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Review

Chromatographic and related techniques for the determination of aromatic heterocyclic amines in foods

Pilar Pais[a,*], Mark G. Knize[b]

[a]*Departament de Química Analítica, Universitat de Barcelona, Diagonal 647, 08028 Barcelona, Spain*
[b]*Molecular and Structural Biology Division, Biology and Biotechnology Research Program, L-452, Lawrence Livermore National Laboratory, P.O. Box 808, Livermore, CA 94551-9900, USA*

Abstract

Some 20 years ago, Japanese scientists discovered a new group of highly toxic compounds, classified as heterocyclic aromatic amines, from broiled and grilled meat and fish products. Numerous studies have shown that most HAs are mutagenic and carcinogenic, and the safety of HA-containing foods has become a concern for the public. To date, more than 20 different mutagenic and/or carcinogenic heterocyclic amines have been identified in foods. This paper reviews the analysis of foods for HAs with 145 references. We survey some of the numerous methods available for the chromatographic analysis of heterocyclic amines and highlight the recent advances. We discuss chromatographic and related techniques, including capillary electrophoresis, and their coupling to mass spectrometry for the determination of these contaminants in foods. In addition, the review summarises data on the content of HAs in various cooked foods. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Food analysis; Heterocyclic amines

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*Corresponding author. Euromed S.A., C/Rec de Dalt 8, 08100 Mollet del Vallès, Barcelona, Spain. Fax: +34-93-5703-237.

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1. Introduction

The first literature on high mutagenic activity in cooked foods appeared more than 20 years ago [1,2]. Since then, more than 20 mutagenic/carcinogenic compounds, known as heterocyclic amines (HAs), have been identified in cooked foods [3–5]. Based on results from long-term animal studies on mice, rats and non-human primates [6–10], several of these compounds have been classified as carcinogens [11]. Epidemiological studies of the relative risk of cancer from cooked foods have shown that frequent consumers of meat run an increased risk of cancer [12–17], which might be explained by their intake of mutagenic compounds such as heterocyclic amines. For a summary of the chemical, biological and epidemiological data and potential contribution of the HAs to human cancer etiology see Ref. [18]. The current knowledge on the formation of HAs in cooked foods and model systems has been recently reviewed by Skog et al. [19].

This paper reviews and summarises the current chromatographic methods for the determination of the mutagenic/carcinogenic HAs present in foods. Fig. 1 shows the structures, abbreviations and chemical names of the known heterocyclic amines found in cooked foods. They are usually divided into two main classes: the pyrolytic amines and the aminoimidazo-azaarenes. The first group is formed at high temperatures, above 300°C, and include the amines: Trp-P-1, Trp-P-2, AαC, MeAαC, Glu-P-1 and Glu-P-2; the compounds harman and norharman are not primary amines nor mutagenic in the Ames test, but have been shown to be co-mutagenic [20] and are frequently included in this class of compounds. The aminoimidazo-azaarenes are formed at the ordinary household cooking temperatures of 100–225°C and are sometimes termed thermic mutagens. Since this class of HAs was found to be extremely mutagenic compared with the other compounds, and more commonly and easily formed during ordinary cooking, they have received more attention over the past decade. The aminoimidazo-azaarenes commonly reported in cooked foods are: IQ, IQx, MeIQ, MeIQx, 4,8-DiMeIQx and 7,8-DiMeIQx, corresponding to the ''IQ-type'' compounds, and PhIP. The number of congeners in this class is still increasing, and more than 10 compounds have been identified in foods, including 4-MeIQx, 4-CH_2OH-8-MeIQx, 7,9-DiMeIgQx, 4′-OH-PhIP, DMIP, 1,5,6-TMIP, 3,5,6-DMIP and IFP. These mutagens are less studied but also present in cooked meat and fish products. According to the chemical behaviour of these compounds, they are sometimes grouped as polar (aminoimidazo-azaarenes together with Glu-P-1 and Glu-P-2) and nonpolar (all others) amines.

It is important to quantify the amounts of these mutagens present in a variety of cooked foods in order to estimate intakes and support studies of their risk to human health. The development of procedures for the quantitative analysis of HA mutagens in foods is also important for studies on the mechanism of mutagen formation under various cooking conditions, and will be useful in assessments of ways to prevent their formation during cooking.

The complex sample matrix of cooked foods makes HA analysis a difficult sample preparation problem. The HAs were originally isolated from pyrolysed proteins of foods cooked at high temperatures, such as broiled sardines or fried beef. Chemical extraction and preparative liquid chromatography and high-performance liquid chromatography (HPLC) were used in combination with the Ames test to measure mutagenic activity to guide purifica-

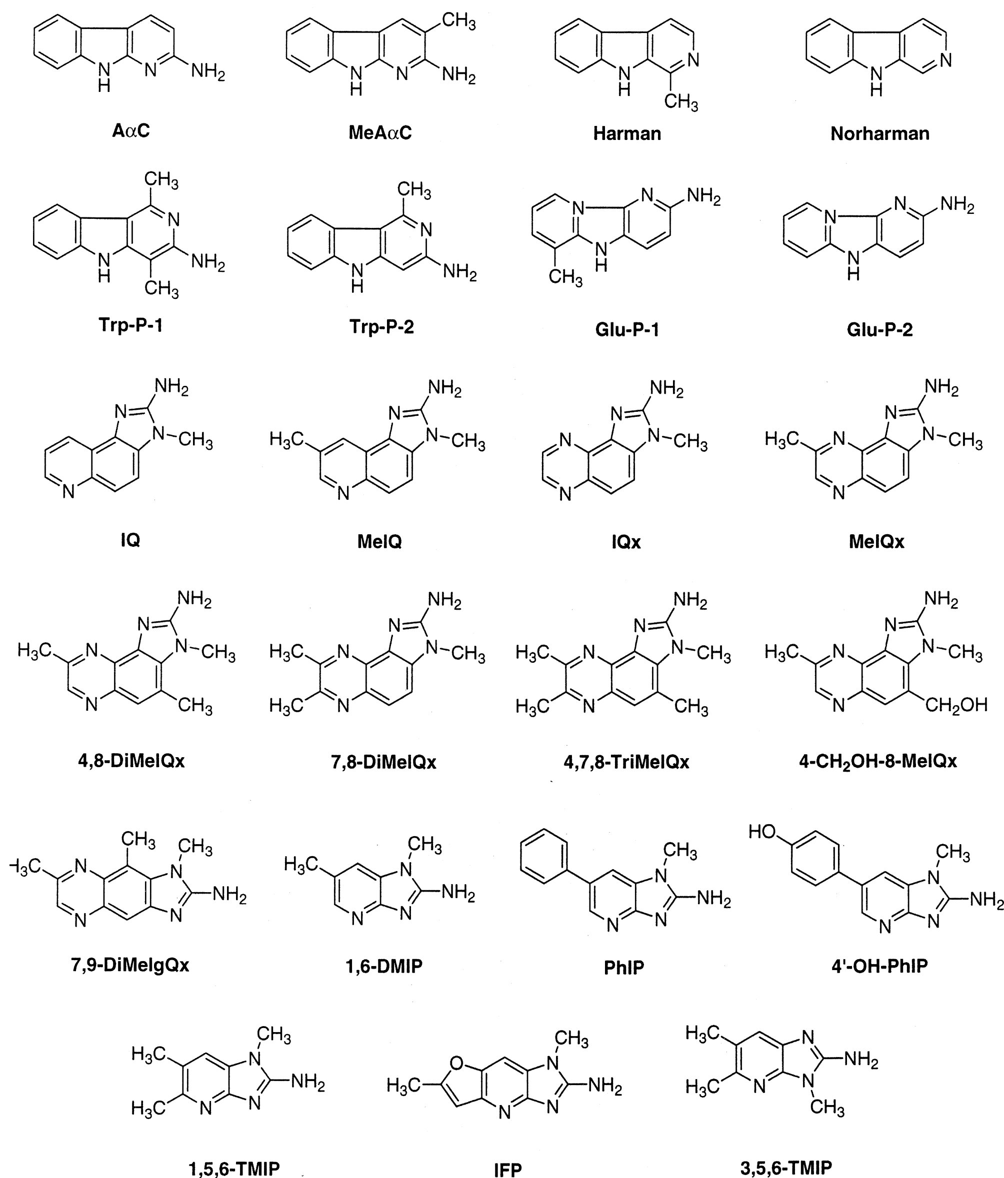

Fig. 1. Heterocyclic amines found in cooked foods. IQ: 2-Amino-3-methylimidazo[4,5-*f*]quinoline, IQx: 2-amino-3-methylimidazo[4,5-*f*]quinoxaline, MeIQ: 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, MeIQx: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, 4-MeIQx: 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline, 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, TriMeIQx: 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline, 4-CH_2OH-8-MeIQx: 2-amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]quinoxaline, 7,9-DiMeIgQx: 2-amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline, PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, 4′-OH-PhIP: 2-amino-1-methyl-6-(4-hydroxyphenyl)-imidazo[4,5-*b*]pyridine, DMIP: 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine, 1,5,6-TMIP: 2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine, 3,5,6-TMIP: 2-amino-3,5,6-trimethylimidazo[4,5-*b*]pyridine, IFP: 2-amino-1,6-dimethylfuro[4,5-*b*]pyridine, AαC: 2-amino-9*H*-pyrido[2,3-*b*]indole, MeAαC: 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole, 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, 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-9*H*-pyrido[4,3-*b*]indole, norharman: 9*H*-pyrido[4,3-*b*]indole.

tion. The structures were mainly deduced from proton nuclear magnetic resonance (NMR) and mass spectral data and proven by chemical synthesis [2,3,21–25].

2. Chromatographic methods for the determination of HAs in foods

The first quantitative data on HAs in various meat and fish products, based on chromatographic techniques, was published in the late 1980s. Earlier literature data on HA levels in foods consist mainly of amounts estimated from the mutagenic activity according to the Ames test [26] analysis of isolated chromatographic peaks. The complex food matrix, the low amounts of HAs present and the need for several isolation steps makes accurate quantification difficult, but effective new methods for the extraction, purification and detection of HAs have recently been developed.

Analysis of HAs from foods involves protein precipitation by homogenisation of the sample with methanol [27–31], dichloromethane [32] or an aqueous acid or basic solution [33–37], or by enzymatic digestion [38]. A first extraction of HAs is performed by acid–base solvent partition with dichloromethane [3,29,30,32,35,37,39–43] diethyl ether [27,44] or ethyl acetate [43,45]. Liquid–solid extraction is performed with XAD-2 resin [3,28,41,42,46] blue cotton or rayon [37,38,47–49]. Liquid–liquid extraction from a solid support is done with Kieselgur [35,37,50] or Extrelut [35,51] diatomaceous earth columns. Further purification is carried out with preparative chromatography [27,30,32,38,40–42,46,52], column chromatography with Sephadex LH20 [40,53] or TSK-Gel CM-650 [54,55] or solid-phase extraction with PRS (propylsulfonic acid) [35,51,55,56], C_{18} [35,51,57], silica [47,48,54] or strong cation-exchange (SCX) [58] cartridges. These extraction, purification and preconcentration techniques are necessary in order to obtain chromatograms free of interfering peaks and the low ppb levels at which HAs are present. These sample preparation techniques are not going to be discussed in this review because extended overview on this topic is presented by Puignou et al. in this same Volume.

During the last decade several analytical methods for the analysis of HAs have been described. Most of them were targeted to the analysis of certain HAs only, such as the IQ-type compounds: IQ, MeIQ, MeIQx or 4,8-DiMeIQx [28–30,32,36,44,45,47, 48,59–62]. A need for a routine screening method able to quantify all known HAs in cooked food generated scientific interest and research papers from many laboratories. The goal was accurate and reproducible analysis methods with detection at the low ppb level that would minimise the operator and instrument time needed. All techniques presently available either require sophisticated and/or expensive equipment, such as enzyme-linked immunosorbent assay (ELISA), liquid chromatography–mass spectrometry (LC–MS) or gas chromatography–mass spectrometry (GC–MS). Some are restricted to the determination of a selected group of compounds. HPLC with UV detection has been found appropriate to determine most of these compounds simultaneously. However, HPLC in combination with electrochemical detection (ED) or fluorescence detection is a convenient option to be considered, since high selectivity and sensitivity are achieved. In addition, capillary electrophoresis (CE) has also been applied to the determination of mutagenic amines in foods.

Co-extracted compounds from the food sample matrix frequently appear. These compounds can interfere in the analysis of HAs and detection limits of the HAs depends more on these co-eluting interferences than on the absolute sensitivity of the detectors. The most important aspect of the determination of HAs is the confirmation of the chromatographic peaks using selective techniques to unambiguously identify the compounds, since numerous co-elutions can occur leading to false peak identification. Investigation of HA formation using model systems or food samples heated at high temperatures or for long times often result in chromatograms containing interfering compounds, making low-level confirmation difficult. HAs in these samples have been reported to be difficult to confirm, and additional purification steps were developed to provide better chromatograms [50,51,58,63–66]. However, confirmation is still a problem when analysing these complex matrices. To solve this problem more selective detectors like mass spectrometers or electrochemical detectors can be used.

2.1. HPLC–UV and fluorescence detection

Most of the analytical methods for the quantification of HAs in foods reported in the literature are based on HPLC with UV detection. After extraction, complex chromatograms are obtained; peak confirmation using ultraviolet absorbance spectra from diode array detection (DAD), comparing the sample spectrum and the reference spectrum, is required. Usually, fluorescence detection is simultaneously recorded with UV to quantify fluorescent compounds. IQ-type compounds give no fluorescence signal under reversed-phase conditions and UV is required for their quantification. Fluorescence provides higher selectivity and sensitivity and, therefore, ''cleaner'' chromatograms are obtained. Due to the fact that no single setting in the excitation and emission wavelength can detect the fluorescent HAs with maximum sensitivity, it is necessary to employ programmable fluorescence instead. Fluorescence results can be 100–400-times more sensitive than UV detection [37,67]. However, no confirmation of the chromatographic peaks is possible with this detector and so DAD is needed. Peak identity is confirmed by retention time and spectral identity matches to reference standards. Peak homogeneity can also be determined with DAD detectors.

Zhang et al. [68] described a method for the isolation, purification and identification of five HAs from fish using TSK-Gel ODS (Tosoh) semi-preparative and analytical columns. Analytical columns packed with this kind of stationary phase have been extensively used for the separation of HAs by HPLC providing excellent results [35,50,65,69–74]. For quantitative purposes, Gross [35] studied a large number of HAs and related compounds: IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP, AαC, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, harman and norharman. Different solid-phase tandem extraction procedures were investigated in meat extracts and HPLC–DAD was used. The optimised HPLC method separated the fourteen compounds using acetonitrile with either of two mobile phases: triethylammonium (TEA) phosphate at pH 3.3 or 7.5.

The separation of all the HAs at trace levels with high resolution requires multicompound analysis methods. In this respect, the TSK-Gel column showed the best peak symmetry and separation efficiency compared with other C_{18} columns tested. Mobile phases conditions allowed the separation of all compounds with a resolution higher than 1.3 excepting Glu-P-1 and TriMeIQx which coeluted at pH 7.5.

A better separation was achieved by using a ternary mobile phase [51] composed of TEA at pH 3.2, TEA at pH 3.6, and acetonitrile. The IQ-type compounds were detected with DAD recording at a wavelength of 264 and 273 nm; the remaining compounds were quantified with a programmable fluorescence detector at different emission and excitation wavelengths. In this method DAD was used for peak confirmation of all the compounds by comparing the UV spectra with reference standards. The method separates the 14 compounds within 32 min and high resolutions (>1.3) are obtained. This HPLC–UV and fluorescence detection technique is the most common method used in the literature for the determination of HAs in cooked foods [37,55,63,75–80]. As an example of the good efficiency provided by this method and the number of peaks that can appear in the analysis of food samples, chromatograms corresponding to fried and barbecued salmon samples are given in Fig. 2.

In addition, some authors have developed HPLC methods based on Gross and Grüter's method [51] with some modifications. Hence, Skog et al. [81] used a modified HPLC method for the analysis of nonpolar HAs in different kinds of foods such as fish and meats and the corresponding pan residues cooked at different temperatures under different cooking conditions. A HPLC–fluorescence system was used for quantification and HPLC–DAD was undertaken for confirmation of the HAs. Polar amines were analysed as in Ref. [51]. The UV chromatograms from the food sample extract containing the nonpolar compounds generally were too complex to allow proper identification and no confirmation with DAD could be achieved. Neither Glu-P-1 nor Glu-P-2 was found in any of the cooked food samples analysed in this study. Coeluting impurities made it impossible to properly identify AαC and MeAαC in the food samples.

Knize et al. [71] adapted the method to quantify a group of compounds less studied: DMIP, 1,5,6-TMIP and 3,5,6-TMIP and IQx together with IQ, MeIQx, 4,8-DiMeIQx and PhIP which were determined by

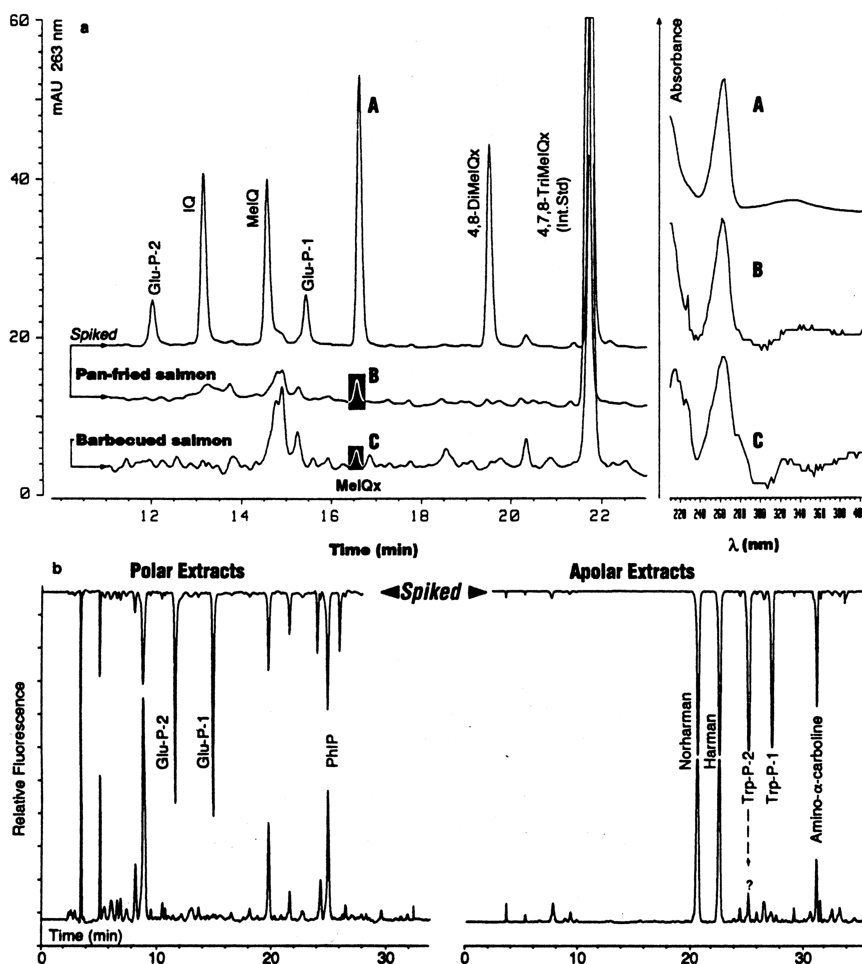

Fig. 2. (a) HPLC–UV chromatogram of a polar extract corresponding to a pan-fried salmon and a barbecued salmon. Pan frying at 200°C produced more MeIQx (peak B) than barbecuing for the same time at 270°C (peak C). On-line recorded UV spectra from MeIQx are shown on the right. (b) HPLC–fluorescence chromatogram of polar and nonpolar extracts of barbecued salmon. A chromatogram of a spiked sample is also shown. Chromatographic conditions: TSK gel ODS80 (Toyo Soda, 25 cm×4.6 mm I.D., 5 μm particle size) column, mobile phase: solvent A, 0.01 *M* TEA phosphate, pH 3.2; solvent B, 0.01 *M* TEA phosphate, pH 3.6; solvent C, acetonitrile. Gradient program: 0–10 min, 5–15% C in A; 10–10.1 min, exchange of A with B; 10.1–20 min, 15–25% C in B; 20–30 min, 25–55% C in B. Figure reproduced from Ref. [51].

the usual published method. The mobile phase was modified for the determination of IQx, DMIP, 1,5,6-TMIP and 3,5,6-TMIP, using TEA at pH 3.6 and acetonitrile running in a gradient mode. IQx was determined with UV detection and DMIP, 1,5,6-TMIP and 3,5,6-TMIP were quantified with fluorescence detection.

Pais and Knize [65] showed remarkably improved spectral library matching in a model system and a process flavour using HPLC separation conditions (mobile phase at pH 7.0) which changed the sample selectivity from the commonly used method of Gross and Grüter [51]. The HPLC method provided one solution to interference problems encountered in complex thermally processed samples. Good UV spectra were difficult to obtain at pH 3.2 and 3.6 as can be seen in the chromatograms of Fig. 3 for a meat model system. One advantage is that the same instrumentation is used. Samples not successfully analysed by the widely used chromatographic con-

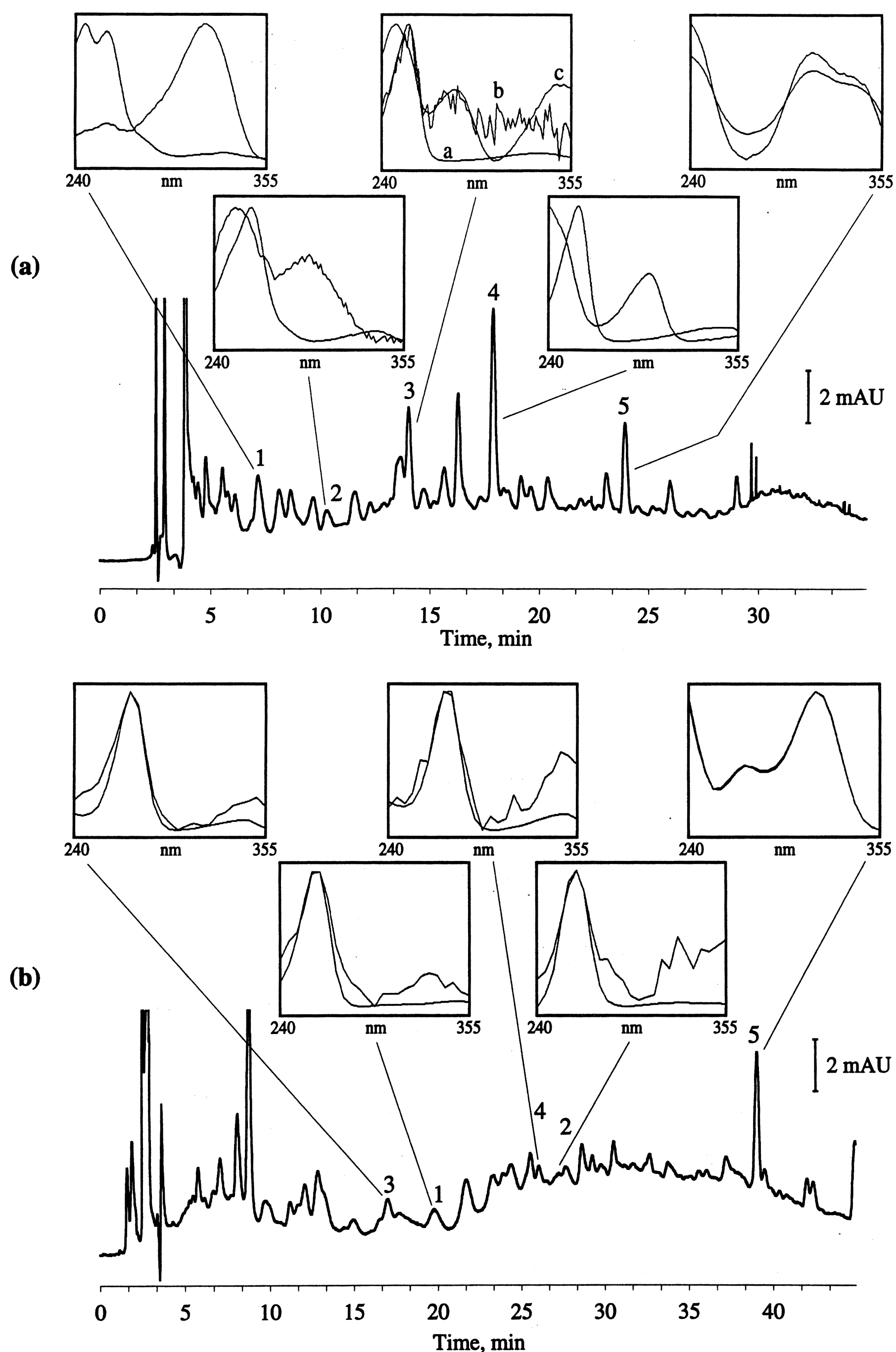

Fig. 3. HPLC–UV chromatogram of a model system of creatine, glucose and various amino acids found in beef steaks obtained with (a) the same conditions as in Fig. 2 and (b) solvent A, 0.01 *M* TEA phosphate, pH 7.0; solvent B, acetonitrile. Gradient program: 0–12 min, 15% B; 12–45 min, 15–50% B. Inset plots show library spectra overlaid with the sample spectra. Detection wavelengths: (a) 262 nm and (b) 273 nm. Peaks: 1. IQ, 2. MeIQ, 3. MeIQx, 4. 4,8-DiMeIQx, 5. PhIP. Figure reproduced from Ref. [65].

ditions can simply be reinjected without further work-up, but using the new selectivity, and the presence of heterocyclic amines can be confirmed with a second chromatographic method. The conditions described by Gross and Grüter do have advantages as a routine method: shorter analysis time, longer chromatographic column life and greater signal to noise at the low pH. Thus, the HPLC method at pH 7.0 would be used best as an alternative when samples contain multiple interfering peaks such as, high temperature meat and fish samples, the corresponding pan residues and model systems [82].

Chen and Yang [67] developed a binary mobile phase consisting of acetonitrile and ammonium acetate at pH 3.6 with gradient elution and UV and fluorescence detections to separate 16 HAs and related compounds. Up to 16 mobile phases of pH ranging from 3.5 to 3.8 were assayed and resolution between peaks was calculated to determine the separation efficiency for each solvent system. Optimum conditions gave resolutions of 1.03–1.24 and separated the 16 compounds within 31 min. The method was successfully applied to the determination of the 16 mutagenic amines in chicken, identifying 12 compounds at concentration of 0.09–0.21 ng/g. This method offered the main advantage of using a binary system and the number of HAs separated is increased compared to Gross and Grüter's method [51]. However, Gross and Grüter's method seemed to produce cleaner chromatograms with more stable baselines at the end of the chromatogram, maybe due to the lower percentage of acetonitrile used in performing the separation.

Other methods used a LiChrospher 100 RP-18 column and a mixture of diethylamine (DEA) and acetonitrile as mobile phase for the determination of IQ, MeIQ, MeIQx, 4,8-DiMeIQx and 7,8-DiMeIQx in meat samples by HPLC–DAD [30]. Gross et al. [53] developed an HPLC–UV method for the determination of IQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx and PhIP using this column. Ammonium acetate (pH 4.5) and acetonitrile under two different gradient conditions were used for separation of IQ, MeIQx, 4,8-DiMeIQx and 7,8-DiMeIQx, and other conditions for the determination of PhIP. The method was developed in order to investigate the purification of HAs in beef products by extraction with Kieselgur followed by HPLC on Sephasorb HP, which resulted in cleaner samples and had wide applicability. Karamanos and Tsegenidis [83] studied the separation of IQ, MeIQx, 4,8-DiMeIQx, TriMeIQx and PhIP by HPLC–UV on a Supelcosil LC-8 column and TEA phosphate buffer (pH 3.2) and acetonitrile as mobile phase. Separation was accomplished under two different isocratic conditions for separating IQ, MeIQx and 4,8-DiMeIQx, and independently TriMeIQx and PhIP. Detection limits were estimated to be 2.3–7.2 ng/g.

In HPLC 4.6 mm I.D. columns are widely used to perform separations. However, smaller diameter microcolumns have the advantages of low solvent consumption, higher sensitivity and good separation at lower flow-rates. This last characteristic makes microcolumns and capillary columns suitable for the liquid chromatography–electrospray mass spectrometry (LC–ESP-MS) techniques and will be discussed separately. While the flow-rate, the volume of the chromatographic peak and the solvent used decrease with the column diameter, the sensitivity increases because microcolumns elute analytes at a higher concentration than conventional columns. If the column diameter is reduced to 2.1 or 1.0 mm, the sensitivity increases 4 or 21 times, respectively.

Gross et al. [69] used a 2-mm I.D. column (Vydac 201HS52) with a ternary mobile phase of TEA phosphate (pH 3.2 and 4.5) and acetonitrile, with a photodiode array detector and a time-programmable fluorescence detector. With these conditions there was approximately a three-fold increase in sensitivity (detection limits of 0.5 ng/g of cooked meat) compared to the conventional column. The method was applied to the determination of 13 HAs and related compounds in cooked beef, fish, bacon and pan residues.

Due to the fluorescent properties of HAs and the resulting high sensitivity a fluorescence method was developed for HA detection in cooked foods. Glu-P-1 and Glu-P-2 were analysed by HPLC with fluorescence detection [84]. A Kaseisorb LC ODS-300-5 column and a mobile phase composed of phosphoric acid and acetonitrile were used. Fractions were collected and UV absorbance, fluorescence emission and mass spectra were obtained for confirmation of HAs in the cigarette smoke samples. Other authors have studied the fluorescence detection of the IQ-

type compounds. Størmer et al. [59] studied the fluorometric detection of IQ and MeIQ and their metabolites in urine samples. In protonic solvents like water (acidic, neutral or basic) and alcohols no fluorescence of the IQ-type compounds is observed, whereas in esters, ethers, ketones and aromatic hydrocarbons, IQ, MeIQ and their *N*-acetyl derivatives showed significant fluorescence. Fluorescence intensity was highest in dimethyl sulfoxide and dichloromethane. Another approach to the determination of the IQ-type compounds by HPLC with fluorescence detection would be fluorogenic labelling of these heterocyclic amines. Schwarzenbach and Gubler [37] tried to derivatise the common amino-group of the amines with a fluorescent reagent, but were not successful.

Sample preparation greatly influences the chromatographic interferences that can appear and, therefore, detection limits depend on the kind of sample analysed and the procedures used in the clean-up of these samples. Detection limits vary with the compound analysed and the complexity of the sample matrix. Adding increasing amounts of the reference standards to known amounts of a sample, the detection limits can be estimated. The HPLC–UV method developed by Gross [35] gave values of 0.03, 0.02 and 0.01 ng/g for MeIQx, 4,8-DiMeIQx and PhIP, respectively, in the crusts of fried beefburgers [85]. Each of these three HAs could be detected and confirmed in the pan residues at 0.02 ng/g. Otherwise, these same compounds were determined by Jackson et al. [63] in beef flavours, and detection limits were estimated in 1–5 ng/g due to the complexity of the samples and the number of interfering peaks present in the chromatograms.

Detection limits of the HAs using the HPLC–UV methods were estimated in fried meat samples to be of 1–2 ng/g [35] and 2–10 ng/g [81], 0.02–0.5 ng in chicken legs [67], 0.015–0.81 ng/g in fried meat or 50 ng/g [37,50] in process flavours when the sample preparation was performed by the solid-phase extraction method proposed by Gross and Grüter [51]. For lightly-cooked meat samples from fast-food restaurants, Knize et al. successful scaled-up the extraction procedures to get greater sensitivity [76]. With further clean-up procedures the detection limits can decrease from 0.1–0.2 ng/g to 0.03 ng/g [75] in fish and meat products, or from 50 ng/g to 1–3 ng/g in process flavours [50]. For the nonpolar HAs, detection limits using HPLC–fluorescence were lower, 0.3–0.4 ng/g in a fried meat sample [81], 0.05–2 pg in chicken legs [67] or 0.2–5 ng/g in process flavours [50,63].

2.2. HPLC–electrochemical detection

HPLC with UV detection has been successful in determining most known HAs. However, HPLC in combination with ED is an option with high selectivity and sensitivity. These attributes are especially important in the analysis of food samples processed at high temperatures. HPLC in combination with amperometric [37,47,48,62,64,70,86] and coulometric [87,88] detectors showed enhanced sensitivity and selectivity for the determination of HAs in foods. For maximum detection signal the usual potentials applied in the electrochemical detector are between 900 and 1000 mV for the oxidation of the HAs. High potentials reduce the selectivity to some extent, but optimise for sensitivity. Mobile phases employed with ED need to work in isocratic mode due to the instability of the baseline in this high sensitivity range, required for the determination of all HAs and related compounds in a single chromatographic run. Hence, different conditions have to be used to determine a large number of HAs using isocratic mobile phases. Using the widely known solid-phase sample purification steps of Gross and Grüter [51], extracts containing polar and nonpolar HAs are obtained. These extracts are analysed using two isocratic mobile phases, and, therefore, no extra injections compared to the HPLC–UV method are required. Additionally, additives such as triethylamine or diethylamine, frequently used for improving peak shapes of HAs with UV or fluorescence detection, can not be incorporated into the mobile phase when working with ED. These additives would increase the background noise and, therefore, detection limits. Tailed peaks can appear under these conditions so sensitivity can be reduced. In addition to the limitations on the choice of mobile phases, ED gives no confirmation of the peaks and additional detection systems such as DAD have to be employed for this purpose [37,64,89,90].

First investigations using HPLC–ED for the determination of HAs were described by Grivas and

Nyhammer [86] and Takahashi and co-workers [47,48]. These methods were focussed on the determination of IQ-type compounds in model systems [86] and beef extracts [47,48].

For the determination of IQ, MeIQ and MeIQx in beef extracts [47] two different columns were used. A TSK ODS-120A column was used to collect IQ, MeIQ and MeIQx fractions, which were further injected on a TSK-Gel SP-2SW column. Isocratic mobile phases composed of acetonitrile and phosphate buffer at pH 3.0 or pH 2.0 were employed in both systems. Different compositions of these mobile phases were used to detect IQ and MeIQ, and separately, MeIQx. Additionally, this group of investigators extended the study to the determination of the mutagenic amines 4,8-DiMeIQx and 7,8-DiMeIQx [48].

IQ-type compounds were also investigated by Schwarzenbach and Gubler [37] using HPLC–ED with a LiChrosorb RP-Select B column and a mobile phase composed of ammonium acetate (pH 4.5), methanol and acetonitrile, resulting in high selectivity for IQ, MeIQx, 4,8-DiMeIQx and 7,8-DiMeIQx in processed flavours. Van Dyck et al. [62] used an ion-exchange chromatographic system with electrochemical detection to determine IQ, MeIQ and MeIQx. Separation was enabled with a Spherisorb SXC 5U column and a mobile phase of sodium phosphate at pH 5.6 and acetonitrile. More recently, a method using a LiChrospher 60 RP-select B column and methanol–acetonitrile–acetic acid–water (pH 5.1) was described for the determination of IQ, MeIQ, MeIQx and 4,8-DiMeIQx in poultry meat by HPLC–ED [89]. Additionally, PhIP was investigated in these samples and in a model system [91] by HPLC with fluorescence detection using the same analytical column but a different mobile phase of TEA (pH 6.5) and methanol. For a positive identification, a second run was carried out using a different voltage. The ratios of the corresponding peak areas were the same as those of the reference materials. Additionally, some samples were analysed by photodiode array detection comparing the UV spectra with those of authentic standards.

Ohe [90] determined MeIQx by HPLC–ED using a QC Pack C_{18} column and ammonium acetate, pH 6.0 and acetonitrile, and Trp-P-1, Trp-P-2 and PhIP by HPLC–fluorescence using the same column but varying the mobile phase. The method provided enough sensitivity and selectivity for the determination of these mutagens in river water. However, the UV and fluorescence spectra of the fractions collected from the HPLC had to be recorded for confirmation purposes.

These cited HPLC–ED methods were restricted to the determination of a selected group of compounds: the IQ-type compounds. In contrast, Billedeau et al. [87] and Galceran and co-workers [64,70] reported the detection of a large number of HAs including IQ-type compounds, nonpolar amines, and related compounds such as PhIP, Glu-P-1, Trp-P-1, Trp-P-2, AαC, MeAαC, harman and norharman. IQ, MeIQx, Glu-P-1, Trp-P-1 and Trp-P-2 were separated in a Synchropak SCD 100 column with ammonium acetate at pH 5.2 and acetonitrile [87]. Fourteen HAs [64,70] were analysed in beef extracts using a TSK-Gel ODS 80T column and ammonium acetate (pH 4.0 and 5.7) and acetonitrile under two isocratic conditions. ED conditions were optimised by obtaining the hydrodynamic voltammograms and optimum working potential was established at +900 mV [87] and +1000 mV [70]. Results from the analysis of beef extracts showed the high sensitivity of ED when working with such complex samples, detecting Trp-P-2, AαC, harman and norharman. HPLC–DAD and fluorescence were used to confirm these compounds, but DAD sensitivity was not high enough to confirm Trp-P-2 and AαC in the beef extract sample.

Since conventional electrochemical detectors cannot confirm the chromatographic peaks seen, a coulometric array detection system coupled to HPLC proved to be a powerful confirmation system [88]. Using a RP-Select B column and an isocratic mobile phase composed of water, ammonia, methanol, acetonitrile and acetic acid, the electrode array detector containing eight coulometric cells detected PhIP between +580 mV and +750 mV. The chromatograms, one from each electrode, were obtained simultaneously, and hydrodynamic voltammograms, acquired with standard solutions and with a cigarette condensate sample, showed maximum sensitivity at +630 mV. The detector shows several advantages compared with single or dual electrochemical detectors. Qualitative information is obtained from the peak shaped hydrodynamic voltammogram, which is available after only one injection of the sample. With single and dual electrode detection, time consuming measurements at various potentials are necessary to

get the hydrodynamic voltammogram. Additionally peak purity can be ascertained by measuring the response ratios of different channels for the sample and the standard compounds. A lower potential gave the maximum sensitivity, decreasing the background noise and the residual current. The applicability of this method to a wide number of HAs and to the analysis of food samples has to be demonstrated.

Good sensitivities and selectivities were achieved with electrochemical methods, with estimated detection limits of about 35–475 pg [37,48,62,86,87], except for the IQ-type compounds found by Galceran and co-workers [64,70], who obtained higher detection limits, from 0.74 to 3.37 ng. These differences can be attributed to the pH of the mobile phase; at higher pH values lower detection limits were obtained but the separation of the compounds was not accomplished. Detection limits are 2–4-times better than the ones obtained by HPLC–UV and higher than values for fluorescence detection. Hence, Grivas and Nyhammer [86] compared the determination of IQ, MeIQ, MeIQx and 4,8-DiMeIQx by HPLC–UV and HPLC–ED and found a 2–3-times lower detection limit for ED (2.5 pmol for UV and 0.5–1.5 pmol for ED). Detection limits in beef extracts and processed flavours reached values of about 0.2–1.6 ng/g [48] and 50 pg/g [37], respectively. The best detection limits of 8 pg were achieved by Murkovic et al. [89] for the IQ-type compounds, and Bross et al. [88] for PhIP with the electrochemical array detector.

2.3. Gas chromatography

The applicability of GC to the analysis of heterocyclic amines is mainly due to the use of GC–MS, combining high separation efficiency of capillary GC with high sensitivity and specificity of MS. Analyte detectability and the possibility of obtaining structural information are the attraction of chromatographic techniques coupled to mass spectrometry. Most GC–MS methods for the determination of HAs in food samples use mass spectrometers with a magnetic sector instrument for positive ion electron ionisation MS. This method yields excellent fragmentation patterns, with further confirmation achieved using chemical ionisation of the samples with a quadrupole instrument. Since chemical ionisation MS is a much softer ionisation technique, it produces less fragmentation and molecular ion is frequently present, which helps with data interpretation. Negative ion chemical ionisation is highly sensitive and selective to electron-capture compounds. In addition, a GC with nitrogen–phosphorous selective detector was developed for the determination of HAs with the advantage of the high response of these compounds in the detector due to the nitrogen atoms in the structure of the HAs.

Most HAs are polar and not volatile, and tend to elute as broad and tailing peaks due to the strong adsorption to the injector and the column during GC analysis. Derivatisation of amines not only reduces the polarity but also improves volatility, selectivity, sensitivity and separation of these amines. Some of the derivatising agents tested for the determination of these mutagens by GC are: 3,5-bistrifluoromethylbenzoyl chloride (for Trp-P-2, MeIQx and 4,8-DiMeIQx), 3,5-bistrifluoromethylbenzyl bromide (for MeIQx, 4,8-DiMeIQx, PhIP, 4′-OH-PhIP and 5-OH-PhIP), heptafluorobutyric anhydride (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP, AαC, Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2), acetic anhydride, trifluoroacetic anhydride, pentafluorobenzyl bromide (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP, AαC, Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2) and *N*,*N*-dimethylformamide dimethyl acetal (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, TriMeIQx, PhIP, AαC, Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2) [36,43,60,92–99]. Advantages and disadvantages of these derivatising agents will be outlined.

Quantification in GC–MS is usually performed by isotope dilution analysis. The advantages of isotope dilution analysis is the reduction of the number of samples to be extracted per determination since the extraction efficiencies are calculated in the same analysis using the different mass/charge ratio of the labelled standards. The disadvantage of this technique is that isotopically labelled standards are not always available for each HA. Using a single standard would give erroneous results since HAs are not extracted with the same recoveries, despite the fact that they are from the same class of compounds.

2.3.1. GC of underivatised HAs

The first studies identifying HAs in cooked foods used direct inlet probe mass spectrometry with both high low and high resolution. Fractions collected from different liquid chromatographic techniques

were obtained to identify and confirm mutagenic amines, but chromatography was not coupled to the mass spectrometer [3,29,39–41,44,47,53,84].

Using a GC column, broiled sardines were analysed for Trp-P-1 and Trp-P-2 [100] and IQ, MeIQ and MeIQx [101] by GC–MS. The method developed for IQ, MeIQ and MeIQx used isotope-labelled internal standards ([$^{2}H_3$]IQ, [$^{2}H_3$]MeIQ and [$^{2}H_3$]MeIQx) to perform the quantification. The MS operated in the selected ion monitoring (SIM) mode for achieving maximum selectivity. A 6-m capillary column coated with SP-2100 was used under a temperature gradient. HAs were not derivatised and injection of such complex sample (even after a clean-up with Sephadex LH-20 column chromatography) into the GC column led to tailed peaks and deterioration of the column, making it difficult to obtain good sensitivity in the detection and reproducible results.

More recently, Skog et al. [102] introduced a GC–MS method for the determination of the nonpolar HAs and related compounds Trp-P-1, Trp-P-2, AαC, MeAαC, harman and norharman without derivatisation. A capillary column Rtx-50 (50% phenyl–50% methyl polysiloxane) was used for the separation. Concentrations in cooked meat products were analysed using this method finding values of 0.04–200 ng/g [102] and 0.4–299 ng/g in processed flavours and pan residues [66], respectively. These HAs have been reported to be difficult to confirm by HPLC–UV in complex matrices when are present at low concentration levels and additional purification steps are needed [50,51,58,63–65]. A chromatogram acquired in SIM mode of a pan residue sample is shown in Fig. 4 illustrating the clean chromatograms and the good selectivity of the method. This method offers high chromatographic efficiency and provides a high specificity and selectivity for the determination of nonpolar HAs in complex food samples. However, authors reported contamination of the ion source through the deposition of non-volatile material, and column lifetime is shorter than the HPLC columns.

2.3.2. Trifluoromethylbenzoyl and -benzyl derivatives

A GC–MS method using derivatisation with detection by negative ion chemical ionisation was developed for the determination of Trp-P-2 from cooked meat and fish [93]. The isotope [$^{2}H_3$]Trp-P-2 was used as internal standard and the bis-trifluoromethylbenzoyl (bis-TFMBO) derivatives were prepared. As a benefit of the derivatisation, high mass ions were monitored eliminating interferences in the final extract. These authors applied this procedure for the determination of MeIQx and 4,8-DiMeIQx in beef [60] and MeIQx in human urine using [$^{13}C,^{15}N_3$]MeIQx as an internal standard [94]. Amounts of derivative equivalent to 1 pg of MeIQx and 4,8-DiMeIQx could be detected [60]. MeIQx and 4,8-DiMeIQx were detected in concentrations that ranged from 0.5 to 2.4 ng/g beef [60]. While the bis-TFMBO derivatives of these HAs showed the molecular ion as the base peak in the mass spectra and hence appeared well suited to SIM work, chromatographic behaviour was so poor as to preclude their use in quantitative analysis. Additionally an alternative derivatisation procedure was examined, using bis-trifluoromethylbenzyl (bis-TFMB), which gave spectra not containing molecular ions but high mass fragment ions suitable for SIM analysis. The high resolving power of capillary gas chromatography in combination with the specificity of SIM mass spectrometry allowed the determination of analytical traces free of interferences. Recovery through the extraction was assessed by radioactivity measurements with [^{14}C]MeIQx. Moreover, these authors [43] determined MeIQx, 4,8-DiMeIQx and PhIP by using the same derivatisation scheme by stable isotope dilution. [$^{13}C,^{15}N_2$]MeIQx and [$^{2}H_5$]PhIP were used as internal standards. Results corresponding to the quantification in fried beef, fried bacon, barbecued pork and chicken, and beef extracts were 0.1–16.4 ng/g. A chromatogram of a fried fatty bacon is given in Fig. 5. The di-bis-TFMB derivatives of MeIQx and DiMeIQx [92] were also prepared and analysed by electron impact GC–MS to evaluate their presence in cooking fumes. For sensitive detection of unmetabolised MeIQx and its metabolites (hydrolysed to the parent MeIQx) in human urine, GC–MS was used in the negative chemical ionisation mode monitoring the di-bis-TFMB derivatives [103].

2.3.3. Heptafluorobutyryl derivatives

Another approach for the determination of HAs by GC–MS was the preparation of the heptafluoro-

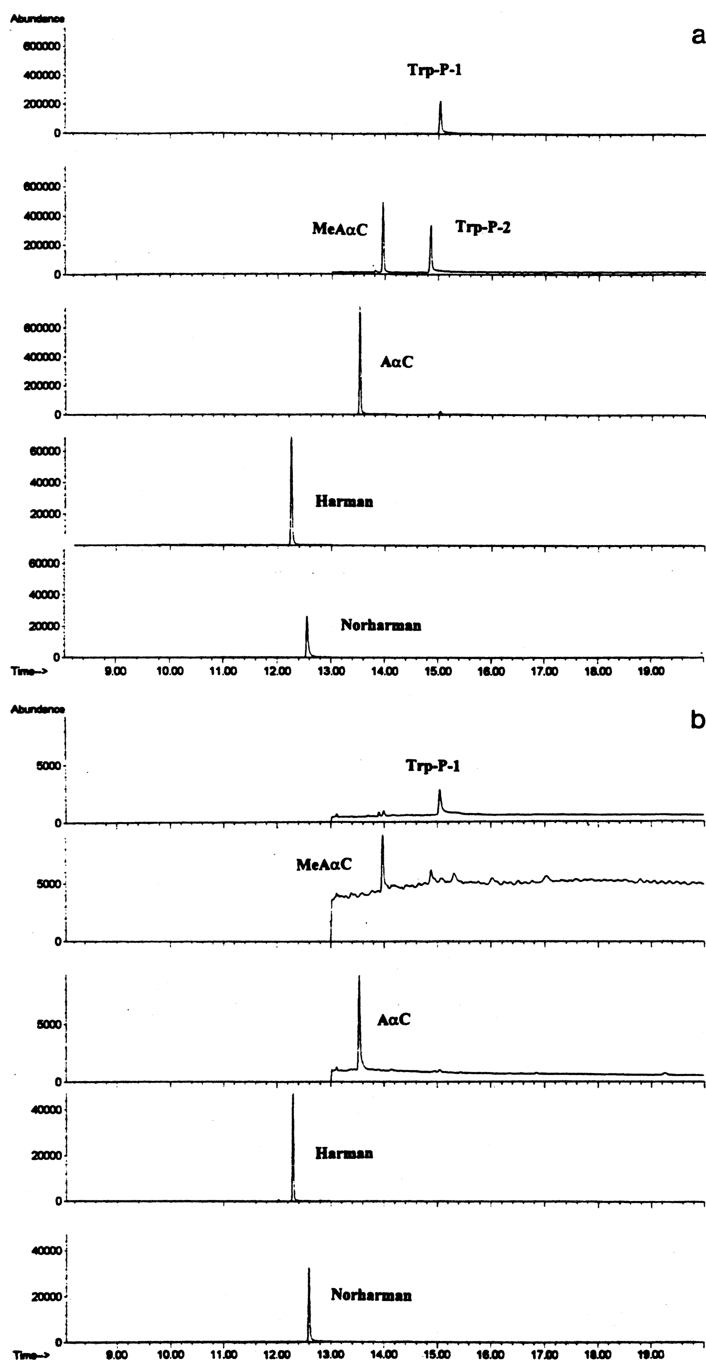

Fig. 4. SIM traces of (a) reference compounds and (b) a meat extract by GC–MS. GC–MS conditions: a Rtx-50 capillary column (30 m×0.32 mm I.D., 0.50 μm film thickness) operating from 100 to 320°C at 20°C/min; helium as carrier gas (1 ml/min and 0.7 p.s.i.); injection in splitless mode at 270°C; mass spectrometer operating in the negative ion mode with an electron energy of 70 eV and an electron impact ion source temperature of 250°C (1 p.s.i.=6894.76 Pa). The m/z monitored were: Trp-P-1 m/z 211, Trp-P-2 m/z 197, AαC m/z 183, MeAαC m/z 197, harman m/z 182, norharman m/z 168. Figure reproduced from Ref. [102].

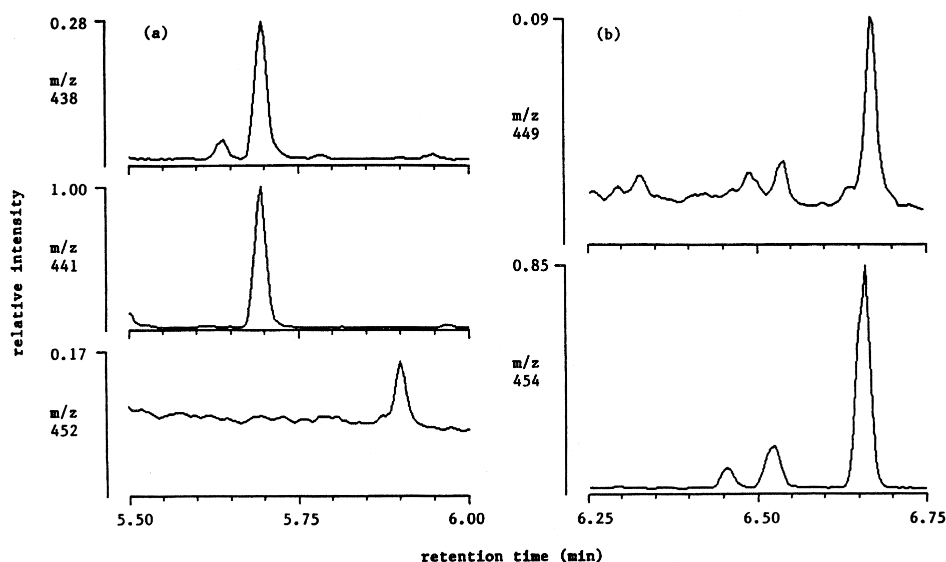

Fig. 5. SIM traces of the di-bis-TFMB derivatives corresponding to the amines MeIQx, DiMeIQx and PhIP in fried fatty bacon. GC–MS conditions: a DB5 capillary column (15 m×0.25 mm I.D, 0.25 μm film thickness) operating from 200 to 320°C at 20°C/min; helium as carrier gas (69 kPa); injection was maintained at 270°C; mass spectrometer operating an ion source pressure of 53 Pa and 150°C. The negative ions were monitored at *m/z* 438 (MeIQx), *m/z* 441 ([^{13}C,^{15}N$_2$]MeIQx), *m/z* 449 (PhIP), *m/z* 452 (DiMeIQx) and *m/z* 454 ([^{2}H$_5$]PhIP). Figure reproduced from Ref. [43].

butyryl derivatives [36] applied to the analysis of IQ and MeIQx in beef and mice tissues in a study investigating the genotoxic/carcinogenic risk to humans from this class of food contaminants. Furthermore, MeIQx, 4,8-DiMeIQx and PhIP in fried meat and their metabolites in human urine were analysed by preparing these derivatives [95]. These three amines, the most common mutagens found in meat and fish products, were investigated as biomarkers for recent exposure to HAs following the intake of a meal prepared by regular home cooking procedures. [^{2}H$_3$]PhIP was used as an internal standard to quantify PhIP, 4′-OH-PhIP and 5-OH-PhIP, while [^{2}H$_3$]MeIQx was used to quantify IQ-type compounds. For the analysis of fried meat, the mass spectrometer operated in the low-resolution electron capture mode with SIM of the $[M]^-$ ions. Urine extracts containing low amounts of HAs showed poor reproducibility under these conditions and were investigated by high-resolution electron impact MS. Good reproducibility was reported by recording the $[M-C_3F_7]^+$ major fragment. MeIQx, PhIP and 4′-OH-PhIP, reported as a mutagenic amine and as a possible detoxification product of PhIP metabolism, were detected in the fried meat and the urine samples. In addition, PhIP was analysed by negative ion chemical ionisation MS in biological samples by preparing the pentafluorobenzyl derivative [96]. The major fragment obtained, $[M-C_6F_5CH_2]$, was monitored in the SIM mode. Good reproducibility and sensitivity were achieved by using [^{2}H$_5$]PhIP as an internal standard.

2.3.4. Comparison of the trifluoromethylbenzyl and the heptafluorobutyryl derivatives

Tikkanen et al. [97] compared the two most widely used derivatisation procedures with heptafluorobutyric anhydride and 3,5-bistrifluoromethylbenzyl bromide, in the analysis of a larger number of HAs: IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx and PhIP. Heptafluorobutyric anhydride derivatisation was rapid and derivatives corresponding to the six HA standards were detected at 5 mg/l levels. It was also possible to separate 4,8-DiMeIQx and 7,8-DiMeIQx derivatives from each other using a non-polar column Ultra 2. However, the sensitivity was insufficient to detect the levels of HAs in cooked foods. The bis-TFMB derivatisation

was suitable only for preparing the MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx and PhIP derivatives, which were sensitively detected. The bis-TFMB derivatives gave narrower chromatographic peaks than in the case of the heptafluorobutyryl derivatives. IQ was not detected by this method and the MeIQ derivative was very unstable. Reproducibility of the method for the detected derivatives was very good, but it was not possible to separate the derivatives corresponding to 4,8-DiMeIQx and 7,8-DiMeIQx. Thus, MeIQx, PhIP and total DiMeIQx were determined by negative-ion chemical ionisation and SIM in heated food samples. Amounts of these HAs found in commercially cooked samples (grilled pork, grilled chicken and flame-broiled fish) [97] and grilled chicken [98] were quantified with values of 0.03–7.6 ng/g. This study reveals that both derivatisation schemes are restricted to several related compounds.

2.3.5. N-Dimethylaminomethylene derivatives

On the other hand, GC with NPD was applied to the determination of 10 HAs (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP, AαC, Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2) [99]. The *N*-dimethylaminomethylene derivatives of these mutagens were prepared by reaction with *N*,*N*-dimethylformamide dimethyl acetal. Structures of the derivatives were confirmed by GC–MS using a fused-silica capillary column containing cross-linked OV-210. Mass spectra showed the molecular ion peak $[M]^+$ and other common ion fragments, which were useful for structure elucidation. Under the GC–NPD conditions, the HAs were separated using two connected fused-silica capillary columns containing DB-1 and DB-17ht.

2.3.6. Detection limits of the GC techniques

The GC–MS methods were shown to be very sensitive and highly specific, with detection limits of 0.5–1 pg. For food samples detection limits were very low: 25 pg/g in beef samples [93], 2.5–100 pg/ml in human urine samples [94,95], 0.01–0.2 ng/g in high temperature cooked meats [43,97] and 0.03–0.20 ng/g in grilled chicken [98]. Detection limits using GC–NPD were estimated between 2 and 15 pg of HA injected [99].

GC–MS has been described to be the most sensitive technique for HA analysis in foods. Despite being sensitive, selective, simple and rapid, most of the GC methods lack the wide application range of a multiresidue method. Only *N*-dimethylaminomethylene derivatives have been prepared for a large number of HAs. However, the method was developed with HA standards and the applicability of the method to food samples has still not been reported.

2.4. Liquid chromatography–mass spectrometry

HAs are not amenable to be analysed by GC without derivatisation procedures being employed. For HAs, LC is the most appropriate separation method, and direct LC–MS analysis is an effective way to obtain both qualitative and quantitative information. As previously discussed, most of HPLC-based methods use common UV, fluorescence or electrochemical detection. However, in the last few years, LC–MS has been successfully applied to the determination of HAs because of its advantage over these conventional detectors: it can provide unambiguous identification. MS has proven to be a valuable technique for the unambiguous identification of contaminants in foods. The development of quantitative LC–MS procedures for HA analysis have undertaken in the last few years in several laboratories [25,32,45,61,69,72,104–110].

We review the application of LC–MS in HA analysis for the three ionisation techniques that have been used: thermospray (TSP), electrospray (ESP) and atmospheric pressure chemical ionisation (APCI). Highly polar and thermally unstable compounds can be separated successfully with HPLC. The mass spectrometer has to cope with the high quantities of mobile phase and low sample concentrations. Further requirements are the high vacuum conditions in the ion source of most mass spectrometers and the requirement that buffers, acids, bases, salts and other additives necessary for chromatographic separations be volatile. Only ESP-MS methods used for HA analyses are limited to low flow-rates requiring microbore or semi-microbore columns for the LC separation. LC–MS, and in particular MS–MS, provides the tools for elucidation of the structure and variety of natural products and other polar analytes directly in complex food samples, therefore opening further dimensions in the field of HA analysis.

The extensive purification schemes used for the isolation of HAs from cooked foods give satisfactory results in most analyses. However, as described previously, samples such as flame-grilled meat and fish and some industrially produced food flavourings, so-called ''process flavours'', show levels of chromatographic interferences which do not allow detection and quantification of the chromatographic peaks [50,51,63]. Also, investigation of HA formation using model systems, containing amino acids, creatine/creatinine and sugars heated at high temperatures often have interfering compounds produced upon heating, making low-level quantification impossible [65,111,112]. As outlined before, fluorescence or electrochemical detection can provide high sensitivity and selectivity for the analysis of these samples but they still do not allow for the necessary peak confirmation, and additional techniques must be used for this purpose. MS has been applied successfully to the analysis of HAs due to the high sensitivity and specificity of MS. The sensitivity of MS is increased by one- or two-orders of magnitude if only a few selected ions are monitored instead of full spectra, as when using the SIM technique [72,73,105–107].

Very few studies on LC–MS for the determination of HAs have been published so far in comparison with the ones that use HPLC–UV, despite the potential of LC–MS in terms of sensitivity and the availability of structural information which is often claimed. Tandem MS or MS–MS provides an elegant means of obtaining structural information, albeit at the cost of an increase in the complexity of the instrumentation. Only a few authors justify their use of MS–MS quantification by providing quantitative data. Nevertheless, the published data strongly suggest that the method may indeed play an important role in HA analysis in the near future.

2.4.1. LC–TSP-MS

Most analysis of HAs in foods by LC–MS has been described using TSP ionisation. In fact, it was the first ionisation technique used for the coupling of LC to MS in the analysis of these mutagens. TSP can work with conventional-size LC columns and mass spectra show abundant quasi-molecular ions, $[M+H]^+$, of the HAs. As the identification capability is often required in HA analysis due to the complexity of the food samples and the low concentrations, TSP has been used for target compound analysis. TSP has been used with reversed-phase columns and volatile buffers. It has been combined to quadrupole MS instruments for the analysis of HAs.

McCloskey et al. published the first work on the analysis of HAs in cooked meat and fish by LC–TSP-MS [32,61]. Stable isotope dilution was first reported for the determination of IQ and MeIQ in salmon, sardine and beef [61]. The internal standards ($[^2H_3]$IQ and $[^2H_3]$MeIQ) were distinguished from IQ and MeIQ by their occurrence 3 mass units higher in each case, thereby permitting simultaneous but independent measurement of the HA mutagen and the internal standard, even though they coelute. The addition of the labelled internal standard also provided chromatographic markers to establish unambiguously the elution positions of IQ and MeIQ. This group of investigators also reported the application of TSP ionisation to the analysis of IQ, MeIQ, MeIQx, AαC, MeAαC, Glu-P-1, Glu-P-2, Trp-P-1 and Trp-P-2 [32]. The applicability of the method to food samples was demonstrated in the analysis of IQ and MeIQ in broiled salmon, using the stable isotope dilution method. In both cases, an Ultrasphere ODS column was used with ammonium formate, diethylamine (pH 4.5), acetonitrile and methanol pumped isocratically at 1.5 ml/min. The mobile phase was directly introduced in the TSP instrument. Additionally a Waters μBondapak C_{18} column was used with the same mobile phase. Estimated values obtained in the quantification of these HAs were 0.2–1 mg/g of cooked food.

Trp-P-1 and Trp-P-2 were investigated in tryptophan pyrolysates [25] by LC–TSP-MS. The conditions used were as follow: a Supelco LC-18DB analytical column; a mobile phase of ammonium acetate, acetonitrile, methanol and TEA used isocratically at 1.3 ml/min. In the same research group, IQ, MeIQx, 4,8-DiMeIQx and 7,8-DiMeIQx were analysed in cooked beef and beef extracts [45]. Two analytical columns were used: a Supelco LC-CN and a Supelco LC-18-DB column. Mobile phases of acetonitrile and ammonium acetate (pH 6.8) were used either isocratically or in a gradient. Deuterated standards of these four HAs were used as both chromatographic markers and as measures of recovery for the quantification of the mutagens.

Amounts of HAs reported were 0.3–52.2 ng/g of heated beef products. These methods accomplished the accurate measurement of HA content with simultaneous determination of recoveries of each individual HA.

IQ, MeIQ and other related hydroxy- and oxo-substituted compounds [113] were determined by LC–TSP-MS using a Synchropak SCD 100 column and ammonium acetate (pH 5.2) and acetonitrile as the mobile phase at a flow-rate of 1.3 ml/min. However, this method has not been applied to the analysis of food samples.

Harman and norharman, products of food heating having a co-mutagenic activity, were determined in alcoholic beverages and uncooked foodstuffs such as vinegar, soy sauce, miso, soybeans, corn starch and rye flour [104]. The analysis was performed by HPLC with fluorescence detection. Due to the impossibility of confirmation with the fluorescence detection, thermospray LC–MS was used for confirmation of these comutagens at 0.1 ng/g levels. Also LC–TSP-MS confirmation was used in the determination of 13 HAs and related compounds (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP, AαC, MeAαC, Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2, harman and norharman) in grilled bacon, beef, fish, and in grill scrapings [69]. This is the LC–TSP-MS method that has been applied to the larger number of HAs. Quantification was performed using UV and fluorescence detection. The column was a TSK Gel ODS 80 TM and used a ternary mobile phase of two buffers of ammonium acetate (pH 3.2 and pH 4.0) and acetonitrile, operating in a linear gradient. The amines MeIQx, 4.8-DiMeIQx, PhIP and AαC were confirmed at concentrations as low as 0.7 ng/g in these complex samples that are difficult to confirm by DAD.

2.4.2. Atmospheric pressure ionisation techniques

As a consequence of the introduction of more robust techniques based on atmospheric pressure ionisation (API), the use of TSP has decreased lately. In the last 5 years, only API methods, ESP and APCI, have been applied to the determination of HAs by LC–MS. In ESP droplet formation and charging take place simultaneously, while in APCI droplets are formed prior to ionisation. Initially, the main distinction between ESP and APCI was in terms of the eluent flow-rates and the molecular mass ranges that can be handled: low flow-rates (up to 20 μl/min) and a high-molecular-mass range in ESP as compared to high flow-rates (up to 2 ml/min) and a low-molecular-mass range in APCI. Now, ESP can be performed at higher flow-rates, by directing a gas flow into the effluent stream (designated "pneumatically-assisted ESP", "high-flow ESP" or ionspray (ISP)). The fact that lower molecular mass compounds already have successfully been subjected to analysis by ESP/ISP makes the distinction between ESP/ISP and APCI less pronounced. Both API techniques provide soft ionisation, providing unfragmented quasi-molecular ions in which little structural information is directly obtained. However, the application of an appropriate voltage difference between two regions of an API source generally induces fragmentation of the primarily formed ions; this mode of operation is termed pre-analyser collision induced dissociation, in-source fragmentation, or cone voltage fragmentation, and has been successfully applied to HA confirmation in beef extracts [72,106].

Only pneumatically-assisted ESP has been applied to the determination of HAs, requiring analytical HPLC columns of smaller I.D. or postcolumn splitting before the analyte enters the ion source. LC columns of 1–2 mm I.D. at flow-rates of 50–200 μl/min have been used and will be discussed. In APCI, both heat and pneumatic nebulization are applied to evaporate the sample solution and to obtain an effluent spray. Under these conditions, reversed-phase LC flows of 0.1–2 ml/min can be handled.

2.4.2.1. LC–ESP-MS. The first work reported in the literature applying an electrospray source was in 1995 [114] for detecting the presence of 14 HAs and related compounds in model systems. The separation was achieved using a TSK ODS-80 column and a ternary mobile phase composed of two ammonium acetate buffers (pH 3.2 and 4.0) and acetonitrile using a linear gradient at 1 ml/min. Half of the mobile phase entered the mass spectrometer by using a split after the LC system and HAs were monitored by SIM. Investigation of HA formation using model systems heated at high temperatures often provide UV chromatograms containing interfering com-

pounds, making low-level confirmation impossible. Confirmation of the HAs was achieved successfully with this method in these complex model systems and quantification was performed by HPLC–DAD and fluorescence detection.

In the analysis of foods, on-line pneumatically-assisted ESP-MS proved to be an attractive approach for the determination of HAs and related compounds in beef extracts [105,106]. These are complex matrices, which in some cases cannot be confirmed using DAD [64]. Trace level quantification and confirmation of HAs were achieved as a consequence of the excellent sensitivity of ionspray MS combined with HPLC analysis. Two different microbore columns of 1 mm I.D. were used: an ODS-Hypersil C_{18} [105] used for the separation of seven HAs and related compounds, and a Hypersil DBS C_{18} [106] separating 14 HAs and related compounds (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP, Glu-P-1, Trp-P-1, Trp-P-2, AαC, MeAαC, harman and norharman). Both columns performed the separation with ammonium acetate (pH 6.7 and 3.5) and acetonitrile at a flow-rate of 50 μl/min, and presented the best separations at low percentages of acetonitrile in the mobile phase. However, ionisation in the mass spectrometer improves when the content of organic solvent in the mobile phase is higher. Taking into account the HPLC resolution and MS sensitivity, the mobile phase chosen must be a compromise between these two parameters. Nevertheless, MS is a selective technique, so a low resolution between two compounds can be compensated for by selecting unambiguous masses to monitor. For quantification purposes the extraction potential applied was 100 V working in SIM. The application of 150 V induced fragmentation of the primarily formed ions $[M+H]^+$, and allowed the confirmation of the detected peaks. In source fragmentation of the molecular ions provided confirmation of Glu-P-1, Trp-P-1, PhIP, harman, and norharman at concentrations of 7.8–60.6 ng/g [106] in a beef extract using TriMeIQx as internal standard. The use of in-source fragmentation provides an easier and less expensive technique than MS–MS for confirmation of the HAs.

Another successful approach is the coupling of ESP to tandem MS with the goal of obtaining relevant structural information. Ionspray tandem MS was successfully applied by Richling and co-workers [108,109] for the analysis of HAs in wine and commercially available meat and fish products. These authors described a method using a 2 mm I.D. LiChrospher 60-RP column and a mobile phase of trifluoroacetic acid, water, methanol and acetonitrile. This method was time consuming requiring two or three injections for the analysis of each sample to reach high sensitivity. Three [108] or two [109] selected reaction monitoring (SRM) experiments with one or two HPLC gradients were performed. Quantification was achieved using $[^2H_3]$PhIP and $[^2H_3]$norharman as internal standards, and $[^2H_3]$MeIQ as external standard to control the final volume in the sample preparation and the ionisation in the electrospray source. Tandem MS collision-induced dissociation (CID) of the molecular ions enhanced the trace analysis of these compounds, providing structural information and selective detection of 14 HAs (IQ, MeIQ, IQx, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP, Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2, AαC and MeAαC) in the wine samples, and 10 HAs (IQ, MeIQ, IQx, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP, Glu-P-1 and Glu-P-2) in meat products down to 0.5–7.5 ng/l [108] and 0.01–0.05 ng/g [109] levels, respectively. A mass chromatogram of a roasted chicken sample is shown in Fig. 6. The low detection limits achieved are in accordance with the specificity of LC–MS–MS under SRM conditions used in both cases. All the analytes displayed simple positive-ion mass spectra, with an intense protonated molecule and no fragment ions of relevant abundance. The use of MS–MS overcame the lack of structural information presented by ISP mass spectra.

Stable isotope dilution quantification was also used by Fay et al. [110] for the analysis of meat products for 15 HAs by LC–ES-MS. The LC–MS conditions are the same as described by Johansson et al. [114]. Additionally, a TSK ODS-Super column and the same mobile phase was used for the separation of the 15 HAs in half of the run time. The content of HAs was evaluated in various meat products using $[^2H_3]$IQ, $[^2H_3]$MeIQx, $[^2H_3]$4,8-MeIQx and $[^2H_3]$7,8-MeIQx as internal standards, added to the sample prior to the extraction procedure. IQx, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx and PhIP were detected and quantified in the meat

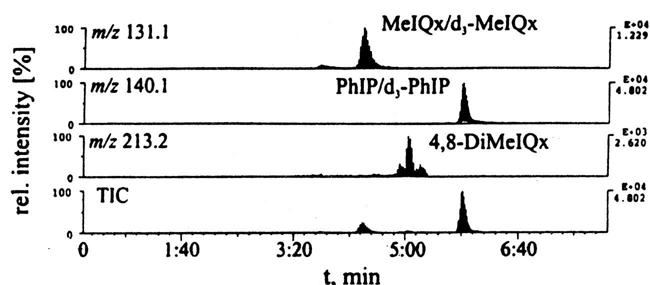

Fig. 6. LC–ESP-MS–MS chromatogram of HAs in roasted chicken extract. LC conditions: LiChrospher 60-RP select B column (100×2.0 mm I.D., 5 μm); mobile phase: solvent A 0.05% trifluoroacetic acid, solvent B: methanol–acetonitrile (1:2); gradient elution: 0–1 min: 20% B, 1–6 min: 20–80% B, 6–8 min: 80% B. SRM conditions: *m/z* 214.3/131.1 (45 V) for MeIQx, *m/z* 217.2/131.1 (45 V) for [$^2H_3$]MeIQx, *m/z* 228.1/213.2 (30 V) for 4,8-DiMeIQx, *m/z* 225.2/140.1 (55 V) for PhIP, *m/z* 228.2/140.1 (55 V) for [$^2H_3$]PhIP. Figure reproduced from Ref. [109].

samples. Additionally, Salmon et al. [115] used LC–ESP-MS for the confirmation of HAs in the study of marinades in complex grilled chicken samples. Complexity of the samples which were heated at temperatures as high as 370°C necessitated such confirmation. A microbore Zorbax C18-BD (100×1 mm) column and a mobile phase composed of methanol and acetic acid at 50 μl/min were used for the detection and confirmation of MeIQx and PhIP. Quantification was carried out by HPLC–DAD and fluorescence detection.

Although API-MS was first developed with quadrupole mass spectrometers, it has now successfully been coupled to an ion trap mass spectrometers. In the case of the HAs all applications described above used quadrupole mass spectrometers. Ion trap MS–MS using ESP as the ionisation source has been applied recently to the confirmation of six HAs detected in restaurant foods [116]. Separation took place on a YMC ODS-A column of 3 mm I.D. with a binary mobile phase of acetic acid–water and methanol running in a linear gradient. The LC–ESP-MS–MS method was applied to the confirmation of HAs using [$^2H_3$]IFP as internal standard and quantifying by HPLC–UV and fluorescence detection.

Related to the analysis of foods for HAs is analysis of biological materials for HAs and their metabolites. An LC–ESP-MS–MS method applied recently to the identification and characterisation of PhIP metabolites in human urine and plasma [117], identified the major PhIP metabolites in human urine and plasma (4′-OH-PhIP, *N*-OH-PhIP, 4′-PhIP-sulfate, *N*-OH-PhIP-N^2-glucuronide, *N*-OH-PhIP-N^3-glucuronide, PhIP-N^2-glucuronide).

2.4.2.2. LC–APCI-MS. An LC–APCI-MS method has been developed for the determination of 15 HAs and related compounds in beef extracts using in-source fragmentation for their identification and confirmation (Fig. 7) [72]. A conventional LC column (TSK-Gel ODS 80T), using a mobile phase of ammonium acetate (pH 5.7) and acetonitrile at a flow-rate of 1 ml/min in gradient mode, was employed for the separation. For maximum sensitivity, 30 V was applied using TriMeIQx as internal standard and the standard addition method for quantification. Proton addition and sodium addition were the common route of ionisation at this voltage. Parameters influencing mass spectra were optimised. The compounds, Glu-P-1, AαC, harman and norharman were detected at 2.8–129.5 ng/g concentration levels. Their confirmation was performed by applying a higher extraction voltage (70 V) to induce fragmentation of the molecular ion, and the most abundant fragments for each analyte were monitored. The fragmentation observed in these conditions was always higher than in ESP [105,106,108,109].

APCI has also been applied to the determination of eight HAs (IQ, MeIQ, MeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP, Trp-P-1 and Trp-P-2) in processed food flavours [73] and pre-processed meat cuts [118]. The familiar TSK-Gel ODS column was employed with a mobile phase of ammonium acetate (5 m*M*) at pH 3.2 or 3.8 and acetonitrile in a

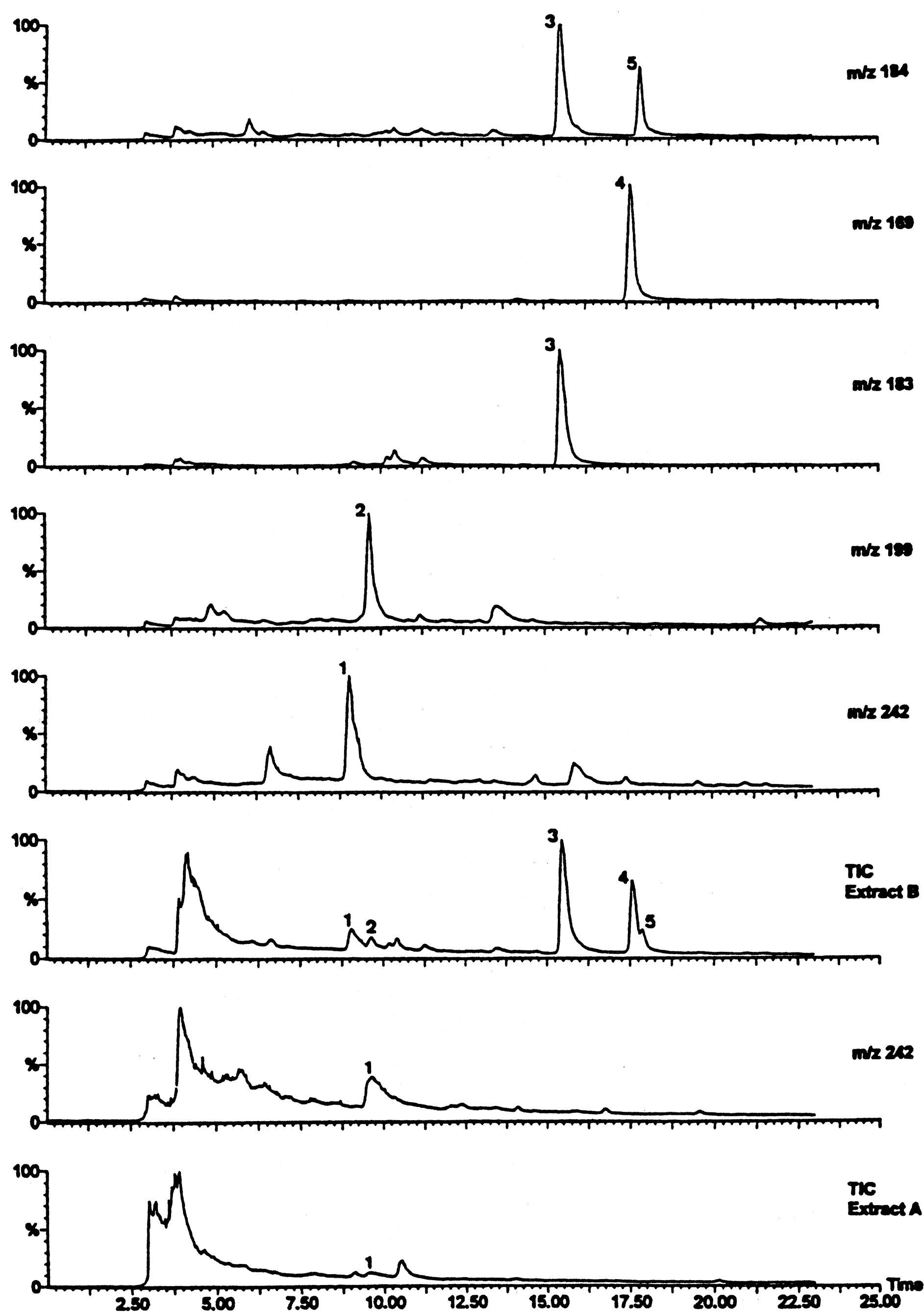

Fig. 7. LC–APCI-MS chromatogram of HAs in a beef extract. LC conditions: TSK-Gel ODS 80T column (250×4.6 mm I.D., 5 μm); mobile phase: solvent A: 50 m*M* ammonium acetate–acetonitrile (80:20), solvent B: acetonitrile; gradient elution: 0–10 min: 100% A, 10–30 min: 100–60% A. Mass spectrometer operating at an extraction voltage of 40 V. The bottom trace of each extract is the TIC obtained by summing all ions above. Peaks: 1. TriMeIQx, 2. Glu-P-1, 3. harman, 4. norharman, 5. AαC. Figure reproduced from Ref. [72].

gradient at 0.7 ml/min. Protonated molecular ions of the eight HAs and three deuterated internal standards ([$^{2}H_{3}$]IQ, [$^{2}H_{3}$]MeIQx and [$^{2}H_{3}$]PhIP) were monitored in SIM in two groups. Quantification was performed using the external standard method or the stable isotope dilution quantification method, which provides more accurate results and monitors the overall recovery of the clean-up procedure. Only IQ was quantified in the processed food flavours at concentrations of 2.1–9.6 ng/g. Only MeIQx was detected in a smoked turkey breast from 16 different cuts analysed.

The amines IQ, MeIQx, PhIP and AαC have been analysed in meat products by LC–APCI-MS–MS using stable isotope dilution quantification and multiple reaction monitoring (MRM) [119]. Deuterated HAs were used as internal standards for the isotope dilution quantification, to compensate for differences in recovery of the HAs through the extraction. Product ion spectra were obtained at several collision energies in order to determine optimal MRM responses. The transitions used for IQ, MeIQx and PhIP involved loss of the *N*-methyl group, and for AαC involved cleavage of the heterocyclic ring structure. Multiple reaction monitoring provided the high degree of sensitivity and selectivity needed for accurate quantification of HAs in complex meat matrices.

An ion trap APCI-MS has also been described for the determination of 14 HAs and related compounds in meat extracts [74]. The analytical column used was a TSK-Gel ODS 80T column using a mobile phase composed of ammonium formate (pH 3.25 and 3.7) and acetonitrile at a flow-rate of 1 ml/min in gradient mode. The $[M+H]^{+}$ ions were monitored in SIM and good results were obtained for the quantification of HAs in complex lyophilised meat extracts spiked with HAs. The LC–MS method simplified the clean-up procedure and reduced the time of analysis due to the specificity and selectivity of mass spectrometry. However, with this simplification, detection limits increased for the analysis of these complex samples [72,106,109]. The applicability of the method to naturally occurring HAs in heat processed food samples still has to be demonstrated.

2.4.2.3. Ionisation and fragmentation of HAs in LC–API-MS. HAs are stronger bases than mobile phase components and therefore, proton addition to form $[M+H]^{+}$ ion is the main route of their ionisation. Both APCI and ESP produce a soft ionisation, supplying little structural information. To obtain that information, two techniques have been applied: in-source fragmentation [72,106] and tandem MS [107–109,119] to induce fragmentation before entering the quadrupole in LC–MS or between the two quadrupoles in LC–MS–MS. Similar fragmentation patterns were observed in ESP-MS, compared with LC–ES-MS–MS. In addition to the protonated molecular ion, in the most sensitive conditions, the sodium ion $[M+Na]^{+}$ is observed in APCI for most of the HAs, which is not observed in ESP. Moreover, the gas phase in APCI can contain cluster ions from the interaction of the analyte with the mobile phase, and several ions containing acetonitrile are observed in the most sensitive conditions. In ESP, ionisation does not take place in the source, and the formation of these clusters is not observed. Both in ESP and APCI, tandem MS or MS, the loss of the *N*-methyl group and the cleavage of the imidazole ring from the protonated molecules are a common route of fragmentation of the aminoimidazo-azaarenes. For the other group of HAs, the pyrolytic amines, the loss of the primary amino group and the cleavage of the heterocyclic ring are the main fragmentations observed. The co-mutagens harman and norharman are cleaved at the methylpyridyl and pyridyl moieties. Fig. 8 shows mass spectra of some HAs when fragmented at high extraction voltages.

2.4.3. Detection limits in LC–MS

Detection limits in the infusion mode showed that APCI [72] was 5- to 40-fold more sensitive than ESP [106]. Otherwise, values are comparable in both ionisations when coupling liquid chromatography to mass spectrometry. Triethylamine is not used in API-MS due to the strong ionisation suppression allowing tailing peaks to decrease the chromatographic efficiency. Therefore, the detection limits increase. Conventional columns are used in APCI and, therefore, detection limits are not as low as expected from the results obtained from the infusion mode. When better columns such as semi-micro-, micro- or capillary columns are employed in the separation, better detection limits are achieved.

The best detection limits are seen using LC–ESP-

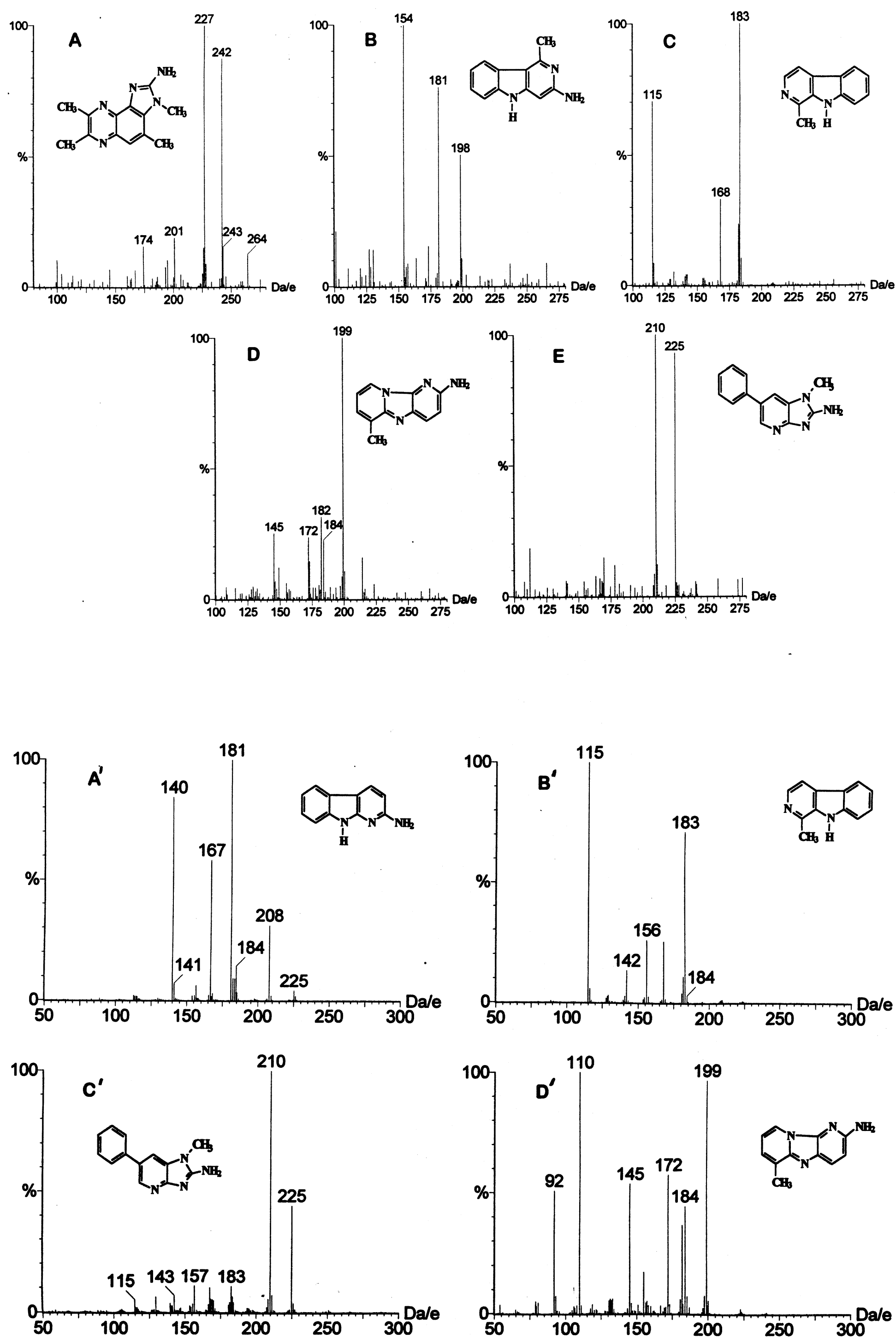

Fig. 8. ESP and APCI spectra obtained at high extraction voltages in order to obtain fragmentation: ESP at 150 V and APCI at 70 V. ESP mass spectra: (A) TriMeIQx, (B) Trp-P-2, (C) harman, (D) Glu-P-1, (E) PhIP. APCI mass spectra: (A′) AαC, (B′) harman, (C′) PhIP, (D′) Glu-P-1. Figures reproduced from Refs. [72] and [106].

MS–MS with values of 0.01–0.05 ng/g for cooked meat or fish [109]. Values were between 8- and 10-fold lower than with LC–MS, because of the specificity of MS under SRM conditions and the use of a semi-micro HPLC column, which offer better concentration factors than conventional columns and hence better sensitivities. With LC–APCI-MS–MS, detection limits varied with sample matrix [119] and were comparable to detection limits with LC–MS. Differences between these tandem methods are probably due to the kind of samples analysed. The better chromatographic efficiency was obtained with the semi-micro column in LC–ESP-MS–MS, and the two SRM experiments employed in LC–ESP-MS–MS achieved maximum sensitivity.

The detection limits estimated in food samples were comparable for the three ionisation techniques using LC–MS. Moreover, the values were similar to those obtained with HPLC and electrochemical detectors, and are lower than detection limits of HPLC–UV systems. LC–TSP-MS gave detection limits of 0.7 ng/g in grilled bacon, beef, fish and in grill scrapings [69]. Detection limits in ESP gave values between 0.1 and 2.3 ng/g for beef extracts [106] and 0.1 ng/g in meat samples [110]. Detection limits of LC–APCI-MS techniques reached values of 0.08–1.4 ng/g for beef extracts [72], 1–3 ng/g for processed flavours [73] and 0.6–1.1 ng/g for meat cuts [118]. Detection limits of LC–APCI-MS–MS varied with the HA and sample matrix but were around 0.1–1 ng/g of meat products [119]. Detection limits obtained with an ion trap LC–APCI-MS system were reported to be 0.8–10.1 ng/g in a lyophilised beef extract [74]. These values were higher than those obtained with other LC–MS methods for determining complex food matrices because sample preparation was simplified with respect to the other methods.

2.5. Capillary electrophoresis

In recent years CE has been widely used for the separation of complex mixtures. The efficiencies of the silica capillaries in the separation can achieve values up to two orders of magnitude greater than HPLC columns. Moreover, the simplicity in method development, the broad working pH range (1.5–13), the rapidity of the analysis, the easy automatisation, and the low operation cost, make CE a powerful technique for the analysis of charged solutes. In the analysis of HAs CE has been applied to the determination of food and environmental samples. HAs are weak bases, and their ionisation, and thus electrophoretic mobility, strongly depends on the pH. In the low pH range, HAs are more effectively converted into the protonated species, providing better separation in shorter migration times. Hence, all CE methods reported for HAs work with buffers at the pH range of 2.7–3.2. Generally, UV has been used for the detection of HAs. However, MS and ED have also been employed for achieving higher selectivity. In the development of CE methods, general parameters affecting the resolution, such as pH, organic modifier, concentration of buffer, capillary conditioning, applied voltage, capillary temperature, and injection mode, are investigated and optimised.

Wu and co-workers [120–122] reported the first work on CE for the optimisation of the separation of 13 HAs and related compounds by orthogonal array design and overlapping resolution mapping [120,122]. The optimum separation was achieved in an uncoated silica capillary (46.4 cm) within 12 min, using sodium phosphate–methanol as buffer, a constant applied voltage of 18 kV, and a photodiode array detector. The method was applied to the determination of HAs in rainwater, not a complex matrix such as a food sample which may present many interferences. Initial work developed for the analysis of HAs in food samples by CE–UV investigated MeIQ, MeIQx, PhIP, Trp-P-2 and Glu-P-1 in a complex meat extract [123]. Optimum conditions use a carrier electrolyte of KCl–HCl (pH 2.20) and 20 kV in the hydrodynamic injection mode. Additionally, Mardones et al. [124] developed a CE–DAD method for the determination of IQ, MeIQx, 4,8-DiMeIQx and PhIP in fried beef, salmon and commercial meat extract. The method separated the amines within 19 min using a fused-silica capillary; sodium phosphate, methanol and sodium chloride at pH 2.0 as the electrolyte; and a constant potential of 19 kV. HAs in salmon and meat extract were below the detection limits. IQ, MeIQx and 4,8-DiMeIQx were detected and confirmed by DAD in fried beef at concentrations from 2 to 12.5 ng/g.

Electropherograms corresponding to beef extracts [123] and fried beef [124] by CE–UV revealed

numerous peaks and indicated the need for selective and sensitive detectors to confirm the identities of the signals. Hence, Olsson et al. [125] developed a micellar electrokinetic chromatography system together with electrochemical detection for the separation and detection of eight heterocyclic amines, which made possible the analysis of HAs in a pan residue from the frying of meat. Fig. 9 shows an electropherogram of the pan residue where Glu-P-2, MeIQx, IQ, 7,8-DiMeIQx and 4,8-DiMeIQx can be detected in the sample at concentrations of 0.011–0.84 mg/l, which correspond to 0.014–1.0 ng/g of meat weight.

A CE–MS method was developed for the determination of 14 HAs, benefiting from the selectivity and sensitivity of mass spectrometry [126]. The method was optimised under CE–UV conditions with polyvinyl alcohol-coated capillary, an ammonium acetate (pH 3.0) and methanol buffer, and a 22 kV potential to perform the separation of 14 HAs and related compounds. The two techniques were coupled to an electrospray ionisation source augmented by methanol–water (9:1) containing 0.1% acetic acid used as the sheath fluid to give the optimum flow-rate of 4 μl/min entering the ESP source. The potential of CE–ES-MS was illustrated by the analysis of six HA standards. CE–MS can not compete with LC–ES-MS in terms of sensitivity and stability, because of the low volume requirement and poorer robustness of CE. The advantage of CE–ES-MS over CE–ED is the unambiguous identification achieved with the mass spectrum.

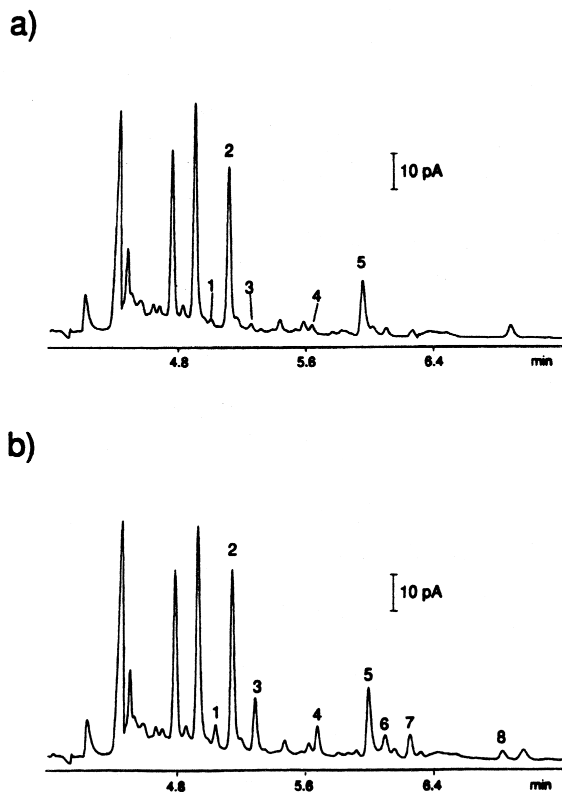

Fig. 9. Electropherograms of a pan residue extract. Amperometric detection at carbon fibber disc electrode 30 μm, 0.6 V vs. Ag/AgCl; buffer: 15 m*M* borax adjusted to pH 9.1 with phosphoric acid, 5 m*M* CTAB; capillary: 60 cm×20 μm I.D.; separation potential: −25 kV. (a) Extract, (b) extract spiked with 0.1 mg/l of each HA. Peaks: 1. Glu-P-2, 2. MeIQx, 3. IQ, 4. 7,8-DiMeIQx, 5. 4,8-DiMeIQx, 6. MeIQ, 7. Glu-P-1, 8. TriMeIQx. Figure reproduced from Ref. [125].

Mendosa and Hurtubise [127] studied literature methods and attempted to improve their efficiency in separating a group of eight HAs and related compounds: IQ, MeIQx, 4,8-DiMeIQx, TriMeIQx, PhIP, AαC, Trp-P-2 and norharman. Their separation was investigated with the phosphate buffer system first reported by Wu and co-workers [120–122] and the KCl–HCl electrolyte system developed by Puignou et at. [123]. Both systems separated all eight compounds. However, the KCl–HCl system had several advantages over the phosphate system. The phosphate buffer produced very high currents of 170 μA, which sometimes led to a breakdown of current in the CE instrument, while the KCl–HCl electrolyte produced a lower background noise, lower detection limits, and better resolution without the use of organic solvent, and required less analysis time. Additionally, the electrokinetic chromatography method developed by Olsson et al. [125] was also studied for these compounds. The system gave sharp peaks only for the IQ-type compounds, broad peaks with long migration times for PhIP and norharman, and no detectable signal for Trp-P-2 and AαC. Differences from Olsson's results may be attributed to the fact that different detectors were used and different compounds were studied. In addition, these authors [128] applied CE for the determined the ionisation constants of these eight HAs, demonstrating CE to be rapid, precise and sensitive.

Using CE–UV, detection limits with standard solutions were estimated to be 0.2–0.8 mg/l

[121,124], or 35–50 ng/g for meat extracts [123]. These values are high considering the low part-per-billion concentration of HAs that may be present in most meats. The micellar electrokinetic chromatography system with electrochemical detection achieved detection limits of 4.0–21 ng/ml [125], which are 2- to 12-times better than the ones obtained by CE–UV and comparable to those obtained by HPLC with electrochemical detection, ranging from 0.4 to 34 ng/ml [37,47,48,62,70,86–88]. For the CE–MS method [126] detection limits were in the order of 50 ppb for the individual HAs. Considering the detectabilities which should be reached, the practical application of the method should include a sample preparation with an enrichment factor of at least 50 to reach the required detection ranges for HAs in foods, which the authors are developing.

2.6. Immunoaffinity chromatography

HAs have been analysed by using monoclonal antibodies, either by immunoassay [46,129,130] or by purification followed by direct quantification using HPLC–UV [28,31]. Immunoaffinity chromatography has been used to purify IQ and MeIQx from complex mixtures such as heated beef products and beef extracts [28]. The limit of detection by HPLC–UV approaches 1 ng/g of beef extract or cooked. This value is comparable to the sensitivity limits obtained for IQ, IQx, 4,8-DiMeIQx, 2-nitro-IQ, MeIQx and 4-MeIQx by competitive ELISA of heated meat products [46]. By its simplicity, rapidity and accuracy, these methods seem to be especially suited for routine analysis of those heterocyclic amines for which monoclonal antibodies are available. However, due to the complexity of the heated food matrix, monoclonal antibodies have only proven useful in limited applications.

3. Quantification of HAs

It is important to emphasise that the use of an internal standard is necessary to accurately quantify HAs, since analyte extraction efficiency is below 100% and amounts of HAs detected have to be corrected for incomplete recoveries. Using a single internal standard for this correction is not ideal because HAs include several classes of compounds which are extracted with varying efficiencies. Even within a class of compounds, significant extraction variation has been reported [51,64,71,102]. The sample matrix greatly influences extraction efficiencies, therefore, the multiple standard addition quantification method is the best way for quantifying the HAs, allowing the recovery of each analyte to be assessed individually in each matrix. Multiple extractions with multiple aliquots of each sample spiked with the standards give sets of concentration data for each analyte. Stable isotope dilution quantification using SIM GC–MS or LC–MS reduces the number of samples to be extracted per determination since the extraction efficiencies are calculated in the same analysis. The disadvantage of this technique is that there are no labelled standards are expensive and not always available.

An internal standard should be used to control the final volume obtained from the purification and preconcentration steps. Generally, a volume of 50–100 μl of a solution containing an internal standard is used for dissolving the final extract and injected in duplicate in the HPLC, GC or CE. A calibration curve of the internal standard is performed in order to control precisely this final volume. The internal standards normally used are, caffeine [50,76,85], 7,8-DiMeIQx [70] and TriMeIQx [67,69,70,77] in HPLC–UV, fluorescence or electrochemical detection. Labelled standard of the heterocyclic amines such as [$^{2}H_{3}$]IQ, [$^{2}H_{3}$]MeIQ, [$^{2}H_{3}$]MeIQx, [^{13}C,$^{15}N_{2}$]MeIQx, [^{13}C,$^{15}N_{3}$]MeIQx, [$^{2}H_{3}$]4,8-MeIQx, [$^{2}H_{3}$]7,8-MeIQx, [$^{2}H_{3}$]PhIP, [$^{2}H_{5}$]PhIP, [$^{2}H_{3}$]Trp-P-2, [$^{2}H_{3}$]norharman and [$^{2}H_{3}$]IFP have been used in the GC–MS and LC–MS techniques to perform stable isotope dilution quantification. Internal standards also monitor for retention time variation. Heavy isotope labelled standards sometimes have a few percent of the natural isotopes, so trace analysis studies need to consider these contaminants [116].

4. Levels of HAs in cooked foods

There is an extensive literature on the presence of HAs in meats cooked at high temperature, but in

many of the reported studies the cooking is not well described, analysis methods vary and might not be comparable, or samples are cooked to maximise the production of HAs, and not to be representative of the ways meats are usually cooked and eaten by general population [69,75,131,132]. Formation of HAs varies depending on the cooking technique and the degree of doneness [79,81] and on uncontrolled variables such as the meat quality and composition and meat geometry.

Table 1 presents a brief summary of quantitative data from the recent literature on the amounts of HAs in cooked meat and fish products from household cooking practice, restaurant samples, precooked products, pan residues, beef extracts, commercial process flavours and bouillon cubes.

HAs have been reported in all kinds of meat and fish products, generally those cooked by frying, grilling, broiling, present higher levels. PhIP and MeIQx are the most constantly reported and abundant compounds, more MeIQx than PhIP is detected at moderate temperatures, but PhIP is the predominant HA in well-cooked samples.

In addition to human exposures through foods, MeIQx, 4,8-DiMeIQx, PhIP and AαC have been detected in cooking fumes at 0.007–1.8 ng/g of cooked meat [55,56,92]. These mutagens have also been identified in cigarette smoke [49,84,139], environmental samples [54,90,121,140,141] beer and wine [104,108,142] and other alcoholic beverages [104].

Epidemiological studies reported to date have not specifically estimated HA exposure, but have used surrogates such as dietary questionnaires of cooking methods and doneness levels of various kinds of meats consumed. The results have been mixed. Although some studies find that the amount of well-done or well-browned red meat, fried or barbecued meats and gravy made from drippings increases the risk of colorectal cancer [13,14,77,143]. Other studies find no association between colorectal cancers and degree of doneness of meat or with various methods of cooking meats [14,144,145]. The conflicting results in the literature may be due to the variability in HA formation, thereby leading to misclassification of exposure. More research is needed to determine processing conditions that minimise the formation of mutagenic HAs in foods.

5. Conclusion

The analysis of foods for HAs is an active area of research, and high quality data are needed. It is important to choose the right technique to analyse these amines to achieve accurate and precise results of their content in cooked foods, based on the availability of instruments, sample type, and experience in sample preparation.

Generally, the most widely used solid-phase extraction method developed by Gross [35,51] followed by HPLC–DAD gives good results for the analysis of HAs in foods. To improve the analysis of difficult samples such as processed flavours and the products of model systems, sample clean-up procedures has been vigorously investigated. The efforts to obtain ‘‘cleaner’’ extracts with good recoveries of HAs have been directed towards developing the best extraction methods and conducting the most efficient purifications using different procedures or columns [50,51,58,64,66]. Fluorescence or electrochemical detection have been used with some success in this kind of samples due the high selectivity and sensitivity they provide, but they still do not allow for the necessary peak confirmation and additional techniques must be used for this purpose. Mass spectrometry coupled to chromatographic techniques, GC or LC, has been applied to the analysis of HAs and good results have been achieved, with detection limits lower than 0.1 ng/g, due to the high selectivity and specificity of mass spectrometry. GC–MS has proven to be very sensitive, specific, selective, simple and rapid for the determination of heterocyclic amines in foods. However, derivatisation schemes are needed and a widely applicable method for all known HAs for food analyses still does not exist. Although, in the most sensitive single-ion monitoring mode, there are frequently no abundant secondary ions for confirmation of the base peak. LC–MS–MS and in-source fragmentation LC–MS approaches have been developed for confirmation of HAs obtaining characteristic fragmentation. A difficulty of the chromatographic techniques coupled to MS is that they require expensive instrumentation not available in most of the laboratories performing routine analysis of HAs. Otherwise, the efficiencies of silica capillaries make CE an attractive technique for the analysis of HAs in foods.

Table 1
Occurrence of HAs in cooked foods (ng/g)[a]

Food sample	HA	Concentration (ng/g)	Reference[b]	Food sample	HA	Concentration (ng/g)	Reference[b]
Beef	IQ	nd–21	1–23	*Poultry meat*	IQ	nd–5	12, 13, 15, 18, 20, 22, 39, 53, 54
	IQx	nd	20, 22		IQx	nd–0.17	20, 22, 54
	MeIQ	nd–0.6	9, 13, 15–23		MeIQ	nd–0.9	12, 18, 20, 22, 39, 53
	MeIQx	nd–16.4, max 80	1, 2, 5–18, 20–37		MeIQx	nd–3.2, max 270	5, 12, 13, 15, 18, 20, 22, 29, 33, 37, 39, 53–55
	4-MeIQx	nd	22		4-MeIQx	nd	22
	4,8-DiMeIQx	nd–15	5, 7–18, 20–22, 24, 27–32, 34, 36, 37–39		4,8-DiMeIQx	nd–4	5, 12, 13, 18, 20, 22, 29, 37, 39, 53, 54
	7,8-DiMeIQx	nd–0.7	5, 15, 18, 20, 27		7,8-DiMeIQx	nd–0.16	5, 18, 20, 54
	TriMeIQx	nd	18, 20		TriMeIQx	nd	18, 20
	PhIP	nd–18.4, max 182	5, 7, 9–12, 14–18, 20–24, 27, 29, 33–38, 40, 41		PhIP	nd–37.5, max 480	12, 13, 15, 18, 20, 22, 29, 33, 37, 39, 53–55
	DMIP	nd–7.2	22, 35		DMIP	nd–5.9	22
	1,5,6-TMIP	nd–1.5	22, 35		1,5,6-TMIP	nd–2.9	22
	IFP	nd–7.6, max 46	22, 35		IFP	0.9–7	22
	4′-OH-PhIP	nq, 21	31, 41		AαC	nd–2, max >100	12, 15, 22, 54
	AαC	nd–21	9, 12, 15, 22, 30, 32		MeAαC	nd	22, 54
	MeAαC	nd	22		Trp-P-1	nd–1.6	22, 18, 29, 54
	Trp-P-1	nd–0.5	9, 18, 22, 23, 29		Trp-P-2	nd–0.14	22, 18, 29, 54
	Trp-P-2	nd–1.7	9, 15, 18, 22, 23, 29, 42		Glu-P-1	nd	20, 54
	Glu-P-1	nd	20, 22		Glu-P-2	nd	20, 54
	Glu-P-2	nd	20, 22		Harman	nq, 0.12	29, 54
	Harman	0.31–28.6	14, 23, 29, 34, 63		Norharman	nq, 0.1	29, 54
	Norharman	0.96–30	14, 23, 29, 34, 63				
Beef extract and process flavour	IQ	nd–15, max 70.3	2, 8, 11, 24, 26, 27, 42–49	*Pan residues*	IQ	nd–1.7	14, 17, 44
	IQx	nd–0.7	44		IQx	2.0	44
	MeIQ	nd–5.8	24, 44, 46–49, 62		MeIQ	nd–1.7	17, 44
	MeIQx	nd–69	1, 2, 10, 11, 24, 26, 27, 38, 43, 44, 46–50, 62		MeIQx	nd–7.3, max 140	14, 17, 29, 30, 34, 44
	4,8-DiMeIQx	nd–11.2	1, 5, 10, 11, 27, 38, 44, 46, 47, 49		4,8-DiMeIQx	nd–14.6	14, 17, 29, 30, 44
	7,8-DiMeIQx	nd–0.7	5, 27, 44, 48		7,8-DiMeIQx	nd	44
	TriMeIQx	nd	48		PhIP	nd–13.3, max 144	14, 17, 29, 30, 34, 44
	4-CH_2OH-8MeIQx	6	41		AαC	nd–0.28, max 76	30, 44, 51
	7,9-DiMeIgQx	53	41		MeAαC	nd–0.08	44, 51
	PhIP	nd–49	5, 10, 11, 38, 44–48		Trp-P-1	nd–0.08	29, 44, 51
	AαC	nd–8.1	44–47, 51, 52		Trp-P-2	nd	29, 44, 51
	MeAαC	nd–20.3	44, 46, 47, 51		Harman	nd–14.0	14, 29, 34, 44, 51
	Glu-P-1	15.5	46, 47, 49		Norharman	nd–52.6	14, 29, 34, 44, 51
	Trp-P-1	nd–13	44–48, 51				
	Trp-P-2	nd–14	44–48, 51, 52				
	Harman	3.3–135, max 755	44–47, 51, 52				
	Norharman	8.3–74, max 200	44–47, 51, 52				

Table 1. Continued

Food sample	HA	Concentration (ng/g)	Reference[b]	Food sample	HA	Concentration (ng/g)	Reference[b]
Pork	IQ	nd–10.5	9, 12–14, 17, 18, 20, 22	*Fish*	IQ	nd–4.9	11–13, 15, 17, 19, 20, 22, 56, 57
	IQx	nd	20, 22		IQx	nd	20, 22
	MeIQ	nd–1.7	9, 13, 17, 18, 20, 22		MeIQ	nd–16.6	13, 17, 19, 20, 22, 56, 57
	MeIQx	nd–3.5, max 45	5, 9, 12–14, 17, 18, 20, 22, 23, 29, 30, 35–37		MeIQx	nd–8.3	11–13, 15, 17, 20, 22, 29, 57–59
	4-MeIQx	nd	22		4,8-DiMeIQx	nd–7.0	12, 13, 17, 20, 22, 29, 57
	4,8-DiMeIQx	nd–12	5, 9, 12–14, 17, 18, 20, 22, 29, 30, 35–37		7,8-DiMeIQx	nd–5.3	20, 58
	7,8-DiMeIQx	nd–0.3	5, 18, 20		TrMeIQx	nd	20
	TriMeIQx	nd	18, 20		PhIP	nd–3.0, max 69.2	11–13, 15, 17, 20, 22, 29, 57, 59
	PhIP	nd–7.4, max 106	9, 14, 17, 18, 20, 22, 23, 29, 30, 35–37, 51		DMIP	0.9	22
	DMIP	nd–37	22, 35		1,5,6-TMIP	2.5	22
	1,5,6-TMIP	nd	22, 35		IFP	2.1	22
	IFP	nd–2.5	22, 35		AαC	nd–2.3, max 109	12, 15, 22, 59, 60
	AαC	nd–trace	22, 30, 51		MeAαC	nd	22
	MeAαC	nd–3.2	5, 9, 12, 13, 22		Trp-P-1	nd–13.3	22, 29, 60, 61
	Trp-P-1	nd–5.3	18, 22, 23, 29, 51		Trp-P-2	nd–13.1	22, 29, 42, 61
	Trp-P-2	nd–7.4	18, 22, 51, 29		Glu-P-1	nd	20, 22
	Glu-P-1	nd	20, 22		Glu-P-2	nd	20, 22
	Glu-P-2	nd	20, 22		Harman	2–130	29, 59, 63
	Harman	nd–2.53, max 200	14, 23, 29, 51		Norharman	2–184	29, 59, 63
	Norharman	nd–10.6	14, 23, 29, 51				
Lamb	MeIQx	nd–1.6	29, 37				
	4,8-DiMeIQx	nd–0.6	29, 37				
	PhIP	nd–11	29, 37				
	Trp-P-1	1.0	29				
	Trp-P-2	nd	29				
	Harman	nq	29				
	Norharman	nq	29				

[a] nd: Not detected, nq: not quantified, max: maximum concentration ever detected.
[b] Numbers corresponding to the following references:

1. Ref. [63]	2. Ref. [28]	3. Ref. [29]	4. Ref. [36]	5. Ref. [43]
6. Ref. [3]	7. Ref. [42]	8. Ref. [60]	9. Ref. [55]	10. Ref. [53]
11. Ref. [133]	12. Ref. [131]	13. Ref. [76]	14. Ref. [77]	15. Ref. [119]
16. Ref. [79]	17. Ref. [84]	18. Ref. [118]	19. Ref. [61]	20. Ref. [109]
21. Ref. [78]	22. Ref. [82]	23. Ref. [80]	24. Ref. [114]	25. Ref. [84]
26. Ref. [46]	27. Ref. [45]	28. Ref. [37]	29. Ref. [81]	30. Ref. [69]
31. Ref. [95]	32. Ref. [56]	33. Ref. [135]	34. Ref. [85]	35. Ref. [116]
36. Ref. [136]	37. Ref. [71]	38. Ref. [35]	39. Ref. [134]	40. Ref. [41]
41. Ref. [49]	42. Ref. [93]	43. Ref. [62]	44. Ref. [66]	45. Ref. [105]
46. Ref. [106]	47. Ref. [72]	48. Ref. [73]	49. Ref. [70]	50. Ref. [40]
51. Ref. [102]	52. Ref. [64]	53. Ref. [89]	54. Ref. [67]	55. Ref. [115]
56. Ref. [32]	57. Ref. [68]	58. Ref. [30]	59. Ref. [51]	60. Ref. [100]
61. Ref. [137]	62. Ref. [48]	63. Ref. [138]		

With the development of LC–MS, accurate methods of measuring the presence of these mutagens in the diet are available and a better assessment of exposure and human health risk towards these chemicals can be made. LC–MS has acquired a role of growing importance in food analysis, as is attested to by the growing number of applications reported in recent years. Nevertheless, photodiode array detection is the most widely used method in the analysis of HAs.

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