24 hours as urinary metabolites when rats and mice were given ^{13}C-AA orally (Sumner *et al.* 1992). AAMA (Metabolite 1 in Fig. 8.6) formed from conjugation of AA with GSH, was the major metabolite for both rats and mice but a much larger fraction of the urinary metabolites was derived from GA in mice compared to rats (Table 8.1).

In order to study the conversion of AA to GA in humans Fennell *et al.* (2005) administered ^{13}C-substituted AA in an aqueous solution orally to male

Table 8.1 Fractions of different urinary metabolites from exposure to AA, measured in experimental studies using stable isotope-substituted AA

	Administrered dose of AA (mg/kg bw)	Fraction (%) of administered dose recovered in the urine (24 h)	Fraction (%) of metabolites derived from GSH-conjugation of AA; Metabolite 1[a]	Fraction (%) of metabolites derived from GA; Metabolites 2, 3, 4, 5[a]	Ratio of metabolites; 2:1[a]	Ratio of metabolites; 2–5:1[a]	Reference
Mouse							
^{13}C-AA	50	51	41	21, 12, 17, 5	0.51	1.35	Sumner *et al.* (1992)
Rat							
^{13}C-AA	50	50	67	16, 9, 6, 2	0.24	0.49	Sumner *et al.* (1992)
^{13}C-AA	3	50 ± 8.6	59 ± 1.5	25, 16, BD[b], BD[c]	0.42	0.69	Fennell *et al.* (2005)
Man							
^{13}C-AA	3	34 ± 5.7	86[d]	BD[c], BD[c], 3, 11	–	0.16	Fennell *et al.* (2005)
^{2}H-AA	1				0.1	–	Boettcher *et al.* (2005a)

[a] Structures of the metabolites 1–5, see Fig. 8.6.
[b] Below detection limit in concentrated sample. Signal from GA observed in some samples prior to concentration.
[c] Below detection limit.
[d] 72% of Metabolite 1 and 14% of its S-oxide.

volunteers. Approximately 86% of the urinary metabolites were derived from conjugation with glutathione and excreted as the metabolite AAMA (72%) and its S-oxide (14%). GA, together with glyceramide and low levels of mercapturic acids derived from GA (GAMA and Metabolite 3 in Fig. 8.6) were detected; in all about 13.5% of the total urinary metabolites. Boettcher *et al.* exposed a male volunteer to a single dose of about 1 mg deuterium-substituted AA in drinking water (Boettcher *et al.* 2005a). After two days a total of about 57% of the administered amount was recovered as AAMA and GAMA in the urine. The ratio GAMA/AAMA was approximately 0.1 as compared to reported values of 0.24–0.42 for rats and about 0.5 for mice (Table 8.1).

Table 8.1 summarises data obtained from experimental studies of urinary metabolites after exposure to AA. The conditions for the experiments are not congruent and therefore the data are not fully comparable, e.g. with regard to species. However, it is strongly indicated that a larger fraction of metabolites from GA are obtained in mice compared to both rats and humans.

The first analysis of AAMA as a biomarker of occupational exposure to AA in humans involved hydrolysis of the urine samples, derivatisation with *o*-phthalaldehyde and quantification by high performance liquid chromatography (HPLC) (Calleman *et al.* 1994; Wu *et al.* 1993). The workers examined in the study, in addition to being exposed to AA were exposed to acrylonitrile. AAMA and the major metabolite of acrylonitrile give both *S*-(2-carboxyethyl)cysteine upon hydrolysis – thus the biomarker measured was not specific to AA, but measured exposure to acrylonitrile as well.

Recently, methods have been developed that may allow routine analysis with accurate and specific quantification of AA-derived mercapturic acids (without further derivatisation) in the urine. These methods are based on LC-MS/MS using ^{13}C- or deuterium-substituted internal standards. Li *et al.* (2005) analysed AAMA using LC-MS/MS coupled with an on-line clean-up system. The detection limit for this method was estimated as $< 5\ \mu$g/l urine on-column. Boettcher *et al.* analysed both AAMA and GAMA (Boettcher *et al.* 2005b). The method included a purification and enrichment step by solid-phase extraction before injection into the LC-MS/MS system. The detection limit for both analytes was down to 1.0 μg/l urine depending on the urinary matrix. This detection limit is well below the median concentrations of AAMA and GAMA, respectively, observed in the general population in recent studies (see Section 8.4.2, Table 8.5).

Besides the reactive compounds themselves, or the various products formed by different routes of detoxification, products indicating reaction with the genetic material are excreted in the urine. Monitoring of adducts with guanine-*N*7 and adenine-*N*3 may be used as a non-invasive approach for the determination of nucleic acid damage caused by exposure to a particular carcinogen during previous days (Shuker *et al.* 1992; Wu *et al.* 1993). Farmer *et al.* (2005) have reported a pilot study where mice were administered AA by gavage and modified DNA bases measured in urine. The concentration of urinary *N*7-(2-carbamoyl-2-hydroxyethyl)guanine increased with increasing dose. Adducts were also detected in urine from control animals.

8.3.3 Protein adducts

Measurement of electrophilically reactive compounds/intermediates as their adducts with blood proteins was initiated in the 1970s. Analysis by GC-MS methods, which allows identification and quantitation, was early applied. Along with the advances of instruments for mass spectrometric analysis the developments of methods for protein adduct measurement have reached a stage where they have been applied to a wide range of compounds (see review Törnqvist *et al.* (2002)). The methods are based on specific detachment and analysis of the adduct (the moiety bound to the protein) or analysis of the adduct as modified amino acid or modified peptide after cleavage of peptide bonds. Adducts with cysteine, histidine, N-terminal valine and carboxyl groups in the protein have mostly been measured, often with specific methods for different classes of compounds. It might be considered as an advantage for the identification of an adduct that the analyte contains a 'tag' from the protein, usually the covalently bound amino acid. This is the case in the methods used for measurement of Hb adducts from AA and GA, which initially were analysed as modified cysteine, and now mostly as modified N-terminal valine.

Advantages with Hb and serum albumin for dose monitoring through adduct measurement are that these monitor molecules are accessible in large amounts, have long lifetimes and known rates of turnover, and that there is no removal of adducts by repair. The Hb molecule has a life span, which is the same as that of the erythrocytes, about 120 days in humans, 40 days in the mouse and 60 days in the rat. Serum albumin has no definite life span, but a half-life which is about 20 days in humans, and a few days in the mouse and the rat. Mostly, blood protein adducts are chemically stable over the whole life span of the monitor molecule, although exceptions occur, such as adducts bound as Schiff bases. This means that at chronic exposure a steady-state level of accumulated stable adducts is reached, cf. Fig. 8.3.

Hashimoto and Aldridge showed with ^{14}C-AA that AA adducts are formed with cysteine-*S* in rat Hb (Hashimoto *et al.* 1970). On the basis of this finding, Bailey *et al.* developed and applied a GC-MS method for analysis of AA adducts with cysteine-*S* in Hb from exposed rats (Bailey *et al.* 1986). The method was based on acid hydrolysis of the protein into amino acids, isolation of the cysteine adduct, derivatisation and analysis by GC-MS. Quantitation was done through comparison with deuterium-substituted internal standard. During the acidic conditions in the procedure *S*-(2-carboxyethyl)cysteine is produced from the initially formed adduct, *S*-(2-carbamoylethyl)cysteine.

Using the same analytical approach, the formation of GA as a metabolite of AA was demonstrated through analysis of its adduct with cysteine-*S* in Hb from AA-exposed rats (Calleman *et al.* 1990). In this case *S*-(2-carboxy-2-hydroxyethyl)cysteine is formed from the initial adduct *S*-(2-carbamoyl-2-hydroxyethyl)cysteine (see Fig. 8.5). Measurement of adducts with cysteine, both from AA and GA, has since then been used in several studies where rats have been exposed to AA or GA (see below).

A more facile method for measurement of N-substituted N-termini in Hb by a modified Edman degradation is now applied to AA-exposure. Valine is the N-terminus in all four globin chains in human Hb, as well as in rat and mouse Hb. This method, the N-alkyl Edman method, involves treatment of globin samples with pentafluorophenyl isothiocyanate, detachment of the formed derivatives of the alkylated N-terminal valines from the globin chain (in contrast to unmodified N-termini which remain bound), and isolation of the derivatised adduct through extraction (Törnqvist *et al.* 1986) (Fig. 8.7). The formed fluorinated derivatives give high analytical sensitivity in GC-MS/MS analysis using negative chemical ionisation. Quantitation is performed by use of isotope-substituted internal standards.

In human Hb the reactivities of AA and GA towards N-terminal valine and towards cysteine are of the same order of magnitude (Bergmark *et al.* 1993), in contrast to the rat Hb with the extremely reactive cysteine. Therefore, the facile N-alkyl Edman method was used when Bergmark *et al.* (1993) applied Hb adduct measurement for studies of occupational exposure to AA. An improvement for the GC-MS/MS analysis of GA adducts was later introduced by Paulsson *et al.* (2003b) through acetonisation of the hydrophilic groups in the GA adduct. Analysis by LC-MS/MS of AA- and GA-adducts with N-termini has also been applied after detachment and derivatisation with phenyl isothiocyanate (Fennell *et al.* 2003; Ospina *et al.* 2005). The mass spectrometric methods based on measurement of adducts with N-terminal valine in Hb by the modified Edman procedure are sufficiently sensitive to measure AA and GA adducts from dietary exposure in humans, see Section 8.5.

An alternative method for the monitoring of adducts from AA to serum albumin has been suggested by Noort *et al.* (2003), who published a procedure for AA measurement based on pronase digestion of the protein and nano LC-MS/MS analysis of adducts with cysteine-34 in human serum albumin.

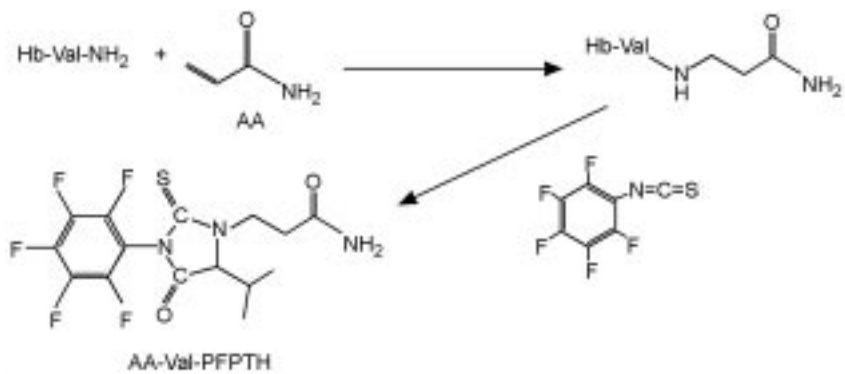

Fig. 8.7 Scheme over the AA alkylation of the N-terminal valine in haemoglobin and the reaction with the Edman reagent leading to the simultaneous detachment of the AA-valine (*N*-(2-carbamoylethyl)valine) from the protein and formation of the pentafluorophenylthiohydantoin, AA-Val-PFPTH.

Relationships between exposure dose and levels of Hb adducts from AA and GA were first studied by cysteine adducts in rats. The initial studies by Bailey *et al.* (1986) indicated a curved relationship between administered dose and AA-adduct level, with increasing slope at higher exposure doses, already at the relatively low exposure doses studied (i.p. injections 0.1–5 mg/kg bw, forming adduct levels up to ca. 0.6 μmol/g Hb).

Bergmark *et al.* (1991) studied the relationships between exposure dose and levels of both AA and GA adducts in Hb in exposed rats. The level of AA adducts (ca. 0.15–8 μmol/g Hb) was approximately linear with administered dose (0.5–100 mg/kg i.p.). The data clearly showed relatively higher GA adduct levels at the lower exposure doses of AA (already observed below 10 mg/kg bw), i.e. at high exposure levels of AA there is a dose rate effect on the metabolism. In an experiment with subchronic treatment it was confirmed that the conversion rate of AA to GA is higher at low administered doses. The GA-adduct level was about five times lower than the AA-adduct level at 3.3 mg/kg and day. Treatment with GA (5–100 mg/kg i.p.) gave a linear increase of the corresponding cysteine-adduct level (ca. 3.2 times lower per administered dose than the AA-Cys adduct levels measured following AA administration). The data obtained was used for estimation of metabolic rates, etc., as illustrated in Section 8.6. It was estimated that about 50% of AA was converted to GA at AA doses of 5 mg/kg in rats.

Barber *et al.* (2001) compared metabolism, toxicokinetics and levels of Hb adducts from AA and GA at subacute (50 mg/kg i.p. per day) and subchronic oral exposure (2.8 mM in drinking water) of AA. They confirmed that subchronic doses with lower daily doses led to higher levels of adducts from GA, i.e. a more efficient conversion of AA to GA. Levels around 8 μmol/g Hb of the AA-Cys adduct and 4 μmol/g Hb of the GA-Cys adduct were measured after 15 days exposure through drinking water. No increase of the adduct level was observed at prolonged exposures.

Crofton *et al.* (1996) measured Hb-adduct levels from AA in a study of the impact of dose rate on neurotoxicity of AA. Acute doses (37.5–150 mg/kg i.p.) and subchronical doses (3.3–30 mg/kg i.p. daily, up to total doses of 900 mg/kg during 90 days) were administered. The level of the AA-Cys adduct increased with exposure doses (up to about 10 μmol/g). The relationship between adduct level and exposure dose deviated from linearity at the highest cumulative doses.

At high exposure doses of AA a very large fraction of the total amount (ca. 30 μmol/g globin) of the highly reactive cysteine$^{125\beta}$ in the β-chains will react with AA and GA, and a very low concentration will remain available for reaction. This condition has certainly influenced the results at the highest exposure doses of AA in the above studies with measurement of cysteine adducts and makes these adduct data less useful for an accurate extrapolation of internal doses of AA and GA to low exposure doses.

The modified Edman degradation method was applied for measurement of AA and GA adducts for the first time in a study of occupational exposure in an AA-production plant in the People's Republic of China (Bergmark *et al.* 1993).

Table 8.2 Levels of Hb-Val adducts from AA and GA in rats, mice, and humans, following different routes of administration and different exposure doses of AA

Species/ strain, sex	Route of adm.	Adm. dose of AA (mg/kg bw)	AA-adduct level (nmol/g per administered dose unit)	GA-adduct level (nmol/g per administered dose unit)	Ratio of adduct levels GA:AA	Reference
Mouse						
CBA, ♂	I.p.	25, 50, 100	9.1, 14, 20	28, 21, 20	3–1	Paulsson *et al.* (2002) (and pers. commun.)
B6C3F1, ♂	Inhalation	2.9 ppm, 24 h	4.0	14	4	Sumner *et al.* (2003)
Rat						
SD, ♂	I.p.	100	26	6.4	0.2	Paulsson *et al.* (2002) (and pers. commun.)
F244, ♂	I.p.	50	21	14	0.7	Sumner *et al.* (2003)
	Inhalation	2.9 ppm, 24 h	16	18	1.1	
	Dermal ($n = 2$)	150	0.8, 2.8	1.2, 4.4	1.3	
F344, ♂	Oral (gavage)	3.0, 50	22, 26	18, 9.9	0.8, 0.4	Fennell *et al.* (2005)
Man						
♂	Dermal	6.0[a]	5.5	3.7	0.7	Fennell *et al.* (2005)
	Oral	0.5, 1.0, 3.0	73, 65, 59	27, 25, 26	0.4	

[a] 2.0 mg/kg bw/d, 3 days.

Different versions of the modified Edman procedure have since then been used for measurement of internal doses of AA and GA in studies of exposed animals and humans. The experimental studies of metabolism of AA to GA, performed through measurement of adducts to the N-terminal valine in Hb are presented in Table 8.2.

Studies of mice treated with relatively high doses of AA by i.p. administration (25–100 mg/kg bw) clearly show a non-linearity in the metabolism to GA (Paulsson *et al.* 2002), in similarity with the above-mentioned studies on rats. The metabolism of AA to GA was compared in mice and rats at an exposure dose of 100 mg/kg bw i.p. by Paulsson *et al.* (2002) and in an inhalation experiment by Sumner *et al.* (2003). At these exposure doses of AA the internal dose of GA per internal dose of AA is 4–5 times higher in mice compared to rats (ratio of adduct levels in Table 8.2). The relation between treatment dose of GA and the GA-Val adduct level was linear both in mice and rats up to the highest treatment doses (ca. 60 and 120 mg/kg bw in mice and rats, respectively) (Paulsson *et al.* 2003a).

Metabolism of AA in humans has been studied experimentally, after exposure to $^{13}C_3$-AA orally or as dermal application, through measurement of Hb adducts to N-termini (see Table 8.2). In parallel experiments rats were administered orally (gavage) an equivalent dose (3 mg/kg bw) of $^{13}C_3$-AA. The comparison between species showed ratios of the levels of the adducts (GA-Val/AA-Val) of 0.4 for humans with oral exposure compared to a ratio of 0.8 in rats.

In conclusion, the data demonstrate that at high doses there is non-linearity in the relationship between administered dose of AA and internal doses of AA and GA, due to saturation kinetics in the metabolism as shown by measurement by cysteine adducts in the rat (already observed below doses of AA of 5 mg/kg) and adducts with N-termini in mice. Furthermore, the Hb-adduct measurements show that a higher internal dose of GA per internal dose of AA is obtained in mice compared to rats, and that humans are more similar to the rat in this respect. The data should be used with some care in quantitative comparisons between species, administration routes and results from different laboratories. It should be considered that most of the experiments have been performed at rather high exposure doses and, also, that intercalibration studies have not been done with regard to the Hb adduct data presented from different laboratories in Table 8.2. Adducts of AA and GA with N-terminal valine in Hb have been analysed in studies in humans with occupational exposure to AA, or with exposure to AA through tobacco smoking or via the diet (see further Section 8.5).

8.3.4 DNA adducts

Detection of DNA adducts is widely used for monitoring of exposure to genotoxic carcinogens. The importance of GA, rather than AA, as a mediator of the genotoxic effects of AA, has been demonstrated in several studies. Therefore, adducts of GA with DNA are commonly considered to be suitable biomarkers of the dose of the genotoxic agent that reaches DNA after exposure to

AA. The direct reaction of AA with DNA is extremely slow as demonstrated by incubation of the compound with DNA *in vitro*. It forms 2-carboxyethyl- and 2-carbamoylethyl-adducts with various reactive sites in the DNA after prolonged incubation (Solomon *et al.* 1985). These adduct structures have not been detected in DNA *in vivo*. In the reaction of GA with DNA *in vitro* three major adducts – *N*7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua), *N*1-(2-carboxy-2-hydroxyethyl)adenosine, (N1-GA-dA), and *N*3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade) have been identified (Gamboa da Costa *et al.* 2003) (Fig. 8.8). Two of these adducts – N7-GA-Gua and N3-GA-Ade – have been detected in experimental animals (Gamboa da Costa *et al.* 2003; Segerbäck *et al.* 1995).

The methods that are currently used for DNA adduct determination include immunoassays, conventional mass spectrometry, and ^{32}P-postlabelling (Farmer *et al.* 2005). Although postlabelling methods have the highest sensitivity, mass spectrometric procedures, particularly tandem mass spectrometry, are more suited for biomonitoring purposes, above all because of the specificity. The method developed by Gamboa da Costa *et al.* (2003) for LC-MS/MS analysis of

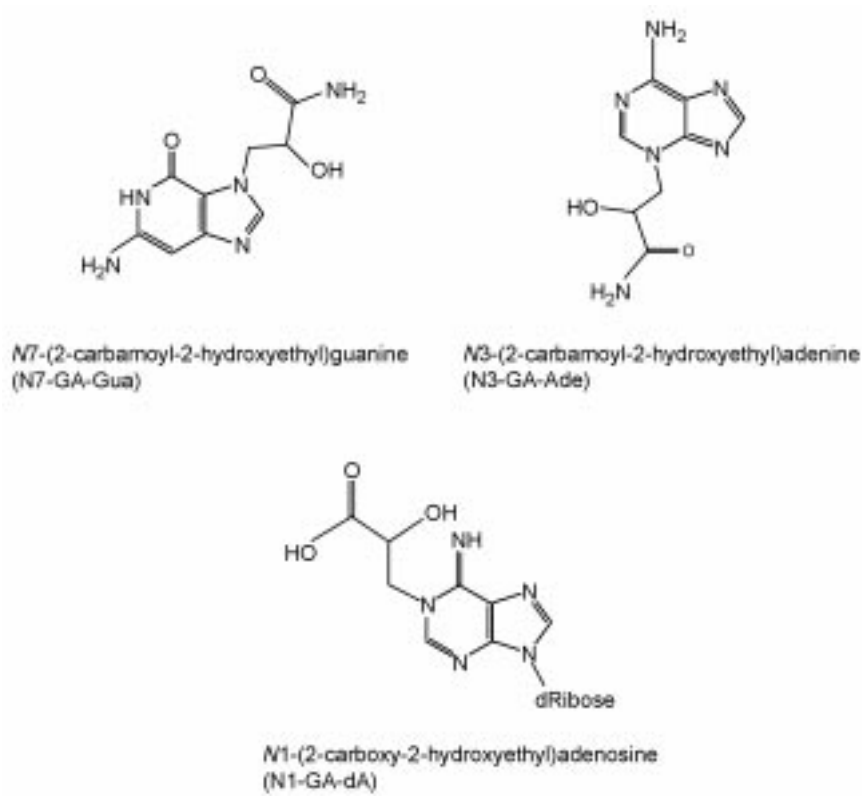

Fig. 8.8 Structures of the three major DNA adducts formed *in vitro* following GA treatment. In treated animals, so far, only N7-GA-Gua and N3-GA-Ade have been found.

adducts of GA with DNA, is based on neutral thermal hydrolysis of DNA, which releases N7-GA-Gua and N3-GA-Ade, followed by addition of ^{15}N-substituted adduct standards, purification and enrichment of the adducts, and, finally, quantification by MS. This method has been used in later studies with minor modification (Doerge *et al.* 2005a,b; Manière *et al.* 2005; Twaddle *et al.* 2004a,b). The limits of detection and quantification are approximately 0.5 and 1 adduct, respectively, per 10^8 normal nucleotides (Doerge *et al.* 2005a).

N7-GA-Gua has been used as a biomarker of internal dose in experimental animals. The DNA adduct levels measured at an early time point after a single exposure to AA, where formation of GA is essentially complete and where the effect of adduct elimination is not yet large, give information on the internal dose of GA in various tissues of the animal. In mice administered AA by i.p. injection, DNA adduct levels in different tissues varied over a rather narrow range (840–2100 N7-GA-Gua in 10^8 nucleotides at 50 mg/kg bw) although the level in leukocytes was significantly lower than in other tissues (Doerge *et al.* 2005a). In rats there was a somewhat larger difference (270–960 in 10^8 nucleotides) between tissues. The dose-response for DNA adduct formation was investigated by Gamboa da Costa *et al.* by treating mice with a single i.p. injection of AA at 0, 1, 10, or 50 mg/kg bw (Gamboa da Costa *et al.* 2003). The levels of N7-GA-Gua and N3-GA-Ade showed similar sub-linear dependency on dose – the levels of N3-GA-Ade being about 100 times lower than those of N7-GA-Gua (cf. Table 8.3).

Untreated mice, used as control animals in the study by Gamboa da Costa *et al.* (2003), consistently showed measurably amounts of N7-GA-Gua. These observations motivated a feeding experiment showing a lower level of adducts in mice fed irradiated laboratory animal diet compared to mice fed autoclaved diet, in agreement with the content of AA in the diets (Twaddle *et al.* 2004a). Data on N7-GA-Gua in liver DNA of AA-treated mice and rats are collected in Table 8.3. The data confirm that formation of GA – resulting in GA-DNA adducts – is more efficient in mice than in rats, and that there is a dose rate effect with a relatively higher efficiency of GA formation at low administered doses of AA.

The half-lives in DNA of N7-GA-Gua and N3-GA-Ade at 37 ºC were shown to be 42 h and 14 h, respectively, *in vitro* (Gamboa da Costa *et al.* 2003), and 50–80 h for N7-GA-Gua and about 20 h for N3-GA-Ade *in vivo* in rats following a single oral dose of AA (Manière *et al.* 2005). Due to the higher reactivity of Gua-*N*7 compared to Ade-*N*3 in combination with the longer half-life of N7-GA-Gua compared to N3-GA-Ade, N7-GA-Gua is preferred for biomonitoring. The steady-state adduct level in female rats administered 1 mg/kg/day AA in drinking water was 150 adducts per 10^8 nucleotides (Doerge *et al.* 2005a). Assuming a similar stability of N7-GA-Gua in rats and humans and about the same *in vivo* dose of GA per exposure dose of AA, the estimated intake of dietary AA in humans, about 0.5 μg/kg/day, would give 0.075 adducts per 10^8 nucleotides. This adduct level is at present below the limit of detection. So far there are no publications on measured DNA adduct levels in AA-exposed humans.

Table 8.3 N7-GA-Gua adduct levels in liver DNA of mice and rats exposed to AA

Species/ strain, sex	Route of exposure	Administered dose (mg/kg bw) or (mg/kg bw/day)	Sampling time (h)	N7-GA-Gua (adducts per 10^8 DNA-P)	N7-GA-Gua (adducts per 10^8 DNA-P per mg AA/kg bw)	Reference
Mouse						
$B6C3F_1$	Gavage	50	8	1200	24	Twaddle *et al.* (2004b)
$B6C3F_1$, ♂, ♀	I.p.	50	6	1700, 1200	34, 24	Doerge *et al.* (2005a)
C3H/HeNMVT, ♂	I.p.	1	6	67 ± 12	60[a]	Gamboa da Costa *et al.*
		10		500	50	(2003) (see also Twaddle
		50		1600	32	*et al.* 2004a)
$B6C3F_1$	Oral	1	14 days	380[b]	68[c]	Doerge *et al.* (2005a)
	Gavage	0.1	8	4.6	46	
Rat						
Sprague Dawley, ♂	Gavage	18	5, 24	250, 300	14, 17	Manière *et al.* (2005)
		54	5, 24	330, 650	6.1, 12	
F 344, ♀	Oral	1	14 days	150[b]	25[d]	Doerge *et al.* (2005a)

[a] Corrected for background from diet.
[b] Steady-state adduct level.
[c] Calculated from the steady-state adduct level observed after about 14 d of exposure and the half-life, 3.9 d, of N7-GA-Gua in liver DNA (380×10^{-8} ln2/3.9 = 68×10^{-8}).
[d] Calculated from the steady-state adduct level observed after about 14 d of exposure and the half-life, 4.2 d, of N7-GA-Gua in liver DNA (150×10^{-8} ln2/4.2 = 25×10^{-8}).

Table 8.4 Examples of genotoxicity studies after AA treatment with measurement of chromosome-related endpoints

Endpoint	Tissues/cells	Administered doses/treatment	Result	References
MN	Mouse peripheral blood (PCE)	1–30 mg AA/kg bw (i.p.)	↑; at ≥ 6 mg/kg bw	Abramsson-Zetterberg (2003)
	Mouse bone marrow	50–125 mg/kg bw	↑	Adler *et al.* (1988)
	Mouse bone marrow, spleen or PCE	≤150 mg/kg bw (i.p.), single or repeated	↑	[a]
	Mouse bone marrow	75 mg AA/kg bw (gavage) x1 or 2	↔	[a]
	Mouse PCE	0.35–1.4 mmol AA/kg bw (i.p.)	↑	[a]
	Rat bone marrow	1.4 mmol AA/kg bw (i.p.)	↔	Paulsson *et al.* (2002)
	Mouse PCE from wild-type and Cyp2E1-null mice	25, 50 mg AA/kg bw (i.p.) per day; 5 days	↑; in wild-type mice	Ghanayem *et al.* (2005)
CA	Mouse bone marrow	100 mg AA/kg bw (i.p.)	↑	[a]
	Mouse bone marrow	50–150 mg AA/kg bw (i.p.) x1	↑	Adler *et al.* 1988
	Mouse bone marrow	500 ppm (~60 mg/kg bw/day) AA in the diet, for 1, 2 or 3 weeks	↑	[a]
SCE	Mouse bone marrow	500 ppm (~60 mg/kg bw/day) AA for 1, 2 or 3 weeks	↑	[a]

[a] Reviewed in the NTP-CERHR Expert Panel Report, June 2004 (NTP-CERHR Expert Panel 2004).
↑ Statistically significant increase in response to treatment.
↔ No significant effect of treatment.

8.3.5 Biological endpoints in biomonitoring

Certain chromosome related endpoints have been suggested for use as biomarkers for genotoxic effects (Aitio 1999; Watson *et al.* 2004). For instance micronuclei (MN), chromosome aberrations (CA) and sister chromatid exchange (SCE) have been used in genotoxicity studies of AA and/or GA *in vivo* (see Table 8.4). MN assays for studies of AA have mainly been used in rodents. Already in the 1980s AA was shown to induce MN in mice (see Table 8.4). Induction of MN has been studied in bone marrow and peripheral blood in mice and rats (in erythrocytes, splenocytes, spermatides). Dose-effect relationships following AA and GA treatment have been demonstrated in mice. In rats MN induction was not observed following exposure to AA. In a study of mice where the induction of MN was studied in relation to the *in vivo* doses (measured as Hb adducts) after exposure to AA or GA, it was concluded that the MN-inducing agent in AA exposure is GA (Paulsson *et al.* 2003a). Treatment with AA has been shown to give a significant induction of CA in mice (bone marrow, lymphocytes, splenocytes, germ cells and embryos). SCE has been studied in mouse bone marrow and spermatids after AA treatment (see Table 8.4).

8.4 Application of biomonitoring in human exposure situations

8.4.1 Acrylamide and glycidamide in body fluids

There are only a few studies reporting measurements of free AA or GA in humans. AA has been detected in the blood of workers exposed to AA (Calleman *et al.* 1994). GA was present in low amounts in the urine of volunteers administered a single dose of ^{13}C-AA in water (Fennell *et al.* 2005). Further, AA has been found in breast milk and urine samples after ingestion of AA-rich food (Sörgel *et al.* 2002). The AA concentrations in breast milk were in the range 5–20 ng/mL in two mothers 3–8 hours after consumption of about 1 mg AA in potato chips. The concentrations before the intake were below the detection limit (5 ng/ml). Nine subjects, who consumed up to 500 g of potato chips or crisp bread, participated in a study of AA in urine. The amount of AA excreted within 8 h was small – not detectable in one subject and in the range 0.5–5 μg in the others. Within a study of the AA content in baby food, Fohgelberg *et al.* (2005) analysed 19 breast milk samples. In all samples except one the AA level was below the limit of quantitation (0.5 ng/ml).

8.4.2 Urinary metabolites

LC-MS/MS methods for specific analysis of the urinary metabolites AAMA and GAMA have been applied for monitoring exposure to AA in individuals of the general population (cf. Fig. 8.6) (Table 8.5). The results confirm that cigarette smoking is an important source of AA exposure, the concentrations being about 2–4 times higher in the smokers than in the non-smokers (Boettcher *et al.* 2005b;

Table 8.5 Concentrations of urinary metabolites from AA and GA, measured in subjects of the general population (cf. Fig. 8.5)

Non-smokers/smokers	N	AAMA	GAMA	Reference
Non-smokers	33	76 ± 30 μg/g creatinine	–	Li *et al.* (2005)
Smokers	37	135 ± 88 μg/g creatinine	–	
Non-smokers	16	29 μg/l (median)	5 μg/l (median)	Boettcher *et al.* (2005b)
Smokers	13	127 μg/l (median)	19 μg/l (median)	
Non-smokers	5	29 μg/l (median)	17 μg/l (median)	Bjellaas *et al.* (2005)
Smokers	1	337 μg/l	111 μg/l	

Li *et al.* 2005). Boettcher *et al.* (2005b) used single spot urine samples and found that the ratio GAMA/AAMA varied largely. Bjellaas *et al.* (2005) found that the metabolite ratio for each individual varied over time within the 48-hour duration of their study. The differences in the metabolite ratio may in part be due to differences in the metabolism of AA between individuals and in part be related to the timing of sampling.

Bjellaas *et al.* (2005) showed that fasting during one day caused about a 50% decrease in the total level of mercapturic acids. After one day on normal diet, the metabolite concentrations increased to prefasting levels. This is in agreement with the proposition that the diet is a major source of exposure to AA. Mercapturic acids have also been analysed by HPLC methods (after derivatisation) for surveillance of occupational exposure to AA (cf. Sections 8.3.2 and 8.5.1).

8.4.3 Haemoglobin adducts

The modified Edman degradation method for measurement of adducts with N-terminal valine in Hb has been applied for surveillance of occupational exposure to AA in several investigations (Table 8.6). Levels of the AA-Val adduct in the range 1–35 nmol/g have been measured in highly exposed workers. In studies of occupational exposure Bergmark demonstrated an increased level of the AA-Val adduct in Hb from the smoking control persons, as well as the occurrence of a signal in the MS analysis corresponding to the AA-Val adduct in samples from the non-smoking controls (Bergmark 1997). It has been estimated that smoking of ten cigarettes a day contributes to an increase of roughly 6 pmol/g Hb to the steady-state level of the AA-Val adduct in Hb (Bergmark 1997; Schettgen *et al.* 2004). A background level of AA adducts was also earlier observed in the studies of Cys adducts. However, at this stage of the work it was unclear whether

Table 8.6 Levels of Hb-Val adducts from AA, measured in blood from AA-exposed workers in different exposure situations

Type of occupational exposure	Number of workers	Range of adduct level (nmol/g globin)	Reference
AA production	41	0.3–34	Bergmark *et al.* (1993)
	11	0.07–1.8	Licea Peréz *et al.* (1999)
Grouting in tunnel work	210	0.02–4.3 (18[a])	Hagmar *et al.* (2001)
	23[b]	0.03–0.89	Kjuus *et al.* (2002)
Sealing work	1	23	Paulsson *et al.* (2006)
Laboratory work	15	0.02–0.12	Bergmark (1997)
Manufacturing and laboratory work	60	ca 0.01–1	Jones *et al.* (2006)

[a] Extreme value.
[b] Blood sampling 63–143 days after discontinuation of grouting work.

this corresponded to a true adduct level or a methodological artifact (Bailey *et al.* 1986; Bergmark *et al.* 1991).

Indeed, it was the repeated observation of a background level of suspected AA adducts in non-smoking control persons in studies of occupational AA exposure that initiated studies of background exposure to AA (Table 8.7). These observations, together with very low levels of the corresponding adduct in Hb from free-living animals (Törnqvist *et al.*, unpublished), initiated the studies of heated food as an unknown source of a background exposure to AA (Tareke *et al.* 2000; Törnqvist 2005). This was also motivated by the relatively high intake of AA calculated from this observed background Hb adduct level (see Section 8.6). Among the reports on the background level of the AA-Val adduct in Hb, a study including 70 non-smokers stratified for dietary intake, comprises the largest group of persons studied so far (Hagmar *et al.* 2005). In the group of non-smokers with high and low dietary intake of AA the adduct levels varied with a factor of 5. In the subgroup of randomly selected non-smoking persons the median AA-Val adduct level was about 30 pmol/g Hb (see Table 8.7).

8.4.4 DNA adducts

To our knowledge, there are no published reports on measurement in humans of DNA adducts from AA exposure. The adduct with guanine-*N*7, N7-GA-Gua, could possibly be used as a biomarker for monitoring of recent high exposures to AA, such as AA in certain work environments. Monitoring of dietary exposure would require analytical methods with an improved sensitivity for the detection of N7-GA-Gua (considering the low amounts of DNA available from humans for biomonitoring). For determination of doses to DNA over longer periods of time there is a need for a more stable DNA adduct biomarker.

Table 8.7 Background levels of Hb-Val adducts from AA measured in blood from non-smoking subjects of the general population

Adduct level (pmol/g globin)	Range (pmol/g globin)	N	Reference
31 (mean)	24–49	8	Bergmark (1997)
~ 40 (mean)	20–70	18	Hagmar *et al.* (2001)
33 (mean)	20–47	6	Kjuus *et al.* (2002)
21 (median)	20–47	25	Schettgen *et al.* (2003)
27 (mean)	20–35	5	Paulsson *et al.* (2003b)
18 (median)	7–31	13	Schettgen *et al.* (2004)
31[a] (median)	20–100[b]	70	Hagmar *et al.* (2005)

[a] A subgroup of non-smoking randomly selected subjects ($n = 20$).
[b] All non-smokers, incl. subgroups with different dietary AA intake ($n = 70$).

8.4.5 Biological endpoints

Kjuus *et al.* (2005) performed a study of chromosome aberrations in tunnel workers exposed to AA and *N*-methylolacrylamide. No increase in chromosome breaks or aberrations was observed for 25 exposed workers compared to 25 unexposed workers. However, an increased frequency of chromatid gaps was found, which the authors interpreted as an indication of a slight genotoxic effect related to exposure to AA and N-methylolacrylamide. Other reports on genotoxic endpoints in humans exposed to AA have not been found.

In a few studies the relation between neurological effects and occupational AA exposure, measured by biomarkers has been studied. In two of these studies dose-response relationships between peripheral nervous symptoms and levels of Hb adducts to N-terminal valine were observed (Calleman *et al.* 1994; Hagmar *et al.* 2001). In the study by Calleman *et al.* of AA-exposed workers in China it was found that Hb adducts were better correlated to the neurological effects than either free AA in plasma or mercapturic acids in urine, most probably due to the fact that Hb adducts reflect the *in vivo* doses of AA during a four-month period.

8.5 Comparison with other methods for exposure assessment

8.5.1 Measurement of airborne acrylamide

Surveillance of chemical contamination in occupational environments is often performed by measurements of air concentration. The majority of countries within the EU have adopted an occupational exposure limit (OEL) value for AA in air of 0.3 mg/m^3 (as an eight-hour time-weighted average, TWA) (EC 2002). Airborne AA is measured after direct sampling onto filters and/or glass tubes filled with absorption material, followed by desorption and analysis by HPLC with UV- or MS-detection (OSHA 1991). In the risk assessment report of AA

from the European Commission (EC 2002) studies concerning measurement/estimations of airborne AA in different occupational settings are reviewed. When exposure data is limited exposure assessments had been performed using predictive models.

Uptake of AA in occupational environments occurs by inhalation but also by ingestion and via dermal uptake. In the case of occupational exposure dermal uptake may occur via surfaces that are contaminated by splashes or condensed vapour or as direct contact with the skin. Dermal exposure is hard to estimate in general, and so also for AA. A disadvantage with exposure assessment by air measurements is that it reflects only exposure via inhalation. Exposure via other routes cannot be estimated and the effect of any protective equipment that might be in use cannot be taken into account. In this section studies where measurement of airborne AA has been performed simultaneously with measurement of biomarkers are discussed.

Jones *et al.* (2006) performed an occupational hygiene survey at a site with potential exposure to AA-monomer and -polymers. The individual airborne exposure was monitored with 2–13 personal full shift air samples. Air was pumped through glass tubes filled with silica gel (with a pre-filter in the case where solid AA was in use). The collected AA was desorbed and analysed by HPLC/UV. All samples showed concentrations below the UK maximum exposure limit (MEL, 0.3 mg/m^3) and the mean exposure was about 0.03 mg/m^3. Two blood samples were collected from each worker, before and after the air monitoring period, approximately three months apart. The Hb-adduct levels were up to ca. 0.1 nmol/g globin. Personal mean airborne AA levels and mean AA-Val adduct levels were well correlated and the calculated linear correlation showed that an exposure at 0.3 mg/m^3 would be expected to give rise to an adduct level of 1.55 nmol/g globin.

Bull *et al.* (2005) compared AA in air (8-h TWA) with *S*-carbamoyl-cysteine (CEC) in urine samples from workers with potential AA exposure (AA in solutions). Air concentrations (cf. method above) were below 0.03 mg/m^3. CEC, measured as mmol/mol creatinine, according to Wu *et al.* (1993), was found in urine from both workers and controls. The levels in more than 43% of the samples were below the detection limit for the method (~1 mmol/mol creatinine). Exposed workers showed only slightly higher mean values than the controls. At low levels of AA exposure, smoking made a significant contribution to the CEC levels. However, a correlation between mean urinary CEC and mean airborne AA was found and a value of 4 mmol/mol creatinine (1 mol creatinine = 113 g) was proposed as a pragmatic biological monitoring guidance value.

8.5.2 Measurement of dietary acrylamide

The finding that high concentrations of AA are formed in certain foods caused worldwide concern. Soon after the discovery several estimations of the average dietary AA exposure in the general population were published. These estima-

tions were mainly based on data of AA in foods from domestic analytical laboratories and exposure calculations from national food surveys, where individual dietary intakes are based on records of current consumption over one or more days or recollections of consumptions over the previous day or to usual intake (food frequency). Based on these studies the daily AA intake in adults was estimated to about 0.4 μg/kg bw and several studies showed higher intake levels (2–3 times higher) for children and adolescents (Dybing *et al.* 2005). The higher intake in young people was not confirmed in a comprehensive study by Hilbig *et al.* (2004) where dietary records from German infants, children and adolescents were evaluated to estimate dietary intakes of six food groups relevant for AA exposure.

Boon *et al.* (2005) calculated dietary exposure to AA in the Dutch population and young children. They used AA levels of different food groups from the IRMM/JRC database with data from several countries (IRMM 2005) and the Dutch National Food Consumption Survey of 1997/98. The median daily intake of AA estimated for a representative sample of Dutch population (1–97 years) was 0.5 μg/kg bw/day, and for children (1–6 years) 1.1 μg/kg bw/day.

The use of Hb adducts for the estimation of the dietary intake of AA and the variation between individuals is being studied, e.g. by Hagmar *et al.* (2005). In the study of 70 non-smokers and 72 smokers stratified for dietary habits, significantly higher levels of the median AA-Val adducts in the non-smoking males and in the smoking women with high dietary intake of AA, compared to the reference groups with low dietary intake of AA, were observed. There were large overlaps in adduct levels between the groups, and a variation among non-smokers with a factor of five (cf. Section 8.4.3). The daily dietary AA intake has been estimated to be about 80 μg, i.e. ca 1.1 μg/kg bw/day, on the basis of an average background AA-Val adduct level of ca. 30 pmol/g Hb (Törnqvist *et al.* 1998; cf. Section 8.6). This means that this initial preliminary estimation of the uptake on the basis of average Hb-adduct levels is compatible with the later estimations of intake of dietary AA from consumption patterns and AA analysis of food.

8.6 Usefulness of biomarkers in risk assessment

In a development of methods for health risk estimation of genotoxic and cancer-risk increasing agents, measurement of protein adducts was introduced as a tool for the determination of the *in vivo* dose (internal dose) of the causative electrophilic compounds (Ehrenberg *et al.* 1974, 1996; Osterman-Golkar *et al.* 1976). It was shown earlier that the relative mutagenic potency for mono-functional alkylating agents could be compared on the basis of the *in vivo* dose (Osterman-Golkar *et al.* 1970; Turtóczky *et al.* 1969), and hence cancer risk estimation should be based on the *in vivo* dose. The dose was defined as the concentration of the compound integrated over time, i.e. the 'Area Under the concentration Curve (AUC)' (Ehrenberg *et al.* 1983) (Fig. 8.9). Furthermore, the

The internal dose (or *in vivo* dose), D [molar × hour, Mh], of an electrophilic compound (RX), is defined as the time integral of the concentration, C:

$$D = \int_t C(t)dt$$

D is calculated from the Hb adduct level, [RY] [mol (g globin)$^{-1}$], from knowledge on the reaction rate for the formation of the Hb adduct, k_y [L × (g globin)$^{-1}$ × h^{-1}], and the lifetime of the erythrocytes, t_{er}, according to:

$$D = (1/k_y) \times [RY] \qquad \text{acute exposure}$$
$$D = (1/k_y) \times [RY] \times (2/t_{er}) \qquad \text{chronic exposure}$$

where D is the dose per day when t_{er} is given in days.
The *in vivo* dose could also be expressed as:

$$D = C_0/\lambda$$

where C_0 is the acutely absorbed amount (mol/kg bw), and λ (1/h) is the first order rate constant for elimination of the electrophilic compound RX (assumption 1 l ≈ 1 kg).

Fig. 8.9 Interrelationship between dose and Hb adduct level, and between dose, uptake and rate of elimination.

dose concept could be applied for estimation of metabolic rates and calculation of uptakes.

Figure 8.9 shows the principle for the calculation of internal dose (strictly the dose in the erythrocytes) from a measured adduct level, formed through reaction with an electrophile (RX) at a specific site in Hb. For the inference to *in vivo* dose from adduct measurements the kinetics of the formation and disappearance of the adducts have to be considered. As shown in Fig. 8.9, the formation of adducts with a monitor protein is determined by the rate of the reaction, i.e. $k_{Y,}$ between the compound and the nucleophilic site(s) (denoted by Y) in the protein. The disappearance of a chemically stable adduct will be determined by the life span of the protein, which for human Hb is equal to the lifetime of the erythrocytes, t_{er} (126 days in humans). In the calculation of the internal dose from a steady-state adduct level in a chronic exposure, t_{er} has to be considered (as well as when the Hb-adduct levels measured a certain time after exposure, or after intermittent exposure, are used for calculations) (cf. Granath *et al.* (1992)). Furthermore, the dose concept is useful for the estimation of the *in vivo* elimination rate (λ) of an electrophile, which could be calculated from the dose when the uptake or absorbed amount (C_0), is known.

Other biomarkers than protein adducts do not allow calculation of *in vivo* dose in humans, with a possible exception of DNA adducts. However, DNA adducts from AA exposure are so far not measured in humans. The short-lived biomarkers, however, could be advantageous in intervention studies in humans

or for measurements of relative exposures, and be used in studies with exposure of animals (cf. Figs 8.2 and 8.3).

A model for cancer risk estimation based on the *in vivo* dose and the relative genotoxic potency has been formulated on the basis of experience of cancer risk models for ionising radiation (Ehrenberg *et al.* 1996; Granath *et al.* 1999; Törnqvist *et al.* 2001). This risk model has been shown to be applicable to cancer test data for ethylene oxide (Granath *et al.* 1999) and this compound has been extensively studied, e.g. with regard to the parameter values according to Fig. 8.9, see, e.g. Ehrenberg *et al.* (1995). In the same way, the cancer risk model has also initially been shown to be adaptable to cancer test data for AA in experimental animals (Granath *et al.* 1999). Most probably GA is the cancer risk-increasing agent in AA exposure (reviewed by Rice (2005)), and therefore internal doses of GA, as well as of AA, are essential for cancer risk estimation.

The parameter values for AA and GA have been studied in humans with occupational exposure to AA, and a value of $0.15\,h^{-1}$ for the elimination (λ) of AA in humans (corresponding to a half-life ($\ln 2/\lambda$) of about 4.6 h) was obtained (Calleman 1996). Using this value, the average background steady-state level of about 30 pmol/g Hb of the AA-adduct in Hb from non-smokers, would correspond to an intake of about 80 μg AA per day (cf. Table 8.7) (see Törnqvist *et al.* (1998)). In this calculation it is assumed that AA is approximately evenly distributed in the tissues, which is supported by the data on biomarkers in Sections 8.3.1 and 8.3.4.

From the studies summarised in this chapter one could conclude that the internal dose (AUC) of GA, associated with a certain uptake of AA in humans, is lower than in the mouse and probably not very different from that observed in the rat. The parameter values for AA and GA are now re-evaluated to obtain a refinement of the calculations at the low exposure levels of AA from dietary intake.

8.7 Future trends

The available chemical biomarkers are already applied for the improvement of the health risk assessment of dietary intake of AA, and the analytical methods are developed towards higher sensitivity and/or faster and cheaper analysis. The comparison of analytical results from different laboratories would gain from inter-calibration tests. A breakthrough for new chemical biomarkers for studies of exposure or internal dose is not expected; however, with regard to genotoxic and neurotoxic effects it is possible that new sensitive biomarkers for AA will be developed and applied for biomonitoring.

It is of importance to quantify the *in vivo* dose of GA and the relation to the intake of AA, the average in different populations and subgroups of the population, and inter-individual differences (e.g., due to dietary habits or genotype). The studies so far published, however, do not indicate very large inter-individual

differences in the internal doses of AA, or that differences in detoxifying enzymes would contribute to large variations. Furthermore, it is essential, by the use of biomarkers, to differentiate between effects from AA and from GA in studies of AA exposure. The biological response to be used for extrapolation of risks from animal tests should be determined in relation to the internal doses in the test species. The extrapolation of risks to the low levels of human AA exposure would be facilitated by studies of the relation between exposure and internal dose, through Hb adduct measurement, e.g. according to Fig. 8.9. Measurement of short-time biomarkers could contribute to the determination of metabolic rates and pharmacokinetic parameters.

In conclusion, there are several methods for measurement of chemical biomarkers with sufficient sensitivity to be used to clarify exposure and health risks with dietary AA exposure. As illustrated in Section 8.1, Hb adduct measurement could be used to infer the average exposure and internal doses over longer periods and other biomarkers (free compounds in plasma and urine, urine metabolites, DNA adducts) could be used to study the relative exposure over short time periods.

8.8 Acknowledgements

This work was financially supported by the European Commission (FOOD-CT-2003-506820, Heat-generated food toxicants – identification, characterisation and risk minimisation), The Swedish Cancer Society, and The Swedish Cancer and Allergy Fund.

8.9 References

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9

Modelling of dietary exposure to acrylamide

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9.1 Introduction

In this chapter we present an overview of dietary acrylamide (AA) exposure calculations performed. We address the implications of reducing AA levels in foodstuffs due to 'new' processing techniques and discuss exposure modelling in relation to toxicity endpoints. At the end of the chapter some concluding remarks are presented regarding future developments in exposure assessment and integration of exposure and effect modelling aiming at further refinements of risk characterisation of AA.

Three different approaches are described to estimate long-term exposure to AA, a genotoxic and carcinogenic compound (Section 9.2). The approaches include the point estimate approach, the deterministic approach and probabilistic modelling of exposure. The probabilistic approach is presented as the most promising tool due to its capability to address uncertainty in the intake estimations. Since the discovery of AA in 2002, many dietary exposure assessments have been performed worldwide, resulting in a large diversity of intakes (Section 9.3). These differences are partly due to differences in the approach used to calculate long-term exposure. We therefore calculated the long-term exposure to AA for the total Dutch population according to different scenarios showing differences in outcome that may indeed partly explain the variation found between countries, next to differences in eating habits, populations addressed and dietary method used.

An important aspect of research into the exposure to AA is efforts to reduce levels of AA in food (addressed in Section 9.4). Several techniques have been developed, and we show that to assess how promising these new techniques are

in reducing overall AA exposure, what-if scenarios can be formulated. Several relevant scenarios are presented and discussed. It is, however, stressed that relatively little is known on how well experiments performed under laboratory conditions are applicable for daily food processing and how achievable possible adjustments in processing techniques are in terms of costs. Also negative side effects of changes in processing techniques should be considered, such as the increase in fat content at lower frying temperatures or possible loss of 'desired' Maillard products.

AA is a carcinogenic and genotoxic compound. Recently the European Food Safety Authority (EFSA) and the Joint Expert Committee on Food Additives (JECFA) have introduced the Margin of Exposure (MoE) as a tool to advise risk managers when dealing with compounds like AA (Section 9.5). We demonstrate how this concept may work by calculating a distribution of MoE of AA for the total Dutch population and young children aged 1–6 years. We show that the MoE calculated was far below the recommended level of 10,000 by the EFSA indicating that AA may pose a problem for human health in the Netherlands. However, this factor of 10,000 is still under discussion, and consensus about which magnitude is acceptable is therefore lacking.

And finally we briefly address two developments which will give new input to risk assessment procedures to toxic compounds such as AA in the near future (Section 9.6). These developments include the establishment of an electronic platform of consumption and residue level databases connected to probabilistic software and the integration of exposure and effect modelling in an integrated probabilistic risk assessment model. In such a model uncertainties associated with consumption data, concentrations, animal dose-response modelling, and inter and intra species variations can be quantified. This point is very important, because the uncertainties in the input data, which can be substantial, will determine the uncertainty in the corresponding MoE.

The aims of this chapter are: (i) to discuss different existing exposure models, (ii) to give an overview of dietary AA exposure calculations performed, (iii) to address the implications of reducing AA levels in foods due to 'new' processing techniques and (iv) to discuss exposure modelling in relation to toxicity endpoints. We end the chapter with some concluding remarks regarding future developments in exposure assessment and integration of exposure and effect modelling aiming at further refinements of risk characterisation of AA.

9.1.1 Acrylamide exposure levels

In 2002 acrylamide (AA), was found to be present in high concentrations in heat treated food products rich in carbohydrates.[1] AA was classified as a possible human genotoxic compound by the IARC. Since then this compound has been the subject of numerous studies and reviews related to, for example, dietary exposure (e.g., refs 2–5), toxicology/carcinogenicity (e.g., refs 6-10), and formation (e.g., refs 1 and 11–15). Extensive amounts of data on AA levels in foods have been collected since then, which, for Europe, have been brought

together in a European database by the Institute for Reference Materials and Measurements (IRMM) of the European Commission. To date (June 2006) about 7,150 data sets have been incorporated in the database after having passed strict quality checks. These data are derived from nine EU Members States and the European food industry, and cover a broad range of food commodities. The database is freely available on the Internet (http://www.irmm.jrc.be/html/activities/acrylamide/database.htm). Also recently a EU-project, titled 'Heat-generated food toxicants, identification, characterisation and risk minimisation' (acronym: HEATOX), has started with the main aim to combine exposure assessment and toxicology to come to a risk characterisation of the intake of AA (and possible other hazardous compounds).

To assess whether a toxic compound forms a risk for a population a risk assessment is performed. This assessment consists of four steps of which exposure assessment forms one of the key parts.[16] To perform an exposure assessment for toxic compounds present in food (such as AA) and to assess whether a population is at risk, information on different aspects is needed, such as levels of the toxic compound in foods and consumption levels of these foods in a certain population. In the case of AA, also information on processing practices is relevant (e.g. does a person consume potatoes after frying or boiling) and the effect of processing on AA levels. To determine the population at risk, calculated exposure levels are compared with a relevant toxicological reference value.

9.2 Different models to estimate dietary exposure to food contaminants

Currently two types of exposure assessments are performed depending on the toxicity of the compound, short-term and long-term assessments. Short-term exposure assessments relate to the dietary exposure to a toxic compound during a period of 24 hours or less. This type of assessment is relevant for compounds that cause acute toxic effects on ingestion. Long-term exposure on the other hand, relates to exposures over a longer period of time, say a couple of months up to life-long. For AA, which is carcinogenic,[17] a long-term exposure assessment should be performed.

To perform a long-term exposure assessment several approaches are available. A recent overview of this is given by Kroes *et al.*[16] One approach is the point estimate approach, which calculates the dietary intake by multiplying a mean concentration level (or median) with a mean consumption level, and then adds the intakes of the different foods containing the contaminant. Point estimates are easy to calculate and relatively easy to understand. For the consumption levels, the average daily consumption per capita as reported in the GEMS/Food database[18] may be used, which is derived from Food Balance Sheet data (trade statistics). This type of data is available in many countries world-wide. Because of the data availability and its simplicity point

estimates are used world-wide as a first screening step within the field of exposure assessment. However, the limitations of this approach have also been recognised. First of all it may overestimate exposures when deriving the mean consumption level from food balance sheet data. These data do not account for food losses during preparation, and for food given to animals. Furthermore, the point estimate approach does not provide insight into the range of possible exposures that may occur within a population or the main factors influencing the results of the assessment.[16] This method also does not provide an opportunity to quantify the different uncertainties related to an exposure assessment.

A more refined method to estimate long-term exposure, referred to as 'simple distributions',[16] makes use of all food consumption levels reported in a food consumption survey. For this, a distribution of food consumption levels using the whole food consumption database is combined with a mean concentration level.[16] The food consumption surveys from which the consumption data are derived are mostly short-term (two to seven days). For the advantages and disadvantages of different ways of collecting food consumption data and on recommendations on how to harmonise this process, see EFCOSUM report.[19] To assess long-term intake based on short-term food consumption data, the resulting estimations of intake per consumption day using the 'simple distributions' approach should be extrapolated to long-term intake estimations, a third and most sophicticated method to assess long-term exposure. For this several methods have been developed as reviewed by Hoffman *et al.*[20] These authors considered the statistical methods developed at Iowa State University[21–23] to be the best approach. In short these models estimate between-subject variation (relevant level of variation in long-term exposure) by correcting the observed total variation for the daily within-subject variation. For this preferably at least two (non-)consecutive days are required within a food survey.[16] With this type of statistical modelling the variation in the long-term intake within a population can be quantified.

However, the distribution of exposure that is calculated in this way cannot be related to the long-term exposure levels at the level of the individual. In epidemiological studies the aim is to relate the intake of a compound at the individual level with biomarkers of exposure and/or measurable health effects. For AA intake in humans it may become important to relate dietary intake levels with biomarkers of exposure, such as Haemoglobin (Hb)-adducts or DNA-adducts and/or with a possible increase or decrease in cancer incidence. Food frequency questionnaires are the most appropriate tool to measure habitual intake at the individual level, but these types of questionnaires have limitations regarding completeness and accuracy,[24–26] which may hamper linking this type of information to biomarkers or effects measured in epidemiological surveys. The aim of the study will determine which method of collecting food consumption will be used, but in general it is assumed that studies using food record or food recall methods prevail above food frequency questionnaires in terms of accuracy.

Another approach to calculate the exposure to toxic compounds is probabilistic modelling as shown in Fig. 9.1. Probabilistic modelling, which can be

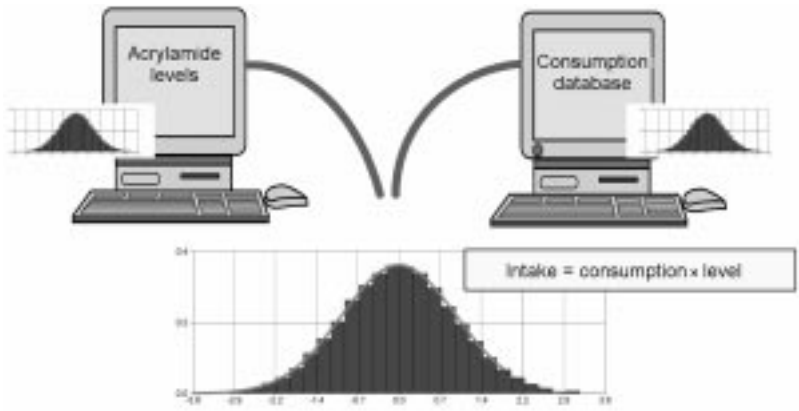

Fig. 9.1 Probabilistic modelling: combining random selections of acrylamide levels from an acrylamide distribution with food consumption levels derived from a distribution of consumption levels resulting in a distribution of acrylamide intake.

integrated into long-term assessments, can account for all the variation in the parameters addressed in an assessment. These models can also address uncertainty. By using what-if scenarios risk managers will be able to make more effective risk management decisions. For AA it may be useful to study the effectiveness of, for example, adjusting food processing techniques, education on preferred household food preparation or selections of cultivars with lower contents of reducing sugars (see also Section 9.3).

9.3 Dietary AA exposure assessments

Since the discovery of AA in heat treated foods, many dietary exposure assessments have been performed. A recent, up-to-date overview of these assessments has been compiled by Dybing *et al.*[5] reproduced in Table 9.1. The results show clearly that intakes differ considerably between countries. During the 64th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in February 2005 AA intake levels were evaluated using national dietary intake data of 17 countries and combining the distribution of consumption with national mean AA occurrence data obtained from national surveys, using the actual reported consumer body weight.[27] Mean AA intake estimates ranged from 0.3 to 2.0 μg/kg bw per day in the general population. For high percentile consumers (P90 to P97.5) intake estimates ranged from 0.6 to 3.5 μg/kg bw per day. Children had AA intakes that were around two to three times those of adult consumers when expressed per kg body weight.[27] JECFA also performed international estimates of AA intake using the point estimate approach. For this, consumption levels derived from the GEMS/Food database were combined with international weighed means of AA levels, assuming a body weight of 60 kg.[27]

Table 9.1 Exposure estimates from 2002–2004 as compiled by Dybing *et al.*[5]

Exposure assessment	Daily intake (μg/kg bw per day) Mean (age group)	95th percentile; *90th percentile	Source
FAO/WHO (2000)	0.3–0.8		http://www.who.int/foodsafety/publications/chem/en/acrylamide_full.pdf
EU, SCF (2002)	0.2–0.4		http://europa.eu.int/comm/food/fs/sc/scf/out131_en.pdf
BfR, Germany (2002)	1.1 (15–18)	3.4	http://www.bfr.bund.de/cm/208/abschaetzung_der_acrylamid_aufnahme_durch_hochbelastete_nahrungsmittel_in_deutschland_studie.pdf
BAG, Switzerland (2002)	0.28 (16–57)		http://www.bag.admin.ch/verbrau/aktuell/d/DDS%20acrylamide%20preliminary%20communication.pdf
AFSSA, France (2002)	0.5 (>15)	1.1	http://www.afssa.fr/ftp/afssa/basedoc/acrylpoint2sansannex.pdf
	1.4 (2–14)	2.9	
FDA (2002)	0.7		http://www.jifsan.umd.edu/presentations/acry2004/acry_2004_dinovihoward_files/frame.htm
FDA (2003)	0.37 (>2)	0.81*	See FDA above
	1.00 (2–5)	2.15*	
SNFA, Sweden (2002)	0.45 (18–74)	1.03	Svensson *et al.*[3]
NFCS, Netherlands	0.48 (1–97)	0.60	Konings *et al.*[2]
	1.04 (1–6)	1.1	
	0.71 (7–18)	0.9	
SNT, Norway (2003)	0.49 (males)	1.01*	Dybing and Sanner[9]
	0.46 (females)	0.86*	
	0.36 (9, boys)	0.72*	
	0.32 (9, girls)	0.61*	
	0.52 (13, boys)	1.35*	
	0.49 (13, girls)	1.2*	
	0.53 (16–30 males)		
	0.50 (16–30 females)		
FDA (2004)	0.43 (>2)		See FDA above
	1.06 (2–5)		

This resulted in international mean intakes ranging from 3.0 up to 4.3 μg/kg bw per day, far higher than the national estimates of intake. These international estimates of exposure were considered very conservative because mean consumption levels were derived from food balance sheets, which are known to overestimate the real consumption at the individual level. Furthermore, consumption levels derived from food balance sheets are the per capita consumption of raw agricultural commodities while the AA levels were from specifically processed foods.[27]

The diversity in AA intake between different countries is largely due to differences in food consumption patterns, cooking traditions and processing techniques. A factor that also explains differences in intake are dissimilarities in the dietary surveys performed. These relate among other things to the dietary assessment method used (food balance sheets, 24-h recall, dietary record method, food frequency questionnaire), the duration of the study (one to seven days), the population addressed and the food coding systems used. Due to these differences it is often difficult to compare intake levels between countries.

To facilitate this comparison and also to be able to merge national databases in a harmonised way to allow for risk assessments involving more than one country or to estimate the intake per country in a standardised and comparable way a harmonised collection of food consumption data is recommended. In two European projects, EFCOSUM[28] and the EPIC project,[29] the 24-h recall method was recommended as the optimal way to collect consumption data at the European level, due to low subject burden and low costs in comparison with the dietary record method. It has also been recognised that combining a 24-h recall with food frequency questionnaires may be useful to cover the intake of contaminants present in infrequently consumed food items.

Disadvantages of the 24-h recall method are, however, that the method depends on memory and that it is usually difficult to perform when addressing food intake of children, while this group is considered to be very important in food safety evaluations. Because of this the dietary record method, in which subjects record all foods and beverages consumed during a specific period (usually one to seven days), may also be a good option, even though the subject's burden will be higher and habitual eating habits may be influenced or changed by the recording process. Apart from harmonising the dietary assessment method, the following aspects also need to be addressed:

- number of days included in the food consumption survey (at least two days, preferably non-consecutive, covering all days of the week)
- population addressed (include the complete population, preferably starting from six months of age)
- food coding.

Differences in national AA intake levels may, apart from differences in culture and the way in which food consumption data are collected, also be due to variations in the way in which long-term exposure levels can be calculated. There are different methods to calculate the exposure (see Section 9.2) which

result in different outcomes. To illustrate this we calculated the exposure to AA in the total Dutch population using AA levels as reported by the IRMM (December 2004 including 3,850 datasets). For this we used data from a Dutch food consumption survey conducted in 1997/1998 in which 6,250 respondents aged 1–97 years recorded during two consecutive days their food consumption, weighing the amounts consumed accurately.[30] To link the Dutch food consumption data to the AA levels, food items analysed were categorised in 14 different food categories. For more details see ref. 31. The exposure calculations were performed with the 'Monte Carlo Risk Assessment' program (MCRA 3.5, available for registered users at the RIKILT website[32]). The following four scenarios were addressed:

1. Scenario 1: daily food consumption patterns randomly linked to a range of AA levels in food categories (= short-term exposure).
2. Scenario 2: daily food consumption patterns linked to mean AA levels in food categories (so one AA level per food category), including statistical modelling for long-term exposure (= long-term exposure).
3. Scenario 3: daily food consumption patterns linked to mean AA levels in food categories, without statistical modelling for long-term exposure (= 'simple distributions').
4. Scenario 4: mean consumption patterns over two consecutive days per respondent linked to mean AA levels in food categories.

For scenario 1, 1,000,000 randomly drawn consumption patterns from the food consumption database were multiplied with randomly selected AA levels per food category. Summing over food categories per consumption pattern resulted in an empirical estimate of the AA intake distribution, representing the daily intake levels that may occur in the Dutch population. To assess the long-term exposure (scenario 2), the 12,500 ($2 \times 6{,}250$) consumption patterns were multiplied with the average AA level per food category, and summed over foods. The resulting set of 12,500 intakes was analysed with statistical methods for usual intake developed at Iowa State University[21–23] (see Section 9.2). This method is implemented in MCRA. For a description of the method see refs 31 and 32. In scenario 3, the same procedure was followed as in scenario 2, with the exception that no statistical methods were applied for usual intake. Scenario 4 is similar to scenario 3 with the exception that the within-person variation in consumption was eliminated by selecting the mean intake over the two reporting days, resulting in a set of 6,250 AA intakes.

The percentages of the population (50%, 95%, 97.5% and 99%) with an intake at or below a certain level are plotted in Fig. 9.2 for each scenario. It is clear that scenario 1 resulted, at the higher percentages (> 95%), in much higher intake levels than the other three scenarios. This can be explained by the fact that individuals can consume a large amount of, e.g. crisps, on an arbitrary day, but are not likely to do this on a daily basis. Also on an arbitrary day individuals may select a food with a high AA level, while in the long run high levels will be levelled out by the selection of foods with lower levels. Scenario 1 is relevant for

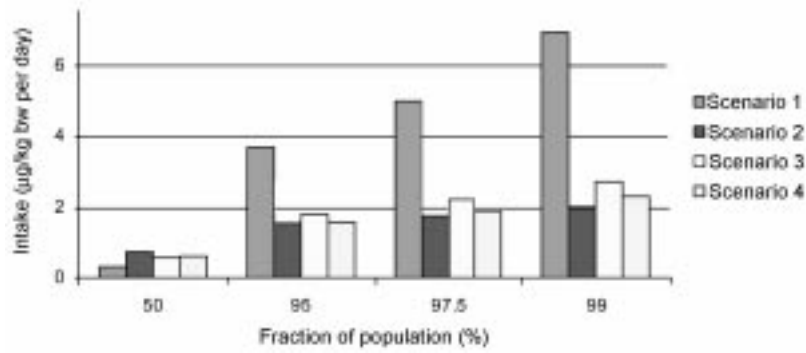

Fig. 9.2 Intake of acrylamide (μg/kg bw per day) as fraction of the total Dutch population (%) following four different scenarios for intake calculations. For details on the scenarios used see text.

compounds that are acutely toxic. Because AA induces toxic effects in the long run, scenario 1 is not suitable to calculate AA intake (see also Section 9.2). Scenarios 2, 3 and 4 resulted in different outcomes at the higher percentiles of the intake distribution. The highest intake levels were calculated with scenario 3 and the lowest with scenario 2. In scenario 3 daily consumption patterns are still included, resulting in higher intakes. In scenario 4 the variation in consumption was levelled out by calculating the mean consumption over two consecutive days, resulting in lower intakes. If the results are expressed as a fraction of scenario 2 (the 'golden standard') all three scenarios underestimated the intake level belonging to a fraction of 50% of the population (60% for scenario 1, 20% for scenario 3 and 15% for scenario 4) and overestimated the P99 with about 250%, 34% and 14%, respectively. It is evident that advanced statistical procedures to model long-term exposure affect the higher percentiles of the intake distribution, and thus the result of the exposure assessment. It should be stressed that this is an example, and that the order of magnitude between the differences of scenario 2, 3 and/or 4 will most likely be different when addressing other compounds, or using other concentrations or food consumption data.

9.4 Reduction of AA levels in foods: implications

Shortly after the discovery of AA an FAO/WHO Consultation was organised[33] covering this subject and the Scientific Committee on Foods expressed their view on the presence of AA in food.[34] Both bodies recognised the severity of the issue and stressed, among others, that possibilities for reducing AA levels in food by changes in formulation, processing and other practices should be investigated. Since then considerable progress has been made in understanding how and from which precursors AA is formed in foods. It is presently clear that asparagine, together with reducing sugars, particularly fructose, is a precursor for the formation of AA in Maillard reactions.[35,36] Numerous studies have since

then been performed studying the effect of different parameters on AA formation in mainly potato products. The parameters studied include heating temperatures,[1,11,12,37–40] duration of heating,[11,37,39,40] sugar content,[11,37,39,41] asparagine content,[11,39,42] pH level,[11,43] addition of additives like citric acid,[43,44] addition of components that bind water,[11,44] and surface-to-volume ratio.[40] These studies have resulted in recommendations of processing to reduce the formation of AA during processing, which were summarised in a report based on a workshop organised by the European Commission Health and Consumer Protection Directorate-General (EU DG SANCO) in 2003.[45] These recommendations include among others that frying temperature for cut potato products should not exceed 175 ºC, and that excessive browning of baked cereal products must be avoided by reducing baking time and/or temperature.

The development of techniques to reduce AA levels in processed foods is not the only option to reduce the AA exposure in a population. Another alternative is the selection of raw materials with low levels of free asparagine and/or reducing sugars such as fructose and glucose. By selecting such materials the precursor present at the lowest level will be the rate-limiting factor in the formation of AA upon processing. A good example of this is potato, the ingredient of two important food groups that world-wide contribute largely to AA intake, French fries and crisps.[5,27] In potato the concentration of reducing sugars strongly determines AA formation on processing.[46] Selecting cultivars with low levels of reducing sugars the AA content could substantially be reduced in foods derived from potato. Also storage of potatoes at moderate temperatures (not below 8–10 ºC due to increase in sugar content at lower temperatures[47]) is important to reduce sugar levels in potatoes. However, reduction of reducing sugar content in potatoes should not be at the cost of the quality (flavour and crispiness) of the processed product. Keeping this in mind Biedermann-Brem *et al.*[41] concluded that potatoes used for roasting and frying should contain levels of reducing sugar between 0.2 and <1 g/kg fresh weight to lower AA levels while preserving the quality of the final processed food product. Another promising approach is the degradation of asparagine using enzyme asparaginase, applicable to potato crisps (AA reduction of 97%) and French fries (AA reduction of 80%), retaining acceptable flavour and colour of the product.[42]

To assess how promising new developments to reduce AA levels in foods will eventually affect the overall AA exposure, probabilistic modelling of exposure in combination with what-if scenario studies is a useful tool. Relevant what-if scenarios related to the quantification of reduction in overall AA exposure are what-if

- the potato processing industry lowers frying temperatures
- the potato processing industry selects potato varieties containing less reducing sugar
- the food industry and/or restaurants apply new or altered processing practices, but the consumer will hardly follow these recommendations in home processing

- only well educated people will follow up the advice of health education boards or food standard agencies relating to new or altered processing practices, and less educated people will not.

What-if scenarios will also be helpful to assess the effect of several mitigation options within food processing. Figure 9.3 pictures several factors in relation to possible reduction strategies in lowering AA exposure via the consumption of crisps. With long-term exposure modelling (scenario 2) the contribution of the separate factors can be quantified and may thus be helpful to prioritise research areas that are most worthwhile to pursue.

Examples of what-if scenario's are given by Boon *et al.*[31] describing the effect of reductions in AA levels on overall intake using adjusted or new processing techniques in comparison with the 'old processing method'. Boon *et al.*[31] studied the effect of reducing frying temperatures of 'potato products, fried' with 10 ºC (35% reduction in AA levels), using a different baking agent to prepare 'gingerbread' (60% reduction in AA levels) or both on the total AA exposure in the Dutch population. Reducing frying temperatures had the largest reducing effect on overall AA exposure, even though the reduction in AA levels due to this was only 35% compared to 60% for levels in 'gingerbread'. The reason for this was that 'potato products, fried' contributed far more to the total AA exposure (about 30%) than 'gingerbread' (about 7%), due to a higher consumption level and somewhat higher AA levels found in 'potato products, fried'. It was concluded that the overall effect on AA exposure of lowering AA levels will depend on the amount and/or frequency in which a food is consumed, the levels present in the food and the influence of processing itself. The largest effect of AA level reduction on the intake is to be expected for those foods that contribute largely to AA exposure in a population due to high levels of consumption and/or high levels of AA present.[31] These examples demonstrate the possibilities, but it should be emphasised that relatively little is known about how well these experiments performed under laboratory conditions, are

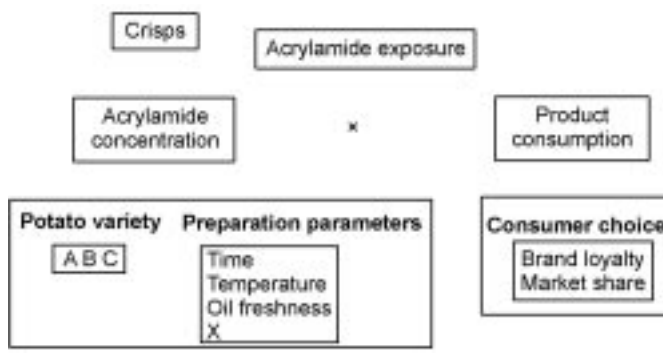

Fig. 9.3 Several factors that influence the intake of acrylamide via the consumption of crisps.

applicable for daily food processing and how achievable possible adjustments are in terms of costs. Also negative side-effects of changes in processing techniques should be considered, such as the increase in fat content at lower frying temperatures or possible loss of 'desired' Maillard products. The CIAA (Confederation of the Food and Drink Industries of the EU) has developed a 'toolbox' approach, which provides a way to help food processors to identify approaches to help control AA in different types of products. The concept highlights the need for specific considerations on natural parameters for crops and ingredients (e.g., agronomic, biological, chemical), product composition, process conditions and finished product characteristics. This 'toolbox' approach was presented during a 'Meeting of Experts and Stakeholders on AA in Food' organised by the European Commission on 14 January 2005.

9.5 Exposure to AA in relation to reported toxicity

The relevant toxicological effect of AA is genotoxic carcinogenicity.[33] Animal studies have clearly shown that AA causes cancer (recently reviewed in ref. 17). However, it is not clear whether this is also true in humans at lower exposure levels via food. For example, epidemiological studies of possible negative health effects due to AA exposure have been inconclusive (recently reviewed in ref. 17). To assess the risk of compounds with genotoxic and carcinogenetic properties data from high-dose animal bioassays are used, requiring the extrapolation to the low levels to which humans are generally exposed.[48] However, the different models used for extrapolation are much debated. In an Opinion of the EFSA on a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic,[49] the two main objections against this type of modelling were:

1. It is rarely known, for a particular compound, whether a model actually reflects the underlying processes.
2. The numerical estimate of risk obtained is critically dependent on which model is used and is very little influenced by the actual data; this can result in estimates of risk for the same compound varying by several orders of magnitude, depending on the model selected.

Because of these drawbacks, which were supported by the JECFA, both regulatory bodies recommend the use of the margin of exposure (MoE) as a tool to advise risk managers when dealing with compounds that are both genotoxic and carcinogenic.[27,49] The MoE is the ratio between a defined point on the dose-response curve for the adverse effect and the human intake and thus quantifies the distance between the level at which a 'measurable effect' occurs and the intake. A small MoE represents a higher risk than a larger MoE. The MoE can thus be used to prioritise different contaminants.[27,49]

Both JECFA[27] and EFSA[49] recommend the use of the benchmark dose (BMD), i.e. 10% tumour incidence above control, as the defined point on the

dose-response curve and to take the lower bound one-sided confidence limit (95%) of the BMD (BMDL) as a reference point of comparison with actual human intake data. The EFSA argues that this lower level will be close to the lowest point that can be measured in most animal studies and would normally require little or no extrapolation outside the observed experimental data.[49] During the JECFA meeting in Rome in February 2005, the Committee derived a lowest range of BMDLs for induction of mammary tumours at AA dose levels of 0.30–0.46 mg/kg bw per day. These analyses were based on available animal studies which were fitted for dose-response modelling using eight different statistical models. The Committee decided to use the lower end of the range of values (0.30 mg/kg bw per day) for comparison with human intake levels of AA.

To illustrate the concept of MoE we calculated the long-term AA intake for the total Dutch population (1–97 years; $n = 6,250$) and for young children (1–6 years; $n = 530$). For this we used data from the Dutch food consumption survey, mentioned earlier (see Section 9.3). The calculations were performed with MCRA following scenario 2 (see Section 9.3), the most appropriate exposure scenario for AA. To calculate the MoE distribution for both age groups, different percentiles of the exposure distribution (50th, 95th, 97.5th and 99th) were related to the BMDL of 0.30 mg/kg bw per day. We also calculated the contribution of the different food groups to the total AA exposure.

In Fig. 9.4 we plotted the distribution of the MoE for both age groups as function of the fraction of the population (50%, 95%, 97.5% and 99%) with a MoE at or above a certain level. It is clear that children had an overall lower MoE than the total population, due to higher exposure levels per kg body weight. For the total Dutch population, 50% had a MoE of 600 or more. For young children this level equalled 273 or more. At 97.5% the corresponding MoE was reduced to 200 and 136, respectively. The JECFA arrived at a MoE of 300 for an average intake of 0.001 mg/kg bw per day using the same BMDL level and at a MoE of 75 for a high percentile (97.5%) intake level (0.004 mg/kg bw per day).

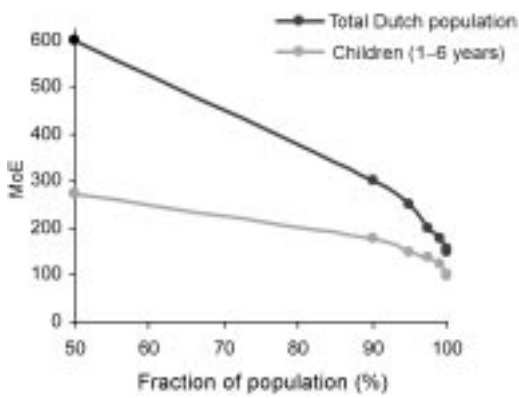

Fig. 9.4 The distribution of the margin of exposure (MoE) for the total Dutch population and young children (1–6 years) as a fraction of the population.

These MoEs were judged to be low for a compound that is genotoxic and carcinogenic and JECFA therefore indicated that there may be a human health concern. At the moment there are no rules for a sufficient magnitude of a MoE to signify low priority of risk management. For genotoxic and carcinogenic compounds, such as AA, the EFSA has proposed in an Opinion that a MoE of 10,000 or higher may indicate low health risk.[49] Using this magnitude we can state on the basis of our results that AA poses a problem for human health in the Netherlands. However, this document is still under discussion, and consensus about which magnitude is acceptable is therefore lacking.

9.6 Discussion and European funded research projects

At the European level there are currently several developments that will give new input to risk assessment procedures to toxic compounds in the near future that are relevant for the dietary risk assessment of AA; below we briefly summarise these developments. Exposure levels to different toxic compounds via the diet, including AA, are well known at the national level. However, as discussed in Section 9.3, exposure assessments are not performed in a harmonised fashion making it difficult not only to compare but also to combine data that are derived from different countries. Recently two initiatives have been taken to improve international comparable risk assessments. The first initiative is the integrated project (IP) SAFE FOODS (http://www.safefoods.nl/), subsidised by the European Commission through the 6th Framework Programme (contract no. Food-CT-2004506-446), which started in April 2004 with a duration of four years. In this IP one research project ('Quantitative Risk Assessment of Combined Exposure to Food Contaminants and Natural Toxins') aims at establishing an electronic platform of six European national databases (The Netherlands, Italy, Denmark, Sweden, Czech Republic and France) and China to be connected to the exposure modelling software package MCRA (see Fig. 9.5). With such an electronic platform, exposure calculations can be performed combining data from different national food consumption databases resulting in a Pan-European exposure assessment according to national demographics. For this the food codes used in the different databases will be harmonised using one common code. Harmonising food coding and food composition information over Europe will be addressed within the 6th Framework Programme project EuroFIR.

The EFSA recognises the need for harmonisation of food consumption data and has discussed the possibility of organising a concise food consumption database. It has been proposed that Member States could be asked to provide EFSA with several percentiles of consumption of several different food categories (e.g., P5, P10, P25, mean, SD, median, P90, P95, P97.5 and P99). Using this information EFSA will compile a European concise food consumption database that can be used to perform preliminary exposure assessments. Such a database will be very useful in covering variation in food

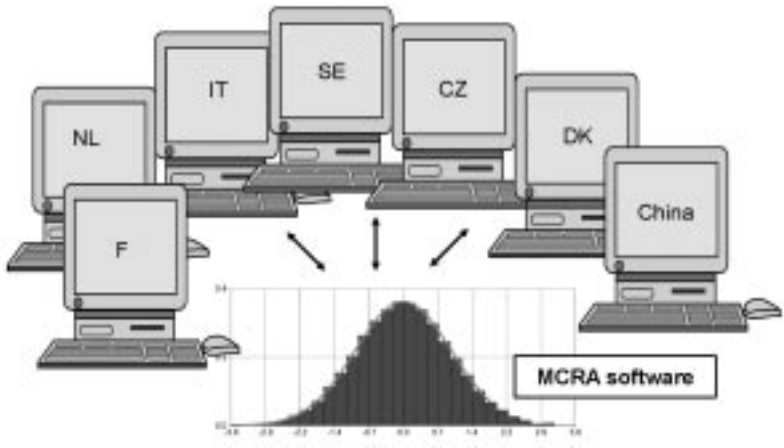

Fig. 9.5 Electronic platform connecting harmonised food consumption and residue databases of several countries to probabilistic software (EU-project SAFE FOODS). The collaborating countries are France (F), The Netherlands (NL), Italy (I), Sweden (SE), Czech Republic (CZ), Denmark (DK) and China.

consumption among European Member States. However, for chemicals with intake concern, which can be found in various foods, refined risk assessment using all relevant and available data will be needed. For this last step the platform established in SAFE FOODS can offer a useful refinement by using the electronic platform.

A second relevant European project is titled 'Heat-generated food toxicants toxicants, identification, characterisation and risk minimisation' (acronym: HEATOX; contract no. Food-CT-2003-506820-STREP; www.slv.se/templatesHeatox/Heatox_default____8425.aspx), which is also subsidised by the European Commission through the 6th Framework Programme. HEATOX will explore mechanisms of formation of AA, impact of raw material composition, inhibiting factors, cooking and processing methods in industry and households with the aim to control and minimise the formation of hazardous compounds. Different hazards will also be explored and characterised in various toxicological models, e.g. genotoxicity, carcinogenicity, neuro-developmental and reproductive toxicity. The exposure assessment and data on hazard characterisation, including data generated outside HEATOX, will be combined in a risk characterisation of AA intake via heat-treated carbohydrate-rich foods. These exposure calculations will be performed using food consumption data from The Netherlands, UK and Sweden in a harmonised way to filter out possible uncertainties regarding the food consumption methodology used as much as possible.

The method to calculate the margin of exposure (MoE) used by JECFA and applied above to AA intake levels of total Dutch population and children can be seen as a semi-probabilistic approach of calculating MoEs; the intake is calculated using daily consumption patterns, while the BMDL is at a fixed level. It is, however, very likely that in reality a BMDL may also vary within a

population. One person may be more sensitive for a toxic effect and may therefore have a lower BMDL than another person. In traditional risk assessment a fixed factor of 10 is applied for threshold chemicals to account for this diversity and another factor 10 for interspecies extrapolation. The EFSA also advises using these two factors (in addition to two additional factors of 10 to account for uncertainties related to the carcinogenic process and the fact that the BMDL relates to a small but measurable response) when addressing compounds that are both genotoxic and carcinogenic (non-threshold compounds).[49]

Instead of using fixed factors it is possible to simulate sensitive and insensitive persons in a population using the probabilistic approach. Recently an integrated probabilistic risk model combining BMD and probabilistic exposure modelling has been defined in which variations in the individual benchmark dose (IBMD), and individual exposure or intake levels (IEXP) are quantified and combined into a distribution of individual margin of exposure (IMoE).[50] This model has been developed for short-term assessments but is conceptually also applicable for long-term assessments. The proportion of the IMoE distribution below unity (where BMD = EXP) can then be equal to the proportion of the population with an AA intake level which results in a cancer incidence as determined in the animal study (e.g., 10% tumour incidence). The magnitude of an acceptable MoE will, among other things, depend on the characteristics of chemicals, e.g. chemicals with threshold and non-threshold concern. In such an integrated probabilistic risk assessment model uncertainties associated with consumption data, concentrations, animal dose-response modelling, and inter and intra species variations can be quantified. This last point is very important, because the uncertainties in the input data, which can be substantial, will determine the uncertainty in the corresponding MoE.

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Assessing exposure levels of acrylamide

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10.1 Rationale of exposure assessment

At the end of April 2002, the Swedish Food Administration reported that Swedish scientists (Tareke *et al.*, 2002) had discovered the presence of acrylamide in heat-treated food products (SNFA, 2002a,b,c). The finding that acrylamide is formed in relatively high concentrations during the preparation of foods was new. Acrylamide is the monomer, from which polyacrylamides are synthesised. The latter are used in the treatment of water, cosmetics and paper packaging. Acrylamide has been detected in various fried, deep-fried and oven-baked foods. It concerned foods like chips (French fries), crisps and bread, and also biscuits, crackers and breakfast cereals (Tareke *et al.*, 2002). Acrylamide has been shown to be neurotoxic in humans and laboratory animals. It has also been shown to induce tumours in experimental animals, and has been classified as 'probably carcinogenic for humans' (IARC, 1994; EC, 2002; SCF, 2002; FAO/WHO, 2002).

After the identification of this potential hazard a process of risk assessment, which includes hazard characterisation, exposure assessment and risk characterisation, was initiated. Questions, like how much acrylamide is present in foods, which foods contain acrylamide and how much of the foods is consumed in the daily diet, what sections of the population are most exposed and at what level, have to be answered. The answers to these questions are used in safety evaluation. Application of animal studies to risk assessment in humans is accompanied by a high degree of uncertainty due both to interspecies and high-to-low dose extrapolations (Hertz-Picciotto, 1995; Samet *et al.*, 1998; Shore *et al.*, 1992). Epidemiology can also play a role in the process of risk assessment.

Epidemiological studies have the advantage that they directly contribute data on risk (or benefit) in humans as the investigated species, and in the full intake range normally encountered by humans or envisaged when ingredient levels (as supplements or in novel foods) are deliberately increased. Observational epidemiological studies can contribute substantially to evidence on the risk of human exposure to dietary non-novel chemicals (van den Brandt *et al.*, 2002). Application of epidemiological studies to risk assessment needs an extensive and accurate exposure assessment of acrylamide.

10.2 Difficulties in exposure assessment of acrylamide

Many difficulties can occur when assessing exposure levels of acrylamide. Difficulties related to its determination in food and measurement of food consumption are discussed below.

10.2.1 Difficulties in the determination of acrylamide in foods

Since 2002, a variety of methods have been employed for the determination of acrylamide in foods including LC-MS/MS, GC-MS, LC-MS and LC-UV. The accuracy and precision of these methods may vary with different extraction and cleanup procedures and other methodological aspects of these methods. Data from proficiency testing studies between July 2002 and June 2004 showed high variability. Acceptable values ranged from 30% for crispbread to 44% for cereal relatively to the values assigned (Owen *et al.*, 2005; Klaffke *et al.*, 2005). Data from a German interlaboratory comparison test confirmed this high variability. CVs (coefficient of variation) of 25–30% for crispbread (184 μg/kg) and mashed potato (7286 μg/kg, which was spiked) were found (Klaffke *et al.*, 2005). Unsatisfactorily performing labs mainly caused high variability. The variability of results of replicate testing using the same analytical method in the same laboratory was generally low (Roach *et al.*, 2003).

The European Commission's Directorate General Joint Research Centre has organised several proficiency tests on the determination of acrylamide in food. A paper presenting the results and outcome of a proficiency test that focused on the determination of acrylamide in crispbread samples was published (Wenzl *et al.*, 2005). One of the goals was the identification of the influence of several parameters, such as analyte extraction and instrument calibration, on the analytical results. A set of samples, containing three different crispbread samples as well as extracts of one crispbread sample and acrylamide standard solutions, was shipped to each participant. A total of 42 European laboratories reported analytical results that were evaluated by applying internationally accepted protocols and procedures. The study found that, for each sample analysed in the proficiency test, acrylamide amounts quantified by 4–8 laboratories were outside the range formed by twice the target standard deviation above or below the target value. In transferring this knowledge to the data of monitoring databases of AA in

food, care must be taken that data are quality controlled, as it is likely that some of them may be biased.

Now, there are generally accepted criteria for the assessment of acrylamide in food. A set of LC-MS/MS and GC-MS methods meet those criteria needs (Acrylamide Workshop, 2004). During a workshop in 2004, critical methodology issues were discussed, e.g. the use of isotopically labelled internal standards, the use of reagent blanks and the recording of multiple ions and relative abundance to distinguish from possible interferences (Acrylamide Workshop, 2004). Available methods can determine levels as low as 20–50 ppb, depending on method and matrix.

The impact of the high inter-laboratory variability may not significantly affect exposure assessment as long as the exposure data set is not dominated by data from poorly performing laboratories. Also, in practice, the impact of inter-laboratory variability on the exposure assessment may be limited, since in many cases the natural and/or process related variability of real acrylamide levels is much wider than the inter-laboratory variability reported, even for one brand in a given product category (Dybing *et al.*, 2005). There is a strong positive relationship between the amount of reducing sugar of potatoes and acrylamide formed during frying. For example, Grob (2005) indicated that 1 g/kg reducing sugar resulted in 500 μg/kg acrylamide. From December 2002 to summer 2003, fresh potatoes on the Swiss market sold as suitable for roasting, baking and frying contained 2–14 g reducing sugar per kg (Grob, 2005). One can imagine what this means for the variability in acrylamide amounts when analysing different kinds of fried potatoes. Studies on the stability of acrylamide in food during storage revealed that acrylamide was relatively stable in most matrices (cookies, cornflakes, crispbread, raw sugar, potato crisps, peanuts) over time (Hoenicke *et al.*, 2005). However for coffee and cacao powder, a significant decrease occurred during storage for three or six months, respectively. Acrylamide concentrations dropped from 305 to 210 μg/kg in coffee and from 265 to 180 μg/kg in cacao powder. Vacuum packs of ground coffee were stored for three months at 10–12 ºC, whereas cacao powder was stored in closed glass jars at 10–12 ºC for six months. So the storage time of these products will influence acrylamide amounts analysed.

Industry has identified several factors that may influence acrylamide amounts in selected foods. In addition to the reducing sugar amount in potatoes as mentioned above, storage conditions of potatoes, processing conditions of fries (temperature/time), and water content may affect acrylamide levels. There seems to be a linear relationship between asparagine concentration in flour and acrylamide level in the finished product. However, the amount of asparagine naturally present in wheat flour varies considerably. The amounts of reducing sugars and ammonium bicarbonate in baking powder also have an impact on acrylamide amounts in the finished product (CIAA, 2004).

Since 2002 a tremendous amount of data on acrylamide levels in foods have been collected by the Confederation of the Food and Drink Industries in the EU (CIAA) and the US Food and Drug Administration. European data were

reviewed for their quality by the Institute of Reference Materials and Measurements, resulting in 3442 suitable data sets out of approximately 5200. The most abundant matrices in the database are potato chips, French fries, crispbread, breakfast cereals, fine bakery products, gingerbread and coffee (Dybing *et al.*, 2005; Lineback *et al.*, 2005). Previously unknown and unsuspected sources of acrylamide in foods are still being identified, such as black olives and prune juice (Acrylamide Workshop, 2004).

10.2.2 Methods in exposure assessment

Exposure of acrylamide through the diet can be assessed in two ways, which will be outlined briefly below. Naturally, the choice for a method strongly depends on the goal for which it is applied.

Direct method

This method is based on collection of a duplicate diet, which means that individuals collect a similar part of everything they eat and/or drink, usually during 24 hours, and put it aside so that it can be analysed for acrylamide. In the case of acrylamide, only one duplicate diet study has been performed up to now, in which 27 Swiss participants collected duplicate portions of their solid foods during two non-consecutive days. Advantages of this method are that the actual food products and brands that were consumed are accounted for and that no assumptions about individual levels are required. Also, the amounts of products consumed are accurately accounted for. However, duplicate diet studies are expensive and require a lot of effort from study participants. Another drawback of this method is that food consumption during 24 h is not likely to reflect long-term dietary patterns. Additionally, the food consumption of participants may be influenced by either their confrontation with the amount of food they collect for laboratory analysis, or free access to food which may be part of the study.

The combination of advantages and disadvantages makes this method suitable for a precise estimation of the cross-sectional dietary acrylamide intake of individuals in a certain population. It can also be used to correlate dietary acrylamide and short-term acrylamide biomarkers, such as urinary metabolites or DNA adducts. It is not feasible, however, to use duplicate diet studies for research on long-term health effects of acrylamide, e.g. cancer, especially when the risk of the disease of interest is low.

Indirect method

This method consists of a combination of acrylamide measurements in various relevant food products and an assessment of the amount of each product that is consumed by the population of interest. The latter assessment is often accomplished by means of a food frequency questionnaire (ffq). Disadvantages of this method are that the actual amounts consumed by consumers and the range in acrylamide levels between product brands and preparation processes are

less accurately accounted for than in the case of duplicate diets. To overcome these disadvantages, the samples for analysis need to be representative for the population in the consumption survey. This means, for example, that for all foods selected the most brands or varieties used, including different production codes or seasonal variations, have to be sampled and analysed (Konings *et al.*, 2003). However, in the case of using questionnaires, this method enables a better estimation of habitual and long-term consumption patterns and thus acrylamide exposure than the direct intake assessment method does. Therefore, this method is suitable for studying the long-term health effects of dietary acrylamide exposure.

It can also be used, for instance, for the Monte Carlo modelling of the distribution of the dietary intake of a population by including information on the distribution of acrylamide levels in food products and on the amount of each product that is consumed. This method also allows the assessment of the contributions of individual food products to the total dietary acrylamide intake, which may give indications for acrylamide reduction strategies in the food industry or counselling on home food preparation procedures. Besides ffqs, also dietary records or 24 h recall methods can be used to assess food consumption. A disadvantage is the short term for assessing food consumption. Although ffqs are less accurate than dietary records, they enable a better estimation of habitual and long-term consumption patterns.

A self-administered dietary questionnaire is often the method of choice in large-scale studies. The validity of such a questionnaire is not self-evident, since it is limited with respect to the foods included and the degree to which portion sizes are quantified. Moreover, each questionnaire needs to be tuned to the specific dietary habits of the study population.

10.3 Overview of dietary acrylamide exposure levels

The methods mentioned above have been applied in various countries all over the world and the results of the exposure estimates have recently been reviewed by Dybing *et al.* (2005). In the meantime three more studies have published results of exposure assessment, which were all based on indirect assessment methods. These studies are summarised in Table 10.1. Mean daily intake from these additional studies ranged from 0.21–1.3 μg/kg bw/day for several age groups. Also, from these additional results it can still be concluded that children and adolescents have a higher dietary acrylamide intake per kg bw than adults. This is probably due to a relatively higher intake of French fries and potato crisps in these age groups compared to adults and also to a greater amount of food intake per kg bodyweight. The major contributing foods to total exposure are French fries, potato crisps, coffee, pastry and sweet biscuits and bread and rolls/toasts. Other food items contribute less than 10% of the total exposure (WHO, 2005).

Despite all difficulties pertaining to methods of analysis, detection limits,

Table 10.1 Exposure estimates of acrylamide intake from 2004–2005

Exposure assessment	Daily intake (μg/kg bw/day)			Source
	Age group	Mean	95th percentile *97.5th percentile	
DONALD/RUB	<1	0.21		Hilbig *et al.* (2004)
Studies	<19	0.43		
Germany (2004)	1–<7	0.61		
FSA, United	19–64	0.3	0.6*	http://www.food.gov.uk/
Kingdom (2005)	15–18	0.5	0.9*	multimedia/pdfs/
	11–14	0.6	1.1*	fsis712005.pdf
	7–10	0.8	1.4*	
	4–6	1.0	1.6*	
	1.5–4.5	1.0	1.8*	
Australia (2004)[‡]	>2	0.4	1.4	Croft *et al.* (2004)
	>2	0.5[†]	1.5	
	2–6	1.0	3.2	
	2–6	1.3[†]	3.5	

[‡] Based on intake of carbohydrate-based foods.
[†] No acrylamide detected set to limit of reporting (50 μg/kg), instead of to 0 μg/kg, as in previous results.

ranges in acrylamide levels within brands and varieties, processing and preparation conditions in addition to exposure assessment methods, consumption patterns and cooking traditions as described above, the daily intake, as assessed by researchers in several European countries and the US, is around 0.4 μg/kg bw/day with a 90th percentile of 0.9 μg/kg bw/day (Dybing *et al.*, 2005). Additional results as presented in Table 10.1, which were published after Dybing's review, are comparable to these values. At the FAO/WHO Consultation (FAO/WHO, 2002) an average long-term exposure of 0.3–0.8 μg/kg bw/day was estimated on the few data available. The findings until now are in good agreement with these first FAO/WHO estimates.

Until now results of only one direct method for the assessment of dietary acrylamide intake have been published. The mean daily intake of acrylamide as assessed by a Swiss duplicate diet study (BAG, 2002) was 0.28 μk/kg/kg bw for an age group of 16–57 years. Generally, this result is lower than results of indirect assessment methods. Although this must be confirmed by more studies, this means that assessment by indirect methods may be overestimated. This might be due to the assumption that samples with acrylamide concentrations lower than the limit of detection are set to a certain amount, e.g. half the limit of detection, for exposure estimations. However for risk assessments this procedure is valid.

10.4 Are the exposure estimates valid?

The exposure assessments made around the world are quite comparable with regard to the levels estimated. However, what do these estimates tell us about safety and/or cancer development? Toxicity and cancer risk estimates in laboratory animals were determined after administering acrylamide via drinking water (Johnson *et al.*, 1986; WHO, 1996). Bioavailability following oral administration in drinking water is good, approximately 50–75% (Barber *et al.*, 2001a; Burek *et al.*, 1980). However, the bioavailability of acrylamide of food matrices is not known. On the basis of data indicating that adducts of haemoglobin with acrylamide and its metabolite glycidamide (which are sensitive biomarkers for acrylamide exposure) can be detected in the blood of non-smoking, not-occupationally exposed people, it must be assumed that acrylamide in foods is at least partially absorbed (JIFSAN, 2002; FAO/WHO, 2002). Haemoglobin adducts of acrylamide are used as biomarkers of exposure covering 120 days (corresponding to the lifespan of red blood cells). The background level of haemoglobin adducts has been estimated to correspond to a daily intake of approximately 1.5 μg/kg bw/day, which is around three times the exposure calculated from acrylamide concentrations found in food (Dybing *et al.*, 2005). This higher estimate might be related to an unknown source of acrylamide exposure, e.g., passive smoking? Or, are there limitations of these biomarkers as indicators of acrylamide intake? However, the study based on these estimates might not be representative of the average population and needs to be verified.

10.5 Bioavailability

The intake estimates mentioned in the previous sections are often used to estimate the exposure of the population to acrylamide through food and to assess the corresponding cancer risk, based on linear extrapolation of the cancer risk in laboratory animals. However, it is not known to what extent acrylamide in food is bioavailable to humans. A study on healthy male volunteers investigated the uptake of acrylamide in an aqueous solution and found that from 88 to 96% (dependent on the administered dose) of the acrylamide dose was taken up (Fennell *et al.*, 2005). The uptake of acrylamide in food, however, is expected to be less, because the food matrix may interfere with uptake and specific food components may increase or decrease the amount that is taken up. For instance, dietary proteins have been shown to interfere with acrylamide intake in a human caco-2 gut model (Schabacker *et al.*, 2004). This is due to the reactivity of acrylamide towards sulfhydryl groups in molecules, which causes acrylamide to bind to proteins during cooking or in the intestines.

In rats, the bioavailability of acrylamide in food was 32–44%, while the uptake after aqueous gavage was 60–98% (Doerge *et al.*, 2005b). In mice, the corresponding percentages were 23% for food and 32–52% for the aqueous solution (Doerge *et al.*, 2005a). These figures show that there are considerable

differences in bioavailability after oral acrylamide intake between species. In a study by Sörgel *et al*. (2002), it was observed that urinary levels of acrylamide increased after consumption of up to 500 g of potato chips or crispbread. The authors stated that their results were the first proof of bioavailability of acrylamide from food in humans. The high between-subject variability of urinary acrylamide excretion that was observed in this study may be caused by differences in bioavailability between subjects.

10.6 Acrylamide metabolism

Calleman *et al*. (1990), in their experiments on rats, discovered that acrylamide was partly converted to its epoxide metabolite glycidamide through oxidation. The part of acrylamide that is not oxidised and also glycidamide, are eliminated to a great extent in urine in the form of mercapturic acids; N-acetyl-S-(2-carbamoylethyl)cysteine, a reaction product of acrylamide and glutathione, and N-acetyl-S-(3-amino-2-hydroxy-3-oxopropyl)cysteine and N-acetyl-S-(1-carbamoyl-2-hydroxyethyl)cysteine, which are derived from the reaction of glycidamide and glutathione (Dixit *et al.*, 1982; Sumner *et al.*, 1992). Glycidamide is also partly hydrolysed to glycideramide (Sumner *et al.*, 1992), for instance by epoxide hydrolases. Some of the acrylamide and glycidamide is excreted in urine in unchanged form. The conversion of acrylamide to glycidamide has been shown to be probably almost exclusively caused by cytochrome P450-2E1 (Sumner *et al.*, 1999; Ghanayem *et al.*, 2005a,b,c). There are considerable species differences in the efficiency of all the steps in acrylamide metabolism. Of the investigated species, mice have the highest efficiency of acrylamide to glycidamide conversion, followed by rats and then humans (Sumner *et al.*, 1997; Fennell *et al.*, 2005). Further downstream in the metabolism, in humans more of the glycidamide is metabolised through hydrolysis than through conjugation with glutathione, as compared to rodents (Fennell *et al.*, 2005).

10.7 Biomarkers of acrylamide exposure

The observations mentioned above indicate that there is probably no completely straightforward relationship between dietary acrylamide intake and subsequent uptake in the intestines. Biomarkers may therefore be a useful indicator for the amount of acrylamide that enters the body. They incorporate total exposure to a substance and are thus not source-specific. For acrylamide there are several biomarkers of exposure.

10.7.1 Plasma and serum levels of acrylamide and glycidamide

Both acrylamide and its epoxide metabolite glycidamide have been measured in plasma and serum in studies in which rats and mice were administered

acrylamide through various routes (Miller *et al.*, 1982; Barber *et al.*, 2001b; Twaddle *et al.*, 2004). However, both substances have a short half-life in plasma and serum, in the order of less than one to a few hours (Miller *et al.*, 1982; Barber *et al.*, 2001b; Twaddle *et al.*, 2004) and therefore reflect very recent exposure. Furthermore, acrylamide levels in human plasma are often below the detection limit, even in acrylamide workers (Calleman *et al.*, 1994), while glycidamide in plasma has not been studied in humans. Calleman *et al.* found significant correlations between a neurotoxicity index for acrylamide-induced neuropathy and the levels of mercapturic acids in urine and haemoglobin adducts in humans occupationally exposed to acrylamide, but no such association was found with the acrylamide level in plasma (Calleman *et al.*, 1994). In conclusion, plasma or serum levels of acrylamide and glycidamide do not seem to be relevant biomarkers for environmental exposure in humans, surely not for long-term exposure.

10.7.2 Haemoglobin adducts

Both acrylamide and glycidamide form covalent adducts with haemoglobin. These adducts, unlike DNA adducts, are not repaired and therefore reflect exposure to acrylamide during approximately four months, the average lifespan of an erythrocyte. Acrylamide forms the adduct N-(2-carbamoylethyl)valine (AAVal), while glycidamide forms two adducts, namely N-(2-carbamoyl-2-hydroxethyl)valine and N-(1-carbamoyl-2-hydroxyethyl)valine (Bergmark *et al.*, 1993; Calleman *et al.*, 1994; Bergmark, 1997). These biomarkers have been measured in rodents that were exposed to acrylamide through various routes (Bergmark *et al.*, 1991; Fennell *et al.*, 2005) and also in humans (Bergmark *et al.*, 1993; Calleman *et al.*, 1994; Bergmark, 1997; Perez *et al.*, 1999; Hagmar *et al.*, 2001, 2005; Schettgen *et al.*, 2002, 2003, 2004a; Fennell *et al.*, 2005; Jones *et al.*, 2005; Kutting *et al.*, 2005).

Fennell *et al.* (2005) showed that the ratio of glycidamide vs. acrylamide haemoglobin adducts was lower in humans (0.44 ± 0.06) than in rats (0.84 ± 0.07). However, at the same dose, the absolute levels of acrylamide haemoglobin and glycidamide haemoglobin adducts were 2.7 and 1.4 times higher in humans than in rats (Fennell *et al.*, 2005). In studies on humans, it was observed that smokers have an approximately fourfold higher level of acrylamide haemoglobin adducts (Schettgen *et al.*, 2004b; Hagmar *et al.*, 2005) and an approximately threefold higher level of glycidamide haemoglobin adducts (Schettgen *et al.*, 2004b) than non-smokers. In another study, haemoglobin adducts of acrylamide correlated positively with the number of cigarettes smoked by the participants (Bergmark, 1997).

Haemoglobin adducts of acrylamide were also investigated in human umbilical cord blood (Schettgen *et al.*, 2004a). This study showed that the umbilical cord blood of the child of a smoking mother contained a higher level of acrylamide haemoglobin levels than that of children of non-smoking mothers. Overall, the levels of these adducts in umbilical cord blood was approximately

50% lower than that in the blood of the mothers, but there was a strong relationship between both ($r = 0.86$). In a study by Hagmar *et al*. non-smokers showed a fivefold range of acrylamide haemoglobin adducts, while considerable overlap in acrylamide haemoglobin levels was found between groups with varying dietary acrylamide intake. For non-smoking men, a significantly higher level of these adducts was found in the high dietary acrylamide intake group compared to the low dietary intake group, but not for non-smoking women (Hagmar *et al.*, 2005). For smokers, however, the same significant difference was found for women, but not for men.

In a study by Kutting *et al*. (2005) no relationship was found between dietary acrylamide intake based on a food frequency questionnaire and acrylamide haemoglobin adducts in ten women soon to give birth. However, ten might be too small a group to show such a correlation, the acrylamide intake estimation was probably too crude, because it seems that they did not take differences in acrylamide levels in the different foodstuffs into account, and this group of participants may not be the best group to study for such a relationship (Kutting *et al.*, 2005). Other reasons for the lack of association, apart from these former likely reasons, may be the inherent difficulties of estimating dietary acrylamide intake through questionnaires (see Sections 10.2.1 and 10.2.2) or differences in bioavailability between people. Yet another reason may be that sources other than smoking or diet dilute the association between dietary intake and internal dose.

Vesper *et al.* (2005) studied the effect of consumption of three ounces of potato chips per day for a week by six volunteers. On average, acrylamide haemoglobin adducts increased up to 46%, and glycidamide adducts up to 79%. However, in five of the participants these levels were actually decreased after the intervention, of which two of these participants showed a lower level of glycidamide adducts post-intervention. None of the participants showed an increase in both acrylamide and glycidamide adducts. The authors concluded that the duration of the intervention was probably too short and the exposure therefore too low to reach a new steady-state level due to increased chips consumption, which is likely to be the reason that no consistent increase in haemoglobin adducts was observed. On the other hand it also shows the limitations of these biomarkers as indicators of intake.

Background levels of acrylamide haemoglobin adduct levels in non-smoking, non-occupationally exposed persons were found to be quite consistent in different study populations. Fennell *et al*. (2005) found levels of 40–200 fmol/mg globin, Schettgen *et al.* (2003, 2004b) found levels in the range of 7–31 fmol/mg globin, with an average of around 20 fmol/mg globin in non-smokers and Bergmark (1997) observed an average of 31 fmol/mg globin. Vesper *et al*. (2005) observed an average baseline level of 43 fmol/mg globin in their experimental study of a chips consumption intervention. Glycidamide haemoglobin adducts are generally in the range of 9–67 fmol/mg globin (Fennell *et al.*, 2005; Schettgen *et al.*, 2004b; Vesper *et al.*, 2005).

10.7.3 Metabolites of acrylamide and glycidamide in urine

Markers of acrylamide exposure in urine reflect exposure during a few days before the measurement. Studies on these biomarkers in humans have been performed, in which the levels of the acrylamide mercapturic acid, and also to a large extent the glycidamide mercapturic acid, were readily detectable in non-smoking, non-occupationally exposed persons (Calleman *et al.*, 1994; Fennell *et al.*, 2005; Boettcher *et al.*, 2005a,b,c; Bull *et al.*, 2005). Fennell *et al*. (2005) have found that after oral administration of acrylamide humans have a lower percentage (12%) of glycidamide metabolites in urine than rats (28%) and mice (59%) (Sumner *et al.*, 1992), albeit at a lower intake level; 3 mg/kg in humans versus 50 mg/kg in the rodents.

In a study on a healthy male volunteer who received acrylamide in drinking water and a study on a sample of the general population, it was observed that the ratio of acrylamide to glycidamide mercapturic acids was in the same order of magnitude as that in rats and significantly lower than in mice (Boettcher *et al.*, 2005b,c). However, there were considerable inter-individual differences in this ratio, which are likely to be caused by differences in the activity of metabolising enzymes (Boettcher *et al.*, 2005c). Similarly to what was observed in studies on acrylamide and glycidamide haemoglobin adducts, smokers had four- to fivefold higher levels of acrylamide and glycidamide mercapturic acids in urine than non-smokers (Boettcher *et al.*, 2005c).

10.7.4 Acrylamide and glycidamide DNA adducts

Due to their reactivity towards –SH groups in proteins, acrylamide and glycidamide will also react to some extent with DNA. Various animal studies have been performed to investigate acrylamide and glycidamide DNA-adducts (Segerback *et al.*, 1995; Gamboa de Costa *et al.*, 2003; Doerge *et al.*, 2005a,b; Ghanayem *et al.*, 2005a; Maniere *et al.*, 2005; Manjanatha *et al.*, 2005). The main adducts that have been investigated in several tissue types are N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua), N3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade), N1-(2-carboxy-2-hydroxyethyl)-2′deoxyadenosine (N1-GA-dA). Glycidamide was reported to be 100–1000 times more reactive with DNA than acrylamide itself (Segerback *et al.*, 1995).

Other indications that the metabolite glycidamide is primarily responsible for the genotoxicity of acrylamide came from studies in which acrylamide was administered to rats and mice through various routes. The oral route, and thus first-pass metabolism of acrylamide, led to higher levels of DNA-adducts than the intravenous route, even though the bioavailability of acrylamide was decreased (Doerge *et al.*, 2005a,b). Another important observation from animal studies was that DNA adduct formation showed a supralinear dose-response curve (Gamboa de Costa *et al.*, 2003), which indicates that at lower acrylamide dosage relatively more adducts are formed than at higher doses. This may be due to saturation of the biotransformation of acrylamide to glycidamide. Also, some *in vitro* studies on the DNA-adduction properties of acrylamide have been

performed (Solomon *et al.*, 1985; Besarati Nia and Pfeifer 2003, 2004). In a study by Besarati Nia and Pfeifer (2003), acrylamide itself was clearly, but weakly, associated with DNA-adducts, but another study by this group showed that equimolar exposure of cells to glycidamide was associated with much higher levels of DNA adducts than exposure to acrylamide (Besarati Nia and Pfeifer, 2005). As yet, no studies have been performed on acrylamide or glycidamide DNA adducts in humans. This may be due to the low exposure of humans as compared to the dose that animals receive in experiments. The level of DNA adducts in human cells is probably too low to detect in current DNA adduct assays. However, Farmer *et al.* (2005) are currently developing a method to analyse N7-Gua-GA adducts in urine of humans.

10.8 Relevance of the biomarkers for exposure and risk assessment

The biomarkers described here represent different levels of acrylamide exposure assessment. Some markers focus on the parent compound acrylamide, while others focus on the metabolite glycidamide. For exposure assessment that is done in order to make a risk assessment for neurotoxicity, acrylamide markers are suitable, because it is assumed that acrylamide itself is responsible for effects on the nervous system (Costa *et al.*, 1995). However, biomarker research on acrylamide has clearly shown that glycidamide, and not acrylamide itself, is responsible for the genotoxic effects of acrylamide exposure. Therefore, for cancer risk assessment it is important to assess the magnitude of internal exposure to glycidamide. This can be done by measuring glycidamide haemoglobin adducts, mercapturic acids of glycidamide and hopefully in the future also glycidamide DNA adducts, since the latter adducts are indications of what part of glycidamide reaches its crucial target for carcinogenesis.

As can be concluded from the biomarker research that was described here, there are likely to be many differences between individuals with regard to how the body will handle acrylamide exposure, starting with uptake and continuing with metabolism, DNA-adduction and repair, and excretion. Therefore, in risk assessment it is important, whenever possible, to combine exposure assessment based on dietary questionnaires with biomarker measurements. In longitudinal observational studies on the risk of dietary acrylamide intake, in which the only way to assess long-term exposure is through questionnaires, it may be very useful to include factors that are likely to influence the internal exposure to glycidamide, for instance, polymorphisms in and inducers of metabolising enzymes.

10.9 References

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11

Assessing human exposure to heterocyclic aromatic amines

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11.1 Introduction

Human exposure to heterocyclic aromatic amines (HAA) can now be estimated with a degree of accuracy because HAA are easily measured in meats and human exposure to them can be determined from food consumption surveys. The difficulty in assessing exposure arises from the number of different heterocyclic amines of concern, variation in their formation depending on meat cooking conditions, and the lack of long-term biomarkers for either intake or biological consequences. Correct exposure estimates are essential for meaningful epidemiology studies to determine if HAAs are involved in human cancer incidence.

The hypothesis that HAA are involved in cancer etiology follows a logical mechanistic progression. HAA are present in some well-done meats, these meats are consumed over a lifetime, and the HAA are absorbed and metabolized to active intermediates that bind to DNA. It is these processes that can cause mutations leading to human cancer. The factors affecting the carcinogenic outcome are the exposure levels, possible susceptible periods during a human lifetime, the internal dose, the details of metabolic activation and detoxification, and repair of damaged macromolecules within the body's cells.

The compelling part of this area of research for population studies is that there are 100-fold variations in human exposure, and, therefore, the current population has enough variation to test the hypothesis that HAA exposures are involved in human cancer etiology. Also compelling is the fact that these exposures can be modified through changes in cooking practices, if changes are warranted from risk assessment, without having to abstain from meat intake. The challenge is to quantify exposure to HAA using the best technology to evaluate

that risk and to make risk-based recommendations about reducing HAA exposures based on the best scientific data available.

The plausibility that HAA are involved in human cancer is derived from the experimental results showing that these compounds are genotoxic rodent carcinogens, initiating tumors in prostate and mammary glands, the colon, and other sites. These animal tumors have many characteristics similar to those implicated in human studies.[1–4] Epidemiological studies relating cancer outcome and meat cooking doneness suggest effects at these same sites plus esophagus, gastric cardia, larynx, lung, blood (lymphoma), stomach, and pancreas. There are 31 such studies compiled by Knize and Felton.[5] Most of these studies show positive associations, but some are negative, as would be expected given the variety of cancer sites evaluated. Improved classification of exposure levels, however, would tend to elevate odds ratios and improve statistical significance if the hypothesis of HAA involvement is true. Thus, improved determination of exposures would refine the risk assessment.

Although accurately classifying exposure levels in individuals in a human population is a key to risk determination, other factors play a role in HAA carcinogenesis. Individual human risk from HAA exposure is likely modified by protective foods like fruits, vegetables, and fiber, possible synergistic effects of other carcinogens, and the usual factors like age and smoking that are routinely adjusted for in epidemiology studies. Individual phenotypic factors like metabolic differences in activation and detoxification and in DNA damage repair may also be involved. With these confounding factors, accurate exposure estimates are especially needed to determine HAA involvement in human cancers.

Compared to non-dietary environmental exposures to toxicants there are some certainties for the HAA exposures. The exposures are internal for HAAs and the amounts in meats have been determined in a great variety of meat samples worldwide, so levels are known to be in a range from the limit of detection, which is typically in the range of 0.1 ng/g, to a few hundred nanograms per gram of cooked meat. A 100 g meat portion with 10 ng/g of HAAs would give an intake of one microgram, so for the known HAA, 'higher' doses are in the microgram range. For our human volunteer feeding studies, we feed 150 g of chicken breast containing approximately 100 ng/g of PhIP for an intake of 15 μg.[6]

Furthermore, the number of HAA compounds of concern is not fixed, and newly identified HAAs are being added to those that have been investigated for many years.[7] All of the mutagenic HAAs appear to be formed by pyrogenesis from the natural precursors in muscle tissue of amino acids, creatine or creatinine, and sugars. Most of the HAAs have the N-methyl-imidazo-amine group, and new derivations of known compound classes have been recently isolated.[7] The HAAs also have common metabolic pathways and mechanisms of action and could reasonably be assessed together as a sum of the known HAAs. Further certainties are the clear genotoxic potential and rodent carcinogenicity of all of the HAAs tested. The possibility of synergistic effects of exposure of

rats to multiple heterocyclic amines has been investigated and synergistic effects were seen.[8]

Understanding how time affects the HAA exposures is also important. The biological half-lives of reactive intermediates are limited, for instance. Most of the dose is cleared from the body in 24 h. Although a bolus dose may be cleared quickly, a large amount of compound in the body may also overwhelm cellular repair mechanisms and initiate tumors differently than a smaller chronic dose. Some of the inherent uncertainty in using HAA exposure assessment as a predictor for cancer initiation is temporal; the relevant exposure may be many years prior to the exposure determination. Monitoring exposure becomes more unreliable the longer the time that passes from exposure to outcome. The amounts of HAAs in foods are relatively low, but tumors can be initiated from damage to only a single cell, suggesting that only a few molecules are enough. There is no experimental evidence for thresholds for the genotoxic effects of heterocyclic amines. Mechanistic data show that, even at low doses, heterocyclic amines form DNA adducts in rodents, primates, and humans.[9–12]

11.2 Biomonitoring

Biomonitoring refers to measuring or estimating the HAA levels in the biologic medium, in this case, the foods in which they are produced. For HAAs, the applied dose is in the same range as the potential dose since food chewed/digested efficiently and the HAAs appear to be readily absorbed and metabolized. Chapter 5 in this book addresses methods of HAA analysis in meats, and HAA formation and methods to reduce the amount of HAAs produced in foods are discussed in Chapter 17.

Studies of the amounts of heterocyclic amines produced in foods as a result of regional cooking practices are reported for Great Britain,[13] Sweden,[14,15] Switzerland,[16] Spain,[17] Japan,[18] Singapore,[19,20] and the United States.[21,22] In most cases, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) tend to be the most mass-abundant heterocyclic amines. Their concentrations in cooked meats typically range from nearly undetectable levels (typically 0.1 ng/g) to tens of ng/g for MeIQx and up to a few hundreds of ng/g for PhIP, depending on the cooking method and food source. Because of their prevalence in cooked meat, these two compounds were used in most studies to understand the mechanisms of mutagenesis and carcinogenesis of HAA. The effects of these two compounds have been explored in model systems using biological endpoints related to mutagenesis in bacteria, cultured cells, and rodents.

The heterocyclic amines have also been reported in beverages, but at low amounts compared to the cooked meats. PhIP was found in all beer and all wine samples analyzed in one study;[23] however, another study reported that PhIP was detected in only one sample of 24 wines.[24] One group of beverages that has potent mutagenic activity consistent with the chemical and biological properties

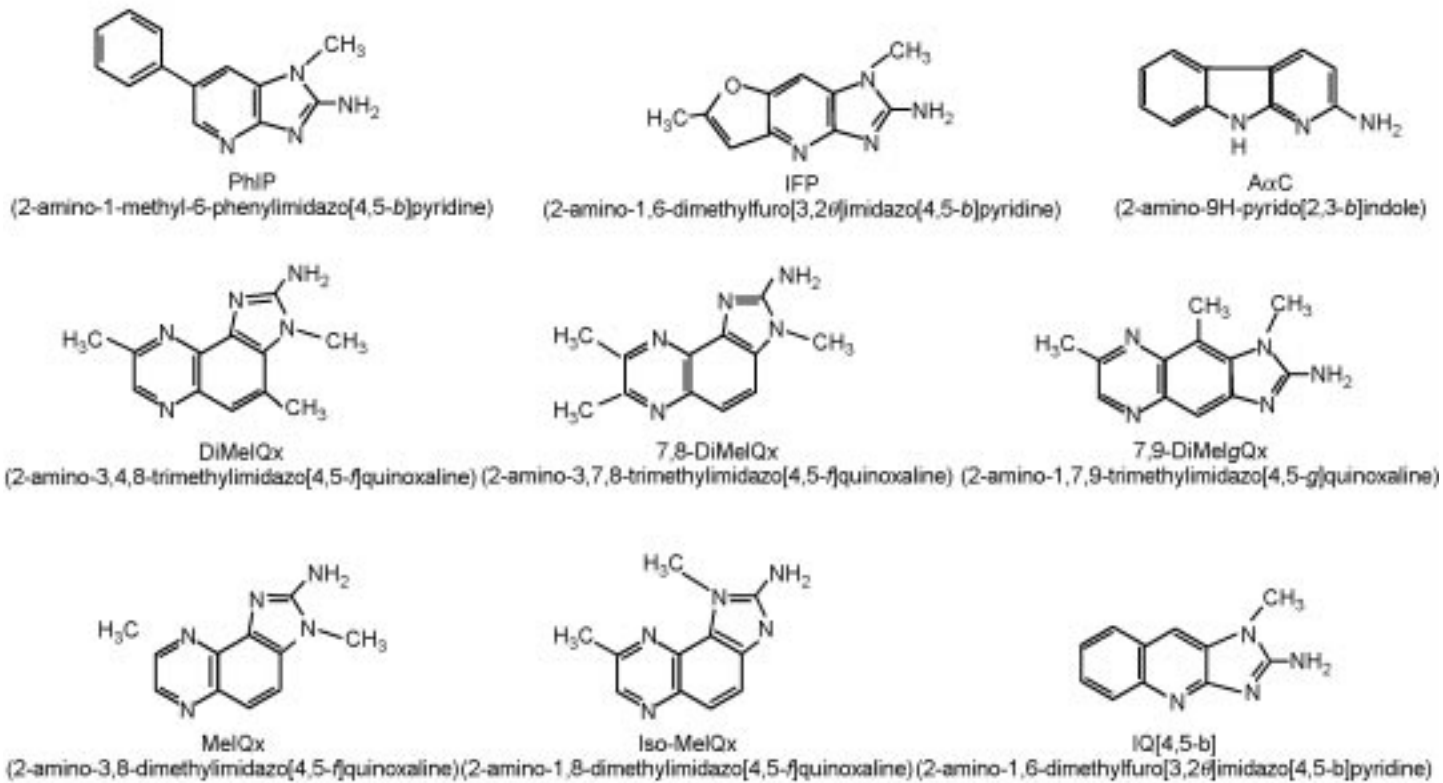

Fig. 11.1 Structures of nine heterocyclic amines found in cooked meat.

of aromatic amines is coffee substitutes, beverages derived from heated grains.[25] The compounds responsible for the mutagenic activity in these beverages have not been identified.

Figure 11.1 shows the structure and chemical name of some of the heterocyclic amines reported in cooked meats. Over 20 heterocyclic amines have been found in meats or model systems that mimic meat cooking, but comprehensive and comparative reports show those in Fig. 11.1 to be among the more abundant and often reported. Two of these, Iso-MeIQx and IQ[4,5-*b*], were recently reported to be found in meat and in human urine from volunteers after consuming cooked meat.[7,26]

An alternative route of HAA exposure is through the respiratory tract via the fumes generated when meat is cooked. Although the HAAs are not volatile as chemicals, collected particles generated during cooking were shown to have mutagenic activity and to contain specific HAA.[27–31] The link between high incidence of lung cancer in non-smoking Chinese women and HAA exposure from cooking fumes has been suggested.[32] The total exposure to HAA appears to be much greater through consumption of well-done meat than cooking fumes, but the tissue exposed differs between the two exposure routes. The consequences of exposures through the respiratory tract need to be investigated further. Occupational exposure to HAA is not expected to be a large factor in human exposure, although the inhalation exposure noted above may be important in some occupational circumstances.

11.3 Food frequency questionnaires and doneness classification

The primary method for assessing dietary intake in epidemiology studies is through food frequency questionnaires. Exposures to HAA have been assessed using questionnaires for frequency of meat consumption, estimates of amounts consumed, and meat doneness preference, often using photographs to aid in assessing doneness.[33–37] Worrisome is that validation of the doneness classification by analyzing foods is typically not done, yet exposure estimates derived from these questionnaires and reported with great certainty often fail to consider meat preparation and cooking factors known to be important in HAA formation.

Measurement error in any exposure classification results in attenuated risk, therefore minimizing those errors is important in determining the scientific validity in the relationship between HAA and human cancer. Questionnaires are not without classification error. Correlation between questionnaire responses and biochemical measures can show weak correlations; for instance, correlation coefficients ranging from 0.14 to 0.42 were seen between estimates from a diet survey and measurements of blood vitamin levels.[38] In self-reported answers on questionnaires, a survey participant's perception of health-related outcome and the specific food has been shown to bias results, especially among those with disease. This bias is a possible factor in case-control studies involving cancer

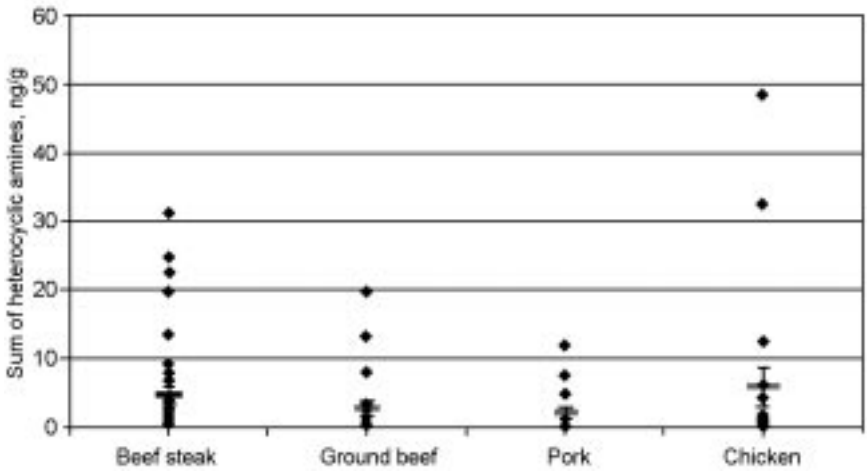

Fig. 11.2 Formation of MeIQx, DiMeIQx, IFP, and PhIP, shown in ng/g cooked weight, in fried beef patties cooked to an internal temperature of 70 ºC at a frying pan temperature of 150, 180, 200, or 250 ºC.

patients and their estimated HAA intake.[39] Color photographs were shown to improve doneness categorization in self-reported doneness specifically of HAA.[36] Despite their limitations, questionnaires are the best current HAA exposure assessment tool for a population, but results need to be used conservatively.

An example of the variation in HAA production during cooking, and, thus, varying exposure from consuming those meats, is shown in Fig. 11.2. The formation of four heterocyclic amines formed from ground beef patties that were cooked to an internal temperature of 70 ºC is shown. Pan temperatures were either 160, 180, 200, or 250 ºC.[40] The amounts of each of the four HAAs increased with increasing pan temperature, with PhIP and IFP becoming the major heterocyclic amines when pan temperature was set at 250 ºC. This figure shows the importance of cooking conditions on the subsequent exposure to HAAs. The correlation of the formation of these four HAAs at each pan temperature is difficult to reconcile with risk assessments that show risk associated with one heterocyclic amine but not the others.[41,42]

Despite the common endpoint temperature for the samples in Fig. 11.2, the sum of the four HAAs varied from 0.14 ± 0.08 ng/g total to 17.05 ± 4.28 ng/g of cooked meat depending on pan temperature. This greater than 100-fold difference shows that the internal temperature reached (which was the same in all cases) may be meaningless for detecting an exposure gradient over the expected range in the human population despite the reported use of endpoint temperature for classification.[43] Asking the correct questions to get accurate HAA exposure information is difficult. Validation of the results from surveys by a measured biomarker is needed.

An investigation of HAA amounts in meats obtained from survey participants demonstrates the limitations of food frequency questionnaires. For a study of

foods cooked under typical household conditions, grilled meat samples were obtained from volunteers in households in the midwestern United States as a part of a published study on pan-fried meats.[36] Participants were volunteers responding to an initial survey that they preferred their meat well done or very well done. The participants were surveyed a second time several years later, and surprisingly, 46% of the participants changed their stated meat doneness preference. To correlate the stated doneness preference with HAA levels, 92 samples of cooked meat were obtained and analyzed by solid-phase extraction and reverse phase HPLC with photodiode-array detection, using published methods.[21] The sum of the amounts of the four detected heterocyclic amines for the four different kinds of cooked meats, their averages and standard errors of the mean are plotted in Fig. 11.3. Surprisingly, in this collection of meat samples thought to be cooked to a well-done or very well-done state, approximately 20% of the samples had undetectable levels of HAAs. The questionnaires would categorize meats with these undetectable levels of HAAs as high exposure samples, according to the meat's reported level of cooking doneness; however, based on chemical analysis, these were actually low exposure samples.

In addition, the quantified HAA content of the meat samples spanned over two orders of magnitude, and the difficulty predicting quantities based on doneness assessments, the variation in HAA formation, and the changing of stated meat doneness preference indicate that exposure estimates using diet surveys need to be made with caution. If misclassifications were reduced, the power of the epidemiology studies would be greatly improved.

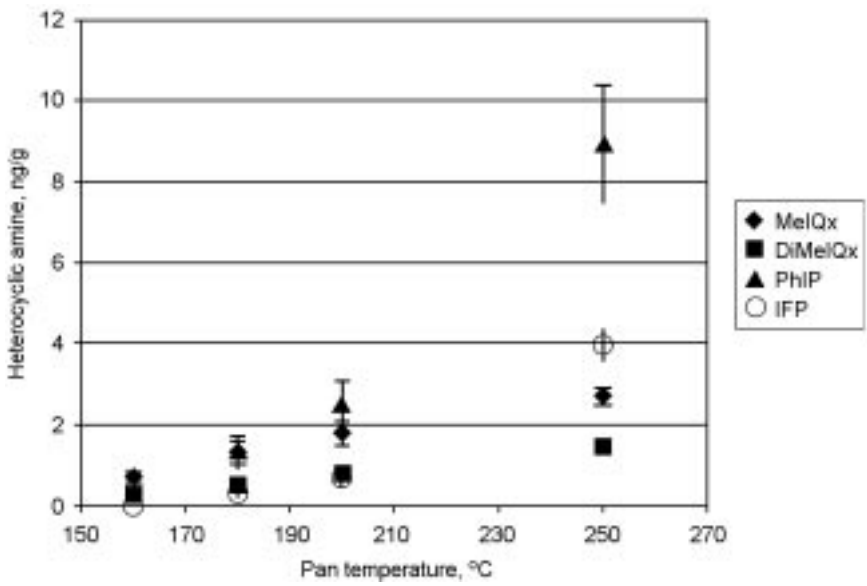

Fig. 11.3 Sum of MeIQx, DiMeIQx, IFP, and PhIP detected in meat samples, in ng/g cooked weight, from households specifying a preference for well-done or very well-done meat.

11.3.1 Chicken and PhIP exposure

Another factor confounding individual HAA exposure assessment is the erroneous assumption that chicken is responsible for almost all of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) exposure. Using high heterocyclic amine values reported in an early study of laboratory-cooked chicken,[44] Byrne *et al.* concluded that chicken prepared by grilling, broiling, or pan-frying most reliably predicts 98% of PhIP exposure from all foods.[45] However, based upon the results presented in Fig. 11.2, as well as analysis of meat cooked in restaurants[46] or in homes in the U.S.,[47] it appears that the levels of PhIP are similar in chicken and beef. Understanding exposure to PhIP from consuming chicken is important because it is the occasional high PhIP values found in very well-done chicken that have led to the seeming contradiction; that chicken consumption is associated with low cancer incidence, yet chicken meat is assumed (wrongly) to be the source of most PhIP exposure.

Another possible exposure source is heterocyclic amines generated from endogenous formation. A laboratory model showed that 37 ºC is warm enough to produce PhIP from a mixture of phenylalanine, creatinine and glucose, or 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) from glycine, creatinine and glucose in aqueous buffers.[48,49] Research in another laboratory showed no PhIP was found in a similar model system left at room temperature for two weeks.[50] This work led to speculation that there is endogenous formation of HAA. Further evidence for endogenous formation of PhIP is the report by Reistad *et al.* that PhIP was found (at low levels) in seven of eight urines from volunteers that were instructed to refrain from ingesting fried meat or fish. No MeIQx, however, was seen in the same samples.[51] The concentration of the precursors is much lower in human tissues than the model systems that were investigated, casting doubt about endogenous formation of HAA. More work is needed to determine the source of the background levels of PhIP seen in some studies. Besides dietary intake, other possible exposures to PhIP are to the developing fetus during pregnancy and in infants through nursing. These inferences have been made in mice using radiolabeled PhIP.[52]

11.3.2 Bioavailability

The bioavailable dose is an even better exposure measurement than the dietary intake, as it reflects the absorption of the dose through the digestive system. Pioneering work was done in 1982 showing that exposure to mutagen-containing fried pork or bacon resulted in a spike of detectable mutagenic activity in urine.[53] Hayatsu *et al.* showed that the ingestion of fried ground beef hamburgers resulted in a mutagenic response in urine that was not detected in urine collected before the hamburgers were consumed.[54] Later, using oral or intra-peritoneal dosing of synthetic HAA, it was shown that in rodents, most of the dose was excreted in urine as metabolites,[55–58] suggesting it would be possible to monitor the exposure in humans as urinary metabolites.

The presence of specific HAAs in human urine after a cooked-meat meal was

shown by Murray *et al.* using a sensitive GC/MS detection method.[59] They showed that only a low percentage of the parent compound was in the urine. Other studies showed four heterocyclic amines were detected in urines from volunteers on their normal diet;[60] PhIP and MeIQx conjugates detected in urine led to conclusions about Phase II conjugation reactions;[61] and PhIP metabolites were detected in a racially diverse population.[62] One drawback of the analysis of urine for HAAs and their metabolites is the short duration of the detectable exposure signal. It has been shown in many studies in humans and rodents that most of the dose is excreted within 12 h, so detecting urinary HAAs or their metabolites is not the desired long-term marker of dose or bioavailable dose.

In 1992 it was discovered that PhIP has a high and selective binding to pigmented tissues.[63] This finding led to detection of PhIP bound to human hair as a marker of dietary exposure and bioavailability.[64] Interestingly, PhIP incorporation appears to vary with hair color and be dependent upon the eumelanin concentration in hair.[65] Because a hair sample may provide a record of exposure over a period of several months, hair analysis may be a promising avenue for determining human exposure.

11.3.3 Biomarkers of the bioactive form of HAAs

Biological markers, or biomarkers, would ideally integrate exposures over a longer time and be a sensitive and specific marker of the active metabolite of the HAA. Blood levels represent the delivered dose of the HAA, but the form of the delivered dose is not known. HAAs measured in blood could be the parent compound that would be activated in the target tissue, or a metabolite formed in the liver that is circulated and can then affect the eventual target tissue. Another possible blood biomarker would be serum protein adducts. Early work in rodents showed adducts were formed after exposure to IQ,[66] MeIQx,[67,68] and PhIP.[69] At present, more work needs to be done to improve the sensitivity of detection of adducts at the low levels present in humans consuming normal dietary doses of HAAs.

DNA adducts at the target site are a measure of the biologically effective dose, which is only a small fraction of delivered dose. The low mass amounts of adducts make detection a challenge. Adducted DNA, an integral part of the mutagenic pathway, is believed to represent a cancer-initiating event, so measurement of DNA adducts is relevant. However, we still need to know whether the number of adducts and the sequence specificity for adduct formation impacts the mutations, and which mutations lead to disease. The measurement of DNA adducts results in information that is close to the biologically effective dose of HAA, but no specific adduct has been linked to any specific clinical outcome in humans for any carcinogen.[70]

Currently, tissue samples are required to obtain enough DNA to measure adducts. At present, methods of DNA adduct analysis use 32P postlabeling or accelerator mass spectrometry (AMS), but are not developed for large-scale studies because of the expense of the analysis for postlabeling,[71] or the need for

dosing with radiolabeled HAA for AMS.[12] A review of HAA-DNA adduct structures and formation by Turesky and Vouros is available.[72] With improved analytical methods, DNA extracted from buccal cell scrapings, or from sloughed bladder cells in collected urine samples, may be sufficient to detect adducts.

A biomarker of the activated form of PhIP, 5-OH PhIP has been reported to be found in model systems and in the urine of rats exposed to PhIP. A degradation product of the reactive PhIP nitrenium ion, 5-OH PhIP offers a biomarker of absorption and activation[73] and has potential as a human biomarker available from urine. This marker for metabolic activation of PhIP could be used to monitor interventions aimed at shifting metabolic processes toward detoxification pathways thereby reducing the genotoxic effects of PhIP.

11.4 Application of exposure assessment to risk

Given that there is human exposure through well-done meats and that absorption does take place, an exposure assessment needs to be quantitative enough to divide a test population into exposure groups. The uptake of HAA has been shown in many studies, but the digestion efficiency and the presence of fiber and other contents in the digestive system may influence the HAA intake. Digestion of meat and the influence of co-digested foods was examined by Kulp *et al.*[74] They find that increased meat doneness reduces the bioaccessible dose of four HAAs investigated, and that bioaccessibility varies with the polarity of each HAA.

Additionally, the exposure duration to HAAs is over a lifetime and there may be important differences in cancer initiation with age during exposure. For instance, the common method of inducing tumors with PhIP in rats uses a protocol of dosing during the animal's age of rapid growth when hormonal influences maximize the number of mammary carcinomas.[75] This suggests that for HAA exposure in humans, the teenage years may be more important than other life periods for the hormone-related cancers. Also, the peak doses may be more important than average dose in evaluating risk.[76] For melanoma patients, recollections of incidents of childhood sun burning – a peak exposure – are common, but were not recalled in the control group without melanomas.

11.5 Conclusion

In determining exposure to HAA, there is a need to use biological monitoring to validate the predicted exposure determined through food frequency questionnaires and to reduce misclassification in exposure assessments by focusing on the most uncertain steps. HAAs are produced in amounts varying over several orders of magnitude depending on meat preparation and cooking, so the portion size, and even the frequency of consumption, may be minor components compared to the HAA content of the cooked meat itself. Because it

is well-established that there is human intake and that metabolism of the compound(s) leads to DNA binding, even at the low doses found in the ordinary diet,[12] the uncertainties in risk assessment are in the dose estimation among the population and in the relationship of the presence of DNA adducts to the initiation of tumors in target tissue.

In rodents the heterocyclic amines are multi-site carcinogens. The number of studies and number of human cancer sites with positive correlations with meat doneness and intake strongly suggest that these compounds may be multi-site carcinogens in humans, as well. Supporting these epidemiology studies is a result showing that women have an increased cancer risk with increasing levels of PhIP-DNA adducts and that the DNA adducts increase with a subject's preference for well-done meat.[77]

Based on these observations it is apparent that quantifying human heterocyclic amine exposure is not a simple task. Formation of heterocyclic amines in meat during cooking is highly dependent upon cooking method and doneness levels. Individual exposure depends upon meat consumption patterns. The compelling conclusion for these meat and cancer studies is that humans are exposed to genotoxic rodent carcinogens over a lifetime. Intake levels are low; still, one microgram of MeIQx (a 200 g steak with 5 ng/g) has 2.8×10^{15} molecules that can be absorbed and then activated or detoxified through metabolic pathways.

Because we do not yet know how many molecules of HAA are needed to produce a disease outcome, accurate exposure assessment over a lifetime is needed to give the definitive answer about dietary exposure to genotoxic carcinogens like the HAAs. Scientists worldwide are working hard to get these assessments.

11.6 Acknowledgements

I thank Cynthia P. Salmon, and Drs Kristen S. Kulp and James S. Felton for reviews of this chapter. This work was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract No. W-7405-Eng-48 and supported by NCI grant CA55861.

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12

Geontoxicity, metabolism, and biomarkers of heterocyclic aromatic amines

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12.1 Introduction

Heterocyclic aromatic amines (HCAs) are potent mutagens and carcinogens in experimental animals; they form in well-done grilled meats, poultry, and fish, and in tobacco smoke condensate, and they also occur in diesel exhaust. The concentrations of HCAs in cooked meats can vary by over a >100-fold range.[1–3] 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methylimidazo[4,5-*b*]pyridine (PhIP) are two of the most abundant HCAs formed in grilled ground beef prepared under common household cooking practices, while 2-amino-9H-pyrido[2,3-*b*]indole (2-AαC) is an abundant mutagen formed in tobacco smoke condensate.[4] The chemical structures of the most prevalent HCAs formed in cooked meats and tobacco smoke condensate are presented in Fig. 12.1.

The genotoxic potencies of HCAs vary by over >1,000-fold in bacterial mutagenicity assays, but these correspondingly large differences in ranges of potency are not observed in mammalian cell assays or in long-term carcinogen bioassays. For example, MeIQx is a potent bacterial mutagen in the Ames reversion assay, while the potency of PhIP is about 100-fold weaker under the same assay conditions.[5] However, both compounds are strong carcinogens and induce tumors at multiple sites in experimental laboratory animals, during long-term feeding studies at comparable doses.[5–7]

Although short-term bacterial mutagenesis assays have been an effective screening tool for the identification of mutagenic HCAs in complex food matrices,[8] they cannot reliably predict carcinogenic potency. HCAs undergo metabolism at the exocyclic amino group by cytochrome P450 (P450) enzymes,

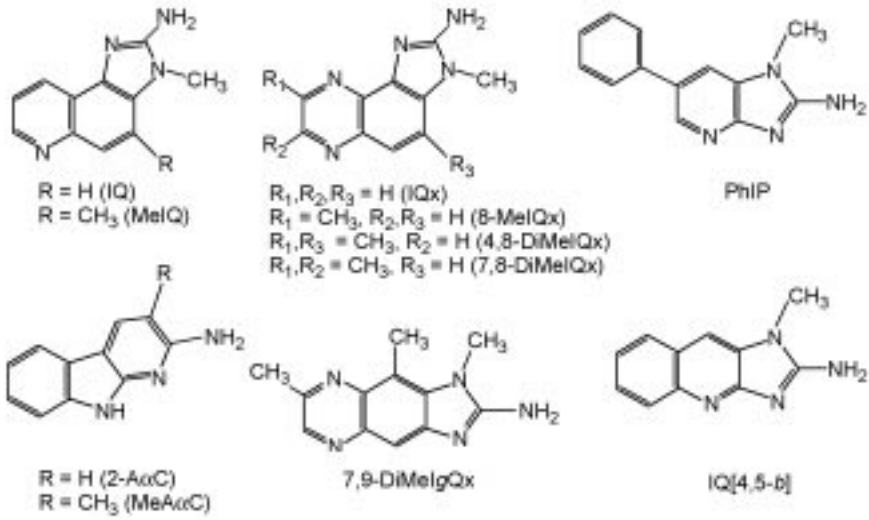

Fig. 12.1 Chemical structures of representative HCAs formed in cooked meats and tobacco smoke condensate.

to form the genotoxic *N*-hydroxylated-HCA metabolites;[9–12] these metabolites can react with DNA or can undergo further metabolism by phase II enzymes to produce unstable esters that form adducts with DNA.[13,14] There are noteworthy interspecies differences in catalytic activity and regioselectivity of the oxidation of HCAs by P450s;[11,15–17] such differences can influence the toxicological properties of these genotoxicants. Moreover, humans, in contrast to rodent species, display large interindividual variation in the expression of cytochrome P450 enzymes[11,18] involved in the bioactivation of HCAs; individuals who display rapid N-oxidation activity of HCAs may be at elevated cancer risk.[19,20] The emergence of highly sensitive mass spectrometry (MS) techniques has enabled the detection of HCAs, their metabolites, and DNA adducts in human tissues.[21-26] These analyses confirm the existence of large interindividual differences in metabolism of HCAs and levels of DNA adduction products and may result in differences in cancer risk among individuals exposed to HCAs.

12.2 Bioactivation of HCAs, DNA adduct formation, mutagenesis, and carcinogenesis

12.2.1 Metabolism and DNA adduct formation

HCAs must undergo metabolism in order to exert their genotoxic effects. The metabolic activation of HCAs occurs by P450-mediated *N*-oxidation of the exocyclic amino group to form the *N*-hydroxy-HCAs;[10,27] these metabolites can react with DNA or can undergo further transformation with sulfotransferases (SULTs) or *N*-acetyltransferases (NATs), to produce unstable esters that also form adducts with DNA (Fig. 12.2).[14,28] HCAs are mainly metabolized by

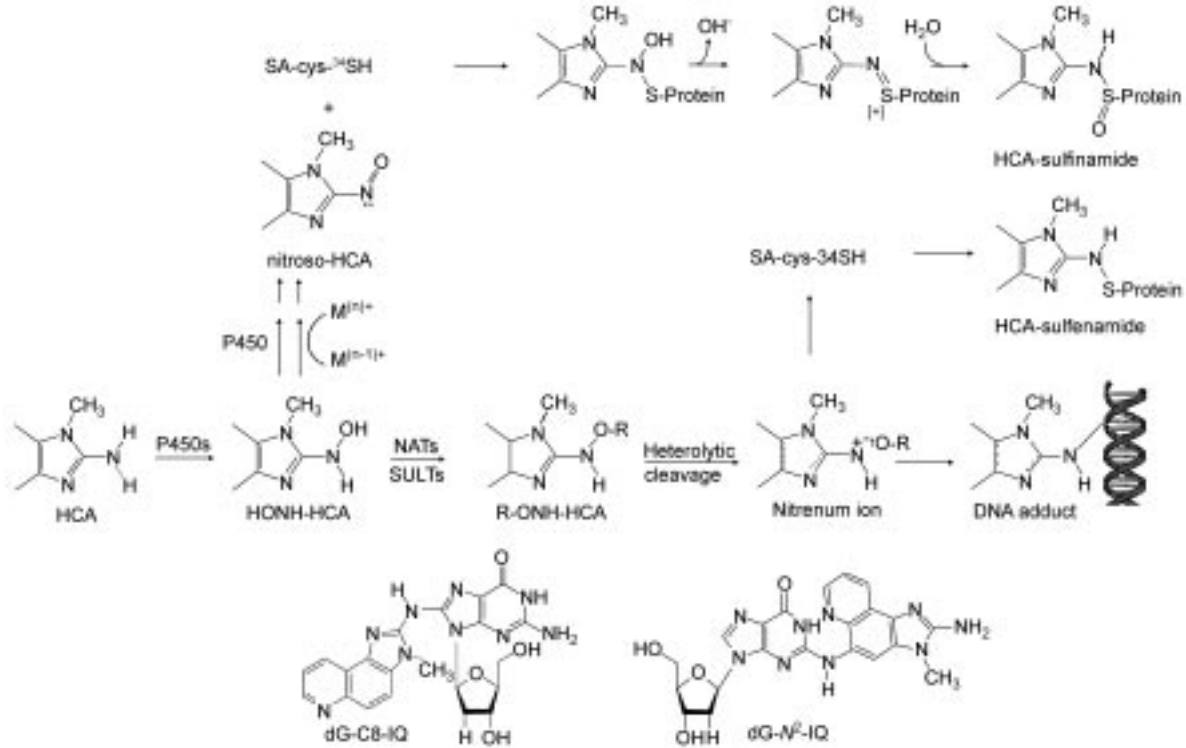

Fig. 12.2 Metabolic activation of HCAs by P450s to the *N*-hydroxy-HCAs, followed by metabolic conjugation with phase II enzymes to produce reactive *N*-O-esters of HCAs. The *N*-O-esters of HCAs can form isomeric dG-C8-HCA and dG-N^2-HCA adducts. The reactive *N*-hydroxy metabolics or their oxidized nitroso metabolites, produced by P450s[112] or formed non-enzymatically through reaction with transition metals,[113] also may react with cys-34 thiol group of SA.[84–86,108,109]

hepatic P450 1A2 in rodents and humans, and by P450s 1A1 and 1B1 in extrahepatic tissues.[10,29–34] The principal HCA-DNA adducts arise from reaction between the C8 atom of dG and the exocyclic amino groups of the HCAs, to produce dG-C8-HCA adducts.[14,21,28] For IQ and MeIQx, adducts also have been reported to form between the N^2 group of dG and the C-5 atom of IQ and MeIQx, indicating charge delocalization of the nitrenium ion over the heteronucleus of the respective HCAs (Fig. 12.2).[35] These adducts are believed to be responsible for the mutagenic effects of HCAs. Protein adduct formation also occurs following metabolic activation of the HCAs to the *N*-hydroxy-HCA species, and is discussed in Section 12.4.3.

12.2.2 Mutagenesis assays

Bacterial mutagenesis assays have been successfully used to identify environmental mutagens and experimental animal carcinogens bearing possible risk for humans.[8] Certain HCAs are among the most potent mutagens ever tested in the Ames bacterial reversion assay.[5,8,36] The high response of these HCAs in inducing frameshift revertant mutations in *Salmonella typhimurium* TA98 and TA1538 tester strains is attributed to a preference for the HCAs to react at a site about nine base pairs upstream of the original CG deletion in the *his*D^+ gene, in a run of GC repeats.[37] Strong genotoxic effects of several HCAs are also observed in strain TA100, which reverts to the wild type through point mutations. The mutagenic potency of HCAs is dependent upon their chemical structures and ability to undergo *N*-oxidation to form the reactive nitrenium ion.[38] The genotoxic potencies of HCAs vary over a >1,000-fold in bacterial mutagenicity assays, but the wide range of potencies is not paralleled in mammalian cell assays, where biological activity is weaker.[39–41] The discrepancies among the biological potencies determined for the HCAs in these *in vitro* assays are due to differing metabolic activation systems, differing DNA adduct repair capacities, differening gene locus endpoints for mutagenicity, and different base sequence contexts and neighboring base effects on the HCA-DNA lesions, all of which affect mutation frequencies.

The risk assessment for HCAs, based upon short-term mutagenesis assays, is tenuous. The complexity of the biological effects and the uncertainties in risk assessment of HCAs will be demonstrated in this review, using as examples, MeIQx and PhIP. The mutagenic potency of PhIP is about 100-fold weaker than that of MeIQx in the Ames reversion assay, when rat liver S-9 is used for bioactivation, even though PhIP undergoes *N*-oxidation (bioactivation) by rat liver microsomes or by rat P450s 1A1 and 1A2 at significantly higher rates than does MeIQx.[11] However, both compounds are strong rodent carcinogens and induce tumors at multiple sites, in studies involving long-term feeding at comparable doses.[5,7]

12.2.3 Carcinogenicity

Despite the large differences in genotoxic potencies of HCAs in various cell assays, the carcinogenic potency of various HCAs in experimental animals is

within approximately ten-fold, depending upon the species or organs affected.[5,7] HCAs are carcinogenic in rodents, and they induce tumors at multiple organs that include the oral cavity, liver, stomach, lung, colorectum, prostate and mammary glands, during long-term feeding studies.[5–7,42,43] IQ is also a powerful liver carcinogen in non-human primates. Tumor induction occurs within several years of treatment, making this compound one of the most powerful carcinogens assayed in cynomolgous monkeys.[44] The inconsistencies between the biological responses of HCAs in *in vitro* assays and *in vivo* in experimental laboratory animals underscore the need for mechanistic studies to assess genetic damage through measurement of DNA adduct formation and elucidation of target genes of mutagenesis in experimental animal models and in humans.[5] Biomarkers of exposure, biologically effective dose, and genetic damage are needed for interspecies extrapolation of toxicity data, and to assess the role of HCAs in the initiation of human cancers.[45]

12.2.4 DNA adducts of HCAs in experimental animals

There are numerous reports in the literature on HCA-DNA adduct formation in experimental laboratory animal models. The principal adducts of HCAs formed *in vivo* have been characterized as dG-C8-HCA or dG-N^2-HCA adducts, by means of ^{32}P-postlabeling techniques.[14,21,28,46–53] Recent studies have assayed HCA-DNA adducts in animals by liquid chromatography/electrospray ionization tandem MS (LC/ESI-MS/MS) techniques, although the number of citations is small.[21,54–58] DNA adducts have been detected both in target and in non-target tissues of animals that succumb to tumors. The level of adduct formation in tissues does not necessarily correlate with tumorigenesis, indicating that other factors, such as cell proliferation, are important in the tumorigenesis process.[14]

Most of the studies on DNA adduct formation have been conducted at elevated doses (1–10 mg HCA/kg body weight); however, DNA adducts of MeIQx have been measured, by accelerator mass spectrometry (AMS), in the livers of rats given MeIQx in doses varying 10^6-fold.[59] Adduct formation was reported to occur as a linear function of dose, and adducts were formed at dose levels approaching those of human exposures.[59] A linear dose response in adduct formation was also observed for the dG-C8- and dG-N^2-IQ adducts in the liver of rats by ^{32}P-postlabeling.[60] The amount of dG-N^2 adducts of IQ and 8-MeIQx formed using biomimetic methods is small relative to the dG-C8 isomers;[35] however, the dG-N^2 adducts persist *in vivo* to become the prominent lesions in slowly dividing tissues of rats and non-human primates given IQ,[49,60–62] or following varying levels of exposure to MeIQx.[57] The contribution of these HCA adducts to mutagenesis requires further investigation.[63,64]

12.2.5 DNA adducts of HCAs in humans

There are several reports on the detection of HCA-DNA adducts in human tissues.[22,26,65-69] Humans given diet-relevant amounts of ^{14}C-radiolabeled-

MeIQx or PhIP had higher DNA adduct levels, detected by AMS, in the colon than did rats given the same size proportional dose of chemical.[66–68,70–72] [^{14}C]PhIP-DNA adduct formation in white blood cells (WBC) of the subjects given an oral dose of PhIP (70 μg/person) reached levels of modification up to 1.5 total adducts/10^9 DNA bases 4–6 hr post-treatment,[67] but decreased to ~0.5 adducts/10^9 DNA bases 24 hr post-treatment. It is noteworthy that the levels of DNA adducts in WBC were higher than the adduct levels formed in the colon of these subjects at 48–72 hr post-treatment with PhIP.

A GC/MS assay of the parent HCAs, based upon alkaline hydrolysis of putative dG-C8-HCA adducts, revealed the presence of PhIP in colorectal mucosa of several individuals at levels of up to several adducts per 10^8 DNA bases, when 100 μg of DNA were used for analysis.[26] Another study detected a base-labile adduct of PhIP, presumably dG-C8-PhIP adduct, in long-lived lymphocytes of colorectal cancer subjects at levels of several adducts per 10^8 DNA bases, when 100 μg of DNA were measured.[22] This adduct was detected in about 30% of the population, and the levels of adduct varied ten-fold between the lowest and highest levels, suggesting differences in the intake of PhIP or interindividual variation in PhIP bioactivation. The adduct levels were not significantly higher in smokers or high meat consumers than in individuals who ate meat less frequently ($\leq$5 servings per week). Unfortunately, the extent of HCA exposure was not known. A second study, using a similar analytical approach, failed to detect PhIP-DNA adducts in the lymphocytes of healthy individuals who consumed a single meal of well-done grilled meat (275 g). This failure to detect PhIP adducts may have been due to the insensitivity of the assay; the lower limit of detection was only ~1 adduct per 10^7 DNA bases, when 500 μg DNA were used.

Two studies have reported DNA adducts of PhIP in human breast tissue. The dG-C8-PhIP adduct was detected in exfoliated epithelial cells from milk of lactating mothers in 30 of the 64 samples analyzed, with a mean value of 4.7 adducts/10^7 nucleotides, by the use of the ^{32}P-postlabeling method.[73] In another study, PhIP adducts, presumably dG-C8-PhIP, were detected in human breast tissues at levels of >1 adduct per 10^7 bases, by means of an immunohistochemistry method, in 82 and 71% of the normal breast tissue sections from the cancer and control patients respectively.[74]

The dG-C8-MeIQx adduct was also detected by the ^{32}P-postlabeling assay in colon and kidney DNA of several individuals at levels estimated at several adducts per 10^9 DNA bases.[65] Collectively, these findings reveal that HCA adduct formation does occur in human tissues, even though the concentrations of HCAs in the diet are generally at low ppb levels.

None of the analytical methods employed for the detection of adducts in all of the studies cited above provides structural information; therefore, the identities of the adducts are ambiguous. Contemporary triple quadrupole mass spectrometers are robust and sensitive instruments that have emerged as a critical analytical tool for the identification and quantitation of DNA adducts of HCAs and other carcinogens at trace levels; these capabilities are a requisite for

measurements in human populations.[21,75-77] Moreover, the problems associated with the uncertain identification of adducts by the ^{32}P-postlabeling or immunodetection methods are circumvented by tandem MS techniques, particularly when stable isotopically labeled internal standards are employed in the analysis.[21,75] The limits of adduct detection by LC/ESI-MS/MS approach the levels that can be measured by ^{32}P-postlabeling.[21,75]

12.3 HCA-protein adduct formation with hemoglobin and serum albumin

Circulating blood protein adducts have been used as an alternative to DNA adducts in the biomonitoring of exposure to some classes of carcinogens. Hemoglobin (Hb) and serum albumin (SA) are the two most abundant proteins in blood. Hb is responsible for binding oxygen in the lung and for transporting the bound oxygen throughout the body, where it is used in aerobic metabolic pathways. Adult hemoglobin is a [α(2):β(2)] tetrameric heme protein found in erythrocytes at abundant concentrations (140–180 mg ml^{-1} whole blood), with a 120-day lifetime.[78] SA is the most abundant protein in plasma (~45 mg ml^{-1}) and has a half-life of about 20 days.[79] The functions of SA include maintenance of osmotic pressure and transport of both endogenous chemicals (i.e., fatty acids, bilirubin, steroids) and exogenous ones (drugs).[79,80] Hb and SA, among other proteins, can trap reactive electrophilic metabolites of a variety of carcinogens that include alkylating agents,[81] aromatic amines,[78,82,83] HCAs,[84–86] aflatoxin B_1,[78,87] sulfur mustards,[88] acrylamide,[89] and polycyclic aromatic hydrocarbons (PAHs).[78]

Much of this work cited above is derived from the pioneering studies of the Millers and is based upon the paradigm of chemical carcinogenesis in which electrophilic species or electrophilic metabolites of carcinogenic compounds react with nucleophilic centers on proteins as well as DNA.[90,91] The advantage of measuring protein adducts, such as those bound to Hb or SA, is that the amount of protein recovered from blood is far greater than the amount of DNA available; thus, protein adducts serve as a more sensitive dosimeter, particularly when the dose exposure is intermittent. Moreover, Hb and SA protein adducts are not subject to repair; thus, if the adduct is stable, chronic exposure should result in accumulation of protein adducts during the lifespan of the protein.[78] The disadvantages of biomonitoring blood protein adducts are: (i) the adduct does not represent genetic damage, and (ii) adduct formation does not occur at the target site of tumorigenesis. However, protein adducts can be used to measure the biologically effective dose of the carcinogen, and in some cases can be correlated to xenobiotic metabolism enzyme (XMEs) polymorphisms that can modulate protein or DNA adduct formation.[92] Hb adducts of 4-aminobiphenyl (4-ABP) and other primary arylamines,[92,93] and SA adducts of aflatoxin B_1,[94,95] have been successfully used in risk assessment of bladder and liver cancer, respectively.

12.3.1 Aromatic amine and HCA sulfinamide adduct formation with Hb

Several investigations have been reported on the reactivity of the genotoxic *N*-hydroxylated HCA metabolites with Hb and SA. Studies in rodents have shown that metabolites of radiolabeled IQ,[84] MeIQx,[96] and PhIP[97] bind to circulating blood proteins at very low levels (<0.01% of the dose), and that a higher percentage of the dose binds to SA than to Hb. In contrast to these HCAs, 4-ABP binds to Hb at levels up to 5% of the dose in the rat model, with lower amounts bound to SA.[78,82,98] The major adduct of 4-ABP formed with Hb has been determined by X-ray crystallography and it was found to be a sulfinamide linkage that occurs at the cys-93 residue of the β chain of human Hb.[99] This adduct occurs by the co-oxidation of oxy-Hb with the genotoxic *N*-hydroxy metabolite of 4-ABP to form met-Hb and the nitrosobiphenyl.[100,101] Thereafter, the nitroso species can react with the β cys-93 residue to form the sulfinamide adduct, or can undergo reduction by reductases within the erythrocyte to re-form the arylhydroxylamine and initiate another round of co-oxidation with oxy-Hb.[78,101] This arylamine-Hb sulfinamide adduct has been successfully used to biomonitor exposure to 4-ABP and other primary arylamines in tobacco smoke and to assess bladder cancer risk.[93,102]

The HCAs studied thus far do not react with Hb at appreciable levels in the rodent or humans to form Hb sulfinamide adducts, in contrast to what has been reported for the 4-ABP and other arylamines.[78,82,103] However, *N*-hydroxy-IQ does catalyze methemoglobinemia in isolated human erythrocytes and forms an acid-labile sulfinamide adduct.[84] These results prove that the *N*-hydroxy-IQ metabolite is capable of penetrating the erythrocyte and reacting with the oxy-Hb to produce the toxic effects that are associated with arylhydroxylamines.[100,101] The absence of this IQ-sulfinamide-Hb adduct in the rodent suggests that very little of the *N*-hydroxy-IQ escapes the hepatocyte. However, the tryptophan pyrolysis mutagen, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), was shown to bind more effectively than IQ, MeIQx or PhIP to these proteins in rats, in which 0.2 and 0.07% of the administered [^{14}C]Trp-P-1 formed stable covalent adducts with Hb and SA respectively.[104] The identities of these adducts remain unknown.

12.3.2 HCA adduct formation with SA

Human serum albumin (HSA) consists of 585 amino acids. The protein is composed of three homologous domains (I,II,III), each of which is the product of two subdomains (A,B), resulting in six distinct regions that can be defined by structure.[79] The cys-34 is one of 35 conserved cysteine residues in SA across species. Thirty-four of these cysteines are involved in 17 disulfide bonds. The single unpaired cys-34 is present either as a free thiol or in an oxidized form, with approximately 30% of this residue present as disulfide linkages with low molecular weight thiols. The cys-34 residue of SA is thought to be responsible for much the antioxidant capacity of SA and accounts for ~80% of the net free thiols in plasma,[105,106] and serves as a major transporter of NO in blood.[107] The

scavenging properties of cys-34 of rat and human SA with reactive carcinogenic metabolites and toxic electrophiles have been reported for IQ,[84] 8-MeIQx,[85] PhIP,[108,109] acrylamide,[89] sulfur mustard,[88] benzene,[110] and acetaminophen.[111] The adducts of IQ, MeIQx, and PhIP bound to SA at the cys-34 residue undergo hydrolysis with dilute acid or base, to produce the parent HCA.[84–86]

The acid-labile adduction product derived from the reaction of *N*-hydroxy-IQ with rat SA was isolated and spectroscopically characterized, following enzymatic digestion with pronase. Based upon MS, ^{1}H NMR, and amino acid analysis, the structure of the adduct was elucidated as a tripeptide containing an IQ-sulfinamide linkage, which formed through the cys-34 residue and nitroso-IQ.[84] This adduct also formed in rodents given IQ and accounted for about 10% of the total adducts bound to SA.[84] A scheme for HCA-sulfinamide-SA adduct formation, based upon studies with IQ,[84] is depicted in Fig. 12.2. The *N*-hydroxy metabolite produced by P450 1A2 in the liver can undergo further oxidation by P450s[112] or it can undergo non-enzymatic reaction with transition metals,[113] to form nitroso compounds that are able to react with the cys-34 residue of SA, producing a sulfinamide adduct. Sulfenamide linkages also may form through reaction of cys-34 with the esterified *N*-hydroxy-HCAs.[109]

One PhIP-SA adduct, which was reported to undergo hydrolysis to form PhIP following acid treatment *in vitro*, was formed in rats as a linear function of dose.[86] However, the structure of this intact PhIP-SA adduct has not been isolated, and the identity remains to be elucidated. Two studies on the reactivity of *N*-oxidized metabolites of PhIP with SA *in vitro* have provided different results on the identities of PhIP-SA adducts. Reistad and colleagues[108] conducted studies on the reactions of *N*-acetoxy-PhIP with rat SA (RSA). They did not identify a PhIP sulfinamide linkage following reaction of *N*-acetoxy PhIP with RSA, but they did detect a C-S linkage at the cys-34 residue, where the cysteine group bound to the C-2 imidazole atom of PhIP. The adduct was proposed to form through nucleophilic displacement of the 2-nitro derivative of PhIP, an oxidation product of *N*-acetoxy-PhIP, by the thiol group of cys.[34,108] This adduct does not release PhIP upon acid hydrolysis of SA.

In another study conducted *in vitro*, Dingley and co-workers[109] identified an adduct formed from reaction of *N*-acetoxy-PhIP and a model peptide with the internal sequence Leu-Gln-Cys-Pro-Tyr-OH as a sulfenamide linkage, with no evidence for oxidation of the sulfhydryl group as would occur for a sulfinamide linkage. They found no evidence to corroborate for the formation of the C-S linked adduct that had been reported by Reistad and colleagues.[108] The proposed structure of adducted peptide reported by Dingley and colleagues is consistent with direct reaction of *N*-acetoxy-PhIP with the SA-cys-34 moiety to form an acid-labile sulfenamide adduct (Fig. 12.2), which was found to be stable for several days at room temperature. The stability of this adduct is surprising, since sulfenamides of aromatic amines and HCAs are usually unstable.[114,115] The differing protein adduction products of PhIP identified in these studies could be due to different reaction conditions, concentrations of reactants, or peptide sequences, any or all of which could affect the reactivity of PhIP oxidation products with SA.

There are limited data on HCA-protein adduct formation in humans. The pilot studies using AMS for HCA-DNA adduct formation revealed that the levels of human PhIP-SA adducts and MeIQx-SA adducts within this small group of subjects (N = 5) were ~30-fold and fourfold higher, respectively, than the HCA-SA adducts formed in rats given comparable doses of these carcinogens.[67,72] The elevated levels of both protein and DNA adducts of PhIP and MeIQx in humans, as compared to the rat, can be attributed to superior catalytic efficiency of human P450 1A2 over rat P450 1A2 in the bioactivation of these HCAs,[11,116–118] resulting in higher levels of the genotoxic metabolite available for adduct formation. The structures of these protein adducts remain to be elucidated.

There is one report that describes the biomonitoring of acid-labile PhIP-SA adducts in humans.[86] Acid-labile PhIP-SA adduct(s) were detected in human subjects on a normal diet, by negative ion chemical ionization/gas chromatography/mass spectrometry (NICI/GC/MS) or LC/EIS/MS, following immunoaffinity chromatography. Adduct levels were tenfold higher in meat-eaters than in vegetarians (6.7 ± 1.6 vs 0.7 ± 0.3 fmol PhIP bound/mg protein; mean $\pm$ SE). We can hypothesize that the acid-labile PhIP-SA adduct is either a sulfinamide, formed by reaction of the cys-34 of HSA with the nitroso-PhIP metabolite, or a sulfenamide linkage, formed by reaction with an esterified *N*-hydroxy-derivative of PhIP, based upon previous studies with HCAs [84,85,109] (Fig. 12.2).

12.4 Analysis of HCAs and their metabolites in human urine

12.4.1 Identification of HCAs in urine

Urine is a useful biological fluid for the measurement of exposure to various classes of carcinogens, since large quantities can be obtained noninvasively.[119] The measurements of HCAs or their metabolites in urine do not shed light on DNA damage; however, these measurements can assess the capacity of an individual to bioactivate and detoxicate HCAs and thus assess the impact of XMEs on health risk.[120] HCAs are rapidly absorbed from the gastrointestinal tract and are eliminated in urine as multiple metabolites, with several percent of the dose present as the parent compounds within 24 hr of consuming grilled meats.[23,24,121,122] In the case of MeIQx, lower levels of the unmetabolized compound were found in urine of individuals with high P450 1A2 activity, indicating that P450 1A2 is an important enzyme in the metabolism of MeIQx *in vivo*.[123]

The contribution of P450 1A2 in the metabolism of PhIP in humans was reported to be less important than the enzyme's contribution in the metabolism of MeIQx.[124] However, glutathione *S*-transferases are able to reduce *N*-hydroxy-PhIP and *N*-acetoxy-PhIP back to the parent amine, a reaction which can obscure the relationship between rapid P450 1A2 activity and PhIP metabolism.[125] The importance of P450 1A2 in the metabolism of MeIQx and PhIP in humans was demonstrated through a pharmacokinetic study with furafylline, a

mechanism-based inhibitor of P450 1A2;[126] in this study, as much as 91% of the MeIQx and 70% of the PhIP following consumption of grilled meat were estimated to undergo metabolism by the enzyme.[121]

HPLC with UV or fluoresence detection has been used to measure MeIQx, PhIP, and tryptophan pyrolysate mutagens in the urine of healthy volunteers on a normal diet.[127] The extraction method required multiple chromatographic purification steps before the HCAs could be identified. A simpler extraction scheme was developed for the isolation of HCAs from urine, followed by chemical derivatization and GC/NICI/MS, for the measurement of MeIQx, 4,8-DiMeIQx, and PhIP.[24,128,129] Other investigators have used immunoaffinity chromatography, followed by analysis of HCAs with either LC/ESI-MS/MS detection,[124] HPLC with fluorescence detection,[130] or GC/NICI/MS.[25] The urinary excretion of MeIQx and total acid-labile phase II conjugates of MeIQx in male African/American subjects was 1.3- and 3.0-fold higher than in Asians and Caucasians, respectively.[131] The urinary levels of MelQx were positively associated with intake frequencies of bacon, pork/ham and sausage/luncheon meats among study subjects. However, the urinary excretion levels of PhIP were not associated with intake frequencies of any cooked meat based on the self-administered dietary questionnaire responses of the same group of subjects.[132] Therefore, urinary excretion levels of a single HCA may not serve as a reliable predictor of other levels of HCAs in estimating exposure to these compounds for humans consuming unrestricted diets.

Recently, we developed a tandem solvent-solid phase extraction procedure to isolate multiple HCAs from urine, and we employed LC/ESI-MS/MS for quantification of HCAs at low parts per trillion (ppt) levels, using less than 1 ml of urine for analysis.[133] MeIQx, PhIP, and 4,8-DiMeIQx were detected, as had previously been reported by GC/NICI/MS analysis,[134] but we additionally identified 2-AαC, a novel isomer of MeIQx,[133] and 2-amino-1-methyl-imidazo[4,5-b]quinoline (IQ[4,5-*b*]), the linear tricyclic ring isomer of the powerful carcinogen IQ (Fig. 12.3).[135] IQ[4,5-*b*] was also detected in urine of several individuals who had refrained from meat consumption, and it was found in urine of vegetarians, following base hydrolysis of urine samples.[135] The amounts of IQ[4,5-*b*] measured in urine of human volunteers who consumed grilled beef ranged from 15 to 135% of the ingested dose, while the amounts of MeIQx and PhIP excreted in urine were on average <2% of the ingested dose.[135] Base treatment of urine at 70 ºC increased the concentrations of MeIQx and PhIP by as much as six-fold, indicating the presence of phase II conjugates; however, the amount of IQ[4,5-*b*] increased by more than 100-fold. The formation of IQ[4,5-*b*], but not IQ, MeIQx, or PhIP, also occurred over time in freshly isolated urine incubated at 37 ºC. The detection of IQ[4,5-*b*] in urine of both meat eaters and vegetarians suggests that this HCA is present in non-meat staples, such as cooked eggs or pasteurized cheese, or that IQ[4,5-*b*] formation occurs endogenously in the urine or other biological fluids. Creatinine and 2-aminobenzaldehyde are likely precursors of IQ[4,5-*b*] formation *in vivo*.[135]

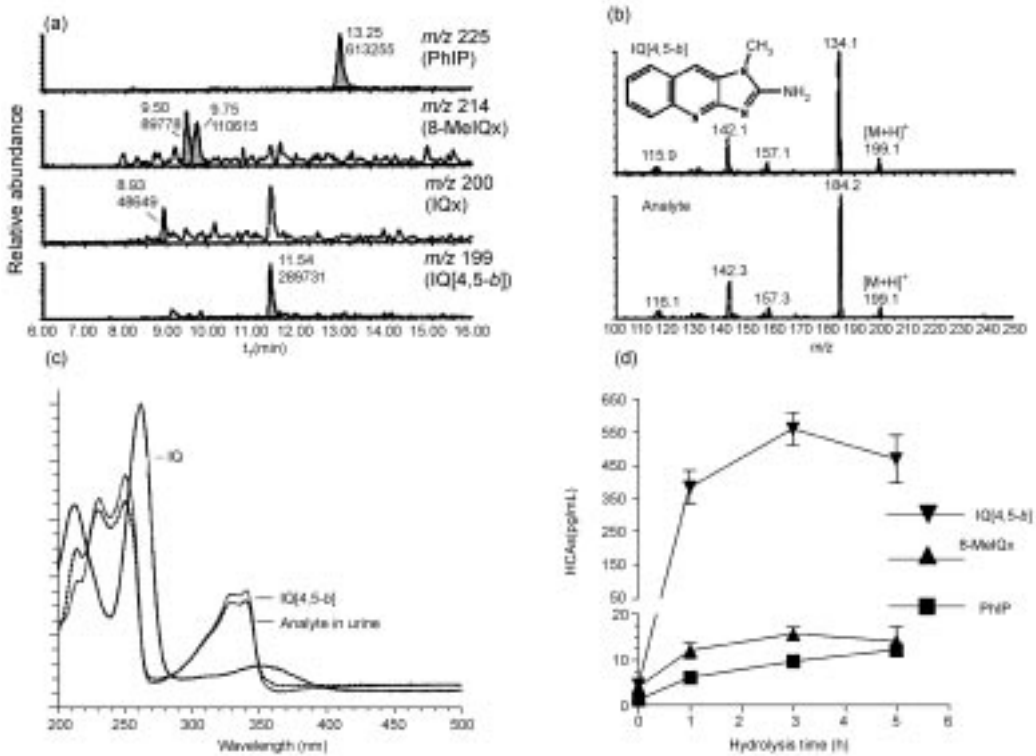

Fig. 12.3 Identification of known and novel HCAs in human urine using the constant neutral loss scan mode $[M+H \rightarrow M+H-CH_3]^+$. (a) Identification of IQx (t_R 8.93 min), isomer of MeIQx (t_R 9.50 min), 8-MeIQx (t_R 9.75 min), IQ [4,5-*b*] (t_R 11.54 min), and PhIP (t_R 13.25 min) in urine of meat-eaters. (b) Product ion spectra of the analyte and synthetic IQ [4,5-*b*] standard. (c) HPLC-UV spectra of the analyte obtained from urine, synthetic standards of IQ[4,5-*b*] and IQ. (d) Kinetics of HCA formation in the urine of a meat-eater as a function of base hydrolysis (1 N NaOH) over time at 70 ºC. Figures adapted from reference.[135]

12.4.2 Analysis of HCA metabolites in human urine

The analyses of urinary metabolites of MeIQx and PhIP, by means of using HPLC with liquid scintillation counting, have been conducted in human subjects as part of the pilot AMS studies for measurement of protein and DNA adducts.[122,136] Other studies have characterized HCA metabolites in urine, following consumption of cooked meats, with HPLC/fluorescence,[130,137] GC/MS[129] or LC/MS used for the detection of analytes.[23,122,134,138] Both MeIQx and PhIP undergo extensive metabolism *in vivo*, so that only several percent of the dose is eliminated in urine as the parent compounds. Direct glucuronidation of these HCAs and glucuronidation of the *N*-hydroxylated metabolites were reported as major pathways of metabolism (Fig. 12.4).

The levels of glucuronide conjugates of *N*-hydroxy-MeIQx and *N*-hydroxy-PhIP in urine were significantly higher than the levels observed in urine of rodents,[23,122,134] a finding consistent with the superior catalytic efficiency of human P450 1A2, relative to the rat orthologue, in N-oxidation of HCAs.[11,15] In the case of MeIQx, oxidation of the C^8-methyl group was the major pathway of transformation, and the detoxicated metabolite, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline-8-carboxylic acid (IQx-8-COOH),[118] accounted for more than 50% of the dose excreted in urine of subjects.[122] All of the oxidation steps to form the carboxylic acid are catalyzed by P450 1A2;[17] this finding underscores the prominent role of P450 1A2 in the metabolism of MeIQx in humans.[121] In contrast to many primary arylamines,[139,140] most HCAs do not undergo direct metabolism by NATs to form detoxicated *N*-acetylated products.

12.4.3 Interspecies differences in P450-catalyzed oxidation of HCAs

There are important species differences in the regioselectivity of HCA oxidation by P450s. For example, the rat P450s 1A1 and 1A2 catalyze the oxidation of MeIQx at the C-5 atom, as the major pathway of detoxication (Fig. 12.5), but unlike human P450s 1A1 and 1A2, neither rat enzyme catalyzes the oxidation of the C^8 atom to form the detoxicated product, IQx-8-COOH.[117,118] In contrast to the rodent P450s, neither human P450 1A1 nor 1A2 catalyzes the 5-hydroxylation of MeIQx.[17,122,141]

Important regioselective differences also exist between rat and human P450s 1A1 and 1A2, in the oxidation of PhIP. The rat P450 orthologues efficiently catalyze the 4′-hydroxylation of PhIP as a major pathway of detoxication, while the human P450s preferentially carry out oxidation at the exocyclic amino group to produce the genotoxic *N*-hydroxylated metabolite.[11,34,142,143]

There are also important differences between the rat and human P450 1A2 orthologues in terms of the catalytic efficiency of *N*-oxidation of MeIQx and PhIP. The human form is considerably more active than is the rat counterpart in formation of the genotoxic *N*-hydroxy-HCA metabolites.[11,15] The differences between human and rat P450s in protein expression, catalytic activity, and regioselectivity of HCA oxidation affect the toxicological properties of these genotoxicants and must be considered in any human risk assessment for HCAs.[11,144]

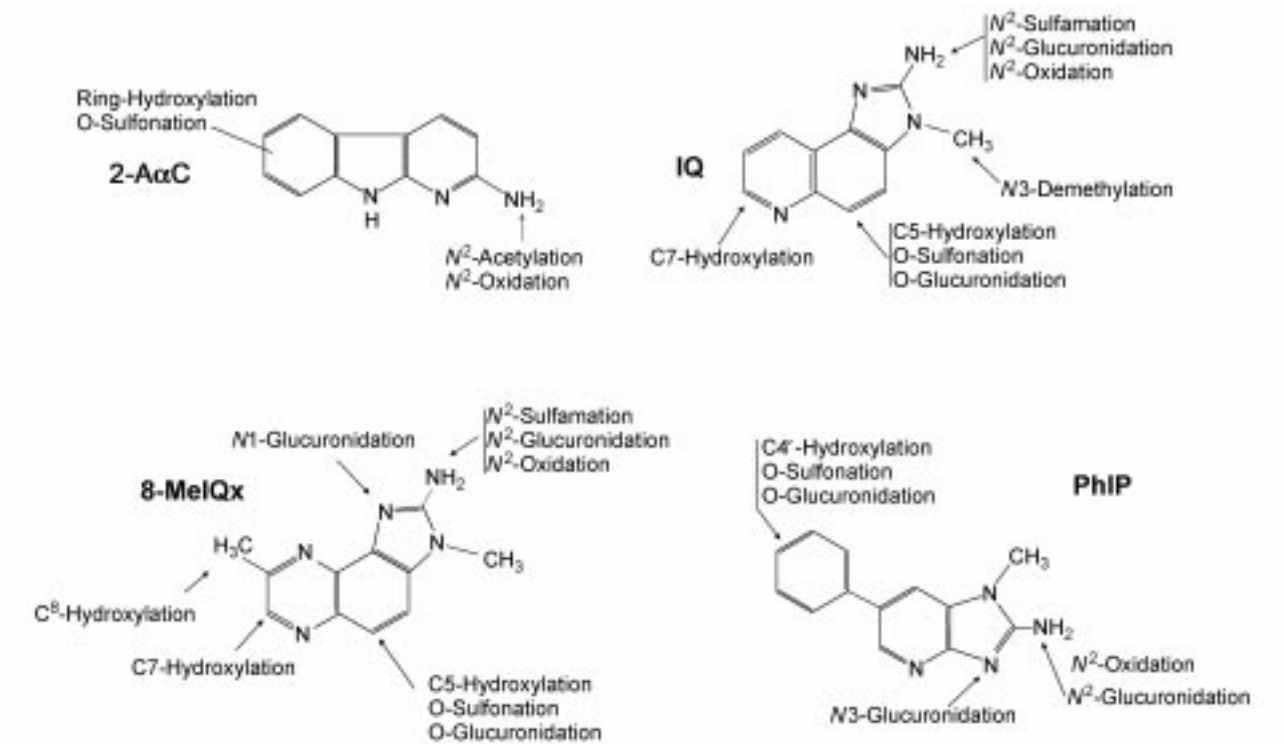

Fig. 12.4 Major pathways of HCA metabolism in experimental laboratory animals and humans.

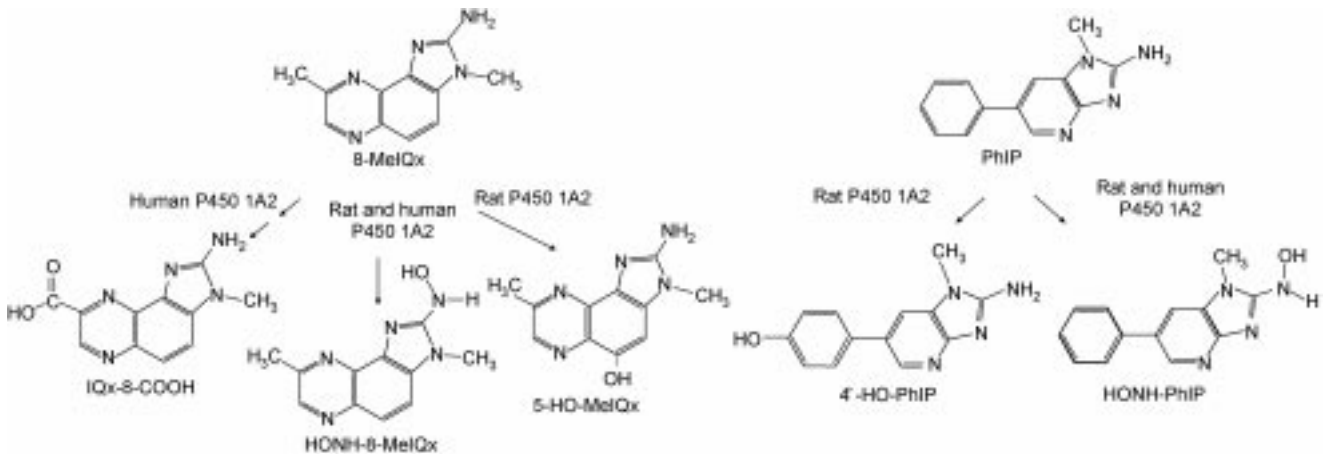

Fig. 12.5 Species differences in regioselectivity of P450-mediated oxidation of 8-MelQx and PhIP.

12.5 Other potential HCA biomarkers

The 5-hydroxylated derivative of PhIP, a decomposition product of *N*-acetoxy-PhIP (one of the ultimate genotoxic metabolites of PhIP) has been proposed as a biomarker of the biologically effective dose.[145] One pilot study reported the presence of this metabolite in human urine at approximately 1% of the ingested dose.[146] Some compounds, including drugs, nicotine and narcotics, bind to melanin in hair and provide information on the exposure history of the toxicants for long time periods. The biomonitoring of hair for the accumulation of HCAs provides a long-term biomarker of HCA exposure, and can be used in conjunction with the analysis of urinary metabolites that capture exposure over the past 24 hr, or determination of HCA-DNA and -protein adducts that can reflect exposure over several weeks.[145]

In mice, PhIP was reported to irreversibly incorporate in a dose-dependent manner into hair,[147] and PhIP was found to accumulate in hair, from <50 to 5,000 pg PhIP/g hair, in human subjects on an ordinary diet in Norway.[145] Another group reported that PhIP was present in 42 out of the 46 hair samples from 23 healthy volunteers in Japan, with PhIP levels ranging from 110 to 3,878 pg/g hair.[148] The use of PhIP in hair as a biomarker of exposure is promising, but requires both validation, and correlation to other endpoints of exposure assessment.

12.6 Future trends

The major pathways of metabolism of MeIQx and PhIP are well characterized in humans.[23,122,124,134,136,138,141,149,150] The findings of these analyses have shown interspecies similarities as well as important differences in the enzymology of HCA metabolism; both factors can influence the toxicological properties of these genotoxicants. As the sensitivity of MS instrumentation continues to improve, the amount of urine or other biological specimen required for assays will decrease; also the purification schemes will likely become more simplified, enabling rapid-throughput studies. One recent study reported the analysis of PhIP metabolites in human urine without any pre-fractionation of the analytes.[138]

The high sensitivity of MS has also permitted the detection of HCA-DNA adducts in experimental animals at levels ~1 adduct per 10^8 DNA bases, with only 100 μg DNA used for analysis.[21] Recent advances in MS instrumentation and ion source interfaces, serving to increase the transmission efficiency of analytes, could soon permit the detection of DNA adducts at even lower levels, or the use of less DNA for measurements. The level of sensitivity of adduct detection by LC/MS is approaching the level that is detectable by ^{32}P-postlabeling (ca. ~1 adduct per 10^9 DNA bases), and LC/MS techniques can be envisioned for biomonitoring of HCA-DNA adducts in human populations. Indeed, indirect methods of HCA-DNA adduct detection in humans, following hydrolysis of DNA to recover PhIP, have already been reported by two different

laboratories.[22,26] The levels of HCAs bound to long-lived blood proteins, such as Hb or SA, are considerably lower than the amounts of 4-ABP or other primary arylamines,[93,102] however, the high level of sensitivity provided by LC/MS could also permit the detection of HCA protein adducts in human populations.[86]

Future studies will require MS analysis of biomarkers of multiple HCAs, since the extent of exposure to various HCAs can vary in the diet. Through a combination of analyses for multiple urinary biomarkers, HCA-protein and HCA-DNA adducts, and other sources of longer-term exposures, such as HCA accumulation in hair, it may be feasible to assess exposure to HCAs, the biologically effective dose of each HCA, and the resultant genetic damage. With the identification of such biomarkers, the interactive effects of genetic polymorphisms of XMEs involved in HCA metabolism (activation and detoxication) may be correlated to the levels of adduction products and cancer risk in human population studies. These analyses should clarify the role of HCAs as dietary factors in the initiation of colorectal and other common human cancers.

12.7 Sources of further information

There are a number of excellent review articles and books that may be used for consultation and additional readings.

The pioneering studies of T. Sugimura and M. Nagao were first reported in *Mutagen-carcinogens in food, with special reference to highly mutagenic pyrolytic products in broiled foods, Origins of Human Cancer*, Book C (editors, Hiatt, H.H., Watson, J.D., Winstein, J.A.), Cold Spring Harbor Publishing, Cold Spring Harbor, NY, 1977, pp. 1561–1577.

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13

Risk assessment techniques for acrylamide

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13.1 Introduction

13.1 Background

Acrylamide is a well-known industrial chemical that has been produced on a large scale for many years and is widely used for the production of organic chemicals as an intermediate and as a monomer for the production of polymers and copolymers. The uses are mainly as a water coagulant, but also in the mining industry, papermaking and electrophoresis gels in biochemistry IARC (WHO, 2005).

With regard to its potential health effects, acrylamide is a known neurotoxicant based on reports of occupational cases and experimental studies since the 1950s (Spencer and Schaumburg, 1974a,b) and was in 1994 classified as a probable human carcinogen (Group 2A) by the International Agency for Research on Cancer (IARC) (IARC, 1994). Similar classifications have been done by other authorities within the European Union and USA (US EPA, 1993).

Until the recent decade only occupational exposure to acrylamide was thought to represent a significant health risk. However, more recently it was discovered that cigarette smoking in 1997 (Bergmark, 1997) and also heated carbohydrate rich foods in 2002 (Tareke *et al.*, 2000, 2002) could represent a low but significant source of acrylamide exposure to the general population.

With this in mind the need for assessing the risk associated with exposure via food is obvious as a background for prioritisation of risk reduction as well as for the identification of gaps in the data base and research needs. Consultations and risk assessments were carried out at national and international levels, the latest being performed by the Joint FAO WHO Expert Committee on Food Additives (JECFA) (WHO, 2005). During recent years extensive research activity has been

put into understanding the chemical processes leading to acrylamide formation in foods and how to reduce its formation as well as exposure assessments, further toxicological studies on adverse effects, their mechanisms and dose response relationships and epidemiological studies.

13.1.2 Risk assessment paradigm

Risk assessment is performed in the framework of risk analysis (Fig. 13.1) where the interaction between science and society is described to ensure a transparent process (WHO, 1995, 2003, 2006). The risk assessment paradigm (Fig. 13.2), which is widely agreed upon, consists of three separate steps:

1. Exposure assessment, which is the description of the exposure and its variation in a given population.

Fig. 13.1 The risk analysis framework, taken from WHO 2006 (2006).

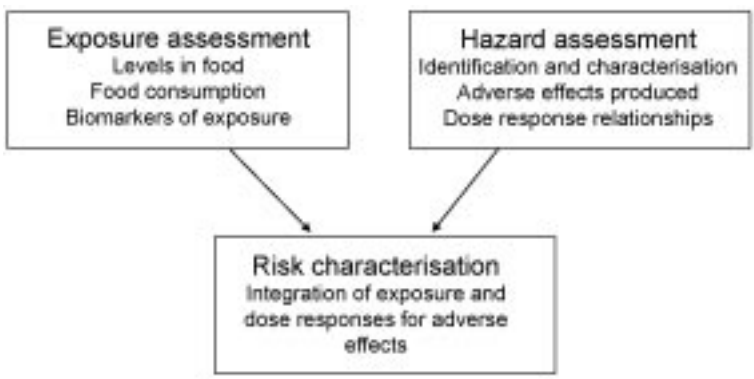

Fig. 13.2 Risk assessment – paradigm.

2. Hazard assessment, which is done in two steps:
   a. identification of the relevant adverse health effects
   b. characterisation of their dose-response relationships from studies in experimental animals and observations in humans.
3. Risk characterisation, which is the combination of the two: the risk of different adverse health effects in a population at a given exposure. The critical effect, which is used in the final risk characterisation, is the effect occurring first upon increasing exposure.

13.2 Exposure assessments

Determination of acrylamide in various foods including brands as well as the estimation of consumers' exposure to acrylamide via food has been a high priority for national food authorities as well as the food industry. A wealth of data has been collected since 2002 and was recently reviewed by an ILSI Europe task force on acrylamide in 2004 (Dybing *et al.*, 2005) (see also Chapters 9 and 10). Generally, the most important food items appear to be fried potato products, various heated cereals products: ready-to-eat breakfast cereals, cookies, pies, cakes and breads, and brewed coffee (Table 13.1). Typically, food surveys show that the levels of acrylamide in the same food category vary considerable, by orders of magnitude, even within the same brand. Main food sources of dietary acrylamide exposure vary with national and regional food habits. For example, in the US French fries and potato products contributed about 35% of the intake whereas coffee and bread were minor sources. In the Netherlands the contribution from potato products was even higher. Coffee and bread including crispbread contribute much more in European countries, e.g. coffee by 28 and 39% of total acrylamide in Norway and Sweden, respectively.

JECFA in their assessment received data on acrylamide levels in different food items from 24 countries comprising 6,752 samples during 2002–2004 (WHO, 2005). National intake data were provided by 17 countries and international intake estimates were prepared by combining the international weighed means of contamination levels with food consumption values reported in the GEMS/Food database. In the JECFA evaluation all regions were represented except Latin America and Africa.

Table 13.1 Contribution of foods to total dietary intake (WHO, 2005)

French fries	16–30%
Potato chips/crisps	6–46%
Coffee	13–39%
Cookies	10–20%
Bread, rolls	10–30%

The acrylamide intake estimates reported at national levels for the average in the general population ranged between 0.3 and 2 μg/kg bw/day. Estimated high level exposure (90 to 97.5th percentile) ranged from 0.6 up to 3.5 μg/ kg bw/day (WHO, 2005) for details see also Dybing and co-workers (Dybing *et al.*, 2005). Using the GEMS/Food regional diets mean intake estimates were between 3.0 and 4.3 μg/kg bw/day. Intake estimates by the Norwegian Food Control Authority were 0.49 (1.01) and 0.46 (0.86) μg/kg bw/day (mean and 90th percentile) for males and females, respectively, and for 13-year-old boys and girls the intake estimates were 0.52 (1.35) and 0.49 (1.2) μg/kg bw/day, respectively. Infants had a lower intake (Scientific Committee of the Norwegian Food Control Authority, 2002a,b). Exposure estimates from Berlin revealed average and 95 percentile intakes among 15–18-year-old youths of 1.16 and 3.24 μg/kg bw/day (Dybing *et al.*, 2005). JECFA decided to use 1 and 4 μg/kg bw/day as representative values for an average and a high intake (WHO, 2005).

The uncertainties associated with dietary exposure estimates for acrylamide are several, both on the underlying data on acrylamide contents in various foods as well as in the methods used in intake estimations. Widely different levels of acrylamide have been reported for similar foods; however, this does not seem to be attributable to inaccuracy of analytical methods even if the methodology including quality control has improved considerably (Dybing *et al.*, 2005). Little is still known about acrylamide in home-cooked foods. Present food consumption surveys were not designed for acrylamide purposes. A small double portion study performed in Switzerland provided an average intake estimate of 0.28 μg/ kg bw/day (Swiss Federal Office of Public Health, 2002), which is at the lower end of the reported range described above.

13.2.1 Biomarkers of exposure

Biomarkers of acrylamide exposure may provide a more accurate measure of exposure at the individual level (see Chapter 8 for a more detailed explanation). Haemoglobin adducts of acrylamide where the adduct formed at the N-terminal valine is determined, integrates the systemic internal acrylamide exposure during 120 days, the lifespan of the red blood cells. Haemoglobin adducts have for more than a decade been used for occupational exposure monitoring, but a low adduct level is also found in the general population with slightly higher levels in smokers (Bergmark *et al.*, 1993; Calleman *et al.*, 1994; Bergmark, 1997; Dybing *et al.*, 2005). Several studies show that the average haemoglobin adduct level is about 30 pmol/g globin. In a recent study the range was 20–100 pmol/g globin in non-smokers consisting of individuals with different food intakes of acrylamide (Hagmar *et al.*, 2005). Törnqvist and co-workers (1998) estimated on the basis of toxicokinetic considerations that an acrylamide adduct level of 30 pmol/g globin would correspond to an intake of about 1.1 μg/kg bw/ day in a 70 kg person (Bergmark, 1997; Dybing *et al.*, 2005). Hence, the range reported by Hagmar *et al.* (2005) corresponds to an intake in the range 0.73 to

4.4 μg/kg bw/day, which is close to the figures used by JECFA in their evaluation (WHO, 2005).

Hence, it appears that the average intake figures reported for the general population are quite good. Importantly, however, Hagmar and co-workers (2005) in their study revealed that upon stratification of the study population into low and high dietary exposure, there were considerable overlaps in haemoglobin adduct levels between groups. Hence, even if some of the variation might be due to inter-individual differences in acrylamide and adduct kinetics, it is reasonable to conclude that food frequency questionnaires not constructed for the purpose of dietary acrylamide intake poorly predict the internal acrylamide dose. Smokers had higher adduct levels (30–430 pmol/g globin).

Due to a short half life of acrylamide in the organism, 3.5 and 10 hours for the biphasic elimination in a male given a single dose of 13.5 μg/kg bw (Boettcher *et al.*, 2006), urinary acrylamide metabolites only reflect exposure one or two days before. In the latter study about 56% of the single dose given was recovered as mercapturic acid metabolites in urine during 46 hours. In a Norwegian study, urinary excretion following fasting decreased to about half the amount indicating food as a major source (Bjellaas *et al.*, 2005). The total urinary excretion of mercapturic acid metabolites ranged from 0.1 to 3 μmol/24 hours with a median value of 0.5 μmol/24 hours in two males and three females both groups being non-smokers. On the assumption that about 60% of the intake is excreted in the urine during 24 hours this amount would correspond to an average intake of 60 μg/day ranging from 12 to 360 μg/day or 0.86 (0.17 to 5.1) μg/kg bw/day. These values are somewhat higher than the estimated dietary exposures in Norway, being 0.41 and 0.42 μg/kg bw/day median intake for males and females, respectively (Scientific Committee of the Norwegian Food Control Authority, 2002a,b; Dybing and Sanner, 2003).

13.3 Hazard identification: neurotoxicity, genotoxicity, development and reproductive toxicity

Following oral administration, acrylamide is rapidly and extensively absorbed from the gastrointestinal tract and distributed in the water phase to tissues as well at to the foetus in experimental animals. Its double bond is oxidised to a reactive epoxide, glycidamide by CYP2E1. Both acrylamide and glycidamide are conjugated to glutathione and the conjugates are further processed to mercapturic acid derivatives, which are excreted. Protein binding occurs with both acrylamide and glycidamide, but glycidamide is much more reactive particularly with DNA and forms adducts with the nucleotides guanine and adenine. The main metabolic steps are similar in experimental animals such as mice and rats and in humans (Calleman *et al.*, 1990; Sumner *et al.*, 1992, 1997, 1999; Calleman, 1996; Friedman, 2003; Twaddle *et al.*, 2004; Doerge *et al.*, 2005a,b) (see also Chapter 8 for further details). In CYP2E1 knockout mice exposed to acrylamide formation of glycidamide and also DNA adducts were

practically abolished (Ghanayem *et al.*, 2005a). Neurotoxicity, reproductive and developmental effects, genotoxicity and carcinogenicity are main effects following acrylamide exposure.

13.3.1 Neurotoxicity

As shown in numerous studies in experimental animals and also in humans the nervous system, both the peripheral and the central, is a principal target for acrylamide toxicity. Neurotoxcity in humans associated with prolonged low-level occupational exposure is characterised by peripheral nerve damage with symptoms of skeletal muscle weakness and numbness of hands and feet including central damage with signs of ataxia (Spencer and Schaumburg, 1974a,b; He *et al.*, 1989; LoPachin, 2004). Similar changes are observed in many laboratory animal species, e.g. rodents, rabbit, dog, cat, Guinea pig, upon exposure to acrylamide at doses of 0.5–50 mg/kg bw (LoPachin, 2004).

Studies on acrylamide neurotoxicity have been on-going since the 1950s. Early studies revealed cerebellar Purkinje cell damage and degeneration of distal axons both in the peripheral and central nervous system (Spencer and Schaumburg, 1974b, 1977a,b; Schaumburg *et al.*, 1989; LoPachin, 2004). However, accumulated evidence shows that particularly nerve terminals are early and primary targets of acrylamide appearing as presynaptic dysfunction and subsequent nerve terminal degeneration and not the earlier held theories of 'dying back' neuropathy following insults to the cell body or direct axon injury (LoPachin *et al.*, 2002, 2003; Friedman, 2003; LoPachin, 2004).

Acrylamide induced nerve terminal degeneration also leads to effects on other nervous functions such as autonomic dysfunction, as well as damage in cerebral cortex, thalamus and hippocampus, all brain areas critical for cognitive functions, i.e. learning and memory (Lehning *et al.*, 2002, 2003a,b; LoPachin, 2004; WHO, 2005). Recently, changes in pain sensation and associated behaviour in rats following acrylamide exposure were observed (Ling *et al.*, 2005). The acrylamide neurotoxicity is cumulative, both the dose rate and time of exposure determine the damage (LoPachin, 2004).

A question still not fully resolved is whether the neurotoxicity is due to acrylamide in itself or its reactive metabolite glycidamide (Friedman, 2003). More recent studies indicate that acrylamide and not glycidamide is primarily responsible for induction of neurotoxicity. Accumulating evidence from *in vivo* and *in vitro* studies (Costa *et al.*, 1992, 1995; Friedman, 2003; LoPachin, 2004; LoPachin and Decaprio, 2005) support the hypothesis of direct interaction of acrylamide, which is a soft electrophile, with soft nucleophilic sulfhydryl groups of nerve terminal proteins being responsible for neurotransmitter uptake in presynaptic vesicles and also membrane fusion proteins involved in transmitter release (Barber and LoPachin, 2004; LoPachin and Decaprio, 2005). It appears as protein adduction of acrylamide and perturbation of presynaptic function is global and not restricted to specific transmitters (LoPachin *et al.*, 2006). Interaction of acrylamide with cytoskeletal proteins, microtubuli, kinesin,

dynein and neurofilaments is another mechanism suggested (Friedman, 2003). The mechanisms of Purkinje cell injury are not known, but dysfunction in cell body membrane fusion proteins has been suggested (LoPachin and Decaprio, 2005; LoPachin *et al.*, 2006). In any event acrylamide and not glycidamide is believed to be the primary agent. A test for this notion could be investigations of acrylamide neurotoxicity in CYP2E1 knockout mice.

Two studies, also covering neurotoxicity, in rats exposed to acrylamide via drinking water for two years had a no adverse effects level (NOAEL) for degenerative nerve changes as determined by light microscopy of 0.5 mg/kg bw/day. Lowest adverse effect level (LOAEL) for these effects was 2 mg/kg bw/day (Johnson *et al.*, 1986; Friedman *et al.*, 1995). In a 90-day study of rats also exposed to acrylamide in drinking water, morphological nerve changes were examined by electron microscopy and the NOAEL and LOAEL were 0.2 and 1 mg/kg bw/day, respectively (Burek *et al.*, 1980).

In a study on human tunnel workers exposed to grouting agents containing N-methylol-acrylamide and acrylamide for a four-month exposure period a NOAEL for slight and reversible peripheral nervous symptoms, i.e. numbness and tingling, was estimated to 510 pmol acrylamide/g globin corresponding to an intake of about 20 μg/kg bw/day (Hagmar *et al.*, 2001). In a group of 41 Chinese workers manufacturing acrylamide for 1–11 years, peripheral neurotoxicity was assessed together with acrylamide haemoglobin adduct levels (Bergmark *et al.*, 1993; Calleman *et al.*, 1994). The adduct levels were between 0.3 and 33.8 nmol acrylamide/g globin with an average exposure level of 9.5 nmol acrylamide/g globin. A dose-response association between the adduct level and a neurotoxic index based on neurophysiological findings and signs and symptoms of peripheral neurotoxicity was observed. On the basis of these data it was not possible to establish any firm threshold, but it was tentatively estimated by Törnqvist and co-workers (1998) to be about 300–1000 pmol acrylamide/g globin. Such a level would correspond to an acrylamide intake in the range of 10–40 μg/kg bw/day.

13.3.2 Genotoxicity

Acrylamide, an α,β-unsaturated carbonyl compound can participate in 'Michael type' addition chemical reactions. As a soft electrophile acrylamide with a preference for soft nucleophiles, e.g. thiol groups, it shows very low reactivity towards DNA bases being hard nucleophiles (Friedman, 2003; Besaratinia and Pfeifer, 2005). As mentioned above, unlike acrylamide glycidamide binds to DNA and forms DNA adducts *in vitro* and *in vivo* (Segerbäck *et al.*, 1995; Gamboa *et al.*, 2003). Hence, acrylamide is not mutagenic in *Salmonella typhimurium* mutagenicity assay, either without or with addition of exogenous metabolic activation system, usually devoid of significant CYP2E1 activity. In contrast glycidamide is clearly mutagenic in this test system. Acrylamide is both clastogenic and mutagenic in mammalian cells *in vitro* and *in vivo* (Abramsson-Zetterberg, 2003; Besaratinia and Pfeifer, 2004; Glatt *et al.*, 2005). Metabolic

conversion of acrylamide to glycidamide apparently is a prerequisite for most of the genotoxic activity of acrylamide both *in vitro* and *in vivo* and the most convincing evidence are studies in wild type and CYP2E1 knockout mice (Ghanayem *et al.*, 2005a,b,c). Whereas acrylamide induced dominant lethal mutation and male germ cell mutagenicity following *in utero* exposure, and caused increased levels of erythrocyte micronuclei and DNA strand breaks in leukocytes, liver and lung cells as well as DNA adducts (N-7 and N-3 guanine) in liver, testes and lung in wild type mice, such effects were generally not observed in CYP2E1 null mice. Only traces of haemoglobin glycidamide adducts and the N-7 guanine adduct were seen in the knockout mice.

Using the sensitive flow cytometer assay a linear dose-response relationship between red blood cell micronuclei and acrylamide down to rather low intraperitoneal doses (1–30 mg/kg bw) was found in mice (Abramsson-Zetterberg, 2003). The mutagenic spectrum of acrylamide has been studied in *cII* transgene Big Blue mouse embryonic fibroblast and an excess of A:T to G:C transitions and G:C to C:G transversions were observed (Besaratinia and Pfeifer, 2003, 2004, 2005). Glycidamide in addition displayed G:C to T:A transversions, which can be explained by the N-7 guanine adduct undergoing depurination. Upon DNA replication the apurinic sites give rise to 2′deoxyadenosine incorporation. Whether the N-7 guanine adduct, which is the dominant one, is also the most important one for mutagenesis remains to be clarified as also the minor adducts are promutagenic and depurinating. The genotoxic metabolite glycidamide is also formed in the human body, at a rate mostly resembling that of the rat (Calleman, 1996; Paulsson *et al.*, 2001), but clear evidence of acrylamide induced mutagenicity in humans has not been reported.

Genotoxicity can lead either to mutations in somatic cells, which in turn could develop into cancer, or mutations in germ cells, which could result in transmissible genetic diseases. Favor and Shelby (2005) recently reviewed the multigenerational studies in mice on heritable mutational events caused by acrylamide exposure. In all studies transmission from the male were investigated. Acrylamide exposed males were mated at different time points after exposure to reveal the stage of spermatogenesis most susceptible to mutagenesis. Increased frequencies of mutational events transmitted to the offspring were results from fertilisation in which spermatozoa or spermatides had been exposed to acrylamide or glycidamide. Whether the mutagenic activity is confined to these stages of sperm development or if stem cell spermatogonia are also targets, is not yet resolved as the results are conflicting. Mutagenic activity in female germ cells of acrylamide or glycidamide has not been investigated.

13.3.3 Developmental and reproductive toxicology

A large number of rodent studies on the reproductive effects, also including multigenerational studies in both rats and mice, of acrylamide have been carried out and these were recently reviewed by Tyl and Friedman (2003). At low doses disturbance of implantation and low litter size have been observed and it appears

that rats are more sensitive than mice. The decreased live litter size can be accounted for by dominant lethal mutations clearly caused by the glycidamide metabolite (Generoso *et al.*, 1996; Tyl and Friedman, 2003). Impaired fertility associated with abnormal sperm morphology and motility as well as neurotoxicity was seen at higher doses. Also disturbance in mating, sperm ejaculation, and depressed body weight gain and food intake were observed. These effects appear to occur at doses above 7 mg/kg bw/day (WHO, 2005). Subchronic exposure to acrylamide in males caused reduced testicular weights, reduced testosterone and prolactin serum concentration (Burek *et al.*, 1980; Ali *et al.*, 1983). In female rodents, no adverse effects on reproduction or fertility have been observed with the exception of slight decreases in the body weight of the off-spring (WHO, 2005). Since it was unclear whether the reduced male fertility was secondary to neurotoxicity, attempts to separate neurotoxic effects from reproductive effects have been done. Neurotoxicity and reproductive toxicity were observed at different doses, and it appears that neurotoxicity may contribute significantly to the reduced male fertility (Tyl *et al.*, 2000).

Foetotoxicity was mainly seen at doses of acrylamide also showing maternal toxicity and no teratogenic effects have been observed in rats or mice (WHO, 2005). Developmental neurotoxicity following acrylamide exposure takes place at doses (>10 mg/kg bw) higher than those causing neurotoxicity in the dam (>0.5 mg/kg bw/day) (Wise *et al.*, 1995; Tyl *et al.*, 2000). The overall NOAEL for reproductive and developmental effects based the effect occurring at the lowest dose, the dominant lethal effect, was 2 mg/kg bw/day in rats (WHO, 2005). There are no relevant human data on reproductive or developmental effects of acrylamide.

13.4 Hazard identification: carcinogenicity

13.4.1 Animal data

Acrylamide has been tested for carcinogenicity both in rats and mice (Rice, 2005). A/J mice exposed at doses of 6.25, 12.5 and 25 mg/kg bw by gavage three times per week for eight weeks and sacrificed seven months after the first exposure, had a significant dose related increase in both incidence and number of lung adenomas per mouse (Bull *et al.*, 1984a). In a similar assay A/J mice intraperitoneally exposed to acrylamide for eight weeks showed both an increased incidence and an increased number of lung adenoma per mouse (Bull *et al.*, 1984a). In initiation-promotion studies, upon topical treatment with 12-*O*-tetradecnoylphorbol 13-acetate (TPA) Sencar mice exposed orally, intraperitoneally and topically to acrylamide all developed skin tumours (Bull *et al.*, 1984a). Skin tumour initiation was also observed in Swiss-ICR mice orally treated with acrylamide followed by topical TPA treatment. In the latter study lung tumours were also induced (Bull *et al.*, 1984b).

Two long-term studies have been conducted in male and female Fischer 344 rats (Johnson *et al.*, 1986; Friedman *et al.*, 1995).The studies have been

Table 13.2 Dose descriptors (BMDL10 and T25) of tumours in rats exposed to acrylamide for two years

Tumour site	Range of BMDL10 (mg/kg bw/day) (Johnson *et al.*)	T25 (mg/kg bw/day)	Range of BMDL10 (mg/kg bw/day) (Friedman *et al.*)	T25 (mg/kg bw/day)
Mammary tum.	0.30–0.46	0.64	0.89–1.1	1.9
Peritest. Mesothel.	0.63–0.97	0.89		3.7
Thyroid adenomas			0.63–0.93	

Adapted from JECFA (WHO, 2005) and Dybing and Sanner (2003).

summarised by Rice (2005) and JECFA (WHO, 2005). In both cases rats were exposed via drinking water for two years. The exposure was 0, 0.01, 0.1, 0.5 and 2 mg/kg bw/day. Corrected for survival in the highest dose group there were in the first experiment significant increases in the incidences of thyroid gland tumours and peritoneal peritesticular mesotheliomas in males, and increased incidences of mammary gland tumours, tumours of the central nervous system, thyroid, oral cavity, uterus and clitoral gland in females (Johnson *et al.*, 1986). In the second study designed to confirm these findings increased incidences of follicular thyroid tumours in both sexes as well as peritesticular mesotheliomas and mammary tumours in males and females, respectively, were observed (Friedman *et al.*, 1995).

JECFA (WHO, 2005) modelled the dose response for selected tumours of the long-term rat experiments and estimated benchmark doses (BMD10) for 10% extra risk of tumours and BMDL10, 95% lower confidence interval for the BMD10 (Table 13.2). Another dose descriptor used is the tumour dose for a 25% extra risk of tumours (Table 13.2) (T25) (Dybing *et al.*, 1997; Sanner *et al.*, 2001), which were used by the Scientific Committee of the Norwegian Food Control Authority (2002a,b) and Dybing and Sanner (2003) in their risk assessment of acrylamide exposure from food.

13.4.2 Observations in humans

Epidemiological studies have been conducted on occupationally exposed cohorts and after it became clear that acrylamide was present in food, several studies on the cancer risk associated with food-borne acrylamide exposure have been carried out on European populations.

Sobel and co-workers (1986) studied mortality among 371 workers that produced acrylamide monomer from the end of the 1950s to the beginning of the 1960s. Total mortality was lower than expected, but there were 11 cancer deaths, slightly more than the 7.9 expected, but not statistically significant. In 1989 Collins and co-workers reported from a cohort of 8,854 male workers of which 2,293 exposed to acrylamide a standardised mortality ratio (SMR) of 203 (95% CI 87-400) for pancreatic cancer and SMR 129 (95% CI 42-300). A follow up

during 1984–1994 of the same cohort was reported in 1999 by Marsh *et al.* (1999). Except for a significant 2.26-fold increase in pancreatic cancer among workers with cumulative exposure to acrylamide above 0.3 mg/m^3, no significant increased or decreased number of cancers was found. The mean length of exposure was 1.7 years, which might seem too short for revealing a cancer risk. Although the authors did not find a consistent exposure response relationship, upon regrouping of the data Schulz *et al.* (2001) were able to calculate a monotonically increasing response with increasing dose. Erdreich and Friedman (2004) pointed out that smoking, which is an established causal risk factor for pancreatic cancer, in this case where reported for all the 14 cases and therefore could obscure the relationship to acrylamide exposure. Marsh *et al.* (1999) concluded that these studies provide little evidence for a causal relationship between acrylamide exposure and cancer mortality.

Mucci *et al.* (2003a) reanalysed a population-based Swedish case-control study originally designed for studying the relationship between heterocyclic amine exposure and cancer of the large bowel ($n = 591$) and urinary bladder ($n = 263$). Healthy controls included were $n = 538$. Dietary acrylamide intake was estimated using a food frequency questionnaire (FFQ) combined with a database of acrylamide content of various foods. They failed to find an excess risk of cancer of the large bowel, urinary bladder or kidney associated with dietary acrylamide. Later, they (Mucci *et al.*, 2003b) included coffee and re-examined the data with respect to a possible relationship between acrylamide intake via coffee and cancer risk, but also in this instance failed to show any relationship. Mucci and co-workers (2004) then used Swedish data contributed to an international collaborative case-control study on renal cell cancer. Acrylamide exposure through food was estimated coupling a FFQ with a database on acrylamide content of various food items. In line with the previous studies, in this study no relationship with acrylamide exposure and risk of renal cell cancer was found.

In 2005 Mucci and co-workers (2005) reported on a study of dietary acrylamide intake and risk of breast cancer in a large cohort of Swedish women, but again failed to find any association. In their most recent prospective study Mucci and co-workers (2006) used women eligible to participate in a population based mammography screening programme. The final cohort consisted of 61,467 women who had answered a questionnaire on dietary habits, including intake of 67 food items, and lifestyle factors. The FFQ was also combined with information of acrylamide content of various foods. Mean intake of acrylamide was 24.6 μg/day, being 12.8 and 37.9 in the lowest and highest quintile, respectively. No evidence was found that acrylamide intake or intake of food high in acrylamide was associated with an increased risk of cancers of the colon or rectum.

Pelucchi and co-workers (2003, 2006) reanalysed data from several large hospital-based case-control studies within Italy and Switzerland to assess the association between consumption of fried potatoes, which is an important source of acrylamide, and cancers of the large bowel, oral/pharynx and oesophagus, breast and ovary. The author failed to find any evidence for an increased risk of any cancer related to fried potato consumption.

There are obvious problems with the epidemiological studies, particularly those addressing low-level exposure through food in the general population. First of all, none of these studies were designed for investigating the carcinogenic risk of acrylamide. There is no validation of the exposure assessments, and in some of them not all dietary sources of acrylamide had been taken into account (Hagmar and Törnqvist, 2003; Beer *et al.*, 2004). Because a comparison of intake estimates based on FFQ with acrylamide haemoglobin adducts showed large overlaps, a major problem is the correct classification according to exposure (Hagmar *et al.*, 2005). Misclassifications would strongly weaken a hypothetical association between acrylamide exposure and cancer. The next limitation is the ability of the dietary studies and even the occupational cohort study to detect the small increases in risk expected from the lowest to the highest exposure groups (Hagmar and Törnqvist, 2003). Generally, occupational cohort studies are not designed to detect small excesses in risk and in the study by Marsh the statistical power to detect even a 1.5 or twofold increase in risk was low (Marsh *et al.* reply in Granath *et al.*, 2001). Indeed, neither of the studies on dietary exposure to acrylamide had the power to detect small increases in cancer risk (Hagmar and Törnqvist, 2003) and hence should be regarded as non-positive and not negative studies.

13.5 Hazard characterisation: dose response analysis for various effects

The most important adverse effects of acrylamide recognised are neurotoxicity, genotoxicity, reproductive toxicity and developmental toxicity, and carcinogenicity. In neurotoxicity studies the most sensitive endpoint is morphological changes determined by the use of electron microscopy and in a 90-day study in rats the NOAEL was 0.2 mg/kg bw/day (Burek *et al.*, 1980). In humans the NOAEL for neurotoxicity was estimated to be 2.5 to 10 times lower, between 10 and 40 μg/kg bw/day based on haemoglobin adduct levels in acrylamide exposed workers (Calleman *et al.*, 1994; Törnqvist *et al.*, 1998; Hagmar *et al.*, 2001). In developmental neurotoxicity studies in rats exposed from gestational day 6 to postnatal day 10 the NOAEL was 10 mg/kg bw/day, which was much higher than the dose causing neurotoxicity in the dam (Wise *et al.*, 1995). The most sensitive effect for reproductive toxicity was male mediated implantation loss with a NOAEL of 2 mg/kg bw/day in a two-generation study in rats (Tyl *et al.*, 2000). Hence, for non-genotoxic and non-carcinogenic effects in experimental animals, the most sensitive effect is neurotoxicity with a NOAEL of 0.2 mg/kg bw/day in rats and a NOAEL in humans about ten times lower, 0.01–0.04 mg/kg bw.

Although the fact that acrylamide induced tumours in hormone sensitive tissues has also led to speculation about non-genotoxic mechanisms involving neuron-endocrine actions, no published studies have linked acrylamide exposure to hormonal changes that could explain the tumourigenic effects. The large body of evidence on *in vivo* genotoxicity supports the notion that the most likely mode

of tumourigenic and carcinogenic action is through genotoxic events. This does not exclude other mechanisms, e.g. hormonal stimulation of cell proliferation, to work in concert with genotoxic mechanisms of action for acrylamide induction of tumours.

Acrylamide, particularly because of its efficient conversion both in rodents and in humans to the reactive and clearly genotoxic metabolite glycidamide, is expected to be genotoxic in humans. It was classified by the International Agency for Research on Cancer (IARC) as probably carcinogenic to humans IARC group 2A (IARC, 1994). Since acrylamide is both carcinogenic and genotoxic and genotoxic mechanisms are likely, assuming non-threshold dose response relationships would be the prudent approach.

There are no human studies on carcinogenicity of acrylamide with quantitative information on which it is possible to base a hazard characterisation. The derived dose descriptors (BMDL10 and T25, see Table 13.2) for the most sensitive tumour response from the rodent bioassays can be used directly as a point of comparison in the risk characterisation. This approach was recently recommended for compounds that are both carcinogenic and genotoxic by European Food Safety Authority (EFSA, 2005) and it was also used by JECFA in its recent evaluation of acrylamide (WHO, 2005).

Another approach for low dose hazard characterisation has been as a default to assume linearity in the extrapolation to low doses from T25 or BMDL10 (Sanner *et al.*, 2001). This approach was used in the assessment of acrylamide by the Scientific Committee of the Norwegian Food Control Authority (Scientific Committee of the Norwegian Food Control Authority, 2002a,b; Dybing and Sanner, 2003). Similarly, the same default assumption has been used for carcinogens that are genotoxic such as acrylamide by other national bodies, e.g. the US Environmental Protection Agency (US EPA, 1993). Generally, it is believed that this is a conservative approach, which overestimates the actual risk, since it is expected that cellular defence mechanisms such as detoxication, cell cycle arrest, apoptosis, DNA repair and immune control would be efficient particularly at low doses. On the other hand a linear dose response relationship for quite low doses of acrylamide, although far above those provided by food, was demonstrated for acrylamide induced micronuclei in erythrocytes in mice (Abramsson-Zetterberg, 2003). Linear dose response relationships have also been demonstrated for other genotoxic carcinogens, however, the shape of the dose response curve outside the observed values has been a matter of discussion for many years and will probably remain so as this is very difficult to prove or disprove in experiments (EFSA, 2005).

In addition to doing a linear extrapolation, a scaling factor $(\text{Weight}_{\text{human}}/\text{Weight}_{\text{animal}})^{0.25}$ is introduced to account for the difference in metabolic rate between rats and humans per kg body weight. The scaling factor for rat to human conversion being $(70/0.25)^{0.25} = 4.1$ (Dybing and Sanner, 2003). Using the lowest BMDL10 of 0.30 mg/kg bw and T25 of 0.64 mg/kg bw (Table 13.2) for mammary tumours and a scaling factor of 4.1 the theoretical human lifetime hazards per μg acrylamide per kg bw per day can be calculated to 1.4 and 1.6×10^{-3}, respectively.

It should be noted that another extrapolation model, the multiplicative model for prediction of cancer risk for genotoxic agents, has also been suggested to be used for acrylamide (Paulsson *et al.*, 2001). This model was developed for use in the field of ionising radiation. According to this model the incremental cancer risk, for low to intermediate exposures, is proportional to the accumulated lifetime target dose of the genotoxic substance and the background risk in control groups (Granath *et al.*, 2001). The surrogate target dose indicator glycidamide haemoglobin adduct level was applied with the multiplicative model to rats and mice and was found to fit better than the linear model using acrylamide exposure (Paulsson *et al.*, 2001). For risk estimations of acrylamide in humans *in vivo* doses of glycidamide at different acrylamide exposure levels are essential as well as data on tumour incidence per unit dose of glycidamide in experimental animals. At present the data needed is not available and risk estimations using this model would, at best, be very uncertain.

13.6 Risk characterisation

Risk characterisation is the step comparing the dose-response relationship for various effects with the exposure to acrylamide from food. The exposure to acrylamide via food has been estimated by combining food content and food consumption as well as using biomarkers of exposure. It seems reasonable to use the average and high estimate of 1 and 4 μg/kg bw/day of acrylamide that were also used by JECFA (WHO, 2005). For non-carcinogenic effects the most sensitive endpoint is neurotoxicity with a NOAEL in experimental animals of 0.2 mg/kg bw/day (Burek *et al.*, 1980). The margins of safety relative to the NOAEL for the average and high exposure were 200 and 50, respectively. The safety margins for the other effects were larger. Comparing the estimated NOAEL for neurotoxicity in humans of 10–40 μg/kg bw/day (Törnqvist *et al.*, 1998; Hagmar *et al.*, 2001) with exposure from food resulted in a safety margin in the range of 2.5 to 40. Given these margins any risks of neurotoxic and other non-carcinogenic effects following dietary acrylamide exposure are likely to be very small.

JECFA compared exposure estimates of 1 and 4 μg/kg bw/day with the BMDL10 of 0.30 mg/kg bw/day and obtained margins of exposure (MOE) of 300 and 75. JECFA considered these exposure margins to be low for a compound being both carcinogenic and genotoxic and that they indicate a human health concern (WHO, 2005). The Scientific Committee of EFSA (2005), also endorsing a margin-of-exposure approach for compounds that are both carcinogenic and genotoxic, is of the view that a margin of exposure of 10,000 or larger would be of low concern from a public health point of view and might be considered as a low priority for risk management actions. The MOEs for acrylamide are clearly below 10,000, but the Scientific Committee of EFSA gives no guidance for risk managers on how to interpret a MOE less than 10,000.

Using the lifetime risk estimates based on extrapolation and inclusion of the scaling factor the average and high exposure estimates (1 and 4 μg/kg bw/day)

are associated with lifetime risks of up to 1.4 and 6.4×10^{-3}, risks that would be considered a public health concern. The theoretical risk estimates given above are compatible with the non-positive outcome for cancer risk of acrylamide in the epidemiological studies.

13.7 Conclusions

Acrylamide is a chemical with a wide spectrum of toxic effects. Except for its ability to induce cancer none of its other hazards is likely to represent risk of adverse health effects at exposures encountered via foods. More knowledge on mechanisms of carcinogenic action and risk of doses associated with low level acrylamide exposure are needed to further assess the actual risk. Generally about 30% of the cancers have been attributed to the diet, be it the composition of the diet, total energy intake, intake of fibres and other macronutrients, micronutrients and possible protective agents as well as foodborne mutagens and carcinogens. The cancer risk associated with foodborne acrylamide exposure is probably low, and can at most explain only a very small fraction (less than 0.5%) of cancers associated with diet.

For other chemicals a risk level to consumers comparable with that of acrylamide would not be ignored. Actions to reduce the risk by reducing acrylamide in foods and thereby foodborne exposure are therefore warranted and generally agreed upon. Work on this is done by academia, other research institutions as well as the food industry in collaboration with national risk managing bodies. Less attention is paid to the significance of home cooking as a source of foodborne acrylamide and possible measures to reduce exposure from these sources need to be addressed. Lastly, as it is known that a number of hazardous compounds, e.g. heterocyclic amines and furan derivatives, can be formed during heat treatment of food, it is important that changes made in food compositions and cooking procedures to reduce acrylamide do not result in increased formation of hazardous compounds other than acrylamide.

13.8 Acknowledgements

This work was financially supported by the Norwegian Research Council and European Commission, Priority 5 on Food Quality and Safety (Contract no. FOOD-CT-2003-506820 Specific Targeted Project), ‘Heat-generated food toxicants – identification, characterisation and risk minimisation’. This publication reflects the author’s views and not necessarily those of the EC. The information in this document is provided as it is and no guarantee or warranty is given that the information is fit for any particular purpose. The user therefore uses the information at his/her own risk and liability.

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14

The possible involvement of mutagenic and carcinogenic heterocyclic amines in human cancer

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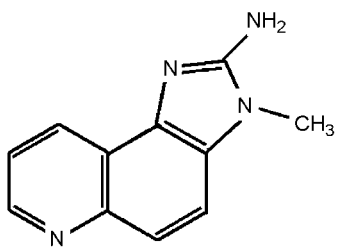
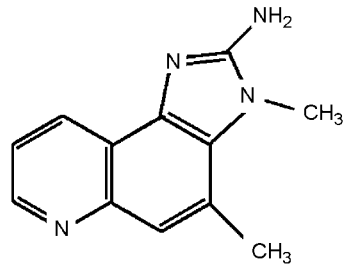
14.1 Introduction

Cancer is the most frequent cause of death in the advanced countries, underlining the need for improvements in prevention, early diagnosis and cure worldwide. While basic carcinogenesis mechanisms have now been well elucidated, most causative factors remain unclear. However, diet has been suggested to be a major contributor to human cancer development (Doll and Peto, 1981; Parkin, 2001) and in the 1970–1980s a series of mutagenic substances, namely heterocyclic amines (HCAs), were discovered in foodstuffs by scientists in Japan and the United States (Becher *et al.*, 1988; Felton *et al.*, 1986; Nagao and Sugimura, 2000; Sugimura, 1986, 1992; Sugimura *et al.*, 2002, 2004; Wakabayashi *et al.*, 1992). More than 20 HCAs are now known to be formed in meat, fish and poultry prepared under common household cooking conditions. Their structures, chemical names and common abbreviations are shown in Fig. 14.1. These food-borne HCAs are divided into two groups. The exocyclic amino groups of group 1 HCAs, exemplified by 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AaC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAaC), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), are readily converted to hydroxyl groups by nitrite treatment, and thereby lose their mutagenicity (Tsuda *et al.*, 1985). These HCAs could be formed by pyrolysis of amino acids (Sugimura *et al.*, 1977; Yoshida and Matsumoto, 1979; Yamamoto *et al.*, 1978;



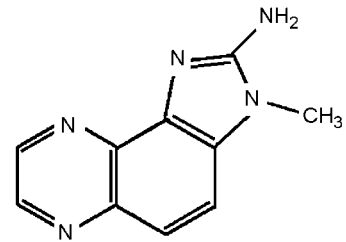
IQ

2-amino-3-methylimidazo[4,5-*f*]quinoline



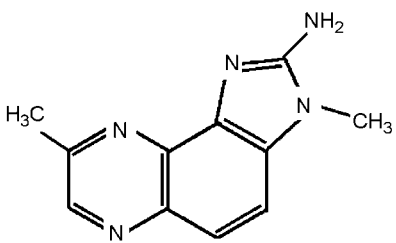
MeIQ

2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline



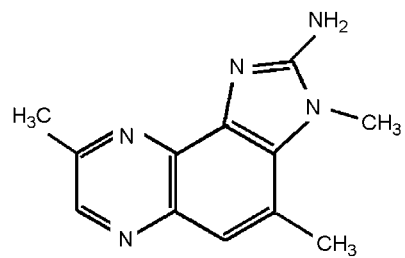
IQx

2-amino-3-methylimidazo[4,5-*f*]quinoxaline



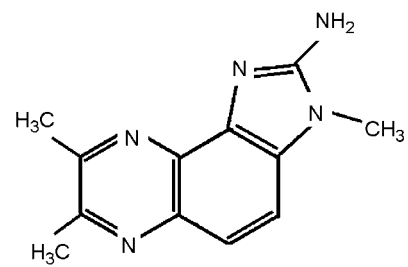
MeIQx

2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline



4,8-DiMeIQx

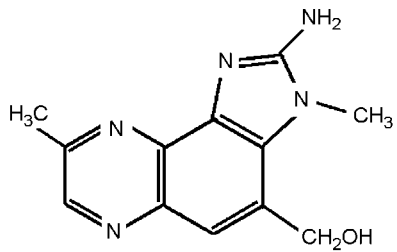
2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline



7,8-DiMeIQx

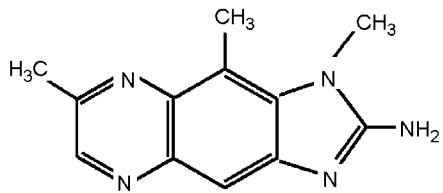
2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline

Fig. 14.1 Structures of HCAs.



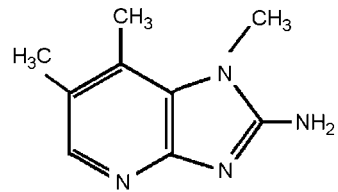
4-CH_2OH-8-MeIQx

2-amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]quinoxaline



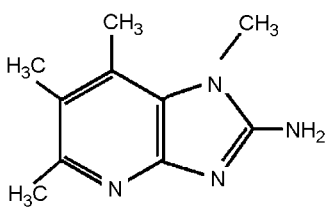
7,9-DiMeI*g*Qx

2-amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline



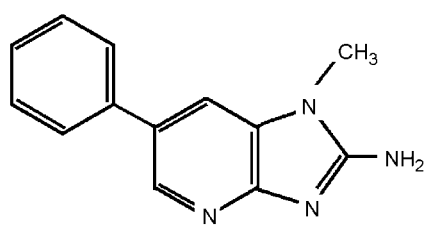
DMIP

2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine



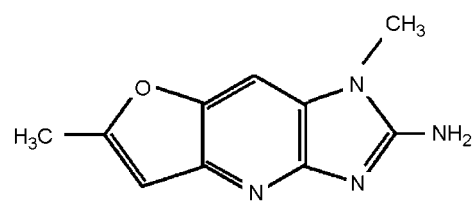
TMIP

2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine



PhIP

2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

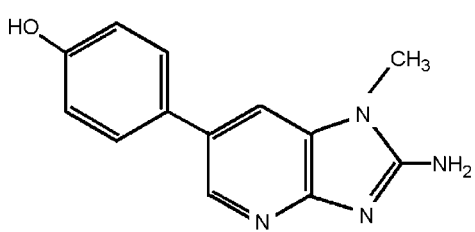


IFP

2-amino-1,6-dimethylfuro[3,2-e]imidazo[4,5-*b*]pyridine

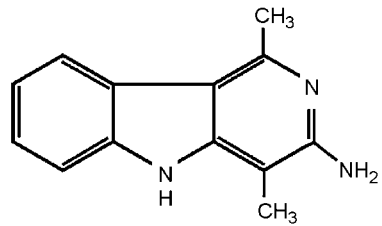
(proposed structure)

Fig. 14.1 Continued



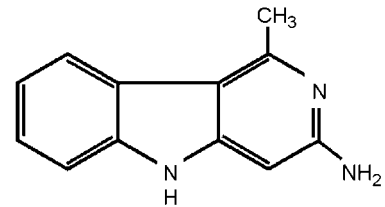
4′-hydroxy-PhIP

2-amino-6-(4-hydroxyphenyl)-1-methylimidazo[4,5-*b*]pyridine



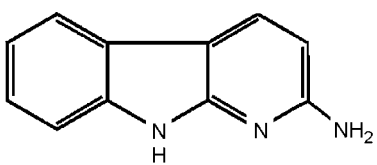
Trp-P-1

3-amino-1-4-dimethyl-5*H*-pyrido[4,3-*b*]indole



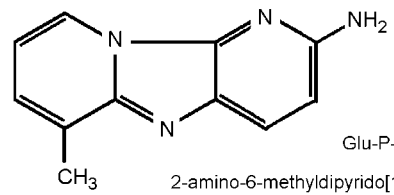
Trp-P-2

3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole



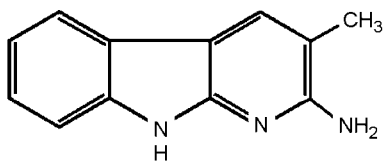
AαC

2-amino-9*H*-pyrido[2,3-*b*]indole



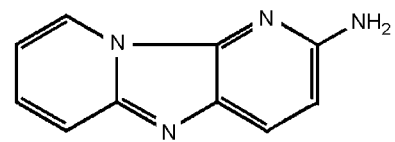
Glu-P-1

2-amino-6-methyldipyrido[1,2-a:3′,2′-*d*]imidazole



MeAαC

2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole



Glu-P-2

2-aminodipyrido[1,2-a:3′,2′-*d*]imidazole

Fig. 14.1 Continued

Yoshida *et al.*, 1978). In contrast, the amino groups of group II HCAs, like 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx), are stable against the 2 mM nitrite treatment, and the Maillard reaction has been suggested to play an important role in their formation (Jägerstad *et al.*, 1991; Tsuda *et al.*, 1985).

Recently, we found a novel type of HCAs, aminophenyl-β-carboline compounds, such as 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (aminophenylnorharman, APNH), 9-(4′-amino-3′-methylphenyl)-9*H*-pyrido[3,4-*b*]indole (aminomethylphenylnorharman, AMPNH), 9-(4′-aminophenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole (aminophenylharman, APH) and 9-(4′-amino-3′-methylphenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole (aminomethylphenylharman, AMPH), produced from β-carbolines and aromatic amines in the presence of metabolic activation systems (Fig. 14.2) (Totsuka *et al.*, 1998, 2004; Sugimura, 1998). The precursors of aminophenyl-β-carboline compounds, β-carbolines (norharman and harman) and aromatic amines (aniline and *o*-toluidine) are known to be distributed widely in our environment, including cigarette smoke and cooked foods, and humans are continuously exposed to both of these during daily life (Bayoumy *et al.*, 1986; DeBruin *et al.*, 1999; International Agency for Research on Cancer, 1982; Luceri *et al.*, 1993; Riffelmann *et al.*, 1995; Totsuka *et al.*, 1999; Ushiyama *et al.*, 1995). Therefore, appreciable amounts of aminophenyl-β-carboline compounds could be formed in human bodies.

As mentioned above, HCAs are produced by cooking meat or fish, so that complete avoidance of intake of these mutagenic and carcinogenic compounds seems impossible. Even if the content of individual HCAs in dishes consumed could be low, clarification of exposure levels, especially in combination, might be important for understanding human cancer causes. This chapter presents recent data on chemical and biological properties and results of risk assessment for HCAs.

14.2 Formation of HCAs

14.2.1 Group I HCAs

The α- and γ-carbolines, including AαC, MeAαC, Trp-P-1 and Trp-P-2, are known to be formed by pyrolysis of either tryptophan or proteins (Sugimura *et al.*, 1977; Yoshida *et al.*, 1978; Yoshida and Matsumoto, 1979). The indole moiety, a common part of their structures, is likely derived from the tryptophan molecule. The dipyridoimidazole compounds, Glu-P-1 and Glu-P-2, can be formed by glutamic acid or protein pyrolysis (Yamaguchi *et al.*, 1979; Yamamoto *et al.*, 1978).

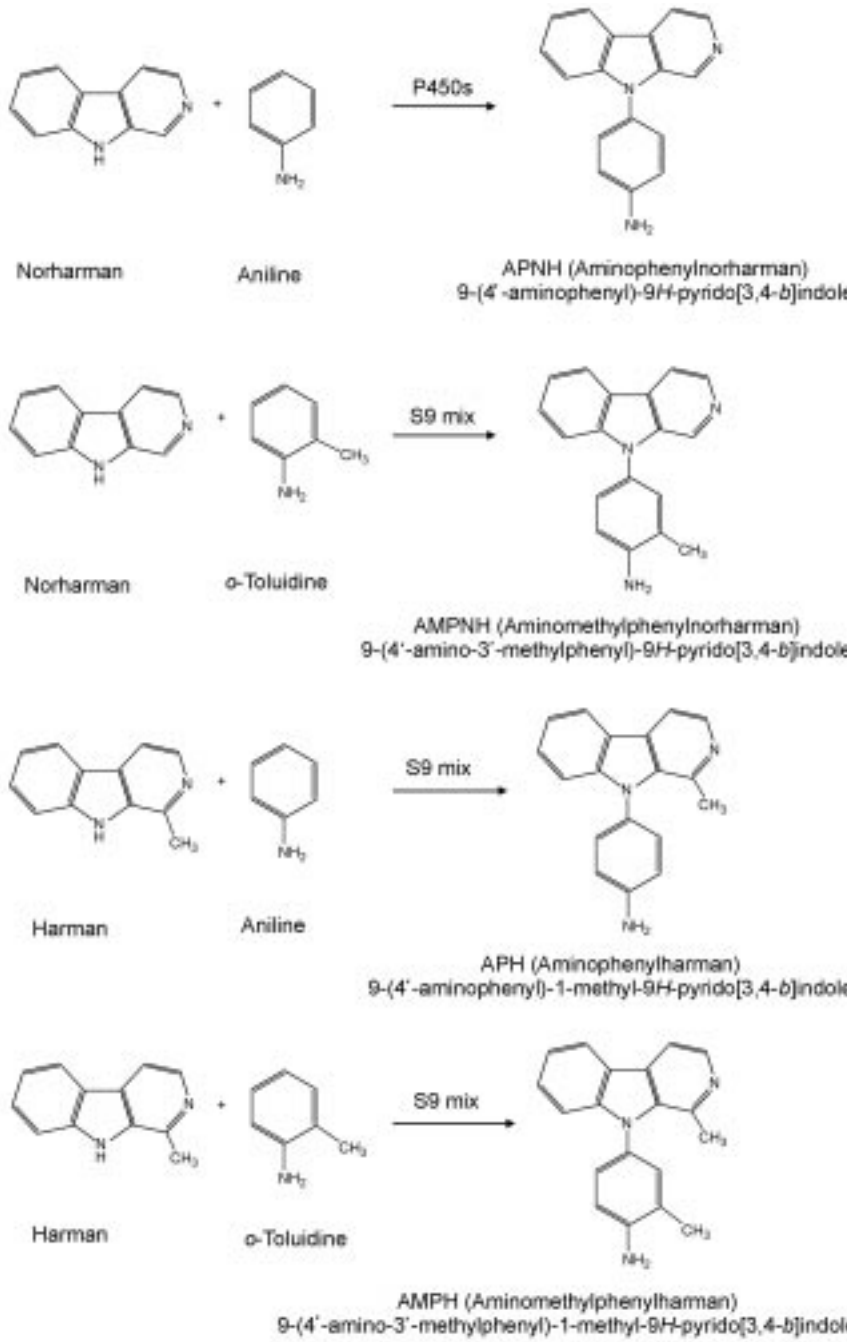


Fig. 14.2 Formation of aminophenyl-β-carboline compounds.

14.2.2 Group II HCAs

The aminoimidazole moieties of IQ-type HCAs may be produced from creatin(in)e, and other moieties are products of Maillard reactions between free amino acids and sugars (Jägerstad *et al.*, 1991). For example, IQ can be formed from heating of creatinine, glycine and fructose. MeIQx and PhIP result from heating mixtures of creatinine, glucose and glycine, and creatinine, glucose and phenylalanine, respectively.

14.2.3 Aminophenyl-β-carbolines

On heating tryptophan, two β-carboline compounds, norharman and harman are produced, together with mutagenic/carcinogenic HCAs, such as Trp-P-1, Trp-P-2, AaC, MeAaC, (Sugimura *et al.*, 1977, 1982). In 1977, Nagao *et al.* reported that norharman is not mutagenic to *Salmonella* strains, but becomes mutagenic to *S. typhimurium* TA98 with S9 mix in the presence of aromatic amines, such as aniline and *o*-toluidine. To identify the mutagenic compound formed by reaction between norharman and aniline in the presence of S9 mix, the reaction mixture was separated and purified by HPLC, and UV, mass and H^1-NMR spectra were determined. Based on spectral data, the mutagenic compound was deduced to be a coupled compound of norharman and aniline, namely APNH (Totsuka *et al.*, 1998, 2004; Sugimura, 1998). This assumption could be confirmed by its chemical synthesis. Recently, we identified the enzymes mediating APNH formation to be CYP3A4 and CYP1A2, (Nishigaki *et al.*, 2004). Other mutagenic aminophenyl-β-carboline compounds, such as AMPNH, APH and AMPH, have subsequently also been found on reaction of norharman or harman with aniline or toluidine isomers in the presence of S9 mix (Hada *et al.*, 2001; Totsuka *et al.*, 2004).

14.3 *In vitro* and *in vivo* mutagenicity of HCAs

14.3.1 *In vitro* mutagenicity

The mutagenic activity of HCAs toward *Salmonella typhimurium* TA98 and TA100 with S9 mix are listed in Table 14.1 (Sugimura, 1992; Wakabayashi *et al.*, 1992; Sugimura *et al.*, 2004; Totsuka *et al.*, 2004). All of the HCAs are more potently mutagenic in TA98, which detects frameshift-type mutations, than in TA100 detecting base-pair change-type mutations. In addition, some of these HCAs show much higher mutagenicities in YG1024 having high *O*-acetyl-transferase activity, than in the parental strain, TA98, indicating this enzyme to be involved in the metabolic activation of HCAs.

In addition, HCAs have be shown to be mutagenic in cultured mammalian cells such as Chinese hamster lung cells with a marker of resistance against diphtheria toxin (Nakayasu *et al.*, 1983; Terada *et al.*, 1986). The mutational spectra induced by some of HCAs, including N-OH-IQ, N-OH-PhIP and PhIP, have been examined with chemically treated plasmids and cultured mammalian cells. Transversions were found to predominate, most occurring at G:C base

Table 14.1 Mutagenicities of HCAs in *Salmonella typhimurium* TA98 and TA100 with S9 mix

	Mutagenicity (revertants/μg)	
	TA98	TA100
Trp-P-1	39,000	1,700
Trp-P-2	104,200	1,800
Glu-P-1	49,000	3,200
Glu-P-2	1,900	1,200
AaC	300	20
MeAaC	200	120
IQ	433,000	7,000
MeIQ	661,000	30,000
MeIQx	145,000	14,000
PhIP	1,800	120
APNH	187,000	1,230
2′-AMPNH	140	16
3′-AMPNH	41,000	2,000

pairs with T:A alterations (Endo *et al.*, 1994; Leong-Morgenthaler *et al.*, 1995; Yadollahi-Farsari *et al.*, 1996; Wu *et al.*, 1995). Furthermore, these HCAs also induce chromosomal aberrations and sister chromatid exchange *in vitro* and *in vivo* (Ishidate *et al.*, 1981; Thompson *et al.*, 1983; Tohda *et al.*, 1980; Sawada *et al.*, 1991, 1994). Especially, APNH shows strong clastogenic activity, at the same order as potent model clastogens, actinomycin D, mitomycin C or 1, 8-dinitropyrene (Ohe *et al.*, 2002).

14.3.2 *In vivo* mutagenicity

In vivo mutation analysis of HCAs has been performed using transgenic murine models featuring shuttle vector transgenes, such as *lacZ*, *lacI*, *cII* and *gpt*Δ (Dashwood, 2003; Masumura *et al.*, 2003a,b). These animal models are convenient for examination of mutational spectra in target organs and several HCAs, such as PhIP, IQ, MeIQ, MeIQx, AaC and APNH, have proved positive in various organs including the liver and colon. As in the case with *in vitro* mutation spectra, these HCAs predominantly cause mutations at G:C base pairs to produce G:C to T:A transversions. Additionally, characteristic mutations of the *Apc* gene have been identified in PhIP-induced colon tumors (one G deletion from the 5′-GGGA-3′ sequence) and in the *lacI* gene of the colon and mammary glands in common from PhIP treated animals (Okochi *et al.*, 1999; Okonogi *et al.*, 1997; Nagao, 1999).

14.4 Metabolism of HCAs

HCAs require metabolic activation to function as mutagens and/or carcinogens (Turesky, 2005). The first step is a phase I hydroxylation catalyzed by

cytochrome P450 enzymes (CYPs). Studies using a variety of approaches have shown that the genotoxic *N*-hydroxylation pathway of these amines primarily involve CYP1A1, CYP1B1, CYP2A3, CYP2C9 and CYP3A4. Among them CYP1A2 most predominantly generates genotoxic metabolites, by *N*-oxidation of HCAs (Hammons *et al.*, 1997; Shimada *et al.*, 1991; Yamazoe *et al.*, 1983). Subsequent metabolism, with *O*-acetylation of *N*-hydroxy-HCAs is known to be catalyzed by *N*-acetyltransferase 1 (NAT1) and/or NAT2. Most *N*-hydroxylated HCAs, including PhIP, IQ and MeIQx, have been reported to be poor substrates for human NAT1, so that *O*-acetylation of *N*-hydroxylated HCAs could be catalyzed by human NAT2 (Hein *et al.*, 1993; Minchin *et al.*, 1992). In addition, sulfotransferases (SULTs) render the other type of activated form, the *N*-sulfoxy metabolite. Recent studies have suggested that sulfation catalyzed by SULT1A1 may be the most relevant pathway of PhIP activation (Muckel *et al.*, 2002). In humans, these phase I and II enzymes, such as CYPs, NATs and SULTs are inducible and exhibit polymorphic variation. CYP1A2 demonstrates 40-fold variation in expression from individual to individual and can be induced by smoking, diet and chronic hepatitis (Lang *et al.*, 1994; Schweikl *et al.*, 1993).

Of particular interest is the finding that HCA-rich diets significantly induce CYP1A2 activity in humans (Sinha *et al.*, 1994). More than ten different allelic variants in the coding region of the *CYP1A2* gene have been reported, and some of these allelic variants demonstrate altered catalytic activities of HCAs (Chevalier *et al.*, 2001; Murayama *et al.*, 2004; Zhou *et al.*, 2004). So far, at least two and ten polymorphic genotypes of human NAT1 and NAT2, respectively, have been reported (Hein, 2000, 2002; Hein *et al.*, 2000). The NAT2 fast acetylator type (*NAT2*4* wild-type allele) has been suggested to bear a relation with increase risk of colorectal cancers. In addition, at least ten human SULT isoforms have been identified and assigned to two major subfamilies, SULT1 and SULT2 (Glatt, 2000). SULT1A1 catalyzes the sulfation of structurally diverse compounds, such as small phenols, steroids, environmental estrogen-like compounds and heterocyclic and aromatic amines (Ozawa *et al.*, 1998; Renskers *et al.*, 1980).

Arylnitrenium ions, which are produced from the metabolically activated exocyclic aminogroup of the HCAs by CYPs and NATs and/or SULTs, are considered as ultimate forms, which react with DNA at the 8-position of carbon of guanine bases (Fig. 14.3) (Turesky and Vouros, 2004). In addition, some of the compounds, such as *N*-acetoxy-IQ and *N*-acetoxy-MeIQx, can bind to the N^2-position of guanine bases (Turesky *et al.*, 1992).

On the other hand, *N*-hydroxylamine metabolites of HCAs may undergo Phase II conjugation reactions via UDP-glucronosyltransferase (UGTs) to form the corresponding *N*-glucuronide conjugates, this being a significant detoxification pathway for *N*-hydroxy HCAs limiting the extent of HCA-induced DNA damage (Kaderlik *et al.*, 1994; Nowell *et al.*, 1999). However, the glucuronide conjugate of PhIP is thought to be cleaved by bacterial β-glucuronidases in the colon lumen to yield *N*-hydroxy metabolites, which might

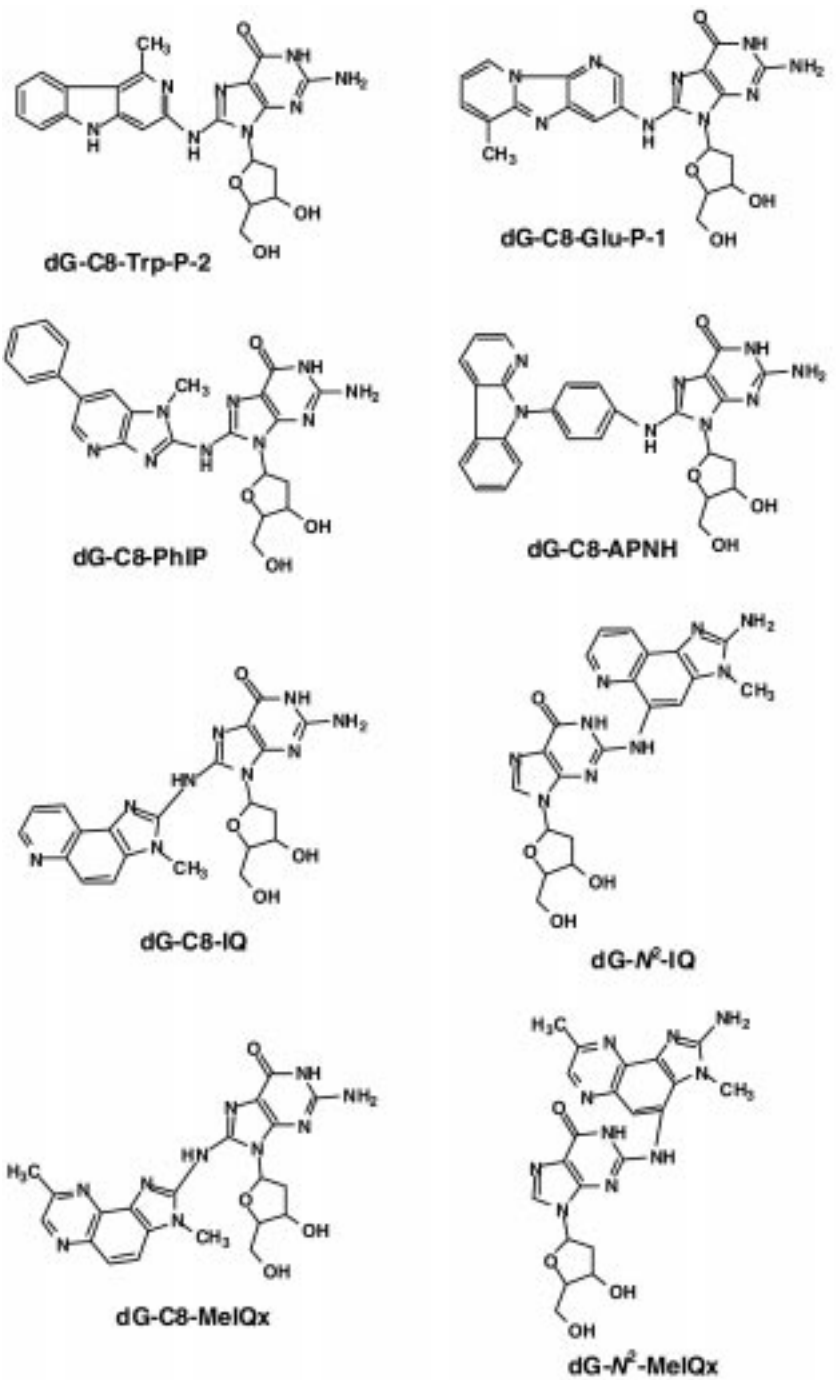


Fig. 14.3 Chemical structures of HCA-DNA adducts.

be further activated by phase II enzymes leading to DNA adduct formation and cancer (Kadlubar *et al.*, 1995; Knasmüller *et al.*, 2001).

14.5 Carcinogenicity of HCAs in rodents

14.5.1 Rats

Long-term carcinogenicity testing of HCAs has been carried out most extensively in F344 rats and CDF_1 mice (Table 14.2) (Kawamori *et al.*, 2004; Sugimura *et al.*, 2004; Wakabayashi and Sugimura, 1998). In the case of rats, most of the HCAs so far tested, such as Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, MeAαC, IQ, MeIQ and MeIQx, but not AαC and PhIP, induced liver tumors predominantly at doses from 100 to 800 ppm, with exposure durations between 52 to 112 weeks (Kato *et al.*, 1988, 1989; Takahashi *et al.*, 1993b; Takayama *et al.*, 1984a,b, 1985; Tamano *et al.*, 1994). Other tumors were observed in the

Table 14.2 Carcinogenicities of HCAs in rats and mice

Chemical	Species	Concentration in diet (%)	Duration of feeding (weeks)	Target organs
Trp-P-1	Rats	0.015	52	Liver
	Mice	0.02	89	Liver
Trp-P-2	Rats	0.01	112	Liver, urinary bladder
	Mice	0.02	89	Liver
Glu-P-1	Rats	0.05	67	Liver, small and large intestine, Zymbal gland, clitoral gland
	Mice	0.05	68	Liver, blood vessels
Glu-P-2	Rats	0.05	104	Liver, small and large intestine, Zymbal gland, clitoral gland
	Mice	0.05	84	Liver, blood vessels
AaC	Mice	0.08	98	Liver, blood vessels
MeAaC	Rats	0.02, 0.01	100	Liver
	Mice	0.08	84	Liver, blood vessels
IQ	Rats	0.03	72	Liver, small and large intestine, Zymbal gland, clitoral gland, skin
	Mice	0.03	96	Liver, forestomach, lung
MeIQ	Rats	0.03	40	Large intestine, Zymbal gland, skin, oral cavity, mammary gland
	Mice	0.04, 0.01	91	Liver, forestomach
MeIQx	Rats	0.04	61	Liver, Zymbal gland, clitoral gland, skin
	Mice	0.06	84	Liver, lung, hematopoietic system
PhIP	Rats	0.04	52	Large intestine, mammary gland, prostate
	Mice	0.04	82	Lymphoid tissue
APNH	Rats	0.004, 0.002	85	Liver, large intestine, thyroid, hematopoietic system, clitoral gland

Zymbal glands, clitoral glands and skin of rats fed diet containing Glu-P-1, Glu-P-2, IQ, MeIQ and MeIQx (Kato *et al.*, 1988, 1989; Takayama *et al.*, 1984a,b). In addition, urinary bladder tumors were also observed in rats treated with Trp-P-2 (Takahashi *et al.*, 1993b). Some HCAs, including IQ, MeIQ and PhIP, can produce tumors in small and large intestine, mammary glands and prostate, these being common sites of neoplasms in most developed countries including Japan, with Westernization of dietary habits (Ito *et al.*, 1991; Hasegawa *et al.*, 1993; Shirai *et al.*, 1997).

Recently, it has been reported that PhIP shows estrogenic activity, e.g. stimulation of cell proliferation, gene expression, and activation of the MAPK signal transduction, in *in vitro* assay systems (Lauber *et al.*, 2004). In addition, it was demonstrated that expression of estrogen (ERα and ERβ) and progesterone receptors were co-upregulated in the nuclei of epithelial cells from PhIP-induced rat mammary carcinomas (Qiu *et al.*, 2005). Based on these findings, it is suggested that the combination of its genotoxicity and estrogenic activity may explain the site-specific carcinogenicity of PhIP. On the other hand, APNH has been demonstrated to induce hepatocellular carcinomas, adenocarcinomas of the colon, thyroid carcinomas, mononuclear cell leukemia and clitoral gland carcinoma in F344 rats at doses of 20 and 40 ppm, when given for 85 weeks (Kawamori *et al.*, 2004).

14.5.2 Mice

As in the F344 rat case, liver tumors are the most common neoplasms observed in CDF_1 mice treated with HCAs (Matsukura *et al.*, 1981; Ohgaki *et al.*, 1984a,b, 1986, 1987, 1991). In addition, tumors of the blood vessels, forestomach, lung, lymphoid tissue and hematopoietic system were found in CDF_1 mice fed diet containing Glu-P-1, Glu-P-2, AaC, MeAaC, IQ, MeIQ, MeIQx or PhIP (Ohgaki *et al.*, 1984a,b, 1986, 1987, 1991; Esumi *et al.*, 1989).

14.5.3 Genetic alterations of tumors induced by HCAs

Analysis of gene alterations in HCA-induced tumors in rodents would be useful for understanding the roles of these environmental carcinogens in the development of human cancers. In case of colon tumors, *β-catenin* and *Apc* gene alterations were commonly detected in the tumors induced by IQ, PhIP or APNH with a high frequency (Table 14.3) (Dashwood *et al.*, 1998; Kakiuchi *et al.*, 1993, 1995; Kawamori *et al.*, 2004; Makino *et al.*, 1994). No mutations in *ras* family and *p53* genes were observed in IQ, PhIP or APNH-induced tumors, although K-*ras* gene alterations were observed in four out of 17 APNH-induced colon tumors (Kawamori *et al.*, 2004). Mammary tumors induced by PhIP have mutations in H-*ras* and *p53* genes with low frequencies, but *β-catenin* mutations were absent in these tumor samples (Dashwood *et al.*, 1998; Ushijima *et al.*, 1994). Therefore, it is suggested that *ras* family and *p53* gene mutations might be rare events during the development of these rodent tumors.

Table 14.3 Genetic alterations in tumors induced by HCAs

	Species	Chemical	Genetic alterations (%)						References
			H-*ras*	K-*ras*	N-*ras*	*p53*	*Apc*	*β-catenin*	
Colon	F 344 rats	IQ	nd	nd	nd	nd	15	100	Kakiuchi *et al.*, (1993, 1995); Makino *et al.*, (1994); Dashwood *et al.*, (1998)
	F 344 rats	PhIP	nd	nd	nd	nd	50	57	Kakiuchi *et al.*, (1993, 1995); Makino *et al.*, (1994); Dashwood *et al.*, (1998)
	F 344 rats	APNH	nd	22	nd	nd	33	44	Kawamori *et al.*, (2004)
Mammary gland	F 344 rats	PhIP	18	nd	nd	10	–	nd	Dashwood *et al.*, (1998), Ushijima *et al.*, (1994)
Liver	F 344 rats	MeIQx	–	–	–	23	–	–	Ushijima (1995a)
	F 344 rats	APNH	nd	nd	nd	nd	nd	24	Kawamori *et al.*, (2004)
	CDF1 mice	IQ	21	–	–	–	–	–	Herzog *et al.*, (1993)
Lung	CDF1 mice	IQ	–	91	–	–	–	–	Herzog *et al.*, (1993)
Zymbal gland	F 344 rats	IQ	57, 56	33	–	27	–	–	Kudo *et al.*, (1991); Takahashi *et al.*, (1993a); Makino *et al.*, (1992a)
	F 344 rats	MeIQ	60	–	–	–	–	–	Kudo *et al.*, (1991)
	F 344 rats	MeIQx	33	–	–	–	–	–	Kudo *et al.*, (1991)

nd = not detected; – = not tested.

On the other hand, H-*ras* and *p53* mutations have been detected in liver tumors induced by IQ and MeIQx (Herzog *et al.*, 1993; Ushijima, 1995a). Moreover, *β-catenin* mutations were found in APNH-induced liver tumors (Kawamori *et al.*, 2004). In lung or forestomach tumors of CDF_1 mice given IQ or MeIQ, H- or K-*ras* mutations were observed (Herzog *et al.*, 1993; Makino *et al.*, 1992b; Nagao *et al.*, 1997). In addition, p53 mutations were also observed in MeIQ-induced forestomach tumors in CDF_1 mice (Ushujima *et al.*, 1995b). In the case of Zymbal gland tumors in F344 rats induced by IQ, MeIQ or MeIQx, mutations of *ras* family and/or p53 genes were observed (Kudo *et al.*, 1991; Makino *et al.*, 1992a; Takahashi *et al.*, 1993a). Most of these mutations were identified to be of the base pair exchange type, occurring at G:C base pairs, suggesting that dG-C8- and/or dG-N^2-HCA adducts could be involved in their induction. Furthermore, a specific flameshift mutation of the *Apc* gene (5′-GGGA-3′ to 5’-GGA-3′) was frequently observed in one series of PhIP-induced colon tumors (Kakiuchi *et al.*, 1995).

14.6 Modulation of carcinogenic activity

A high-fat diet may enhance the carcinogenicity of PhIP and IQ in target organs, such as the mammary glands and colon of rats (Ghoshal *et al.*, 1994; Ubagai *et al.*, 2002). Tanaka *et al.* (2005) reported colonic adenocarcinomas to be induced rapidly by combined treatment with PhIP or MeIQx and dextran sodium sulfate, a tumor-promotor causing colitis. Administration of caffeine caused a significant increase of aberrant crypt foci, putative precursor lesions for intestinal adenocarcinomas, via increased levels of CYP1A2 (Tsuda *et al.*, 1999).

There have been many reports describing suppression and prevention of HCA-induced *in vivo* mutagenicity and/or carcinogenicity. Dietary fibers and tetrapyrrole compounds, such as hemin and chlorophyllin, a stable and soluble derivative of chlorophyll, have been shown to reduce the mutagenicities of HCAs, possibly by adsorbing or interacting with these carcinogens (Arimoto *et al.*, 1980; Ferguson *et al.*, 1996; Guo *et al.*, 1995a,b; Kada *et al.*, 1984). Wheat bran and chlorophyllin suppressed the development of aberrant crypt foci (ACF) induced by administration of IQ or PhIP with gavage (Ferguson *et al.*, 1996; Guo *et al.*, 1995a,b). In addition, chlorophyllin caused inhibition not only of IQ-induced liver, small intestine, Zymbal gland tumors, but also PhIP-induced mammary adenocarcinomas in rats (Hasegawa *et al.*, 1995). Indole-3-carbinol, a component of cruciferous vegetables including broccoli, cabbage and cauliflower, has also demonstrated suppression of ACF development in rats given IQ or PhIP (Guo *et al.*, 1995b; Xu *et al.*, 1996). It has been reported that indole-3-carbinol induces CYP1A1 to a much greater extent than CYP1A2 in the liver and colon mucosa of rats, so that this enzyme is likely to be more involved in detoxification of these HCAs than their metabolic activation (Xu *et al.*, 1996, 1997).

Docosahexaenoic acid (DHA), a polyunsaturated ω3 fatty acid, was shown to reduce ACF formation in the colons of rats given PhIP, probably due to

decreasing levels of prostaglandin E_2 (Takahashi *et al.*, 1997). It has also been reported that the intestinal microflora may play a key role in the detoxification of HCAs (Knasmüller *et al.*, 2001). Lactobacillus strains, such as *Lactobacillus bulgaricus* and *Bifidobacterium longum*, in yogurt can prevent HCA-induced DNA damage in the colon and liver of rats (Zsivkovits *et al.*, 2003). Moreover, *Bifidobacterium longum* has been found to inhibit the formation of IQ-induced colon, mammary and liver tumors (Reddy and Rivenson, 1993). Recently, bovine lactoferrin, a milk protein, was found to exert preventive effects against development of ACF induced by PhIP, correlating with decreased levels of the metabolic activation enzyme, CYP1A2 (Tsuda *et al.*, 2002). Moreover, it has been reported that freeze-dried beer samples show anti-mutagenic effects against MeIQx, PhIP, Trp-P-2, Glu-P-1 and IQ in *Salmonella typhimurium* TA98 in the presence of S9 mix (Nozawa *et al.*, 2004), also causing reduction of the number of ACF of the colon and inhibition of mammary carcinogenesis in rats given PhIP (Nozawa *et al.*, 2004, 2006). Since suppression of CYP1A2 activity was also found, inhibition of HCA activation might partly explain the observed anti-mutagenic/carcinogenic effects.

14.7 Estimation of human intake and exposure to HCAs

14.7.1 Levels of HCAs in cooked foods and human biological samples

HCAs are formed from pyrolysis of amino acid and proteins, or creati(ni)ne, amino acids and sugars via the Maillard reaction, during the cooking of foods (Jägerstad *et al.*, 1991; Skog *et al.*, 1992; Sugimura *et al.*, 1977; Yamamoto *et al.*, 1978; Yoshida *et al.*, 1978). HCA amounts in several kinds of foods are summarized in Table 14.4 (Felton *et al.*, 2000, 2002; Kataoka *et al.*, 2002). The most abundant HCAs in cooked foods are PhIP and MeIQx. Based on the available data, human exposure has been estimated to be between 43–110 ng for PhIP, and 14–47 ng for MeIQx per day (Cantwell *et al.*, 2004; Delfino *et al.*, 2000; Sinha *et al.*, 2000a,b). To elucidate exposure levels of HCAs in humans, direct measurements have been performed using various human biological samples, such as urine, breast milk and hair. Some HCAs, including MeIQx, PhIP, Trp-P-1 and Trp-P-2, have been detected in urine samples from volunteers consuming a normal diet with the range between a few pg to the ng order (Ushiyama *et al.*, 1991) and PhIP has been found in the milk of healthy women (DeBruin *et al.*, 2001). However, no HCAs have been detected in patients receiving parental alimentation (Ushiyama *et al.*, 1991).

With only 0.5–6% excreted unchanged in the urine, the majority of ingested HCAs, such as PhIP and MeIQx, are detoxified and converted to glucuronides and/or glutathione conjugates in the body then excreted into the urine (Reistad *et al.*, 1997). Since several HCAs, including PhIP, bind strongly to melanin-rich tissues, such as hair, this has been successfully employed for biomarker studies (Brittebo *et al.*, 1992). When hair from volunteers was analyzed, PhIP could be found in the range from <50 to about 5000 pg per g using deuterated PhIP as an

Table 14.4 Amounts of HCAs in cooked foods

Food	Cooking method	HCA (ng/100 g cooked food)								Reference
		PhIP		MeIQx		4,8-DiMeIQx		7,8-DiMeIQx		
		Flesh	Skin	Flesh	Skin	Flesh	Skin	Flesh	Skin	
Salmon	Grilled	29	593	10	59	0	0	0	414	Kataoka *et al.* (2002)
Salted fish	Grilled	37	700	8	59	0	9	0	446	Kataoka *et al.* (2002)
Bacon	Fried	30–450		nd–2,370		20–140		nd		Felton *et al.* (2000)
Pork	Barbecued	420		40		10		nd		Felton *et al.* (2000)
Chicken breast	Grilled	2,700–4,800		nd–900		nd–200		nd		Felton *et al.* (2000)
Beef steak	Grilled	18,200		300		nd		nd		Felton *et al.* (2002)

nd = not detected

internal standard (Hashimoto *et al.*, 2004; Reistad *et al.*, 1999). In addition, MeIQx was detected in human hair samples at levels of <50 to about 500 pg per g (Hashimoto *et al.*, 2004).

14.7.2 Measurements of DNA- and protein-adducts in human tissues

The detection of HCA-DNA adducts as critical biomarkers is thought to be essential for extrapolation for accurate human risk assessment. Several HCA-DNA adducts have already been characterized in experimental animals, and the major DNA adducts are due to binding of the exocyclic amino groups of HCAs with C8 atoms of guanine (dG-C8-HCA). In addition to these dG-C8 adducts, minor adducts are formed by N2 atoms of guanine binding to the C5 position of IQ or MeIQx. dG-C8-MeIQx has been detected by ^{32}P-postlabeling in three of 38 DNA samples extracted from colon, rectum and kidney specimens derived from surgical and autopsy samples (Totsuka *et al.*, 1996), with adduct levels estimated to be around 2–20 per 10^{10} nucleotides. Similarly, another report documented dG-C8-PhIP in two of six colon samples at levels of 2.9 ± 0.5 adducts per 10^8 nucleotides, although the same adduct was not detected in the pancreas or urinary bladder (Friesen *et al.*, 1994).

One report demonstrated that 30 of 64 samples contained PhIP-DNA adducts, ranging from 1 to 500 adducts per 10^8 nucleotides in human breast ductal epithelial cells analyzed by the ^{32}P-postlabeling method in combination with an adduct enrichment technique using Sep-Pack columns (Gorlewska-Roberts *et al.*, 2002). Accelerator mass spectrometry (AMS) has been used to measure radiocarbon isotopes due to its atto mole sensitivity. This technique has been employed to analyze MeIQx- and PhIP-DNA adducts in surgical specimens resected from patients administered ^{14}C-labeled MeIQx or PhIP before surgery. Very small amounts of MeIQx-DNA (26 ± 4 adducts/10^{12} nucleotides) and PhIP-DNA (26–480 adducts/10^{12} nucleotides) were detected in colon and/or breast tissues (Dingley *et al.*, 1999; Lightfoot *et al.*, 2000; Turteltaub *et al.*, 1997). However, AMS could not determine the nature of the adducts. In another approach, the formation of PhIP-DNA adducts in lymphocytes from colorectal cancer patients has been measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Magagnotti *et al.*, 2003). Positive results have been obtained for 24 of 76 samples, with levels up to 18.96 adducts per 10^8 nucleotides.

Protein adducts have been used successfully as biomarkers for many compounds, including aromatic amines (Skipper *et al.*, 1994). When human blood samples were examined for human exposure of Trp-P-1, derivatives of Trp-P-1-haemoglobin adducts were identified in all samples, ranging in concentration from 0.23 to 4.33 pmol per g haemoglobin (Umemoto *et al.*, 1992). Moreover, detection of serum protein adducts by AMS has been described for humans exposed to a small dose of ^{14}C-labeled PhIP or MeIQx (Turtletaub *et al.*, 1999). The mean albumin adduct levels were 17.4 and 11.4 fmol PhIP or MeIQx per g protein per pmol PhIP or MeIQx per kg body

weight, respectively. Binding to haemoglobin was much lower. Serum albumin and globin adducts of PhIP have been detected in humans without giving any labeled synthetic compound and, interestingly, the level of PhIP adducts was lower in vegetarians in comparison with meat eaters (Magagnotti *et al.*, 2000).

14.8 Epidemiological studies

Several case-control studies have provided evidence of positive associations between higher consumption of well-done red meat and risk of colon (Sinha *et al.*, 1999; Probst-Hensch *et al.*, 1997; Gerhardsson de Verdier *et al.*, 1991), breast (Zheng *et al.*, 1998; Sinha *et al.*, 2000a), lung (Sinha *et al.*, 1998) and gastric cancer (Ikeda *et al.*, 1983; De Stefani *et al.*, 1998; Ward *et al.*, 1997). In a very large population European prospective study, colorectal cancer risk was also found to be positively associated with high consumption of red and processed meat (Norat *et al.*, 2005). Moreover, it should be borne in mind that individuals with rapid variants of either CYP1A2 or NAT2 activate HCAs from meat more efficiently and therefore could potentially be at the greatest risk (Lang *et al.*, 1986, 1994; Roberts-Thomson *et al.*, 1996; Welfare *et al.*, 1997). The available reports are thus compatible with the hypothesis that high HCA exposure is associated with an elevated risk of colorectal cancer, especially in subgroups which are genetically susceptible to metabolic and detoxification enzymatic activity.

An early study showed a significant twofold risk of colorectal cancer with consumption of well-done meat and a significant threefold risk in those with a rapid phenotype for CYP1A2 and NAT2. Combination of CYP1A2 and NAT2 rapid phenotypes and well-done meat consumption resulted in a sixfold increased risk in one investigation (Lang *et al.*, 1994). In a recent population-based case-control study, it was found that subjects who preferred well-done red meat with the NAT2 and CYP1A2 rapid phenotypes were at an 8.8-fold increased risk of colorectal cancer (Le Marchand *et al.*, 2001). On the other hand, reviews have concluded that NAT1 alone was only associated with risk of colorectal cancer in one of nine studies and that the results with NAT2 are not consistent (Bell *et al.*, 1995; Brockton *et al.*, 2000). Uncertainty over the role of NAT2 has been further fuelled by a case-control study that did show a significant increased risk of colorectal cancer with red meat intake but this was not modified by the NAT2 genotype (Barrett *et al.*, 2003). Reasons for these inconsistencies regarding red meat consumption and cancer development include the large variability in estimated dietary HCA intake due to different cooking conditions, questionnaire makeups, and study populations. Clearly, the relationship between dietary HCA intake and cancer risk should be further detailed with the aid of available surrogate markers for long-term exposure to HCAs such as adducts in DNA or protein samples.

14.9 Risk of development of human cancer from HCAs

Since environmental carcinogens, HCAs, can be produced by cooking meat and fish under standard conditions, serious consideration must be given to the potential risks, even if exposure levels are very low, given the continuously almost unavoidable presence in the environment. Among the HCAs, amino-phenyl-β-carboline derivative, such as APNH, is produced from norharman and aniline in the presence of CYP3A4 and CYP1A2, being found in urine samples collected from F344 rats treated with norharman and aniline. This thus could be thought of as endogenous HCA. Recently, APNH was detected in 24-hr urine samples from healthy volunteers (our unpublished data) and therefore could play an important role in human carcinogenesis as a new type of endogenous mutagen.

In general, it is accepted that about one-third of all cancers are related to dietary factors. Several lines of evidence indicate that cooking conditions and dietary culture play important roles in determining ingestion of food-borne mutagens/carcinogens, such as HCAs. A number of other mutagenic compounds including polycyclic aromatic hydrocarbons, *N*-nitrosoamines, plant alkaloids, mycotoxins, oxidative agents and nitroarenes have been reported to be present in our environment, and humans are exposed to these as well as HCAs in combination. Although single carcinogenic factors may not be sufficient to induce cancers, genotoxic effects due to multiple agents could accumulate and lead to the multiple genetic alterations, being characteristic of neoplasia. Moreover, the effects of these genotoxic compounds on human carcinogenesis will be influenced by the genetic background regarding metabolic activation, detoxification and cellular responses to DNA damage. Consumption of a high fat diet, and the existence of chronic inflammatory conditions may modify the activities of HCA mutagens. It must also be regarded that mutations lead to genomic instability because of impaired control of DNA replication and DNA repair. Under these conditions, accumulation of genetic alterations would be rapid, therefore it might be more susceptible to development of cancer by HCAs. Actually, various types of genomic instabilities were found in PhIP-induced rat mammary and colon tumors (Kitazawa *et al.*, 1994; Nagao *et al.*, 1997; Okochi *et al.*, 2002; Watanabe *et al.*, 2001). On the other hand, it is reported that threshold levels for the carcinogenic potential of HCAs possibly exists (Fukushima *et al.*, 2002, 2004). Therefore determination of the real risk assessment of these food-borne mutagens/carcinogens must take into account a myriad of factors.

As mentioned above, humans are exposed to mutagens/carcinogens HCAs as both exogenous and endogenous agents. Therefore, knowledge of how to minimize the formation of HCAs during cooking, and to suppress their *in vivo* production and carcinogenicity by modification of their metabolic activation or detoxification activities is needed. Moreover, it is a high priority to develop suitable approaches to estimate the impact of these food mutagens on human cancer development based on evidence gained with realistic animal models.

14.10 Acknowledgements

The studies described in this chapter were supported by Grants-in-Aid for Cancer Research, the Third-Term Comprehensive 10-Year Strategy for Cancer Control, and research on Advanced Medical technology from the Ministry of Health, Labour, and Welfare of Japan; a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (J.S.P.S).

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15

Health risks of 5-hydroxymethylfurfural (HMF) and related compounds

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Abbreviations

ACF	aberrant crypt focus
CMF	5-(chloromethyl)-2-furfural
CYP	cytochrome P450
FDCA	furan-2,5-dicarboxylic acid
Hb	haemoglobin
HbS	aberrant (sickling) haemoglobin
HMF	5-(hydroxymethyl)-2-furfural
HMFA	5-hydroxymethyl-2-furoic acid
HMFG	*N*-(5-hydroxymethyl-2-furoyl)glycine
LD_{50}	lethal dose for 50% of the treated animals
NTP	National Toxicology Program
OAT	organic anion transporter
PAPS	3′-phosphoadenosine-5′-phosphosulphate
SMF	5-(sulphooxymethyl)-2-furfural
SULT	sulphotransferase
Sult	lower case: rat or murine form of sulphotransferase
SULT	italics: sulphotransferase gene

15.1 Introduction

One of the numerous compounds resulting from the heating of foods is 5-(hydroxymethyl)-2-furfural (HMF, CAS No. 67-47-0). It is formed by acid-catalysed dehydration and in the Maillard reaction from reducing sugars. It is

found at high levels in numerous foods and other consumer products. Estimates of the mean daily intake are in the range 30–150 mg per person. Various studies conducted in the 1960s and 1970s consistently showed low acute and chronic toxicity of HMF in mice and rats. No case reports on adverse effects of HMF in man are available in the literature. Likewise, HMF is inactive in various standard *in vitro* genotoxicity tests. It was therefore concluded that HMF formed in food or as a result of sterilisation of parenteral solutions does not seem to pose any significant toxicological problem. However, HMF has a number of structural alerts (furan ring, α,β-unsaturated carbonyl group and allylic hydroxyl group) that point to a possible risk of genotoxic and carcinogenic activity. Indeed, HMF initiated and promoted preneoplastic lesions, aberrant crypt foci (ACFs), in rat colon (Zhang *et al.*, 1993). The initiation of these lesions implies the induction of gene mutations in colon mucosa.

Numerous carcinogens/mutagens are not active as such, but require metabolic activation to chemically reactive metabolites. The enzymes involved in this activation vary between different compounds and often are expressed only in specific tissues and cell types. Since targets cells of standard *in vitro* genotoxicity tests largely lack toxifying enzymes, external activating systems are normally used. However, the common activating systems are primarily optimised for detecting relatively large aromatic mutagens activated via cytochrome P450-dependent pathways. HMF is a small molecule and already contains two functional groups, and thus might be toxified by enzymes other than those used in standard tests. Thus, the discrepancy between genotoxicity results *in vivo* (ACFs) and standard *in vitro* tests might have metabolic reasons. Indeed, in subsequent studies using non-standard activating systems, it was demonstrated that rat and human sulphotransferases (SULTs) could convert HMP to a chemically reactive metabolite, 5-(sulphooxymethyl)-2-furfural (SMF), which is mutagenic and carcinogenic.

The exceptionally high human exposure, the induction of ACFs and the genotoxicity in modified tests prompted the National Toxicology Program (NTP) of the USA to conduct long-term carcinogenicity studies with HMF in mice and rats. These studies are still in progress and no results have been published to date. In this chapter we review occurrence, biotransformation and toxicology of HMF and discuss possible health risks. We focus on newer aspects, such as the SULT-mediated bioactivation. Findings with structurally related compounds are presented, in as much as they might be relevant for judging fate and effects of HMF in biological systems.

15.2 Occurrence of HMF in foods and other consumer products

HMF is an intermediate in the Maillard reaction, which occurs when reducing hexose moieties are heated in the presence of amino acids or proteins (Mauron, 1981). An alternative source of HMF involves direct thermal dehydration of

fructose, sucrose and, to a lesser extent, glucose (Antal *et al.*, 1990). This reaction does not require the presence of amino groups. It is strongly enhanced under acid conditions. Food chemists primarily know HMF as an indicator that honey has been heated, stored under inappropriate conditions or adulterated with high-fructose syrup. The legal limit for HMF in honey amounts to 40 mg/kg in Germany; the major beekeepers association sets stricter standards ($\leq$ 15 mg/kg). Honey is a negligible source for intake of HMF compared with other food items. Particularly high levels of HMF have been detected in caramel products, dried fruit (especially plum), old Port and Madeira wines, and balsamic vinegar (Table 15.1). Bread and heat-sterilised milk contain lower levels of HMF, but are consumed in much higher quantities. Likewise, coffee is a dominating source for HMF.

Simonyan (1971) estimated that humans may ingest up to 150 mg of HMF daily. Janzowski *et al.* (2000) consider an average daily intake in the range of 30–60 mg per person as a more realistic estimate. Nevertheless, the intake of HMF in a single day may sporadically exceed one gram, e.g. in persons liking plum juices and liquor wines. Significant levels of HMF have also been detected in sterilised glucose solutions used for parenteral nutrition (Hryncewicz *et al.*, 1996). Moreover, HMF has been found in cigarette smoke condensate (Black, 1966; Crump and Gardner, 1989), wood smoke, and smoke aromas (http://

Table 15.1 Content of HMF in some foods

Food	HMF content, mg/kg or mg/L (number of samples)	Reference
Honey	< 0.1–57 (94)	Bachmann *et al.* (1997)
Caramel products	110–9500 (8)	Bachmann *et al.* (1997)
Plum (dried)	1600–2200 (2)	Murkovic and Pichler (2005)
Plum jam	1100–1200 (2)	Murkovic and Pichler (2005)
Juices made from dried plums	510–2850 (13)	Bachmann *et al.* (1997)
Pear (dried complete fruit)	56–3500 (14)	Bachmann *et al.* (1997)
Breakfast cereals	4–193 (23)	García-Villanova *et al.* (1993)
Bread crust	6.8–410 (12)	Bachmann *et al.* (1997)
Liquid infant milk formulae	2.2–8.1 (8)[a]	Mulchandani *et al.* (1979)
Coffee (ground, roasted)	300–1900 (22)	Murkovic and Pichler (2006)
Liquor wines (Malaga, Madeira)	620–840 (3)	Bachmann *et al.* (1997)
Sweet sherry wine	27–1245	Guerra-Hernández *et al.* (1988)
Balsamic vinegar ('Aceto Balsamico di Modena')	316–3251 (16)[b]	Theobald *et al.* (1998)
Solutions for parenteral nutrition	0.2–1200[c]	Ulbricht *et al.* (1984)

[a] Different modes of processing.
[b] A traditionally produced balsamic vinegar sample ('Aceto Balsamico di Modena tradizionale') even reached a value of 5500 mg/L (Theobald *et al.*, 1998).
[c] Reviewed from various studies.

www.leffingwell.com/smoke.htm). Even deliberate exposure may occur. Thus, it has been claimed that HMF has 'refreshing' and 'vitalising' activities, making it an 'active ingredient' of an Austrian 'CYL (change your lifestyle) drink' (http://www.cyl.at). Clinical trials are being conducted with HMF in tumour patients before and during chemotherapy with the aim to reduce adverse side effects (http://www.cyl-pharma.com). Others have proposed HMF as a drug for the treatment of sickle cell disease (Section 15.4).

15.3 Absorption, biotransformation and elimination of HMF

15.3.1 General considerations on biotransformation pathways

HMF contains three structural components, the furan ring, the allylic alcohol group and the aldehyde group. The structural formulae of identified and hypothetical metabolites are presented in Fig. 15.1. They are indicated by standard abbreviations or bold numbers in the figure and the subsequent text. Unsubstituted furan can be oxidised, leading to cleavage of the ring (Section 15.8.3). Ring oxidation and opening has not been observed with HMF. The reason may be that the alcohol and aldehyde groups are easy targets for biotransformation and sterically hinder attack at the ring. These groups may be converted into each other by alcohol dehydrogenases. Thus, HMF might be metabolised to a symmetrical bis-aldehyde (**2** in Fig. 15.1) or a symmetrical bis-alcohol (not shown). Under the redox conditions of the cell, the thermodynamic equilibria of most alcohols and aldehydes are on the side of the alcohols. However, the net flux will be determined by the rate of further metabolism of the hydroxyl and carbonyl functions.

In competition with oxidation, alcohols may undergo conjugation reactions, primarily glucuronidation by UDP-glucuronosyltransferases and sulphonation by SULTs. Glucuronidation reactions have not been observed in the metabolism of HMF. However, HMF is a good substrate of SULTs (Section 15.3.3), but the resulting conjugate, SMF, is chemically reactive, making its detection in biological samples difficult. The sulphate group, located in an allylic position, may be spontaneously cleaved off, as the resulting cation is resonance-stabilised. Thus, allylic and benzylic sulphates are electrophiles that may react with numerous nucleophiles via S_n1 or S_n2 mechanisms (Landsiedel *et al.*, 1996; Glatt, 2000). The most abundant nucleophile in the cell is water. Reaction of SMF with water regenerates HMF. The half-life time of SMF in water amounts to 120 min at 37 ºC (Sommer *et al.*, 2006). It is somewhat shortened in the presence of various electrolytes. For this reason it is not surprising that SMF has only been detected as an HMF metabolite *in vitro*, but not *in vivo*, especially as no attempts have been made to search for it and to adjust sample collection and preparation to its instability.

Glutathione is another potent nucleophile, present at high levels in cells. Conjugation of glutathione with numerous electrophiles is strongly enhanced by glutathione transferases. Many glutathione conjugates are processed to, and excreted as, mercapturic acids. Some hypothetical mercapturic acids that might

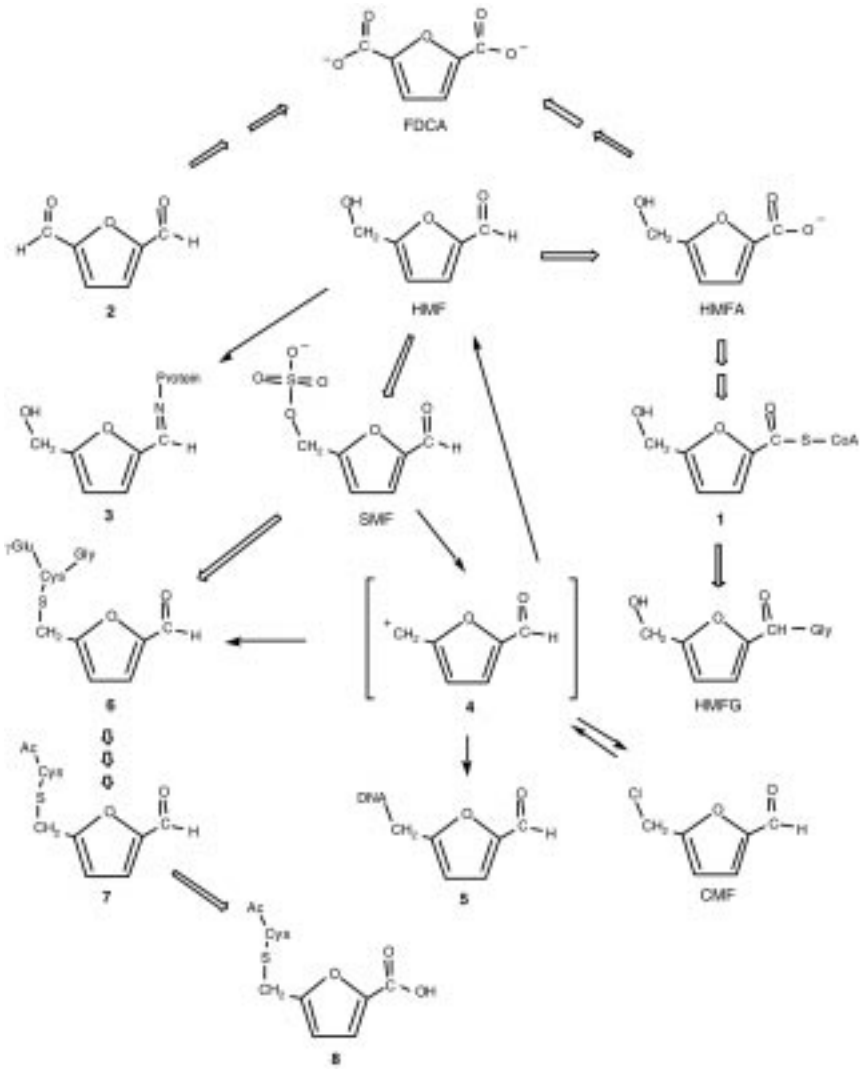

Fig. 15.1 Possible biotransformation pathways of HMF, HMFA, HMFG and FDCA have been detected in urine of laboratory animals and humans exposed to HMF. Conversions of HMF to SMF, and of SMF to **6** have been noted *in vitro* in the presence of human and rodent enzymes. The protein adduct **3** has been detected in blood of HMF-treated mice. Formation of DNA adducts by SMF was demonstrated *in vitro*. The remaining structures represent hypothetical intermediates and products. Simple and broad arrows indicate spontaneous and enzyme-mediated reactions, respectively. Schiff's bases (analogous to **3**) and reactive sulpho conjugates (analogous to SMF) might also be formed from intermediates other than HMF, the prerequisite being the presence of a carbonyl or allylic hydroxyl group, respectively. HS-CoA, coenzyme A; γGlu-Cys(SH)-Gly, glutathione; Ac-Cys-SH, *N*-acetylcysteine, GlyH, glycine.

be formed from HMF via SMF are depicted in Fig. 15.1 (**7** and **8**). Whereas reactions of benzylic and allylic sulphuric acid esters with water and glutathione reduce the chemical reactivity, substitution reactions with other small nucleophiles, such as the chloride anion, may enhance the reactivity. Thus, chloride is a better leaving group than sulphate in its deprotonated form, as present in the organism. Moreover, as chlorides, such as 5-(chloromethyl)-2-furfural (CMF), are not charged, they may penetrate cell membranes more readily than their sulphate congeners. More important than the reaction of SMF and CMF with small nucleophiles is their covalent binding to macromolecules, such as proteins and DNA (**5**).

HMF as an aldehyde may form Schiff's bases with amino groups, e.g. those present in proteins (**3**). This reaction may be reversible, unless it is followed by further reactions. HMF is unsaturated in the α,β-position. Such aldehydes often undergo Michael addition reaction, e.g. with glutathione. However, this reactivity is reduced in HMF, as the furan ring has aromatic character. Aldehydes tend to be short-lived in biological systems not only due to their reactivity, but also due to the presence of reducing and oxidising enzymes, such as alcohol and aldehyde dehydrogenases, respectively. In most cases, reduction to the alcohol is reversible, whereas oxidation to the carboxylic acid is irreversible.

Carboxylic acids are water soluble and anionic under physiological conditions and, therefore, could be excreted. However, they may also be conjugated with amino acids (Steventon and Hutt, 2002). For this conjugation, the carboxylic acid has to be activated in two steps. At first it forms an acid anhydride with AMP donated from ATP. Subsequently, the acid anhydride is used to form a thioester with coenzyme A (e.g. **1** in Fig. 15.1), from which the carboxylic acid residue can be enzymatically transferred to an amino acid, forming a peptide bond. The amino acid utilised depends on the animal species and the structure of the carboxylic acid. In many species, including man and rat, conjugation with glycine is particularly common. The coenzyme A conjugates of various xenobiotic carboxylic acids (such as **1**) may not only be used for conjugation with amino acids, but also, for example, for side chain elongation with acetate or for transfer to glycerol, followed by integration of atypical triglycerides into membranes. Such reactions have not been reported for HMF, but side chain elongation has been detected with furfuryl alcohol (NTP, 1999) and furfural (Flek and Sedivec, 1978) leading to the formation of 2-furanacrylic acid and its glycine conjugate, 2-furanacryluric acid. Another relatively common pathway of carboxylic acids involves their conjugation with glucuronic acids. Some acyl glucuronides are chemically reactive and may form protein adducts. However, glucuronidation has not been observed with carboxylic acids derived from HMF and similar furan derivatives.

15.3.2 Toxicokinetics of HMF in laboratory animals

Germond *et al.* (1987) administered [U-^{14}C]-HMF to rats. Urinary elimination was similar after intragastral and intravenous administration, indicating high

oral bioavailability. Over the wide dose range studied (0.08 to 330 mg/kg body mass), 85% of the radioactivity was eliminated in urine within 8 h and less than 1% was found in body-cavities organs and faeces after 24 h. The two major metabolites detected in urine, 5-hydroxymethyl-2-furoic acid (HMFA) and its glycine conjugate, *N*-(5-hydroxymethyl-2-furoyl)glycine (HMFG) (Fig. 15.1), were formed at similar levels at low doses of HMF. The ratio was shifted in favour of the free acid with increasing dose levels, an effect that could be mitigated by prior administration of glycine. A third metabolite, detected at lower levels, was more polar. Its structure was not elucidated in this study. Later studies suggest that it might have been furan-2,5-dicarboxylic acid (FDCA).

Surh and Tannenbaum (1994) reported that HMF is converted to SMF in the presence of rat hepatic cytosolic fraction fortified with 3′-phosphoadenosine-5′-phosphosulphate (PAPS). They also showed that chemically synthesised SMF is mutagenic (Section 15.6.2). Its mutagenicity was enhanced in the presence of chloride anions, suggesting some conversion to CMF. Although this conversion was not directly demonstrated, it appeared plausible based on previous findings with 1-sulphooxymethylpyrene, whose mutagenicity is also strongly enhanced in the presence of chloride anions; in this case, the corresponding 1-chloromethylpyrene could be isolated from the incubation mixture (Glatt *et al.*, 1990).

SMF is a good substrate of rat glutathione transferase T2, leading to the formation of metabolite **6** shown in Fig. 15.1 (H. Schneider and H. R. Glatt, unpublished result). This finding on the activation of HMF by rat enzymes *in vitro* and the observation that HMF induces and promotes ACFs in the mouse (Section 15.7) prompted Godfrey *et al.* (1999) to conduct further pharmacokinetic studies with [U-^{14}C]-HMF in mice and rats. They used single oral doses of 5 to 500 mg/kg body mass. They confirmed the rapid absorption and – primarily urinary – elimination previously found by Germond *et al.* (1987). In the mouse the elimination was somewhat slower than in the rat. The principal urinary metabolite in both species was HMFA (77.5–80.9% of urinary ^{14}C). HMFG (1.3–7.9%) and FDCA (2.0–5.9%) were found at lower levels. Approximately 10% of urinary ^{14}C was not structurally identified. However, it did not contain 5-(*N*-acetyl-L-cysteine-*S*-methyl)furoic acid (**8**), the mercapturic acid that might be formed after glutathione conjugation and oxidation of SMF. The elimination in the faeces (within 24 h) amounted to 8.5–25.5% of the dose of [U-^{14}C]-HMF. This material was not further analysed.

Abdulmalik *et al.* (2005) orally administered 50–200 mg HMF per kg body mass to mice. They detected high levels of unchanged HMF in blood serum, up to 305.7 mg/L at the highest dose. Volume of distribution and half-life time amounted to 0.45–0.65 L/kg and 0.83–1.5 h, respectively, depending on the dose employed. While the mice used in that study were transgenic for human sickle haemoglobin (resulting effects are described in Section 15.4), it is unlikely that this trait affected the pharmacokinetics.

15.3.3 Toxicokinetics of HMF in man *in vivo* and biotransformation by human enzymes *in vitro*

In the human, unlike in the rat, oxidation of the hydroxyl group appears to be an important biotransformation reaction of HMF. Jellum *et al.* (1973) detected high levels of HMF metabolites in urine of two infants receiving intravenous nutrition, 50 mg HMFA and 25 mg FDCA per day in a one-month-old girl, and 75 mg of each acid in a nine-month-old boy. The amount of these acids corresponded to approximately 50% of the level of furan compounds (primarily HMF) present in the infused solutions. HMF was not detected in urine, and the fate of the remaining amount of the dose administered was not elucidated. Glycine conjugates would not have been recovered with the method used. The same group detected FDCA in urine from all 20 normal adults investigated (0.5–8.2 μg per mg creatinine, equivalent to about 1–15 mg per day) (Pettersen and Jellum, 1972). It is probable that most of this FDCA was formed form HMF ingested with food.

cDNA-expressed human SULTs efficiently catalyse the sulpho conjugation of HMF (Table 15.2). Calculated V_{max} values as well as actually observed maximal conjugation rates of HMF markedly exceed the corresponding values for reference substrates of several SULT forms. However, the K_m values for HMF are unusually high, 2.5 to 15 mM for the three most active human SULT forms. These concentrations are far above those reached in the human circulation and tissues. Thus, the catalytic efficiency (V_{max}/K_m) should be the appropriate parameter for identifying critical SULT forms. Based on this criterion, human SULT1A1 should be particularly important (Table 15.2). Moreover, SULT1A1 is the most abundant SULT form in the human organism. It is expressed in numerous tissues and cell types (Glatt *et al.*, 2001). Particularly high levels of SULT1A1 have been detected in liver and intestinal mucosa (Glatt *et al.*, 2005b). Interestingly, rat and murine Sult1a1 are markedly less efficient in the activation of HMF than human SULT1A1. Species-dependent differences in tissue distribution of SULTs may also be momentous. Expression of most rodent

Table 15.2 Conjugation of HMF by human SULTs: kinetic parameters

	HMF			Standard substrate	
	V_{max} nmol/mg/min	K_m mM	V_{max}/K_m L/mg/min/10^6	v nmol/mg/min	S μM
SULT1A1	520 ± 70	2.5 ± 0.2	230 ± 20	73	4
SULT1A2	250 ± 80	15 ± 3	17 ± 3	46	10
SULT1B1	15 ± 9	7 ± 2	2.7 ± 0.6	5.7	1

Note: human SULTs were expressed in *S. typhimurium* TA1538 as described previously (Meinl *et al.*, 2006). Activities (v) were determined in total cytosolic fractions and expressed per mg SULT protein. Standard substrates (4-nitrophenol for SULT1A1 and SULT1A2, and 1-naphthol for SULT1B1) were used at the concentration S, which leads to nearly maximal activity and is somewhat above the K_m value. Data from Sommer *et al.* (2006).

Sult forms is focused on the liver and usually is low in extrahepatic tissues, in contrast to various human SULTs.

15.4 Reaction of HMF with amino acids and protein

HMF induced a concentration-dependent decrease in glutathione in various cells in culture (Janzowski *et al.*, 2000). The underlying mechanism has not been elucidated. However, the effect was similar in V79 cells, which are SULT-deficient, and in primary hepatocytes, which probably were SULT-proficient. Sickle cell disease is caused by abnormal haemoglobin (HbS) that polymerises under hypoxic conditions. Some chemicals that form Schiff's base with the terminal valine residue of the α chain can stabilise HbS. Abdulmalik *et al.* (2005) have shown that HMF can exert this anti-sickling effect in a transgenic mouse forming human HbS. Almost 100% of human Hb α chain as well as the endogenous murine Hb α chain were modified 1 h after oral administration of 100 mg HMF per kg body mass. This level sustained for 3 h, followed by a gradual decrease to undetectable levels at 6 h. Thus, the adducts disappeared shortly after the elimination of HMF from blood, suggesting reversibility of this Schiff's base. Similarly, orally administered Schiff's base of lysine (via the ϵ amino group) with HMF was found to be fully available as lysine source in the rat (Finot *et al.*, 1977).

After treating rats and mice with [U-^{14}C]-HMF, Godfrey *et al.* (1999) determined covalent binding in tissues. As an indicator of protein binding they used the radioactivity that could not be removed from tissue homogenates by extensive washing with various organic solvents and repeated precipitations. Twenty-four hours following a 500 mg/kg dose of HMF to mice and rats, levels of non-extractable radioactivity in liver, kidney and gut tissues were in the range of 328–857 pmol/mg protein. Using a similar protocol, the parent structure of HMF, furan (8 mg/kg body mass), was found to produce 2200 pmol non-extractable radioactivity per mg protein in rat liver (Burka *et al.*, 1991). Thus, the binding per molar dose unit was 90–370 times higher with furan than with HMF. Here we have to remember that the average daily intake of HMF by humans from the diet is approximately 1000 times higher than that of furan. However, the value of these data is limited, as the nature of the non-extractable radioactivity is unknown for HMF as well as for furan. Covalent binding is only a hypothesis, formation of atypical lipids from HMF and conversion of fragments of furan into normal cellular constituents are alternative possibilities.

15.5 Acute and chronic toxicity of HMF and SMF

15.5.1 HMF

Simonyan (1969) determined the LD_{50} (lethal dose for 50% of the treated animals) at 1910 and 3100 mg/kg body mass in the mouse and rat, respectively,

after single oral administration of HMF. LD_{50} values of 751, 842 and >2000 mg/kg body mass were reported by Czok (1970) for HMF administered via the intravenous, intraperitoneal and oral route, respectively. In other studies, HMF was administered daily to rats or mice for four days to 11 months without causing serious toxicity (reviewed by Ulbricht *et al.*, 1984). For example, rats receiving HMF in the feed (0.25 g/kg body mass/day for 40 weeks) showed normal weight gain and food consumption as well as normal histology of the tissues investigated (liver, kidney, heart, spleen and testis) (Lang *et al.*, 1970).

15.5.2 SMF

SMF is much more toxic in animals than HMF. As preparatory work for carcinogenicity studies, we administered SMF intraperitoneally to male FVB/N mice. In the initial experiment, groups of 35 mice received a single dose of SMF (250 mg/kg body mass) or the solvent only (0.9% saline). Twenty dosed animals died spontaneously between days 5 and 11 after the treatment, other moribund animals were euthanised during this period and underwent thorough pathological examinations. The most dramatic effects were seen in the kidneys with abundant acute necrosis and proteinaceous casts in the proximal tubules. Distal tubules were affected to a lesser extent. Glomerular lesions were mild. Other serious histopathological alterations were observed in the livers. All SMF-dosed animals, but no control animal, showed scattered acute necrosis of hepatocytes and swelling of Kupffer cells. Details will be published shortly (F. Taugner, S. Florian, Y. Sommer, A. Seidel and H. R. Glatt, manuscript in preparation).

This organotropism is similar to that previously observed with another reactive sulphuric acid ester, 1-sulphooxymethylpyrene. After intraperitoneal administration of this compound to rats, the highest levels of DNA adducts were formed in kidney followed by liver (Glatt *et al.*, 2003). These organs mediate the urinary and biliary excretion of sulpho conjugates, processes that often involve concentrative uptake into cells. Uptake into renal proximal tubule cells is primarily performed by organic anion transporters (OAT) 1 and/or 3. We recently demonstrated that heterologous expression of human OAT1 and OAT3 strongly enhances 1-sulphooxymethylpyrene-induced formation of DNA adducts in a human embryonic kidney cell line (Bakhiya *et al.*, 2006). Likewise, SMF inhibited the uptake of *p*-aminohippuric acid by OAT1-expressing cells, suggesting that it is a competing substrate.

15.6 Genotoxicity of HMF, SMF and CMF

15.6.1 Genotoxicity in conventional *in vitro* system

Results with HMF from conventional genotoxicity test systems were negative or were weakly positive (Table 15.3). The weakly positive and equivocal results were obtained at concentrations of at least 12 to 120 mM in *in vitro* test systems.

Table 15.3 Genotoxicity results for HMF from conventional test systems

Effect studied	Result	Reference
Gene mutation, *Salmonella typhimurium* (reversion of various *his*$^-$ strains, including TA98 and TA100, in the presence and absence of rat liver S9, 'Ames' test)	Negative[a]	Florin *et al.* (1980); Aeschbacher *et al.* (1981); Kasai *et al.* (1982); Lee *et al.* (1995)
DNA repair, *Bacillus subtilis* (rec assay)	Positive (20 μmol/disk)	Shinohara *et al.* (1986)
SOS response, *S. typhimurium* (umu assay)	Positive (12 mM)	Janzowski *et al.* (2000)
Chromosomal aberrations, Chinese hamster V79 cells	Weakly positive (16 mM)	Nishi *et al.* (1989)
DNA damage detected by single-cell gel electrophoresis (Comet) assay	Negative in V79 and Caco-2 cells; weakly positive/equivocal in primary rat hepatocytes (40 mM) and human colon cells (80 mM)	Janzowski *et al.* (2000)
Gene mutations (*hprt* locus), V79 cells	Weakly positive/equivocal (120 mM)[b]	Janzowski *et al.* (2000)
Gene mutations (*tk* and *hprt* loci), TK6 cells	Negative	Surh and Tannenbaum (1994)
Micronuclei in peripheral blood cells, male and female mice	Negative	http://ntp-server.niehs.nih.gov/ (study A40470)

Note: *in vitro* experiments were conducted in the absence of an external activating system, unless specified otherwise. For positive and equivocal results, the minimum concentration is added in parentheses for producing this result. V79, fibroblastoid line from Chinese hamster lung; Caco-2, epithelial line derived from a human colon carcinoma; TK6, human lymphoblastoid cell line.

[a] An aberrant result was published by Shinohara *et al.* (1986). A particularly strong increase in the number of revertants was observed in strain TA100, tested in the presence of liver S9. Strangely, the lowest dose used produced the strongest effects. The study is not well described, although it appears that standard procedures, similar to those described in the studies leading to negative results, were used. However, in Japan it is relatively common to add ATP to the S9 preparation. Under this condition, PAPS can be synthesised and SULTs are active. Furthermore, a weakly positive result was reported in the NTP study (http://ntp-server.niehs.nih.gov/ – study A22124) for strain TA100 in the absence of S9. The results were negative for TA100 in the presence of liver S9 and with all other strains used under all conditions.

[b] Fivefold increase in mutant frequency (statistically not corroborated) at markedly cytotoxic concentration levels (120–140 mM); no increase at 100 mM. Similar experiments were conducted in our laboratory. We used lower exposure concentrations but much longer exposure periods (72 h). The result was completely negative.

Table 15.4 Influence of expression of human SULT1A1 in target cells on the genotoxicity of HMF

Test system	Lowest effective concentration, μM		
	HMF, standard cells	HMF, SULT1A1-expressing cells	SMF, standard cells
S. typhimurium TA100, gene mutations (*his* reversion)	>20000	400	20
V79 cells, gene mutations (*hprt* locus)	>6200	1600	300
V79 cells, sister chromatid exchange	900	20	40

Note: SMF was investigated in standard cells for comparison. The indicated concentration of HMF or SMF was required to induce 40 revertant colonies per plate, ten mutants per million cells, or two sister chromatid exchanges per metaphase above the solvent control level. HMF did not induce any gene mutations in standard *S. typhimurium* and V79 cells even at the highest concentration studied. Data from Glatt *et al.* (2005a) and unpublished results from our laboratory.

These concentrations are very high, as regulatory requirements are such that new compounds should be tested to 10 mM or the limit of solubility, whichever is lower.

Surh *et al.* found that an HMF metabolite, SMF, induces gene mutations in *S. typhimurium* (forward mutations in strain TM677 and reverse mutations in strains TA104 and TA100) as well as human TK human lymphoblastoid cells (forward mutations at the *tk* and *hprt* loci), whereas HMF was inactive in the same models (Surh *et al.*, 1994; Surh and Tannenbaum, 1994; Lee *et al.*, 1995). This mutagenic activity of SMF in bacterial and mammalian cells in culture was confirmed in our laboratory (Table 15.4). As an additional effect we demonstrated the induction of sister chromatid exchange by SMF (Fig. 15.2).

The bacterial mutagenicity of SMF was enhanced approximately twofold in the presence of an extra chloride ion (154 mM) in the exposure medium, and chemically synthesised CMF was 100 times more potent than SMF in inducing mutations in bacteria (Surh and Tannenbaum, 1994). The effect may be due to a better permeation of bacterial membranes by uncharged molecules.

Due to the positive results with SMF it would be important to know the SULT status of the models used for testing HMF. The target cells used in the *in vitro* models listed in Table 15.4 are either SULT-deficient (bacteria, V79 cells) or their SULT level is very low (Caco-2). Standard liver S9 preparations lack the cofactor PAPS for SULTs. The primary hepato- and colonocytes used may have been SULT-proficient, but the status was not examined. This would have been critical as this enzyme system is prone to lose its activity. For example, most *Sult* genes cease expression in rat hepatocytes within a few hours after their isolation (Liu *et al.*, 1996). Moreover, continuous synthesis of the cofactor PAPS is an additional prerequisite for SULT activity (Glatt and Meinl, 2005).

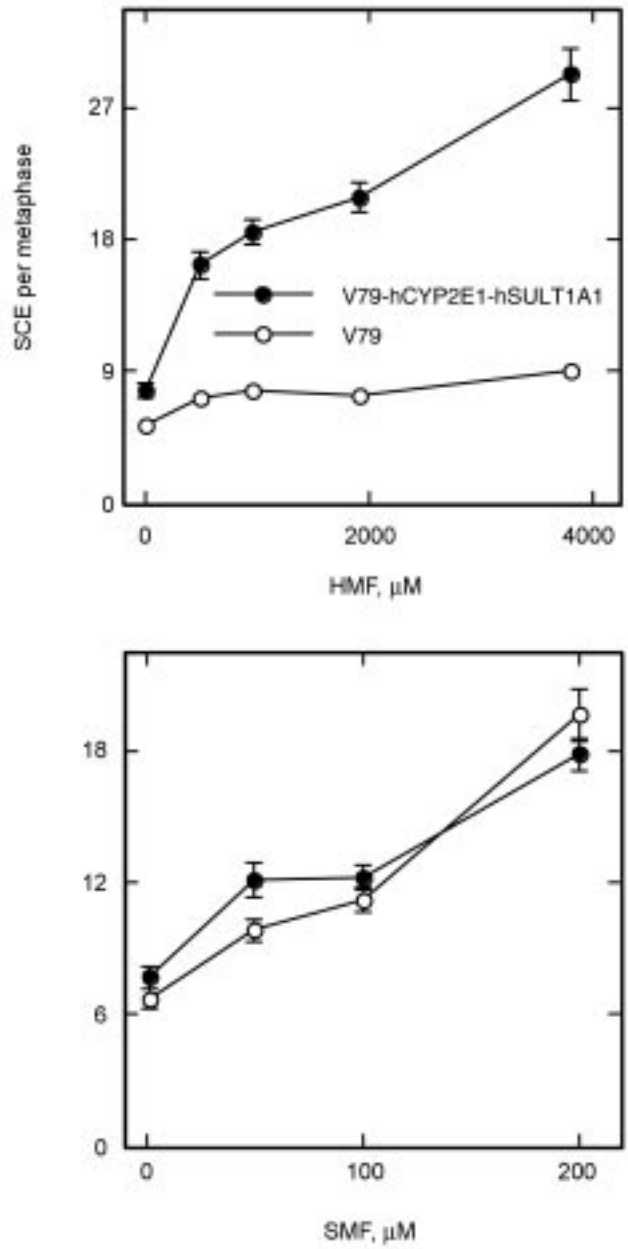

Fig. 15.2 Induction of sister chromatid exchange by HMF and SMF in normal V79 cells and V79-derived cells engineered for expression of human CYP2E1 and SULT1A1. Values are means and SE of 50 metaphases. Data for HMF are from Glatt *et al.* (2005a), where additional details have been presented. Data for SMF have not yet been published elsewhere.

15.6.2 Genotoxicity of HMF in SULT-proficient *in vitro* systems

HMF demonstrated mutagenic activity in bacteria when tested in the presence of rat hepatic cytosolic fraction supplemented with PAPS, the cofactor for SULTs (Lee *et al.*, 1995). In this model, the active metabolite has to permeate bacterial membranes to reach its target, a situation that may affect its sensitivity. Moreover, it might be difficult to study human enzymes and to identify the critical forms in this model, as standardised human enzyme systems and purified enzymes are not readily available. To avoid these difficulties, we have constructed a number of SULT-proficient bacterial and mammalian target cells during the last decade (Glatt and Meinl, 2005). The data presented in Table 15.2 indicate that SULT1A1 should be the most efficient human form with the substrate HMF. Therefore, we conducted genotoxicity studies with HMF in bacterial and mammalian target cells expressing this form and in SULT-deficient control cells (Table 15.4, Fig. 15.2). The SULT expression strongly affected the test result in all models. HMF induced gene mutation in bacterial and mammalian cells when SULT was expressed. Induction of sister chromatid exchange, an endpoint that required lower exposure concentrations than gene mutations, was also observed in control cells, but drastically enhanced (45-fold) in SULT1A1-expressing cells. Interestingly, HMF (in SULT expressing cells) was a stronger inducer of sister chromatid exchange than its metabolite, SMF, studied in control cells. Thus, cellular uptake of SMF may not be powerful. This situation was reversed for the endpoint gene mutations, possibly due to the requirement of substantially higher levels of active metabolite and capacity problems for bioactivation in the cells.

15.6.3 *In vivo* genotoxicity studies with HMF and SMF

Both HMF and SMF have been investigated for the formation of micronuclei in peripheral erythrocytes in mice *in vivo*. HMF gave negative results (Table 15.3), whereas SMF was positive in this test (Dahlberg, 2004). Since different mouse strains and treatment protocols were used, a comparison is possible only with reservations. It appears that SMF, after its direct administration, reached the bone marrow, where it damaged the DNA of haematopoietic cells. Due to its high volume of distribution (Section 15.3.2), it is likely that HMF also reached the bone marrow. Yet, HMF lacks intrinsic activity and murine bone marrow exhibits insufficient SULT activity for its conversion to SMF. However, it is probable that some SMF is formed from HMF in other tissues, primarily the liver. The level of this formation is unknown, but was insufficient under the experimental conditions used to produce detectable genotoxicity in a remote tissue such as the bone marrow.

15.6.4 Formation of DNA adducts

Incubation of DNA with SMF in a cell-free system led to the formation of DNA adducts that could be detected by the ^{32}P-postlabelling technique. The major

adduct, D1, co-migrated with adducts formed in incubations of SMF with desoxyadenosine-3′-phosphate. No adducts were formed in incubations with HMF instead of SMF. In subsequent experiments we searched for these adducts in mammalian and bacterial cells treated with SMF and in SULT-proficient cells treated with HMF. Although mutations were induced, adducts were not seen under the same conditions. With many other compounds, DNA adducts were the more sensitive endpoint than mutations. Thus, mutagenicity of SMF was not mediated via DNA adducts (which appears rather improbable) or the adducts were processed in a way that obviated their detection. For example, the free aldehyde group may have formed crosslinks within the DNA or with proteins or other cellular constituents. Therefore, we are exploring other approaches for detecting SMF-induced DNA adducts.

15.7 Carcinogenicity of HMF, SMF and CMF

One out of five rats treated shortly after birth with a single subcutaneous dose of HMF (200 mg/kg body mass) showed a lipomatous tumour of the kidney when the animals were killed at the age of 25 months (Schoental *et al.*, 1971). Due to the small number of animals and the lack of a negative control group, it is not possible to deduce a carcinogenic activity of HMF from this finding. Nevertheless it is interesting, in as much as kidney was the major target tissue of the acute toxicity of an HMF metabolite, SMF (Section 15.5.2), and of histopathological and neoplastic effects after chronic exposure to an HMF congener, furfuryl alcohol (Section 15.8.1).

Corpet *et al.* (1990) treated mice and rats with a single dose of the colon carcinogen azoxymethane and then maintained the animals under different diets. Animals fed a diet containing 20% caramelised sucrose had three times as many large ACFs at 100 days than animals fed untreated sucrose. The HMF content of the caramelised sugar was ~1%. In a follow-up study, using the same general treatment scheme, two additional diets were used (Zhang *et al.*, 1993). One involved caramelised sucrose freed from HMF by butanol extraction, the other diet contained 19% untreated sucrose plus 1% HMF. After the extraction of HMF, caramelised sucrose lost its ACF-promoting activity, whereas HMF supplementation to untreated sucrose enhanced the average size of the ACFs similarly to the caramelised sugar. In another part of the same study, rats received HMF, dissolved in water by gavage (two doses of 100 to 300 mg/kg body mass, with a one-week interval). Animals were killed 30 days after the second treatment. HMF led to statistical significant increases in the fraction of ACF-bearing animals and in the average number of ACFs per animal. Thus, these studies demonstrate that HMF has ACF-initiating and -promoting activity. Initiation strongly suggests that HMF induces mutations in colonic stem cells.

Miyakawa *et al.* (1991) studied various carbohydrate pyrolysis for tumour-initiating activity in mouse skin, using 12-*O*-tetradecanoylphorbol-13-acetate as the promoter. A single animal developed a skin tumour (a papilloma) in the

solvent control group. This value was increased to four tumour-bearing animals (each with a single papilloma) in the HMF treatment group and to five tumour-bearing animals (with a total of seven papilloma and one carcinoma) in the group receiving furfural. Only the effect of furfural was statistically significant. However, the (moderate) papilloma-initiating effect of HMF in mouse skin was reproduced and found to be statistically significant in a later study (Surh *et al.*, 1994).

Surh *et al.* (1994) treated newborn mice with single intraperitoneal doses of HMF (0.25 and 1 mol/kg body mass) and CMF (0.1 and 0.25 μmol/kg body mass). High incidences (74 and 93%) and multiplicities (2.1 and 3.6) of hepatomas were detected at both doses of CMF at month 10, but not in the control group (two tumour-bearing animals, each with a single tumour, in a total of 29 animals). Thus, CMF is a potent hepatocarcinogen. In contrast, HMF showed no statistically significant effect (five tumour-bearing animals among the 33 animals treated with the higher dose). Long-term carcinogenicity studies of HMF in mice and rats are in progress (Table 15.5), but results are not available to date. No epidemiological studies on HMF intake and cancer incidence exist, and no case reports are available in the literature.

15.8 Other furan derivatives formed from carbohydrates

15.8.1 Furfuryl alcohol

Furfuryl alcohol (CAS No. 98-00-0) is well absorbed in the lungs and the alimentary tract of rats and mice, extensively metabolised and then primarily eliminated in urine (Nomeir *et al.*, 1992; NTP, 1999). Furoylglycine was the major metabolite detected in urine. Free furoic acid, the side-chain elongated compound furanacrylic acid and its glycine conjugate were minor metabolites. Two-year carcinogenicity studies in mice and rats were conducted with furfuryl alcohol administered by inhalation (NTP, 1999) (Table 15.5). The incidences of a variety of non-neoplastic lesions of the nose were increased in all animal groups exposed to furfuryl alcohol compared to chamber controls. In male rats, furfuryl alcohol led to statistically significant increases in the incidences of neoplasms of the nose. A similar, but weaker, trend was observed in female rats, but not in mice of either sex. However, male mice exposed to furfuryl alcohol showed increased incidences of renal tubule neoplasms. Exposure associated increases in the incidence of renal tumours were not observed in female mice and male rats, and were marginal in female rats. Yet, the severity of nephropathy (which is seen even in untreated old mice or rats) was enhanced by furfuryl alcohol in both sexes of the rat as well as in male mice.

In the same study, the genotoxicity of furfuryl alcohol was investigated in various test systems (NTP, 1999). All results from *in vivo* tests (micronuclei, chromosomal aberrations and sister chromatid exchanges in bone marrow of mouse) were negative. Negative results were also obtained in the Ames test (reversion of various *his*$^-$ *S. typhimurium* strains in the presence and absence of

Table 15.5 Two-year carcinogenicity studies of furan derivatives in mice and rats

Compound (reference)	Treatment	Neoplastic effects
HMF (NTP study C95011B)	Gavage: 0, 375, 750 and 1500 mg/kg for rats; 0, 188, 375 and 750 mg/kg for mice	Results not yet available, see: http://ntp-server.niehs.nih.gov
Furfuryl alcohol (NTP, 1999)	Inhalation: 0, 2, 8 and 32 ppm for rats and mice	Kidney • male mice: some evidence (renal tubule adenomas and carcinomas: 0/50, 0/49, 0/49, 5/50) • female mice: equivocal (renal tubule adenomas and carcinomas: 0/50, 1/49, 2/49, 2/50) Nose • male rats: some evidence (tumours at all sites: 0/50, 1/50, 1/50, 4/50) • female mice: equivocal (tumours at all sites: 0/49, 0/50, 1/48, 0/49)
Furfural (NTP, 1990)	Gavage: 0, 30, and 60 mg/kg for rats; 0, 50, 100 and 175 mg/kg for mice	Liver • male mice: clear evidence (hepatocellular adenomas: 9/50, 13/50, 11/49, 19/50; carcinomas: 7/50, 12/50, 6/49, 21/50) • female mice: some evidence (hepatocellular adenomas: 1/50, 3/50, 5/50, 8/50) • male rats: some evidence (cholangiocarcinomas: 0/50, 0/50, 2/50; historical negative control: 3/2145; and two other animals showing bile duct dysplasia with fibrosis, the precursor of cholangiocarcinomas, at the high dose) Kidney • male mice: equivocal (renal cortical adenomas or carcinomas: 0/50, 1/50, 1/49, 1/50; historical negative control: 8/2183) Forestomach • female mice: equivocal (squamous cell papillomas: 1/50, 0/50, 1/50, 6/50)

Furan (NTP, 1993)	Gavage: 0, 2, 4 and 8 mg/kg for rats; 0, 8, 15 mg/kg for mice	Liver • rats: clear evidence (cholangiocarcinomas: 0/50, 43/50, 48/50, 49/50 in males; 0/50, 49/50, 50/50, 48/50 in females; hepatocellular adenomas and carcinomas combined in males: 1/50, 5/50, 22/50, 35/50; hepatocellular adenomas in females 0/50, 2/50, 4/50, 7/50) • mice: clear evidence (hepatocellular adenomas: 20/50, 33/50, 42/50 in males, 5/50, 31/50, 48/50 in females; hepatocellular carcinomas 7/50, 32/50, 34/50 in males; 2/50, 7/50, 27/50 in females) Haemopoietic system • rats: clear evidence (mononuclear cell leukaemia: 8/50, 11/50, 17/50, 25/50 in males, 8/50, 9/50, 17/50, 21/50 in females) Adrenal gland • mice: clear evidence (benign phaeochromocytomas 1/49, 6/50, 10/50 in males, 2/50, 1/50, 6/50 in females) Forestomach • male mice: some evidence (squamous cell papillomas: 0/50, 1/50, 3/50)

Note: The studies were conducted in Fischer 344/N rats and B6C3F_1 mice, 50 animals per group, for two years with five treatments per week (gavage of the indicated dose per kg body mass, or inhalation for 6 h at the indicated ppm level). Numbers in the result column indicate number of tumour-bearing animals per number of surviving animals observed at the various treatment groups, starting with the negative control, followed by low-, mid- (if applicable) and high-dose groups. Carcinogenic effects were not noted in tissues, species and sexes that are not listed.

liver S9), in a chromosomal aberration test in Chinese hamster ovary (CHO) cells in the absence of liver S9 (with an equivocal result in its presence) and in a sister chromatid exchange test in the same cell line in the presence of liver S9. However, in the absence of S9, furfuryl alcohol induced a statistically significant increase in sister chromatid exchanges in this cell line. The absolute increases in exchanges were modest, similar to those observed with HMF in parental V79 cells (like CHO a fibroblastoid cell line from Chinese hamster) (Fig. 15.2).

Furfuryl alcohol is a substrate for rat arylsulphotransferase IV (Sult1a1and/or Sult1c1) (Binder and Duffel, 1988). Surh and Tannenbaum (1994) synthesised the corresponding sulphuric acid. In contrast to SMF, it did not show any mutagenic activity in bacteria. Furthermore, furfuryl alcohol, unlike HMF, was not activated to a bacterial mutagen in the presence of PAPS-fortified rat hepatic cytosolic fraction (Lee *et al.*, 1995). This finding was somewhat surprising, as furfuryl sulphate is more reactive than SMF, whose aldehyde group is electron-withdrawing and thus should decrease the reactivity of the allylic sulphuric acid ester. However, furfuryl alcohol is mutagenic in *S. typhimurium* strains engineered for expression of human SULTs (Fig. 15.3). Thus, furfuryl sulphate may only reach the target DNA when it is generated within the cell, but not when it has to penetrate the bacterial cell wall and membrane before. SMF may

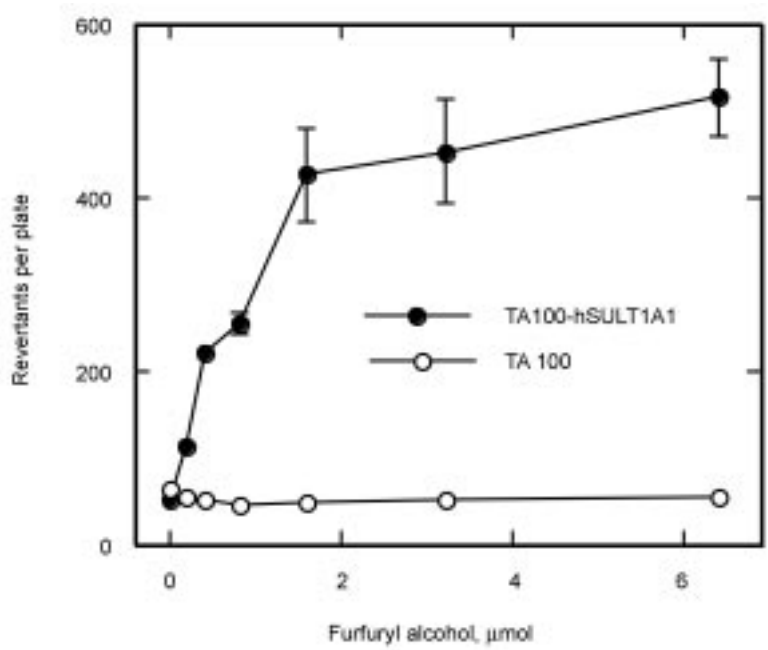

Fig. 15.3 Mutagenicity of furfuryl alcohol in the conventional *S. typhimurium* strain TA100 and in a TA100-derived strain expressing human SULT1A1. Unpublished result from our laboratory. Mutagenicity was determined as described elsewhere (Meinl *et al.*, 2006). Values are means and SE of 3 plates.

have some ability to permeate these barriers due to its lower reactivity and longer lifespan. The half-life times of furfuryl sulphate and SMF in water at 37 ºC amount to 20 s and 120 min, respectively (H. Schneider, Y. Sommer and H. R. Glatt, unpublished result).

We suspect that the renal toxicity and carcinogenicity of furfuryl alcohol in animal models may be mediated by its metabolite furfuryl sulphate. This hypothesis is proposed on the basis of the high renal toxicity of SMF (Section 15.5.2) combined with the findings described in the preceding paragraph. Nasal damage might also be mediated by the sulpho conjugate, as high expression of several Sult forms has been detected in rodent nasal mucosa, where they may terminate the effects of odorants (Tamura *et al.*, 1997; Tamura *et al.*, 1998). However, irritation by furfuryl alcohol itself or its oxidation product, the aldehyde, would also be a plausible mechanism.

15.8.2 Furfural

Furfural (CAS No. 98-01-1) lacks an allylic hydroxyl group, and thus could not undergo the SULT-mediated biotransformation unless it were reduced to the corresponding alcohol. However, oxidation, rather than reduction, of the side-chain dominates the metabolism of furfural, as deduced from the pattern of urinary metabolites, which is similar in rat (Nomeir *et al.*, 1992; Parkash and Caldwell, 1994), mouse (Parkash and Caldwell, 1994) and man (Flek and Sedivec, 1978). Moreover, similar patterns of urinary metabolites are formed from furfural and furfuryl alcohol, as deduced from comparative biotransformation studies following oral administration of the [^{14}C]-labelled compounds to rats (Nomeir *et al.*, 1992). However, there was a difference in the amount of ^{14}C label exhaled as CO_2 in the air, none with furfuryl alcohol, up to 7% of the dose with furfural. The mechanisms underlying decarboxylation are not known, but are thought to involve ring cleavage (Adams *et al.*, 1997).

The main target of acute and chronic toxicity following oral exposure of rats and mice to furfural is the liver (NTP, 1990; Arts *et al.*, 2004; Adams *et al.*, 1997), whereas nasal effects dominate the toxicology after inhalation exposure (Arts *et al.*, 2004). Indeed, local irritating effects have also been found with many other aldehydes and appear to be due to their reactivity with amino and thiol groups.

Two-year carcinogenicity studies in mice and rats were conducted with furfural administered by gavage (NTP, 1990) (Table 15.5). Clear evidence for carcinogenic activity was observed in liver of male mice. Some evidence for hepatocarcinogenicity was also found in female mice and male rats. These increased tumour incidences were only seen at the highest dose level. Renal cortical tumours in male mice and squamous cell papillomas of the forestomach in female mice may have been related to exposure to furfural, but their incidences were too low for statistical corroboration. In another study, furfural demonstrated some tumour-initiating activity in mouse skin (Miyakawa *et al.*, 1991). After intratracheal instillation to Syrian hamsters, it was not carcinogenic

when used alone, but it shortened the latency period for benzo[*a*]pyrene-induced tracheobronchial neoplasms (Feron, 1972). There was no evidence for a carcinogenic or co-carcinogenic activity of furfural in Syrian hamsters exposed to furfural vapour for 52 weeks alone or in combination with intratracheal instillation of benzo[*a*]pyrene or subcutaneous injection of *N*-nitrosodimethylamine (Feron and Kruysse, 1978). Supplementation of the diet with furfural did not lead to an induction of preneoplastic lesions in rat liver, but enhanced the number of 2-acetylaminofluorene-induced lesions (Shimizu, 1986).

Furfural has shown genotoxic activity in several *in vitro* test systems, usually with only moderate potency (reviewed by Adams *et al.*, 1997). The results of *in vivo* tests in rodents were negative; furfural did not induce sister chromatid exchanges or chromosomal aberrations in bone marrow cells of $B6C3F_1$ mice, the strain used in the carcinogenicity studies (NTP, 1990). Furthermore, it did not induce unscheduled DNA synthesis, an indicator of DNA repair, in hepatocytes of male and female $B6C3F_1$ mice and male Fischer 344 rats after oral treatment with the maximum tolerated doses (Lake *et al.*, 2001). Thus, genotoxicity may not be a major mechanism of the carcinogenicity of furfural. Adams *et al.* (1997) have postulated that the observed hepatocarcinogenicity was secondary to pronounced hepatotoxicity.

15.8.3 Furan

Furan (CAS No.110-00-9) is lipophilic and volatile (boiling point 32 ºC). It is a planar cyclic diene with some aromatic character. In contrast to benzene, it readily reacts with alkenes to form cyclohexene derivatives (Diels-Alder reaction) and it can be easily hydrogenated to tetrahydrofuran. Furan is well absorbed by the inhalation and gastro-intestinal routes (Egle and Gochberg, 1979; Burka *et al.*, 1991). It is extensively metabolised in the liver (Burka *et al.*, 1991; Kedderis and Held, 1996). Studies using selective enzyme inducers and inhibitors suggest that cytochrome P450 (CYP) 2E1 plays a prominent role, but that other CYP forms are also involved in the biotransformation of furan. Burka *et al.* (1991) treated rats with [2,5-^{14}C]-furan by gavage. Expired air, urine and faeces were all significant routes of elimination of furan-derived ^{14}C. Within 24 h, 14% of the dose was expired as unchanged furan (chiefly in the first hour) and 26% was expired as $^{14}CO_2$, implying ring opening and oxidation.

Radioactivity in urine (at least ten separate peaks in an HPLC radiochromatogram) and faeces (not further analysed) accounted for 20 and 22% of the dose, respectively. After 24 h, some radioactivity was still present in tissues, with the highest level in liver (13% of the dose), followed by kidney (0.45%) and intestine (0.28%). Approximately 80% of the radioactivity in liver was not extracted by organic solvents and was associated with proteins. The nature of this material has not been elucidated. It may involve covalent binding of reactive metabolites or utilisation of breakdown products of furan in the endogenous metabolism (legend to Fig. 15.4). No binding to DNA was detected. Apart from CO_2 no furan metabolites were identified in this study. Later, *cis*-2-butene-1,4-

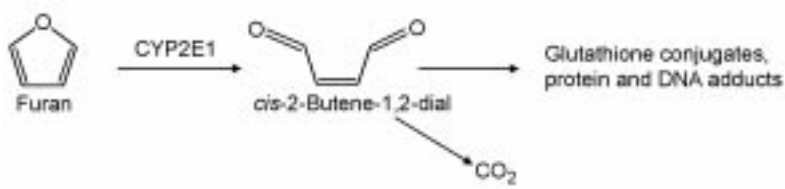

Fig. 15.4 Biotransformation of furan. Conversion of furan to *cis*-2-butene-1,4-dial has been detected *in vitro* in the presence of rat hepatic microsomal systems. The product spontaneously reacts with numerous nucleophiles, such as glutathione, proteins and nucleic acids. CO_2 is a major furan metabolite in rodents *in vivo*. Burka *et al.* (1991) proposed that it may be formed via *cis*-2-butene-1,4-dial and maleic acid, which could be hydrogenated to fumaric acid or hydrated to malic acid (which both are intermediates of the citric acid cycle).

dial was identified as a microsomal metabolite of furan (Chen *et al.*, 1995) (Fig. 15.4). This bis-aldehyde is highly reactive. It readily reacts with proteins and glutathione (Chen *et al.*, 1995; Peterson *et al.*, 2005). It can also form DNA adducts at least *in vitro* (Byrns *et al.*, 2004).

Furan evokes histopathological changes in various tissues of laboratory animals; in general, the most pronounced effects are observed in the liver and the biliary tract; in the rat, kidney is another important target (Masuda *et al.*, 1984; Wiley *et al.*, 1984; Elmore and Sirica, 1991; NTP, 1993). Acute toxic effects were prevented or markedly reduced in animals treated with inhibitors of CYP, such as piperonyl butoxide (McMurtry and Mitchell, 1977), phenylimidazole (Mugford *et al.*, 1997) and aminobenzotriazole (Fransson-Steen *et al.*, 1997). Mugford *et al.* (1997) detected damage to mitochondria and decoupling of oxidative phosphorylation in rat liver early after administration of furan. Fransson-Steen *et al.* (1997) observed dose-related increases in apoptosis and cell proliferation in the liver of furan-treated mice. Wilson *et al.* (1992) noted hepatocellular necrosis and a sharp increase in the frequency of hepatocytes in S-phase 48 h after treatment of rats and mice with furan, indicative of restorative cell proliferation secondary to cytotoxicity. After six weeks of furan administration, male and female rats, but not mice, exhibited bile duct hyperplasia (Wilson *et al.*, 1992).

In long-term studies, furan induced neoplasias in several different tissues (Table 15.5). The most prominent effect involved the induction of cholangiocarcinomas in rats. At all three dose levels used, nearly 100% of the rats (both sexes) had developed this tumour in the two-year study. The study also involved two subgroups of animals killed for an interim evaluation after 9 and 15 months of exposure (data not included in Table 15.5). Even in these animals the incidences of cholangiocarcinomas were very high, whereas the other carcinogenic effects of furan were not detected at these early time points.

Furan did not induce gene mutations in *S. typhimurium* strains TA100, TA1535, TA1537 and TA98 in the presence and absence of rat liver S9 (NTP, 1993). Its metabolite *cis*-2-butene-1,4-dial showed some direct mutagenic

activity in strain TA104, but not in TA97, TA98, TA100 and TA102 (Peterson *et al.*, 2000). In the absence of liver S9, furan induced gene mutations at the *tk* locus in mouse L5178Y lymphoma cells (NTP, 1993), but not at the *hprt* locus of normal Chinese hamster V79 cells or V79-derived cells expressing high levels of human CYP2E1 (unpublished results from our laboratory). No induction of unscheduled DNA synthesis was detected in hepatocytes isolated from furan-treated Fischer rats and $B6C3F_1$ mice (Wilson *et al.*, 1992). Furan administered intraperitoneally to male $B6C3F_1$ mice induced chromosomal aberrations, but not sister chromatid exchanges in bone marrow cells (NTP, 1993). Yet, sister chromatid exchanges were induced by furan in Chinese hamster CHO and V79 cells (NTP, 1993; Glatt *et al.*, 2005a). The concentration–response curve in these cell lines was unusually flat. In V79 cells, essentially the same increase in sister chromatid exchanges was induced over an extremely wide concentration range of furan (3-16,000 μM); moreover, this response was unaffected by cDNA-mediated expression of human CYP2E1 in the cells (Glatt *et al.*, 2005a).

Overall, the mechanism of genotoxicity of furan is unclear in most models. The negative results in gene mutations assays (with the exception of the L5178Y *tk* assay, which also detects large chromosomal damage) and the lack of induction of unscheduled DNA synthesis in liver argue against a significant role of covalent binding of furan or a metabolite to DNA. Furthermore, the dose-response curves of the induction of gene mutations in L5178Y cells and of micronuclei in bone marrow of mice were clearly biphasic; no effect over a wide dose range, followed by a strongly hyperlinear response at the highest dose levels. Such curves might be indicative of an indirect mechanism, involving a primary target other than DNA. For these reasons it is unlikely that primary genotoxic effects are important in furan-induced carcinogenesis. However, furan-induced hepatic tumours in mice display mutation profiles in oncogenes different from those found in spontaneous tumours (Reynolds *et al.*, 1987). It is unknown how these mutations are induced. In any case, the high cytotoxicity and the resulting mitogenesis appear to play a major role in the carcinogenicity, and possibly also in the oncogene activation, by furan.

15.9 Conclusions

Human intake of HMF from food is exceptionally high, 10^3–10^5 times that of other pyrolysis products, such as acrylamide, furan, heterocyclic amines or polycyclic aromatic hydrocarbons. Fortunately, acute and chronic toxicities of HMF are very low. Likewise, HMF shows low genotoxicity in standard *in vitro* test systems. However, this is also the case for acrylamide and furan, established carcinogens in rodent models. Indeed, many small molecules are activated to reactive metabolites by enzyme systems that are poorly represented in standard activation systems. HMF can be activated to an unambiguous mutagen *in vitro* in the presence of active SULT enzyme. Furthermore, there is circumstantial

evidence for a mutagenic activity of HMF *in vivo* from the induction of ACFs in rat colon.

A long-term carcinogenicity study in animals has been initiated only lately and results are not yet available. Although this study is most desired, it can hardly give final answers with regard to human risks. Additional information will be useful for the evaluation of positive and negative results and their extrapolation to humans under actual exposure situations. For example, we need to know more about the formation of reactive metabolites and primary damage (such as DNA adducts) *in vivo*, preferentially in situations relevant for man.

The profiles of HMF metabolites detected in animals are not a concern. A high percentage of the dose is excreted primarily in urine in the form of inconspicuous metabolites whose formation does not appear to involve any reactive intermediates. However, reactive intermediates are usually minor metabolites even with potent carcinogens. Since dietary intake of HMF is so much higher than that of many established human carcinogens (such as aflatoxin B_1), conversion of a minute fraction of the dose (e.g., 0.001 to 0.1%) could still be relevant. Analysis of metabolites in this range is possible only if it is guided by hypotheses on the nature of active metabolites. SMF is such a metabolite. It has been detected *in vitro*, but we have no doubts that it is also formed *in vivo* from HMF. However, we have no idea on the level of its formation and the rate of its disposition, either at human exposure levels or at high doses used in animal experiments. Possible ring-opened metabolites, as observed with furan, may also be of interest with regard to toxification.

Covalent binding is another indicator of the formation of reactive intermediates. The amounts of [^{14}C]-HMF that remained in the organism for extended periods or could not be washed out from cellular constituents were low if related to the dose administered, but substantial in absolute terms. The resulting risk at human exposure levels could range from nil to high, depending on the nature of the non-extractable material (which has not been elucidated). With regard to carcinogenesis, DNA adducts are most relevant in general. SMF forms DNA adducts in cell-free systems, but the corresponding adducts have not yet been observed in tissues of HMF or SMF-treated animals. This negative finding is not reassuring, in as much as adducts were not detected even in cell and animal models in which SMF clearly induced various genotoxic effects (gene mutations, sister chromatid and micronuclei). This points to technical problems rather than the absence of adducts in these models.

The major target tissues for congeners (unsubstituted furan, furfural and furfuryl alcohol) and active forms of HMF (SMF and CMF) are liver and kidney. They will deserve particular attention in future studies with HMF. Moreover, local effects of these other compounds were observed at the sites of applications, such as the airways after inhalation of furfural and furfuryl alcohol and forestomach after gavage of furfural and furan. Diet represents the most relevant exposure of man to HMF. Thus, the alimentary tract may constitute another target for HMF-induced effects. Indeed, oral administration of HMF has led to the initiation and promotion of ACFs in the colon of rats (Zhang *et al.*, 1993).

SMF is the only genotoxic metabolite of HMF identified to date. It is formed by SULTs. Some human SULTs, in particularly SULT1A1, show high enzymatic activity towards HMF, whereas the orthologous murine and rat Sult1a1 enzymes are much less active with this substrate. Moreover, extrahepatic expression of many Sult forms, including Sult1a1, is low in rat and mouse compared to man. Thus, if SMF constitutes an important active metabolite of HMF, then humans may be more susceptible to its adverse effects than standard rodent models. We recently introduced human SULT genes (*1A1, 1A2* and *1B1*), including their regulatory sequences, into new mouse lines. These mice express human SULTs with a human-like tissue distribution. For example, human forms are highly expressed in small and large intestine of these novel mouse lines. It will be interesting to see whether this genetic manipulation affects the susceptibility for HMF-induced effects such as the initiation and promotion of ACFs in colon. Thus, these humanised models should be useful in the risk assessment.

15.10 Sources of further information

HMF levels in a total of 500 food samples representing numerous different products have been determined by Bachmann *et al.* (1997). A comprehensive review of the toxicology of HMF dates back to 1984 (Ulbricht *et al.*, 1984). With regard to acute and chronic toxicity, no major additional studies have been published in the meantime. However, various genotoxicity investigations were carried out. Janzowski *et al.* (2000) competently reviewed these findings, but only briefly addressed the SULT pathway. We have reviewed the SULT-mediated activation of genotoxicants, but without specifically treating HMF (Glatt, 2000, 2005). Technical aspects of the detection of SULT-mediated mutagenicity are in more detail in a further article (Glatt and Meinl, 2005). Furfural, a congener of HMF, is used as a flavour ingredient. The safety of this usage was recently reviewed by Adams *et al*. (1997). The toxicology of furan has been reviewed by Garcia and James (2000); this study also includes a risk assessment. Furan, furfural and furfuryl alcohol have been investigated within the National Toxicology Program of the USA for carcinogenicity in long-term studies with mice and rats. The reports also contain comprehensive information on the occurrence, biotransformation and genetic toxicology of these compounds (NTP, 1990, 1993, 1999). A corresponding study with HMF is in progress (http://ntp-server.niehs.nih.gov – study C95011B). The results have not yet been published but will be extremely important for the risk evaluation.

15.11 Acknowledgement

Our work on HMF has been carried out with support from the European Commission, Priority 5 on Food Quality and Safety (Contract no. FOOD-CT-

2003-506820 Specific Targeted Project), *Heat-generated food toxicants – identification, characterisation and risk minimisation*. This publication reflects the authors' views and not necessarily those of the EC. The information in this document is provided as is and no guarantee or warranty is given that the information is fit for any particular purpose. The user thereof uses the information at his/her sole risk and liability.

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16

Metabolic factors affecting the mutagenicity of heterocyclic amines

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Abbreviations

acetyl-CoA	acetyl coenzyme A (cofactor for NAT)
CYP	cytochrome P450
CYP-R	reductase
DNP	name component for bacterial strains, indicating deficiency in endogenous acetyltransferase (OAT)
GST	glutathione transferase
HCA	heterocyclic aromatic amine
isoIQ	2-amino-1-methylimidazo[4,5-*f*]quinoline
MAPP	2-amino-3-methyl-5-phenylpyridine
NAT	(mammalian) *N*-acetyltransferase
NI	2-amino-3-methylnaphtho[1,2-*d*]imidazole
nitro-Glu-P-1	2-nitro-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole
nitro-IQ	2-nitro-3-methylimidazo[4,5-*f*]quinoline
nitro-PhIP	2-nitro-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
nitro-Trp-P-2	3-nitro-1-methyl-5*H*-pyrido[4,3-*b*]indole
nitroso-Glu-P-1	2-nitroso-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole
nitroso-Trp-P-2	3-nitroso-1-methyl-5*H*-pyrido[4,3-*b*]indole
OAT	bacterial acetyltransferase
PAPS	3′-phosphoadenosine-5′-phosphosulphate (cofactor for SULT)
Phe-P-1	2-amino-5-phenylpyridine (phenylalanine pyrolysate 1)
S9	postmitochondrial fraction from tissue (usually liver) homogenates fortified with a system generating NADPH, the cofactor for CYPs
SULT	sulphotransferase
UGT	UDP-glucuronosyltransferase

16.1 Introduction

In toxicology, the term heterocyclic aromatic amine (HCA) normally designates compounds composed of an aza-heterocyclic aromatic system and an exocyclic amino group. HCAs can be formed during common food preparations, especially frying and broiling, from nitrogen-rich constituents, primarily free amino acids and creatinine. All HCAs examined have demonstrated carcinogenic effects in animal experiments as well as strong mutagenic activity in various test systems. These effects are due to the metabolic formation of electrophilic intermediates that can covalently bind to tissue constituents, such as DNA.

The target organs for carcinogenicity and DNA adduct formation can differ between various HCAs and animal models. Target organs of carcinogenicity consistently show high levels DNA adducts, implying high exposure to the active metabolites. The dominant pathway of metabolic activation of HCAs consists of two steps. The first step involves the hydroxylation of the exocyclic amino group, usually by cytochromes P450 (CYPs). In the second step, a good leaving group is created by esterification, most often by acetyltransferases (NATs) or sulphotransferases (SULTs). Alternative toxification pathways may involve peroxidative activation of the exocyclic amino group, other esterification reactions of *N*-OH metabolites and formation of reactive benzylic sulphuric acid esters.

Various detoxification pathways, such as ring hydroxylation (catalysed by CYPs) and glucuronidation (catalysed by UDP-glucuronosyltransferases (UGTs)), compete with the activation pathways. Likewise, reactive intermediates may be metabolically inactivated, for example by glutathione transferases (GSTs). Each of these enzyme classes is present in mammalian organisms in several individual forms, which can differ widely in their substrate and/or product specificities, kinetic properties and tissue distributions. Substantial differences may even occur between orthologous forms from rodent models and man and between human alloenzymes encoded by polymorphic genes. These characteristics may play a decisive role in the different organotropisms observed between various HCAs and animal models. Understanding of the role of individual human enzymes in the toxification and detoxification of HCAs may help to predict organotropisms, genetic risk factors as well as protective or synergistic interactions of other chemicals with HCAs. In this article, we review the bioactivation of HCAs to genotoxicants and their detoxification with a focus on individual human enzymes mediating these processes.

16.2 Genotoxicity and carcinogenicity of HCAs in standard models

16.2.1 Relationships between genotoxicity and carcinogenicity: general aspects

Tumorigenesis is a long-lasting, complex process, which usually involves the induction of mutations in various oncogenes and tumour suppresser genes, with

clonal expansions of the altered cells between the individual mutation steps. The important role of somatic mutations in tumorigenesis explains why many mutagens are strong carcinogens. In addition, DNA damage (and damage to other cellular constituents by the same reactive intermediates) may alter the cell cycle and induce apoptosis and necrosis with compensatory cell proliferation, processes that can facilitate clonal expansion of preneoplastic cells.

16.2.2 Detection of mutagenicity and carcinogenicity of HCAs

At the beginning of the 1970s, a number of simple and sensitive *in vitro* mutagenicity test systems were developed, such as the 'Ames test' (involving reversion of various *his*$^-$ *Salmonella typhimurium* strains). This test (presented in Section 16.2.3) rapidly became the most widely used assay in genetic toxicology. Sugimura's group observed that smoke condensate and extracts from the surface layer of broiled fish and meat exert high mutagenic activity in this test and identified HCAs as the major active principles (Nagao *et al.*, 1977). Subsequently, approximately 20 different HCAs were isolated from broiled fish, fried meat and/or model reactions containing free amino acids, proteins and creatinine (Sugimura *et al.*, 1977; Felton *et al.*, 1986). The structural formulae of some HCAs are depicted in Figs 16.1 and 16.2. Many HCAs are extraordinarily strong mutagens to *S. typhimurium*. Thus, the number of revertants induced per nmol IQ and MeIQ in strain TA98 amounted to 86,000 and 140,000, respectively, far above the corresponding values for standard carcinogens, such as benzo[*a*]pyrene (81) and aflatoxin B_1 (1900) (Wakabayashi *et al.*, 1992) – to facilitate comparisons, we consistently use the unit 'revertants per nmol'; other data from the literature were converted into this format.

16.2.3 Ames test: metabolic and other characteristics relevant to the study of HCAs

The Ames test (Maron and Ames, 1983) was pivotal in the detection of HCAs and the characterisation of their mode of action as mutagens. Therefore, a description of the test may be useful for some readers.

Target cells

The *S. typhimurium* tester strains have lost their ability to synthesise histidine due to small sequence changes in the histidine operone. Thus, these bacteria can form colonies on histidine-deficient agar plates only after a reverse mutation. An increase in the number of colonies on plates in the presence versus the absence of a test chemical indicates that this chemical is mutagenic. The various tester strains differ in the original *his*$^-$ mutation or in other traits, such as the DNA repair system. Strains TA1538, TA98 and TA100 are most often used for studying HCAs. TA1538 and TA98 carry the same *his* D3052 mutation, a deletion of a C, and require frameshift mutations for reversion. Fuscoe *et al.* (1988) have detected a total of 13 distinct sequence changes in spontaneous and

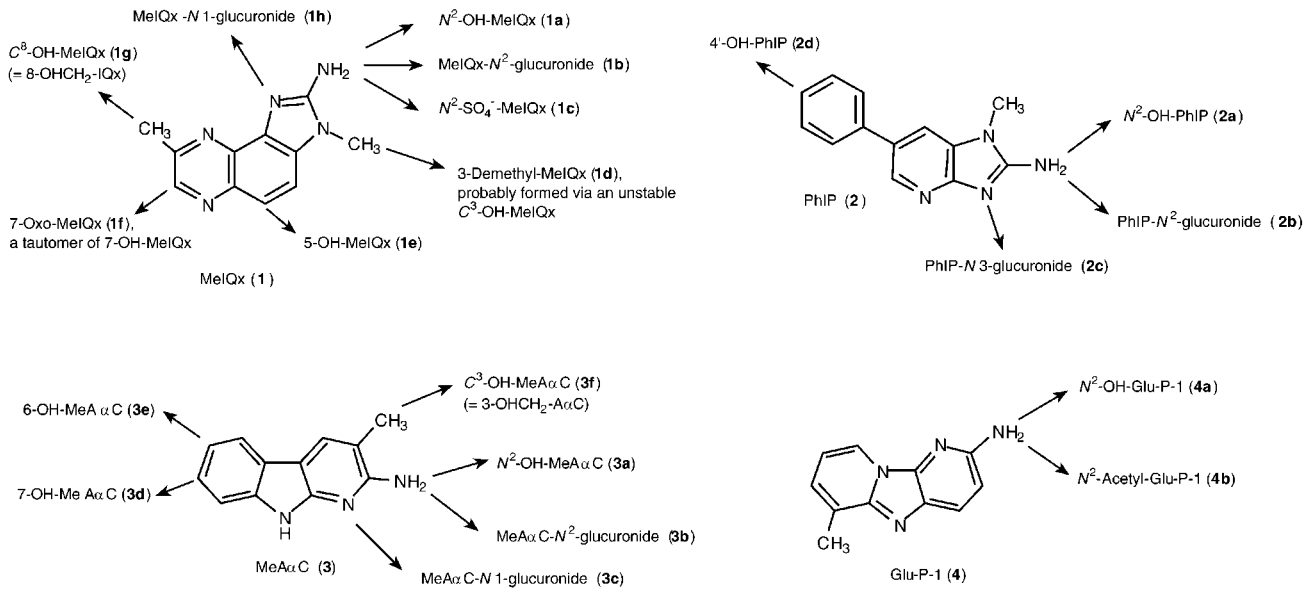

Fig. 16.1 Initial steps of the biotransformation of MeIQx (**1**), PhIP (**2**), MeAαC (**3**) and Glu-P-1 (**4**). The data are primarily based on the following studies: for MeIQx (Turesky *et al.*, 2001; Langouet *et al.*, 2002), for PhIP (Alexander *et al.*, 1994; Turesky *et al.*, 1998), for Glu-P-1 (Ishii *et al.*, 1981; Negishi *et al.*, 1986), and for MeAαC (Frederiksen and Frandsen, 2002, 2004). The structures of metabolites **3b** and **3c** are tentative.

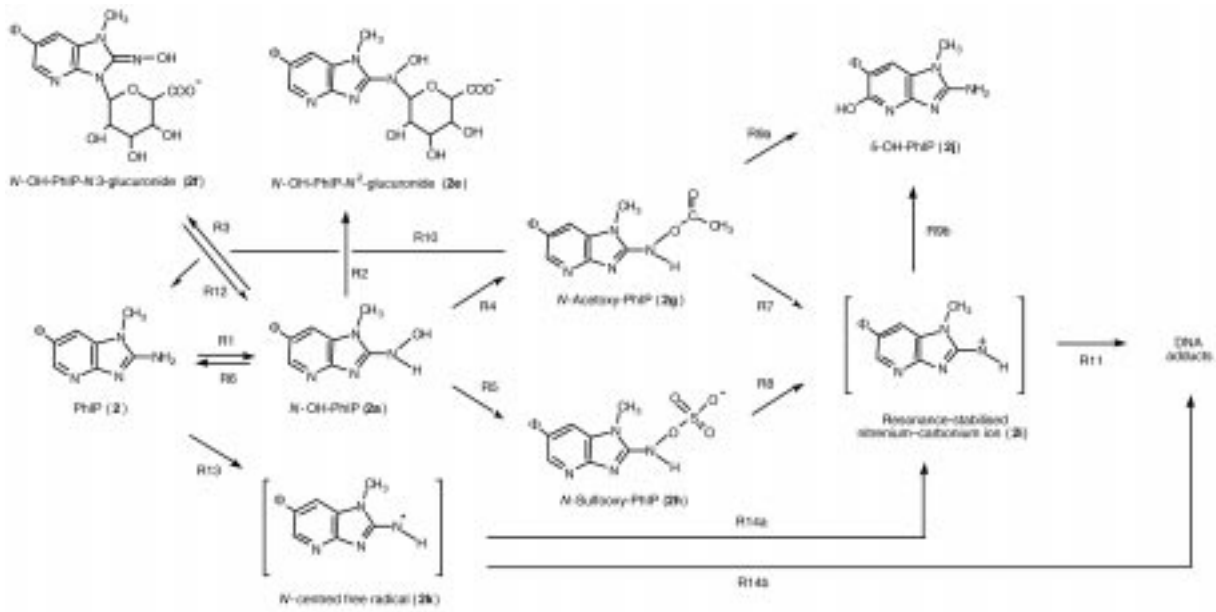

Fig. 16.2 Metabolic generation of a reactive centre at the exocyclic nitrogen atom of PhIP (Φ = phenyl) and some competing pathways. In general, it is not known whether the reactive esters **2g** and **2h** react with nucleophiles via an S_n1 mechanism (involving the occurrence of a distinct cation **2i**) or an S_n2 mechanism. The reactions are mediated by the enzymes or other factors indicated in parentheses: R1 (CYP); R2 and R3 (UGT); R4 (NAT); R5 (SULT); R6 (microsomal enzymes, including cytochrome b_5 and cytochrome b_5 reductase (King *et al.*, 1999), not discussed in the main text); R7, R8, R11 and R14a (or R14b) (spontaneous); R9a or R9b (sulphhydryl groups); R10 (GST); R12 (β-glucuronidase); R13 (peroxidase).

chemically induced *his* D3052 revertants; however, all 21 revertants induced by HCAs (IQ, MeIQ and PhIP) exhibited the same change, a 2-base (CG) deletion situated ten bases upstream from the original C deletion, marking a hotspot for HCA-induced mutagenesis. TA100 is histidine-dependent due to a base substitution and is usually reverted by another base substitution (and sporadically by an in-frame deletion) (Maron and Ames, 1983). Strains TA98 and TA100, but not TA1538, contain the pKM101 plasmid carrying an *umuC* gene, which encodes a translesion DNA polymerase; this polymerase enhances the sensitivity towards some mutagens. However, TA1538 and TA98 normally show similar responsiveness towards HCAs. During the last decade new traits, such as the expression of human mutagen-metabolising enzymes, were introduced into target strains using plasmid-mediated gene-transfer. This transfer is complicated with recipient strains that already contain a plasmid due to interferences between different plasmids and the need of a separate selection marker for each vector. In particular, pKM101 contains a gene conferring resistance to ampicillin and thus this convenient resistance marker can no longer be used. For this reason, most recombinant strains used for testing HCAs are derived from the plasmid-free strain TA1538.

External activating system

Most environmental mutagens are not active as such, but require metabolic activation. This need is taken into account in various standard *in vitro* genotoxicity tests by the addition of an external activating system in the form of a postmitochondrial supernatant (S9) or microsomal fraction from liver homogenate fortified with a system generating NADPH, the cofactor for CYPs. In general, liver S9 is prepared from rats treated with Aroclor 1254 (a mixture of polychlorinated biphenyls), a potent inducer of various CYPs.

It was detected early that Aroclor 1254-induction drastically enhances the activation capacity of S9 for various HCAs, e.g. for Trp-P-2 (Yamazoe *et al.*, 1980) and Glu-P-1 (Niwa *et al.*, 1982). These old findings are illustrated in Fig. 16.3 by results from our own experiments using a fixed amount of IQ and PhIP with varying levels of S9 (in the standard mutagenicity tests, a fixed amount of S9 is used with varying dose levels of the test compound). The amount of S9 fraction leading to a given mutagenic response with IQ and PhIP could be reduced by a factor of 30 to 100 when S9 from Aroclor 1254-treated rather than control rats was used. Aroclor 1254 induces many different CYPs, and suppresses others, in rat liver (Gemzik *et al.*, 1992; Guengerich *et al.*, 1982). It roughly combines the effects of the more selective inducers 3-methylcholanthrene and phenobarbital, which primarily enhance the levels of Cyp1 and Cyp2b enzymes, respectively. Kato and his co-workers detected that 3-methylcholanthrene, but not phenobarbital, strongly induces enzymes that activate HCAs, such as Trp-P-2 (Yamazoe *et al.*, 1980) and Glu-P-1 (Niwa *et al.*, 1982). Then they purified high-spin CYPs (termed MC P-448 and PCB P-P448 IIc at that time) that retained this high activation capacity from the liver of 3-methylcholanthrene and Aroclor 1254-treated rats, respectively. These

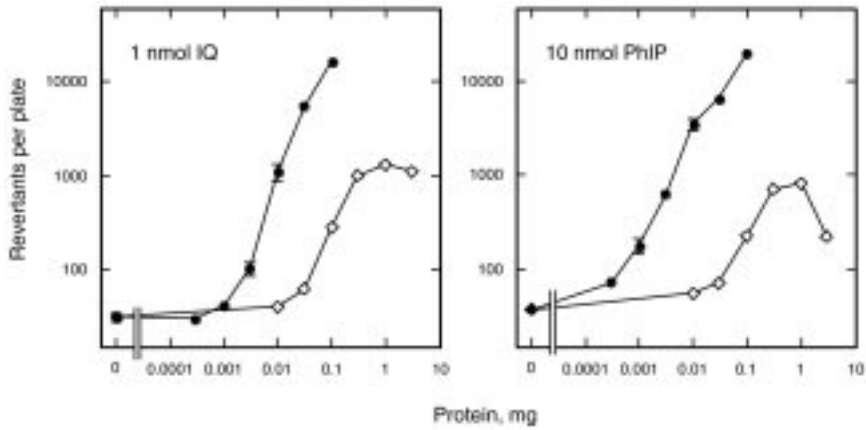

Fig. 16.3 Influence of the amount of S9 enzyme and of enzyme induction on the mutagenicity of HCAs in *S. typhimurium.* Liver S9 was prepared from control (open symbols) and Aroclor 1254-treated (solid symbols) male Fischer 344 rats. Mutagenicity of IQ and PhIP was studied in strains TA1538/DNP-NAT2*4 and TA1538-SULT1A1*1, respectively. Values are means and SE of three plates (unpublished data from our laboratory).

enzymes are identical with each other and correspond to Cyp1a2 in the present nomenclature.

It is important to know that liver S9 does not reflect the full biotransformation system of mammalian organisms. Primarily, it is a source of CYP activity, due to their induction by Aroclor 1254 (in standard tests) and the selective supplementation of the cofactor NADPH. Enzymes requiring other cofactors are essentially inactive, unless their cofactors are added (what is not done routinely). Shortly after the detection of the S9-dependent mutagenicity of HCAs in bacterial tests, it was found that the metabolites hydroxylated at the exocyclic amino group (such as **1a**, **2a**, **3a** and **4a**) were formed by microsomal preparations or reconstituted CYP system from HCAs and that these metabolites did not require the addition of S9 for the induction of mutations in *S. typhimurium* (Yamazoe *et al.*, 1980; Kato *et al.*, 1983).

Bacterial enzymes involved in the activation of HCAs and related promutagens

The observation that a compound is mutagenic in the presence of an external activating system, but inactive in its absence, might lead to the premature conclusion that the entire activation is mediated by the external system. This is not always true. McCoy *et al.* (1983) selected a mutant strain from *S. typhimurium* TA98 with strongly reduced sensitivity to the mutagenic and bacteriotoxic actions of 2,8-dinitropyrene. This strain, TA98/1,8-DNP$_6$, was found to lack an endogenous *O*-acetyltransferase (OAT) activity. Analogous OAT-deficient variants, often carrying the suffix DNP, are also available for other standard strains (Watanabe *et al.*, 1990; Espinosa-Aguirre *et al.*, 1999). They show drastically mitigated mutagenic responses towards various HCAs

and *N*-OH-HCAs. However, the influence of OAT varies from very strong (e.g. $\geq$ 100-fold difference in mutagenicity with IQ, MeIQx and MeIQx) to negligible (e.g. with Trp-P-2 and PhIP) (Table 16.1, columns headed TA1538 and TA1538/DNP; other data of the table will be commented on later).

Seryl-tRNA synthetase purified from baker's yeast (Yamazoe *et al.*, 1981) and prolyl-tRNA synthetases partially purified from the liver of rat and other mammalian species (Yamazoe *et al.*, 1985) strongly reinforced the covalent binding of *N*-OH-Trp-P-2 to DNA in cell-free systems. Like all organisms, *S. typhimurium* contains tRNA synthetases for the proteinogenic amino acids, but it is not known whether any of them could use *N*-OH-HCAs as aminoacyl acceptors and whether this reaction would take place in the intact cell containing high levels of tRNAs, the natural acceptor substrates.

Amino- and nitroarenes are activated to the same proximate mutagens, the hydroxylamines. As described in the preceding sections, enzymes that hydroxylate aminoarenes are not naturally present in *S. typhimurium*. However, several nitroreductases have been found in this species. Rosenkranz and Mermelstein (1983) isolated mutant strains (TA98NR and TA100NR) that lack a major endogenous nitroreductase; these strains are resistant towards the mutagenic action of many, but not all, nitroarenes. The nitro derivatives of various HCAs are potent mutagens in *S. typhimurium* TA98 not requiring the addition of S9. The mutagenicity of nitro-Trp-P-2, nitro-Glu-P-1 and nitro-PhIP was markedly decreased in strain TA98NR, defective in the 'classical' nitroreductase (Saito *et al.*, 1983b; Wild *et al.*, 1991) (Table 16.2). On the contrary, the mutagenicity of 'nitro-IQ' was virtually unchanged in TA98NR, but drastically decreased in TA98/1,8-DNP_6, indicating that OAT was involved in the activation (Dirr and Wild, 1988). It is probable that OAT can act only after reduction of nitro-IQ to *N*-OH-IQ, implying that it is a substrate for a non-classical nitroreductase present in strain TA98 as well as TA98NR.

Conventional *S. typhimurium* strains do not appear to express any xenobiotic-metabolising SULTs and UGTs, but have demonstrated GST activities (Meijer *et al.*, 1980). In conclusion, *S. typhimurium* expresses various enzymes that may mediate some steps in the activation and inactivation of HCAs and structurally related compounds. In order to avoid false positive results, it is important to know whether bacterial toxifying enzymes have a correlate in mammalian organisms.

External versus internal formation of mutagenic metabolites

Saito *et al.* (1985) studied the binding of *N*-OH-Glu-P-1 to DNA in a cell-free system. Addition of purified acetyltransferase (OAT) from *S. typhimurium* and its cofactor, acetyl-CoA, drastically boosted the binding. Likewise, OAT knockout decreased the mutagenic activity of *N*-OH-Glu-P-1 in *S. typhimurium* (Saito *et al.*, 1985) (Table 16.1). Conversely, overexpression drastically intensified its mutagenicity (Watanabe *et al.*, 1990). However, external addition of purified OAT and acetyl-CoA had the opposite effect; it decreased the mutagenicity of *N*-OH-Glu-P-1 (Saito *et al.*, 1985). These results imply that the

Table 16.1 Influence of expression of SULTs and NATs on the mutagenicity of HCAs and *N*-OH-HCAs in *S. typhimurium* TA1538-derived strains

Substance	Revertants per nmol						Reference
	S9	TA1538	TA1538/DNP	TA1538/DNP-NAT2[a]	TA1538/DNP-SULT1A1[b]	TA1538-SULT1A1[b]	
IQ	+	39,400	<400	86,500	nt	nt	Wild *et al.* (1995)
IQ	+		130	185,000	110	nt	c
N-OH-IQ	–	nt	10,100	1,200,000	15,300	nt	Muckel *et al.* (2002)
isoIQ	+	207,000	10,900	261,000	nt	nt	Wild *et al.* (1995)
MeIQ	+	396,000	<1,000	196,000	nt	nt	Wild *et al.* (1995)
MeIQ	+	nt	180	340,000	100	nt	c
MeIQx	+	43,100	<200	21,300	nt	nt	Wild *et al.* (1995)
MeIQx	+	nt	120	48,000	90	nt	c
N-OH-2,4-DiMeIQx	–	34,000	2,100	90,000	7,200	nt	c
NI	+	17.9	3.3	23.3	nt	nt	Wild *et al.* (1995)
Glu-P-1	+	9,300	303	40,700	nt	nt	Wild *et al.* (1995)
Glu-P-1	+	nt	3,300	120,000	9,500	nt	c
Glu-P-2	+	203	8.8	1,134	nt	nt	Wild *et al.* (1995)
AαC	+	55	33	250	820	450	c
MeAαC	+	45	10	450	3,100	800	Glatt *et al.* (2004)
N-OH-MeAαC	–	9,000	2,200	6,400	150,000	550,000	Glatt *et al.* (2004)

PhIP	+	396	329	273	nt	nt	Wild *et al.* (1995)
PhIP	+	450	280	230	1,100	4,000	c
N-OH-PhIP	–	16,000	10,400	7,100	102,100	380,000	Muckel *et al.* (2002), c
N-OH-Phe-P-1	–	4	<7	12	4,100	3,900	Meinl *et al.* (2002), c
N-OH-AMPP	–	4,000	6,000	4,500	nt	430,000	c
Trp-P-2	+	7,300	7,410	6,360	nt	nt	Wild *et al.* (1995)
Trp-P-2	+	38,000	60,000	55,000	83,000	40,000	c

Note: Standard strain TA1538 expresses an endogenous acetyltransferase (OAT). This enzyme is knocked out in TA1538/DNP and replaced by human enzymes in TA1538/DNP-NAT2 (NAT2, wild-type sequence) and TA1538/DNP-SULT1A1 (SULT1A1, wild-type sequence), respectively. TA1538-SULT1A1 was transformed using the same vector, but without prior knockout of OAT. HCAs were tested in the presence liver S9 from male Aroclor-1254-treated rats (0.4–0.6 mg protein per incubation). Several different dose levels of the test compounds were used. Mutagenic potencies (revertants per nmol) were calculated from the initial part of the dose-response curve. Results from independent repeat experiments usually are within a factor of 2. Different conditions (e.g., buffer, incubation times, and numbers of bacteria) were employed in the different studies. Moreover, Wild *et al.* (1995) and our laboratory used different plasmids expressing human NATs, pKEN2 and pKK223-3, respectively (Glatt and Meinl, 2005). For these reasons, the table is primarily designed for comparisons within rows. nt, not tested.

[a] A strain, expressing human NAT1 (wild-type sequence) was additionally used in all studies. Activation by this enzyme was always much weaker than by NAT2, or not detected at all.

[b] Strains expressing other human SULT forms were used in a case-by-case basis (examples in Figs 16.4 and 16.5(b)). Sporadically, expression of SULT1A2 (which however is a minor form in human tissues unlike SULT1A1) resulted in a stronger activation than expression of SULT1A1 (e.g. with *N*-OH-Phe-P-1, Fig. 16.4(a)). To date, we have observed only minor activation of HCAs by the remaining human SULT forms.

[c] Unpublished results from our laboratory using essentially the same protocols as in the cited studies.

Table 16.2 Mutagenicity of HCA metabolites other than hydroxylamines in *S. typhimurium* strains

Metabolite	Strain	Revertants per nmol[a]		Comments, references
		Direct	+ S9	
Nitro-IQ	TA98	68,900	n.t.	Wild *et al.* (1991)
	TA98/1,8-DNP_6	1,820	n.t.	
	YG1024[b]	2,170,000	n.t.	
7-OH-IQ	TA98	2,000	2,000	IQ, concurrently tested was inactive in the direct test, but five times more potent than 7-OH-IQ in the presence of S9 (Carman *et al.*, 1988)
	TA100	0	0	
N^2-SO_3H-IQ	TM677[c]	0	0	Turesky *et al.* (1986)
8-$OHCH_2$-IQx (**1g**)	NM2009[d]	n.t.	+[d]	60 times higher concentration required than of MeIQx for equal response (Langouet *et al.*, 2001)
8-COOH-IQx	NM2009[d]	n.t.	0[d]	Langouet *et al.* (2001)
4-$OHCH_2$-8-MeIQx	TA98	0	28,000	1/4 and 1/10 of the activity of 4,8-DiMeIQx in the corresponding strains (Wakabayashi *et al.*, 1995)
	YG1024[b]	0	92,000	
N^2-Acetyl-Glu-P-1 (**4b**)	TA98	0	3,800	1/4 and 1/20 of the activity of Glu-P-1 in the corresponding strains (Negishi *et al.*, 1986)
	TA100	0	48	
Nitro-Glu-P-1	TA98	1,200	n.t.	1/4 and 1/30 of the activity of *N*-OH-Glu-P-1 in the corresponding strains (Saito *et al.*, 1983b)
	TA98NR	300	n.t.	
Nitroso-Glu-P-1	TA98	1,200	n.t.	1/2 of the activity of *N*-OH-Glu-P-1 in the corresponding strains (Saito *et al.*, 1983b)
	TA98NR	300	n.t.	
3-$OHCH_2$-AαC (**3f**)	TA1538-SULT1A1	32	n.t.	Much less active than *N*-OH-MeAαC in the same strain (Glatt *et al.*, 2004)

Nitro-PhIP	TA98	5,970	n.t.	Wild *et al.* (1991)
	TA98NR	2,210	n.t.	
	TA98/1,8-DNP_6	3,910	n.t.	
	YG1024[b]	6,630		
4′-OH-PhIP (**2d**)	TA98	0	0.43	Much less active than PhIP (Wakabayashi *et al.*, 1995)
N-OH-PhIP-*N*3-glucuronide (**2e**)[e]	TA98	+	n.t.	18-fold increase in mutagenicity in the presence of β-glucuronidase from *E. coli* (Alexander *et al.*, 1991)
N^3-Acetyl-Trp-P-1	TA98	0	132	1/80 of the activity of Trp-P-1 in the corresponding strains
	TA100	0	5.26	(Nagao *et al.*, 1980)
N^3-Acetyl-Trp-P-2	TA98	0	1,060	1/25 of the activity of Trp-P-2 in strain TA98 (Nagao *et al.*, 1980)
	TA100	0	0	
Nitro-Trp-P-2	TA98	1,200	n.t.	1/50 and 1/200 of the activity of *N*-OH-Trp-P-2 in the corresponding strains
	TA98NR	300	n.t.	Saito *et al.* (1983b)
Nitroso-Trp-P-2	TA98	1,200	n.t.	1/2 of the activity of *N*-OH-Trp-P-2 in the same strains (Saito *et al.*, 1983b)
	TA98NR	300	n.t.	
Glutathione conjugate of *N*-OH-Trp-P-2	TA98	3,820	n.t.	Exact structure unknown, six times more potent than *N*-OH-Trp-P-2; two other glutathione conjugates of *N*-OH-Trp-P-2 were inactive Saito *et al.* (1984)

[a] Original data presented in other formats were converted into revertants/nmol. Some values were estimated from figures. n.t, not tested.
[b] TA98-derived strain with plasmid-mediated over-expression of OAT.
[c] Forward mutations at the *hgprt* locus.
[d] 8-$OHCH_2$-IQx and 8-COOH-IQx were tested in the presence of human liver microsomes for *umuC* induction in NM2009, a strain with plasmid-mediated over-expression of OAT.
[e] Originally, it was suggested that this glucuronide is *N*-OH-PhIP-N^2-glucuronide; based on later studies, it appears to be the *N*-OH-PhIP-*N*3-glucuronide (Kaderlik *et al.*, 1994c).

putative active metabolite, *N*-acetoxy-Glu-P-1, did not rapidly equilibrate between the extra- and intracellular compartments and that a substantial portion of the extracellular *N*-acetoxy-Glu-P-1 decomposed (to Glu-P-1 (Saito *et al.*, 1986)) before it reached the intracellular target.

N-Sulphooxy derivatives are other major ultimate mutagens of homo- and heterocyclic aromatic amines. *N*-Sulphooxy-2-acetylaminofluorene, the ultimate hepatocarcinogen of 2-acetylaminofluorene, shows negligible mutagenicity in *S. typhimurium* (Smith *et al.*, 1986). The presence of SULT and PAPS enhances the covalent binding of *N*-hydroxy-2-acetylaminofluorene to DNA in cell-free systems, but attenuates its bacterial mutagenicity (Mulder *et al.*, 1977). However, direct expression of SULT in bacterial target cells reinforces the mutagenicity of *N*-hydroxy-2-acetylaminofluorene up to 1000-fold (Meinl *et al.*, 2002). These findings demonstrate that extracellularly generated reactive sulpho conjugates may not readily penetrate into target cells.

16.3 Biotransformation pathways

16.3.1 Biotransformation of genotoxicants: general aspects

HCAs are 'pro-genotoxicants' and thus require metabolic activation to their 'ultimate genotoxicants'. Most ultimate genotoxicants react chemically with DNA and other cellular nucleophiles. In general, they are more or less short-lived, are not excreted in urine or faeces, and are difficult to detect in *in vitro* incubations using standard chemical analyses. To our knowledge, no ultimate genotoxicants of HCAs have been isolated from biological systems (with the exception of *N*-OH-HCAs, which may show minor intrinsic genotoxic activity but normally are further activated to much more potent genotoxicants). The activation of some pro-genotoxicants involves several biotransformation steps. Intermediates between the original pro-genotoxicant and the ultimate genotoxicants are termed 'proximate genotoxicants'.

In general, the nature of the ultimate genotoxicants has to be deduced indirectly from the structure of the pro-genotoxicants, the enzyme classes required for the activation, and the structures of DNA adducts and stable metabolites formed via the ultimate genotoxicants. Competing pathways may sequester pro-genotoxicants by transforming them on routes that do not involve reactive intermediates.

16.3.2 Metabolites formed from HCAs: products sufficiently stable for isolation and characterisation

It was detected early that *N*-hydroxylation is a common biotransformation reaction of mutagenic HCAs and that the resulting hydroxylamines are major proximate genotoxicants (Yamazoe *et al.*, 1980; Ishii *et al.*, 1981; Niwa *et al.*, 1982; Kato *et al.*, 1983). Whereas this specific biotransformation step has been investigated extensively, systematic studies on the different alternative bio-

transformation pathways of HCAs are less common. They are essentially limited to MeIQx and PhIP (references in Fig. 16.1, Fig. 16.2 and Table 16.3) and, for selected systems, to the α-carbolines AαC and MeAαC (Niwa *et al.*, 1982; Raza *et al.*, 1996; Frederiksen and Frandsen, 2002, 2003, 2004). Findings with other HCAs are sporadic. With these limitations, it appears that the principal biotransformation pathways of HCAs are direct conjugation, hydroxylation at various sites, and conjugation after hydroxylation.

Figure 16.1 contains lists of metabolites formed via a single enzymatic reaction from MeIQx (**1**), PhIP (**2**), MeAαC (**3**) and Glu-P-1 (**4**) in various human or rodent systems. Metabolites of MeIQx and PhIP detected in humans *in vivo* or by human enzyme preparations *in vitro* are listed in Table 16.3. Hydroxylation of HCAs may occur at the exocyclic amino group (metabolites **1a**, **2a**, **3a** and **4a** in Fig. 16.1; **2g**, **2h**, **2e** and **2f** in Fig. 16.2; and *N*-OH-MeIQx-N^2-glucuronide and *N*-OH-C^8-OH-MeIQx in Table 16.3), a ring carbon (**1e**, **1f**, **2d**, **2j**, **3e** and **3d**) or a methyl group (**1g**, **3f**, *N*-OH-C^8-OH-MeIQx, MeIQx-C^8-aldehyde and MeIQx-C^8-carboxylic acid). It is probable that *N*-demethylated metabolites of HCAs (**1d** and 7-OH-3-demethyl-MeIQx) are formed via hydroxylation of the methyl group (as known for numerous dealkylation reactions with other xenobiotics). Hydroxylation of methyl groups attached to a ring-C yields benzylic alcohols (**1g**, **3f** and *N*-OH-C^8-OH-MeIQx), which may be enzymatically oxidised to carboxylic acids. Sequential oxidations at multiple sites of HCAs may also occur (*N*-OH-C^8-OH-MeIQx, 7-OH-3-demethyl-MeIQx).

Various types of direct conjugation reactions have been observed with HCAs, such as sulpho conjugation of the exocyclic amino group leading to the formation of a sulphamate (**1c**), glucuronidation of the exocyclic amino group (**1b**, **2b** and **3b**) and glucuronidation of a ring nitrogen atom (**1h** and **2c**). Direct *N*-acetylation is a common initial biotransformation of some homocyclic aromatic amines. Shinohara *et al.* (1984) studied the *N*-acetylation of various HCAs in the presence of hepatic cytosol from various species (but not from humans). They noticed large species- and substrate-dependent differences in this activity. Overall, the rates of *N*-acetylation were much lower for the HCAs studied (Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC, MeAαC and IQ) than for the homocyclic aromatic amine, 2-aminofluorene. Among the HCAs, the highest rates of *N*-acetylation were found with Glu-P-1 and Glu-P-2. Minchin *et al.* (1992) reported that Glu-P-1 is conjugated by recombinant human NAT2. PhIP was a poor substrate, whereas IQ and MeIQx were not conjugated by this enzyme. No activity was detected with recombinant human NAT1 towards any of these four HCAs. In another study, N^2-acetyl-Glu-P-1 (**4b**) was the major metabolite found in bile of rats treated with Glu-P-1 (Negishi *et al.*, 1986). Likewise, N^2-acetyl-Glu-P-1 and N^2-acetyl-Glu-P-2 were detected in urine, bile and liver and kidney samples from human subjects (Kanai *et al.*, 1988).

In general, conjugation reactions are facilitated after hydroxylation of HCAs. Hydroxylamines of HCAs can form glucuronides via ring (**2f**) or exocyclic (**2e** and *N*-OH-MeIQx-N^2-glucuronide) nitrogen atoms. Phenolic metabolites, such

Table 16.3 Metabolites formed from HCAs in man *in vivo* and by human enzyme systems in *vitro*

HCA	Metabolite	System
MeIQx	*N*-OH-MeIQx (**1a**)	Liver microsomes, CYP1A2 (Turesky *et al.*, 2001)
	N-OH-MeIQx-N^2-glucuronide	Hepatocytes (Langouet *et al.*, 2001); *In vivo:* urine after HCA-rich diet (Stillwell *et al.*, 1999)
	C^8-OH-MeIQx (**1g**)	Liver microsomes, CYP1A2 (Turesky *et al.*, 2001)
	MeIQx-C^8-aldehyde	Liver microsomes, CYP1A2 (Turesky *et al.*, 2001)
	MeIQx-C^8-carboxylic acid	Liver microsomes, CYP1A2 (Turesky *et al.*, 2001); Hepatocytes (Langouet *et al.*, 2001); *In vivo:* principal metabolite in urine after HCA-rich diet (Langouet *et al.*, 2001)
	N-OH-C^8-OH-MeIQx	Liver microsomes, CYP1A2 (Turesky *et al.*, 2001)
	7-OH-MeIQx (**1f**)[a]	Hepatocytes (Langouet *et al.*, 2001)
	7-OH-3-demethyl-MeIQx[a]	Hepatocytes (Langouet *et al.*, 2001)
	MeIQx-N^2-glucuronide (**1b**)	Hepatocytes (Langouet *et al.*, 2001)
	MeIQx-N^2-SO_3^- (**1c**)	Hepatocytes (Langouet *et al.*, 2001)
PhIP	*N*-OH-PhIP (**2a**)	Liver microsomes, CYP1A2 (Turesky *et al.*, 1998); CYP1A1, CYP1A2 and CYP1B1 (Crofts *et al.*, 1998)
	N-OH-PhIP-N^2-glucuronide (**2e**)	Formed from *N*-OH-PhIP by liver microsomes, and also by various recombinant UGTs (1A4, 2B10) (Nowell *et al.*, 1999; Malfatti and Felton, 2004; Girard *et al.*, 2005); *In vivo:* major metabolite in serum and urine after dosing of [^{14}C]-PhIP (Lang *et al.*, 1999; Malfatti *et al.*, 1999); in urine after HCA-rich diet (Kulp *et al.*, 2000)
	N-OH-PhIP-*N*3-glucuronide (**2f**)	Formed from *N*-OH-PhIP by liver microsomes, and also by various recombinant UGTs (1A4, 2B10) (Nowell *et al.*, 1999; Malfatti and Felton, 2004; Girard *et al.*, 2005); *In vivo:* serum and urine after dosing of [^{14}C]-PhIP (Lang *et al.*, 1999; Malfatti *et al.*, 1999); in urine after HCA-rich diet (Kulp *et al.*, 2000)
	Glucu1[b]	Formed from *N*-OH-PhIP by liver microsomes, and also by some recombinant UGTs (1A4, 2B10) (Malfatti and Felton, 2001; Girard *et al.*, 2005)
	Glucu2[b]	Formed from *N*-OH-PhIP by liver microsomes, and also by some recombinant UGTs (1A4, 2B10) (Girard *et al.*, 2005)
	4′-OH-PhIP (**2d**)	CYP1A1, CYP1A2 and CYP1B1 (Crofts *et al.*, 1998)
	4′-Sulphooxy-PhIP	*In vivo:* serum and urine after dosing of [^{14}C]-PhIP (Lang *et al.*, 1999; Malfatti *et al.*, 1999); in urine after HCA-rich diet (Kulp *et al.*, 2000)
	2-OH-deamino-PhIP	CYP1B1 (Crofts *et al.*, 1997); possibly formed via autoxidation of *N*-OH-PhIP to nitro-PhIP followed by spontaneous hydrolysis (Alexander *et al.*, 1991)

Table 16.3 Continued

HCA	Metabolite	System
	5-OH-PhIP (**2j**)	Liver S9 supplemented with acetyl-CoA; thought to be a spontaneous decomposition product of *N*-acetoxy-PhIP (Frandsen and Alexander, 2000)
	PhIP-N^2-glucuronide (**2b**)	Liver microsomes, and also by recombinant UGTs (Nowell *et al.*, 1999); *In vivo:* in serum and urine after dosing of [^{14}C]-PhIP (Malfatti *et al.*, 1999) *In vivo:* urine after HCA-rich diet (Kulp *et al.*, 2000)
	PhIP-*N*3-glucuronide (**2c**)	Liver microsomes, and also by recombinant UGTs (Nowell *et al.*, 1999); *In vivo:* serum and urine after dosing of [^{14}C]-PhIP (Malfatti *et al.*, 1999)

Note: additional metabolites were detected in other species, such as the rat (*in vivo* or *in vitro*) (Alexander *et al.*, 1994; Langouet *et al.*, 2001, 2002; Turesky *et al.*, 2001).
[a] Present as the corresponding tautomeric 7-oxo compounds.
[b] Probably isomers of **2e** and **2f**, structures not elucidated.

as 4′-OH-PhIP, are excellent substrates for UGTs and SULTs (Alexander *et al.*, 1994). The same types of biotransformation pathways for HCAs have been observed in humans and rodent models. However, the relative contribution of the individual pathways, and the regio selectivity of oxidative or conjugative metabolism can substantially vary between different enzyme systems, tissues and species.

16.3.3 Proximate and ultimate genotoxicants of HCAs

Most *N*-OH-HCAs show only low reactivity towards DNA in cell-free systems, even under moderately acidic conditions (pH 5) that boost the reactivity of some homocyclic aromatic hydroxylamines (Turesky *et al.*, 1991). The situation may not be equal for all *N*-OH-HCAs and conditions, as a marked enhancement of DNA binding has been observed with *N*-OH-Trp-P-2 at pH5.5 versus 7.0 by Hashimoto *et al.* (1980), but not by Yamazoe *et al.* (1981). In any case, the reactivity of *N*-OH-HCAs can be strongly enhanced in the presence of cytosolic fractions from mammalian tissues supplemented with one of the following cofactor systems: acetyl-CoA, PAPS, ATP or ATP plus an amino acid (usually proline) (Yamazoe *et al.*, 1985; Kato and Yamazoe, 1987; Davis *et al.*, 1993; Lin *et al.*, 1995). Likewise, expression of an acetyltransferase (OAT or NAT) or a SULT in bacterial target cells can vigorously enhance the mutagenicity of various *N*-OH-HCAs (Table 16.1). These findings suggest that acetic, sulphuric, phosphoric and aminoacylic esters of hydroxylamines are ultimate genotoxicants of HCAs. However, none of these esters has been isolated from biotransformation reactions with HCAs or *N*-OH-HCAs. And chemical standards have been prepared only for selected *N*-acetoxy-HCAs (Brown *et al.*, 2001). Nevertheless, formation of reactive sulphuric and acetic acid esters is plausible, as these conjugation reactions are also known from numerous stable metabolites.

In contrast, phosphorylation and aminoacylation (not to be confused with conjugation of carboxylic acids with the amino group of amino acids) appear to be exotic reactions in xenobiotic metabolism. Moreover, activation via these pathways has been observed only in cell-free systems using high concentrations of *N*-OH-HCA substrates. They may represent biochemical curiosities rather than significant activation pathways in intact cells.

Various *N*-OH-HCAs tend to autoxidise to the corresponding nitroso and nitro derivatives. Standards of these compounds have been synthesised and found to be direct mutagens in bacteria, but less potent than the corresponding *N*-OH-HCAs (Table 16.2). The nitroso compounds are rapidly reduced to *N*-OH-HCAs in the presence of ascorbic acid, NADPH or sulphhydryl compounds (Saito *et al.*, 1983a,b). The nitro derivatives are markedly less potent than the congeneric *N*-OH-HCAs and their activity is usually strongly decreased in strains lacking the 'classical' nitroreductase, although non-classical nitroreductases may also contribute to the activation (Section 16.2.3).

Only a small number of other HCA metabolites have been investigated for mutagenic activity (Table 16.2). Hydroxylation or demethylation at another site may not prevent activation at the exocyclic amino group. Thus, 4′-OH-PhIP, 4-$OHCH_2$-8-MeIQx and *N*-OH-C^8-OH-MeIQx are mutagenic in bacteria, but markedly less potent than the corresponding HCAs or *N*-OH-HCAs, respectively.

7-OH-IQ, a metabolite of IQ (**9**) formed by intestinal bacteria (such as *Eubacterium moniliforme*), is mutagenic in *S. typhimurium* (Bashir *et al.*, 1987; Weisburger *et al.*, 1994). It does not require the presence of an external activating system (which is difficult to understand from the structure of this metabolite). *In vivo*, phenolic hydroxyl groups are efficiently used for conjugation reactions, such as sulphonation and glucuronidation, largely preventing activation at the exocyclic amino group. This property may explain why 7-OH-IQ, unlike IQ, did not induce any tumours in adult rats and showed only marginal hepatocarcinogenicity when administered to newborn mice (Weisburger *et al.*, 1994).

Numerous benzylic alcohols of carboxylic compounds are activated to mutagens by SULTs (Glatt, 2000, 2005). This pathway is analogous to the SULT-mediated activation of hydroxylamines (Fig. 16.2). Many HCAs contain benzylic methyl groups (e.g. Trp-P-1, Trp-P-2, Glu-P-2, MAPP, MeAαC, MeIQ, MeIQx and 4,8-DiMeIQx). In man, oxidation of the 8-methyl group (**1g**) is the major metabolic pathway of MeIQx (**1**) (Langouet *et al.*, 2001). Likewise, 3-hydroxymethyl-AαC (**3f**) is a metabolite of MeAαC (**3**) (Frederiksen and Frandsen, 2002). We have shown that 3-hydroxymethyl-AαC is activated to a mutagen by a human SULT expressed in bacterial target cells, but the resulting mutagenic effect was modest compared to that observed with the corresponding hydroxylamine, *N*-OH-MeAαC (**3a**), in the same test system (Glatt *et al.*, 2004). Other benzylic alcohols of HCAs have not been investigated for SULT-mediated activation. *In vivo*, this activation pathway may be competed by oxidation of the alcohol to the corresponding aldehyde and carboxylic acid.

N^2-Acetyl-Glu-P-1, N^3-acetyl-Trp-P-1 and N^3-acetyl-Trp-P-2 are activated to bacterial mutagens by liver S9, but are less potent than their deacetylated congeners. Their carcinogenic potential has not been explored. The widely used model carcinogen 2-acetylaminofluorene is a weaker bacterial mutagen than 2-aminofluorene, although the carcinogenic activity of both compounds is similar (Heflich and Neft, 1994). The pathway of activation of *N*-acetyl-HCAs has not been elucidated. It might involve deacetylation prior to oxidation, *N,O*-transacylation after *N*-hydroxylation or formation of reactive metabolites that retain the *N*-acetyl group – all these pathways have been detected with 2-acetylaminofluorene (Heflich and Neft, 1994; Glatt and Meinl, 2005).

Various glucuronides of xenobiotics are substrates of mammalian and intestinal bacterial β-glucuronidases or are labile at low pH values. Thus, glucuronides may act as transport forms of procarcinogens/promutagens. Acid-catalysed hydrolysis of glucuronides is thought to play a role in the bladder carcinogenicity of aromatic amines (Babu *et al.*, 1996; Bock, 2002). Likewise, deconjugation of HCA metabolites may be involved in the colon carcinogenicity of HCAs. Indeed, *N*-OH-PhIP-*N*3-glucuronide is a weak direct mutagen in *S. typhimurium* and its activity is enhanced in the presence of β-glucuronidase from *E. coli* (Alexander *et al.*, 1991). Moreover, higher levels of DNA adducts in colon mucosa were formed in conventional versus germ-free rats by PhIP (Hollnagel and Glatt, 2003) as well as IQ (Kassie *et al.*, 2001).

CYPs are not the only enzymes that can mediate oxidative metabolism of HCAs. Various peroxidases, such as prostaglandin H synthase, horse radish peroxidase, bovine lactoperoxidase and human myeloperoxidase, can form DNA-binding metabolites and bacterial mutagens from HCAs (Wild and Degen, 1987; Wolz *et al.*, 2000; Williams *et al.*, 2000; Gorlewska-Roberts *et al.*, 2004). The DNA adducts formed in cell-free systems in the presence of peroxidases are the same as those formed by CYPs, but are not dependent on the presence of conjugating enzymes (Wild and Degen, 1987; Wolz *et al.*, 2000; Williams *et al.*, 2000; Gorlewska-Roberts *et al.*, 2004). It is expected that peroxidases form nitrogen-centred free radicals directly from HCAs by abstraction of an electron (R13 in Fig. 16.2). The free radical may immediately react with nucleophiles or disproportionate to the nitrenium ion and the parent amine.

Incubation of HCAs with peroxidases yielded hydrazo-HCAs, azo-HCAs and nitro-HCAs as stable metabolites (Wolz *et al.*, 2000; Gorlewska-Roberts *et al.*, 2004). Using microsomes from ram seminal vesicles (a rich source of ram prostaglandin H synthase 1) and arachidonic acid (the cofactor for prostaglandin H synthase) for the activation, IQ was mutagenic in bacteria, and this mutagenicity was strongly dependent on the expression of OAT in bacteria, like with CYP-dependent activation systems (Wild *et al.*, 1995). In this model, the nitro-HCA may have penetrated into the bacteria (what might not be possible for the free radical and the nitrenium ion), where it was reduced by a nitroreductase to the hydroxylamine and then conjugated by OAT to a reactive ester. It has been suggested that peroxidases may be involved in the bioactivation of HCAs in some extrahepatic tissues that only express low levels of CYPs, such as the mammary gland or the colon mucosa.

16.3.4 Comparison of reactive acetic and sulphuric acid esters of HCAs

The same DNA adducts are formed by different esters of a given *N*-OH-HCA. This is plausible as the same nitrenium moiety (e.g. **2i** in Fig. 16.2) is transferred to the nucleophilic acceptor molecule. Nevertheless, different esters may vary in their reactivity and other physico-chemical properties. Acetic acid esters are uncharged, whereas sulphuric acid esters are anionic under physiological conditions. The charge may impede the penetration of cell membranes within and between cells. In agreement with the fact that sulphuric acid (pK_a -3 and 1.92 for the first and second proton, respectively) is a stronger acid than acetic acid (pK_a 4.74), sulphuric acid esters are usually more reactive than the corresponding acetic acid esters. For example, $t_{1/2}$ of 1-sulphooxymethylpyrene amounted to about 2.8 min in water at 37 ºC, whereas no significant decomposition of 1-acetoxymethylpyrene was detected within 24 h under the same conditions (Landsiedel *et al.*, 1996).

Finally, reactive acetic and sulphuric esters may differ as substrates for subsequent enzymes. Thus, the addition of glutathione to human hepatic cytosols decreased the acetyl-CoA-dependent (NAT-mediated), but not the PAPS-dependent (SULT-mediated) activation of *N*-OH-PhIP to DNA adduct forming species (Lin *et al.*, 1995).

16.3.5 Detoxification of reactive metabolites of HCAs

Two mechanisms of detoxification should be distinguished, (i) the sequestration of pro-genotoxicants preventing the formation of the ultimate genotoxicants and (ii) the inactivation of ultimate genotoxicants after they have been formed. Glucuronidation may be a good example for the first mechanism. Thus, a high percentage of the dose of PhIP is excreted as PhIP and *N*-OH-PhIP glucuronides in rats (Kaderlik *et al.*, 1994b) and humans (references in Table 16.3). Accordingly, inhibition of UGTs enhanced the genotoxicity of PhIP in primary cultures of rat hepatocytes (Kaderlik *et al.*, 1994b), and cDNA-mediated expression of human UGT1A1 protected CYP1A2-proficient cells against the cytotoxicity and mutagenicity of PhIP (Malfatti *et al.*, 2005).

Numerous ultimate genotoxicants (electrophiles) are detoxified by conjugation with glutathione (Sheratt and Hayes, 2002). Indeed, depletion of glutathione and inhibition of its resynthesis resulted in a 15-fold increase in PhIP-induced DNA adducts in isolated rat hepatocytes (Kaderlik *et al.*, 1994b). The underlying mechanisms may be complex. In aqueous solutions, various *N*-OH-HCAs can autoxidise to the corresponding nitroso and nitro derivatives. This autoxidation of *N*-OH-HCAs, such as *N*-OH-Trp-P-2 (Saito *et al.*, 1984), is prevented, or reversed, in the presence of cysteine or glutathione. A number of labile intermediates [N^2-hydroxy-N^2-(glutathion-*S*-yl)-Glu-P-1, its rearrangement product N^2-(glutathion-*S*-yl)-Glu-P-1 S-oxide, and N^2-(glutathion-*S*-yl)-Glu-P-1] have been identified in the glutathione-mediated reduction of the nitroso derivative of Glu-P-1 to the hydroxylamine (Saito and Kato, 1984). In contrast, metabolically formed *N*-acetoxy-Glu-P-1 is readily converted to Glu-P-1 in the presence of thiols (Saito *et al.*, 1986).

In a similar system, thiols catalysed the hydrolysis of *N*-acetoxy-PhIP to 5-OH-PhIP (**2j**) (Frandsen and Alexander, 2000); the binding of the hydroxyl group from water to the *C*5 position may due to the delocalisation of the charge in the nitrenium/carbonium moiety. Another metabolite of PhIP, nitro-PhIP undergoes a substitution reaction in the presence of glutathione; in the product, the sulphur atom of glutathione is directly attached to *C*2 atom of PhIP (Alexander *et al.*, 1991). The situation is somewhat different for reactions with glutathione in the presence of GST. Saito *et al.* (1984) observed the formation of three different glutathione conjugates from *N*-OH-Trp-P-2 in the presence of rat liver cytosol or purified GST. One conjugate (CH-1) was stable, whereas the others, CH-2 and CH-3, decomposed with release of Trp-P-2 and *N*-OH-Trp-P-2, respectively. Whereas CH-1 and CH-2 were not mutagenic, CH-3 was six times more potent than *N*-OH-Trp-P-2 in *S. typhimurium* TA98. Thus, glutathione and GSTs may not play purely detoxifying roles in the biotransformation of HCAs.

16.4 Overview of enzyme super-families involved in the biotransformation of HCAs

16.4.1 CYPs

A total of 57 functional CYP genes have been detected in the human genome (http://drnelson.utmem.edu/CytochromeP450.html). Nearly half of the CYP forms are primarily involved in the endogenous metabolism, such as the synthesis of steroid hormones. The remaining forms are important in xenobiotic metabolism and show broad, partially overlapping substrate tolerance. Induction of CYP1 enzymes (comprising CYP1A1, 1A2 and 1B1) strongly induces the bioactivation of various HCAs in rodents *in vivo* (Iwata *et al.*, 1990) and by rodent enzymes *in vitro* (section 16.2.3). For this reason, primarily human CYPs of the family 1 have been investigated for their ability to metabolise and activate HCAs. In general, CYP1A2 showed the highest activity, but CYP1A1 and 1B1, and sporadically other forms, were also active towards some HCAs (Table 16.4). Sometimes conflicting results were obtained in different studies.

The differences might be due, at least in part, to large differences in the enzyme levels and substrate concentrations used. Although CYP1A2 appears to be particularly important in the activation of HCAs, some findings indicate a role for other forms: (i) PhIP demonstrated similar carcinogenic activity in wild-type and Cyp1a2 knockout animals (Section 16.6). (ii) Frederiksen and Frandsen (2002) studied the phase-I metabolism of AαC and MeAαC in the presence of liver microsomes from control and Aroclor 1254-treated rats. Enzyme induction substantially enhanced the ratio of *C*-hydroxylation (detoxification) versus *N*-hydroxylation (toxification). These results indicate that constitutive enzymes direct these α-carboline-type HCAs more strongly into the toxification pathway than do Cyp1 and other inducible enzymes. Likewise, human liver microsomes (which normally are low in CYP1 enzymes) preferentially metabolised AαC and MeAαC at the exocyclic amino group. (iii) Rat Cyp4b1 was more efficient than nine other rat

Table 16.4 Biotransformation of HCAs by individual human CYP forms

Substrate	Product or effect studied		Influence of CYPs
IQ	*N*-OH-IQ[a]	+	CYP1A2
		0	CYP1A1, 3A4
IQ	Bacterial mutagenicity, external activation[b]	+	CYP1A2 >> 1A1
IQ	Bacterial mutagenicity, external activation[c]	+	CYP1A2 >> 3A4 > 2C9 > 3A5 (trace)
		0	CYP2B7, 2C8, 2D6, 2E1
IQ	Genotoxicity in bacteria, external activation[d]	+	CYP1A2 >> 1B1, 1A1
IQ	Genotoxicity in CYP-expressing bacteria[e]	+	CYP1A2 > 1A1
		0	CYP1B1, 2C9, 2D6, 2E1, 3A4
IQx	Bacterial mutagenicity, external activation[c]	+	CYP1A2 >> 3A4
		0	CYP2B7, 2C8, 2C9, 2D6, 2E1, 3A5
MeIQ	Bacterial mutagenicity, external activation[f]	+	CYP1A2
		0	CYP1A1
MeIQ	Bacterial mutagenicity, external activation[b]	+	CYP1A2 >> 1A1
MeIQ	Bacterial mutagenicity, external activation[c]	+	CYP1A2 >> 3A4, 2C9 > 3A5 (trace)
		0	CYP2B7, 2C8, 2D6, 2E1
MeIQ	Genotoxicity in bacteria, external activation[d]	+	CYP1A2 > 1A1 > 1B1
MeIQ	Mutagenicity in CYP-expressing bacteria[g]	+	CYP1A2 >> 1A1
		0	CYP1B1
MeIQ	Genotoxicity in CYP-expressing bacteria[e]	+	CYP1A2
		0	CYP1A1, 1B1, 2C9, 2D6, 2E1, 3A4
MeIQx	*N*-OH-MeIQx[a]	+	CYP1A2
		0	CYP1A1, 3A4
MeIQx	Bacterial mutagenicity, external activation[f]	+	CYP1A1 ~ 1A2
MeIQx	Bacterial mutagenicity, external activation[b]	+	CYP1A2 >> 1A1
MeIQx	Genotoxicity in bacteria, external activation[d]	+	CYP1A2 > 1A1 > 1B1
MeIQx	Mutagenicity in CYP-expressing bacteria[g]	+	CYP1A2 >> 1A1 >> 1B1
MeIQx	Genotoxicity in CYP-expressing bacteria[e]	+	CYP1A2
		0	CYP1A1, 1B1, 2C9, 2D6, 2E1, 3A4
2,4-DiMeIQx	Bacterial mutagenicity, external activation[c]	+	CYP1A2 >> 3A4
		0	CYP2B7, 2C8, 2C9, 2D6, 2E1, 3A5
Glu-P-1	Genotoxicity in bacteria, external activation[d]	+	CYP1A2 >> 1A1
		0	CYP1B1
Glu-P-1	Genotoxicity in CYP-expressing bacteria[e]	+	CYP1A2
		0	CYP1A1, 1B1, 2C9, 2D6, 2E1, 3A4
AαC	*N*-OH-AαC[h]	+	CYP1A2 > 1A1, 2C10 (now identified as a particular alloenzyme of CYP2C9)
AαC	Total metabolism in cell-free systems[i]	+	CYP1A2 > rat Cyp1a1 > rat Cyp1a2 > CYP1A1 (trace)
		0	CYP3A4
	N-OH-AαC plus protein-bound products in cell-free systems (for ranking: % of total metabolites)[i]	+	rat Cyp1a1 > CYP1A2 > rat Cyp1a2
		0	CYP1A1, 3A4

Table 16.4 Continued

Substrate	Product or effect studied		Influence of CYPs
MeAαC	Total metabolism in cell-free systems[i]	+	rat Cyp1a1 >> CYP1A2 > rat Cyp1a2 > CYP1A1
		0	CYP3A4
	'Activated products' (dimers and protein adducts) in cell-free systems (for ranking: % of total metabolites) for ranking: % of total metabolites[i]	+	human liver microsomes >> rat Cyp1a1 > rat Cyp1a2, CYP1A2 (trace)
		0	CYP1A1, 3A4
PhIP	*N*-OH-PhIP[j]	0	CYP2S1 positive control: CYP1A2
PhIP	*N*-OH- PhIP[a]	+	CYP1A2, 1A1
		0	CYP3A4
PhIP	Formation of *N*-OH-PhIP and 4′-OH-PhIP[k]	+	V_{max} (*N*-hydroxylation): CYP1A2 > 1A1 > 1B1 Regio selectivity (*N*-hydroxylation): CYP1A2 > 1A1, 1B1 V_{max}/K_m (*N*-hydroxylation): CYP1A1 > 1A2, 1B1
PhIP	Genotoxicity in bacteria, external activation[d]	+	CYP1A1 > 1A2 > 1B1
PhIP	Bacterial mutagenicity, external activation[b]	+	CYP1A2 = 1A1
PhIP	Genotoxicity in CYP-expressing bacteria[e]	+	CYP1A2
		0	CYP1A1, 1B1, 2C9, 2D6, 2E1, 3A4
PhIP	Mutagenicity in CYP-expressing bacteria[l]	+	CYP1A1 = 1A2
		0	CYP2A6, 2C8, 2C9, 2C19 2D6, 1E1, 3A4, 3A5
Trp-P-1	Genotoxicity in bacteria, external activation[d]	+	CYP1A1, 1B1 > 1A2
Trp-P-1	Genotoxicity in CYP-expressing bacteria[e]	+	CYP1A1 > 1A2, 1B1, 2C9, 2D6, 2E1, 3A4
Trp-P-2	Genotoxicity in bacteria, external activation[d]	+	CYP1A2, 1B1 > 1A1
Trp-P-2	Mutagenicity in CYP-expressing bacteria[g]	+	CYP1A2 > 1A1 > 1B1
Trp-P-2	Genotoxicity in CYP-expressing bacteria[e]	+	CYP1A1, 3A4 > 1A2, 1B1, 2C9, 2D6, 2E1, 3A4
Trp-P-2	Bacterial mutagenicity, external activation[f]	+	CYP1A2 > 1A1

Note: unless specified otherwise, human CYPs were used. In most studies, results were not adjusted for differences in expression levels between various CYP forms.

[a] CYPs expressed in *E. coli* and human lymphoblastoid cells (Hammons *et al.*, 1997).

[b] CYPs expressed in COS-1 cells (McManus *et al.*, 1990).

[c] Lysates of recombinant HepG2 cells (Aoyama *et al.*, 1990).

[d] Microsomes from recombinant yeast fortified with rabbit CYP-R (Shimada *et al.*, 1996).

[e] Reporter assay for *umuC* activation in *S. typhimurium* strains expressing human CYP-R, the indicated CYP form and high levels of OAT (Oda *et al.*, 2001).

[f] Microsomes from recombinant yeast (also expressing human CYP-R) (Sengstag *et al.*, 1994).

[g] Reversion of *lacZ*⁻ *E. coli* strains expressing CYP reductase, the indicated CYP form and high levels of OAT (Josephy *et al.*, 2001).

[h] CYPs expressed in *E. coli* (Raza *et al.*, 1996).

[i] Microsomes from recombinant insect cells (Frederiksen and Frandsen, 2003) or from rat and human liver for comparison (Frederiksen and Frandsen, 2002).

[j] Microsomes from recombinant insect cells (Wang *et al.*, 2005).

[k] Microsomes from recombinant insect cells (Crofts *et al.*, 1998).

[l] Mutagenicity in *S. typhimurium* TA1538-derived strains co-expressing the indicated CYP with human CYP-R (Yamazaki *et al.*, 2004).

Cyp forms (including Cyp1a1 and Cyp1b1) in the activation of a series of homocyclic aromatic amines to genotoxicants (Imaoka *et al.*, 1997). Cyp4b1 is highly expressed in bladder tissue. To the best of our knowledge, no data have been published on the biotransformation of HCAs by human or rodent CYP4B1 enzymes.

The situation is further complicated by the observation that orthologous CYPs from different species may vary in their regio selectivities. For example, human CYP1A2 converts PhIP (**2**) nearly exclusively to *N*-OH-PhIP (**2a**), whereas rat Cyp1a2 generates high levels of 4′-OH-PhIP (**2d**) in addition to *N*-OH-PhIP (Turesky *et al.*, 1998). Recombinant human CYP1A2 converted MeIQx (**1**) to its hydroxylamine, *N*-OH-MeIQx (**1a**), and its benzylic alcohol, C^8-OH-MeIQx (**1g**) (Turesky *et al.*, 2001). Rat Cyp1a2 substantially differed in the rate and regio selectivity of MeIQx metabolism from human CYP1A2 (Turesky *et al.*, 2001). Its rates of N^2 and C^8 hydroxylation were 10–15 times lower than with human CYP1A2. Further oxidation of the C^8-OH group, leading to the aldehyde and the carboxylic acid, was extensive with human CYP1A2, but negligible with the rat enzyme. Furthermore, rat Cyp1a2 formed substantial levels of the phenol, 5-hydroxy-MeIQx (**1e**), a metabolite not generated by human CYP1A2 or human liver microsomes from MeIQx. Recombinant rat Cyp1a1 displayed higher *N*-hydroxylation activity for a number of HCAs than its human orthologue (Kanazawa *et al.*, 1999); this species-dependent difference was moderate for PhIP, but large for IQ and Trp-P-2.

16.4.2 NATs

Two NAT forms have been detected in humans (NAT1 and NAT2), whereas three forms (Nat1, Nat2 and Nat3) occur in rats and mice (Boukouvala and Fakis, 2005) (http://www.louisville.edu/medschool/pharmacology/NAT.html). Little is known about Nat3. Human NAT2 appears to be the orthologue of rodent Nat1 and vice versa. NAT2 is high in liver, detected at lower levels in some extrahepatic tissues such as colon, and absent or very low in many other tissues. NAT1 shows a much broader tissue distribution. NAT2 is much more efficient in the activation of HCAs and *N*-OH-HCAs than NAT1, based on the enhancement of mutagenic effects observed in recombinant bacterial and mammalian target cells (Tables 16.1 and 16.5). Formation of *N*-acetoxy-HCAs cannot be determined directly in metabolic systems. However, Saito *et al.* (1986) have developed a simple and sensitive assay for measuring the *O*-acetylation of *N*-OH-Glu-P-1, exploiting the fact that *N*-acetoxy-Glu-P-1 is rapidly converted to Glu-P-1 in the presence of sulphhydryl compounds. The assay may also work with other *N*-OH-HCAs, but has not been used with defined human NAT forms to the best of our knowledge.

16.4.3 SULTs

Eleven SULT forms have been found in human tissues (Glatt, 2002); two additional forms (SULT1C3 and 6B1) have been detected on the gene level, but not as proteins. Most SULTs are capable of activating various pro-genotoxicants

Table 16.5 Mutagenicity of HCAs in Chinese hamster V79 cells engineered for expression of various human xenobiotic-metabolising enzymes

Substance	Result (with lowest effective or highest ineffective concentration, μM)				
	CYP1A2 only	CYP1A2 + NAT1	CYP1A2 + NAT2	CYP1A2 + SULT1A1	CYP1A2 + SULT1A2
IQ	– (30)	+ (1)	+ (0.01)	– (30)	nt
MeIQx	– (100)	– (30)	+ (0.1)	– (30)	nt
Glu-P-1	– (100)	– (30)	+ (0.3)	– (30)	– (100)
AαC	– (30)	– (30)	– (30)	+ (0.1)	+ (0.3)
MeAαC	– (10)	– (10)	– (10)	+ (0.1)	+ (3)
PhIP	– (30)	– (30)	+ (10)	+ (0.3)	+ (1)
Trp-P-2	– (3[a])	– (3[a])	– (3[a])	– (3[a])	– (3[a])

Note: data from Glatt *et al.* (2004) and unpublished results from our laboratory. A similar model was used by Yanagawa *et al.* (1994). They found that IQ and MeIQx are mutagenic in a Chinese hamster liver (CHL)-derived cell line co-expressing human CYP1A2 and human NAT2 (requiring about ten times higher substrate concentrations than in our model), but not in cell lines expressing CYP1A2 alone or together with human NAT1. n.t., not tested.

[a] Trp-P-2 was more cytotoxic in all cell lines than any other HCA studied. Adequate mutagenicity testing was possible only up to a concentration of approximately 3 μM.

(Glatt, 2005). However, they differ in their substrate specificity and tissue distribution. Several human SULT forms are highly expressed in certain extra-hepatic tissues, whereas the expression of most rat and mouse Sults is more strongly focused on the liver.

16.4.4 UGTs

A total of 21 UGTs have been detected in humans (Bock, 2002) (http://som.flinders.edu.au/FUSA/ClinPharm/UGT/). Nine forms belong to the UGT1A subfamily, which usually has a preference for planar substrates; six other xenobiotic-metabolising UGT forms are members of the UGT2B subfamily, which often is involved in the conjugation of sterically more complex molecules. Glucuronides have been detected in the biotransformation of various HCAs using rodent systems. In man, biotransformation data are primarily available for MeIQx and PhIP. Both compounds are excreted in part as glucuronides in humans *in vivo* (Table 16.3). PhIP is the sole HCA for which the activities of individual human UGT forms have been investigated (Section 16.5.5).

16.4.5 GSTs

At total of 15 soluble GSTs have been detected in humans (Sheratt and Hayes, 2002). In addition, some membrane-associated enzymes (MAPEG) exist that mainly have endogenous functions but also may conjugate some xenobiotics (Sheratt and Hayes, 2002). Only a small number of these enzymes have been studied for a possible role in the biotransformation of HCAs, using a rather limited selection of HCA metabolites (Section 16.5.5, last paragraph).

16.4.6 Other enzyme classes and host factors

Virtually no data are available on the role of specific forms of other enzymes classes (such as peroxidases) and further host factors (such as transmembrane transporters) in the toxicokinetics of HCAs.

16.5 Identification of specific human enzyme forms involved in the activation and inactivation of individual HCAs

16.5.1 Specific findings with IQ, IQx, MeIQ, MeIQx and 4,8-DiMeIQx

In nearly all studies conducted, human CYP1A2 was more efficient than any other human CYP form in the *N*-hydroxylation or bioactivation of these HCAs (Table 16.4). In a single study, CYP1A1 was as active as CYP1A2 in the activation of MeIQx, but in five other studies it was either inactive or clearly less active than CYP1A2 (Table 16.4). Peroxidative bioactivation by human enzymes has been observed with IQ and MeIQx (Williams *et al.*, 2000; Wiese *et al.*, 2001; Gorlewska-Roberts *et al.*, 2004), but not investigated with the remaining congeners. Oxidation of the 8-methyl group is the predominant biotransformation pathway of MeIQx in humans but is low in the rat (Langouet *et al.*, 2001; Turesky *et al.*, 2001). This pathway probably leads to detoxification, with the limitation that it has not been studied whether the intermediate benzylic alcohol might be bioactivated to a reactive sulpho conjugate.

All these IQ and IQx congeners are potent mutagens in *S. typhimurium* TA1538 and TA98, but their effects are drastically diminished (by a factor of 15 to 400) when the bacterial acetyltransferase, OAT, is knocked out (Table 16.1). Human NAT2 expressed in target bacteria enhanced the bacterial mutagenicity with equal or higher efficiency than OAT. Likewise, human NAT2, co-expressed with CYP1A2 was essential for the activation of these HCAs in mammalian target cells (Table 16.5). Human NAT1 showed very low activity in these systems compared to NAT2. The exceptions are studies using DNA adduct formation in a cell-free system as the endpoint (Table 16.6) (Minchin *et al.*, 1992; Hein *et al.*, 1994). In these studies *N*-OH-IQ was used at a concentration of 100 μM, whereas 1 pM is sufficient to induce mutations in NAT2-expressing bacteria (see Fig. 16.5(c)). At high substrate concentrations many enzymes become promiscuous, and we are inclined to believe that these effects are irrelevant for real human exposures.

Expression of SULTs in bacterial or mammalian target cells did not affect the mutagenicity of any IQ or MeIQ derivative or their *N*-hydroxylated metabolites studied (Tables 16.1 and 16.5). Negative results were also obtained in cell-free systems with individual human SULT forms (Table 16.6). However, PAPS, the cofactor for SULTs, enhanced the DNA adduct formation by *N*-OH-MeIQx and *N*-OH-IQ in the presence of cytosolic preparations from various tissues of monkeys and rats (Davis *et al.*, 1993). A high substrate concentration (2 μM *N*-OH-HCA) along with non-human enzyme sources were used in this study.

Table 16.6 Bioactivation of *N*-hydroxy-HCAs to DNA binding species by individual enzymes in cell-free systems

Test compound	Influence on level of DNA adducts	Reference
N-OH-IQ (100 μM)	Enhanced by: NAT1 ~ NAT2 (several alloenzymes)[a]	Hein *et al.* (1994)
N-OH-IQ (100 μM)	Moderately enhanced by: NAT1 ~ NAT2[b]	Minchin *et al.* (1992)
N-OH-IQ (1–100 μM)	Unaffected by: SULT1E1[a]	Lewis *et al.* (1998)
N-OH-MeIQx (100 μM)	Enhanced by: NAT2 (several alloenzymes) > NAT1[a]	Hein *et al.* (1994)
N-OH-MeIQx (100 μM)	Moderately enhanced by: NAT2 (but not by NAT1)[b]	Minchin *et al.* (1992)
N-OH-MeIQx (1–100 μM)	Marginally enhanced by: SULT1E1[a]	Lewis *et al.* (1998)
N-OH-AαC (100 μM)	Enhanced by: NAT1 > NAT2[a]	King *et al.* (2000)
N-OH-Glu-P-1 (100 μM)	Enhanced by: NAT2 > NAT1[b]	Minchin *et al.* (1992)
N-OH-PhIP (100 μM)	Enhanced by: NAT2 (several alloenzymes) > NAT1[a]	Hein *et al.* (1994); King *et al.* (2000)
N-OH-PhIP (100 μM)	Enhanced by: NAT2 > NAT1[b]	Minchin *et al.* (1992)
N-OH-PhIP (100 μM)	Enhanced by: human SULT1A1 ~ human SULT1A2 > rat Sult1b1 > rat Sult1c1 > rat Sult1a1[c]	Ozawa *et al.* (1994)
N-OH-PhIP (1–100 μM)	Enhanced by: SULT1E1[a]	Lewis *et al.* (1998)
N-OH-Trp-P-2 (3.77 μM)	Enhanced by: seryl-tRNA synthetase (purified from baker's yeast)	Yamazoe *et al.* (1981)
N-OH-Trp-P-2 (3–6 μM)	Enhanced by: prolyl-tRNA synthetase (partially purified from liver of rat and other species)	Yamazoe *et al.* (1985)

Note: unless specified otherwise, human enzymes were used. Binding in the absence of the corresponding cofactor served as a negative control, unless specified otherwise.

[a] Expressed in *E. coli*.

[b] Enzymes expressed in COS-1 cells, except SULT1A2 (expressed with modified N-terminus in *E. coli*). Results adjusted to expression level.

[c] Enzymes expressed in COS-1 cells; binding in the absence of enzyme, but in presence of the acetyl-CoA (which enhanced the binding) was used as a negative control.

Direct sulpho conjugation of the exocyclic amino group of HCAs yields sulphamates (such as **1c** in Fig. 16.1). Formation of such sulphamates in human hepatic systems has been detected for IQ (Ozawa *et al.*, 1994) and MeIQx (Langouet *et al.*, 2001), but was much lower than in corresponding systems from rat liver (Ozawa *et al.*, 1994). Recombinant rat Sult1a1 and Sult1c1 mediate these reactions, whereas human SULT1A1 and SULT1A2 were ineffectual in this regard (Ozawa *et al.*, 1994). The findings suggest that SULTs represent detoxifying rather than toxifying enzymes for this group of HCAs. This pathway is significant in the rat *in vivo* (Turesky *et al.*, 1986), but may be rather unimportant in man.

16.5.2 Specific findings with Glu-P-1 and Glu-P-2

In the study of Aoyama *et al.* (1990), Glu-P-1 was activated to a bacterial genotoxicant by CYP1A2, but not by any of six other human CYPs examined (Table 16.4). In a second study, low activity was additionally observed with CYP1A1. The genotoxicity was furthermore dependent on acetyltransferase. Human NAT2 appeared to be markedly more efficient in the activation of Glu-P-1 and Glu-P-2 than the endogenous enzyme of *Salmonella* (OAT), at least when the latter enzyme was expressed at its natural level (Table 16.1). The relatively low efficiency of OAT is also reflected in the observation that high over-expression of this enzyme above the endogenous level in the target bacteria strongly enhances the mutagenicity Glu-P-1 (Watanabe *et al.*, 1990).

The second human NAT form, NAT1, supported the activation of Glu-P-1 and Glu-P-2, but not of IQ and IQx compounds, to bacterial mutagens in the Ames test (Wild *et al.*, 1995). NAT1 was much less effective than NAT2 in this study. However, in the study of Oda *et al.* (1999), who used *umuC* activation in *Salmonella* as the endpoint, NAT1 was nearly as efficient as NAT2 in the activation of Glu-P-1 (in the presence of S9); in this assay higher substrate concentrations (1.5–50 μM) were used than in the Ames test conducted by Wild *et al.* (1995) (approximately 0.01–2 μM). Expression of human SULT1A1 in bacterial target cells also enhanced the mutagenicity of Glu-P-1 somewhat (Table 16.1). Likewise, Chou *et al.* (1995) reported that the addition of PAPS enhanced the formation of DNA adducts by *N*-OH-Glu-P-1 (used at a high concentration, 20 μM) in the presence human hepatic cytosol preparations. However, Glu-P-1 only demonstrated mutagenicity in mammalian (V79) cells when human NAT2 was co-expressed with CYP1A2, but not when NAT1 or SULT1A1 were co-expressed with the same CYP (Table 16.5).

In conclusion, it appears that Glu-P-1 and probably also Glu-P-2 have less specific requirement for the enzyme catalysing the second activation step than the IQ and IQx compounds. Nevertheless, NAT2 was clearly more efficient than the other human phase-II enzymes studied. Moreover, NAT2 did not only *O*-acetylate *N*-OH-Glu-P-1, but it also *N*-acetylated (at a lower rate) Glu-P-1 (but not IQ or MeIQx) (Minchin *et al.*, 1992). Indeed, *N*-acetyl-Glu-P-1 is a significant Glu-P-1 metabolite in humans *in vivo* (Kanai *et al.*, 1988).

16.5.3 Specific findings with AαC

Biotransformation of AαC has been investigated with only four individual human CYP forms. CYP1A2 manifested the highest *N*-hydroxylation activity, but some activity was also seen with CYP1A1 and CYP2C10 (an alloenzyme of CYP2C9, originally thought to be encoded by a separate gene), but not with CYP3A4 (Table 16.4). The observation that the ratio of *N*- versus *C*-hydroxylated metabolites is higher with liver microsomes than with CYP1A2 may suggest the presence of an additional human hepatic CYPs with high regio selectivity for the exocyclic amino group of AαC (Frederiksen and Frandsen, 2002, 2003).

King *et al.* (2000) studied the formation of DNA adducts by *N*-OH-AαC in the presence of hepatic cytosolic fraction from various subjects in the presence and absence of acetyl-CoA and PAPS, the cofactors for SULTs and NATs, respectively. The acetyl-CoA-dependent adduct formation correlated with the NAT activity towards sulphamethazine ($r = 0.997$), a characteristic substrate of NAT2, but not with the activity towards *p*-aminobenzoic acid, a marker substrate for NAT1 ($r = -0.04$). However, recombinant NAT1 activated *N*-OH-AαC somewhat more efficiently than did recombinant NAT2. The discrepancy may be due to the fact that NAT2 is expressed in liver at much higher levels than NAT1. PAPS-dependent activation was also substantial in these liver samples, but showed substrate inhibition at *N*-OH-AαC concentrations above 10 μM. This activation was diminished in the presence of 2,4-dichloronitrophenol, an inhibitor of SULT1A1.

Only SULT1A1, SULT1A2 and NAT2, but not NAT1, expressed in target bacterial strains, enhanced the mutagenicity of AαC (using S9 for the first activation step) (Table 16.1). In the V79/CYP1A2 model, mutagenicity of AαC was detected only when one of these SULTs, but not when NAT1 or NAT2, was additionally expressed (Table 16.5). Thus, depending on the experimental model used, different phase-II enzymes are found important in the activation of AαC. I suspect that SULTs may be more critical than NATs in human exposures, as their influence was enhanced from cell-free systems to bacteria to intact mammalian cells. However, this hypothesis requires corroboration (section 16.8).

16.5.4 Specific findings with MeAαC

In rat and human hepatic microsomal systems, [^{3}H]-MeAαC (**3** in Fig. 16.1) was converted to two phenols (**3e**, **3d**) and a benzylic alcohol (**3f**) (Frederiksen and Frandsen, 2002). *N*-OH-MeAαC (**3a**) could not be isolated from the incubations, but the presence of some dimers as well as well as protein adducts indicated the formation of 'activated metabolites', which appeared to be formed via *N*-OH-MeAαC. Interestingly, enzyme induction by Aroclor 1254 resulted in a decrease in the ratio of 'toxified' and *C*-hydroxylated metabolites. The cDNA-expressed CYPs studied (human CYP1A1, 1A2 and 3A4 as well as rat Cyp1a1 and 1a2) formed negligible levels of 'activated metabolites', suggesting that other hepatic CYPs should be involved in the activation of MeAαC (Frederiksen and

Frandsen, 2003). Nevertheless, MeAαC was activated to a mutagen in recombinant V79 cells co-expressing human CYP1A2 with a human SULT (Glatt *et al.*, 2004) (Table 16.5). This finding suggests that either CYP1A2 formed some *N*-OH-MeAαC in this system or, less likely, that the mutagenic effects were induced by the benzylic sulphuric acid ester (discussed below).

MeAαC (in the presence of S9) and *N*-OH-MeAαC (in the direct test) are relatively weak mutagens in the standard *S. typhimurium* strain TA1538 (Table 16.1, Fig. 16.4(c)). Whereas knockout of OAT (resulting in strain TA1538-DNP) moderately decreased the mutagenicity of MeAαC and *N*-OH-MeAαC, expression of some human SULT and NAT enzymes enhanced it (Table 16.1, Fig. 16.4(c)). Expression of SULTs resulted in much stronger activation than expression of NATs. SULT1A1 was the most active human SULT; among the human NATs, activation was observed with form NAT2, but not with NAT1. The same activation characteristics were mirrored in the V79 mammalian cell system. MeAαC only manifested mutagenicity in these cells when human CYP1A2 was co-expressed with human SULT1A1 or SULT1A2, but not in cells that express CYP1A2 alone or together with a human NAT (Table 16.5).

The benzylic alcohol 3-OHCH$_2$-AαC (**3f**) is a major primary metabolite of MeAαC (Frederiksen and Frandsen, 2002). We have demonstrated that it can be activated to a mutagen by human SULT1A1 expressed in *S. typhimurium* TA1538 (Glatt *et al.*, 2004). The resulting mutagenicity was very weak compared to that of *N*-OH-MeAαC (Tables 16.1 and 16.2), but these data may be biased, since 3-OHCH$_2$-AαC was available in very small amounts and thus could only be tested in selected models without opportunities for any optimisation.

16.5.5 Specific findings with PhIP

Species-dependent differences in biotransformation

The high interest in PhIP resides in its absorbing target tissues for tumorigenesis in the rat. However, biotransformation of PhIP differs between species. Conjugates of *N*-OH-PhIP are the major PhIP metabolites formed by isolated human hepatocytes and found in human urine (references in Table 16.3), whereas conjugates of 4′-OH-PhIP predominate in isolated rat hepatocytes (Alexander *et al.*, 1994) and in the rat *in vivo* (Alexander *et al.*, 1989; Kaderlik *et al.*, 1994b). The high regio selectivity in oxidative metabolism of PhIP in humans is due to a chief role of CYP1A2; unlike its rat orthologue and various other CYPs it coverts PhIP nearly exclusively to *N*-OH-PhIP (Wallin *et al.*, 1990; Crofts *et al.*, 1998; Turesky *et al.*, 1998). This metabolite is preferentially glucuronidated at the N^2 position in humans (references in Table 16.3), but at the *N*3 position in the rat (Kaderlik *et al.*, 1994a; Kaderlik *et al.*, 1994c). In humans 4′-OH-PhIP is essentially excreted as its sulpho conjugate; in other species substantial levels of its glucuronide were found in addition (Alexander *et al.*, 1989, 1994; Kaderlik *et al.*, 1994a).

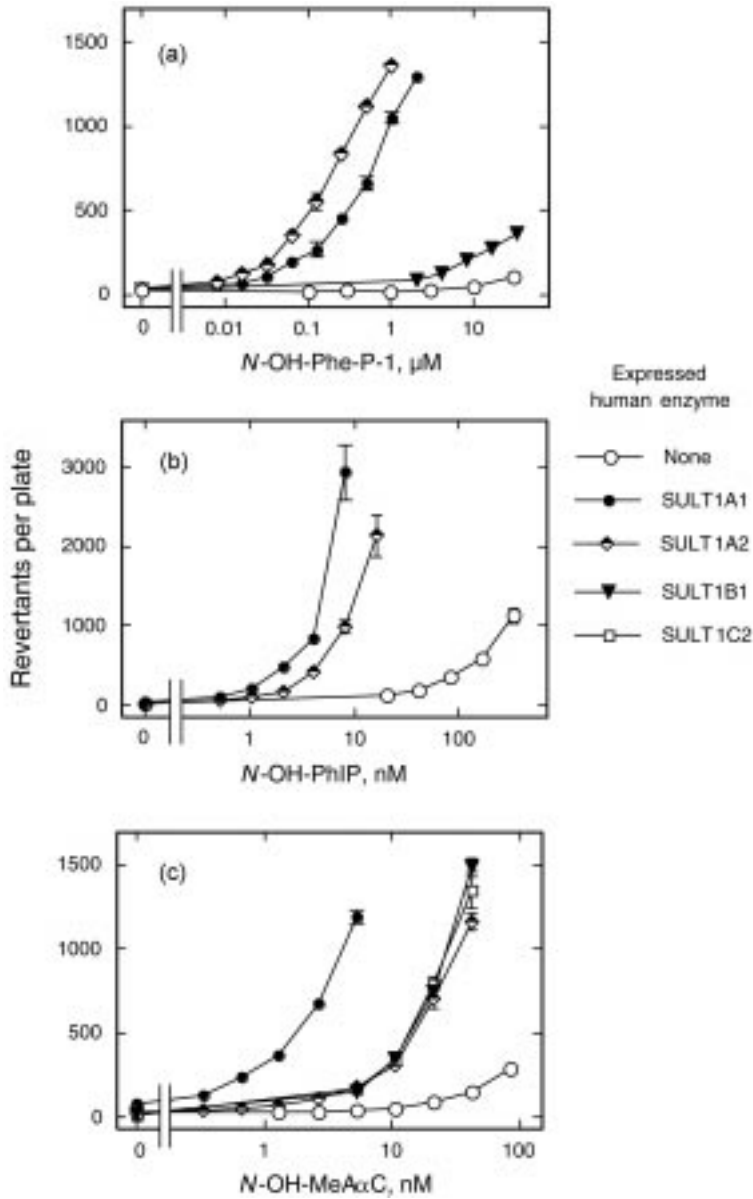

Fig. 16.4 Influence of expression of human SULTs on the mutagenicity of *N*-OH-Phe-P-1 (panel **A**), *N*-OH-PhIP (**B**) and *N*-OH-MeAαC (**C**) and in *S. typhimurium* TA1538. All compounds were investigated in strains engineered for expression of wild-type forms of SULT1A1, 1A2, 1A3, 1B1, 1C1, 1C2, 1C3, 2A1, 2B1a, 2B1 and 4A1. Results are only shown for the standard strain TA1538 and those strains with deviating responses. Values are means and SE of 3 plates. Information on the expression levels (Meinl *et al.*, 2006) and details of the studies (Meinl *et al.*, 2002; Glatt *et al.*, 2004) have been published elsewhere.

N-OH-PhIP (in the direct test) and PhIP (in the presence of liver S9) showed elevated mutagenicity in a *S. typhimurium* TA1538-DNP-derived strain expressing rat Nat1 (unpublished result from our laboratory), but not in corresponding strains expressing human NAT1 or NAT2 (Table 16.1). However, human SULTs were very efficient, much more than rat Sults, in mediating the second activation step of PhIP. Despite these various differences in the biotransformation between rats and humans, PhIP-induced DNA adducts have been detected in the colon mucosa of both species. Turteltaub *et al.* (1999) treated human volunteers diagnosed with colon cancer with low doses of [^{14}C]-PhIP prior to resection of their tumours. Levels of adducts were then determined in colon DNA using accelerator mass spectrometry. Similar studies were conducted in rats. Expressed per dose unit, the levels of DNA adduct were approximately ten times higher in human colon than in rat colon.

Hydroxylation of PhIP by individual CYPs

Several human CYPs convert PhIP to *N*-OH-PhIP. Detailed kinetic studies have been performed with CYP1A1, 1A2 and 1B1 (Crofts *et al.*, 1998). PhIP was converted to *N*-OH-PhIP as well as 4′-OH-PhIP by all three CYP forms. CYP1A2 showed the highest V_{max} value and the highest selectivity for *N*-hydroxylation (60-fold excess over 4′-hydroxylation). However, the catalytic efficiency (V_{max}/K_m) for *N*-hydroxylation was similar or greater with CYP1B1 and 1A1, respectively. While the liver is the principal site of expression of CYP1A2 in man, CYP1A1 and CYP1B1 are primarily found in various extrahepatic tissues.

Further activation of N-OH PhIP by individual conjugating enzymes

Mutagenicity results with recombinant bacterial and mammalian target cells expressing various human enzymes consistently demonstrated strong activation by human SULT1A1 and SULT1A2 (Tables 16.1 and 16.5; Figs 16.4(b), 16.5(a) and 16.5(b)). Human NATs and all the other human SULTs showed negligible influence in the *Salmonella* model. Mutagenicity in V79 cells was somewhat enhanced by NAT2 (much less than by SULT1A1 or SULT1A2), but not by NAT1. Other working groups studied the bioactivation of *N*-OH-PhIP to DNA adduct forming species in cell-free systems. They found ATP-, PAPS- as well as acetyl-CoA-dependent activation by cytosolic fractions from human tissues (Lin *et al.*, 1995; Agus *et al.*, 2000) and activation by cDNA-expressed human enzymes (NAT2, SULT1E1) that were ineffective or showed very low activity in mutagenicity assays in our recombinant target cells (Table 16.6).

Most of these differences might be due to the different substrate concentrations used. In the cell-free systems, *N*-OH-PhIP was used at concentrations of 1–100 μM (Table 16.6), whereas concentrations of less than 1 nM were sufficient to induce strong mutagenic effects in TA1538-SULT1A1 strains (Figs 16.4(b) and 16.5(a)); at concentrations above 100 nM significant mutagenicity was also detected in control strains (Fig. 16.4(b)), suggesting direct reaction of *N*-OH-PhIP with DNA or, more likely, activation by unknown

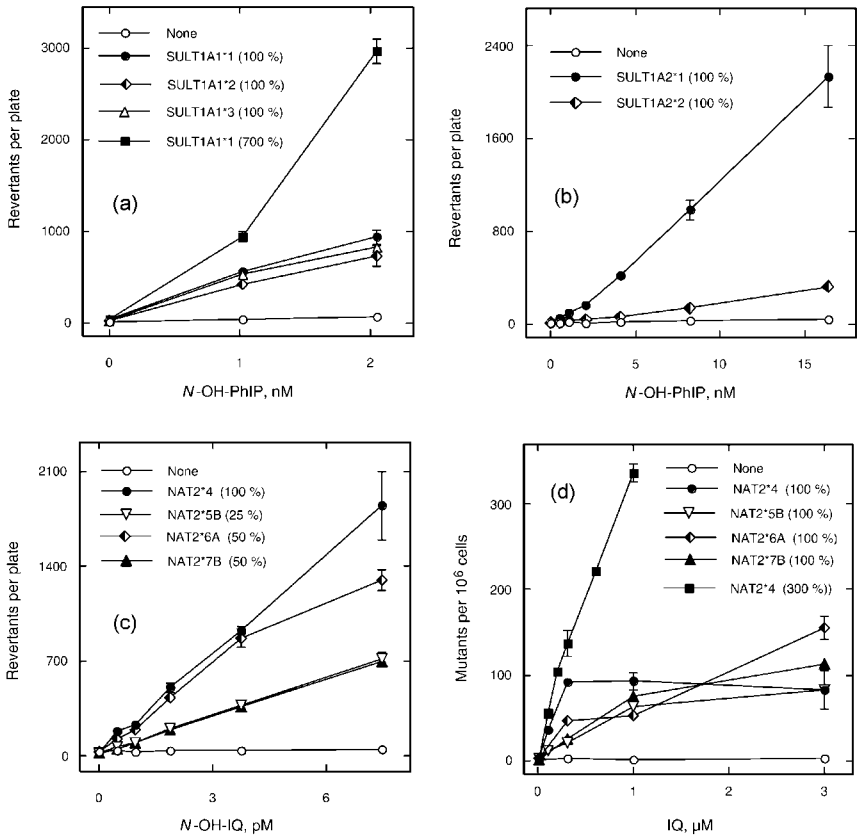

Fig. 16.5 Comparison of genetic variants of human SULT1A1 (**A**), SULT1A2 (**B**) and NAT2 (**C**, **D**) on the activation of HCAs and or their *N*-hydroxy derivatives to mutagens. Since expression can vary between different cDNAs and transformed cell clones, an estimate of this level is given in parenthesis. The level in the standard strain expressing the wild-type alloenzyme (*1 for SULT1A1 and SULT1A2, and *4 for NAT2) was set at 100%. The enzymes were expressed in *S. typhimurium* TA1538 (**A**, **B**), *S. typhimurium* TA1538/DNP (**C**) and a Chinese hamster V79-derived cell line already engineered for expression of human CYP2A1 (Wölfel *et al.*, 1992) (D). Values are means and SE of 3 plates (**A**-**C**) or 2 cultures (**D**). Unpublished results from our laboratory.

bacterial enzymes. A similar situation was found in the V79 model. Strong mutagenicity was seen in V79-CYP-SULT1A1 cells at a concentration of 10 μM. At this concentration, < 10% of the substrate was metabolised during the entire exposure time, implying that the concentration of *N*-OH-PhIP was much below 1 μM. *N*-OH-PhIP, tested directly, was mutagenic to control V79 cells at concentrations above 3 μM. Therefore, we think that activation of HCAs at low substrate concentrations, relevant in human exposures, is much more dependent on specific enzyme forms than at high concentrations, where enzymes become promiscuous.

Conjugation by human UGTs

PhIP itself was conjugated in the order with descending activity by UGT1A4 > 1A1 > 1A9, but not by UGT1A6 (Malfatti and Felton, 2004). Whereas UGT1A4 and 1A1 predominantly formed the N^2-glucuronide, UGT1A9 preferred conjugation at the *N*3 position. *N*-OH-PhIP was a much better substrate for all UGT forms investigated than PhIP. Although conjugation of *N*-OH-PhIP by UGT1A1 was undetected in the initial study (Nowell *et al.*, 1999), particularly high activity and affinity were observed with this form in all subsequent investigations (Malfatti and Felton, 2001, 2004; Girard *et al.*, 2005). UGT1A1 is highly expressed in liver, and Girard *et al.* (2005) observed a good correlation ($r^2 = 0.76$) between the UGT1A1 level and the rate of formation of *N*-OH-PhIP-glucuronides in hepatic microsomal samples from 48 subjects. Thus, UGT1A1 appears to be the principal UGT catalysing the conjugation *N*-OH-PhIP in human liver. Co-expression of human UGT1A1 and CYP1A1 in CHO-derived cell lines reduced the cytotoxicity and the mutagenicity of PhIP compared to cells expressing CYP1A1 alone (Malfatti *et al.*, 2005).

However, various other UGT forms were also able to catalyse the glucuronidation of *N*-OH-PhIP, namely UGT1A4, 1A9, 1A8, 1A10, 2B10, 1A7 and 1A3 (approximately in this order, with some variation in the different studies and at different substrate concentrations), whereas UGT1A5, 1A6, 2B4, 2B7, 2B11, 2B15, 2B17 and 2B28 were ineffectual (Nowell *et al.*, 1999; Malfatti and Felton, 2001, 2004; Girard *et al.*, 2005). Some forms other than UGT1A1 may be important for the conjugation of *N*-OH-PhIP in tissues with low levels of UGT1A1. For example, seven UGT1A forms, including two forms not found in liver (UGT1A8 and1A10), have been detected in human colon (Strassburg *et al.*, 1998).

Four different glucuronides have been isolated from incubations of *N*-OH-PhIP with liver microsomes, the N^2 and *N*3 glucuronides with (**2e** and **2f** in Fig. 16.2) and two unidentified conjugates ('Glucu1' and 'Glucu2') (Girard *et al.*, 2005). The individual UGTs differ in their product specificity (Nowell *et al.*, 1999; Malfatti and Felton, 2001, 2004; Girard *et al.*, 2005). UGT1A4 forms all four products, but with a preference of the N^2glucuronide (Malfatti and Felton, 2001; Girard *et al.*, 2005). Whereas UGT1A1, 1A8 and 1A10 strongly prefer the N^2 position, UGT1A3, 1A7 and 1A9 favour the *N*3 position. UGT2B10 exclusively produces Glucu2 (Girard *et al.*, 2005). The structures of the products are of interest, as the *N*3, but not N^2, glucuronides of PhIP and *N*-OH-PhIP are cleaved by β-glucuronidase from *E. coli* (Styczynski *et al.*, 1993; Kaderlik *et al.*, 1994c). Thus, *N*-OH-PhIP-*N*3-glucuronide excreted into the gut might be cleaved by bacteria in the large intestine and then be activated in the mucosa by SULTs or NATs.

Human GSTs in the metabolism of PhIP

Several human GSTs (GST A1-A1 > A1-A2 > P1-P1 >> A2-A2) were able to reduce the formation of DNA adducts in a cell-free system by *N*-acetoxy-PhIP, but not by *N*-acetoxy-IQ and *N*-acetoxy-MeIQx (Lin *et al.*, 1994). No glutathione conjugates were detected in incubations of *N*-acetoxy-PhIP,

glutathione and GST A1-1. Instead, parent PhIP and oxidised glutathione were found, indicating the occurrence of redox reactions, whose details are unknown.

16.5.6 Specific findings with Phe-P-1 and AMPP

Little is known about the biotransformation and the biological activities of Phe-P-1 (Kosuge *et al.*, 1978) and AMPP. Expression of human SULT1A1 or SULT1A2 in bacterial target cells strongly enhanced the mutagenicity of their *N*-hydroxy derivatives, whereas human NAT2 showed little effect (Table 16.1, Fig. 16.4(a)).

16.5.7 Specific findings with Trp-P-1 and Trp-P-2

Trp-P-1 and Trp-P-2 may be activated to proximate or ultimate genotoxicants by several different human CYP forms (Table 16.4). Moreover, purified human myeloperoxidase converts Trp-P-2 to metabolites that irreversibly bind to protein (Yamada *et al.*, 1980). Trp-P-2 is substantially more potent as a bacterial mutagen (Nagao *et al.*, 1980) and therefore is more often studied than Trp-P-1. Its strong mutagenicity of in *S. typhimurium* is virtually unchanged by knockout of the endogenous acetyltransferase (OAT) or the expression of human SULTs or NATs (Table 16.1). Nevertheless, purified OAT (Saito *et al.*, 1985) and hepatic preparations from some mammalian species (hamster and rat) (Shinohara *et al.*, 1986) enhance the DNA adduct formation by *N*-OH-Trp-P-2 in cell-free systems, although to a lesser extent than with other *N*-OH-HCAs, such as *N*-OH-Glu-P-1.

Trp-P-2 is much more cytotoxic than other HCAs in V79 cells engineered for expression of human CYP1A2, and this toxicity is barely affected by the additional expression of human NATs or SULTs (Table 16.5). Thus, *N*-OH-Trp-P-2 may directly react with cellular target structures or be activated via special mechanisms. Several aminoacyl-tRNA synthetases from different species enhanced the DNA binding of *N*-OH-Trp-P-2 in cell-free systems (Table 16.6), and such enzymes are present in every cell. Furthermore, Saito *et al.* (1984) reported that *N*-OH-Trp-P-2 may be converted by GST to a semistable glutathione conjugate that is more potent as a mutagen in *S. typhimurium* than *N*-OH-Trp-P-2. GSTs have been detected in all bacterial and mammalian cells studied. In conclusion, the activation of Trp-P-2 deviates from the activation of most other HCAs, as neither NATs nor SULTs play a prominent role.

16.6 Knockout and transgenic mouse models for HCA-metabolising enzymes

Snyderwine *et al.* (2002) have studied the influence of *Cyp1a2* knockout on the formation of DNA adducts by HCAs in the mouse. Levels of IQ-induced DNA adducts were markedly attenuated in liver and kidney, moderately decreased in

colon and unaffected in mammary gland. The effect of *Cyp1a2* knockout was stronger with PhIP. No DNA adducts were detected in liver and kidney of knockout mice and adduct levels in mammary gland and colon were decreased by > 90% compared to wild-type mice (adducts levels were determined 3 h after a single dose PhIP). The same *Cyp1a2*$^{-/-}$ mouse line was later used in a carcinogenicity study (Kimura *et al.*, 2003). PhIP administered to newborn mice induced lymphomas and tumours of the lung and the liver in wild-type as well as homozygous *Cyp1a2*$^{-/-}$ mice. Differences in the effects between genotypes were small, and Cyp1a2 even tended to be protective. Thus, other enzymes must have been involved in the activation of PhIP in this model. The discrepancies with the DNA adduct results are surprising. Possibly, biotransformation was slowed down, but not principally shifted into other pathways, in *Cyp1a2*$^{-/-}$ mice.

Cheung *et al.* (2005) introduced the human *CYP1A* gene cluster (*CYP1A1* and *CYP1A2*) into *Cyp1a1*$^{-/-}$ and *Cyp1a2*$^{-/-}$ mice, resulting in the novel *mCyp1a1*$^{-}$-*hCYP1A1*$^{+}$-*hCYP1A2*$^{+}$ and *mCyp1a2*$^{-}$-*hCYP1A1*$^{+}$-*hCYP1A2*$^{+}$ mouse lines. Up to date, no genotoxicity and carcinogenicity data have been published for these lines. However, the oxidative biotransformation pathways for PhIP were shifted in hepatic microsomal preparations as well as *in vivo* by these manipulations. Elimination of mouse Cyp1a2 as well as well expression of human CYP1A2 increased ratio of N^2- to $C4'$-hydroxylation.

It is likely that much competition between different enzymes (NATs, SULTs and UGTs) occurs after the *N*-hydroxylation of HCAs. Recently, a double knockout mouse line for *Nat1* and *Nat2* was created (Sugamori *et al.*, 2003). Moreover, mouse lines expressing human SULT1A1 and/or SULT1A2 with a human-like tissue distribution have been constructed in our laboratory (unpublished work). It will be interesting to see whether and how these manipulations in conjugation enzymes will affect the biological activities of HCAs. Leff *et al.* (1999) have constructed a mouse line that expresses a high level of human NAT2 specifically in the prostate. The level of PhIP-induced DNA adducts in prostate and other tissues was unchanged in this mice compared to wild-type animals. As described in section 16.1, PhIP may be preferentially activated by SULTs, rather than NATs, *in vivo.*

16.7 Genetic polymorphism of human enzymes involved in the activation and inactivation of HCAs

CYP1A2 is genetically polymorphic (http://www.imm.ki.se/CYPalleles/cyp1a2.htm), but it is probable that variable exposure to inducing agents has a much higher impact on CYP1A2 levels and activities than these polymorphisms. Moreover, the minor influence of Cyp1a2 knockout on the carcinogenicity of PhIP in mice (Section 16.6) may also argue against a prominent role of CYP1A2 polymorphism in modifying risks from HCAs. However, this is a preliminary opinion open to revision when more information is available.

The SULT and NAT forms that are primarily involved in the activation of HCAs – SULT1A1, SULT1A2 and NAT2 – show common genetic polymorphisms involving amino acid exchanges, which might affect the intrinsic activities and expression levels of the enzymes (Glatt and Meinl, 2004; Boukouvala and Fakis, 2005). The variant enzyme SULT1A2*2 exhibited much lower intrinsic activity in the activation of *N*-OH-Phe-P-1 (Meinl *et al.*, 2002) and *N*-OH-PhIP (Fig. 16.5(b)) than its wild-type form, SULT1A2*1. On the contrary, variants of SULT1A1 (Fig. 16.5(a)) and NAT2 (Fig. 16.5(c) and (d)) did not differ greatly in the activation of HCAs from the wild-type form when expressed at equal levels. However, alteration of the expression levels in the *in vitro* models had a substantial impact. Therefore, it would be interesting to know how these polymorphisms affect the expression levels in human tissues. Subjects homozygous for SULT1A1*2 showed much lower SULT activity (Raftogianis *et al.*, 1997) and SULT1A1 protein levels (unpublished data from our laboratory) in their platelets than subjects homozygous for SULT1A1*1; however, data are missing for liver, colon and other potential target tissues of HCA-induced carcinogenicity. Likewise, it is not known whether and how NAT2 polymorphisms affect expression levels in human tissues.

Human GST A1-1, which inactivated *N*-acetoxy-PhIP in a cell-free system (Section 16.5.5), is genetically polymorphic. However, GSTs did not protect against DNA adduct formation in cell-free systems by *N*-sulphooxy-PhIP, *N*-acetoxy-IQ and *N*-acetoxy-MeIQx, which are the primary candidates for the ultimate genotoxicants of corresponding HCAs in humans (Sections 16.5.1 and 16.5.5). Studies with other specific human GST forms, HCA metabolites and more complex test systems (e.g., target cells of toxic effects) are largely missing. Therefore, any discussion of the role of GST polymorphisms in human risks from HCAs (Coles *et al.*, 2001) has to be preliminary.

UGT1A1 is the major UGT form catalysing the glucuronidation of *N*-OH-PhIP in human liver microsomes (Section 16.5.5). Several common polymorphisms (number of TA repeats in the TATA box, –3156G/A and –3279G/T) were associated with decreased UGT1A1 expression levels and decreased conjugation rates for *N*-OH-PhIP in liver microsomes (Girard *et al.*, 2005). Thus, many enzymes involved in the biotransformation are genetically polymorphic and/or can substantially vary in their levels due to induction. These variations directly demonstrated impacts on activation and inactivation of HCAs and HCA metabolites in *in vitro* systems using subcellular preparations from human tissues and recombinant enzymes. Therefore they may modulate risks to HCAs in humans. However, a simple translation is difficult, as the toxicokinetics are determined by numerous factors, such as enzyme, substrate and co-factor concentrations, and the presence of functionally redundant and competing enzymes. Furthermore, decreased activity of an enzyme in a given tissue may lead to a shift of the biotransformation to other sites, with possible alterations in target tissues for toxic effects.

16.8 Conclusions

Genotoxicity appears to be the principal mode of action of the carcinogenicity of HCAs. During the last decade much insight has been gained into the human enzyme forms mediating the activation and inactivation of HCAs. These processes can vary between different HCAs. The differences are subtle for the first activation step, the *N*-hydroxylation, a reaction catalysed particularly well by CYP1A2. However, findings with knockout models demonstrate that Cyp1a2 is dispensable for HCA carcinogenicity, at least in the mouse. Biotransformation by other CYPs appears to vary markedly between different HCAs. In 1987, Kato and Yamazoe noticed that clear species-dependent differences in the *N*-hydroxylation of HCAs occur, and that further and greater species- and tissue-dependent differences are found in the subsequent enzymatic activation of *N*-OH-HCAs by various types of esterification.

Based on their mutagenicity in test systems engineered for various human enzymes, HCAs can be classified into three groups. Group 1 (IQ, IQx, MeIQ, MeIQx, 4,8-DiMeIQx, Glu-P-1 and Glu-P-2) is primarily activated by human NAT2. Group 2 (AαC, MeAαC, PhIP, Phe-P-1 and AMPP) is efficiently activated via human SULT1A1 (and often also by SULT1A2, which however is a minor enzyme in human tissues). Group 3 (Trp-P-2) is highly mutagenic even in the absence of NAT and SULT; *N*-OH-Trp-P-2 is either directly effective or is activated by processes that have not been elucidated sufficiently (Section 16.5.7). Other HCAs cannot yet been classified into these groups due to the lack of data.

Separation into these groups is not absolute. For example, some SULT-dependent activation has also been seen with *N*-OH-Glu-P-1, and some acetylation-dependent activation has been observed with all members of groups 2 and 3 – usually at very high concentrations of the *N*-OH-HCAs or with non-human enzymes. UGTs may sequester HCAs and *N*-OH-HCAs. However, specific data are available only for PhIP, with UGT1A1 playing a prominent role in liver microsomes. Data from animal studies suggest that conjugation with glutathione is another detoxification pathway for HCAs. Indeed, *N*-acetoxy-PhIP (which may be only a minor active PhIP metabolite in humans in my opinion) was inactivated by human GST A1-A1 and with lesser efficiency by other GSTs studied. All enzymes found to play a major role in the metabolism of HCAs are encoded by polymorphic genes, and it is evident from *in vitro* studies that these polymorphisms have an impact on the activation or inactivation of HCAs. However, it will be necessary to specify the role of these polymorphisms in the complex toxicokinetics occurring in intact organisms, humans or humanised animal models.

16.9 Sources of further information

Dashwood (2003) has reviewed the use of transgenic and mutant animal models in the study of HCA-induced mutagenesis and carcinogenesis. Turesky (2005)

recently reviewed the biotransformation of HCAs with special consideration of differences between species. A recent article from our laboratory deals with technical aspects in the study of NAT- and SULT-mediated mutagenicity (Glatt and Meinl, 2005).

16.10 Acknowledgements

Our work on HCAs has been carried out with support by the Commission of the European Communities RTD programme *Quality of Life and Management of Living Resources*, contract QLK1-1999-01197. This publication reflects the author's views and not necessarily those of the EC. The information in this document is provided as it is and no guarantee or warranty is given that the information is fit for any particular purpose. The user thereof uses the information at his/her sole risk and liability.

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Part III

Minimising the formation of hazardous compounds in foods during heat treatment

17

Modifying cooking conditions and ingredients to reduce the formation of heterocyclic amines

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17.1 Introduction

Research leading to the discovery of a series of mutagenic and carcinogenic heterocyclic amines (HCAs), around 30 years ago, was inspired by the idea that since cigarette smoke was carcinogenic, smoke produced during grilling of food, especially meat or fish, might also be carcinogenic (Sugimura *et al.*, 1977). Today, there are literature reports of more than 20 derivatives of HCAs, actually produced by cooking or heating of meat or fish. After evaluations based on high-dose, long-term animal studies and *in vitro* and *in vivo* genotoxicity tests, the International Agency for Research on Cancer (IARC 1993) concluded that several HCAs present in cooked foods are possibly (2A) or probably (2B) carcinogenic to humans. Yet, there is insufficient scientific evidence that these toxicants really cause human cancer, and no limits have been set for their presence in cooked foods. However, the relevant authorities in most Western countries recommend minimising their occurrence in our diet.

17.2 Chemical structures

The chemical structures of several HCAs have been elucidated, most of them being previously unknown compounds (Felton and Knize, 1991; Sugimura, 1997). The molecular weights of the HCAs are around 200 g/mol (Jägerstad *et al.*, 1998). The chemical structure and trivial names of some HCAs are shown in

Appendix II. Depending on their structures, HCAs can be divided into subgroups: imidazo-quinolines (IQ), imidazo-quinoxalines (IQx), imidazo-pyridines (IP), furu-imidazo-pyridines and amino-carbolines (α, β and γ). For full names, see the list of abbreviations (Appendix I). The IQ and IQx compounds are derivates of imidazo-quinolines or imidazo-quinoxalines, for example 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ).

The IP compounds include 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP) and 2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine (TMIP). The HCA 2-amino-1,6-dimethylfuro[3,2-e]imidazo[4,5-*b*]pyridine (IFP) is the only one containing an oxygen atom. The structure of IFP was long unknown, but Knize *et al.* (1990) suggested a tentative structure which was later confirmed (Pais *et al.*, 2000). The amino-carbolines differ from the other groups in that the amino group is attached to a pyridine ring instead of an imidazo group. The β-carbolines 1-methyl-9H-pyrido[3,4-*b*]indole (Harman) and 9H-pyrido[3,4-*b*]indole (Norharman) have no exocyclic amino group and lack mutagenic activity but enhance the mutagenic activity of other compounds.

17.3 Precursors

Model systems are useful tools to use when studying the formation of HCAs regarding precursors, inhibition agents and kinetics. In 1983, Jägerstad and co-workers (1983) suggested a pathway for the formation of IQ and IQx compounds from the precursors free amino acids, creatine or creatinine and sugars via the Maillard reaction. This route of formation has been supported in many studies based on model systems (see Skog *et al.*, 1998). The three groups of precursors are naturally occurring in meat and fish muscle tissues, where free amino acids and sugars are metabolic products from muscle protein and glycogen, respectively, while creatine is an energy metabolite. Creatine is present at significant levels only in muscle cells, which explains the general lack of HCAs in other common fried protein-rich foods (e.g., pancakes). MeIQx is one of the most abundant HCAs and can be formed from almost any amino acid when heated together with creatine or creatinine and glucose in a model system (Skog *et al.*, 1998). The amino-carbolines have been shown to arise through pyrolysis of amino acids and proteins at temperatures above 300 ºC (Jägerstad *et al.*, 1998). They are assumed to be formed via free-radical reactions, but little has, in fact, been done to determine the mechanisms and pathways leading to their formation. Amino-carbolines have also been found in meat and model systems heated at normal domestic cooking temperatures (Borgen *et al.*, 2001; Skog *et al.*, 1997), which suggests that another mechanism of formation exists. Harman and Norharman are the most common amino-carbolines and tryptophan is an important precursor; for a review see Skog *et al.* (2000).

17.4 HCA levels in cooked foods

HCAs are generally formed at ppb levels during cooking of meat and fish. Table 17.1 shows typical amounts of some HCAs formed in cooked foods taken from the literature. MeIQx is commonly found in amounts up to 10 ng/g, 4,8-DiMeIQx below 5 ng/g and PhIP commonly below 35 ng/g. There are large variations between the reported HCA concentrations, probably due to different cooking conditions and meat composition and ingredients. In general, pan-frying and grilling produce high yields of HCAs at cooking temperatures from 200 ºC and above, boiling yields little or no HCAs, and deep-fat frying, and roasting give variable yields.

The formation of MeIQx in fried meat has been shown to increase with cooking temperature and time in the intervals 150–230 ºC, and 2–10 min (Skog *et al.*, 1995). Several studies have shown the presence of HCAs in meat extracts, flavouring products or flavouring ingredients produced by heating various mixtures containing, e.g., creatine, protein or amino acids, carbohydrates and fat or fatty acids, for several hours at temperatures of about 100 ºC. HCAs may also be formed at temperatures below 100 ºC. Bouillon cubes contain very low or undetectable amounts of HCAs (Solyakov *et al.*, 1999). Johansson and Jägerstad (1994) reported the presence of HCAs in salmon and flounder smoked at low temperatures (80–85 ºC). This is surprising, but the low water activity at the surface of the product and/or the relatively long heating time (more than 24 hours) may favour the formation of HCAs.

The amounts of HCAs are generally higher in cooked meats than in fish, and in pure meat than in mixed meat products, e.g. meatballs or sausages. The levels of HCAs in pan residues after frying different meat and fish products are generally the same as in the corresponding food products, but in some cases the amounts are considerably higher (Skog *et al.*, 1998). Industrially processed 'ready-to-eat' food products generally contain very low or undetectable amounts of HCAs (Skog *et al.*, 1998), with a few exceptions. Fast-food products are reported to contain low levels of HCAs (Klassen *et al.*, 2002; Knize *et al.*, 1995; Zimmerli *et al.*, 2001), which may be explained by the high fat content or the incorporation of non-meat ingredients.

17.5 Daily intake of HCAs

Exposure to HCAs can be estimated from the amounts excreted in the urine, general levels in foods and a combination of data on dietary habits and levels of HCAs in foods. Data from some of the most recent studies estimating mean daily intake of HCAs range from around 60 up to 1820 ng per person (Augustsson and Steineck 2000; Butler *et al.*, 2003; Layton *et al.*, 1995; Nowell *et al.*, 2002; Sinha *et al.*, 2001). The variations in HCA intake can partly be explained by variations in eating habits among populations, different study designs, variations in HCA analysis and the number of HCAs included. Maxi-

Table 17.1 Literature data: HCAs (ng/g cooked weight) in some heat-treated meat samples

Cooking conditions				MeIQx	PhIP	Harman	Reference
Sample	Method/ equipment	Temperature, ºC	Time, min				
CHICKEN							
Chicken	Boiled	100	240	nd	nd	0.2	Solyakov and Skog (2002)
Breast	Pan-fried	140–225	12–34	0.1–1.8	nd–38.2	0.1–6.9	Solyakov and Skog (2002)
Breast	Pan-fried	160–220	15–25	nd–10.4	0.5–19.4	na	Krul *et al.* (2000)
Breast	Pan-fried	150–225	30	nd–0.5	nd–10.0	nq	Skog *et al.* (1997)
Breast	Pan-fried	197–211	14–36	1–3	12–70	na	Sinha *et al.* (1995)
Breast	Roasted	175–240	25–40	nd–1.7	nd–3.0	nq–3.3	Solyakov and Skog (2002)
Breast	Broiled	79–86	9–17	nd–3	6–150	na	Sinha *et al.* (1995)
Breast	Fried	175–200	12	nd	46.9	7.5	Busquets *et al.* (2004)
Legs	Fried	200	10	0.13	0.21	0.12	Chiu *et al.* (1998)
Chicken	Roasted	Com.		2.2–3.2	2.4–5.3	na	Richling *et al.* (1998)
Chicken	Grilled	220	40	0.11	1.4–7.6	na	Tikkanen *et al.* (1996)
Turkey breast	Pan-fried	140	20	1.4	3.8	na	Murkovic *et al.* (1997)
Turkey breast	Pan-fried	190	12	4.4	64.9	12.0	Brockstedt and Pfau (1998)
BEEF							
Patties	Pan-fried	150–230	2–10	nd–7.3	nd–32	na	Knize *et al.* (1994)
Patties	Pan-fried	180	6	0.03–2.8	1.2	na	Johansson and Jägerstad (1994)
Patties	Pan-fried	180–190	6–20	1.3–8.2	1.9–23.2	na	Sinha *et al.* (1998a)
Patties	Pan-fried	180–200	12	3.5	4	na	Reistad *et al.* (1997)
Patties	Fried	175–200	11.2	0.7	0.6	1.9	Busquets *et al.* (2004)
Patties	Pan-fried	180–220	3–6	0.05–2.76	0.65–8.08	na	Persson *et al.* (2003b)
PORK							
Chops	Pan-fried	160–200	6	0.08–0.86	0.05–3.27	nd–1.08	Olsson *et al.* (2005)
Chops	Pan-fried	150–225	8–9.5	nd–2.6	nd–4.8	na	Skog *et al.* (1995)

na = not analysed; nd = not detected.

mum values of around 5,000 ng/person per day have been reported (Augustsson *et al.*, 1999; Nowell *et al.*, 2002).

17.6 Factors affecting the yield of HCAs

The formation of HCAs depends on a combination of several factors such as the presence and relative amounts of precursors, cooking methods and ingredients, e.g. antioxidants or compounds that affect the water-holding capacity.

17.7 Effects of varying levels of natural precursors in meat

The concentrations of the three major groups of natural HCA precursors: creatine, sugars and free amino acids and dipeptides, have been reported to vary in meat samples from different organs (Laser Reuterswärd *et al.*, 1987), between different animal species (Borgen *et al.*, 2001; Pais *et al.*, 1999; Vikse and Joner, 1993), between animals of the same species and within a species (Olsson *et al.*, 2002) (see Table 17.2). This natural variation may affect the formation of HCAs during cooking. For example, poultry products differ from beef and pork products in that extremely high levels of PhIP have been detected in cooked chicken, above 100 ng/g (Sinha *et al.*, 1995; Solyakov and Skog, 2002; Holder *et al.*, 1997).

Table 17.2 Precursor composition (average values in micromoles per g wet weight)

Meat sample	Creatine (μmol/g)	Free amino acids (μmol/g)	Glucose (μmol/g)	Total HCAs (ng/g tissue)	Reference
Pig meat	39.7	10.7	75.3	(2.0)	Olsson *et al.* (2002)
(carriers of RN^- allele)	37.7	9.4	55.6	(0.49)	Olsson *et al.* (2005)
Pig meat	36.4	10.1	17.2	(4.2)	Olsson *et al.* (2002)
(non-carriers of RN^- allele)	34.6	12.0	10.9	(4.13)	Olsson *et al.* (2005)
Beef meat	33.0	30.0	12.0	NAm	Laser Reuterswärd *et al.* (1987)
Liver (bovine)	~2	100	183	NAnm	Laser Reuterswärd *et al.* (1987)
Pork	34	42.5	28.6	47	Pais *et al.* (1999)
Beef	45	26.6	38.0	3.0	Pais *et al.* (1999)
Poultry	26–32	51.5 (50–70)	2.6 (1.9–3.2)	9–54	Pais *et al.* (1999)
Fish	52	16.2	1.16	12	Pais *et al.* (1999)
Chicken breast	24–28	29–34	8–27	14.4–38.8	Solyakov and Skog (2002)

Nam = not analysed, but mutagenic; NAnm = not analysed, no mutagenic activity.

17.7.1 Creatine

The physiological role of creatine in muscles is to serve, in its phosphorylated form, as a reservoir of high-energy phosphate for ATP generation during muscle contraction. This explains the relatively high content of creatine in muscles (0.3–0.6%) in comparison with other tissues (Dvorak, 1981). In muscle cells, almost all creatine (>90%) is in a free state a few hours after slaughter (Sulser, 1978). Olsson and co-workers (2002) studied the chemical composition of meat samples from 26 different pigs varying in sex (females or castrated males), feeding regime (conventional feed or feed composed according to organic standards) and genotype (carriers or non-carriers of the RN^- allele), and reported that the creatine concentration ranged from 3.8 to 5.8 mg/g raw meat wet weight (29.1–44.2 μmol/g). However, this variation did not correlate with the concentration of HCAs (4,8-DiMeIQx, MeIQx, PhIP, Harman and Norharman) after pan-frying.

Using the Ames test, Laser Reuterswärd *et al.* (1987) showed the mutagenic activity of fried minced patties of different bovine tissues (muscle, heart, tongue, liver and kidney) to be most associated with creatine content. The sum of creatine and creatinine levels in raw muscle, heart and tongue samples varied between 19 and 33 μmol/g raw wet tissue. In contrast, liver and kidney both showed very low levels of creatine plus creatinine (about 2 μmol/g raw wet tissue) and produced no detectable mutagenic activity (Ames test) upon pan-frying (150–200 ºC). Furthermore, pan-fried chicken liver was shown not to contain any detectable amounts of HCAs (Solyakov and Skog, 2002). In a study of 16 different animal species, the creatine and creatinine levels in meat samples ranged between 40 and 50 μmol/g, except for one species (rabbit) which contained 75–80 μmol/g (Vikse and Joner, 1993). In spite of this twofold difference, the mutagenic activity in the pan-fried minced meat samples was not correlated to the creatine content. However, the mutagen activity of cooked fish was found to be approximately related to creatinine level (Marsh *et al.*, 1990). It seems that although creatine is a key precursor for HCAs, it is not rate limiting at concentrations found in raw meat.

17.7.2 Free amino acids

As the principal component of the dry matter content, the amount of protein can constitute between 16 and 22% of the muscle mass. Free amino acids and dipeptides (e.g., anserine and carnosine) are small molecules present at low concentrations in muscles and other tissues, and their concentration may depend on the metabolic type of muscle, physical activity and nutrition (Cornet and Bousset, 1999; Essén-Gustavsson and Blomstrand, 2002). The concentration of free amino acids and dipeptides in meat may also be affected by post-mortem handling of the meat, including refrigerated storage, curing or fermentation when micro-organisms and their enzymes hydrolyse proteins. The contents of total free amino acids reported in five studies are compiled in Table 17.2. An overall twofold variation in total free amino acids or dipeptides can be seen, both

between different animal species and within a species. In the study by Olsson *et al.* (2002) including 26 pigs, the concentration of total free amino acids in raw meat varied between 8 and 16.7 μmol/g wet weight. The dipeptides anserine and carnosine also varied about twofold, in the ranges of 0.5–1.1 and 20.3–35.2 μmol/g wet weight, respectively.

There are significant variations in the levels of single amino acids and consequently also the proportions of the different free amino acids. From Table 17.2 it is obvious that total free amino acids and dipeptides are present at approximately equimolar amounts as creatine, and it could be concluded that this group of precursors probably does not limit HCA formation, but that they are the principal regulators determining which HCA derivative is formed. The high amounts of phenylalanine in chicken are thought to be the reason for the high levels of PhIP found in this meat (Borgen *et al.*, 2001; Pais *et al.*, 1999; Skog *et al.*, 2000). However, no correlation between the amount of phenylalanine and the level of PhIP was observed in fried minced pork (Olsson *et al.*, 2002).

17.7.3 Sugars

Naturally occurring sugars in meat originate mainly from glycogen, a homopolymer of glucose. Glycogen is the most abundant carbohydrate in the muscle, comprising approximately 0.5% of the muscle weight. Other carbohydrates are glycosaminoglycans (associated with connective tissues), some mono- and disaccharides, as well as intermediates of the glycolytic metabolism (e.g., phosphorylated monosaccharide derivatives) (Hedrick *et al.*, 1993). Glycogen serves as an important source of energy for contracting muscle under both aerobic and anaerobic conditions (Lawrie, 1992). Breed, genotype within breed, muscle type and stress, exercise as well as the amount and type of feed affect the muscle glycogen level both in the short and long term. Immediately following slaughter, post-mortem glycolysis starts. This process plays an important role in the quality of the raw meat through its effect on pH and thereby water-holding capacity and colour (Briskey, 1964; Rosenvold *et al.*, 2001). The end products of glycolysis are determined as residual glycogen (sum of glycogen, glucose and phosphorylated intermediates).

Residual glycogen is the precursor that varies most among the three groups of HCA precursors (see Table 17.2). In pork (average of 26 animals) a 13-fold range in residual glycogen (7–91 μmol/g) was observed, and the major factor behind this variation was the genotype, e.g. the occurrence of the RN^- allele (Olsson *et al.*, 2002). Pork originating from RN^- carriers had significantly higher concentrations of residual glycogen than non-carriers ($p = 0.001$). As can be seen in Table 17.2, this had a pronounced effect on the proportions of various precursors. The carriers of the RN^- allele had twice the amount of residual glycogen, on a molar basis, than creatine and free amino acids/dipeptides. The increased level of residual glycogen resulted in about 50% lower amounts of total mutagenic HCAs in cooked meat compared with cooked meat from normal pigs. In a follow-up study on carriers and non-carriers, the residual glycogen varied fivefold in raw pork chops which upon pan-frying resulted in ten times

lower HCA concentrations in carriers of the RN^- allele than in the non-carriers (Olsson *et al.*, 2005).

In chicken breasts, high natural concentrations of glucose were shown to decrease the amount of PhIP, while the amount of MeIQx was increased (Solyakov and Skog, 2002). In addition, when glucose, lactose, milk powder or honey was added to minced patties, reductions in the amounts of HCAs were observed (Skog *et al.*, 1992; Shin *et al.*, 2003a).

In conclusion, among the key precursors of HCAs, the amino acids, dipeptides and creatine seem to be available in amounts that do not limit the formation of HCAs in cooked meat. Moreover, their natural variation does not generally exceed twofold between and within animal species. One exception is organs such as the liver and kidneys, which contain relatively low concentrations of creatine (Laser Reuterswärd *et al.*, 1987). Residual glycogen concentrations in meats, on the other hand, seem to vary substantially (Table 17.2). Glucose has a dual effect on HCA yield, it enhances the HCA yield when present up to equimolar concentrations compared with creatine. However, when the glucose concentrations exceed that of creatine, on a molar basis, glucose shows inhibiting effects on the HCA yield. Interestingly, however, given the concentrations of natural precursors on mol levels, it is remarkable to notice that the HCAs are normally present only in nmoles (in model system) or pmoles (in cooked meat). Consequently, it is evident that the thermally induced reaction route behind HCA formation is already blocked to more than 99%!

17.8 Cooking methods and ingredients

Investigations of various cooking methods have shown pan-frying and grilling/barbecuing to generally yield higher levels of HCAs than oven roasting, deep-fat frying, boiling or microwaving (Sinha *et al.*, 1995, 1998a,b; Skog *et al.*, 1997; Skog and Solyakov, 2002). High concentrations of HCAs are formed during pan-frying, especially at temperatures, above 225 ºC. Turning beefburgers more frequently during frying was found to greatly reduce the formation of HCAs, especially at high temperatures (Salmon *et al.*, 2000). The large variations in the literature data on the amounts of HCAs formed after grilling reflect the difficulty in controlling the temperature during grilling/barbecuing (Knize *et al.*, 1996; Sinha *et al.*, 1995; Solyakov and Skog, 2002). During roasting and broiling, the heat is transferred by air, which means that the heat transfer is less efficient than during pan-frying where heat is transferred by conduction. The heat transfer may be the explanation of the lower amounts of HCAs formed during oven cooking. During boiling where the temperature did not exceed 100 ºC, low or no detectable amounts of HCAs were formed (Solyakov and Skog, 2002). Microwave pre-treatment of beef patties resulted in a loss of water and precursors, and when the beef patties were fried afterwards they showed lower mutagenic activity than normally fried beef patties, however, nothing was reported on the juiciness of the fried meat (Felton *et al.*, 1994).

17.8.1 Temperature and time

Of the various physical parameters influencing the formation of HCAs, temperature is the most important. It is well established that both the varieties and amounts of HCAs increase with increasing cooking temperature (Chiu *et al.*, 1998; Knize *et al.*, 1994; Skog *et al.*, 1995, 1997), and particularly PhIP is often formed at higher amounts at high temperatures (Skog and Solyakov, 2002). To ensure microbiological safety, the cooking time is commonly regarded as the time required to reach a certain centre temperature, for example, 72 ºC for pig meat. Since the inner temperature is generally not measured during domestic cooking, the cooking time is often associated with the degree of doneness.

Prolonged cooking time may result in increased formation of HCAs (Knize *et al.*, 1994; Sinha *et al.*, 1995, 1998a,b). The cooking time is related to the temperature of the pan or surrounding medium. At lower cooking temperatures, only a slightly longer time is required to reach a specific internal temperature (Dagerskog, 1979). In cooked foods, a decrease in HCA content has been observed with longer cooking times in pan-broiled salmon (Gross and Grüter, 1992). Frying at 160 ºC resulted in higher amounts of HCAs than frying at 200 ºC, due to longer cooking time at the lower temperature (Salmon *et al.*, 2000). However, the eating quality of the meat may decrease if the meat is over-cooked.

The heat and mass transport in meat during frying are complex. Due to chemical reactions in the meat, the crust will be more porous than the inner part of the meat, and act as an insulating layer. Inside the crust, a zone of water evaporation moves inwards, while water and juices, released through protein denaturation, move outwards. At low frying temperatures, the formation of HCAs in pan residues seems to be favoured by a long cooking time, due to the mass transport of either precursors or HCAs from the meat to the pan, resulting in less HCAs in the crust of the product and a higher amount in the pan residue (Skog *et al.*, 1997). In addition, meat juice leaking out from meat products during thawing and cooking enhances transportation of the low-molecular-weight and water-soluble precursors. Thus, the concentrations of HCA precursors in meat juice are tenfold higher than in raw meat samples. The higher precursor concentration combined with the high temperatures to which the meat juice is exposed during frying explain the tenfold higher concentrations of HCAs in pan residue compared with fried meat.

17.8.2 Fat

The presence of fat may influence the formation of HCAs, both chemically and physically, and it is difficult to distinguish between these two types of mechanisms during the cooking of meat. There are few studies on the relation between fat content and the amounts of HCAs in cooked meat products. It seems that IQ is formed at higher levels in high-fat than in low-fat meat (Barnes and Weisburger, 1983; Johansson and Jägerstad, 1994). Conversely, MeIQx, PhIP, Norharman, Harman and Trp-P-2 were all found at higher levels in fried beef patties containing 5% fat than in those with 15% fat (Abdulkarim and Scott

Smith, 1998). In another study, minced beef patties with 15 and 30% fat content were cooked to an internal temperature of 100 ºC on a propane grill, and the beef patties with the low fat content showed higher levels of PhIP, but lower levels of AαC than those with the high fat content (Knize *et al.*, 1996). When beefburgers with different fat contents (6.7, 16.1 and 39%) were fried from the frozen state until the centre temperature had reached 72 ºC, the amounts of HCAs were highest in the beefburgers with the lowest fat content, but the variations between the different beefburgers were not significant (Persson *et al.*, in press).

17.8.3 Antioxidants

Since the Maillard reaction and the mechanism for the formation of HCAs may involve free radicals, it has been proposed that antioxidants may scavenge these free radicals and decrease the formation of HCAs (Kikugawa, 1999). Various antioxidants, spices and other compounds have been tested as additives in minced beef patties in several studies (Balogh *et al.*, 2000; Shin *et al.*, 2002, 2003b; Murkovic *et al.*, 1998; Britt *et al.*, 1998). However, the additives are often poorly characterised and may be spread on the surface or mixed with the meat, leading to poor knowledge of the exact concentration. Some compounds showed a pronounced inhibitory effect on the formation of HCAs, some compounds did not show any effect, while others had an increasing effect.

The application of various spices (rosemary, thyme, sage and garlic) to the surface of ground beef patties reduced the content of HCAs to below 60% of the control patties (Murkovic *et al.*, 1998). Vitamin E added to ground beef at 1 and 10% decreased the amount of PhIP by 59 and 72%, respectively, while the reduction of MeIQx was smaller and more variable (Balogh *et al.*, 2000). Oleoresin rosemary was found to reduce the concentration of PhIP by 44% (Balogh *et al.*, 2000). Also organosulphur compounds as well as minced garlic inhibited the formation of HCAs in fried minced beef patties, (Shin *et al.*, 2002). The results of these studies are contradictory, which reflects the fact that several parameters, e.g. concentration, hydrophobicity and environment, modify the action of the antioxidants.

17.8.4 Fats containing antioxidants

A significantly higher amount of MeIQx was found in pan residues after frying beefburgers in butter compared with frying in oil. This finding was explained by the presence of antioxidants in oil that may have interfered with HCA formation (Johansson and Jägerstad, 1994). In another study, the type of frying fat was shown only to have a minor effect on the formation of HCAs in beefburgers, while the pan residue was more affected (Johansson *et al.*, 1995). The total amounts of HCAs in beefburger and pan residue were lowest after frying in margarine or sunflower seed oil, which was explained by differences in oxidation status and antioxidant content. After frying beefburgers in different olive oils with and without added rosemary extract, it was shown that when

using virgin olive oil instead of refined olive oil, the formation of HCAs was reduced, an effect probably due to the content of phenols in the virgin olive oil. The HCA-reducing effect of virgin olive oil decreased during storage, but the addition of rosemary extract may prevent this decrease (Persson *et al.*, 2003a).

17.8.5 Water-holding capacity

During cooking, the amounts of HCA precursors at the meat surface may be enhanced by the transport of water and water-soluble precursors from the inner parts of the meat. This mass transport may be influenced by water-binding ingredients (Persson *et al.*, 2003b), however, this may also be a result of over-cooking. A high cooking loss has been found to be related to the formation of large amounts of HCAs (Persson *et al.*, 2002; Skog *et al.*, 1992, 1995).

In the food industry, a mixture of NaCl and sodium tripolyphosphate is often added to meat products to improve texture, taste and water-holding capacity, which are of great financial importance (Schmidt, 1988). The addition of NaCl and sodium tripolyphosphate to beefburgers has been found to reduce the cooking loss and decrease the formation of PhIP, MeIQx and 4,8-DiMeIQx. These results clearly show that it is possible to modify cooking practices to minimise the formation of HCAs.

There is an increased interest in the use of polysaccharides to improve textural characteristics such as tenderness, juiciness and cooking loss, as well as taste and aroma in meat products. Polysaccharides often used are different kinds of starch, gums and dietary fibre (Desmond *et al.*, 1998; Shand *et al.*, 1993; Troutt *et al.*, 1992). In pan-fried beefburgers containing 1.5% polysaccharides, 1.5% NaCl and 0.3% tripolyphosphate, it was found that the addition of polysaccharides reduced both the weight loss and formation of HCAs during cooking. Of eleven different polysaccharides tested, potato starch was most capable of inhibiting the formation of HCAs (Persson *et al.*, 2004). When 1.5% fructo-oligosaccharides, galacto-oligosaccharide, isomalto-oligosaccharide or inulin was added to beefburgers fried at 225 ºC for 10 minutes, the formation of PhIP, MeIQx, and DiMeIQx was reduced by 46–54% (Shin *et al.*, 2003a). Thus, adding small amounts of a complex carbohydrate is a simple and effective way of reducing the amount of HCAs and can easily be applied in households and commercial preparation of beefburgers. Moreover, certain indigestible carbohydrates have the capacity to bind HCAs and thereby reduce the uptake from the small intestine (Sjödin *et al.*, 1985; Vikse *et al.*, 1992).

17.8.6 Coating

Coating foods with breadcrumbs before frying may also reduce the formation of HCAs due to the insulating effect of the coating. However, HCAs were found in the crust of fried fish coated with golden breadcrumbs, probably due to the very thin coating (Augustsson *et al.*, 1997).

17.8.7 Marinating

Marinating chicken before grilling or frying is a common way of enhancing the flavour and aroma of the meat. Marinating is another method that can modify the concentrations of HCAs, and some studies have shown reduced levels of HCAs in chicken that was marinated before grilling (Tikkanen *et al.*, 1996). Chicken breasts marinated in a marinade containing olive oil, brown sugar, cider vinegar, lemon juice, crushed garlic, salt and mustard, and unmarinated chicken breasts were grilled for 10 to 40 minutes, and marination was found to strongly reduce the amount of PhIP, while the amount of MeIQx increased (Salmon *et al.*, 1997). When marinating chicken in one ingredient at a time, sugar was found to be responsible for the increase in MeIQx, however, the reason for the decrease in PhIP was unclear. In another study, a commercial marinade was found to reduce the mutagenic activity as well as the amount of HCAs (Tikkanen *et al.*, 1996). The antioxidant properties of red wine may reduce HCA formation in wine-marinated fried chicken (Busquets *et al.*, in press).

17.9 Conclusions and recommendations

Over the past two decades an increasing number of HCA species has been detected in cooked foods. There is no general agreement on the role of HCAs regarding human health and thus there is an interest in minimising our intake of them. Special risk groups are people who are more susceptible to the toxic effects of HCAs due to genotype/genetic polymorphism than others and/or those with HCA intakes in the 75th–90th percentile and above, e.g. more than 1 μg/day. Knowledge on the precursors, their reaction routes and factors affecting the HCA yield has been gained, but more work remains to be done in this area. However, the results of research efforts so far point to several ways of reducing the amount of HCAs in our diet.

The influence of pig genotype on HCA formation has been well documented. Similar results have been obtained for beef (Skog, unpublished). However, information on genotype is not available to the consumer. Increased residual glycogen levels in meat from pigs that were carriers of the dominant RN^- allele, resulted in browner crust colour and reduced yield of total HCAs after frying than meat of non-carriers. The brown colour may also act as a signal to the consumer to reduce the cooking time.

The choice of different cooking methods offers variation of meat and fish dishes. Controlling the cooking time and temperature is important to minimise the amount of HCAs formed. One simple piece of advice is to avoid over-cooking. Boiling is a traditional cooking method that deserves more attention as a way of reducing HCA formation. Boiled foods normally do not contain significant amounts of HCAs. Oven roasting normally results in foods with a low amount of HCA. However, it should be noted that pan drippings that have dried out may contain very high amounts of HCAs and should be discarded.

Grilling may generate high amounts of HCAs and during grilling it is

important not to use a very high cooking temperature. This is often easier said than done, but dark brown parts of grilled meat should be avoided. One way of reducing the HCA content may be to use different marinades as this has been shown to reduce some HCAs. However, it is important to wipe off the marinade before grilling so that it does not cause flames that may generate other hazardous compounds. Pan-frying is normally a fast method for home cooking. When frying, it is advisable to fry quickly to obtain an appetising crust (with colour and aroma compounds), and then add water and braise until ready.

Butter or a margarine containing milk provides a simple temperature indicator – as a result of protein denaturation. When the frying fat has reached the correct frying temperature, it turns golden brown and stops bubbling. As a large proportion of the HCAs are found in the pan drippings, a simple means of reducing the HCA content of a meal is not to include the pan drippings. Sauces or gravy based on industrially prepared stock cubes or concentrated bouillons should be used instead.

There is a tendency for a high weight loss during cooking to result in increased formation of HCAs. The transport of water-soluble precursors to the meat surface can be lowered by the addition of water-binding ingredients, for example common salt or polysaccharides. In addition, this will yield a juicier, tastier and more tender product. Recipes for making meat balls often recommend the addition of a small amount of common salt and this will be enough to reduce the HCA formation. Ingredients such as potato starch, wheat bran and potato fibre can be added at low concentrations to decrease the formation of HCAs in beefburgers. This would be easy to implement in domestic, industrial and restaurant cooking. In addition, various polysaccharides have been shown to bind HCAs and thus reduce their bioavailability.

Using a frying fat with a high content of antioxidants or the addition of antioxidants to the meat may be other ways to reduce the formation of HCAs. The role of antioxidants and their effects in the formation of HCAs are still unclear. The concentration, heat stability and polarity of the antioxidant are characteristics which influence HCA formation and must be further investigated. However, there is a risk that the use of antioxidants could change the taste and aroma of the food. The suitability of additives and their effects on HCA formation require further investigation.

The formation of HCAs should also be taken into consideration when designing food processing equipment, for example thermostat-controlled heating devices for both domestic and restaurant cooking, to reduce the risk of overcooking. The use of such equipment is motivated from both food quality and food safety aspects.

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18

Dietary compounds which protect against heterocyclic amines

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Abbreviations

ACF	aberrant crypt foci
CA	chromosomal aberration
C+K	cafestol and kahwehol
CHL	chlorophyllin
CHO	chinese hamster ovary cells
CYP 450	cytochrome P 450
CLA	conjugated linoleic acid
GST	glutathione-S-transferase
HCA	heterocyclic aromatic amine
HepG2	human derived hepatoma cells
I3C	indole-3-carbinol
IQ	2-amino-3-methylimidazo[4;5-*f*]quinoline
LAB	LAB (lactic and bacteria)
MeIQ	2-amino-3;4-dimethylimidazo[4;5-f]quinoline
MeIQx	2-amino-3;8-dimethylimidazo[4;5-f]quinoxaline
MN	micronucleus
NAT	N-acetyltransferase
PhIP	2-amino-1-methyl-6-phenylimidiazo[4;5-*b*]pyridine
SCGE	single cell gel electrophoresis
SULT	sulfotransferase
Trp-P-2	3-amino-1-methyl-5*H*-pyrido[4;3-*b*]indole
UDPGT	UDP-glucuronosyltransferase
V79	hamster fibroblasts

18.1 Introduction

The increasing evidence for the possible involvement of heterocyclic aromatic amines (HCAs) in the etiology of various forms of cancer in humans has stimulated intense efforts aimed at identifying dietary compounds which protect against the adverse health risks caused by these components. Already in 1978, two years after the isolation of the first HCAs (tryptophan and phenylalanine pyrolysis products) by Sugimura and co-workers,[1,2] a Japanese group described the antimutagenic action of a 'vegetable factor' towards these compounds. In a recent review in 2000[3] we found data on more than 500 complex dietary mixtures and individual components which had been investigated for potential protective effects towards HCAs. Also in recent years, numerous studies on the protective effects of dietary factors have been published. We estimate that at present data on approximately 600 complex food mixtures and food components have been published in more than 250 articles.

While in the 1970s and 1980s mainly bacterial mutagenicity assays were performed to identify antimutagens, more reliable models were developed and have been used during the past two decades. Examples are the establishment of cell lines which reflect the metabolism of HCAs in humans, the development of animal models in which antimutagenic and anticarcinogenic effects can be detected and intervention studies designed to provide information on protective effects in man. Also the knowledge of the molecular mechanisms of protection, which is required to assess if beneficial effects can be expected in humans, has increased gradually over the years. The different modes of action of anti-mutagens/anticarcinogens are described in the next paragraph. On the basis of this information the advantages and shortcomings of different methods used to identify HCA protective factors in the human diet are discussed. The following sections summarize the current state of knowledge on selected food items and their components such as vegetables, lactic acid bacteria, fibers and non-digestible starch, pigments, fats and fatty acids and vitamins which have been extensively studied for protective effects.

18.2 Mechanisms of protection

Several attempts have been made to develop classification schemes for anti-mutagens and anticarcinogens. Kada *et al.*[2,4] designated compounds, which act extracellularly and inactivate mutagens either directly or indirectly (via enzymatic inhibition or inhibition of activation) as 'desmutagens' and agents which act via interaction with DNA-repair and replication as 'bio-antimutagens'. Wattenberg and co-workers[5,6] designed a scheme for cancer-protective agents. Compounds which prevent the formation of tumors before/during carcinogen treatment were termed as 'blocking agents' whereas compounds which are protective when given after the carcinogen administration were categorized as 'suppressing agents'. De Flora and Ramel[7,8] developed a more detailed scheme which was used to design Fig. 18.1; examples for the different modes of action,

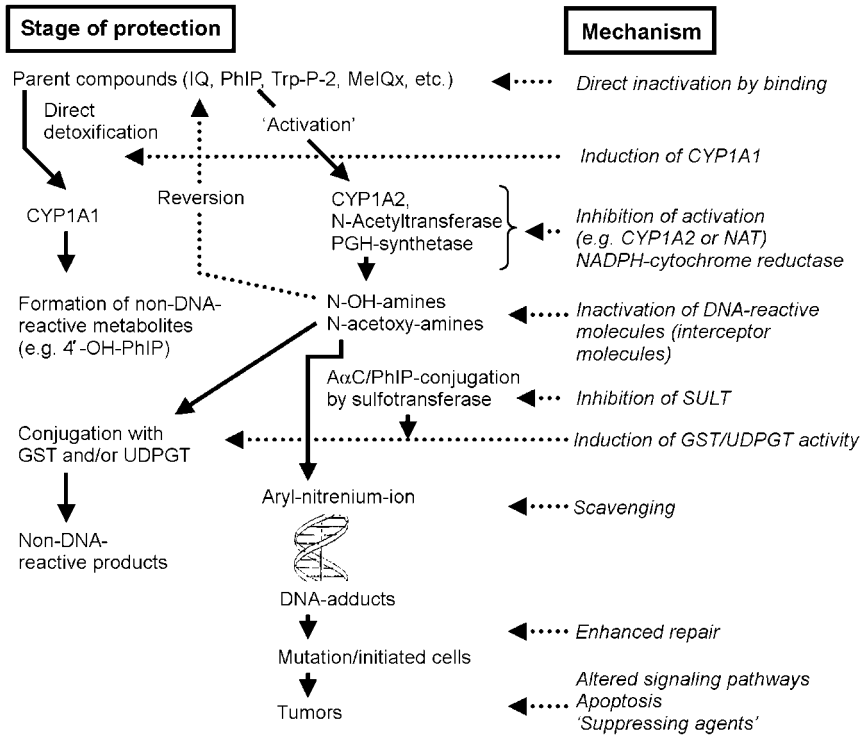

Fig. 18.1 Scheme for cancer-protective agents.

by which the DNA-damaging and clastogenic effects of HCAs are prevented, are listed in Table 18.1. 'Avoidance strategies', i.e. the development of specific cooking methods and the mechanisms by which food components inhibit the formation of HCAs, are described in a separate chapter in this book.

The metabolism of HCAs is very complex and a variety of enzymes are involved in their activation and detoxification. Moreover, recent studies showed that the activation of different amines differs substantially. In the case of tryptophan pyrolysates (Trp-P-1, Trp-P-2) only conversion by cytochrome P (CYP) 1A2 is required to lead to formation of DNA-reactive metabolites. Quinolines and quinoxalines require subsequent O-acteylation catalyzed by N-acetyltransferases (NAT) whereas sulfotransferases (SULT) are essential for the activation of PhIP (2-amino-1-methyl-6-phenylimidiazo[4,5-*b*]pyridine) and aminocarbolines.[44,45]

Many protective compounds are active at different levels; for example, green tea causes alterations of CYP 450 1A isoenzymes, induction of UDP-glucuronosyl-transferase (UDPGT) and leads to inactivation of free radicals and electrophiles,[22] The coffee diterpenoids cafestol and kahwehol (C+K) act on multiple stages, i.e. they inhibit several CYP 450 isoenzymes[46,47] and NAT, and increase the levels of detoxifying enzymes such as glutathione-S-transferase (GST) and UDPGT.[48,49]

Table 18.1 Different protective mechanisms towards HCA-induced DNA damage

	Mechanism	Method	Example	Reference
(1)	Direct inactivation by binding	Changes of the absorbtion spectrum by HCAs (HPLC) – in parallel reduction of DNA damage *in vivo/in vitro*	Chlorophyllin – inactivation of different HCAs; the same mechanism is probably also active *in vivo*	[3]
			Lactobacilli – binding of HCAs mainly by adsorption to the cell walls	[43, 10]
			Fibers – binding demonstrated for various HCAs, no firm evidence from animal experiments	[2, 11–13]
(2)	Preferential induction of CYP 1A1	Quantification of metabolites in urine, DNA adduct formation	Demonstrated for 13C in experiments with rats, preferentially CYP 1A1 induction leads to increased detoxification	[15]
(3)	Inhibition of CYP 1A2	Quantification of N-OH-metabolites/enzyme inhibition studies, use of transgenic cell lines expressing individual CYP2	Inhibition of PhIP mutagenesis in genetically engineered Salmonella bacteria expressing human CYP 1A2 by EGCG, inhibitory effects of menadion and retinol	[16, 17] [18]
			Inhibition of IQ-induced strand breaks in THL cells by sulforaphane	[19]
			Inhibition of PhIP mutagenesis by diallyl-sulfide, genistein, tannic acid, ethoxyquine	[20]
			Inhibitory effects of hop-flavonoids and protective effects of citrus flavonoids	[21]
(4)	Inhibition of prostaglandin H-synthase	Comparison of prostaglandin levels in colon cells of rats with/without treatment	*In vitro* inhibition of IQ mutagenesis by conjugated linoleic acid	[22, 23]
(5)	Inhibition of NADPH-cytochrome c reductase	Enzyme measurements and electron spin resonance	Teas (green, black and decaffeinated)	[24]

(6)	Reversion of the hydroxyl-amine to the parent compound	HPLC-analysis	Re-conversion of N-OH-Trp-P-2 to Trp-P-2 by 2,6-di-tert-butyl-8-hydroxy-di-benzofuran-1,4-quinone (a reaction product of butylated hydroxyanisole)	[25]
(7)	Inhibition of PhIP-induced damage by inhibition of SULT	Measurement of SULT-gene expression and enzyme activities/ correlation with reduced DNA migration *in vitro* and in human intervention trials with lymphocytes	Inhibition of N-OH-PhIP binding to mammary-cell lines by estrone	[26]
			Inhibition of DNA-damage in lymphocytes of humans after consumption of Brussels sprouts	[27]
(8)	Direct inactivation of N-hydroxy-HCAs	Changes in the absorbance spectrum, experiments with N-OH derivatives	Inhibition of PhIP-mutagenesis in bacteria by EGCG and conjugated linoleic acid	[24, 28]
			Inhibition of N-OH-PhIP mutagenicity in V-79 by red beets and spinach	
			Inhibition of N-OH-IQ mutagenicity in bacteria by vitamin K	[29]
			Inhibition of N-OH-IQ by spearmint	[30]
(9)	GST-induction	Enzyme measurements (spectrophotometry, ELISA)	Induction of GST and GST-α by coffee diterpenoids (C+K), reduction of PhIP-DNA adducts in the colon of rats	[31]
			Induction of GST-π by white tea in rats	[32]
			Inhibition of PhIP mutagenicity by oltipraz (a GST inducer) of PhIP-induced lymphoma formation in F-344 rats	[33]
(10)	Induction of glucuronosyl-transferase (UGT)	Enzyme measurements with different substrates (UGT1/UGT2), quantification of glucuronides in urine	Induction of UDPGT by *Lepidium sativum* in rats (no other enzymes altered!) paralleled by a decrease of ACF induction by IQ	[34]
			Induction of UGT by polyphenolics in tea	[32, 35]
			Increased excretion of PhIP-glucuronides in humans after consumption of Brussels sprouts	[27]

Table 18.1 Continued

	Mechanism	Method	Example	Reference
(11)	Electrophile scavenging of nitrenium ions	Indirect evidence	Inhibition of the mutagenic activity of N-OH-IQ in Salmonella by spearmint	[28]
(12)	Enhanced DNA-repair	Indirect evidence/addition of putative modifiers after mutagen treatment	Protection of human hepatoma cells towards IQ, MeIQx, TrpP2, and PhIP by vanillin, coumarin, caffein	[36]
			Protection studies with *Saccharomyces cerivisiae* and with *Drosophila melanogaster*	[37, 38]
(13)	'Suppression of tumor formation'	Administration after the carcinogen comparison with tumor yields or preneoplastic foci formation/ determination of apoptosis, COX2, etc.	Inhibition of IQ-induced ACF formation in rats – paralleled by induction of apoptosis and increased cell proliferation by chlorophyllin	[39, 40]
			Inhibition of mammary gland carcinogenesis caused by PhIP by nimesulide – a COX2 inhibitor in rats	[41]
			Alteration of apoptosis in APC min mice by curcumin – paralleled by reduction of PhIP induced tumors in the small intestine	[42]

It is notable that the mode of action of specific compounds may depend strongly on the dose administered. This has been shown, for example, for indole-3-carbinol (I3C), a degradation product of the glucosinolate glucobrassicin, which is contained in cruciferous vegetables. I3C increased the level of DNA adducts when given at low dose levels to rats treated with IQ, whereas at higher doses protective effects were seen. This could be explained by dose dependency of the induction of the cytochrome P450 isoenzymes CYP 1A1 and 1A2. At low doses (10–50 ppm in the diet), CYP 1A2 was preferentially induced whereas at higher exposure levels CYP 1A1 protein expression was enhanced, leading to increased ring hydroxylation and formation of detoxification products.[15,22] The protective properties of I3C depend also on the administration scheme. I3C protects against DNA adduct formation and induction of preneoplastic lesions in the colon by IQ and PhIP when administered before and during the amine treatment. On the other hand, it promotes the formation of aberrant crypt foci (ACF) when it is given after treatment with chemical carcinogens.[50–52]

A number of studies show that individual components may possess protective effects towards HCAs whereas complex mixtures which contain these components may be inactive. For example, the coffee diterpenoids C+K prevented formation of HCA adducts in the colon of rats whereas coffee which contained these diterpenoids was not protective (Knasmüller *et al.*, unpublished). Also, in studies with other cruciferous vegetables the inhibition of IQ-induced preneoplastic lesions in colons and livers of rats could not be correlated with their glucosinolate contents.[53]

18.3 Methodological aspects

On the basis of the evaluation of the currently available literature we estimate that approximately 250 studies on DNA-protective effects of dietary components and mixtures towards HCAs have been published. In more than 80% of these investigations, bacterial indicator cells were used to detect antimutagenic effects, most other studies were *in vivo* experiments with rodents. Only few (less than 30 studies) were conducted with mammalian or human cells. In addition, we found a few investigations with fruit flies[54] and in total 35 studies in which either prevention of preneoplastic lesions or inhibition of HCA-induced tumor formation were used as endpoints.

18.3.1 *In vitro* mutagenicity studies

We stressed earlier that the predictive value of bacterial *in vitro* assays for the detection of compounds which protect against HCAs is very limited.[3,55] Most of these studies were carried out with *Salmonella typhimurium* strains TA98 (which is particularly sensitive towards HCAs and detects frameshift mutations) and TA100 which enables the detection of base substitutions. In all these experiments, enzyme homogenate (S9-mix) is added which contains phase I

enzymes (CYP1A) required to convert HCAs to DNA-reactive metabolites. Part of the metabolic activation of the HCAs is catalyzed by bacterial enzymes. We have stressed earlier that only compounds which bind directly to HCAs (pigments, fiber and lactobacilli) can be detected in bacterial assays, whereas compounds which interfere with the metabolism of the amines are likely to give misleading results.[55] Several compounds which were protective under *in vitro* conditions in bacterial assays[3] gave negative results in *in vivo* experiments with rodents and some even caused an increase of DNA damage and/or tumor formation.[3] Typical examples are anthraquinones, anthraflavic acid, oleic acid and various other fatty acids and whole and refined wheat.[3,56]

The main reason for the poor predictive value of bacterial assays and *in vitro* experiments with mammalian cell lines such as Chinese hamster ovary (CHO) cells or hamster fibroblasts (V79), which also require metabolic activation by exogenous enzyme addition, is that any compound which causes shifts in pH or the molarity of the incubation mix will decrease the activity of the activating enzymes (for details see ref. 3). Also compounds which cause precipitation of proteins, for example catechins and tannins, are likely to give false positive results. Furthermore, it was stressed by Rutten and Gocke,[57] that compounds which cause division delay in bacteria (which is not monitored under standard conditions) will mimic antimutagenic effects which are also seen when the cultivation medium is deprived of nutrients.

Despite the shortcomings of bacterial assays, numerous studies have been published over recent years.[17,21,58–66] The results of these investigations have only little, if any, relevance for humans. In some of these experiments, additionally attempts were made to characterize the mode of action of the putative protective compounds by addition of liver homogenates and subsequent enzyme measurements (for example see refs. 17, 19, 21 and 62). However, such investigations do not provide evidence that these compounds are active intracellularly, and many agents which act as enzyme inhibitors *in vitro* may even cause an increase of the same enzyme under *in vivo* conditions. A typical example is ethanol which inhibits the activation of nitrosamines in subcellular liver fractions via interaction with CYP 2E1, whereas chronic administration to rats leads to a strong induction of this isozyme.[67]

Animal experiments in which enzymes are isolated and measured after extended feeding periods are definitely more reliable to study protective effects towards HCAs. These approaches allow also the detection of induction of phase II enzymes which are not represented in enzyme homogenates used for *in vitro* experiments, such as GST and UDPGT (for examples see refs 15, 22, 34, 48, 49, 68 and 69).

Another possibility to overcome the disadvantages of conventional *in vitro* assays is the use of genetically engineered bacterial or mammalian indicators which possess different phase I or phase II enzymes involved in the activation of amines. Typical examples are Salmonella strains expressing the human CYP 1A2, which were used to investigate the effects of flavonoles[20] or V79 cells expressing human CYP 1A enzymes, NATs and/or SULTs in experiments with fruit and vegetable extracts.[29] However, one of the disadvantages of these

models is that induction effects of phase I or phase II enzymes, which play a key role in chemoprevention towards HCAs (see Section 18.2) cannot be detected in these modified cell lines.

It was shown by Anderson and co-workers,[70,71] in single cell gel electrophoresis (SCGE) assays, that HCAs cause DNA migration in peripheral human lymphocytes and sperm cells. These observations indicate that these cells are able to convert the amines to DNA-reactive metabolites. The same group used this approach to investigate the potential protective effects of different dietary components such as flavonoids and vitamin C.[70,71] A similar approach was used by Edenharder *et al.*[72] who used cultured lymphocytes to study the chemoprotective properties of different vitamins on Trp-P-2 induced sister chromatid exchanges.

One of the most promising methods to identify HCA-protective dietary components is the use of human derived hepatoma (HepG2) cells and standardized protocols for SCGE-assays[73,74] and for micronucleus (MN) tests[75] have been developed. It was shown that these cells can detect genotoxic effects of HCAs[76,77] and numerous other promutagens without addition of exogenous enzyme homogenate, since these cells have retained the activities of phase I and phase II enzymes.[78,79] A major advantage of this cell line is that CYP 1A isozymes as well as GST, UDPGT and NAT are inducible (for a review see ref. 80). We showed in model experiments that the enzyme induction patterns caused by the flavonoid chrysin (contained in propolis) and the coffee diterpenoids C+K are similar in HepG2 cells and animal studies. Furthermore, pronounced DNA-protective effects were found with both compounds in combination experiments with PhIP in rats and also in human derived cells.[81,82] Also many other dietary components have been tested for protective effects towards HCAs in HepG2 cells (for reviews see refs 79 and 80).

18.3.2 *In vivo* genotoxicity test with rodents

The upper part of Fig. 18.2 gives an overview on different endpoints that have been used in genotoxicity experiments with rodents. 'Classical' endpoints used in *in vivo* assays, such as chromosomal aberration (CA) analyses in peripheral blood cells and MN-assays in bone marrow, were only rarely employed in antimutagenicity studies as they are quite insensitive towards HCAs. For example, only weak and/or negative results are obtained with quinolines and quinoxaline-compounds in the latter tests.[83]

DNA adduct measurements enable monitoring of protective effects in a variety of organs and have been used in a number of studies. For example, Huber *et al.*[31] measured prevention of PhIP-adduct formation by a variety of dietary components in colons of rats. Another working group observed a reduction of IQ-induced DNA adducts in rat liver after oral administration of green and black tea.[84] A much cheaper approach uses SCGE-assays, which are based on the determination of DNA-migration in an electric field.[85] We used this method to study the protective effects of cruciferous vegetables,[86] probiotics[87] and lactic acid bacteria.[88] We also demonstrated that prevention of DNA-migration in livers

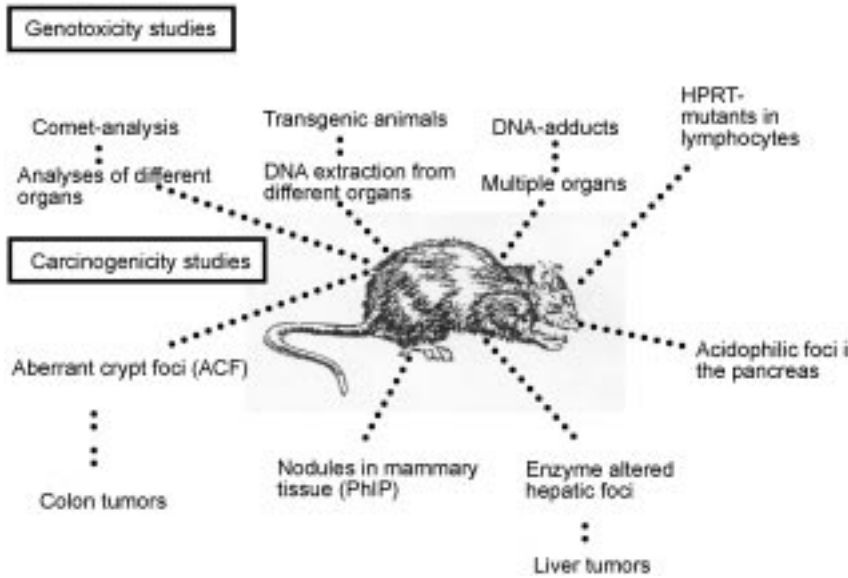

Fig. 18.2 Overview on different endpoints used in genotoxicity and carcinogenicity studies in rodents.

and colons of rats is paralleled by a decrease of preneoplastic lesions (ACF) and $GSTp^+$-foci in these organs. Recently Montgomery and co-workers[69] reported protective effects of antioxidant vitamins towards PhIP, IQ, MeIQ (2-amino-3,4-dimethylimidazo[4,5-f]quinoline), MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline) in the rat lymphocyte mutation assay (hypoxanthine guanosine phosphoribosyl transferase (Hprt-) locus). However, in contrast to the other methods described above, this test does not provide information about protective effects in inner organs which are the targets for tumor induction by HCAs.

In recent studies transgenic animals were used in antimutagenicity trials. These animals carry target genes (e.g., lac-Z Muta Mouse/Muta Rat or lac-I Big Blue mice and rats) which can be isolated from different organs of chemically treated and control animals. After plasmid transfer to bacteria, the mutations are monitored and the mutations spectra are determined by sequencing if desired.[89,90] De Boer[90] used Big Blue mice and found protective effects of conjugated linoleic acid (CLA) towards PhIP in the prostate and in the distal (but not in the proximal) part of the colon. The same group[91] published another study on the protective effects of CLA and 1,2-dithiole-3-thione towards PhIP. Interestingly, they found an increase of the mutation frequency by CLA in the caecum, whereas protective effects were observed in the distal colon.

18.3.3 Carcinogenicity studies with rodents

Figure 18.2 depicts different endpoints used in carcinogenicity studies with rodents. Preneoplastic foci are morphologically or biochemically altered groups

of cells which may transform into tumors. These premalignant lesions are frequently used in experimental cancer research (for a review see ref. 92). In contrast to tumor formation as an endpoint, assessment of aberrant crypt foci are less time consuming and lower numbers of animals are required. Foci can be detected 2–4 months after treatment with the carcinogen and usually 8–10 animals are used per treatment group. Since the colon is one of the most important target organs for HCA-induced cancer, mainly aberrant crypt foci (ACF) experiments have been conducted with the amines. ACF can be easily detected after staining with methylene blue; a description of their morphology and histochemical properties can be found in the review of Ehrlich *et al.*[93]

One of the main problems in HCA-ACF experiments is that the amines cause only moderate increases of the ACF-frequencies even when high doses are administered. Therefore, protocols have been developed in which the animals received a high-fat, fiber-free diet, which leads to a substantial increase of the foci yields.[53] Also strain differences play an important role. For example, it was shown that BuF/Nac rats are more sensitive in regard to ACF induction as F344-animals.[94] Recently, ACF-formation was investigated in a modified model in which rats were initiated with the colon carcinogen dimethylhydrazine (DMH) and subsequently dosed with PhIP. Upon co-treatment with putative protective dietary compounds during PhIP treatment it was possible to investigate protective effects on the post-initiation level.[95,96] Recent observations suggested that ACF may not be a reliable tool for the detection of colon cancer-protective compounds, since certain known promoters of colon cancer (e.g., secondary gallic acids) caused a reduction of ACF (for a review see ref. 93). However, in a comprehensive review article of Corpet and Pierre,[97] it was stressed that many compounds which led to a reduction of ACF in rat models also reduced tumor formation in intestinal cancer models with mice as well as regression of polyps in human studies.

A number of ACF studies have been published with HCAs in which, for example, protective effects of vegetables,[53] teas,[98] CLA,[23] wheats,[56] and lactic bacteria[99] were investigated. Another important foci-model are enzyme altered hepatic foci. In most studies with HCAs, foci which express the placental form of GST ($GSTp^+$) were used as an endpoint. $GSTp^+$-foci experiments have been conducted, for example, with cruciferous plants, glucosinolates, the quercetin, diallyl sulfide, vanillin, glutathione and vitamins (for details see refs 3 and 93). Recently, we showed in experiments with IQ, that it is possible to simultaneously evaluate $GSTp^+$-foci formation in the liver and ACF in the colon of IQ-treated animals,[53,100] and a Japanese group demonstrated that both organs can also be evaluated after combined treatment with HA-mixtures.[101]

In a number of experiments, the effects of putative chemopreventive compounds on the development of PhIP-induced mammary carcinomas were studied. Protective effects were observed, for example, with β-naphtoflavone, I3C, vitamin E and caffeine.[102–104] In addition, a few studies have been published in which other target organs of HCAs were investigated. Rao *et al.*[33] reported protective effects of a lignan towards formation of PhIP-induced

acidophilic pancreatic foci and inhibition of PhIP-induced lymphoma incidence with oltipraz.

It is interesting that some compounds cause conflicting effects in different organs. For example, caffeine was found to protect against PhIP-induced mammary tumors in rats but it increased the number of colon tumors.[103] Similarly, chlorophyllin (CHL) was protective against IQ in different organs, but shortened the time of tumor development in the skin.[105]

An important new development is the increasing use of transgenic animals which are highly sensitivity towards HCAs. Some of these models were used to study the effects of HCAs (see the review of Dashwood[106]). At present only data from a few anticarcinogenicity studies with transgenic animals are available but it is likely that the use of such models will increase in the future.

18.3.4 Human studies

Humans are exposed only to low doses of HCAs via the diet. Therefore it is not possible to monitor the impact of dietary factors on HCA-specific DNA adducts in man. Measurements of adducts have been used successfully in chemoprevention studies with other DNA-reactive substances such as polycyclic aromatic hydrocarbons. Several attempts have been made to draw conclusions on protective effects of vegetable diets towards HCAs with urinary mutagenicity experiments. In these studies the impact of vegetables and other dietary factors was monitored in intervention trials in which mutagenic effects in urine were induced by consumption of HCA-enriched meat diets. Protective effects were seen, for example, with red cabbage[107] whereas black tea had no effect on the urinary mutagenicity.[108] However, the results of such experiments do not provide firm evidence for protective effects but are only indicative of alterations of the metabolism.

A more fruitful approach might be the chemical identification of specific metabolites in urine. For example, Frandsen *et al.*[109] suggested monitoring the formation of 5-OH-PhIP in urine, which reflects the endogenous formation of the most important DNA-reactive intermediate of this amine. Walters *et al.*[110] showed that the excretion of PhIP-glucuronides in urine is strongly enhanced after consumption of Brussels sprouts. These findings strongly indicate that these vegetables increased the detoxification of HCAs in humans.

Very recently, we developed protocols for '*ex vivo*' tests in which the SCGE-technique was used to monitor differences of the sensitivity of peripheral human lymphocytes towards HCA-induced DNA-migration. At present, results from two intervention studies are available: one of them showed that coffee consumption (600 ml/person/day over five days) has a moderate effect towards Trp-P-2 induced damage. On the contrary, with Brussels sprouts (300 g/person/day over five days) no protection towards the tryptophan pyrolysate was observed, whereas PhIP-induced damage was strongly reduced. Subsequent enzyme measurements indicated that this effect is due to inhibition of the SULT-isoenzyme 1A1 which is required for the activation of this amine.[27]

18.4 Protective effects of different foods and of individual food components

In view of the large number of complex dietary mixtures and individual compounds which have been investigated for potential protective effects towards HCAs, it is not possible to give a comprehensive overview on the different studies within the framework of this chapter. In the following sections, selected results of studies with vegetables, beverages and other dietary constituents are described, for which strong evidence exists that they protect against DNA damage and cancer induction by the amines. For more detailed information readers are referred to the reviews of Schwab *et al.*[3] and Dashwood.[111]

18.4.1 Vegetables

Numerous vegetable juices have been tested for antimutagenic effects towards HCAs in *in vitro* models (mainly in bacterial assays) and many of them were found protective (for a review see ref. 3). However, due to the limited predictive value of these experimental models it is not possible to draw firm conclusions on potential protective effects for humans. This is also true for many secondary plant constituents such as phenolic acids and flavonoids. Only some compounds which were antimutagenic in *in vitro* assys, e.g. quercetin,[112] tannic acid[31] and certain vitamins,[113] were also found protective under *in vivo* conditions. The antimutagenic and anticarcinogenic properties of plant constituents such as chlorophyllins and other pigments and fibers are described separately in the following paragraphs.

A group of vegetables, which is highly protective towards HCA-induced DNA damage are cruciferous plants which include a broad variety of Brassica species, such as cabbage, radish, Brussels sprouts and broccoli. These plants contain specific constituents, namely glucosinolates, which are released upon destruction of the cellular structure and converted enzymatically (either by the plant enzyme myrosinase or by the intestinal flora) to highly active breakdown products (nitriles, isothiocyanates and thioles). These metabolites interact with phase I and phase II enzymes involved in the activation/detoxification of various chemical carcinogens (for a review see ref. 114). We demonstrated in a model study with garden cress that oral administration of small amounts of juice to rats inhibits the formation of IQ-induced DNA damage in colon and liver cells. These DNA-protective effects were paralleled by a significant reduction of the formation of ACF in the colon. Enzyme measurements showed that the only enzyme which was significantly induced by garden cress juice was UDPGT and on the basis of this observation we concluded that this may be the main protective mechanism of Brassicaceae.[86]

In subsequent experiments with different commonly consumed Brassica vegetables (red and white cabbage, broccoli, Brussels sprouts) strong protective effects towards induction of ACF and $GSTp^+$-foci by IQ were observed, which were again paralleled by induction of UDPGT.[53] The assumption that Brassicas are also effective in humans is confirmed by studies which show that the con-

sumption of these vegetables leads to an increase of the excretion of glucuronidation products of PhIP.[110] In a recent intervention trial we could demonstrate that the sensitivity of human lymphocytes to PhIP (but not to Trp-P-2) induced DNA damage is strongly reduced after consumption of Brussels sprouts.[27] This effect could be explained by inhibition of SULT isoenzymes (1A1 and 1A3), which play a key role in the activation of PhIP and also of aminocarbolines.[77,114–118]

Although it was repeatedly emphasized that glucosinolates and/or their breakdown products are responsible for the chemoprotective properties of cruciferous vegetables it was not possible in our studies to correlate the protective effects of Brassicas with the concentrations of individual glucosinolates. However, it was evident that cultivars, which had high levels of total glucosinolate contents, were more effective than varieties with lower concentrations.[53] Also in experiments with garden cress it was not possible to attribute the effects of the plant extract to glucotropeolin (the only glucosinolate contained in this plant) or to its breakdown product benzylisothiocyanate. Both compounds were active, but protection was seen only at high dose levels, which exceeded by far the concentration contained in the plant juice.

A large number of studies have been carried out with glucosinolates and their breakdown products. For example, potent inhibition of IQ and PhIP induced ACF-formation was observed in rats with I3C, and the effects were related to induction of CYP 1A1 and 1A2.[22] Also, in adduct formation experiments with I3C, protective effects were observed.[9,119–121] It is interesting that the effects of I3C are obviously organ specific and also depend on the dose and administration period. In contrast to the protection seen in the colon, no significant reduction of PhIP-induced mammary tumors was observed,[102] and it was emphasized by Dashwood and Xu,[15] that administration of low doses may even lead to enhanced formation of IQ-adducts. Several investigations indicated that breakdown products of glucosinolates may act as tumor promoters (for details see ref. 100). However, no indication for an increase of the size and multiplicity of IQ-induced ACF and/or $GSTp^+$ foci was seen in animal experiments with fresh juices from different Brassica vegetables, indicating that adverse effects take place only when high doses of the pure compounds are given to the animals.

18.4.2 Lactobacilli and fermented foods

A number of *in vitro* studies showed, that lactic acid bacteria (LAB) prevent DNA damage caused by HCAs (for a review see ref. 14). Probably the most important mechanism that accounts for these effects is direct binding of the amines to components of the cell membrane.[122,123] Since less pronounced effects were seen with heat-inactivated bacteria, compared to those observed with living bacteria, it was postulated that in addition other mechanisms, for example inactivation of HCAs by short chain fatty acids, may play a role (for details see ref. 80). The question whether LAB might also alter the metabolism of HCAs via modulation of the activities of drug metabolizing enzymes has been

studied by Tavan *et al.*[99] and no indication for such interaction were observed. Since strong protective effects were seen immediately after the administration of the bacteria, it is likely that induction of detoxifying enzymes plays no (or only a minor) role.

The antimutagenic properties of LAB are species specific and depend also on the chemical structure of the amine. The strongest binding effects were observed in a number of studies with tryptophan pyrolysates. Several findings suggest that LAB are also protective under *in vivo* conditions. Zsivkovits *et al.*[88] found that different LAB strains used for the production of yogurt reduced DNA damage in livers and colons of rats treated with a HCA-mix representative of fried meat. These effects took place under conditions which are relevant for humans (i.e., after administration of bacteria at concentrations contained in commercial yogurt) and were seen not only immediately after treatment of the animals, but also persisting for up to 12 hours. Similar observations were made by Tavan and co-workers[99] who reported protection of HCA-induced DNA damage in rats after administration of fermented milk containing different LAB strains. In the same study, they also found a significant reduction of HCA-induced ACF. However, the results of the later experiments are not conclusive as protection was also observed with unfermented milk.

A number of investigations indicate that the absorption of HCAs in animals is strongly reduced upon simultaneous administration of LABs and it was also shown that the urinary excretion of HCAs in rats is significantly reduced under conditions that prevent induction of DNA damage.[124] Interestingly, such effects were also observed in humans; consumption of freeze dried *Lactobacillus gasseri* (3×10^{10} cells/person) led to a pronounced (on average 47%) reduction of bacterial urinary mutagenicity caused by consumption of ground fried beef.[125] A similar observation was reported by Lidbeck *et al.*[126]

The assumption of a protective role of LAB in humans is also supported by the results of a recent study in which intestinal floras of vegetarians and meat consumers were established in germ-free rats. It is known that the microflora of vegetarians contains increased amounts of LAB and a significantly lower extent of IQ-induced DNA-migration was observed in livers and colons of animals which harbored floras from the vegetarians.[124] Apart from yogurt studies, a report on fermented soy-milk containing a *Bifidobacterium breve* strain has been published by Ohta *et al.*[127] The authors found that the induction of PhIP-induced mammary tumors was significantly reduced when the animals received fermented milk and concluded that the effect is due to increased amounts of isoflavones contained in the fermented product.

18.4.3 Beverages

Different types of teas as well as their constituents have been investigated for protective effects towards HCAs. Green tea is produced by withering, pan frying and drying and contains higher concentrations of epigallocatechin gallate (EGCG) and other bioactive catechols than black teas which undergo

fermentation. Significant inhibition of IQ-induced formation of ACF was observed with black and green teas in a number of animal studies[35,84,113,128–131] and also prevention of DNA adduct formation is well documented.[84] Furthermore, it was shown in more recent experiments, that also white tea (which is the least processed type of tea and produced without withering) possesses potent antimutagenic potential *in vivo* and prevents formation of PhIP-induced ACF in rats.[32]

It is conceivable that the effects of teas are due to alterations of the metabolism of the amines. This assumption is supported by observations which showed that green and black teas decreased the urinary bacterial mutagenicity of IQ.[132] Since no such effects were seen with decaffeinated teas, the authors concluded that they are mainly due to caffeine. Embola *et al.*[133] analyzed the impact of green tea on the urinary excretion of IQ-metabolites in rats and found a significant increase of the concentration of glucuronides. In this context it is notable that tea specific catechins were also shown to induce the activity of UDPGT in rodents (for a review see ref. 22).

It is assumed that mainly polyphenolics such as EGCG and other structurally related catechins are responsible for the multiple chemoprotective effects of teas (for a review see ref. 134). These compounds cause a variety of effects, which may be causally related to the cancer protective properties of teas, including protection against radicals, alteration of signaling pathways as well as changes in the activity of drug metabolizing enzymes.[135] In this context it is notable that catechin-enriched fractions of green tea inhibited Glu-P-1-induced formation of liver foci in rats,[136] but apart from this study there is no evidence for protective effects of catechins towards HCAs in animals. Since all other data come from *in vitro* studies (for details see ref. 3), it cannot be excluded that other substances (caffeine, theaflavins and gallates) account for the effects of teas.[137,138]

Huber and co-workers[31] compared the efficiency of different dietary components to reduce the formation of PhIP-induced DNA adducts in the colon tissue of rats. The most potent inhibitors were the coffee diterpenoides C+K which are found in unfiltered coffee. Subsequent studies showed that C+K are potent inducers of phase II enzymes involved in the detoxification of HCA-metabolites (e.g., GST, UDPGT) and also reduce the activity of NAT, which catalyzes the activation of the amines.[48,49] Also, in subsequent studies with human-derived hepatoma cells, pronounced protective effects towards PhIP-induced MN formation were observed.[82] These observations indicate that coffee may be protective towards HCA-induced DNA damage. This assumption is also supported by results of epidemiological studies, indicating that coffee consumption is inversely related to the incidence of colon cancer in man. However, no protection was seen in rat experiments in which the impact of coffee on IQ-induced DNA damage was monitored in livers and colons with the SCGE technique (Knasmüller, unpublished) or in human studies, in which the sensitivity of lymphocytes towards DNA-migration caused by Trp-P-2 and PhIP was measured.[139] Urinary analyses of PhIP-metabolites showed that the excretion of 5-OH-PhIP (which reflects the formation of DNA-reactive

metabolites) is even enhanced by coffee consumption (Knasmüller and Frandsen, unpublished). The reason for this discrepancy may be due to the fact that C+K do not affect the activities of CYP 1A1 and 1A2, while coffee leads to a substantial induction of these enzymes, which play a key role in the conversion of the amines to DNA-reactive metabolites (Knasmüller *et al.*, unpublished).

Caffeine is contained not only in tea and coffee but also in a variety of soft drinks. In bacterial *in vitro* experiments pronounced protective effects were observed with methylxanthine.[140–142] Also in host-mediated assays (in which bacterial indicator cells are injected into chemically treated host animals and recovered after short exposure periods) a pronounced reduction of mutants was seen in cells recovered from the liver of MeIQx treated mice.[141] However, the results of foci experiments are less promising. In one study the incidence and multiplicity of mammary tumors was slightly reduced after caffeine feeding to PhIP treated rats, whereas the number of colon-tumors was significantly increased.[103] In another study, an increase of PhIP-induced ACF was observed[101] and the authors postulated that the co-carcinogenic effects of caffeine may be primarily due to induction of CYP 1A2.

Arimoto-Kobayashi and co-workers[143,144] reported repeatedly that beer inhibits the mutagenicity of Trp-P-2 and other amines like PhIP, MeIQx, Glu-P-1 and IQ in bacterial assays.[143,144] Also *in vivo* experiments with rats provided evidence for protective effects; i.e., the formation of Trp-P-2 and MeIQx adducts in the liver of mice was significantly reduced by non-volatile beer components.[143,144] Additionally protective effects towards the quinoxoline were observed in other organs and in a further study a significant protective effect towards induction of ACF by PhIP was detected.[145] In the case of Trp-P-2 it was shown that the antimutagenic effects of beer components are due to inhibition of the formation of the hydroxy-derivative.[144] It is not known at present which of the many components contained in the non-volatile fraction of beer account for the effects; interesting candidates are, for example, prenylflavonoids, which were shown to inhibit the activation of IQ in Salmonella strains expressing human CYP 1A2[20] and xanthohumol, which was protective towards IQ in HepG2 cells.[146]

18.4.4 Pigments

One of the most intensely studied protective compounds in regard to prevention of DNA damage and cancer induction by HCAs is chlorophyllin (CHL). This water-soluble derivative of chlorophyll is easily available. It is notable in this context that Dashwood *et al.* emphasized that commercially available CHL preparations may contain high amounts of impurities.[10,147] The protective effects of CHL were initially discovered by Hayatsu and co-workers in experiments with bacterial indicators and it was postulated that they are due to covalent binding (for a review see ref. 148); also with other tetrapyrole pigments (such as hemin) antimutagenic effects were detected by Japanese groups.[149,150]

A number of subsequent studies showed that CHL is also highly protective in *in vivo* experiments when administered simultaneously with the amines, e.g. reduction of formation of IQ-adducts by CHL was observed in liver and colon of rats.[147,151] Dashwood and co-workers[128] studied the dose and time dependency of the effects of CHL in detail. They found the most pronounced protection (reduction of ACF) when CHL and PhIP were given simultaneously and when the pigment was given at dose levels between 0.01% and 1% in the diet. However, no effects were seen with an initiation protocol.[128] In post-initiation experiments, protection against IQ-induced ACF was seen in the range between 0.01% and 0.1% CHL in drinking water but at the lowest dose (0.001%) even an increase in the number of preneoplastic lesions was observed.[39] On the basis of mechanistic investigations the authors postulated that these latter effects are due to a deregulation of the homeostatic balance between cell birth and apoptosis.

There is also increasing evidence that other pigments reduce the genotoxic and carcinogenic effects of HCAs. For example, inhibitory effects were seen with alizarin and purpurin from madder root (*Rubia tinctorium*) in bacterial tests and in Drosophila.[129] Furthermore it was shown that these pigments inhibit different human CYP P450 isozymes including those involved in the activation of HCAs.[152] Other promising pigments are natural anthocyanins such as purple corn colour which decreased the frequencies and multiplicity of colorectal adenomas and carcinomas in rats treated with dimethylhydrazine and PhIP and caused a reduction of PhIP-induced ACF.[95] Also sweet potato and red cabbage colour were effective inhibitors in this experimental model.[96]

18.4.5 Dietary fiber and non-starch polysaccharides

A number of studies showed that fibers bind HCAs and reduce their mutagenic effects *in vitro*.[11,56,153–155] The inactivation depends on the type of fiber, on the hydrophobicity of the HCA and also on the pH value of the incubation mix. Ferguson and co-workers reported that suberized cell walls from plants prevent the formation of ACF in rats,[156,157] but only marginal protective effects were observed in the same experimental model with fiber-rich unrefined wheat.[56] The assumption that fibers are protective towards HCA-induced DNA damage are supported by experiments which showed that they inhibit the activity of CYP 1A enzymes. This effect explained the reduced mutagenicity of IQ seen in Salmonella experiments with enzyme fractions from rats which had been fed with wheat bran derivatives.[158] Also retarded metabolism as well as reduced intestinal absorption and decreased urinary excretion of amines were seen in experiments with rats fed wheat bran and IQ are indicative for cancer protective effects.[159]

It was also shown that resistant starches and non-starch polysaccharides affect the metabolism and excretion as well as the bioavailability of IQ,[160,161] but it is not known at present if these changes lead to protection against HCA-induced mutagenesis and cancer. However, some results are available from experiments with inulin and long and short chain oligofructose. Humblot *et*

al.[162] found that these compounds, which are used as prebiotics for yoghurt production, reduce IQ induced DNA migration in colon and liver tissue of IQ treated rats.

18.4.6 Fats and fatty acids

It is generally assumed that fats act as epigenetic colon carcinogens, in particular the increased formation of secondary bile acids as a consequence of fat consumption is considered to play an important role.[163] As described above, animal studies provided evidence that high-fat, fiber-free diets lead to an increase of HCA-induced foci yields in animal studies, but it is not known which one of the two factors is more important.

Hayatsu *et al.*[137,164] reported in the 1980s that various fatty acids, in particular oleic acid inhibit the bacterial mutagenicity of HCAs. However, in subsequent *in vivo* studies (animal host mediated assays in which indicator bacteria were injected into chemically treated animals, recovered from several organs and analysed for genetic damage) no evidence for protective effect was found.[165]

Josyula and Schut[166] investigated the impact of a ω-3 fatty acid ethyl-esther concentrate (O3C) on PhIP induced DNA adduct formation in various mouse organs and found pronounced reduction in target (spleen) and non-target organs (liver, GI-tract) of tumorigenesis, whereas no protection was observed in similar experiments with rats. The authors hypothesized that O3C may, apart from interaction with CYP 1A enzymes, cause reduction of PhIP-induced tumors via inhibition of the generation of arachidonic acid, which is a substrate for prostaglandin H synthetase. It is known that prostaglandin H activates IQ (and possibly other HCAs) in extrahepatic tissues by one electron co-oxidation. However, the possible protective effects of ω-3 fatty acids towards PhIP carcinogenesis were not confirmed in a recent study in which intestinal tumor formation was investigated in Min mice (Alexander *et al.* unpublished).

Conjugated linoleic acid (CLA) is a collective term for a mixture of positional and geometric isomers of linoleic acid. Dietary sources include dairy as well as meat products and daily consumption in the US has been estimated to be ~1 g/person/day. CLA has been investigated for protective effects in a number of chemoprevention trials with HCAs and the results are highly promising. For example, a significant reduction of IQ-DNA adducts in different organs was seen in feeding studies with mice and rats,[23,167] also PhIP-adducts were significantly reduced in livers and in mammary glands of rats.[168] Furthermore, it was also shown that CLA inhibits the formation of IQ-induced ACF in rats. More recent studies with lac-I transgenic animals showed that the protective effects of CLA against PhIP are tissue specific; pronounced reduction of mutations was seen in particular in the prostate and in the distal colon.[90,91] Interestingly, it was also found that CLA alters the mutation spectrum of PhIP and it was postulated that the compound may interact with DNA repair.[91] Other mechanisms which may play a role are modulation of CYP 1A enzyme levels and inhibition of prostaglandin H synthetase (for details see ref. 22).

18.4.7 Vitamins

The chemoprotective properties of vitamin A and structurally related compounds have been studied intensely in *in vitro* experiments,[17,72,169–171] but to our knowledge no firm data are available on protective properties from *in vivo* experiments. However, it was shown that provitamin A (β-carotene) inhibits IQ-induced adduct formation in rats[112] and in experiments with GluP-1, inhibition of liver foci formation was observed.[112] Also with vitamin E, antimutagenic effects were found *in vitro*[72,136] and protective properties were also seen in the animal models described above.[112] Additionally, a weak protective effect, namely reduction in the number of liver tumors, was reported in a study with PhIP whereas no protection was observed in the colon in the same experiments.[103,172]

Also numerous other vitamins were investigated for putative protective effects towards HCAs, but to our knowledge no reliable results concerning antimutagenic/anticarcinogenic properties are available from *in vivo* studies, however, some synthetic derivatives of ascorbic acid were shown to be protective in animal models.[136,173]

18.4.8 Miscellaneous compounds

Numerous other compounds have been tested for prevention of HCA-induced preneoplastic lesions and tumor induction. For example, Tsuda *et al.*[112] reported protective effects of ellagic acid, decosahexanoic acid, vanillin, glutathione and diallylsulfide towards induction of GSTp+ foci in rats by IQ. Also several synthetic antioxidants inhibited formation of these preneoplastic lesions.[173,174] Newer interesting studies concern the protective effects of soy isoflavones on PhIP-induced prostate cancer,[175] prevention of PhIP-induced mammary, colon and pancreatic cancer by arctin (a lignan isolated from burdock (*Arctium lappa*) seeds);[104] the protective effects of propolis (an MeIQx promotion of rat hepatocarcinogenesis)[176] and the prevention of PhIP-induced colon carcinogenesis by roselle (*Hibiscus sabdariffa*)[177] to name just a few.

18.5 Conclusions and implications for food producers

As described in previous chapters, the results of animal studies support the assumption that a number of dietary components protect against DNA damage and cancer induction by heterocyclic amines. Typical examples are lactic acid bacteria, brassica vegetables, teas, and pigments such as chlorophyllin as well as conjugated linoleic acid. For other components, such as phenolics, fatty acids, certain vitamins (E and A) and fibers only limited evidence is available and further experimental work is required to further substantiate potential protective properties. Based on these considerations, it can be recommended that industries that market 'ready to eat' meals should design products that contain combinations of meats with vegetables or plant foods which are rich sources of the aforementioned protective factors and/or fermented products which

contain lactic acid bacteria. In this context it is notable that detailed chemical analytical studies are available, in which the impact of processing procedures on the glucosinolate contents of Brassicas have been studied (see for example refs 178 and 179).

The results of these investigations indicate that storage of chopped plant material leads to alterations of the glucosinolate profiles and that the cooking time and temperature are important parameters. The production of functional foods containing putative HCA protective components, e.g. supplementation with green tea concentrates or chlorophyllin chitosan, warrants further studies concerning the proof of protective effects in humans and prevention of possible adverse effects.[180,181]

18.6 Future trends

As described in Part II of this book, a number of investigations support the assumption that exposure to HCAs leads to increased health risks to humans. In addition, epidemiological data show that consumption of foods which contain compounds that are protective towards HCAs is inversely related with different forms of human cancer, in particular with the incidence of cancer in the colon, which is considered to be the primary target for HCAs. This is, for example, true for fibers,[3,137] green vegetables that contain glucosinolates[182] and fermented dietary products that contain LAB.[183] The results of these studies are in many cases not equivocal and further verification is required and it should also be desirable to focus specifically on HCA exposure in combination with consumption of putative protective factors.

Another important task in the future will be the development of experimental approaches that provide information if protective effects of dietary compounds take place in humans. As described above, chemical analyses of metabolites, as well as design of human intervention trials in which the impact of dietary factors on HCA-induced DNA damage are monitored, are promising approaches. Such experimental systems could be complemented by data obtained with advanced molecular techniques, such as gene expression analyses and proteomics. These efforts will also contribute to clarifying if, and to what extent, specific modes of protection that have been identified in animal studies are operative in humans.

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19

Controlling acrylamide formation during baking

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19.1 Introduction

In 2002 the detection of acrylamide in a broad range of heated food[1] prompted health authorities, food companies, and research institutions such as universities to start a large number of research projects. The neurotoxic and carcinogenic properties of acrylamide,[2] its widespread occurrence in food, and its concentrations sometimes exceeding 1000 μg/kg made it a new potential health hazard. In 2005 the World Health Organisation (WHO) emphasised that acrylamide levels in food should be reduced because of public health concerns.[3] The free amino acid asparagine was rapidly identified as the key precursor delivering the backbone of the acrylamide molecule when reacting with sugars and carbonyls in Maillard type reactions.[4–6]

In general, acrylamide formation requires a minimum temperature of 120 °C which is often accompanied with the drying of the matrix.[1,5,7] However, acrylamide formation also occurred at lower temperatures in some products and models.[8–10] During preparation of food, and particularly in bakery products, the formation of acrylamide is rather complex and influenced by the composition of the product (matrix) and the baking process. All these factors have to be taken into account to understand the formation of acrylamide in bakery and to develop means for its mitigation.

The present chapter will focus on bakery products and give an overview of the key factors for acrylamide formation and possible ways to reduce the acrylamide content in bakery.

19.2 Acrylamide formation and ways to reduce its content in bakery products

In terms of food technology, bakery products comprise bread as the largest segment and biscuits, cakes, crackers, pastries, and pies as a segment with a wide variation of recipes and manufacturing procedures. All products are based on cereal flour as the most important ingredient, varying amounts of water, salt, and in most cases a leavening agent. Leavening can be achieved through fermentation by baker's yeast and lactic acid bacteria, or by the action of added chemical raising agents such as sodium hydrogencarbonate and ammonium hydrogencarbonate. Fat, sugar, egg, honey, nuts, spices, and other ingredients are added depending on the type of product. Almonds present a popular ingredient as they develop a well balanced flavour profile upon baking.

The process for all bakery products starts with mixing flour, water, and the rest of the ingredients and kneading to obtain a visco-elastic dough. The dough preparation is followed by various steps of proofing and forming. During baking oven temperatures may be as high as 260 ºC. For further information the reader is directed to the comprehensive review on bakery which is presently being finalised by Hui *et al.*[11]

Cereal flours contain free asparagine and reducing sugars (glucose, fructose, maltose) in appreciable amounts[12] and temperatures above 120 ºC are applied during baking. Thus, all requirements for the generation of acrylamide are fulfilled and as a consequence acrylamide is found in virtually all bakery products. After an overview of the products of potential concern, the key factors for acrylamide formation are presented and ways to reduce the acrylamide content will be discussed.

19.2.1 Products

Acrylamide is found in all baked goods and the most important products are bread, crispbread, gingerbread, crackers, cookies, and biscuits. Table 19.1 gives an overview of the acrylamide content of bakery products from these categories. Breads usually contain low amounts of acrylamide (< 50 μg/kg) which is almost exclusively located in the crust.[14,26,27] Nevertheless, bread was calculated to

Table 19.1 Overview of acrylamide contents of bakery products

Product category	Acrylamide content (μg/kg)	References
Bread	10–50	1, 13–15
Crispbread	40–1700	1, 14, 16, 17
Toast	50–300	13, 15, 18
Gingerbread products	50–8000	9, 15, 19–21
Crackers	25–1600	15, 21, 22
Cookies and biscuits	25–1600	15, 21, 23–25

contribute about 10% to the total dietary exposure of acrylamide because of its frequent and large consumption.[28,29] In total, about 30% of the dietary exposure originates from bakery products.[28] The highest amounts were found in gingerbreads that were over baked or prepared with ingredients rich in free asparagine.[9,20] The large variation in all categories points to differences in the composition and in the baking process of the individual products. This becomes particularly evident for crispbread, gingerbread, and biscuits.

19.2.2 Critical factors for acrylamide formation

A detailed knowledge of the variation of the process is crucial before critical factors for acrylamide formation can be identified. Otherwise, changes of the acrylamide content may be attributed to an applied measure (e.g., a changed recipe) while they reflect only the process variations. Studies with pilot plant scale production[9] as well as with industrial production[14,22,23] showed that variations of at least 10% (relative standard deviation) have to be expected. The variation of the acrylamide content of a potato cracker produced at industrial scale even amounted to 55%. Springer *et al.* observed that the acrylamide content of crispbread also depended on the location of the product on the oven belt.[14] Thus, the process variation and the place of sampling may be critical and should be checked first in order to obtain a reliable basis for data interpretation.

The content of free asparagine in the dough before baking determines the extent of acrylamide formation and therefore it often correlates with the acrylamide content of the baked product. This was observed in gingerbreads (see Fig. 19.1) where free asparagine was intentionally added to the flour,[9] in bread,[26] crispbread,[14,30] and in model systems.[8,14]

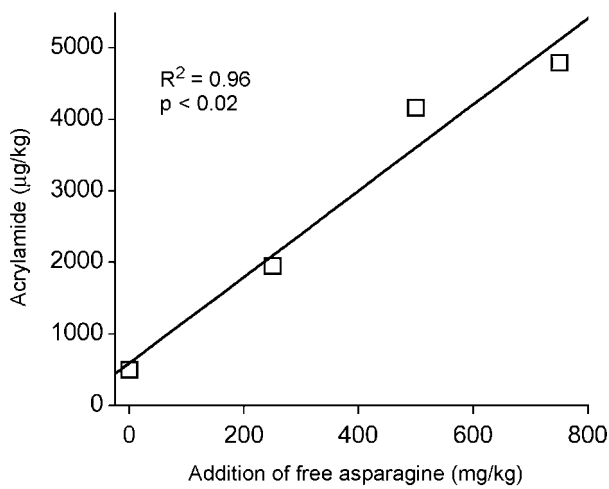

Fig. 19.1 Acrylamide contents of gingerbread with different addition of free asparagine to the dough. Data taken from Amrein *et al.*[9]

Flour is usually the main source for free asparagine[9,14,22] but other ingredients such as honey, almonds, and potato flakes may considerably contribute to the pool of free asparagine in the dough.[9,12,22,24,31] The content of free asparagine in flours depends on the type of cereal and the extraction during milling. Rye often contains more free asparagine than wheat. For whole rye flour 600 to 1100 mg/kg free asparagine were reported[12,32] while in whole wheat flour 180 to 500 mg/kg were determined.[12,32] In addition, differences between cultivars as well as agricultural aspects (fertilisation, farming system) may play a role in this context.[14,25] Apart of these agricultural aspects, the milling process is important as well. A higher extraction rate during milling (i.e., the inclusion of outer kernel layers such as aleurone, bran, and germ) leads to higher concentrations of free asparagine in the flour.[14,32] In rye bran about ten times more free asparagine was determined than in the endosperm (125 mg/kg).[14]

However, fermentation by yeasts and/or lactic acid bacteria decreased the amount of free asparagine in the raw dough. After about 2 h of fermentation over 80% of the free asparagine was consumed[32,33] and a longer fermentation led to lower acrylamide contents in breads because of almost complete removal of free asparagine by yeasts.[32] Lactic acid bacteria decreased free asparagine less efficiently than yeasts and even partially inhibited yeasts with respect to asparagine consumption.[32]

Since asparagine needs a reducing sugar to form acrylamide efficiently[34] the content of glucose, fructose, and maltose as well as the enzymatic degradation of starch play a role as well. Cereal flours contain up to 16,000 mg reducing sugars (glucose + fructose + maltose) per kg with maltose being most abundant.[12] As for free asparagine, the amount of reducing sugars depends on the type of cereal (variety and cultivar) and extraction during milling.[21] However, in sweet bakery such as biscuits or gingerbread, the flour is a negligible source, because over 90% of the reducing sugars originate from inverted sugar syrup, honey, or caramel colourings.[9,22,23] In contrast to free asparagine, addition of glucose or fructose did not increase the acrylamide formation in bread and crispbread.[26,30]

However, in the presence of the baking agent ammonium hydrogencarbonate (NH_4HCO_3, E503, 'ABC') the reducing sugars play an important role. The replacement of the relevant sources for reducing sugars (e.g., inverted sugar syrup) by sucrose solutions strongly decreased the acrylamide content of products containing NH_4HCO_3.[9,22,23] This baking agent was shown to strongly enhance acrylamide formation.[8,9,31] In gingerbread the amount of NH_4HCO_3 added to the dough correlated with the acrylamide formation in the baked product (see Fig. 19.2).

Experiments with ^{15}N-labelled NH_4HCO_3 showed that the nitrogen atom was not incorporated into acrylamide and that amidation of acrylic acid did not take place in gingerbread.[9] Other hypotheses for the promoting effect of NH_4HCO_3 on the acrylamide formation are 'activation' of reducing sugars by ammonia[8,31] or the formation of reactive sugar fragments by reaction of ammonia and reducing sugars.[9] In contrast, Levine *et al.* have found that NH_4HCO_3 tended to increase the elimination of added ^{13}C-labelled acrylamide in a cracker model,

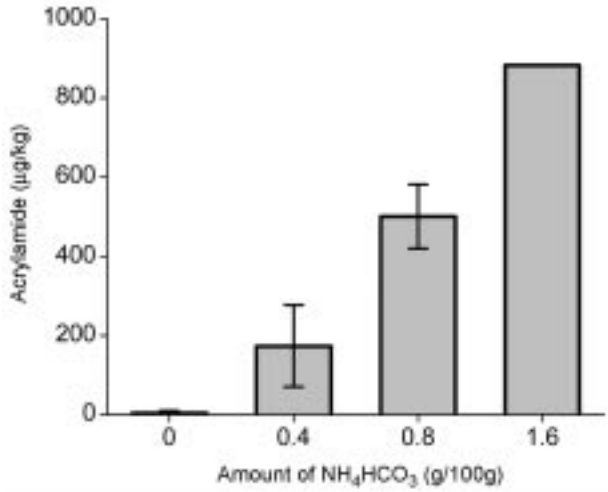

Fig. 19.2 Acrylamide content of gingerbread prepared with different amounts of NH_4HCO_3 as baking agent (error bars are standard deviation). Adapted from Amrein *et al.*[9]

particularly in the early phase of baking.[35] However, the recipe of that cracker contained no sources of reducing sugars (apart from the flour), which may explain the absence of the promoting effect of NH_4HCO_3 in this study.

Other additives such as acids (e.g., citric acid, amino acids), other baking agents (e.g., $NaHCO_3$), salt, etc., may influence acrylamide formation as well. The addition of citric or tartaric acid lowered the acrylamide content of gingerbreads,[9] biscuits,[23] and crackers,[35] possibly by inhibited formation rather than enhanced elimination.[35] Similar effects were observed upon addition of glycine, glutamine, cysteine, or lysine. Less acrylamide was determined if one of these amino acids was added prior to baking, while browning was often enhanced.[9,35–37]

The baking agents $NaHCO_3$ and Na_2CO_3 do not promote acrylamide formation as NH_4HCO_3 does (see Fig. 19.3) as observed in various products[8,9,22,23,31,35,38] but seem to favour elimination of acrylamide.[35] Baking temperature and time obviously influence acrylamide formation. In general, high temperatures and prolonged baking increase the acrylamide contents in bakery.[9,26,30,35,39] As an example, the acrylamide contents in gingerbread baked at different temperatures are shown in Fig. 19.4. In the first ten minutes the largest acrylamide contents were determined in the samples baked at the highest temperatures. These differences were less clear for 180 ºC and 200 ºC in the second ten minutes, when the products started to become over baked. After about 18 min, decreasing acrylamide concentrations were observed which indicates that elimination of acrylamide exceeded new formation.

Regarding baking temperature, one has to take into account that the temperature within the product stays remarkably below that of the oven.[9,25] In

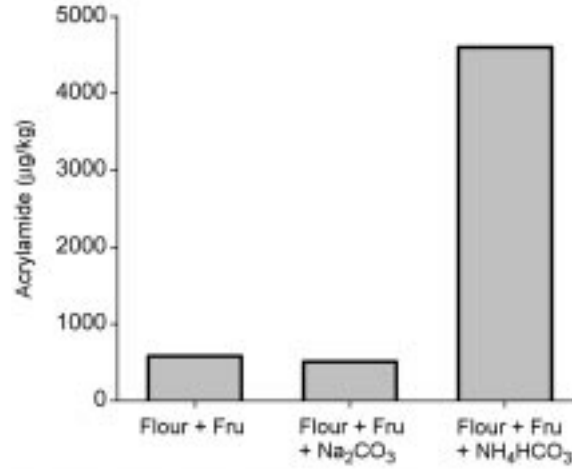

Fig. 19.3 Effect of type of baking agent on acrylamide formation in a flour model (Fru = Fructose). Data taken from Biedermann and Grob.[8]

the centre of the product, the temperature stayed below 100 ºC in the first minutes of baking,[9,35] while the crust approached the oven temperature.[25,35] The development of temperature depends on oven temperature, initial water content of the dough, product shape, and product composition.[25,35] Therefore, comparisons between different products regarding the effect of temperature are difficult. The depletion of asparagine by fermentation and the low temperature in the dough during baking prevent the formation of acrylamide in the breadcrumb. In contrast, acrylamide was found in substantial amounts in the crumb of

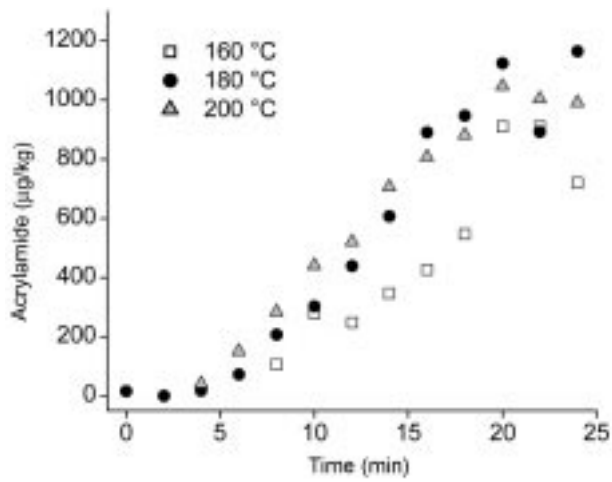

Fig. 19.4 Acrylamide contents of gingerbreads baked at 160 ºC, 180 ºC, and 200 ºC. Adapted from Amrein *et al.*[9]

gingerbread although the temperature never exceeded 110 ºC.[9] A similar effect was also reported for a wheat biscuit cereal, where acrylamide was formed at 80 ºC inside the product.[25] This demonstrates that the composition and the shape of the dough have an influence on the impact of temperature.

Water content is another factor in the formation of acrylamide. On one hand, water content influences the product temperature, and on the other hand it controls reaction rates at equal temperatures. As long as water evaporates, the temperature in the product will not exceed 100 ºC. Temperatures above 100 ºC are reached only when the product is almost completely dry.[40] Elmore *et al.* found that acrylamide formation was relatively low when the moisture levels in cakes were above 5%. Below 5% they observed a linear relationship between moisture content and acrylamide.[12] A similar relation was also reported in a study with gingerbread but the authors suggested that the correlation was rather coincidental than causal.[9] However, a higher water content keeps the product temperature lower so that less acrylamide is formed.[25] Sadd and Hamlet reported that low moisture in the crust was a key factor controlling acrylamide levels. The lower the moisture was in the crust, the more acrylamide was formed at a given temperature.[27] The moisture in crusts depends on the initial water content, the shape and composition of the product, and the baking temperature whereby moisture, temperature, and acrylamide formation are linked. Furthermore, the water content or the water activity may influence the acrylamide formation by affecting mobility and concentration of reactants and by physical changes of the matrix as it also was observed for Maillard reaction in general.[41–43]

Since elimination of acrylamide begins from the first minutes of baking,[8,9,35] decreases in acrylamide concentration were observed in strongly baked products.[9,35,39] Browning often correlated with the acrylamide content as observed in gingerbread, breads (see Fig. 19.5), and model systems[9,26,44] which demonstrates that acrylamide formation and development of flavour and colour are linked.[5,41,44] However, Sadd and Hamlet reported that colour was not a reliable predictor of acrylamide levels in different UK cereal products. They stated that the moisture content, particularly in the crust, was the effective key factor for acrylamide formation.[27] Thus, the correlation of browning and acrylamide concentration may apply to sets of the same product baked under different conditions[9,30] or to particular product categories such as breads[26] or biscuits[27] but not to bakery products in general.

Altogether, the formation of acrylamide in bakery appears to be rather complex, and numerous factors influence this process. Most of these factors present starting points to find ways to reduce the acrylamide content of bakery products, which will be discussed in Section 19.2.4.

19.2.3 Modelling versus direct measurement

To characterise acrylamide formation in bakery and to find ways to limit it, model systems are often applied.[8,31,35,39] These models offer advantages such as reproducibility, simplicity, and feasibility to identify the key factors for

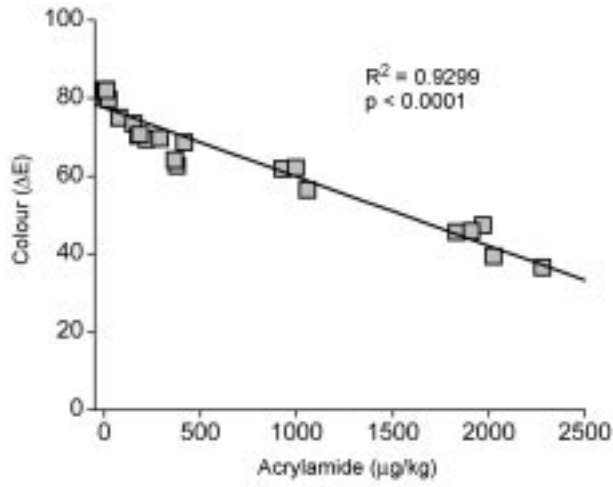

Fig. 19.5 Correlation between colour and acrylamide in bread crusts. Data taken from Surdyk *et al.*[26]

acrylamide formation. However, they cannot take product characteristics such as flavour, colour, or texture into account because no real product is produced. From that point of view, investigations on real products provide more information and facilitate the implementation of the results into industrial processes. Since every product has its individual composition and character and some effects depend on the presence of particular components, e.g. baking agent or reducing sugars, it is advisable to perform experiments for each product.[31]

Mathematical models are useful tools to elucidate and demonstrate the relation between acrylamide formation and process parameters, e.g. temperature (T) or time (t). In this respect, the range of technological relevance has to be kept in mind and the selection of data used for modelling is of crucial importance for the outcome of the calculations. This is demonstrated in Table 19.2 (gingerbread) and in Fig. 19.6 (roasted almonds). Table 19.2 contains data from gingerbread baked at 200 °C for different times (t). With three different subsets covering three time ranges, completely different mathematical relations between acrylamide content and baking time are obtained. In all three cases the coefficient of correlation (R^2) is high. In the early phase the relation to

Table 19.2 Relation between acrylamide content and baking times for different data sets from the same experiments with gingerbread. Data taken from Amrein *et al.*[9]

Time range (min)	Correlation	Equation	R^2
2–10	Quadratic	$y = 5.3 \cdot x^2 - 8.0 \cdot x$	0.9972
6–16	Linear	$y = 66 \cdot x - 244$	0.9934
14–24	Negative quadratic	$y = -4.6 \cdot x^2 + 206 \cdot x - 1296$	0.9294

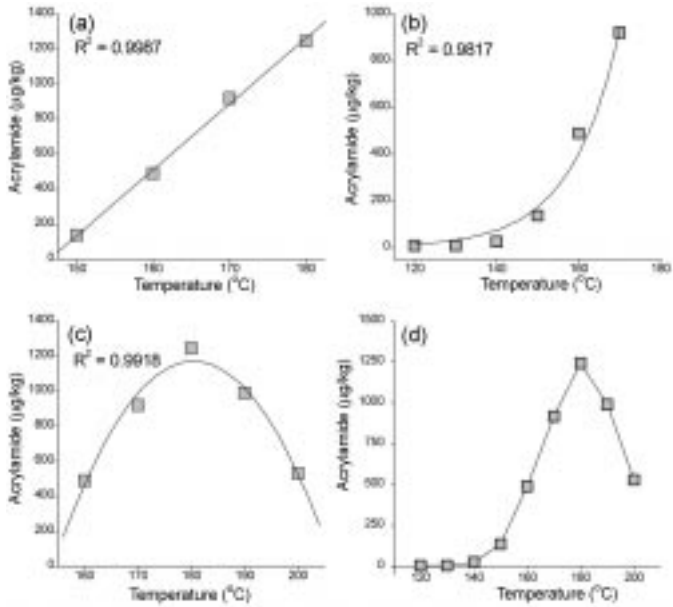

Fig. 19.6 Relation between roasting temperature and acrylamide content of roasted almonds ($t = 10$ min). A: linear ($y \sim x^1$), B: exponential ($y \sim e^x$), C: negative quadratic ($y \sim x^2$), D: whole data set. Data taken from Amrein *et al.*[45]

acrylamide is proportional to t^2, in the middle phase it is linear, and towards the end it is proportional to $-t^2$.

Similar phenomena were also observed during roasting of almonds. Figure 19.6 shows correlations between the roasting temperature and the acrylamide content of almonds after roasting for 10 min.[45] The correlations are significant ($p < 0.05$) and the values for R^2 are high, but completely different relations are obtained. The almonds roasted for 10 min at 120 ºC and 140 ºC were only slightly roasted and had not the desired roast character. The samples roasted at 190 ºC and 200 ºC were considered over roasted and tasted bitter and burnt. The range of technological relevance at which a product of desired quality is produced covers only the range of 140 ºC to 180 ºC. From this point of view, only the linear correlation (A) and probably the exponential correlation (B) are relevant. Therefore, feasible information is obtained only if the data set covers a range of technological processes where products of acceptable quality are obtained.

Results from extreme process conditions with products containing a few mg acrylamide per kg may lead to confusing results such as that acrylamide concentration in bread may be proportional to $-t^2$ or $-T^2$.[39] Although this is mathematically correct and is in accordance with enhanced elimination of acrylamide at drastic conditions, it may not be relevant in practice, because the products are over baked.[9,26] Altogether, model systems and calculations of the relation between acrylamide and process parameters should take the following points into account:

- The model used should resemble as much as possible a real product. In the best case a real product is prepared under industrial conditions.
- The parameters tested (e.g., time and temperature) should cover a realistic process range.
- The selection of the data set has a strong influence on the calculation. Thus, the data set must reflect realistic conditions and the products produced at these conditions should be of acceptable quality.

19.2.4 Approaches for mitigation

The main starting points for mitigation of acrylamide in bakery comprise the following ingredients and parameters:

- free asparagine
- baking agent
- reducing sugars
- baking process (t, T)
- special additives (e.g., organic acids).

The amount of free asparagine in the dough is a key factor for acrylamide formation in bakery. Thus, this parameter is the starting point for various approaches for mitigation. The content of this amino acid can be decreased by selecting flours with less free asparagine, e.g. wheat instead of rye or flours with a low extraction during milling[14] or by omitting potato flakes in the product.[22] Fermentation of yeast leavened dough can be optimised with respect to enhanced consumption of free asparagine whereby less acrylamide is formed during baking.[32] A very elegant way to eliminate free asparagine in unfermented dough is the application of an asparaginase which hydrolyses asparagine to aspartic acid and ammonia. Incubation of mashed potato, potato flakes, rye flour, and wheat flour with asparaginase prior to heating decreased the acrylamide content in model systems by over 90%.[6,31] The large effect of the enzyme in these models was facilitated by the aqueous suspension of the product during the incubation. This is not practicable for most products and therefore, the enzyme may not exhibit full activity or maximum effects in matrices such as dough. Nevertheless, the use of this enzyme reduced the acrylamide content of gingerbread by 55%,[9] and by over 70% in two different types of crackers.[22] By using asparaginase, the prescription and the baking process remain unchanged and thus, the sensory properties of the product are the same.

Because the baking agent NH_4HCO_3 strongly enhances the acrylamide formation in sweet bakery, its replacement by $NaHCO_3$ presents an important and feasible approach for chemically leavened products. It was successful in model systems[8,31,35] as well as in gingerbread,[9,38] crackers,[22] and biscuits.[23] This approach may be combined by replacing the main sources of reducing sugars (e.g., inverted sugar syrup) by sucrose solutions whereby the acrylamide content is even further decreased (see Fig. 19.7)

The promoting effect of NH_4HCO_3 is particularly strong in products

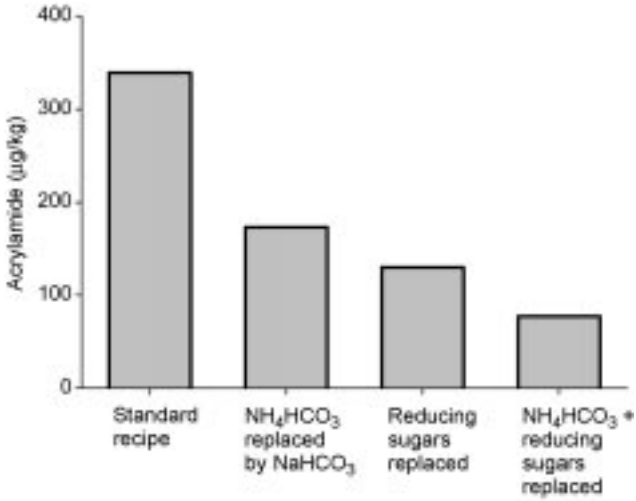

Fig. 19.7 Acrylamide content in wheat crackers prepared with different formulations regarding baking agents and sugars. Adapted from Vass *et al.*[22]

sweetened with glucose and fructose (e.g., by the use of honey or inverted sugar syrup). The replacement of the main sources of reducing sugars by sucrose solutions of equivalent concentration decreases the acrylamide content to a large extent, even if NH_4HCO_3 is present.[9,22,23] The application of isomalt (5–10%) and sorbitol as sweeteners was reported to reduce the acrylamide content in model systems and gingerbread.[46]

A rather general approach is the prevention of over baking or enhanced browning, respectively. The optimisation of the time-temperature combination,[9] the change of the baking profile,[14] or to decrease the temperature toward the end of baking[22] are measures that lowered the acrylamide content in various bakery products. However, no general rule is at hand and therefore experiments have to be performed for each product category to find the optimal conditions. This is demonstrated by the following examples: gingerbread baked at 160 ºC for 20 minutes showed the same browning as a sample prepared at 200 ºC for 10 minutes, but the acrylamide contents were 910 and 440 μg/kg, respectively. Thus, a shorter baking time at a higher temperature led to lower acrylamide concentrations compared to longer baking at a lower temperature.[9] In contrast, experiments with wheat bread and short pastry showed that the highest acrylamide contents were determined in the samples baked at the highest temperature.[21,26] A recent study focused on air humidity in the oven and on product moisture during baking. Higher humidity and product moisture might be a further option to limit the formation of acrylamide in baked goods.[46]

In various studies, the addition of organic food grade acids (e.g., citric acid and tartaric acid) or inorganic acids to the dough decreased acrylamide contents.[9,22,23,35] The addition of acids lowers the pH of the dough with several effects. The α-NH_2 group of asparagine becomes more protonated whereby the

first step of acrylamide formation, i.e. the formation of the imine, is hindered. The more acidic matrix also reduces the release of ammonia from NH_4HCO_3 whereby the acrylamide formation cannot be enhanced. Levine *et al.* observed that citric acid hindered formation of acrylamide, but did not affect the elimination of $^{13}C_3$-acrylamide.[35]

Several research groups showed that addition of glycine lowered the acrylamide contents and enhanced the browning.[9,36,37] As an example, the effect of glycine on the acrylamide of breadcrust is shown in Fig. 19.8. Similar effects were reported for gingerbread. The addition of 10 g glycine per kg dough decreased the acrylamide by over 60% and the browning was enhanced while the pH was not affected.[9] This is an interesting feature of this approach; less acrylamide combined with stronger browning. Normally, the browning is positively correlated with the acrylamide concentration. Addition of 1 g of cysteine to 150 g flour reduced the acrylamide content of a cracker model by about 50% while the recovery of deuterated acrylamide was strongly decreased.[35] The effect of added amino acids on the acrylamide content is not fully understood yet. Possible explanations are the competition between glycine and asparagine for the reactive components and/or the removal of acrylamide by reaction with the amino or sulhydryl group of amino acids via a Michael type addition.[2] The latter is supported by the observation that addition of cysteine enhanced elimination of $^{13}C_3$-acrylamide.[35]

Levine *et al.* also tested a series of other ingredients for their effect in a cracker model. They found that sodium bisulphite, sodium ascorbate, and sodium chloride decreased the formation of acrylamide, but had little or no effect on its elimination.[35] Habel and coworkers also reported that the addition of 0.5 to 2.0% (w/w) NaCl or KCl lowered the acrylamide content in model

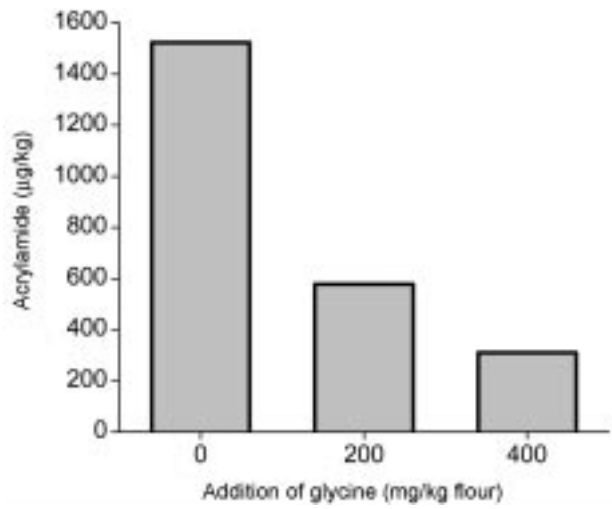

Fig. 19.8 Effect of added glycine on the acrylamide content in breadcrust, data taken from Bråthen *et al.*[36]

systems and gingerbread.[46] The effect of added chloride salts was not explained by the two groups, but the added salts probably lowered the a_w-value which might have affected acrylamide formation. The addition of protein, e.g. gluten or casein, somewhat decreased the acrylamide content of the cracker which was attributed to enhanced elimination of acrylamide.[35] Addition of different vegetable oils affected the acrylamide content in crackers, but the effect may be only physical by altering the heat transfer within the product.[35]

19.2.5 Problems encountered

A general problem is the sensory properties of the product. If a measure negatively affects the colour, texture, taste, or flavour of the product, the feasibility of this approach is limited. As acrylamide is formed in the Maillard reaction, browning or formation of aroma active compounds such as pyrazines may correlate with the acrylamide content.[44] Problems encountered with the above-mentioned ways to decrease acrylamide contents are now discussed.

The use of other ingredients (e.g., wheat instead of rye, white flour instead of whole flour) may affect the sensory and nutritional quality as well as the character of the product. The acrylamide content of a potato cracker was substantially reduced by interchanging the amount of wheat flour and potato flakes in the dough, but the new product was no more a potato cracker.[22] The application of asparaginase is not feasible for industrial purposes yet because the costs for this enzyme are still very high. However, a GMO-derived asparaginase is to be released soon at lower costs. Experiments with asparaginase also demonstrated that at least some 40% of the free asparagine has to be eliminated to obtain a significant reduction of the acrylamide concentration.[9,22] If the optimisation of ingredients with respect to free asparagine does not comply with that limit then this measure may not be effective. Furthermore, such recipe changes have to be balanced with sensorial and nutritional aspects (e.g., content of dietary fibre).

Replacing reducing sugars by sucrose effectively decreased the acrylamide contents. However, a reducing sugar is needed in the Maillard reaction for the formation of melanoidins (brown colour) and flavour compounds. Thus, this measure may impair browning and development of desired flavour. The feasibility of this approach depends on the product character. It was not acceptable for gingerbread where browning is an important quality factor[9] and only satisfactory for wheat crackers[22] while it was successful in a semi-finished biscuit.[23] The use of sucrose is therefore feasible only if the browning is not very important for the product character. To date, little is known about the effects of alternative sweeteners such as sorbitol and isomalt regarding acrylamide in bakery. More investigations are necessary to assess the suitability of these compounds to reduce the acrylamide content of sweet bakery products.

When the baking agent NH_4HCO_3 was replaced by $NaHCO_3$ the acrylamide concentration was reduced in all studies. However, these baking agents behave differently; NH_4HCO_3 reacts at higher temperatures and releases double the gas volume per mole compared to $NaHCO_3$. In addition, $NaHCO_3$ needs a proton

donor (e.g., citric acid) to release gas efficiently and to prevent an alkaline taste. Good results are obtained if this replacement is done in a way whereby the same volume of gas is released from the baking agent and some organic acid is added together with $NaHCO_3$.[23] Replacing NH_4HCO_3 by $NaHCO_3$ led to a decreased product volume, finer pores, and an alkaline taste in gingerbread.[31,38] The use of larger amounts of acidic sodium pyrophosphate (SAPP 10) largely eliminated these negative effects and the product was faultless.[38]

SAPP 10 was also successfully used to improve the leavening and taste of gingerbread prepared with $NaHCO_3$.[38] The use of SAPP instead of citric or tartaric acid in combination with $NaHCO_3$ presents an advantage because SAPP is less reactive and the gas release is retarded and slower during baking. However, for some products, e.g. certain gingerbreads, ammonia from NH_4HCO_3 contributes to the typical flavour of the product. Then the replacement by $NaHCO_3$ might be critical. The use of NaCl or KCl to limit acrylamide formation might be limited for two reasons. Firstly, a salty taste may develop. Secondly, the increase in salt uptake has to be considered and therefore the acceptance of this approach may be limited.

The optimisation of the baking process with respect to time and temperature may help but different products behave differently as discussed above. For many bakery products a minimum temperature is needed to prepare typical and safe products of good quality. Product moisture is a critical factor for product quality (e.g., texture) as well as for microbial stability and safety. Therefore, approaches with higher product moisture must respect this constraint.

Mixing citric or tartaric acid with the dough reduces the acrylamide content of the product. But at the same time, browning is also decreased and an acidic taste may result. Thus, the use of organic acids is limited to an extent at which the sensory properties are not negatively affected. In gingerbread the addition of 1 g/kg citric acid to the dough was an upper limit regarding taste, but the browning was already insufficient.[9] In a semi-finished biscuit 2.44 g tartaric acid per kg (normal amount, 1.95 g) dough was consistent with sensory quality standards including colour.[23] This demonstrates that the feasibility of this measure is product-specific.

Although amino acids may decrease the acrylamide content and enhance the browning at the same time, their impact on sensory properties may be negative. Cysteine may decompose during baking and cause off-flavours as observed in gingerbread.[9] Furthermore, the products formed from added amino acids are yet to be identified. Some amino acids (e.g., aspartic acid, valine, and phenylalanine) may form other vinylogous compounds (e.g., acrylic acid, methylpropene, styrene) during heating as shown in model systems.[47,48] Thus, this promising approach needs to be checked for the formation of other potentially toxic compounds. Addition of proteins (e.g., gluten or casein) to dough led to the formation of a hard outer shell in crackers resulting in volume expansion from the trapped steam.[35] Thus, the effect of added proteins on the texture of the product may be negative, although the acrylamide content might be decreased to some extent.

19.3 Conclusions

The formation of acrylamide in bakery appears to be rather complex and some aspects are yet to be clarified. The feasibility of an approach for mitigation is often product-specific. This section will summarise the most feasible approaches to reduce the acrylamide content of bakery and identify some knowledge gaps.

19.3.1 Most feasible approaches for mitigation

Because free asparagine controls the extent of acrylamide formation in bakery products, its minimisation in the raw dough obviously presents a potential approach for mitigation. But fermentation by yeast or addition of asparaginase is not always suitable, because the product character might change. In general, it might be difficult to significantly decrease the concentration of free asparagine by optimisation of ingredients. Therefore, avoiding NH_4HCO_3, replacing reducing sugars by sucrose, and the optimisation of the baking process are presently the most feasible approaches.

The optimisation of temperature and time has to be product-specific, because different products will respond in different ways to changed baking conditions. The addition of organic acids, e.g. citric acid and/or tartaric acid, presents another option. Both compounds are known and accepted as ingredients and were used before acrylamide became an issue, so there is considerable knowledge regarding their application. However, the development of an acidic taste and the impaired browning limit the use of organic acids.

Knowledge of the critical factors for acrylamide formation in baking must be adopted for the development of new products. For such novelties problems like changed flavour or colour, which may arise from adoption of processes or recipes, are avoided if this knowledge is implemented right from the beginning. Thus, for new products, the following guidelines should be kept in mind:

- omitting NH_4HCO_3 as baking agent
- avoiding excessive browning
- minimisation of ingredients rich in free asparagine, e.g. potato flakes, ground almonds, whole cereal flours
- using sucrose solution instead of inverted sugar syrup.

Irrespective of the effect on acrylamide content, quality aspects such as safety (microbiological, toxicological), culinary quality, product originality, and consumer acceptance have to be respected as well. Therefore, a balance between measures to limit acrylamide formation and product quality has to be found.

19.3.2 Knowledge gaps and research needs

The use of the baking agent NH_4HCO_3 has a long tradition for bakery and thus may be hard to replace. Its promoting effect on acrylamide formation is still not fully understood. In-depth knowledge of the effect of this compound on

acrylamide formation may help to find ways to limit its negative effects when it is used. Since amidation of acrylic acid by ammonia can be excluded and reducing sugars are needed for the promoting effect of NH_4HCO_3, investigations should focus on the reactions of ammonia with reducing sugars and amino acids. Preliminary experiments have shown that the formation of reactive sugar fragments such as α-dicarbonyls may play an important role in this context.

Up to now, no data are available on the occurrence of 3-propaneamide (3-APA) in cereal flours and on its eventual formation during baking. 3-APA is a very potent precursor for acrylamide which can form acrylamide already at 60 °C.[10] Furthermore, it may be a key intermediate in the formation of acrylamide from free asparagine in food.[49] Thus, it is imperative to know, if 3-APA is also present in raw materials for bakery and if this compound is formed during baking. In addition to its biochemical generation by decarboxylases, the thermal formation in bakery products should be investigated as well.

Since flour is the main source for free asparagine in dough, investigations on factors that influence the content of this amino acid are necessary. The selection of cultivars, breeding programmes for crops with low content of free asparagine, and the influence of fertilisation are some research areas. These measures, particularly breeding programmes, are long-term projects and cannot contribute to acrylamide mitigation on a short-term basis. Furthermore, they also have to retain other crop qualities such as yield, suitability for baking, and nutritional aspects. Therefore, it might take great effort and a long time to make substantial progress in this field. Nevertheless, a substantial decrease in the content of free asparagine in cereal flours is one of the very few approaches that 'solve rather than fight the problem'.

The addition of glycine and other amino acids can reduce the acrylamide content and enhance browning at the same time. Thus, this presents a very attractive option for mitigation. But the impact on flavour, the new compounds formed, and the elimination products of acrylamide are unknown and need to be investigated. In this context the decay of acrylamide during baking is of interest as well.[27] The formation of other vinylogous compounds is particularly interesting because this was shown in model systems.[47,48] Therefore, the formation of other potentially hazardous compounds should be largely excluded if amino acids are added to reduce the acrylamide content.

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20

Novel techniques to prevent the formation of acrylamide in processed food

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20.1 Introduction

This chapter will look at potential strategies to reduce the formation of hazardous compounds during food processing. With emphasis on acrylamide, it will review the principles that may serve as the theoretical basis for method selection. This chapter presents the general considerations for process improvement and selection, based on reaction kinetics as well as the effect of exogenous and endogenous parameters such as temperature and formulation respectively. Existing technology, as well as technologies under development, will be reviewed for two purposes: introducing the technology and its principles, and shedding light on the specific applications. We will deal not only with unit operation but also with other approaches such as controlling the physical state of the materials, etc. Initially, we will discuss the possible solutions through formulation changes and adaptations. Where applicable, the effect of the specific techniques on food quality will be reviewed as well as the commercial potential.

20.2 General considerations

When choosing the appropriate technological solution to reduce the formation of hazardous compounds formed by thermal treatment of the food, one should analyse the literature for the various parameters affecting their formation, and the effect of chemical and technological conditions on their formation. We will follow this line for acrylamide as the leading example.

For the specific case of acrylamide, since the early reports on its formation during industrial and domestic food processing, much attention was given to the quantification of this substance in various food products. Some key rules of thumb were drawn as to the circumstances, which enhance the formation of acrylamide (Rydberg *et al.*, 2005; Stadler, 2005). These can be generally classified to composition factors and processing conditions principles, which may serve as the theoretical basis for method selection. Thus, the key points in the development of preventive technologies are the chemistry of acrylamide formation, and the kinetics of the specific reactions. Furthermore, it is of utmost importance to understand the dependence of the reactions on environmental factors, starting with temperature, followed by pH, oxygen concentration and the state of the material. To choose appropriate technological solutions, these relations should be drawn in quantitative terms in order to enable a knowledge-based approach to provide adequate solutions.

The chemistry dominating acrylamide formation is the key for product formulation, aimed at reducing its formation. Acrylamide in food is mainly formed by a reaction between asparagine and reducing sugars (Becalski *et al.*, 2003; Mottram *et al.*, 2002; Stadler *et al.*, 2002; Weisshaar and Gutsche, 2002). Two mechanisms, varying in details, have been proposed (Becalski *et al.*, 2003). The most plausible scheme would include the formation of acrolein from the thermal degradation of glycerol, oxidation of acrolein to acrylic acid, and finally reaction of acrylic acid with ammonia, which potentially could be generated by pyrolysis of nitrogen-containing compounds, leading to the formation of acrylamide. The second hypothesis is that acrylamide could be formed alone, by rearrangement from nitrogen-containing compounds already present in foods. Zyzak *et al.*, (2003) presented the mechanism for the formation of acrylamide from the reaction of the amino acid asparagine and a carbonyl-containing compound at typical cooking temperatures, involving formation of a Schiff base followed by decarboxylation and elimination of either ammonia or a substituted imine under heat to yield acrylamide. The suggestion that acrylamide can be formed from lipid-rich foods by a reaction between ammonia and acrolein was supported by Yasuhara *et al.* (2003), and some data indicate that the type of oil used for deep-frying can influence the formation of acrylamide (Becalski *et al.*, 2003). However, evidence clearly points to the reaction between asparagine and reducing sugars as the main route.

Apparent kinetics of acrylamide formation is usually considered as an apparent first-order reaction. These parameters can be used to determine the time range that can be allowed for the thermal treatment. As with any chemical reaction, there is always a short 'window of opportunity' that enables a thermal treatment for either cooking or preservation, with the minimal formation of hazardous reaction products. However, most of the data shows that as the temperature and time increase there is a reduction in acrylamide content in a reaction sample (Becalski *et al.*, 2003; Bråthen and Knutsen, 2005; Wedzicha *et al.*, 2005).

Claeys *et al.* (2005a) performed kinetic analysis of the process of AA formation in foods. The AA yield, which appears to be the net result of

simultaneous formation and elimination reactions, could be modelled by two consecutive first-order reactions according to the following simplistic scheme (Claeys *et al.* 2005a):

$$\underset{\text{(reactants)}}{\text{R}} \xrightarrow{k_F} \text{AA} \xrightarrow{k_E} \text{D} \begin{matrix}(\text{AA-protein complex/} \\ \text{AA degradation products/ } \ldots\end{matrix} \tag{20.1}$$

in which k_F and k_E are the first-order rate constants of AA formation and elimination at the temperature studied. At time $t = 0$, the concentrations of AA and of the AA elimination products D can be considered to be zero.

Perhaps the most important factor governing the rate of the reaction is temperature. Equally important is the temperature dependence of the reaction, as expressed by its activation energy (E_a) (for a small temperature range one can also use Q10). The effect of temperature on the reaction rate constant k can be expressed by the Arrhenius relation, in which the temperature dependence of the rate constant k is quantified by the activation energy E_a (J/mol) according to

$$k = k_{ref} \exp\left(\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right) \tag{20.2}$$

with R the universal gas constant (8.314 J/mol·K), T the temperature concerned (K), and k_{ref} the reaction rate constant at reference temperature T_{ref}. By using this approach, Claeys *et al.* (2005a) determined acrylamide (AA) formation and elimination kinetics in an asparagine-glucose model system (0.01 M, pH 6) heated at temperatures between 140 and 200 ºC. They reported values of k_{Fref} 0.451 ± 0.023 ($\times 10^3$ min^{-1}) and k_{Eref} 111.1 ± 8.9 ($\times 10^3$ min^{-1}), and temperature dependence expressed by Ea_F 168.25 ± 3.80 (kJ/mol) and Ea_E 167.21 ± 4.30 (kJ/mol). The overall concept is in generally good agreement with the proposed model by Knol *et al.* (2005).

Most important are the various observations suggesting that for the formation of acrylamide, the thermal treatment of the food should be at temperatures above 100 ºC (Tareke *et al.*, 2002). Interestingly, heating for ~20 minutes at 120 ºC was sufficient to cause an increase in acrylamide content. This observation implies that under conventional commercial sterilisation conditions, given the appropriate food composition, acrylamide can be formed in measurable amounts.

Apart from reaction time and temperature, the major difference between thermal procedures leading to high or low amounts of acrylamide from asparagine is the water content of the reaction system, which directly influences its physical state. The water activity has been shown to be one key factor to consider in the Maillard reaction (Labuza and Saltmarch, 1981; Ames, 1990). In particular, it is critical for the shelf-life quality of industrially processed dry food products (Roos, 2003; Karel *et al.*, 1994). Robert *et al.* (2005) investigated the formation of acrylamide in model systems based on asparagine and glucose under low moisture as a function of reaction temperature, time, physical state, water activity, and glass transition temperature. Acrylamide release in amorphous and crystalline glucose/asparagine models indicated the importance of the physical state in acrylamide formation. In amorphous systems, acrylamide

was generated in higher concentrations and at lower temperatures as compared to the crystalline samples. Interestingly, and somewhat unexpectedly, the water activity and glass transition temperature do not seem to be critical parameters for acrylamide formation in the systems studied by Robert *et al.* (2005). Lowering water activity at the surface of a product for pre-frying was proposed by Franke *et al.* (2005) as a means of acrylamide reduction.

20.3 Technological approaches for reducing acrylamide and other hazardous materials

Hazardous compounds formed during thermal processing of foods span beyond acrylamide, and thus encompass a wide range of time-temperature combinations in which these compounds are formed. As the chemistry of their formation differs as well, changes in product formulations may be equally diverse. Therefore, possible solutions will be presented to demonstrate the principle, with no intent to provide a comprehensive review of all hazardous compounds or formulations. Reviewing the abundance of acrylamide in food products, it appears that it is formed mainly during frying and baking processes with ultra-high temperatures, but is also formed during more moderate conventional thermal treatments. This variety of processes and technologies in which it is formed call for equally diverse approaches for its reduction.

20.3.1 Formulation and process modification solutions

The most elegant way that reduction of acrylamide content in potato products might be accomplished is merely by the selection of the proper potato variety (Hebeisen *et al.*, 2005; Olsson *et al.*, 2004). In one study, tubers from several ware and processing varieties, originated from two locations and two harvest seasons 2002 and 2003, were stored at 8 ºC. Standardised processing tests were performed twice, early and late within the storage period respectively. The average acrylamide content in crisps of eight processing varieties was 777 mg/kg in harvest 2002 and 31% higher in harvest 2003, respectively. Reducing sugar contents in tubers were lower in 2003 due to very dry and hot growing conditions. Both parameters were significantly influenced by variety, year and location. Lowest acrylamide contents were determined in potato varieties Panda, Lady Claire and Markies. The varieties Agria, Markies and Fontane had low acrylamide contents in French fries and hash browns. The authors concluded that excellent raw material is a prerequisite for a lower acrylamide formation during frying and roasting.

Changes in the sugar and amino acid contents of potato tubers during short-term storage and the effect on the acrylamide level in chips after frying were investigated by Ohara-Takada *et al.* (2005). There was a strong correlation between the reducing sugar content and acrylamide level for fructose and glucose. The sucrose content had less correlation with the acrylamide content.

The contents of the four amino acids, i.e., asparatic acid, asparagine, glutamic acid and glutamine, showed no significant correlation with the acrylamide level. These results suggest that the content of reducing sugars in potato tubers determined the degree of acrylamide formation in chips.

Despite the promise offered by the use of proper raw materials, this approach is not always feasible, in particular where the product attributes stem from specific quality of a particular raw material. The most common solutions offered for the reduction of acrylamide formation are pre-treatment by washing, blanching, or the addition of simple chemical inhibitors. For fried potatoes, several papers demonstrated that pre-treatments such as immersing in water, blanching in hot water, citric acid solution or sodium pyrophosphate solution could decrease acrylamide formation during frying (Pedreschi *et al.* 2004, 2006). Jung *et al.* (2003) showed that lowering pH by 0.2% citric acid treatments induced 82.2% and 72.8% inhibition of acrylamide formation in fried and baked corn chips, respectively. Dipping potato cuts in 1% and 2% citric acid solutions for 1 h before frying showed 73.1% and 79.7% inhibition of acrylamide formation in fries. In addition, by lowering the pH from 7.0 to 4.0, 99.1% inhibition of acrylamide formation was achieved, and thus offered an effective, simple, and practical way to limit acrylamide formation in real foods.

The effect of amino acids on the content of acrylamide in potato crisps, French fries, flat breads, and bread crusts were investigated by Bråthen *et al.* (2005). Addition of glycine or glutamine during blanching of crisps reduced the amount of acrylamide by ~30% compared to no addition, however, no effect was found in French fries. Addition of glycine during dough making significantly reduced acrylamide in both flat breads and bread crusts. Kim *et al.* (2005) also used free amino acids to reduce acrylamide, with lysine, glycine, and cysteine having the greatest effects in the aqueous system. Lysine and glycine were effective at inhibiting the formation of acrylamide in wheat-flour snacks. In potato snacks, the addition of 0.5% glycine to pallets reduced acrylamide by more than 70%. Soaking potato slices in a 3% solution of either lysine or glycine reduced the formation of acrylamide by more than 80% in potato chips fried for 1.5 min at 185 ºC.

Fiselier *et al.* (2004) demonstrated the ability of an edible coating to reduce acrylamide formation. Coating potato croquettes with egg/breadcrumbs resulted in stronger browning and at the same time in reduced acrylamide formation. It shields the potato from the heat by a material the browning of which is not linked with acrylamide formation. Croquettes prepared from fresh potato confirmed that coating with egg/breadcrumbs improves the product quality while strongly decreasing the acrylamide content. Granada *et al.* (2004) performed vacuum frying experiments at 118 ºC, 125 ºC, and 140 ºC and a vacuum pressure of 10 Torr. Vacuum frying reduced acrylamide formation by 94%, showing that modified frying systems can play an important role in reducing acrylamide formation in fried potatoes. As the frying temperature decreased from 180 ºC to 165 ºC, acrylamide content in potato chips reduced by 51% during traditional frying and by 63% as the temperature decreased from 140 ºC

to 125 ºC in vacuum frying. In another study, acrylamide formation was studied by use of a new heating methodology, based on a closed stainless steel tubular reactor (Mestdagh *et al.*, 2005). Different artificial potato powder mixtures were homogenised and subsequently heated in the reactor. Surprisingly, when artificial mixtures did not contain vegetable oil, significantly lower concentrations of acrylamide were detected, compared to oil-containing mixtures.

When Amrein *et al.* (2004) examined the influence of ingredients, additives, and process conditions on acrylamide formation in gingerbread, they found that the use of sodium hydrogen carbonate as a baking agent reduced the acrylamide concentration by >60%. Acrylamide content could also be lowered by replacing reducing sugars with sucrose or by adding organic acids. Recently, the suppressive effect of various saccharides on the formation of acrylamide in solution was investigated by Kazuyuki (2005). In general, as expected, non-reducing sugars with the exception of sucrose did not yield acrylamide with asparagine. Interestingly, trehalose and neotrehalose inhibit the formation of acrylamide by 75% and 76% respectively. Moreover, trehalose inhibited both the degradation of glucose as well as the conversion of the reaction intermediates to acrylamide. These findings offer an exciting new route in preventing the formation of acrylamide.

20.3.2 Innovative technologies

Very few studies, if any, were performed on the use of alternative food processing technologies for the reduction of acrylamide in food. However, the close relation of acrylamide formation to the Maillard reaction is well described (Mottram *et al.*, 2002; Stadler *et al.*, 2002, 2004). Therefore this section will introduce the techniques, and their implication on other known potentially hazardous substances with emphasis on Maillard reaction products. Innovative food processing technologies are categorised using various parameters, which in our case will be thermal, and non-thermal. On one hand there are techniques such as high hydrostatic pressure, used for non-thermal preservation, and on the other hand, for example, ohmic heating technology, which provides an improved technique for thermal treatments. We will review these technologies based on their mode of action, thermal or non-thermal. Our focus will be on those techniques aimed at replacing ultra-high-temperature treatments.

Non-thermal innovative preservation technologies

Among the non-thermal technological solutions for processing and preservation two techniques appear to be most promising: high pressure processing and pulsed electric field (excluding irradiation which requires specialised facilities). High-pressure processing (HPP) is also described as high hydrostatic pressure (HHP), or ultra-high pressure (UHP) processing. During this process, the food is being subjected to pressures between 100 and 800 MPa, under controlled temperature from below 0 ºC to above 100 ºC. The exposure times can range from a millisecond pulse up to 20 minutes. In relation to chemical reactions,

foods subjected to HPP treatment at or near room temperature will very likely not undergo significant chemical transformations due to the pressure treatment itself (Tauscher 1998, 1999). HPP may be combined with heat to achieve an increased rate of inactivation of microbes and enzymes, and this may induce some chemical changes in the food.

Among the innovative food preservation technologies, HHP was most thoroughly investigated for its effects on biological matter. Most relevant to our discussion are the studies of the Maillard reaction as affected by HHP. A study on the effect of a combination of heating and pressurisation showed that high hydrostatic pressure (HHP) up to 400 MPa retarded the Maillard reaction in a temperature range of 100–115 ºC. The HHP treatment also suppressed the browning of the white sauce, which was heated at 115 ºC for 30 minutes under 300 MPa (Okazaki *et al.*, 2001).

High hydrostatic pressure (100 MPa) treatment suppressed the formation of intermediate and final products of sugar (glucose or fructose)-amino acid (leucine, lysine or glutamate) solution models. HMF was the most abundant compound among the detected compounds; its content was increased with the increase in temperature, but decreased in high-pressure treatment (Komthong *et al.*, 2003). In a model system consisting of amino acids or β-casein and sugars, pentosidine was found to correlate positively with the increase of pressure, while the formation of pyrraline was reduced. As the Maillard reaction is a complex scheme of linked reaction cascades, the authors suggested that the effect of high pressure cannot be studied using sum parameters, but has to be regarded for each single reaction product (Schwarzenbolz *et al.*, 2000, 2002). Indeed, yields of all volatile compounds were suppressed at 600 MPa compared to prolonged incubation at atmospheric pressure in a glucose-lysine model system. Many of the compounds reported may be formed by, or subsequently react via, aldol condensation. Due to the observed differences among the systems in the profiles and yields of volatile compounds it was suggested that aldol condensations increase in rate in the systems under pressure (Hill *et al.*, 1996a, 1999). As for temperature effects, the activation energies for the high pressure and atmospheric pressure were not significantly different (Hill *et al.*, 1996b).

Studying the influence of high hydrostatic pressure (HHP) on the formation of selected odour-active compounds in D-glucose and L-proline solutions treated either at 0.1 MPa or 650 MPa showed that while the roast-smelling odorants 2-acetyl-1-pyrroline and 2-acetyltetrahydropyridine were much decreased at HHP, the caramel-like smelling odorants 4-hydroxy-2,5-dimethyl-3(2H)-furanone and 2-hydroxy-3-methyl-cyclopenten-1-one were significantly increased at HHP compared to atmospheric pressure (Deters *et al.*, 2003). These findings merely indicate the complexity of the data, where HHP affects different paths of the reactions in a different manner. Another study on browning products formed in Maillard-type reactions at 100 ºC showed that the effect of HHP may be inverse when the amino acid in xylose solution was changed from alanine to proline (Heberle *et al.*, 2003; Schieberle and Deters, 2003). This observation demonstrates the different effects of HHP on the various reaction pathways.

The data for high intensity pulsed electric field (PEF) processing is unfortunately scarce. The technology is based on the application of pulses of high voltage (up to 80 kV/cm) to foods placed between two electrodes. It is conducted for less than one second at ambient, sub-ambient, or slightly above ambient temperature, and energy loss due to heating of foods is assumed to be minimal. The PEF technology is considered superior to traditional heat treatment in terms of food quality attributes, because it greatly reduces the detrimental changes of the sensory and physical properties of foods due to thermal abuse (Quass, 1997). Effects of PEF on the chemical and nutritional aspects of foods must be better understood to enable its proper use in food processing (Qin *et al.*, 1995).

In one study, the effects of commercial-scale pulsed electric field (PEF) processing on the flavour and colour of tomato juice during storage at 4 ºC for 112 days were studied (Min and Zhang, 2003). Tomato juice was prepared by hot break at 88 ºC for two minutes and then thermally processed at 92 ºC for 90 s or PEF processed at 40 kV/cm for 57 ms. PEF-processed juice had significantly lower non-enzymatic browning than thermally processed or control juice. However, sensory evaluations indicated that the flavour of PEF-processed juice was preferred to that of thermally processed juice.

Alternative thermal technologies

Perhaps the most promising and commercially applicable new technologies for thermal treatment of foods are ohmic and microwave heating. Ohmic heating of food products involves the passage of alternating current through them, thereby generating internal heat as a result of electrical resistance (Reznick, 1996). This technology provides rapid and uniform heating, resulting in less thermal damage to the product (Sastry and Barach, 2000). In addition, the absence of a hot surface with ohmic heating reduces fouling problems and thermal damage to the product. Therefore, a high-quality product with minimal structural, nutritional or sensorial changes can be manufactured in a short operating time (Rahman, 1999). The key to the successful implementation of an ohmic process is the rate of heat generation, the electrical conductivity of the food material, and the way the food flows through the heater. Changes in electrical conductivities of vegetable samples and meat were studied and shown to be affected by a number of factors, e.g. field strength, soluble solids, melting of fats and cell structure changes (Halden *et al.*, 1990; Palaniappan and Sastry, 1991a; Wang and Sastry, 1997). Palaniappan and Sastry (1991b) also determined the electrical conductivity of orange and tomato juices using a static device. They concluded that electrical conductivities of tomato and orange juice increase linearly with temperature and decrease with solids content. In addition, they determined that electrical conductivity tended to increase as particle size decreased, but general conclusions cannot be reached without accounting for particle shape and orientations. Although the technology of ohmic heating appears to be promising and highly effective, there is little information concerning the effects of this technique on specific food products compared to conventional pasteurisation.

Recent work studied the effect of ohmic heating thermal treatment on liquid fruit juice made from oranges (Leizerson and Shimoni, 2005a,b). Effects of ohmic heating on the quality of orange juice were examined and compared to those of heat pasteurisation at 90 ºC for 50 s. Orange juice was treated at temperatures of 90, 120, and 150 ºC, for 1.13, 0.85, and 0.68 s in an ohmic heating system. The ohmic heated orange juice maintained higher amounts of the five representative flavour compounds than in heat-pasteurised juice. Sensory evaluation tests showed no difference between fresh and ohmic heated orange juice. However, the browning of the ohmic heated product was higher than the conventionally pasteurised, although the sensory panel could not identify this difference.

Unlike ohmic heating, where direct electrical current passing through the product generates heat, microwave heating refers to the use of electromagnetic waves of certain frequencies to generate heat in a material (Metaxas, 1996; Metaxas and Meredith, 1988; Roussy and Pearce, 1995). Typically, microwave food-processing uses the two frequencies of 2450 and 915 MHz. Of these two, the 2450 MHz frequency is used for home ovens, and both are used in industrial heating. It is worthwhile to note that outside the United States, frequencies of 433.92, 896 and 2375 MHz are also used. Heating with microwaves involves primarily dielectric and ionic mechanisms. Water in the food is usually the primary component responsible for dielectric heating due to its dipolar nature. Water molecules try to follow the electric field associated with electromagnetic radiation as it oscillates at the very high frequencies, and thus produce heat. The second major mechanism of heating with microwaves and radio frequency is through the oscillatory migration of ions in the food that generates heat under the influence of the oscillating electric field.

The dielectric properties of the food (representing the material's ability to absorb the wave) depend on the composition (or formulation) of the food, where moisture and salt are the two primary determinants of interest (Datta *et al.*, 1994). Subsequently the temperature rise in the food depends on the duration of heating, the location in the food, convective heat transfer at the surface, and the extent of evaporation of water inside the food and at its surface. To calculate the rate of heat generation per unit volume, Q, at a particular location in the food during microwave heating one can use the following equation (Buffler 1993; Datta, 2000):

$$Q = 2 \cdot \pi \cdot f \cdot \epsilon_0 \cdot \epsilon'' \cdot E^2 \qquad (20.3)$$

where E is the strength of electric field of the wave at that location, f is the frequency of the microwaves or the radio frequency waves, ϵ_0 the permissivity of free space (a physical constant), and ϵ'' is the dielectric loss factor (a material property called dielectric property) representing the material's ability to absorb the wave.

As with ohmic heating, microwave heating for thermal treatments is superior to conventional heating mainly since it is rapid and therefore requires less time to come up to the desired process temperature. Oruna-Concha *et al.* (2002)

compared two cultivars of potato cooked by three different procedures, i.e. boiling, conventional baking and microwave baking. Analysing the flavour compounds generated, they found that the ratio (yield derived from lipid)/(yield derived from Maillard reaction and/or sugar) decreased from 8.5–9.1 (boiling) to 2.7–3.4 in microwave baking and to 0.4–1.1 in conventional baking. They suggested that indeed these differences among the cooking procedures are explained in terms of the variations in heat and mass transfer processes that occurred. In milk, however, experiments carried out at controlled temperatures of 80 ºC and 90 ºC, at holding times up to 420 minutes showed that none of the reaction products showed significant differences between microwave heating and conventional cooking methods (Meissner and Erbersdobler, 1996). Indeed, even Villamiel *et al.* (1996) who found some rate enhancement in lactose isomerisation, Maillard reaction and protein denaturation during microwave treatment, attributed these differences, at least to some extent, to uneven heating of the milk in the microwave oven.

The differences between conventional and microwave thermal treatments are not always limited to the quantity of the heat-generated compounds. Several studies showed that the chemical nature of the products might differ according to the heat-generating technology. In one study, a mixture of proline/glucose was reacted at 150 ºC and the volatiles were compared to those of a similar system reacted in a microwave oven. While qualitatively, similar compounds were found in both systems, quantitatively, carbohydrate decomposition products predominated in the microwave system while larger amounts of N-heterocyclic compounds were present in the thermal system (Parliament, 1993). Zamora and Hidalgo (1995) showed that when the model system of lysine/(E)-4,5-epoxy-(E)-2-heptenal is irradiated in a microwave oven, 1-alkyl-2-(1′-hydroxy-propyl)pyrroles play a role in the development of colour and fluorescence in this system.

Apparently, however, the kinetics of the browning in microwave heating reaction is in good agreement with data obtained during conventional heating treatments. This was demonstrated in equimolar concentrations of L-proline and D-glucose in propylene glycol at added moisture contents of 0, 2.5, and 5% (Peterson *et al.*, 1994). The browning rate followed a zero-order reaction, with the rate constants drastically reduced with the addition of a small amount of water. The temperature dependence of the browning reaction was adequately described by the Arrhenius equation, with activation energies (36.03 $\pm$ 1.73 kcal/mol) consistent with the literature.

20.4 Conclusion

As noted in this chapter, providing adequate solutions and reducing the formation of hazardous compounds in food during thermal treatments and preservation processes should be based on a solid knowledge base of the circumstances in which each specific compound is being formed. In the case of acrylamide,

despite the vast effort in the past four years, still more research is required to fully elucidate the reactions leading to acrylamide formation as well as elimination during heating. Furthermore, the relation of water activity (or water content) to acrylamide formation, and its interaction with the state of the food matrix should be explored in detail. In foods, and in particular for reactions occurring in an aqueous environment, the mobility is governed by the chemical potential of the solvent (water) commonly called water activity. At a given temperature, under a critical water activity, the matrix is in its glassy state with highly restricted mobility, thus reducing the reaction rate to practically zero. Some solutions using these concepts already exist in the form of an edible coating forming a glassy, non-acrylamide-forming film, that reduces its formation in the product during thermal treatments.

Formulation modifications are likely to be the most powerful tool in acrylamide reduction. That is because simple acrylamide-containing products have typical textures and forms, as well as sensory attributes, and dramatic changes in such products will cause them to lose their identity and appeal for the consumer. Raw material selections, the addition of competing amino acids and/or non-reducing sugars, are therefore likely to lead this trend.

Some innovative processing technologies have been presented in this chapter. None can replace the preparation technologies applied traditionally in the processing of high-acrylamide food products. However, these technologies may become extremely useful when developing new products and processes with the reduction of acrylamide in mind. It is very likely that techniques such as HHP, PEF, ohmic heating and microwave heating will be used for speciality products in liquid food where the reduction of acrylamide and Maillard reaction products is of importance.

20.5 Sources of further information

As outlined in this chapter, the re-design of the product or its processing conditions to achieve a lower concentration of AA in the final product should rely on the chemistry, physical state as well as unit operation considerations. A comprehensive overview on most aspects is given in *Chemistry and Safety of Acrylamide in Food* edited by Mendel Friedman and Don Mottram (2005). Beyond the biological effects and importance of AA, the book is an excellent source on the chemistry of AA formation, and factors affecting its formation, as well as directions for reducing its content in foods.

For innovative and alternative food processing technologies consult the various books on this topic, such as *Innovations in Food Processing* (Barbosa-Canovas, Gustavo V., Gould, Grahame W., Strauss, Steven, June 2000); *Novel Food Processing Technologies* (Barbosa-Canovas, Gustavo V., Tapia, M. Soledad; Cano, M. Pilar, Aug. 2004), or *Nonthermal Preservation of Foods* (Barbosa-Cánovas, Gustavo V., Jan. 1998). A detailed update on issues related to the Maillard reaction can be obtained from *The Maillard Reaction: Chemistry*

at the Interface of Nutrition, Aging, and Disease (John W. Baynes, Vincent M. Monnier, Jennifer M. Ames, Susan R. Thorpe, eds., New York Academy of Sciences, July, 2005).

FAO/WHO 'Acrylamide in Food Networks' functions as a global resource and inventory of ongoing research on acrylamide in food. It includes formal research, surveillance/monitoring and industry investigations, etc. This network website currently comprises an interactive database, and references for research. The network also serves as a discussion forum for active researchers in the field. Information on these aspects is posted on this network website: http://www.acrylamide-food.org/index.htm.

Other web sources provide links to variety of info pages and databases. These include, among others, the Institute of Food Science & Technology (IFST) Acrylamide Information and News page at http://www.ifst.org/acrylmd.htm; the HEATOX project: 'Heat-generated Food Toxicants – Identification, Characterisation and Risk Minimisation' at http://www.heatox.org; and the FDA website at http://www.cfsan.fda.gov/.

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Appendix I

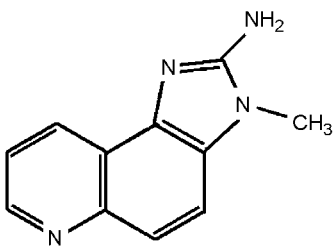
List of abbreviations of heterocyclic amines

IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
MeIQ	2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline
IQx	2-amino-3-methylimidazo[4,5-*f*]quinoxaline
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
4,8-DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
7,8-DiMeIQx	2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline
4-CH_2OH-8MeIQx	2-amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]-quinoxaline
7,9-DiMeI*g*Qx	2-amino-1,7,9-trimethylimidazo[4,5-*f*]quinoxaline
DMIP	2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine
TMIP	2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
IFP	2-amino-1,6-dimethylfuro[3,2-*e*]-imidazo[4,5-*b*]-pyridine
4-OH-PhIP	2-amino-6-(4-hydroxyphenyl)-1-methylimidazo[4,5-*b*]-pyridine
Trp-P-1	3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole
Trp-P-2	3-amino-1-methyl-5H-pyrido[4,3-*b*]indole
AαC	2-amino-9H-pyrido[2,3-*b*]indole
MeAαC	2-amino-3-methyl-αtrimethylimidazo[4,5-*f*]-quinoxaline
Glu-P-1	2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole
Glu-P-2	2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole
Harman	1-methyl-9H-pyrido[3,4-*b*]indole
Norharman	9H-pyrido[3,4-*b*]indole
HAA/HCA	heterocyclic aromatic amines/heterocyclic amines

Appendix II

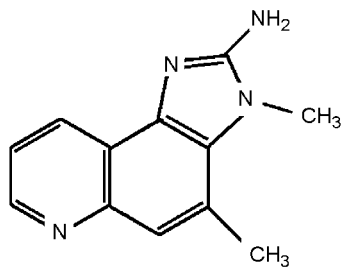
Molecular structures of heterocylic amines

The following pages show the molecular structures of the heterocyclic amines



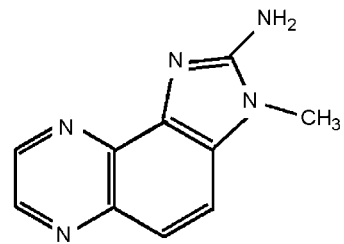
IQ

2-amino-3-methylimidazo[4,5-*f*]quinoline



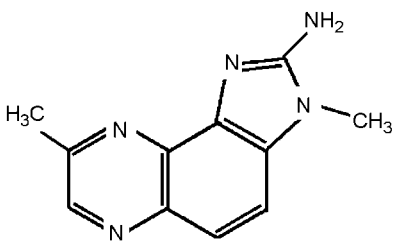
MeIQ

2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline



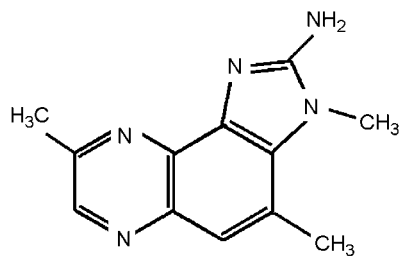
IQx

2-amino-3-methylimidazo[4,5-*f*]quinoxaline



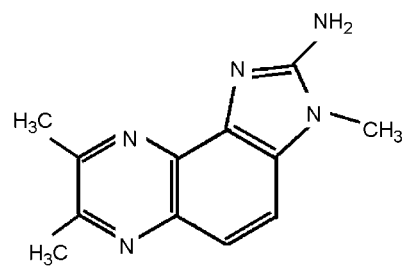
MeIQx

2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline



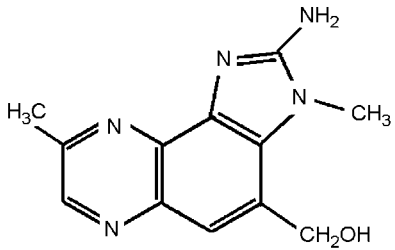
4,8-DiMeIQx

2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline



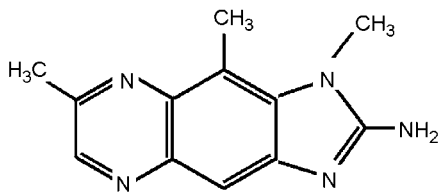
7,8-DiMeIQx

2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline



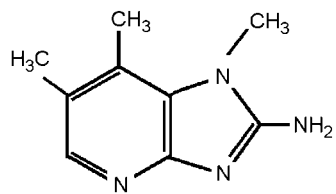
4-CH_2OH-8-MeIQx

2-amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]quinoxaline



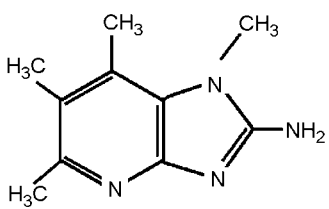
7,9-DiMeI*g*Qx

2-amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline



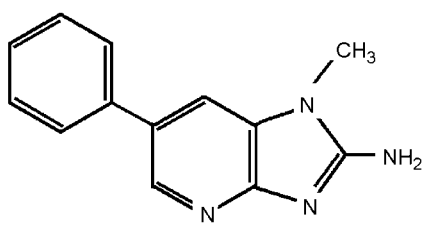
DMIP

2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine



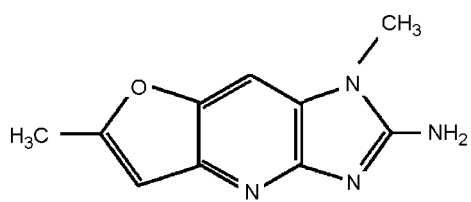
TMIP

2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine



PhIP

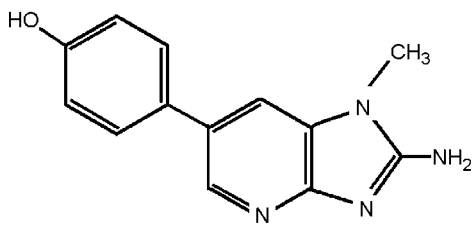
2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine



IFP

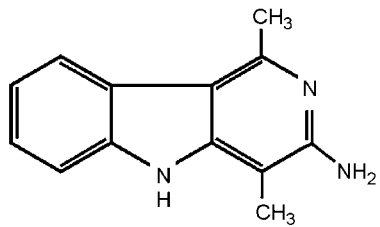
2-amino-1,6-dimethylfuro[3,2-e]imidazo[4,5-*b*]pyridine

(proposed structure)



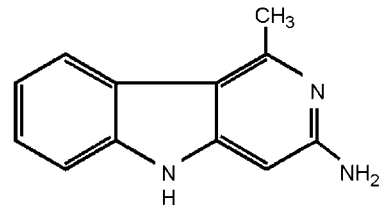
4′-hydroxy-PhIP

2-amino-6-(4-hydroxyphenyl)-1-methylimidazo[4,5-*b*]pyridine



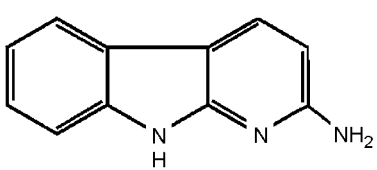
Trp-P-1

3-amino-1-4-dimethyl-5*H*-pyrido[4,3-*b*]indole



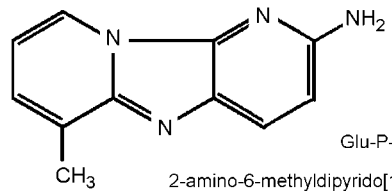
Trp-P-2

3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole



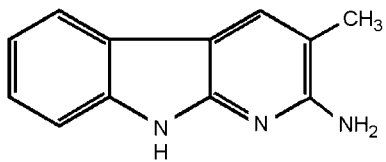
AαC

2-amino-9*H*-pyrido[2,3-*b*]indole



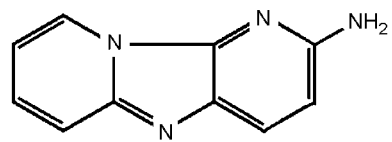
Glu-P-1

2-amino-6-methyldipyrido[1,2-a:3′,2′-*d*]imidazole



MeAαC

2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole



Glu-P-2

2-aminodipyrido[1,2-a:3′,2′-*d*]imidazole

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