Review

Heterocyclic amines: Chemistry and health

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Heterocyclic amines (HAs) occur at the ppb range in foods. Most of them demonstrate potent mutagenicity in bacteria mutagenicity test, and some of them have been classified by the International Agency for Research on Cancer as probable/possible human carcinogens. Their capability of formation even during ordinary cooking practices implies frequent exposure by the general public. Over the past 30 years, numerous studies have been stimulated aiming to alleviate human health risk associated with HAs. These studies contribute to the understanding of their formation, characterization, and quantification in foods; their mutagenesis/carcinogenesis, mechanisms of antimutagenesis by chemical or phytogenic modulators; and strategies to inhibit their formation. The chemistry of HAs, their implications in human health, factors influencing their formation, and feasible ways of suppression will be briefly reviewed. Their occurrence in trace amounts in foods necessitates continuous development and amelioration of analytical techniques. Various inhibitory strategies, ranging from modifying cooking conditions to incorporation of different modulators, have been developed. This will remain one of the foremost areas of research in the field of food chemistry and safety.

1 Introduction

Genetic predisposition and environmental factors are both implicated in the etiology of cancers. The latter is where most scientific research groups in the field of food and chemical toxicology have been working on with the primary aim of modulating human cancer risk. Thus far, it is generally accepted that diet (especially those rich in animal products) is an important contributor to environmental exposure which increases cancer risk, precluding those cases where occupational exposure is significant [1]. Since the late 1970s when the Japanese scientists identified potent

mutagenic activity in the charred surface of broiled beef and fish [2], a substantial amount of studies in the areas of cooking-induced mutagens and carcinogens have been stimulated, which gather researchers from a wide spectrum of areas. These food-derived mutagens were later isolated and characterized as heterocyclic aromatic amines (HAs) which are in essence polycyclic aromatic molecules. They can be regarded as byproducts of food processing, which is essential for food safety and improvement of organoleptic properties of foods. Further studies showed that HAs are mainly formed in muscle foods (*i. e.*, meat and fish), which provide creatin(in)e in addition to other precursors, amino acids, and sugars or other aldehydes [3–5]. More than 25 HAs have been isolated from different food samples and their structures elucidated [6]. Like most chemical mutagens/carcinogens, HAs also form DNA adducts. Some of them have been demonstrated to exhibit strong mutagenicity based on the Ames *Salmonella*/mutagenicity test [7, 8]. Several of them, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethyl-imidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6–phenylimidazo-[4,5-*b*]pyridine (PhIP), 2-amino-9H-dipyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9H-dipyrido[2,3-*b*]indole (MeAαC), 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), and 2-amino-dipyrido[1,2-a:3′,2′-*d*]imidazole (Glu-P-2) have been classified by the International Agency for

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Abbreviations: AαC, 2-amino-9H-dipyrido[2,3-*b*]indole; **AIA,** aminoimidazo-azaarene; **AO,** antioxidant; **APCI,** atmospheric pressure chemical ionization; **Glu-P-2,** 2-amino-dipyrido[1,2-a:3′,2′-*d*]imidazole; **HA,** heterocyclic amine; **IQ,** 2-amino-3-methyl-imidazo[4,5-*f*]quinoline; **IQx,** 2-amino-3-methyl-imidazo[4,5-*f*]quinoxaline; **MeAaC,** 2-amino-3-methyl-9H-dipyrido[2,3-*b*]indole; **MeIQ,** 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, **MeIQx,** 2-amino-3,8-dimethyl-imidazo[4,5-*f*]quinoxaline; **PhIP,** 2-amino-1-methyl-6-phenyli-midazo[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; **TSI,** thermospray ionization

Research on Cancer as possible, and one of them, 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) as a probable human carcinogen. Moreover, the observation that Westernization of dietary habits in some populations has led to an increasing trend in the rates of certain cancers (colon, mammary glands, and prostate) [9] provides useful clues, at least to the major sources of dietary mutagen/carcinogen exposure from epidemiological point of views. Some epidemiological studies reported a positive correlation between dietary exposure and risk of certain cancers [10–13].

Although numerous reports have pointed out the existence of HAs at the ppb range in foods, the fact that these food-borne mutagens may be produced even during ordinary household cooking practices has raised global concerns as this implies frequent exposure by the general public [14, 15]. High temperature and prolonged heat treatment such as frying, grilling, and roasting are even more detrimental in the sense that HA formation is substantially enhanced in these processes [16–18]. Therefore, a general conception is that the total HA-induced cancer risk depends on the amount and type of muscle-based food in the diets, frequency of consumption, processing conditions, and proportion of physical and/or chemical modulators in the diets [19].

Over the past 25 years, extensive studies have been conducted which can be broadly classified into three categories. First is the chemical and analytical approach which mainly focuses on isolation, characterization, and quantification of HAs formed in model systems and foods [20, 21]. Second is the mechanistic approach which studies the kinetic and mechanisms of their formations [22, 23], possible mechanisms of mutagenesis/carcinogenesis [24], and possible mechanisms of antimutagenicity by chemicals or phytochemicals [25]. Third is the preventive approach aiming at developing strategies to inhibit their formation [26, 27]. These previous works have made great contribution to the database of food-derived HAs. The aim of this review is to give an overview of the chemistry of HAs, their implications in human health, factors influencing their formation, and feasible ways of suppression. Considering the fact that even in systems which attempt to boost the final concentrations of target HAs, they are still present in trace amounts, establishment of a robust and reliable analytical method remains the foremost important requirement, especially regarding the hindrance of the complex plethora of closely related and/or unrelated intermediates and end-products in the matrices that embrace the HAs. Therefore, this part will be addressed in greater details with major focuses on the most popular analytical tools applied. For more detailed information specifically on the occurrence of HAs, their formation, genotoxicity/carcinogenicity, and mechanisms of modulations by natural and synthetic compounds, please refer to some reviews given in ref. [3, 28, 29].
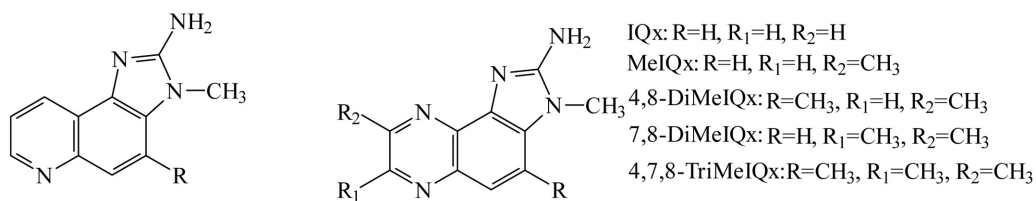
2 Chemistry of heterocyclic amines (HAs)

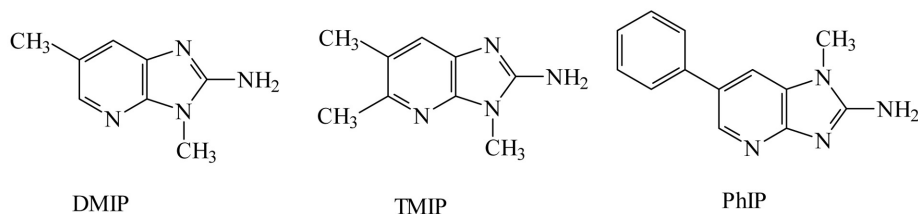### 2.1 Classification, structural features, and chemical properties

According to Miller [30], heating temperature of about 300°C is a critical boundary for formation of different classes of mutagens from proteinaceous food, such as meat and fish. Those formed above 300° are protein pyrolysates and characterized as 2-amino-pyridine-mutagens (or amino-carbolines) [31, 32], while those formed at moderate temperatures (below 300°C) are 2-amino-imidazole-type mutagens (or aminoimidazo-azaarenes (AIAs)) [3, 33]. Kataoka [34] further divided pyrolytic mutagens into five groups, pyridoindoles, pyridoimidazoles, phenylpyridines, tetraazafluoranthrene, and benzimidazole; and the AIAs into three groups, IQ, 2-amino-3-methyl-imidazo[4,5-*f*]quinoxaline (IQx), and imidazopyridine. Structures for 2-amino-pyridine and 2-amino-imidazole HAs and their corresponding subclasses can be found in a previous review by Kataoka [34]. Based on polarity (Figs. 1a and b), HAs can also be divided into polar which are mainly of the IQ- and IQx-type as well as the imidazopyridine type, and non-polar which have a common pyridoindole or dipyridoimidazole moiety [35].

All HAs have at least one aromatic and one heterocyclic structure which give them another name, heterocyclic aromatic amines. Most of them have an exocyclic amino group, except β-carbolines, harman, and norharman [3]. In the β-carbolines, it is the lack of such functional group that causes them to be nonmutagenic in the Ames/*Salmonella* mutagenicity test [36]. The amine groups or nitrogen atoms may have different pK_a values. This together with different positions and number of these ionizable moieties will, therefore, affect the behavior of HAs during chromatographic separation. As an example, Bianchi *et al.* [37] found that that PhIP and AαC were strongly retained *via* electrostatic interactions between deprotonated silanols and protonated HAs at higher pH (4.7), while at pH 2.8, late elution was due to their capability of having both N–H-acceptor and N–H-donor groups on the same part of the molecule to form hydrogen bonds with SiOH and SiO⁻ groups. The fact that most HAs are stronger based than the components of the mobile phase suggests that protonation to form [M + H]⁺ is their common mode of ionization using soft ionization techniques [38].
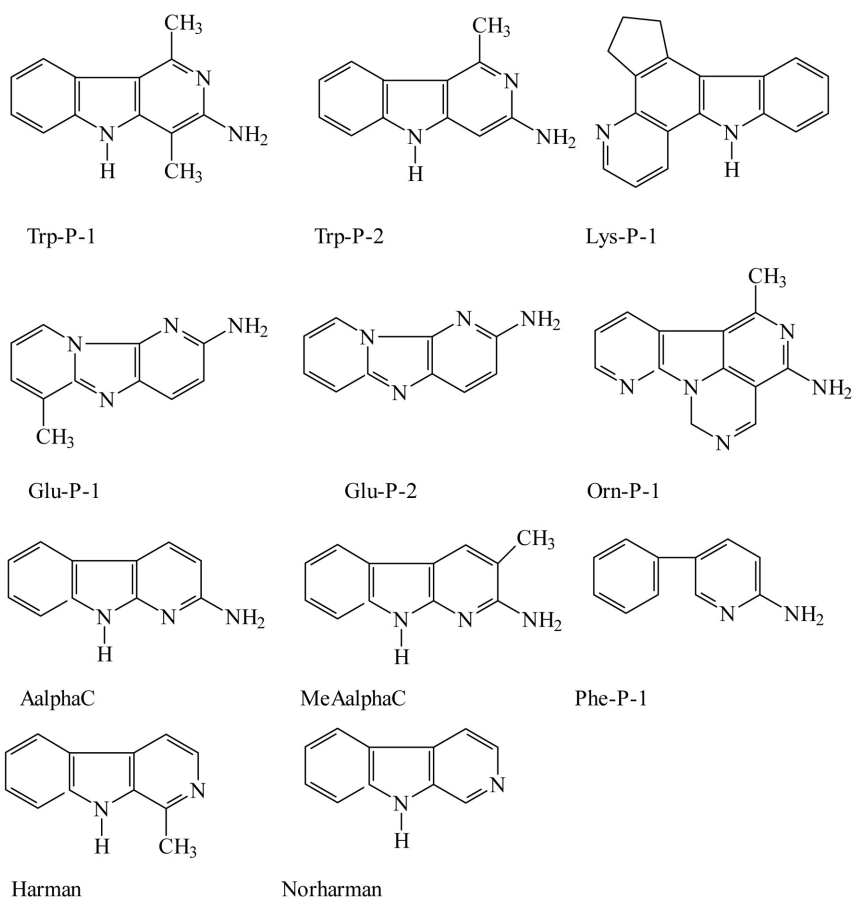
For the amino-carbolines, the five-membered heterocyclic aromatic ring is sandwiched between two six-membered aromatic rings, one or both of which can be pyridine, in contrast to AIAs. All AIAs have an N-attached methyl group at the imidazole ring of the molecule, whereas for amino-carbolines, they either lack a methyl group or the methyl group is attached to a six-membered ring. Different

IQ: R=H
MeIQ: R=CH₃

IQx: R=H, R₁=H, R₂=H
MeIQx: R=H, R₁=H, R₂=CH₃
4,8-DiMeIQx: R=CH₃, R₁=H, R₂=CH₃
7,8-DiMeIQx: R=H, R₁=CH₃, R₂=CH₃
4,7,8-TriMeIQx: R=CH₃, R₁=CH₃, R₂=CH₃

DMIP   TMIP   PhIP

1a.

Trp-P-1   Trp-P-2   Lys-P-1

Glu-P-1   Glu-P-2   Orn-P-1

AalphaC   MeAalphaC   Phe-P-1

Harman   Norharman   **1**

1b.

Figure 1. (a) and (b) Structures of polar and nonpolar HAs, respectively (adapted from [35]).

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number and positions of methyl groups largely increase the number of this class of mutagens and it is, therefore, likely that new mutagens will be identified [24, 39, 40].

2.2 Formation of HAs

2.2.1 Amino-carbolines

Major precursors of amino-carbolines are amino acids or proteins and as their formation are not dependent on cretin(in)e as a precursor, they may be produced in foods of both animal and plant origins [3]. However, as addition of excess creatinine to a model system was found to increase harman and norharman formation [3], it is probable that creatinine may serve as an extra source of structural fragments. The most popular hypothesis for the formation of amino-carbolines under such drastic thermal environment has been a pathway *via* free radical reactions, which produce many reactive fragments [41]. These fragments may then condense to form new structures [41]. However, due to their much lower occurrence in normally cooked foods and greater difficulty in manipulation of the high temperatures required in experimental set up, relatively little investigation has been carried out to verify the above hypothesis compared with the AIAs.

2.2.2 Aminoimidazole-azaarenes

AIAs have been found to be responsible for most of the mutagenic activity in cooked foods, especially in Western diets [24]. IQ- and IQx-types HAs and their derivatives (methylated forms) are suggested to be formed from creatin(in)e, sugars, free amino acids, and some dipeptides through Maillard reaction and Strecker degradation upon heating [4, 42] (Fig. 2). Weisburger [43] found that addition

of extra creatinine to the surface of meat prior to frying increased the yield of HAs, indicating the important role of creatinine. On the other hand, as several amino acids may give rise to a common HA, though likely with different yields, no definite amino acid precursor-HA relationship has been drawn. Findings of studies intending to identify the limiting factor have also been inconsistent. Some studies suggested sugar as a limiting factor based on the observation that glucose concentration decreased to undetectable level after only 2.5 min of heating, while levels of amino acids and creatinine, though decreased quickly at the very beginning, they then leveled off [23, 44]. Other studies found that HAs can also be formed in dry-heated mixtures of amino acids and creatine [7, 45]. It appears that HA formation proceeds *via* different reaction kinetics under the above different sets of parameters whose variation has strong influence on the rate limiting step.

It was postulated and later demonstrated that creatine formed the amino-imidazo part of IQ and IQx by cyclization and water elimination, while the Strecker degradation products such as pyridines or pyrazines formed in the Maillard reaction between amino acids and hexose contributed to the remaining part of the molecule, probably *via* aldol condensation [3, 46] (Fig. 1). This reaction is especially favored at temperatures above 100°C [16]. However, yield of pyrazines and pyridines from the Maillard reaction and Strecker degradation is still low and Arvidsson *et al.* [23] proposed this as a causal factor for low yield of HA in foods or model systems. Despite evident involvement of the Maillard reaction, a precious study has shown suppression of IQ compound formation in model systems by addition of preformed Maillard reaction products [47]. The underlying principle has not been elucidated.
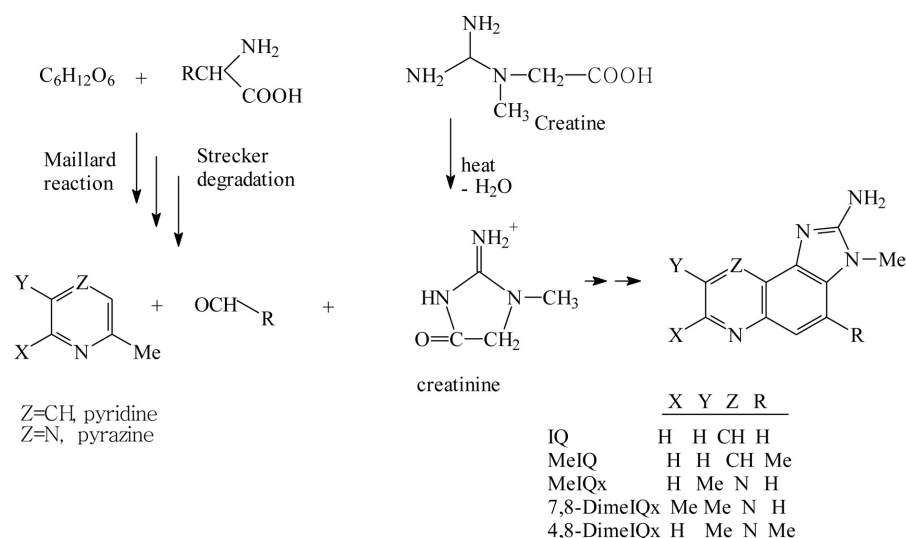

	X	Y	Z	R
IQ	H	H	CH	H
MeIQ	H	H	CH	Me
MeIQx	H	Me	N	H
7,8-DimeIQx	Me	Me	N	H
4,8-DimeIQx	H	Me	N	Me

Figure 2. Suggested pathway for the formation of imidazo-quinolines and imidazo-quinoxalines (adapted from [3]).
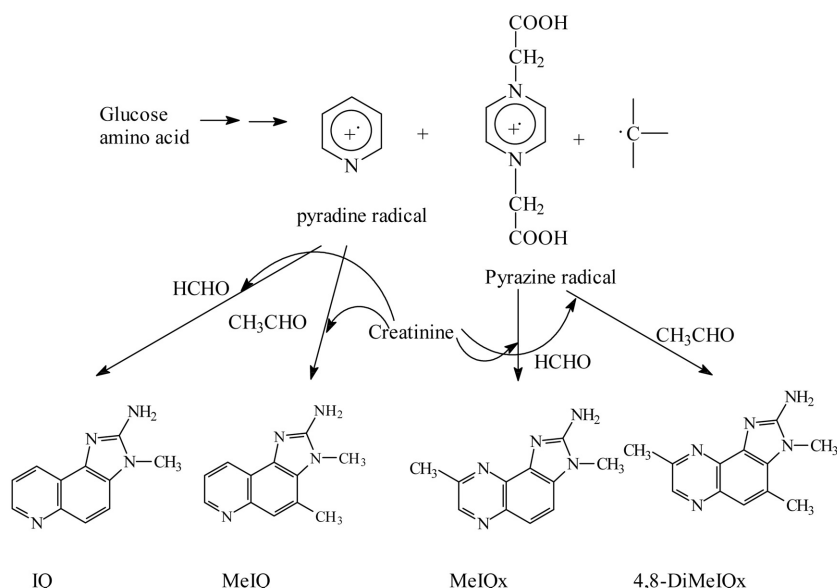
Figure 3. Suggested pathway for the formation of imidazo-quinolines and imidazo-quinoxalines (adapted from [22, 26]).

Free radical mediated pathway has also been proposed. Hayashi and Namiki [48] showed that, prior to Amadori rearrangement, free radicals may be formed *via* fragmentation reactions. Further studies suggested the involvement of pyridine and pyrazine radicals [22, 49] (Fig. 3).

2.3 Model systems for studying HA formation in foods

In complex meat matrices, there may be a large number of concurrent reactions that would make the investigation of those reactions in relation to HA formation difficult [50]. With the development of model systems, which simulate some or most aspects of the target food samples, the data obtained have been shown to agree between chemical- and meat-based systems [23, 51], and more importantly with realistic cooking in terms of yield and pattern of HAs formed [52, 53]. Moreover, modeling experiments can be used to study the influence of physical and chemical parameters and precursors on HA formation [54]. Therefore, they are important in the development of methods for preventing or inhibiting HA formation in foods [54].

Most studies using model systems have been performed in the temperature range of 125–300°C [55]. In model systems, the samples are usually suspended in diethylene glycol to promote heat transfer [56] and contained in sealed tubes, most commonly stainless test tubes, quartz test tubes, or glass vials. Uniform and effective heat transfer through the tube walls is achieved using heating block with oil-filled cavity or using oil bath [23, 44]. Temperature of the set up is well controlled with a regulator equipped with thermo-

couple and samples are collected at specific time points for further analysis [23].

2.3.1 Model system using mixtures of pure precursor compounds

In these systems, major precursors such as creatinine, reducing sugars, and amino acids are incorporated in proportions similar to those found in target food systems, but usually at much higher concentrations to facilitate detection and analysis [23]. It has been suggested to have pilot studies to determine whether such systems are capable of forming HAs [23]. Due to the simpler and defined compositions of these types of systems, they are particularly suitable for evaluating the kinetic of HA formation [23], and for providing preliminary data on the effects of various food components and additives [44, 53, 57]. Most of these studies involved addition of the purported modulators at the beginning of the heating process. PhIP, some imidazoquinolines, and imidazoquinoxalines have been the major HAs investigated [44, 53, 57, 58].

2.3.2 Model systems using meat juice

Although the systems were composed only of the few major precursors, they have been suggested to be satisfactory surrogates for studying the formation of certain HAs in meats [53]. A more complete profile of various food components should be combined to give a better reflection as these components interact, resulting in enhancing or inhibiting effects on HA formation. In this regard, meat juice provides a convenient and economic, yet near complete medium for evaluating formation of the full set of HAs in the corresponding

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meat samples. Arvidsson *et al.* [59] have developed an efficient method for extracting juices from meats and these can be freeze-dried and stored in freezers until used for analysis [60]. With different preparation paradigms, HA formation under different heating conditions can be analyzed. To mimic wet heating, freeze-dried juice is mixed thoroughly with distilled water to obtain a concentrated mixture [41]. Alternatively, distilled water can be added to the sample chamber before heating [60]. For dry heating, freeze-dried meat juice is heated in test tubes without being mixed with water [60]. Depending on whether the test tubes are sealed or open during the heating process, such model system can also be applied to simulate HA formation under open or closed conditions [41].

2.3.3 Model systems using homogenized freeze-dried fresh meats

Perhaps the best surrogate for investigating HA formation during realistic cooking of meats and fish is samples of the target food which are processed into a uniform shape and size. However, it has been realized that for these systems, parameters such as heat and mass transfer, vaporization of water, and crust formation would be difficult to control during heating [41]. To facilitate control of these parameters, Messner and Murkovic [56] have proposed the use of homogenized freeze-dried fresh meat to achieve uniform chemical composition throughout the experiment. As an example of imitating frying of meat, homogenized freeze-dried samples can be heated with diethylene glycol in vials using thermostatically controlled heating block equipped with magnetic stirring [56]. Effects of food additives such as antioxidants (AOs) can also be studied by adding them to the vials together with the meat samples before heating [56].

2.4 Quantitative and qualitative analysis of HAs

2.4.1 General rundown for quantitative and qualitative analysis

For preliminary studies and for facilitating measurement, levels of HAs formed in model systems can be boosted. This can be achieved by using multiple concentrations of precursors. However, the amount of HAs formed may still be in the nanogram *per* gram order. Therefore, efficient and robust analytic techniques are essential, especially when analyzing complex real food matrices which contain lots of interfering substances, which may compromise detection limits and complicate spectral interpretation. Prior to quantitative and/or qualitative analysis, liquid–liquid extraction (LLE) [61, 62], or solid phase extraction (SPE) [20, 21] has been regarded as a critical step, both for cleaning and for concentration of target chemical species. SPE has been much more widely practiced due to its convenience, efficiency of purification, and greatly reduced solvent require-

ment. Moreover, it is a nonequilibrium process, in contrast to LLE, whose extraction depends on an equilibrium established among the extraction solvents applied, and thus it is hard to predict when complete extraction has been reached for the latter [63]. The only important limitation may be the requirement for the sample to be manipulated in a liquid form [63]. The nature and variability of the heating processes during food preparation together with the complex and diverse food matrices render sample preparation an ever challenging task. Therefore, improvement in extraction methods is equally important as optimization of the subsequent separation (chromatographic) and detection techniques.

LC has been the most popular chromatographic technique for separation of HAs following extraction/purification and reconstitution in compatible solvents. Moreover, SPE-LC has been compared and validated through interlaboratory studies [64]. Subsequent identification of known compounds can be achieved by coupling LC to UV photodiode array-detector [54, 65, 66], in addition to confirmation by their retention time. Quantification of fluorescence HAs (nonpolar HAs and PhIP) can be achieved by simultaneous programmable fluorescence detection, which has been found to be 100–400 times more sensitive than UV detection [67, 68]. These detectors are satisfactory for samples taken from simple chemical model systems or those subject to low to moderate heat treatments. With applications of electrochemical detectors (ECD) [69] and mass spectrometers [70], the challenge of ultracomplicated spectra arising from complex sample matrices has largely been overcome due to their high selectivity and sensitivity. However, EC detection is limited by the lack of on-line peak confirmation [71]. Kataoka [34] pointed out that simultaneous analysis of all HAs in one run is difficult using ECD as gradient elution cannot be applied in the high sensitivity range. Moreover, LC-ECD was also found to be less sensitive than LC-MS [64]. Applications of LC-ECD, LC-UV, and LC-fluorescence detections in HA analyses were reviewed by Pais and Knize [15]. Highly specific detectors based on immunosorbent or immunoaffinity assays have also been developed [72, 73]. However, these have been restricted to analyses of HAs for which antibodies are available [20]. GC has also been used for separation of HAs. Despite the requirement of a derivatization step, its simplicity, separation efficiency, and sensitivity and specificity when coupled to MS have granted it another valuable analytical tool for HAs, especially in laboratories where more sophisticated techniques like LC-MS/MS are not accessible [74]. For unknown mutagens, identification has to be based on NMR and mass spectroscopy spectra and proof of the proposed structures by chemical synthesis. Chemical synthesis also increases the possibility of obtaining sufficient amounts of certain mutagens for biological or biochemical testing [24].

These analyses, together with data obtained from various *in vitro* and *in vivo* assays on the mutagenicity and carcinogenicity of HAs, are important not only for estimating dietary intakes and thus human exposure, but also valuable for mechanistic studies aiming to explore their mechanisms of formation and/or plausible inhibitory strategies [15].

2.4.2 Sample preparation and SPE
2.4.2.1 Sample preparation

SPE applied for analysis of HAs is a gross cleaning process which serves to selectively concentrate target analytes from a complex mixture [75]. This offers enhanced sensitivity which may facilitate subsequent detection [63]. Most procedures have been based on those developed by Gross [21] and Gross and coworkers [20, 65]. For simple model systems that incorporate chemical precursors with molar ratios similar to target food products, several solvent systems such as diethylene glycol (containing varying proportion of water) [26, 58], dilute hydrochloric acid (~pH 4.5) [23], water [44], and phosphate buffer [76] have been commonly employed. For SPE using blue cotton, rayon, or chitin, the model system mixture usually requires dilution before being treated with the rayon [58, 77]. A comparison of these three types of sorbents for analyses of aromatic compounds was detailed in a review by Skog [78]. With Oasis MCX cartridge, an aliquot of the model system is usually dissolved in 0.1 M HCl before spiking with authentic standards [50, 57]. With diatomaceous earth followed by PRS and then C_{18} cartridge, an aliquot is commonly dissolved in 5 N NaOH [44].

Model systems that use real meats or meat juices require more sample preparation work. Homogenization in sodium hydroxide solution is commonly used in studies that chose diatomaceous earth as the medium for preliminary cleaning [54, 79]. For studies that used blue cotton to absorb HAs from the food matrices, homogenization is often carried out in HCl [80]. This is followed by mixing with trichloroacetic acid and centrifugation to remove protein. Protein binding or failure to release the analyte into the liquid part will greatly affect the analyte's adsorption properties and its recovery in SPE [81]. Analysis of apolar HAs requires neutralization of the supernatant [80], whereas that of polar ones requires alkalinization (to pH ~ 9) [77] before purification by blue cotton treatment.

2.4.2.2 SPE for HAs

Most SPE of HAs use a combination of a strong cationic exchange cartridge (PRS) and an RP (C_{18}) cartridge. Conditioning of the cartridge prior to sample application is important for improving analyte recovery, and it is recommended to use the cartridge as soon as possible [82, 83]. This is followed with sample application, cartridge washing, cartridge drying, and elution. Cartridge drying is particularly impor-

tant if GC is used for the final analysis as too much water in the extract may damage the injection liner and columns, or disrupt the derivatization step [74, 83, 84]. Several SPE clean-up procedures based on lyophilized meat extracts were recently evaluated by Toribio *et al.* [85]. Toribio *et al.*'s [86] comprehensive study in 2000 also provides valuable information about the suitability of different commercial brands and structures of sorbents (PRS and C_{18} cartridges), in terms of simplicity of operation and recoveries of analytes from a lyophilized beef extract.

Although another SPE method (employing the so-called LiChrolut EN material), which enables very quick determination of trace HAs in meat extracts with reduced requirement for some toxic organic solvents such as methylene chloride, has also been applied [87], the recoveries for nonpolar HAs, harman and norharman were poor (20–30%). The major advantage lies in its tremendously high surface area (made of stryrene divinyl benzene polymers) [88]. Therefore, this type of sorbents may be most suitable for routine screening of food samples and the traditional SPE protocol, which enables isolation of both polar and nonpolar HAs with satisfactory recoveries is preferred for studies aiming to investigate the effects of external intervention during cooking on mutagen production. Automated/robotic sample extraction is still in its infancy and its application for analyzing complex food samples raises several major considerations: possibility of clogging during period of unsupervision; robustness and reliability of the delivery system; and parameter setting for the pressure or vacuum system [89]. The much well-established methodological and experimental foundation of the manual version will very likely remain an important driving force for maintaining its popularity in the near future.

Recoveries of target analytes should always be checked to ensure the effectiveness and reliability of the available extraction and cleaning process. The usual practice is by spiking the sample with specific amounts of known HA standards at the beginning, and allowing the spiked and unspiked samples to go through the same processes [66]. Concentration of the spiked samples should be sufficiently high to allow estimation of recoveries even in the presence of interfering peaks, yet should not be so high that it exceeds the capacity of the sorbent [82]. An alternative is by adding a known quantity of isotopically labeled standards to samples prior to spectroscopic analysis, and comparing the response to samples where standards are added before the extraction process [40].

2.4.3 LC-MS and GC-MS

Over the past 30 years, LC-UV has undoubtedly been the most commonly coupled technique for the analysis of HAs formed in foods. In recent years, hyphenation to MS has been proven to perform incomparable functions, especially

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when characterizing complex samples. As HAs are stable during the ionization process, the protonated molecular ion peak may thus serve as the marker in mass selective detection of HAs [79]. However, if conditions are set to allow fragmentation, subsequent fragmentation patterns are different between AIAs and carbolines [90]. For AIAs, major fragmentation occurs at the aminoimidazole moiety with loss of the methyl group (dominant), C_2NH_3 and CN_2H_2 groups, and further fragmentation occurs at heterocyclic rings with losses of HCN and CH_3CN; for carbolines, the most important fragmentation occurs with loss of ammonia, except for harman and norharman, which may lose a methyl group for methylated carbolines or lose diverse fragments from the heterocyclic rings [90].

Coupling of LC and GC to MS enables satisfactory separation and detection with minute starting materials; and LC-MS and GC-MS have been principal analytical techniques for HAs. Contribution of MS and applications of LC-MS and GC-MS for analysis of HAs have been reviewed [15, 34, 91]. A summary of some liquid chromatographic techniques coupled to various detectors for the analysis of HAs in test solution and in meat extracts, can also be found from an interlaboratory study conducted in 2004 [64]. The intent of this part of the review is to present an overview and add some updated information.

2.4.3.1 LC-MS

LC-MS, by virtue of its elegant combination of LC's gentle separation and high sensitivity and selectivity of MS, is suggested as the most suitable technique for the analysis of multiple HAs, especially in complex food matrices [34, 64]. The latter also helps reduce laborious sample preparation steps required. Superior selectivity can be achieved with LC-MS/MS, which has also been shown to be most satisfactory for analyzing samples containing trace amount of HAs even in interlaboratory studies [64].

Different types of RP columns are available, but many laboratories now use ODS column as it gives the most satisfactory values of peak height, peak symmetry, and requires shorter equilibration time [64, 66, 92]. Various types of interactions like hydrophobic interaction, electrostatic interaction, and hydrogen bonding determine the chromatographic behavior of the solutes, whose relative retention, therefore, depends not only on their structural features, but also on their chemical environment. For coupling to MS, it is required that the solvents used for LC are volatile [15]. Ternary and binary mobile phase under gradient elution have both been applied. An example of the gradient program with a ternary mobile phase is performed with solvent A (water with 10 mM ammonium acetate at pH 3.2), solvent B (water with 10 mM ammonium acetate at pH 4), and solvent C (ACN) [93]. Solvent A and solvent B can also be ammonium formate at similar pH values [90]. Binary gradi-

ent elution program has been more popular: (1) pH buffer as solvent A and ACN as solvent B [38, 92]; (2) methanol/ACN/water/acetic acid in different proportions as solvent A and ACN as solvent B [56]. If the interface to MS is an electrospray type which requires analytes to be in aqueous ionic states, it is important that the pH of the mobile phase should be kept lower than pKa values of the target HAs (<pH 5) in order to protonate their amino group [92].

Only scarce attention has been paid to the effects of mobile phase composition and conditions on LC of HAs and their subsequent detection by MS [37]. It is suggested that addition of an ion-pairing agent may increase the affinity of a nonpolar sorbent for analytes that ionize in solution [81]. Presumably, the buffer anions will form ion pair with HAs. Bianchi *et al.* [37] recently compared the effectiveness of formate and acetate in achieving better separation by RPLC and detection by MS, or MS/MS for seven HAs, IQ, MeIQ, MeIQx, PhIP, AαC, harman, and norharman from different subclasses. They found that analytes usually exhibited better peak shape when using formate as the pairing ion reagent. pH of the mobile phase also has important effects on solute retention. However, optimal elution does not occur at the same pH under isocratic and gradient conditions. For the former, Bianchi *et al.* [37] showed that pH higher than 3.7 produced broad peaks (especially for IQ compounds) probably due to strong interaction with residual silanols of the stationary phase, while decreasing pH from 4.7 to 2.8 reduced retention of the HAs with improved peak shape which helped improve sensitivity of detection with MS. Using gradient elution, Barceló-Barrachina *et al.* [92] determined it to be at pH 4. One probable explanation is that in Barceló-Barrachina and coworkers' study, the optimal pH was for a much larger number of HAs (including the seven HAs in Bianchi and coworkers' study) which represented a much larger number of amine groups or nitrogen atoms with more diverse pK_a values, and thus electrostatic and hydrophobic interaction behavior. Another factor of concern is the concentration of ACN and buffering solution used. It was found that retention of HAs correlated negatively with increasing percentage of ACN, while the effect of formate concentration varied for different HAs [37]. Moreover, high ammonium ion concentration (from ammonium formate) may reduce the effective surface area of the stationary phase owing to competition with the analytes for binding to the stationary phase [37]. Therefore, depending on the specific purpose and design of the experiment, fine adjustments for pH and mobile phase concentration are always recommended to achieve optimal separation of HAs.

To produce a clearer spectrum, it may be beneficial to pass the standards and samples through an appropriate filter (*e.g.*, 0.45 µm) before introducing into the LC-MS system [94]. It is important to ensure that the internal standard chosen is not present in the target sample. 2-Amino-3,4,7,8-te-

tramethyl-imidazo[4,5-*f*]quinoxaline (TriMeIQx) has been the most common choice of internal standard which is added to the purified sample before being injected into the LC-MS system [56, 64]. Some studies also used isotopically labeled internal standards, typically with $^2H_3$, the so-called isotope dilution technique [79]. Although the latter can improve quantitative analysis, only few isotope-labeled analogues are commercially available [64].

Different types of soft ionization interfaces have been developed for coupling LC to MS in the analysis of HAs: thermospray ionization (TSI), atmospheric pressure chemical ionization (APCI), and ESI. Depending on the purpose of LC-MS analysis, data acquisition can be performed in full scan mode or selected/single ion monitoring mode. For those samples of low concentration levels, selectivity can be enhanced using MS/MS whose sensitivity can be improved with selected daughter ion scan mode instead of full scan mode [92]. Quadrupole and ion trap (IT) have been the most popular mass analyzers for LC-MS of HAs [79, 95, 96]. For quantitative purpose, peak area is suggested to be a better parameter [64]. However, mass accuracy (>0.1 Da) of these conventional mass analyzers may not be high enough for unequivocal differentiation between ions that have the same nominal mass but different elemental compositions. Recently, quadruple TOF (Q-TOF) instrument, which enables data acquisition with much higher mass accuracy (<2 mDa) has also been applied in both V-Optics™ and W-Optics™ configurations [94]. The former can achieve better sensitivity and lower detection limits, while the latter much higher mass resolution (approximately two times the former), thus valuable for quantitation and studying fragmentation of HAs, respectively [94].

Early studies used TSI which was able to accomplish low detection limit with small amounts of samples from fish, beef (0.3 ng/g), and tryptophan pyrolysates (0.1 ng/g) [34, 70, 97, 98]. Kataoka [34] pointed out that LC-MS with this interface was restricted to the detection of a single HA. In recent years, other soft ionization techniques, ESI and APCI, have largely replaced TSI in the analysis of HAs, with the former being most popular. ESI imparts charge to sample molecules by spraying the sample solution across a high potential while APCI uses a corona discharge [99]. The former is suitable for analyzing more polar HAs while the latter is suitable for less polar ones, so they may be considered complementary in the analysis of HAs, although APCI may lead to a high degree of fragmentation compared to ESI [99, 100]. A comparison of the dominant ions formed form 14 HAs at different extraction voltages (30 and 70 V) can be found in the study of Pais *et al.* [100]. ESI produces lots of protonated molecular ion $[M + H]^+$ which forms the base peak in the spectrum of the HA of interest. Using LC-ESI-MS, 15 HAs were simultaneously measured and their identity confirmed [79]. In the past, ESI-MS was

limited to low flow rates which necessitated the use of microbore or semimicrobore columns for LC separation [15]. With the application of pneumatically assisted ES interface (usually by directing heated nitrogen into the capillary region), higher flow rates can be used [15, 38].

2.4.3.2 GC-MS

GC-MS is a powerful, yet inexpensive tool for the analysis of volatile compounds. Separation takes place in capillary column which offers high separation efficiency. Both low and high resolution GC-MS have been developed and applied for HA analysis in different types of samples ranging from standard HA solutions, to food matrices, to cooking fumes, or process flavors [50, 101–104]. However, as most HAs are nonvolatile and many are polar, gas chromatographic separation in their native form will result in broad and tailing peaks due to strong absorption in the injector and the GC column [15]. Although direct determination without prior derivatization is possible in the presence of isotopically labeled internal standards, appropriate standards may not always be available [15, 40, 105]. Therefore, derivatization has been the most popular solution for GC analysis of these HAs. Derivatization may imply extra sample preparation; nevertheless, this step actually offers multiple advantages, improved selectivity, sensitivity, and separation, in addition to reduced polarity and improved volatility [34, 106].

Many derivatizing agents have been tested for the conversion of HAs into their volatile derivatives and some of these have been reviewed [15, 34]. Derivatization reactions involved can be broadly divided into four types: alkylation, acylation, condensation to the Schiff base, and halogenation [34, 105]. The third type has been most popular for analysis of HAs from real food samples [74, 105]. Common alkylating agents used are halides of benzyl or benzoyls such as 3,5-bistrifluoromethylbenzoyl chloride, 3,5-bistrifluoromethylbenzylbromide, and pentafluorobenzyl bromide which produce HA derivatives with much better GC properties than those derived from acylating agents such as acetic, trifluoroacetic anhydride, and heptafluorobutyric anhydride [34, 104]. While acylated HAs have been criticized of having acidic property, alkylating derivatives are usually in a mixture of mono- and di-alkylated forms and alkylation is efficient only for a limited number of HAs [74]. The application of Schiff base-type reaction (based on the condensation between a primary amine and a carbonyl compound) for derivatization of HAs was developed by Kataoka and Kijima [107] who used DMF dimethyl acetal (DMF-DMA) as the derivatization agent, giving rise to *N*,*N*-dimethylaminomethylene derivatives of HAs. However, this reagent may also react with the carboxyl groups of fatty acids and amino acids in food samples to form esters [106]. This further emphasizes the importance of the extraction and

cleaning steps. Apart from DMF-DMA, other dimethyl for-mamide dialkyl acetals (DMF-diethylacetal, DMF-diiso-propylacetal, DMF-di-*tert*-butylacetal) have also been tested and it was found that derivatization with DMF-di-*tert*-butylacetal provided the best yield, and thus highest sensitivity in mass spectrometric determination [74]. Although it was previously suggested to add excess deriva-tization reagent (3,5–bistrifluoromethylbenzoyl bromide) for obtaining a maximum yield [104], Barceló-Barrachina *et al.*'s study [74] obtained an optimal reagent volume with respect to a given mass of HA. Optimal derivatization tem-perature and time were also determined in this study. Deri-vatization to the Schiff-type base also works well with a nitrogen-phosphorus selective detector, and detection limits of 2–15 pg *per* injection have been reported [107]. In spite of some limitations with acylation and Schiff-base type condensation, both processes are fast, simple, economical (low reagent requirement), and form stable derivatives (for 6 months at 4°C) [105]. Halogenation is a relatively new derivatization method for HAs. To the best of our knowl-edge, only iodine has been tested [105]. However, results have been unsatisfactory for this multistage derivatization method, and further tests are required before its actual application in analyses of food samples [105].

Positive ion electron ionization (EI) and negative ion che-mical ionization (CI) are popular for ionizing derivatized HAs. Only few negative ion EI analyses have been reported [108]. EI produces excellent fragmentation patterns under the given ion source temperature and bombarding electron energy [15]. As HAs are all aromatic compounds, ioniza-tion with EI may be able to retain their molecular ion peak in their mass spectra in addition to providing information about their characteristic fragmentation patterns [109]. Using GC-EI-MS, five AIAs previously determined with HPLC from ten meat samples were qualitatively confirmed based on their retention time and mass spectra (as PFPA derivatives) [84]. However, GC-MS interpretation of PhIP was found to be problematic in the study and they attributed this to the very low yield of the PhIP derivatization reaction and to the presence of coeluted interfering substances. CI is a softer technique without causing much fragmentation, and the molecular ion peak is usually the dominant one in the mass spectra [109]. Negative ion CI (NICI) is highly sensitive and selective to electron capture compounds and with this method, Murray and coworkers obtained mass spectra which contained intense, high mass ions, facilitating SIM with detection limits of 0.05–0.2 ng/g for high-tem-perature-cooked meats [15, 101]. Similar detection limits (0.03–0.2 ng/g) was also obtained for grilled chicken [103]. Nevertheless, NICI has poor reproducibility of the meas-urements, which forms the bottleneck to its wide adoption in this application.

Data acquisition in GC-MS can be in total ion scanning mode or in SIM mode. For the latter, only the base peaks are chosen to allow maximum sensitivity [34]. Apart from ser-ving as a qualitative confirmation tool, GC-MS is also very effective for quantification of HAs, usually with isotope-labeled internal standard. Such isotope-dilution analysis is based on the fact that isotope-labeled analogues have differ-ent mass/charge ratio from target HAs, thus enables calcu-lation of the extraction efficiency in the same analysis and reduces number of samples to be extracted [15].

Concerning optimization of the GC-MS system for the analysis of HAs, most studies have focused on the derivati-zation process and no efforts have been directed on the elu-tion programme which is also a critical stage. In fact, it was previously reported that with HP Ultra 1 column, MeIQ and MeIQx could not be resolved due to their similar retention times [105].

3 HAs and human health

HAs have been recognized as an important class of muta-gens associating predominantly with thermally processed foods. Over the past 25 years, a lot of studies have been car-ried out which contribute to our better understanding of their relationship with human health. HAs have been found, after metabolic activation, to be capable of forming DNA adducts which may be important in exerting their muta-genic/carcinogenic effects [110, 111]. In addition to their *in vitro* mutagenic activity, which in the Ames test was reported to be 100 to 100 000 times higher than the two most well-known classes of food toxicants during the 1960s and 1970s, polycyclic aromatic hydrocarbons (PAHs) and *N*-nitrosocompounds (NOCs) [2, 111, 112], 10 of them have been shown to be carcinogenic in mice models [113, 114]. IQ, an amino-imidazo araarene, has even demon-strated carcinogenic activity in monkeys [115]. It is not the intention of this part of the review to address in details the potential toxic effects of HAs. Instead, an overview from their bioavailability to their probable terminal impacts on human health in the long term will be given.

3.1 Bioavailability and bioactivation

It is generally accepted that in order for a toxicant to exert its toxicity, the prime factor is its ability to overcome biolo-gical barrier(s) such as the gastrointestinal barrier and blood-tissue barrier (for those with target-specific effects). Information related to the potential mutagenic and carcino-genic effects of HAs is contained in ample literature. Quite extensive research efforts have also been stimulated to esti-mate the so-called relative cancer risk index. However, rela-tively scarce attention has been paid to investigate the bio-availability of this group of food-borne mutagens, which presumably being variable for different HAs, may signifi-

cantly influence their ultimate effective concentrations in target physiological sites.

While it is unfeasible to undergo real-time monitoring of digestion processes in human subjects, Kulp *et al.* [116] developed a surrogate *in vitro* model which mimicked the three major digestion phases (in the mouth, stomach, and intestine) responsible for releasing HAs from meat matrices, and it was found that even under optimal conditions, accessibility of HAs could never achieve the level from conventional laboratory analysis of undigested cooked meat, which involves physical grinding of the meat to a liquid. This, together with the consideration that the absorption process across intestinal walls (bioavailability) was not simulated, raised doubt about the reliability of using laboratory analysis of HA content in epidemiological estimation of the biologically effective dose [116].

Most HAs are not mutagenic/carcinogenic in their native form but acquire the capability of forming DNA adducts after metabolic activation [117–119]. The major activation pathway of HAs involves phase I *N*-hydroxylation followed by phase II esterification, both of which take place at the exocyclic amino group. For example, while treatment *in vitro* (45 µM, 1 h, 37°C) of isolated rat mammary epithelial cells with *N*-hydroxy-PhIP produced PhIP-DNA adducts in cell DNA, no adducts were detected for incubation using bare PhIP, even at nearly five-fold dosage and with much longer incubation [120]. In cynomolgus monkeys given MeIQx, a correlation between a low DNA-adduct level and low capacity of hepatic phase *N*-hydroxylation was observed [121]. For some HAs, phase II activation may also be critical stemming from many previous studies which reported multifold enhancements in DNA-adduct formation compared with that achieved using phase I enzyme(s) alone [122–125]. Metabolism of some HAs appears to be extensive as it was observed that radiolabeled HAs were rapidly eliminated with only a small amount excreted unchanged in urine [126–128]. In contrast, it was reported that for PhIP, wide interindividual differences were observed for the proportion of ingested dose being excreted as urinary metabolites, and this could be brought about by factors such as absorption or metabolic rates in addition to level of exposure [129]. Such comparison between the amounts of metabolites in urine and the original amounts ingested has also been the usual approach for assessing HA bioavailability in humans [116].

Substantial progress has been made in elucidating the metabolic pathways for both AIAs and carbolines as well as in the structural characterization of their metabolites [119, 130–135]. Numerous studies converge on the recognition of cytochrome P450 1A2 as the most important phase I oxygenase for conversion of these aromatic amines to their corresponding hydroxylamines [136–139]. However, contribution by other cytochromes P450 should by no means be

neglected when assessing the total risk [119, 132, 137, 140–146]. Four mammalian phase II enzymes have been identified, *N*-acetyltransferase (NAT), sulfotransferase, prolyl tRNA synthetase, and phosphorylase which produce *N*-acetoxy, *N*-sulfonyloxy, *N*-prolyloxy, and *N*-phosphatyl ester derivatives, respectively [117, 120, 122, 130, 138, 147, 148]. Among these, NAT, expressed both in rodents and humans (predominantly in the liver), appears to play a dominant role, at least in phase II bioactivation of IQ, MeIQx, and PhIP [120, 149]. It was suggested that rapid acetylator individuals may be more susceptible to HA toxicity [149]. Studies taking the phenotype of both phase I and phase II enzymes into consideration further suggested that individuals with both highly active NAT (NAT2) and CYP 1A2 may be predisposed with earlier onset of colon cancer than the general population (odd ratio = 2.86) [149, 150]. Over 50 variant alleles have been identified for NAT (NAT1 and NAT2), and this implies the existence of a large number of phenotypes and probably widely varied susceptibility in terms of HA-induced carcinogenecity [151]. Esterification reactions catalyzed by these four enzymes generate momentary metabolites whose ester moieties are better leaving groups, thus greatly favor the formation of arylnitrenium ions (the generally considered principal carcinogenic form of HAs) compared with their corresponding hydroxylamines [147, 152]. Although the liver is the largest organ of HA metabolism, bioactivated metabolites of HAs and/or their resultant DNA-adducts have also been reported in a number of extrahepatic tissues such as the lung, kidney, mammary gland, colon, and pancreas [131, 153–157]. It was suggested that migration *via* bloodstream, *in situ* bioactivation in extrahepatic tissues, and the presence of alternative enzymatic systems [147] such as prostaglandin H-synthetase [125, 158] and peroxygenase [159] could contribute to their detection in these tissues [147].

Until recently, there seems no doubt that mutagenic/carcinogenic potency of HAs could be enhanced if the diet also contains inducer(s) of the aforementioned metabolic enzymes.

Latest findings appear to support the existence of much more complex interacting/counteracting factors. This is best illustrated with caffeine, a known CYP 1A2 inducer in rats [160], and it was reported that incorporation of caffeine into a diet rich in MeIQx did not have significant promotion effects on MeIQx-induced hepatocarcinogenesis [161]. Further mechanistic investigation led to their proposition that up-regulation of CYP 1A2 and NAT2 by caffeine may be counter-acted by suppression of Sdc2 and induction of cell cycle arrest through p21 pathway [161].

3.2 DNA adduct formation and genotoxicity

Development of cancers involves several critical stages, namely initiation, promotion, and progression [162]. Muta-

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tion induction has been suggested to participate in one or more of these events [163], and DNA-adduct formation is considered a biomarker for assessing mutagenic/carcinogenic potential of genotoxic chemicals [164, 165]. Many HAs have been demonstrated to be capable of forming DNA adducts whose roles in triggering mutagenesis/carcinogenesis are supported by both *in vitro* [7, 8, 166] and *in vivo* evidence [139, 167–169]. The principal reaction involved in the formation of these adducts is believed to be mediated *via* electrophilic attack of guanine base at N-2 or C-8 position by the HA nitrenium ions [165], which can induce very different levels of DNA damage depending on the parent HA [139]. It was found in bacterial and mammalian cells that reactivity of the AIA nitrenium ions and consequently the quantity of DNA-adducts formed (from MeIQ, IQ, and MeIQx) were principal factors in determining the quantitative damage incurred on genetic matter [170]. Such emphasis on the association quantitatively between DNA-adduct formation and mutagenicity, apparently does not apply for carbolines stemming from the observation that in both C57Bl/*lucZ* and c-*myc*/*lacZ* mice strains the mutant frequencies in the *lucZ* gene induced by AαC were 30–40% lower, despite having a two- to three-fold higher level of DNA adducts formation compared to either IQ or MeIQx [171]. Moreover, it was found that nontarget tissues could have high adduct levels in spite of their resistance to carcinogenic effects occurred in target tissues [172–174]. Further studies are required to establish the relation between DNA-adduct formation and subsequent induction of genotoxic events for different categories of HAs *in vivo*.

Detection and evaluation of the roles of the HA-DNA-adducts in their pathogenic processes were particularly facilitated with the application of accelerator MS, which offers enormous sensitivity in detecting radiolabeled compounds [175]. Moreover, with the availability of an array of analytical tools such as ^{32}P-HPLC, UV/VIS spectroscopy, MS, and NMR, the structures of DNA-adducts formed from many HAs, including Glu-P-1 [176], Trp-P-2 [177], IQ [178], MeIQ [179], MeIQx [180], 2-amino-3,4,8-trimethyl-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) [181], PhIP [182], and AαC [139, 183] have been characterized.

3.2.1 Mutagenicity

The Ames mutagenicity test, developed in the 1970s [184], was first used to assess the *in vitro* genotoxicity of HAs in prokaryotic cells. Requirement for metabolic activation of HAs led to modification of the method to incorporate a liver extract, conventionally called S9 mix [185]. Strain TA 98, which contains a frameshift mutation in the histidine gene, has been most widely applied and quantification of the relative mutagenic activities among HAs is based on the linear portion of their corresponding dose–response curves [185]. Exponential variation (>1 million times) was observed

between the most potent and the weakest HAs in these tests. Apart from *Salmonella* strains, some genes (*lacZ*, *lacZa*, and *lacI*) of *Escherichia coli* have also been used to analyze the mutational specificity of some HAs [186–188].

Several types of mammalian cells have been used to derive information relating to mutagenicity of HAs in eukaryotic cells. For some HAs, completely different results were obtained. This is exemplified by PhIP, which in bacterial cells, exhibited weak mutagenicity unequal to that observed in eukaryotic cells [7, 126, 189]. Variations in mutagenicities of HAs in *Salmonella typhimurium* and in mammalian cells could arise from their different genotoxic behaviors in these cells (primarily frameshift in bacteria and base substitution in mammalian cells) [147], in addition to other factors such as the relative efficiency of metabolic activation systems and the repair mechanism. Different genes like hypoxanthine-guanine phosphoribosyl transferase (*hgprt*), dihydrofolate reductase (*dhfr*), and adenine phosphoribosyltransferase (*aprt*) have been selected to study the mutational characteristics of HAs in mammalian cells. These are manifest in studies relating to PhIP and IQ or their metabolites. For example, using Chinese hamster ovary (CHO) cells, it was found that *N*-OH-PhIP, PhIP (in cells capable of expressing P450 1A2), and IQ primarily induced single-base transversion, mostly GC → TA, but also AT → TA, and CG → AT (for PhIP-induced mutants) [190–192]. Moreover, the observation that 75% of the PhIP-induced mutations occurred at the 3′ end of exon 2 and the beginning of exon 3 of the *aprt* gene, suggested higher susceptibility to mutation in genes (involved in directing cell replication and controlling cell survival) which harbor these sequences [191]. Ability of PhIP to cause single-base transversion was further revealed in human lymphoblastoid cells, more specifically at the thymidine kinase and *hgprt* loci with unequal distribution between the coding and the noncoding sequences (60% *vs.* 40%) [193]. Interestingly, HA-induced-mutation in these mammalian cell lines appears to be bias to the nontranscribed strand of the gene [190, 193, 194].

While HAs frequently demonstrate potent mutagenic effects *in vitro*, results from *in vivo* studies have been less consistent likely due to a number of factors such as the types and sex of animal model, and target organ(s)/tissue(s) chosen for evaluation of the mutational spectra. In studies that use the parent HA(s) (instead of their activated metabolites) as putative mutagens, one of the contributive factors to the distinct genotoxicity induced in different animal models may be their differential ability in activating the administered HA(s). For more information on this aspect, readers may refer to a recent review by Gooderham *et al.* [126] who gave a detailed account for the substantial species differences in metabolism of HAs. Duration of exposure, time allowed for DNA-adduct formation, and for conversion of such adducts into detectable mutation events also affect the

final mutagenic activities quantified. While 24 h is proposed as sufficient for DNA-adduct formation, at least a few weeks time is suggested for the adduct(s) to induce a permanent base sequence change [171]. This further emphasizes the increased risk associated with regular consumption of well-done muscle foods on a daily basis compared with those people with much lower frequency of consumption. Therefore, reducing the proportion and the frequency of well-done meats in the diets is an important protective measure against HA-associated mutagenesis, especially in light of previous findings that some dietary components such as vegetables, fruits, and rice bran may alleviate HA burden [195–197], that our body possesses metabolic machinery which could help detoxify some activated HAs [198], and that some HAs may require threshold levels of DNA-adducts for their genotoxicity to effect [147].

3.2.2 Carcinogenicity

Carcinogenicity of HAs has been well documented in a wide range of organs/tissues in long-term animal studies [14, 113] and this led to the classification of eight HAs (MeIQ, MeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2, and Glu-P-2) by IARC (1993) as possible (Group 2B) and IQ as probable human carcinogen (Group 2A). The liver (rat and mouse) has been shown to be susceptible to carcinogenesis induced by many HAs, including Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC, MeAαC, IQ, MeIQ, and MeIQx [9]. This may be partly attributed to its predominant role in metabolic activation of HAs, especially for those HAs whose carcinogenicity significantly correlates with their levels of metabolites and/or DNA-adducts in corresponding organs/tissues. One AIA, IQ has even been shown to induce hepatocarcinoma in nonhuman primates [199]. Other organs and tissues identified using rat and mouse models at least include the alimentary canals, blood vessels, mammary gland, prostate gland, Zymbal gland, clitoral gland, lung, lymphoid tissue, skin, and urinary bladder. Moreover, in an approach to compare the relative carcinogenicity of eight HAs, Trp-P-1, and 4,8-DiMeIQx were found to exert promoting effect on pancreatic duct carcinogenesis [200]. The large number of carcinogenic HAs and the wide range of target sites may, therefore, imply a much higher overall cancer risk for the human population as these HAs usually coexist in the diet, let alone the probable presence of other categories of mutagens/carcinogens such as acrylamide, PAHs, and NOCs.

Species, sex, and age variation in susceptibility to HA-induced carcinogenesis has also been evident [9, 201]. For example, in CDF_1 mouse, AαC-induced tumors in the liver and blood vessels, but no tumors were detected in F344 rat [9]. MeIQx, one of the most abundant HAs in muscle foods, was found to cause hepatocellular carcinoma only in male mice which were intervened with a diet containing 0.06% w/w of this HA for 30 wk [201]. It is generally agreed that

neonatal animals are more sensitive to exogenous chemicals. With a much lower dosage than the standard chronic bioassay, HAs were found to cause tumor development in neonatal mice [202]. Further, in a two-generation exposure study, PhIP increased the risk of mammary carcinogenesis in the second generation, expectedly *via* its transplacental transport to the fetus and its excretion into the milk [203]. Age-dependent susceptibility to PhIP-induced carcinogenesis was further exemplified in a recent study, which detected mammary gland cancer in adolescent (43-day-old) female rats but not in mature (150-day-old) rats [204]. More in-depth mechanistic investigation attributed this phenomenon to the differential induction of gene expression in these two groups of rats [204]. In particular, expression of genes associated with differentiation was enhanced in mature rats but inhibited in the adolescent which was accompanied with enhanced cellular proliferation. On the other hand, it was reported that *c-myc* overexpression in c-*myc*/λ*lacZ* mice produced a synergistic effect on MeIQx hepatocarcinogenicity [201]. Therefore, it is anticipated that gene expression and HA carcinogenicity is more than a one-way relation which for different genes (*e.g.*, *Apc*, β-catenin, Ha-*ras*, and p53) and HAs could vary widely.

Epidemiological studies provide the most direct link between HA genotoxicity and human health in the long term. Although the correlation has not been consistent, population-based data appear to favor a positive correlation between HA exposure and risk of certain cancers, especially colon cancer [205–207]. As being pointed out previously that the terminal genotoxic effects of HAs on humans should be the integrated outcome of multiple factors, epidemiological data are most valuable in this respect and more such studies should be launched to confirm the correlations in different populations.

4 Factors affecting HA formation and postulated strategies of inhibition

The findings that HAs formed in foods are potent mutagens and some are probable human carcinogens have stimulated ample interests in developing strategies to reduce risk of certain cancers in relation to this group of chemicals. There are mainly two branches. The first one is related to mitigation or abrogation of mutagenic/carcinogenic activity after entry of the purported mutagens into the biological system. According to a recent review [29], these protective agents can be divided into nine groups based on their mechanisms of interaction with the mutagens, which range from the first line of defense (affecting bioavailability), to enzyme systems involved in metabolic activation of HAs, and to interaction at genetic levels. The second one is related to inhibition of HA formation in food and this is where more prudent, direct, and secure strategies should be directed at. As

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cooked (especially high temperature heated) meat and fish are considered principal sources of HAs [35], reducing intake of these foods [29] is suggested as one of the feasible protective strategies in populations that have many different primary food choices. The occurrence of HAs in different cooked food has been well investigated and related information can be sought from previous reviews [14, 55]. In the past, combination of temperature, pH, cooking time, water activity, and precursor compositions/concentrations were central determinants for the final Maillard reaction products formed. In other words, these have been targets for developing inhibitory strategies. However, the view point has changed ever since the growing popularity of incorporating various food additives, especially phytochemicals or plant extracts or tissues into different cuisines. Therefore, it is no wonder that searching for potent inhibitors has become one of the dominant directions in relation to protection from food-derived mutagens. This part of the review will be dealing mainly with studies aiming to establish effective strategies for minimizing HA occurrence in foods.

4.1 Types and concentrations of precursors

It is known that creatinine or creatine, sugars, and amino acids are principal precursors of HAs in food [208]. However, regarding the similarity, yet significant difference in the structures and chemical properties of HAs, different types of precursors, especially amino acids can give rise to fundamentally different HAs. It is therefore expected that different HAs may predominate in different types of meat and fish under similar heating conditions [54].

Despite the critical roles of precursor types in HA formation, it is impractical to specifically modulate concentrations of certain precursors prior to cooking. Alternatively, strategies targeting at modulating the overall precursor profiles have been proposed as it was observed that a higher concentration of precursors may favor formation of HAs in foods, especially polar ones [35]. For example, it was found that meat extracts and gravies had higher concentrations of most of these compounds [56, 5, 209, 210], and high intake of gravy has been associated with an increased risk of colon cancer [211]. Taking into account the convenience of preparation and retaining of sensory properties, especially texture, microwave pretreatment of meat for 1–2 min and discarding the drippings before high temperature cooking is probably the best approach in reducing precursor concentrations [51, 212]. Moreover, as different meat components differ in their rates of leaking out of the meat matrix, there may be a pronounced change in the relative concentrations (profile) of precursors after microwave pretreatment, which may also contribute to the reduction in HAs formation as observed in previous studies [51]. This may further confirm the benefit of using microwave for dethawing frozen meats

and fish, as this practice is not only microbiologically safe, but also causes loss of meat juices and reduces time of contact with a hot surface which has been shown to increase HA formation [210, 55].

Modulating concentrations of reducing sugars is another feasible strategy. Effect of reducing sugars on HA formation is concentration dependent. It was found that addition of reducing sugars such as glucose, fructose, and lactose beyond a certain concentration range caused reduction in mutagen formation in ground beef and beef patties heated between 150 and 200°C [213, 214]. However, such strategy seems to apply only for high temperature cooking (≥150°C). In another study conducted at about 100°C, it was found that HA production increased with increasing levels of sugars added although heating time was different [215]. Despite the possible temperature dependent effects of glucose, increasing sugar levels prior to high temperature heating could be a recommended strategy as it is such drastic thermal treatments like grilling, frying, and broiling that are of most concern in relation to mutagenic HA formation. This can be accomplished by marinating meats or fish with sauces containing relatively high sugar content prior to heat treatment. This proposition is only tentative as contradictory results have been obtained in different studies. Marinating beef steaks with honey barbecue sauce produced a promoting effect on the formation of HAs [216], whereas a later study observed an inhibitory effect in fried pork chops premarinated with honey formulation [217]. It is thus probable that other food components and/or the way of increasing sugar levels have a modifying effect on the final outcome.

4.2 Temperature, time, and water content

Many studies suggested that both time and temperature had strong impact on formation of HAs [23, 218] and that the amounts of HAs generally increased with increasing temperature and time [65, 219, 220]. Effect of temperature was especially important according to a function developed by Bjeldanes *et al.* [218], where the temperature term was raised to a much higher exponent than the time factor. In a liquid model system with creatinine, phenylalanine, and glucose as the major constituents, yield of PhIP was found to increase when the temperature was raised from 180 to 225°C [221], while at low temperatures, this HA has rarely been produced in significant quantity [219]. Abdulkarim and Smith [16] also reported pronounced enhancement in formation of many HAs upon increase in temperature from 190 to 230°C.

On the other hand, heating at much lower temperature (~100°C) may also give rise to HAs if duration of heat treatment is prolonged [3]. For example, Arvidsson *et al.* [59]

reported the formation of harman and norharman in a meat juice system at 100°C heat treatment for 150 min; Manabe *et al.* [222] reported the presence of PhIP in a similar liquid system heated at 37 and 60°C for 4 wk. Although there have not been conclusions drawn on the definite effect of temperature, it would be prudent to maintain low cooking temperatures, avoid sudden increment in temperature [55], and avoid unnecessary prolonged heat treatment in terms of minimizing mutagen formation. One such strategy could be mixing of ground meat with water binding compounds such as soy protein, starch, or other polysaccharides, which help lower surface temperature and hinder transport of water soluble precursors [55, 223, 224]. Moreover, this practice minimizes water loss during cooking and the resulting high water content may also suppress formation of intermediary pyrazine cation radicals [223, 225].

4.3 Content and types of fat

The amounts of HAs were found in most cases to be higher in meats than in fish [55], except IQ and MeIQ [54, 226, 227]. One possible reason may be the presence of much higher concentration of unsaturated fatty acids in fish which help trap radical intermediates involved in HA formation, with themselves being converted to more stable radicals. Despite the possible involvement of lipid/fatty acids-derived radicals, their exact effects on mutagen formation in meats and fish have been controversial: while some studies have reported enhancing effects [4, 228, 229], others reported no effects [229, 230], or even inhibitory effects [231, 232]. It was observed that fats had an enhancing effect on the yield of HAs in model systems, probably by free radicals formed during thermally induced fat oxidation [4]. Addition of Fe^{2+} or Fe^{3+} to a model system containing creatinine, glycine, and glucose almost doubled the amount of MeIQx formed [233, 234], probably due to iron-catalyzed lipid peroxidation, and thus, formation of free radicals [4]. Surprisingly, Johansson and Jagerstad [233] found that the degree of oxidation of fat did not affect the yield of MeIQx in model systems. Further studies are thus demanded before specific recommendation can be made on the types and concentrations of fat/fatty acids to be added during cooking.

4.4 Presence/addition of inhibitors

Despite the possibility that HA formation during cooking and thus mutagenic activity in food products may be reduced *via* manipulation of some food components, it is strongly conceivable that more effective inhibition can be accomplished through addition of potent inhibitors at certain stages of the heating process. Desirable inhibitors should fulfill the following criteria: (a) capable of causing significant reduction in total HA content at low doses; (b) do not lead to formation of new HAs (based on analysis of HA profiles); (c) do not lead to formation of new or more potent mutagens. Various synthetic and natural agents have been reported to have modulating effects on the formation of HAs [29, 235]. The importance of the former has been demonstrated through identification of inhibitors of this type [18, 225]; nevertheless, the ultimate goal should be directed at identification of inhibitors from plants, preferably dietary plants or their products. As antioxidative activity has been the most popular mechanism of inhibition proposed, phenolic compounds occupy the largest proportion of putative phytochemicals investigated [27, 29, 58].

4.4.1 Synthetic antioxidative agents

Synthetic AOs that have been extensively tested for inhibition of HA formation include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ). These AOs showed widely varied effects on HA formation under different systems and at different concentrations. In real food systems, BHA, PG, and TBHQ were found to reduce formation of HAs at a concentration of 100 ppm [236]. In another study using a real food system at boiling temperature, it was found that BHT had a net minor inhibitory effect [18], whereas in simple model systems constituting of pure HA precursors, these synthetic AOs and BHT exerted opposite effects, particularly TBHQ, which increased MeIQx formation by more than 200% at 100 ppm [53]. The enhancing effect of BHT has been attributed to its role as an alkylating agent, which increased formation of HA precursors in these systems [22, 235]. Investigation of BHA and PG in simple model systems showed that they suppressed formation of IQx-type HAs dose-dependently [225]. Their inhibitory effects might be attributed to the conversion of their aromatic ring-attached methoxy or hydroxyl group to a quinone-like compound, and thus potently scavenges free radicals [22].

4.4.2 Phytogenic inhibitory agents

A wide range of natural agents have been tested for their effects on formation of HAs in model systems and in real food. However, few data on those isolated from dietary plants are available and the mechanism of inhibition has been abridged to antioxidation [26, 237]. These phytochemicals mainly include AO vitamins, phenolic compounds, and carotenoids.

4.4.2.1 Studies based on pure phytochemicals

Vitamin C and α-tocopherol did not demonstrate consistent effects when added to different real food systems [213, 238]. Results for different phenolic compounds have also been divergent as also their roles in the formation of differ-

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ent types of HAs. Lee *et al.* [239] found that flavones inhibited IQ-type mutagen formation in simple model systems. In a later study employing different kinds of phenolic AOs, contradictory observations were obtained for ellagic acid, syringic acid, and nordihydroguaiaretic acid (NDGA) [58]. The authors showed that ellagic acid and syringic acid reduced MeIQx formation in chemical model systems, but at the same time, significantly enhanced formation of PhIP. Active principles from spice plants have also been targets in several studies [240, 241]. In particular, curcumin from turmeric exhibited dose-dependent inhibition of formation of mutagenic Maillard reaction products in model systems. Tea phenolics, especially green tea catechins, (−)-epigallocatechin gallate, and caffeic acid have also been reported to be capable of inhibiting formation of IQx-type HAs [43, 58]. However, the effects of most of these phytogenic active components lack corroboration from real food system-studies.

4.4.2.2 Studies based on plant extracts/tissues

Apart from adding pure phytochemicals, addition of plant extracts/tissues is another approach to derive benefits from purported inhibitors. This also provides a means to study the possible synergistic effects among different potential inhibitors. Vitaglione and coworkers reported that carotenoid extracts from tomato reduced IQx and MeIQx formation in both chemical system and meat juice system, probably due to their AO capacity, but significant inhibition was achieved only at a concentration of 1000 ppm [25, 27]. Anthocyanonin is well known for its strong AO activity and berries, especially blackberry, chokeberry, and cherry are principal dietary sources [242–244]. Although these fruits together with many other major fruit and vegetable sources have been examined to confirm their protective effects on genotoxic activity of the most abundant HA, PhIP [245, 246], only limited information has been obtained for their roles in HA formation [247]. Addition of soy protein prior to high temperature heating can be bifunctional. Apart from serving as a layer of physical insulator, its phenolic components may also interfere with HA formation [235, 248]. However, findings have not been consistent among studies that used soy proteins and those that used soy sauce or soybean oil [215, 238, 249]. Further studies are required to reveal the relative importance of water and lipid soluble phytochemicals in these soy extracts. It is also probable that other components in soy sauce such as minerals interact with the phytochemicals to produce a different net effect. Tea extracts have been extensively studied, but mainly for their effects on HA-induced mutagenicity/carcinogenicity [250, 251] and their bioavailability [252]. Moreover, the fact that tea or its extract is a rare ingredient in culinary dishes needs to be considered when extrapolating these findings to practical benefits for humans. With reference to a recent review, a number of spices such as thyme, marjoram and rosemary have diverse effects on the formation of HAs [235]. As an example, Murkovic *et al.* [253] reported that application of dried rosemary, thyme, sage, and garlic to the surface of meat prior to heating resulted in significant reduction in HA content, but to various extents with respect to different HAs. A recent study by Ahn and Gruen [254] also demonstrated the effectiveness of the extracts from rosemary and the bark of another plant, Pycnogenol, in suppressing formation of both polar and nonpolar HAs, though changes in the total mutagenicity was not evaluated. On the other hand, extracts from many of these spices were shown to exert an enhancing effect on formation of PhIP in chemical model systems [57].

The large discrepancy in the effects of AO phytochemicals on HA formation further emphasize their ability to switch between an AO and a pro-oxidant role, depending on their specific chemical environments. Alternative interpretation could be that in addition to AO activity, they possess other concurring modulating activities which may dominate or become insignificant under certain conditions and which may not have been taken into consideration during analysis of the experimental results.

4.4.3 Organosulfur compounds

Organosulfur compounds are another group of compounds that are receiving increasing attention in view of the findings that they may be effective in inhibiting nonenzymatic browning reaction [174, 255]. It was postulated that such modulating effect could be mediated *via* one or both of the following mechanisms: (i) suppression of free radical formation through trapping by thiols [256, 257]; (ii) interaction of sulfhydryl compounds with Maillard intermediates thus preventing their further reaction to form final Maillard reaction products [257, 258]. The most well studied sulfur containing compound in relation to inhibition of HA formation is sodium bisulfite ($NaHSO_3$). Addition of $NaHSO_3$ at a level of 0.5% was demonstrated to significantly inhibit the formation of HAs in canned foods [259]. Sodium bisulfite is well known to inhibit Maillard reaction by nucleophilic attack on the carbonyl group of reducing sugar or α, β-unsaturated carbonyl intermediates, thus may inhibit their further reaction to produce HAs [260]. Inhibitory effects of some organosulfur compounds such as diallyl sulfide, dipropyl disulfide, diallyl disulfide on HA formation have also been evidential based on model system [44, 261] and real food system studies [258]. However, the only proposition these previous studies arrived at was that there was a competitive reaction between the organosulfur compounds and amino acids tested for glucose, which was thus suggested as the limiting reactant [44]. Detailed mechanisms of inhibition remain to be clarified. These compounds probably exert their inhibitory effect *via* different mechanisms from $NaHSO_3$ as sulfur atom in the former molecules

is not bonded to electronegative oxygen atoms. On the other hand, the finding that cysteine did not significantly inhibit formation of IQx, MeIQx, and PhIP in model systems [44] also implies the requirement for specific structural features to effect desired intervention.

5 Summary and concluding remarks

It is important to understand the basic chemistry of HAs before efficient analysis can be performed. Knowing the different surrogate systems for studying HA formation in foods not only introduces flexibility, but also facilitates experimental design in a more systematic approach. The fact that HAs are formed at nanogram levels under most conditions necessitates the requirement for an effective clean-up and concentration procedure and at present time, SPE is most popular. Development and amelioration of various chromatographic techniques (GC and LC) together with their coupling to an array of detectors enable both quantitative and qualitative analyses of HAs formed under much more diverse conditions to be accomplished.

The growing body of *in vitro* and *in vivo* evidence for the potential genotoxicity of HAs implies that development of strategies to inhibit mutagen formation will continue to be one of the most significant areas in the field of food-derived mutagens. In particular, more efforts should be focused on the following three areas. First is the search for more potent inhibitors from dietary sources which are not yet sufficiently exploited. Second, is to examine the mechanism of inhibition. The differential effects of adding the phytochemicals at different stages of the heating process should be emphasized. This also helps in deciding their dominant inhibitory mechanism(s). As an example, modulators which primarily interact with HA precursors or intermediates will not be effective in alleviating HA burden in foods if added toward the end of the cooking process. Third, is to analyze the structure–activity relationship of purported inhibitors. Phenolics have been the most promising candidates. It is well known that different number and position of hydroxyl groups can affect their stability or stability of their reaction products, implying their different reactivity toward susceptible HA intermediates or the HAs themselves. In all these cases, mutagenicity tests are required to corroborate that the total mutagenicity of the system under investigation is significantly attenuated.

6 References

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