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Review

Analytical methods applied to the determination of heterocyclic aromatic amines in foods

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Abstract

Analytical aspects concerning the heterocyclic aromatic amines (HAAs) determination in foods are reviewed. Sample pre-treatment procedures such as liquid–liquid extraction (LLE), supercritical fluid extraction, solid-phase extraction (SPE), solid-phase microextraction (SPME), and the mainly used LLE–SPE tandem extraction are discussed. The analytical methods used for the identification and quantification are HPLC, HPLC combined with single or tandem MS detection (HPLC-MS, HPLC-MS/MS), GC–MS and capillary electrophoresis. Advantages and figures of merit for each technique are discussed.

Keywords: Heterocyclic aromatic amines; Foods; Extraction methods; Chromatographic methods/mass spectrometry; Capillary electrophoresis

Contents

1. Introduction

To date, more than 25 HAAs have been isolated as potent mutagens in the Ames/*Salmonella* test, and have been characterized. Table 1 shows their chemical and abbreviated names, molecular weights and some properties. All these heterocyclic amines contain from two to five (generally three) condensed aromatic cycles with one or more nitrogen atoms in their ring system and, usually, one exocyclic amino group.

HAAs are formed during the heating process of organic products containing nitrogenous compounds, mainly proteins. The achieved temperature has an important influence on the kind of HAAs formed. The HAAs formed at temperatures between 100 and 300 °C are known as "thermic HAAs", IQ type or aminoimidazoazarenes, and the others formed at higher temperatures, above 300 °C, are known as "pyrolytic HAAs", or non-IQ type. The thermic HAAs are generated from the reaction of free

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Table 1
Classification of HAAs

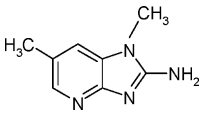
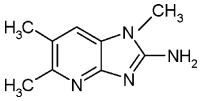
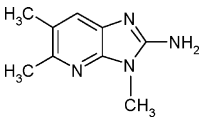
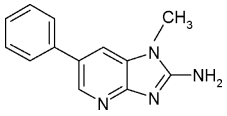
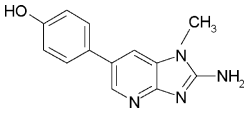
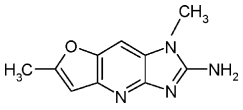
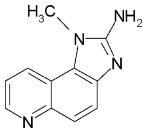
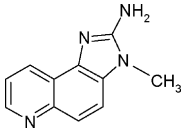
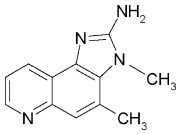
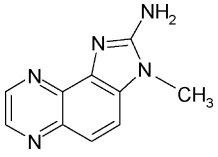
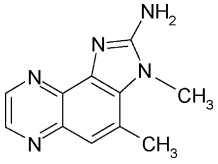
Chemical name	Abbreviated name	Structure	Molecular mass and properties
Isolated thermic HAAs: aminoimidazoazarenes			
Imidazopyridine derivatives			
2-Amino-1,6-dimethylimidazo [4,5-b]-pyridine	DMIP		162.2, polar
2-Amino-1,5,6-trimethylimidazo[4,5-b]-pyridine	1,5,6-TMIP		176.2, polar
2-Amino-3,5,6-trimethylimidazo[4,5-b]-pyridine	3,5,6-TMIP		176.2, polar
2-Amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine	PhIP		224.3, pK_a = 5.6, polar
2-Amino-1-methyl-6-(4′-hydroxyphenyl)-imidazo[4,5-b]-pyridine	4′-OH-PhIP		240.3, polar
2-Amino-1,6-dimethyl-furo[3,2-e]imidazo[4,5-b]-pyridine	IFP		202.3, polar
Imidazoquinoline derivatives			
2-Amino-1-methyl-imidazo[4,5-f]-quinoline	iso-IQ		198.2, polar
2-Amino-3-methyl-imidazo[4,5-f]-quinoline	IQ		198.2, pK_{a1} = 3.5, pK_{a2} = 6.1, polar
2-Amino-3,4-dimethyl-imidazo[4,5-f]-quinoline	MeIQ		212.3, pK_a = 6.4, polar
Imidazoquinoxaline derivatives			
2-Amino-3-methyl-imidazo[4,5-f]-quinoxaline	IQx		199.3, polar
2-Amino-3,4-dimethyl-imidazo[4,5-f]-quinoxaline	4-MeIQx		213.3, polar

Table 1 (*Continued*)

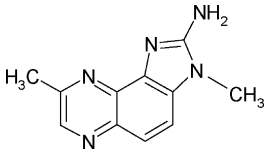
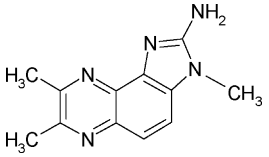
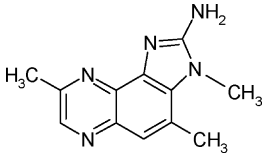
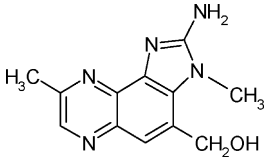
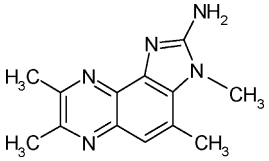
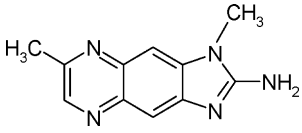
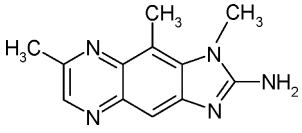
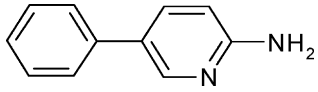
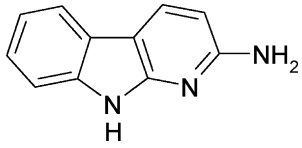
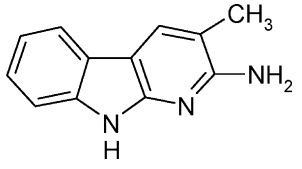
Chemical name	Abbreviated name	Structure	Molecular mass and properties
2-Amino-3,8-dimethyl-imidazo[4,5-f]-quinoxaline	8-MeIQx		213.3, pK_a = 5.95, polar
2-Amino-3,7,8-trimethyl-imidazo[4,5-f]-quinoxaline	7,8-DiMeIQx		227.3, pK_a = 6.5, polar
2-Amino-3,4,8-trimethyl-imidazo[4,5-f]-quinoxaline	4,8-DiMeIQx		227.3, pK_a = 5.8, polar
2-Amino-4-hydroxymethyl-3,8-dimethyl-imidazo[4,5-f]-quinoxaline	4-CH_2OH-8-MeIQx		243.3, polar
2-Amino-3,4,7,8-tetramethyl-imidazo[4,5-f]-quinoxaline	TriMeIQx		241.3, pK_a = 6.0, polar
2-Amino-1,7-dimethyl-imidazo[4,5-g]-quinoxaline	7-MeIgQx		213.3, polar
2-Amino-1,7,9-trimethyl-imidazo[4,5-g]-quinoxaline	7,9-DiMeIgQx		227.3, polar
Isolated pyrolytic HAAs: carbolines			
Phenylpyridine derivatives			
2-Amino-5-phenylpyridine	Phe-P-1		170.2, non-polar
Pyridoindole derivatives: α-carbolines			
2-Amino-9H-pyrido[2,3-b]indole	AαC		183.2, pK_a = 4.4, non-polar
2-Amino-3-methyl-9H-pyrido[2,3-b]indole	MeAαC		197.2, non-polar

Table 1 (*Continued*)

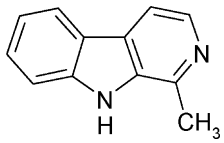
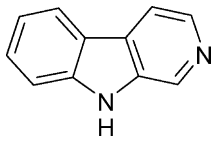
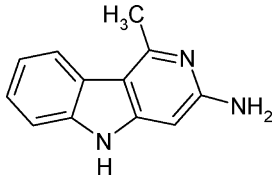
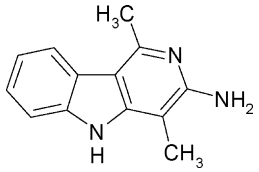
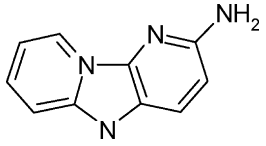
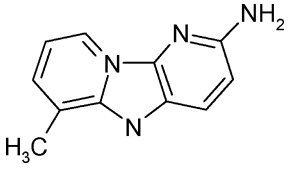
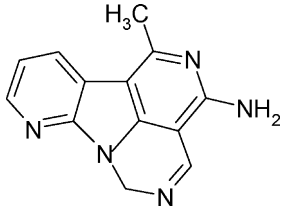
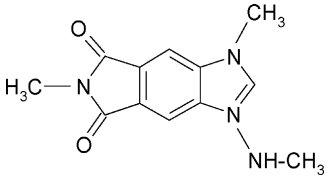
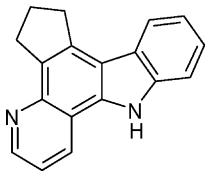
Chemical name	Abbreviated name	Structure	Molecular mass and properties
β-Carbolines			
1-Methyl-9H-pyrido[3,4-b]indole	Harman		182.3, non-polar co-mutagenic
9H-Pyrido[3,4-b]indole	Nor-harman		168.2, pK_a = 6.8, non-polar co-mutagenic
γ-Carbolines			
3-Amino-1-methyl-5H-pyrido[4,3-b]indole	Trp-P-2		197.4, pK_a = 8.5, non-polar
3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]-indole	Trp-P-1		211.3, pK_a = 8.6, non-polar
Pyridoimidazole derivatives δ-carbolines			
2-Aminodipyrido-[1,2-α:3′,2′-d]imidazole	Glu-P-2		184.3, pK_a = 5.9, non-polar
2-Amino-6-methyldipyrido-[1,2-α:3′,2′-d]imidazole	Glu-P-1		198.3, pK_a = 6.0, non-polar
Tetraazafluoranthene derivatives			
4-Amino-6-methyl-1H-2,5,10,10b-tetraazafluoranthene	Orn-P-1		237.3, non-polar
Benzimidazole derivatives			
4-Amino-1,6-dimethyl-2-methylamino-1H,6H-pyrrolo-[3,4-f]benzimidazole-5,7-dione	Cre-P-1		244.3, non-polar

Table 1 (*Continued*)

Chemical name	Abbreviated name	Structure	Molecular mass and properties
Carbazole derivatives			
3,4-Cyclopenteno-pyrido[3,2-a]carbazole	Lys-P-1		246.3, non-polar

amino acids, creatin(in)e and hexoses. The precursor undergoes further dehydration and cyclization to form the observed pyrrole and pyridine derivatives. The heterocyclic pyridines and pyrazines formed in the Maillard reaction between hexose and amino acids, undergo further transformation with participation of Strecker aldehydes and creatin(in)e to produce imidazoquinoxalines, perhaps through free-radical reactions. However, at temperatures as high as 225 and 250 °C, these compounds seem to degrade or react with other compounds [1]. In the case of the non-IQ type, the formation takes place through pyrolytic reaction among amino acids and proteins. Pyrolysis occurs at temperatures higher than 300 °C, and produces many reactive fragments through radical reactions. These fragments are believed to condense to form new heterocyclic structures, and pyrolytic mutagens might be formed via free-radical reactions. The mechanisms of formation of Trp-P-1, Trp-P-2, AαC and MeAαC, are unknown. TriMeIQx is a synthetic substance formed in model systems but not in heated foods [2]. An isomer of 8-MeIQx was discovered in grilled meat and human urine [3]. Recently, the compounds IQ[4,5-*b*], 7-MeIgQx and 7,9-DiMeIgQx have been identified and quantified in meats cooked under common household conditions [4,5]. In addition, six novel compounds that appear to contain the IQx skeleton have also been detected. One of them, 7-MeIgQx, has the same nominal molecular weight as 4-MeIQx and 8-MeIQx [5]. Other four are likely to be isomers of DiMeIQx [4].

The HAAs are mutagenic not only for bacteria, but also for some mammalian cell systems and can produce chromosomal aberrations and sister chromatid exchanges in cultured cells. In addition, some of them show higher mutagenic activity in bacteria and certain animals than typical mutagens/carcinogens such as benzo{*a*}pyrene or aflatoxin B_1. In 1993, the International Agency for Research on Cancer (IARC) [6] considers eight of the HAAs tested (MeIQ, MeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2 and Glu-P-1) as possible human carcinogens (class 2B) and one (IQ) as a probable human carcinogen (class 2A) and recommends a reduced exposure to these compounds. These results are based on the conclusions of long-term animal feeding studies. To assess the intake of HAAs it is important to collect data on the content of these contaminants in different types of foods prepared in various ways.

Many of these HAAs have been isolated from proteinaceous foods including cooked meats and fish, meat extracts or process flavours. They are also present in cooking fumes [7,8], several foods [9,10], coffee [11], alcohol beverages [10,12], and from environmental sources, such as cigarette smoke [13–17], air [13], river and rainwater [18,19]. Also, some HAAs have been detected in human tissues [20], hair [21] and in biological fluids, such as plasma, urine or bile [3,22–27], as well as in milk of healthy women [28,29].

These facts have generated great interest on HAAs, which have been widely investigated. In relation to the determination of HAAs in foods, some reviews on sample treatment [30–32] and analytical methods applied [31,33–38] have been performed.

This paper belongs to a series of reviews on different aspects of HAAs, which cover the period from 1992 up to 2007. The reviewed aspects are: their occurrence and formation during the foods cooking, mainly meat and fish products [39]; the relation between HAAs intake and human cancer risk [40]; and the relation among HAAs intake, genetic predisposition and human cancer risk [41]. This paper refers to the main steps of the most important analytical methodologies proposed for the HAAs determination in food samples.

2. Sample preparation and clean-up

HAAs are present at trace quantities (ng g^{-1} level) in complex matrices, such as food samples, and a high number of matrix interferences can be present. The determination of HAAs is commonly carried out by means of chromatographic or electrophoretic techniques using different detection systems. The sample matrix greatly influences the clean-up procedures and many peaks with the same retention times as those of HAAs are often present in the chromatograms of real samples.

The first step for the sample preparation usually consists of a solubilization step, where the sample is homogenized and dispersed using different solvents. The solvents used are organic, such as acetone, ethyl acetate, methanol, hydro-alcoholic mixtures, or aqueous solvents, such as water, hydrochloric acid or, more frequently, sodium hydroxide. After solubilization, it is usually accustomed to eliminate proteins by precipitation using conventional procedures and to make their separation by centrifugation or filtration.

In order to remove interferences, several techniques have been applied to achieve the suitable pre-concentration of HAAs, and to isolate the analytes in different fractions. The most significant ones are stated below:

In the liquid–liquid extraction (LLE), if an organic solvent has been used to homogenize the sample, the analytes must be extracted with HCl. But when an aqueous solvent is used, the acidic solution obtained is directly extracted with an organic solvent, such as dichloromethane [8,24,42–46], ethyl acetate [7,24,43,45,47], or diethyl ether in order to remove acidic or neutral interferences. If the obtained solution is basic, HAAs can be extracted with dichloromethane in their neutral form [12,48,49]. Frequently, further purification is carried out by consecutive acid–base partition processes with dichloromethane [44,46,50–52] or by combination with extraction using sorbents, such as Kieselgur [42], Extrelut NT [45,49,53–55], diatomaceous earth, or with Blue Rayon [46]. These materials can be added to the liquid in the batch mode or, more frequently, as a support in a chromatographic column.

Supercritical fluid extraction (SFE) has only been applied to extract HAAs from cooking fumes [8]. Supercritical CO_2 was inefficient in extracting HAAs spiked onto a solid matrix, whereas supercritical CO_2/10% methanol at 6000 psi and 55 °C resulted in good recoveries of quinolines and quinoxalines. SFE is an efficient and reliable technique that presents certain advantages. It allows the extraction and concentration of volatile compounds in one-step, minimizing potential loss of the compounds, and providing a methanolic extract that can directly be analyzed by GC–MS. One disadvantage is that the flow restrictor is subject to plugging when the samples are wet or contain high amounts of extractable material and particulate matter. Therefore, the bead trap condensate and the filters were extracted with conventional liquid solvents [8].

The solid-phase extraction (SPE) has been widely used [20,56–57] with different sorbents, such as Blue Rayon [18,58], Blue Cotton [15,24,42,44,45,54,55,59–61] or Blue Chitin [62,63]. Using Blue Cotton, some HAAs that are not commonly found in fried foods have been identified, for example 4-OH-PhIP [24,59], 4-CH_2OH-8-MeIQx [60] and 7,9-DiMeIgQx [61]. Blue Cotton is cotton bearing covalently linked copper phthalocyanine trisulphonate as ligand. It can adsorb selectively aromatic compounds having three or more fused rings. The adsorption takes place in aqueous media, involving 1:1 complex formation between the ligand and the aromatic compound. Desorption can be done by elution with organic solvents, although a treatment with methanol containing ammonia is usually more efficient. Probably the ammonia helps to dissociate the complex by coordinating itself to the central metal ion in the ligand. Several cycles of adsorption can be carried out, and with this repetition of the cycle, an efficient concentration is achieved. Oleic acid interferes with the detection of mutagenicity of various compounds in the Ames *Salmonella* test, and this inhibition seems to occur by entrapping the mutagens in micelles of the fatty acid. As these fatty acids are adsorbed very poorly on Blue Cotton, samples prepared by the Blue Cotton method are usually free from this problem [64].

An improved method uses Blue Rayon as the supporting material instead of cotton. Blue Rayon is, similarly, rayon bearing covalently bound copper phthalocyanine trisulphonate, but it can contain two to three times more blue pigment, making Blue Rayon a more efficient adsorbent than Blue Cotton [64]. The extraction procedure using Blue Rayon is the same as for Blue Cotton. Blue Rayon has mainly been used for the adsorption of HAAs from river water [19]. Both Blue Cotton and Blue Rayon can be packed into glass or plastic columns, which facilitate the extraction and purification procedure [24,45,64]. Packing Blue Rayon columns in a standardized way is difficult, and this led to try pre-packed Blue Chitin columns [62]. Chitin (poly-*N*-acetylglucosamine) can also covalently link copper phthalocyanine trisulphonate as ligand. By using chitin powder as the supporting material, the content of the blue pigment can be doubled when compared with rayon and increased by four times compared with cotton [65]. Methods based on Blue Chitin columns are simpler and less-time consuming than methods based on Blue Cotton or Blue Rayon, and allow us to obtain higher HAAs recoveries for compounds having more than three rings. However, compounds with two or one-ring structures gave little or no adsorptions [62,63,65].

In order to remove interferences, to pre-concentrate the HAAs, and to isolate the analytes in different fractions, column chromatography on XAD-2 resin [2,7,8,45] or other sorbents [59,61], in-tube solid-phase microextraction [58], and preparative HPLC [2,59,61] have also been applied.

2.1. Tandem extraction procedures

Analytical sensitivity and selectivity can be optimized by combining different sorbents and eluents, or by coupling different sorbents in tandem [1,5,7,8,11,12,18,24,42,44,45,48,49,51–56,58,66–111]. Some methods are based on the HAAs extraction by sample alkalinisation and subsequent extraction with kieselgur [109] or with diatomaceous earth (Extrelut-20) [1,5,8,9,11,41,45,53–56,66,67,69–74,77–96,98–112]. Then, the extract is undergone to purification on Bond-Elut propylsulphonyl silica gel (PRS) [1,8,9,11,12,18,45,48,49,51–53,56,66,67,69–74,76–96,98–104,106,107,111,112], octadecylsilane (C_{18}) [1,8,11,18,20,45,48,49,51–56,66,67,69–74,76–94,96,98–104,106–109,111] benzene sulfonic acid silica (SCX) [56,73,87,89,109], Oasis MCX LP SPE extraction cartridge [5,105,110], cationic exchange-HPLC column [95], or carboxypropyl silica (CBA) [86,89,108] columns. The tandem extraction requires few sample transfers and evaporation steps. Other advantages are the time-saving and the high recoveries achieved. The elution from diatomaceous earth seems to improve when toluene or phenol are added to dichloromethane [4,5,8,45,56,69,76,86,87,89,98,107,112]. Also, ethyl acetate improves slightly the recoveries of some HAAs in meat samples [83,92,99,101,110]. Nevertheless, in model systems and pan residues, the recoveries did not increase significantly, and more interferences were co-extracted [87].

Cationic exchanger columns have been used for purification. Gross and Grüter [53] separated a series of HAAs into a polar group and an apolar group by the optimization of the PRS step in the solid-phase extraction with Extrelut-PRS-C_{18} coupled cartridges. One of the main advantages of this technique is that allows the elution of all the fluorescent compounds in the same fraction. However, although the method worked well for some process flavours, it was inadequate for the analysis of

the more complex ones, such as those produced at high temperatures [73,75]. Recoveries of PhIP were erratic and too low [8,49,53,95]. Gross et al. [66] have dealt with these clean-up problems and recommended an extra clean up on TSK gel, but this material is not available in pre-packed columns. A study was made to modify the Gross procedure [53] to improve its reliability, to determine PhIP and to facilitate the analysis of very complex process flavours [73].

Different SPE procedures are compared in Fig. 1 to establish the best conditions for the determination of HAAs.

Method A [53,75]. Alkalinised samples are loaded onto Extrelut column that is coupled on-line with PRS, and dichloromethane is passed [8,11,45,48,49,53,67,70,74,78,79,81,82,84,87,90,91,95–98,102,107,111–113] in order to extract HAAs and other basic organosoluble materials. PRS can retain other dichloromethane-soluble compounds, which may interfere with the HAAs during HPLC analysis. In order to remove most of these co-extracted interfering peaks, the PRS cartridge is dried and rinsed with methanol–water [11,97,111,113] or with HCl [70,84,98,107,112] to activate the ion-exchange process, and it is then washed with methanol/HCl solution and water [8,45,48,49,53,67,70,74,79,81,82,84,87,90,91,96–98,102,111,112]. Rinsing of PRS with methanol–HCl selectively desorbs the less polar analytes, such as some carbolines and PhIP. Elution can be carried out with ammonium acetate at pH 8.0 [45,48,67,70,74,79,82,84,90,102,107], or at pH 8.5 [11,49,97,102,111,113]. The cationic exchanger is coupled with a C_{18} cartridge, and after washing and eluting with methanol/NH_3 an extract A is simply obtained [11,45,48,49,67,70,74,79,90,92,95,97,98,102,107].

It is also possible to obtain two extracts of different polarity, if after washing the PRS cartridge with methanol/HCl and water, a neutralisation with NH_3 is performed. The fraction eluted in the washing step contains the apolar amines (extract A_1). The fraction retained in the PRS contains the polar amines, which are all aminoimidazoazarenes and the δ-carbolines that were strongly bound to PRS (extract A_2). These mixtures passed through C_{18} cartridges. The acid extract A_1 was neutralized and carefully eluted with methanol–ammonia [1,8,53,75,80–82,84,86–88,90,91,96,97,111]. Moreover, to separate the extract A_2 the PRS cationic exchanger column was coupled to a C_{18} column and ammonium acetate was used as eluent at pH 8.0 [1,8,53,75,80–82,84,86–88,90,91,96] or pH 8.5 [97,111], in order to achieve a preconcentration prior to chromatography. Finally, methanol/ammonia passed through the C_{18} cartridge to elute the extract A_2, which contains the polar HAAs that were adsorbed [8,53,80–82,84,87,88,90,91,96,97,111]. Extracts A_1 and A_2 were concentrated and redissolved in order to detect the HAAs.

Method B. In method A, some imidazopyridine and indolpyridine derivatives are not recovered [67]. The different steps are studied in order to improve the recoveries reported previously [72]. The changes introduced are increases in the dichloromethane volume in the Extrelut step and in the percentage of methanol in the PRS stage. The increase of methanol in the solution allows the collection of PhIP in the less polar extract [71,72,77,78,90,99–101,103–104,106,108,111]. Likewise as in method A, the cation-exchange column coupled with Extrelut is a PRS cartridge and the lesser polar compounds are washed with HCl, MeOH/HCl and water. These less polar HAAs are neutralized and pre-concentrated in a Bond Elut C_{18} column. Finally, the reversed-phase mini-column is washed and the analytes are eluted with methanol/ammonia. A less polar extract (B_1) is obtained. On the other hand, a Bond Elut C_{18} cartridge is coupled on-line with the PRS cartridge, and the most polar amines are eluted with ammonium acetate at pH 8.5. The C_{18}

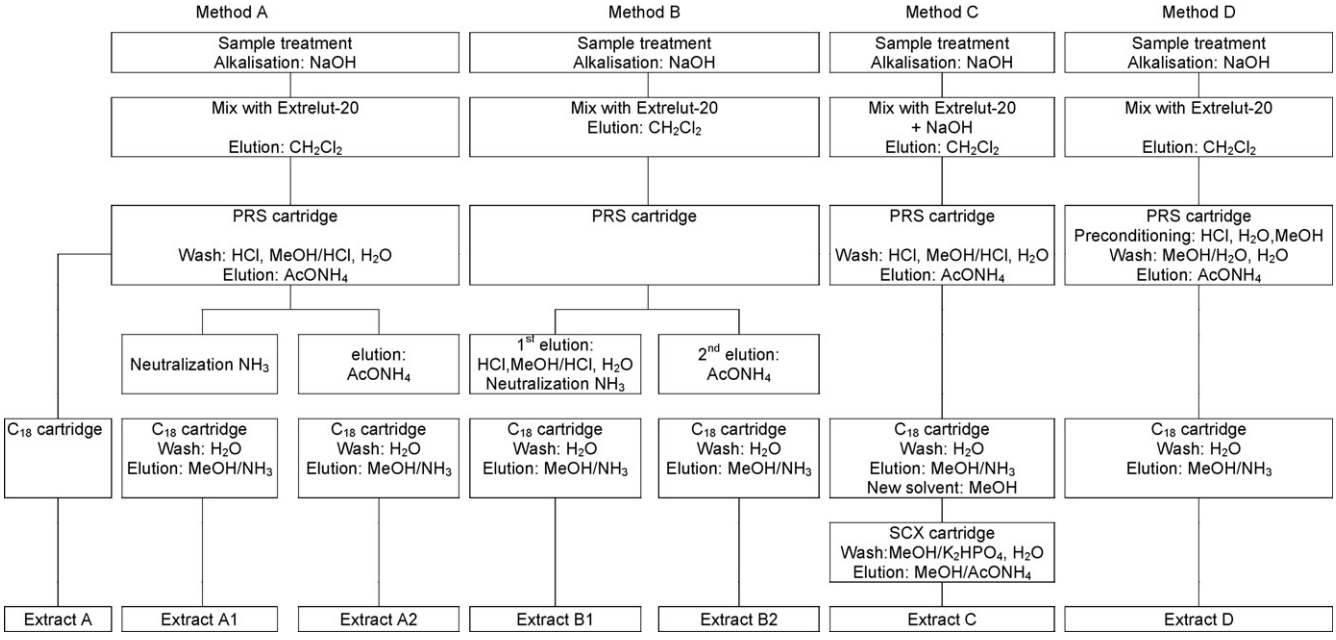


Fig. 1. Solid-phase extraction procedures (modified from Ref. [90]).

cartridge containing the most polar analytes is rinsed and the sorbed HAAs are eluted using methanol/ammonia (extract B_2) [99–101,104,108].

Method C [73]. It is very similar to method A, but it shows some modifications that can affect, for example, to the analysis of the complex process flavours. The first modification involves a change in the way the process flavour is applied to the Extrelut column. The second modification requires the development of an additional clean-up step using a benzenesulfonate SPE column, which can substitute the TSK gel column. The reason is that TSK gel cartridges are not available in prepacked columns, and they must be prepared manually, but such manual preparation may increase the possibility of introducing experimental errors [73,89]. Consequently, unlike method A, the sample is mixed with Extrelut-20 column and NaOH is added to it. After elution from Extrelut, PRS and C_{18} cartridges, the extract is introduced into SCX column for purification. After rinsing with MeOH-K_2HPO_4, the adsorbed HAAs were eluted with methanol-ammonium acetate at pH 8 (extract C).

Method D. In contrast to method A, the PRS column is preconditioned with HCl, water and methanol, and this column is rinsed with methanol–water instead of MeOH–HCl solution [90,93,94,97].

Methods A, B, and D provided similar recovery values (60–90%) with confidence intervals of about 10% [90]. The modified method A is less time consuming, requires fewer materials and gives slightly higher recovery values. Method C provided the most efficient clean-up procedure for some polar HAAs although lower recoveries and higher standard deviations were obtained. Therefore, some polar and non-polar HAAs were not recovered [89,90] and interfering co-eluting compounds were present [89]. When using methods A and C, the less-polar amines were completely lost in the clean-up process [90]. The modified NaOH treatment allows that all the Extrelut packing be wet, and this essentially improves the PhIP recovery and reduces the coefficients of variation [73]. Considerable amounts of PhIP are lost during SCX clean up, but PhIP is easily determined by fluorescence detection before SCX purification. If PhIP must be determined by UV detection, PhIP recovery can be improved by either decreasing the volume of the rinsing solution used on the SCX column or increasing the pH of the rinsing solution [73].

Method B appeared to be the most consistent for samples containing both polar and non-polar HAAs [90]. Method D is a faster and suitable procedure for screening unknown materials [90,93,94].

Method A has been automated by using a robotic workstation, in which all the peripherals and the robotic arm were computer controlled [75]. As significant drawbacks were the relatively important consumption of solvents to keep the tubings of the system clean, and detectable memory effects.

Two extraction and purification methods are compared [111]. The first one is the classic two extracts method developed by Gross and Grüter [53] and modified by Galcerán et al. [72]. The second procedure is a low-time consuming method, in which an unique extract is obtained. This method is based on the coupling of liquid–liquid with solid-phase extraction applying propil-sulphonic and octadecil silica cartridges, according Toribio et al. [97]. The purification of the samples is achieved with this less-time consuming method than those often used. The main differences are found for DMIP, δ-carbolines and 8-MeIQx, whose extraction is improved using the single extract method, and for Trp-P-2, which presents a higher recovery by applying the reference method. The two sample treatment procedures provide no significant differences among HAAs contents. This indicates that the validated method supplies accurate results, constituting a reliable and more simply alternative to the reference method [111].

Some authors [4,5,29,105,110,114] replace the PRS and C_{18} cartridges with a cation exchange Oasis MCX extraction cartridge, which is connected in series to the Extrelut-20 resin. This allows the recovery of all the HAAs in one fraction.

2.2. Other extraction methods

Some HAAs (DMIP, 1,5,6-TMIP and 3,5,6-TMIP) are not recovered by method A. For this reason, a new extraction method is proposed in order to separate these HAAs from IQ, IQx, MeIQx, 4,8-DiMeIQx, and PhIP [56]. Cooked meat is homogenized and extracted in HCl–methanol and the extract is applied to a C_{18} cartridge coupled to a SCX column. This SCX column exhibits a dual mode of action, a cation exchange and an apolar mechanism, and it is washed with HCl–methanol, methanol and water. The analytes are eluted with ammonium acetate, methanol (pH 8) and then, passed through a C_{18} cartridge, likewise as in method A [56].

Various extraction and purification procedures have been studied and compared for HPLC determination of aminoimidazoazarenes [45]: In this way, liquid–solid extraction with column ion-exchange chromatography, ultrasonic extraction or Soxhlet extraction, SPE, LLE, Soxhlet or ultrasonic extraction combined with LLE, were applied. To achieve the selective isolation of aminoimidazoazarenes in methods based on ultrasonic extraction, Soxhlet extraction and SPE, a SPE with cation-exchange phase was applied, while a two step LLE with the use of organic solvents and acid–base system was used in the rest of those methods. With the exception of the SPE method, a third step was needed to clean the aminoimidazoazarene fraction with Blue Cotton or by SPE with C_{18} cartridge. SPE, similar to method A [53] allows the smallest losses and it is the most effective and faster [45]. Calbiani et al. [115] used extraction into acetone followed by a clean-up on a SCX-solid-phase extraction column.

An interlaboratory study on analysis of HAAs has been carried out [116]. Pure methanolic solution and mixtures of unknown identity and concentrations, prepared in a beef extract spiked with known amounts of four HAAs. Whereas the analytical determination of HAAs in the beef extract appeared to be satisfactory, the procedures of isolation and purification required further improvement [116]. Consequently, in the case of processed food flavours, pan residues, bouillon concentrates, etc., some authors [42,66,70,73,80,89,95] have recommended additional steps and improvements in the clean-up procedure. Stavric et al. [50–52] used a LLE procedure, in which, samples were acidified with HCl and extracted with dichloromethane and

further purification was carried out by consecutive acid–base (HCl–NaOH) partition processes with dichloromethane. The obtained extracts were undergone at additional clean-up, due to the complexity of sample matrices in order to facilitate identification and quantification by LC/MS analysis. In this latter clean-up procedure, HAAs were eluted from the PRS cartridge with acetonitrile-ammonium acetate. Partly purified concentrated extract was dissolved in ammonium acetate and was passed through a second column (C_{18} cartridges). The HAAs were eluted with MeOH-aqueous NH_3 and then, with MeOH. Both fractions were collected in the same test-tube and evaporated to dryness. Residue was redissolved in the LC mobile phase. Very fine particulates from the packing material of the high capacity cartridges were often present in the eluate. Since these particulates from the cation-exchange SPE cartridges have characteristics that differ entirely from those of the LC C_{18}-column packing material, traces of them would have a severe adverse effect on the chromatography. In the same way, Stavric et al. [51,52] compared the SPE and Extrelut cartridges for the extraction of HAAs with the LLE procedure. Despite the fact that the Extrelut cartridge procedure was much faster, and the LLE was time consuming, this LLE method was preferred because processed food flavour samples spiked with HAAs standards produced a "cleaner" extract with comparable or slightly better recoveries, and the weights of solids obtained were considerably lower [51,52]. Pais and Knize [83] performed an additional clean-up of a heated model system extract using a strong cation-exchange cartridge procedure [73] for the process flavour and a TSK gel procedure for the model system, before HPLC analysis [83,92].

In some cases, this additional step in method A is carried out using a Fractogel TSK CM column, a weak cation-exchange gel [8,9,66,68,69,80,81,85] or a Nucleosil column [95].

Other alternative method, with at least the same degree of recovery and reproducibility as the TSK gel method was proposed [86,89,108]. This procedure consists in dissolving the polar and non-polar extracts in methanol, adding ammonium acetate buffer (pH 6) and applying to a carboxypropyl silica (CBA) columns. To remove interfering co-eluting compounds, the CBA columns were rinsed with methanol in the ammonium acetate buffer in two equal portions. The analytes were eluted into vials with methanol–concentrated ammonia (4:1). Two modes of the CBA column additional purification have been assayed. In mode 1, polar and non-polar HAAs were pooled before additional clean-up, while in mode 2 the polar and non-polar HAAs were treated separately. As similar efficiencies were found for both modes, mode 1 was considered the best, although for certain samples with extremely complex matrices it may be advisable to separate the polar and non-polar fractions in order to reduce the amount of possible cross-contaminants [86,89,108].

A quick SPE method using only one cartridge is proposed [57]. The method comprises extraction with methanolic NaOH (pH 10), centrifugation, and SPE using a commercially available polystyrene copolymer cartridge. After different washing steps with hexane and ethanol, the eluate (pH 3) was analyzed by HPLC. In addition, the reduced requirement of organic solvents, automation of the preparation and separation procedures are practicable [57].

The effects of changes of commercial brand and structure of sorbents were studied [94]. For the PRS step, the most suitable cartridge was Isolute PRS 200 mg, due to the elimination of the acidic activation, which simplified the procedure, and because the recovery of all the analytes was higher than 50% without significant differences in the obtained cleanness, compared with the rest of cartridges. In the case of C_{18} adsorbents, higher recoveries were obtained when monofunctional Isolute C_{18} was used [94].

SPME. Traditional extraction techniques such as LLE and, in particular, SPE are, however, characterized by intrinsic disadvantages like the use of toxic solvents and plugging of the cartridges. These drawbacks can be avoided by using solid-phase microextraction (SPME) technique. It enables simultaneous extraction and pre-concentration of analytes from gaseous, aqueous, and solid matrices. The principle of SPME is equilibration of the analytes between the sample matrix and an organic polymeric phase usually coating a fused-silica fiber; the amount of the analyte absorbed by the fiber is proportional to the initial concentration. In order to apply to non-volatile or thermally unstable compounds, SPME can be performed in combination with HPLC [117–119], or capillary electrophoresis. The difference between SPME-GC and SPME-HPLC is the desorption step. Four kinds of fiber coatings are compared for the extraction efficiency of HAAs from beef extracts. The most polar fiber studied (CW-TPR) exhibits better extracting efficiency and is recommended [117]. Factorial designs were used to optimise variables affecting the microextraction process [118]. The high fat content of the samples used led to low recoveries, probably due to the fiber coating poisoning. To minimize the fat content in the extract, it was frozen between −18 and −20 °C for 1 h [119,120]. Besides the simplification of the clean-up step, this method eliminates different solid-phase extraction stages required in the analysis of HAAs reducing the time and the amounts of organic solvents needed [117–119].

In-tube solid-phase microextraction method (in-tube SPME) is suitable for the extraction of less volatile or thermally labile compounds not amenable to GC or GC–MS, such as HAAs [58]. Food sample is treated with HCl. After centrifugation, the supernatant is neutralized with NaOH and the HAAs are extracted by the blue-rayon adsorption method. This method can selectively adsorb compounds having polycyclic planar molecular structures, such as HAAs, in order to concentrate them from aqueous solution. The extract is passed through a syringe microfilter, and a capillary column is used as a SPME device. This column is placed between the injection loop and the injection needle of the autosampler. The method is simple, rapid, automatic, and gives 3–20 times higher sensitivity in comparison with the direct liquid injection method [58]. A review on SPME and in-tube SPME methods applied to food analysis has been performed [30].

Extraction of HAAs from meat extracts has been carried out using a focused microwave system [120]. The optimum extraction was performed under a maximum radiation of 20 W (microwave oven power). Stirring samples with methanol–NaOH as extractant phase, were exposed to

Table 2
Figures of merit for the determination of thermic HAAs by gas chromatography

Method	Figure of merit	PhIP	IQ	MeIQ	IQx	MeIQx	7,8-DiMeIQx	4,8-DiMeIQx	Ref.
GC-NPD	D.L. (pg)	15	2	4		8		10	[121]
GC-EI-MS	D.L. (ng)	6	50	50		6.0		7.5	[107]
GC-HREI/LREC-MS-SIM	D.L. (pg)	0.5				0.5		1	[24]
	Recovery (%)	≥60				10		<10	
GC-EI-MS-SIM	D.L. (ng/g)	0.12	0.09	0.09	0.05	0.05	0.06	0.08	[11]
	RSD (%)	20.7	8.8	7.1	12.7	10.2	11.8	12.6	

microwaves until the temperature reached 80 °C, and then kept like this during 1 min. The total extraction time took around 5 min. The optimisation of this method, applied for first time to HAAs, was carried out by means of the experimental design. The selected optimum temperature was 80 °C, because higher temperatures can cause HAAs formation during heating and losses of the extractant phase by volatilisation [120].

3. Analytical methods for the determination of HAAs

3.1. Gas chromatography

Most HAAs are polar and non-volatile, and tend to elute as broad tailing peaks due to their strong adsorption to the column and injector. Therefore, they cannot be detected in low concentrations. Because of this, derivatization steps are needed to detect them in the usually lower concentrations. Derivatization of amines may be employed not only to reduce the polarity but also to improve the volatility, selectivity, sensitivity and separation of these amines [34–121].

A GC procedure with nitrogen-phosphorous selective detector (NPD) was developed for the determination of HAAs with the advantage of the high response of these compounds in the detector due to the nitrogen atoms present in the structure of the HAAs. Kataoka and Kijima [121] developed a simple and rapid derivatization method for GC analysis of mutagenic HAAs. Ten HAAs were converted into their *N*-dimethyl-amino-methylene derivatives with *N*,*N*-dimethylformamide dimethyl acetal and measured by GC with NPD using two-connected fused-silica capillary columns in order to improve the separation of HAAs. However, overlapping of AαC and Glu-P-2 could not be improved in many cases. The structures of the HAAs derivatives were confirmed by GC–MS analysis.

Tables 2 and 3 show the figures of merit for the determination of thermic and pyrolytic HAAs by gas chromatography with NPD and MS detectors.

3.2. Gas chromatography–mass spectrometry

GC–MS is one of the best on-line identification systems because it combines the high separation efficiency of capillary GC with the selectivity and relatively high sensitivity of MS (Tables 2 and 3). Identities of HAAs peaks could be verified by GC–MS [85,86,107]. However, GC-NPD and GC–MS techniques require a derivatization step and have been applied to determine a few HAAs. Several derivatizing agents, such as acetic, trifluoroacetic, pentafluoro-propionic and heptafluoro-butyric anhydrides, pentafluoro-benzyl bromide, 3,5-bistrifluoro-methylbenzyl bromide and 3,5-bistrifluoromethylbenzoyl chloride have been tested for the analysis of some HAAs [7,24,43,88,107,122–123]. Acylation with acid anhydrides yielded derivatives with very poor GC properties, perhaps due to the acidity of the acylated aminoimidazoazarenes. Nevertheless, good results were obtained by acylation with heptafluoro-butyric anhydride followed by methylation of the acidic amide proton with diazomethane [24] or with dimethylformamide dimethylacetal [16]. Aminoazarenes were derivatized to amides by acylation with pentafluoropropionic anhydride [45,107,123]. A molecular ion peak $M^+ = [(M_{HAA} - M_H) + M$ of the COC_2F_5 group] was observed for each of the derivatives. The abundant fragment (base peak) in the amides mass spectra was [$M^+ - M$ of the C_2F_5 group] [45]. Other procedures for derivatization, such as iodination and condensation to *N*-dimethylaminomethylene derivatives, have been applied. These last derivatives were identified as amides and quantified [123]. Quantification in GC–MS is usually performed by isotope dilution analysis, although isotopically labelled stan-

Table 3
Figures of merit for the determination of pyrolytic HAAs by gas chromatography

Method	Figure of merit	Glu-P-2	Glu-P-1	Harman	Norharman	AαC	MeAαC	Trp-P-2	Trp-P-1	Ref.
GC-NPD	D.L. (pg)	14	8			9		14	3	[121]
GC-NICI-MS-SIM	D.L. (ng)			0.1	0.1	0.5	0.1	2	0.1	[85]
	Recovery (%)					52	58	44	42	
GC-EI-MS-SIM	D.L. (ng/g)				0.02	0.05	0.07	0.29	0.35	[11]
	RSD (%)				5.0	6.9	9.2	15.0	9.7	

dards are not always available for each HAAs. In addition to this, using a single standard would give erroneous results since HAAs are not extracted with the same recoveries, despite the fact that they are from the same class of compounds [36].

Since chemical ionization MS is a much softer ionization method, it has the advantage of producing far less fragmentation of the compound and thus allows a greater chance of the molecular ion being present, which can aid interpretation. Negative ion chemical ionization (NICI) is highly sensitive and selective to electron-capture compounds. The GC–MS can usually be operated in two modes, total ion scanning and selected-ion monitoring (SIM). For SIM, only the base peaks are chosen to obtain the highest possible sensitivity [34]. GC–MS-negative ion mode-SIM offers high chromatographic efficiency and provides an alternative method of analyzing non-polar HAAs in complex samples [85,88]. However, it causes contamination of the ion source through the deposition of non-volatile material. Trp-P-1, Trp-P-2, AαC, MeAαC, harman and norharman, due to their low polarity can be directly analyzed without previous derivatization. The quantification of harman and norharman is tentative, due to varying degrees of recovery of these compounds [85]. MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx and PhIP were determined by NICI-MS-SIM as their 3,5-bistrifluoromethylbenzyl derivatives [43,122]. The alkylation products with 3,5-bistrifluoro-methylbenzyl bromide, 3,5-bistrifluoromethylbenzoyl chloride and pentafluoro-benzyl bromide, had good GC properties for some HAAs. However, these methods gave a mixture of mono- and di-alkylated forms and can be used for GC–MS analysis but not for conventional GC analysis [34,43]. Although the spectra of the 3,5-bistrifluoromethylbenzyl derivatives possess high mass fragment ions suitable for SIM work, it was not possible to separate the 4,8- and 7,8-DiMeIQx derivatives, therefore total DiMeIQx was determined [122]. MeIQx and 4,8-DiMeIQx were analyzed as the same derivatives by electron ionization MS-SIM [7]. The detection limits for these compounds are 2 pg [7]. A NICI-MS-SIM method was developed for the determination of PhIP after conversion into an electron-capturing pentafluoro-benzyl derivative. Quantification was carried out by selected-ion monitoring of the $[M - C_6F_5CH_2]^-$ ions of the derivatives of PhIP (*m/z* 403) and d_5-PhIP (*m/z* 408) as an internal standard [22].

Derivatization with heptafluorobutyric acid anhydride was made [24] and good selectivity and sensitivity were reported by recording the $[M - C_3F_7]^+$ main fragment. The mass spectrometer was operated in either the low-resolution electron capture (LREC) mode or the high-resolution electron impact (HREI) mode, both with SIM. In the LREC mode, no fragmentation takes place, and the complete molecule plus one electron $[M^-]$ is recorded. In the HREI mode, the basic peak $[M - C_3F_7]^+$ is recorded [24].

A two-step derivatization with heptafluoro-butyric anhydride followed by alkylation with *N,N*-dimethylformamide dimethylacetal (DMF-DMA) is proposed as well [16]. In this procedure, one proton on the primary amine is replaced with the heptafluorobutyryl group. The DMF-DMA methylates the remaining proton from the initial primary amine as well as protonated ring nitrogens if they are present. It is considered that each HAA gives a single derivative by the reaction with DMF-DMA. Analysis was carried out by GC-NICI-MS-SIM using CH_4 as a CI gas [16].

In spite of the above-mentioned derivatization procedures, the direct formation of *N,N*-dimethylformamide dialkylacetal derivatives has been proposed, offering some advantages. We can mention as examples, the derivatization reaction is performed in a single step, the excess of reagent can be easily removed by evaporation, and the derivatives present high stability. In this way, a comparative study of several HAAs derivatization procedures based on the formation of Schiff bases using *N,N*-dimethylformamide dialkylacetals reagent was performed [101]. Among the derivatization reagents, *N,N*-dimethylformamide di-*tert*-butylacetal (DMF-DtBA) was selected because it provided the best yield and, therefore, sensitivity in the GC–MS method. The most important condition to achieve a suitable yield in the derivatization is to avoid the presence of moisture in both the methanolic solutions of HAAs and in the reagents. It was observed that the molecular ion $[M]^+$ of all derivatives was the base peak of the spectra except for Trp-P-1 and Trp-P-2 derivatives, in which the base peak was $[M - 15]^+$. Confirmation ions for the determinations correspond to the $[M - 56]^+$ fragment due to the loss of $–C–N(CH_3)_2$. On the other hand, *N,N*-dimethylformamide dimethylacetal, reagent proposed in the bibliography, was the one that provided the lowest yield in the derivatization reaction [101].

Silylation is probably the most versatile GC derivatization technique. Besides improving volatility and stability, the introduction of the silyl group can also serve to enhance mass spectrometric properties. Consequently, a derivatization method in a one-step reaction with *N*-methyl-*N*-(*tert*-butyl-dimethyl-silyl) trifluoroacetamide for the analysis of 12 HAAs by GC–EI-MS analysis with SIM quantification was developed [11]. The derivatives are characterized by easy-to-interpret mass spectra due to the prominent ion $[M - 57]^+$ by loss of a *tert*-butyl-dimethyl-silyl group. The derivatization of the pyridoimidazoles Glu-P-1 Glu-P-2 and the β-carboline harman is incomplete for all the temperatures tested, and a tailed peak due to the underivatized compound is observed. The procedure is simple, rapid and accurate. However, the instability of the imidazolquinoline and imidazo-quinoxaline derivatives, requiring their injection on the same working day, is a further drawback.

3.3. High-performance liquid chromatography

Highly polar, non-volatile, and thermally unstable compounds can be separated successfully with HPLC. Besides, the derivatization step required in GC is not necessary, and several detection systems can be applied: All the HAAs have characteristic UV spectra and high extinction coefficients, and they are also electrochemically oxidizable. Some of them, the less polar HAAs and PhIP, fluoresce in polar solvents (IQ, MeIQ and MeIQx have no fluorescence). In this way, these compounds can be measured with UV [1–3,8,15,42,45,46,53,56,57,59,61,66,68,69,73,76,80,83,89,90,92,95,96,98,107,117,124,125], fluorescence [1–3,8,11,15,42,44,46,53–56,61,66,68,69,72,73,76,80,83,87,89,92,98,125], or electrochemical [14,42,44,54,55,

Table 4
Figures of merit for the determination of thermic HAAs by liquid chromatography

Method	Figure of merit	PhIP	IQ	MeIQ	IQx	MeIQx	7,8-DiMeIQx	4,8-DiMeIQx	Others	Ref.
HPLC–UV/FD	D.L. (ng)	0.4	4	10		2		4		[80]
	Recovery (%) (meat samples)	20 ± 9	66 ± 16	63 ± 20		68 ± 15		72 ± 15		
	Recovery (%) (pan residues)	45 ± 17	62 ± 22	57 ± 16		76 ± 14		68 ± 14		
HPLC–UV/FD Clean-up C	RSD (%)	8.2	5.3	7.0		4.7		3.4		[73]
	Recovery (%)	87.6	90.4	88.1		83.3		86.5		
HPLC–FD SPME (CW-TPR fiber)	D.L. (ng/mL)	1.13								[119]
	Precision (%)	6.6								
	Recovery (%)	75.5–109.6								
	Linearity (ng/mL)	0.70–7.00								
HPLC–FD-DAD	D.L. (ng)	4			2	1	1	1		[2]
	Recovery (%)	79 ± 8			88 ± 7	92 ± 8	88 ± 8	89 ± 9		
HPLC–FD-DAD	D.L. (ng)	0.001				0.1		0.1		[46]
	Recovery (%)	45.3 ± 4				68.7 ± 6		62.3 ± 5		
HPLC–FD-DAD	D.L. (ng/g)	0.01				0.03		0.02		[69]
	Recovery (%) (fried meat samples)	66 ± 16				71 ± 14		63 ± 14		
	Recovery (%) (pan residues)	51 ± 16				76 ± 10		63 ± 12		
HPLC–FD-DAD	D.L. (ng/g)	0.08	0.81	0.28		0.06				[1]
	Recovery (%)	45.7	66.8	48.2		72.5		42.0		
HPLC–FD-DAD	D.L. (ng)	1.5			15	1.5	3	1.5		[3]
	Recovery (%)	82 ± 4	90 ± 3	79 ± 7	83 ± 2	92 ± 2	91 ± 3	89 ± 1		
HPLC–FD-DAD	Recovery (%) TSK gel ODS column	60 ± 15	68 ± 15	71 ± 10	34 ± 14	75 ± 13	81 ± 14	79 ± 12		[89]
	Recovery (%) CBA column	68 ± 15	75 ± 14	72 ± 5	55 ± 1	73 ± 11	75 ± 5	72 ± 2		
HPLC–FD-DAD	Recovery (%)	77 ± 16				81 ± 12		78 ± 16		[98]
HPLC–FD-DAD (tandem extraction)	Recovery (%)	31	63			63		68		[8]
HPLC–FD-DAD (supercritical fluid extraction)	Recovery (%)	1.2–22.1	90.5–95–3	93.3–102.0		83.2–87.0		82.6–89.5		
HPLC–UV	D.L. (ng/g)	0.1	0.1	0.1		0.1	0.1	0.1		[95]
	RSD (%)	15	6	15		15	6	6		
	Recovery (%)	58.8–64.4	82.5–104.5	98.2–123.6		100.2–100.7	103.1–113.1	87.9–99.4		
HPLC–DAD	RSD (%)	10	7	8		12		6		[45]
	Recovery (%)	50	85	50		46		62		
HPLC–DAD	D.L. (ng/g)	9.1	4.6	4.3		3.4	3.3	3.1	TriMeIQx: 2.8	[57]
	RSD (%)	3.1	4.2	4.1		3.1	3.2	3.4	TriMeIQx: 2.9	
	Recovery (%)	91.1	62.3	64.5		95.1	86.8	83.6	TriMeIQx: 87.9	
HPLC–DAD, clean-up A	Recovery (%)	16.9 ± 3.2	71.8 ± 2.7	63 ± 10		87.2 ± 0.2	82.3 ± 2.3	82.1 ± 1.9		[90]
HPLC–DAD, clean-up B	Recovery (%)	70.0 ± 4.4	69.7 ± 5.7	56.4 ± 8.6		77.2 ± 4.8	71.0 ± 4.6	72.3 ± 2.9		
HPLC–DAD, clean-up C	Recovery (%)	NR	28 ± 12	47 ± 11		37.9 ± 6.3	49.7 ± 9.6	53.0 ± 10		
HPLC–DAD, clean-up D	Recovery (%)	70.1 ± 2.7	74.9 ± 0.74	70.3 ± 1.3		85.6 ± 3.4	82.5 ± 2.5	82.0 ± 1.5		
HPLC–DAD	D.L. (ng)	0.03	0.13	0.15		0.30		0.06		[96]

	Recovery (%)	62.1 ± 4.3	88.6 ± 2.7	88.7 ± 6.2	91.6 ± 3.1	89.7 ± 6.2	84.6 ± 5.1	86.4 ± 5.3		
HPLC–DAD	D.L. (ng)	2.0	0.4	0.4		0.8		1.0		[107]
	RSD (%)	8–10	7–11	8–9		6–12		4–6		
	Recovery (%)	61.7 ± 10.4	83.7 ± 8.1	77.5 ± 14.5		60.6 ± 5.7		77.0 ± 12.1		
HPLC–DAD SPME (PA fiber)	D.L. (ng/mL)			5		3.6				[117]
	RSD (%)			3.9		3.1				
HPLC–DAD SPME (CW-TPR fiber)	D.L. (ng/mL)		2.6	2.2		14				[117]
	RSD (%)		16	22		11				
	Recovery (%)		ND	19.1		28.5				
HPLC–DAD SPME (CW-TPR fiber)	D.L. (ng/mL)	23.8	9.81	11.3		16.8				[118]
	RSD (%)	9.65	16.6	5.78		28.3				
	Recovery (%)	57.2 ± 17.6	77.3 ± 24.8	80.2 ± 2.4		115.4 ± 16.5				
	Linearity (ng/mL)	40.0–154	40.0–154	40.0–154		40.0–154				
HPLC–ECD, extraction with focused microwave system	D.L. (ng)	0.98	1.66	1.15		0.83				[120]
	RSD (%)	6.1	10.4	6.9		12.8				
	Recovery (%)	36.0–77.3	58.1–77.2	57.9–83.3		73.2–101.3				
HPLC–ECD	D.L. (ng)	0.26	0.74	1.72		1.34		3.37		[67]
	RSD (%)	4.57	2.91	2.58		3.01		3.78		
	Recovery (%)		68.28	77.37		62.93		66.26		
	Linearity (ng)	1.65–164.8	3.14–156.8	3.36–163.2		4.35–222.4		6.46–161.6		
HPLC–ECD/FD-DAD	Recovery (%)	55 ± 3	82 ± 7	99 ± 4		87 ± 12		78 ± 7		[72]
LC-ECD	D.L. (ng/g), fried chicken	2	1.6	2		0.7	2	0.2	DMIP: 0.6	[108]
	D.L. (ng/g), beef extract	10	8	30		8	2	30	DMIP: 15	
	RSD (%)	1.2	4.7	6.2		7.6	5.4	1.2	DMIP: 6.1	
HPLC–ECD	D.L. (pg)		37	70		35				[70]
	RSD (%)		2.20	2.42		1.66				
	Recovery (%)		36.14	50.72		27.75				
	Linearity (ng)		10–20	2.5–20		2.5–20				
HPLC–ECD	D.L. (ng/g)		4.8	8.4		4.6				[74]
	Recovery (%)		54 ± 2	80 ± 1		60 ± 4				
	Linearity (ng)		6.5–65.0	11.5–115.0		7.2–72.1				
HPLC–ECD/FD-DAD	D.L. (pg)	2	8	8		8		8		[44,54]
	RSD (%)	3.2	4.4	24		4.5		9.7		[54,55]
	Recovery (%)	67–69	74–77	69–71		70–77		61–62		
HPLC–ECD (coulometric electrode array detect.)	D.L. (ng/g)		1.4	2.1		1.5		2.5	DMIP: 0.8	[104]
	RSD (%)		6.4	10.7		10.6		19.1	DMIP: 17.9	
Ion-pair chromatography (coulometric electrode array detect.)	Quantific. limit (ng/g)		0.2			0.2	0.3	1.5		[112]
	Recovery (%)		54.8–73.2			67.0–81.6	79.2–91.9	65.7–100.9		
	RSD (%)		4.3–9.5			3.2–7.2	3.4–6.0	4.4–8.1		

67,70,72,74,104,108,112] detectors. HPLC with fluorescence (FD) or electrochemical detection (ECD) presents high selectivity and sensitivity, although these detectors are restricted to the determination of selected groups. Sensitivity of HPLC with UV detection is not high, around 100–400-fold lower than fluorescence, but fluorescence detection does not allow to confirm the chromatographic peaks, and for that reason, the detection method most commonly used is diode array detection (DAD) [1–3,8,11,15,42,44–46,53–57,66,68,69,83,87,89,90,92,96,98, 107,117,125], which allows the on-line identification of the analytes by spectral library matching. Usually fluorescence detection is used as a complement to diode array detection in order to eliminate interferences produced when using UV detection, or to confirm the peaks obtained. The figures of merit for the determination of thermic and pyrolytic HAAs by HPLC are shown in Tables 4 and 5 .

In a model system [83], only PhIP could be confirmed. The presence of IQ, MeIQ, MeIQx and DiMeIQx could not be determined because coextracted compounds masked their HPLC-UV-diode array detection. Good separation of these and other HAAs was achieved by means of a change in the pH of the mobile phase from pH 3.2 to 7.0 and a modification in the gradient elution [83,87]. IQ, MeIQx, 4,8-DiMeIQx, 4,7,8-TriMeIQx, and PhIP were analyzed from standards mixtures [124]. Increasing the concentration of acetonitrile from 15% to 30% (v/v) results in higher and more reproducible peaks for the more retarded 4,7,8-TriMeIQx and PhIP. In higher acetonitrile concentration, TriMeIQx is completely resolved from PhIP, but MeIQx is coeluted with IQ [124]. A binary mobile phase consisting of acetonitrile and ammonium acetate at pH 3.6 with gradient elution and UV-fluorescence detections to separate 16 HAAs and related compounds is assayed [6]. Optimum conditions gave high resolutions and separated the 16 compounds within 31 min.

Gross and Grüter [53] separated simultaneously 12 mutagenic HAAs and two non-mutagenic pyrolysis products, harman and norharman, using a TSK gel ODS column with a ternary gradient elution system within 32 min. Many reversed-phase silica columns have been tested, but the TSK gel ODS column showed the best peak symmetry and separation efficiency [11,15,16,34,42,45,46,51,53,56,59,61,66–68,72,74,78,83,87, 96,98,107,110,117]. A ternary gradient including pH switching from pH 3.2 to 3.6 during the run, solved problems of co-elution of Glu-P-1 and MeIQ, and not baseline separation of Trp-P-2 and PhIP [53]. Other authors have proposed the application of two different HPLC separation systems for the quantitative determination of HAAs. Through pH shifting of the mobile phase a change of the elution order of the HAAs was achieved, which was used for identification and confirmation purposes [95]. Schwarzenbach and Gluber [42] tried the possibility of fluorogenic labelling of HAAs, but the derivatization of the amino group with a fluorescent reagent has not been successful. Some problems were found with the internal standard employed [106]. When spiking the samples with high amounts of HAAs in presence of 2-aminofluorene as internal standard, a decrease of the peak area of 2-aminofluorene was observed. This phenomenon was caused by a quench effect of the unrealistically high amounts of HAAs. Naphthalene is not affected by quenching and can be separated from the signals of all the other analytes. For this reason, it was used as internal standard [106].

IFP quantification was performed using the extinction coefficient corresponding to PhIP and the IFP absorbance maximum at 323 nm because a synthetic standard was still not available. Confirmation of the IFP peak by UV-absorbance spectra was difficult, so further confirmation was achieved by HPLC-MS–MS using ion-trap instrument using $^{2}H_{3}$-IFP as internal standard [92].

Electrochemical detection (ECD) is based on the oxidization of the HAAs at the operating potential. These detectors offer increased sensitivity compared with UV detectors, but are limited by the absence of on-line peak confirmation, which is a crucial step in HAAs analysis at the low concentrations present in cooked foods. The selectivity of electrochemical detection results from the fact that HAAs are oxidized at lower potentials than other compounds [34]. Most of the impurities detected as overlapping peaks with UV detection are not oxidized at the working potential and do not perturb the detection. From the voltammograms it can be observed that at electrode potentials lower than +750 mV no detectable response was obtained for any compound except for the aminoindol derivatives Trp-P-2 and MeAαC, which gave high responses at this potential [67]. High responses were also obtained for all compounds at +1150 mV, but at higher potentials an increase occurred in both the background noise and the residual current [67]. Isocratic conditions of mobile phase are needed due to the instability of the baseline in this high sensitivity range [124]. The gradient system is difficult to perform when electrochemical detection is used and different conditions have to be used to determine a large number of HAAs using isocratic mobile phases. For example, good separations between all the compounds using isocratic mode can only be achieved with two different conditions of mobile-phase: (a) ammonium acetate (pH 6)–acetonitrile (70:30, v/v) as mobile phase for the analysis of Trp-P-2, PhIP, MeAαC, norharman and harman; and (b) ammonium acetate (pH 4 or 5.25)–acetonitrile (90:10, v/v) as mobile phase for the analysis of Glu-P-1, IQ, MeIQ, MeIQx, 4,8-DiMeIQx and 7,8-DiMeIQx, using a TSK gel OSD column [67,72,108]. Determination of polar fraction was carried out at +1000 mV [108], and at +950 mV for the less polar fraction [42,108].

Van Dyck et al. [70] achieved low detection sensitivity using a Spherisorb (ion-exchange type) column. Separations on an ion-exchange stationary phase are dependent on the ionic strength of the mobile phase and on the ionization state of the different amines. So, IQ, MeIQ, and MeIQx can be determined at conditions of mobile phase acetonitrile—80 mM Na_2HPO_4 (30:70, v/v), at pH 5.6 and 1050 mV (vs. Ag/AgCl electrode) [70].

Additives such as triethylamine or diethylamine, frequently used for improving peak shapes with UV or fluorescence detection, cannot be incorporated into the mobile phase when working with ECD [36]. These additives would increase the background noise and, therefore, the detection limits. As ECD gives no confirmation of the peaks, additional detection system, such as diode array, must be employed [42,44,72,74]. Also, a coulo-

Table 5
Figures of merit for the determination of pyrolytic HAAs by liquid chromatography

Method	Figure of merit	Glu-P-2	Glu-P-1	Harman	Norharman	AαC	MeAαC	Trp-P-2	Trp-P-1	Ref.
HPLC–UV/FD	D.L. (ng)					5	5	0.3	0.3	[80]
	Recovery (%)			66 ± 13	57 ± 13	7 ± 8	25 ± 14	37 ± 15	45 ± 23	
HPLC–DAD SPME (CW-TPR fiber)	D.L. (ng/mL)			0.38	0.59	0.43	0.28	0.51	1.12	[119]
	Precision (%)			8.0	8.9	5.8	5.7	8.1	6.1	
	Recovery (%)			61,4–68.2	56.2–116.5	41.1–66.8	21.5–51.6	49.4–109.4	59.2–132.8	
	Linearity (ng/mL)			0.345–3.45	0.39–3.90	0.30–3.00	0.285–2.85	0.30–3.00	0.328–3.28	
HPLC–FD-DAD	D.L. (ng)					0.001				[46]
	Recovery (%)					27 ± 4				
HPLC–FD-DAD	D.L. (ng/g)			0.16	0.54			0.015	0.03	[1]
	Recovery (%)			70.0	77.0			30.5		
HPLC–FD-DAD	Recovery (%)			88 ± 10	87 ± 10	53 ± 5	76 ± 3	88 ± 2	91 ± 3	[3]
HPLC–FD-DAD	Recovery (%) TSK gel ODS column clean-up			Erratic (5–100)	Erratic (5–100)	55 ± 24	52 ± 12	66 ± 8	49 ± 5	[89]
	Recovery (%) CBA column clean-up			75 ± 21	72 ± 16	58 ± 9	68 ± 13	65 ± 5	55 ± 6	
HPLC–FD-DAD	Recovery (%)			60.4	63.3					[15]
HPLC–FD-DAD	Recovery (%)					33		51		[8]
HPLC–DAD	D.L. (ng/g)			5.3	5.1		3.8			[57]
	RSD (%)			5.2	4.9		3.7			
	Recovery (%)			31.5	24.7		78.9			
HPLC–DAD, clean-up A	Recovery (%)	83.5 ± 4.2	87.0 ± 4.6	NR	NR	NR		NR	NR	[90]
HPLC–DAD, clean-up B	Recovery (%)	75.5 ± 5.0	84.3 ± 8.2	83.2 ± 4.4	75.2 ± 7.6	57.9 ± 2.3		72.5 ± 8.2	72.8 ± 6.3	
HPLC–DAD, clean-up C	Recovery (%)	NR	3.38 ± 0.67	NR	NR	NR		NR	NR	
HPLC–DAD, clean-up D	Recovery (%)	87.2 ± 3.4	90.6 ± 4.4	55.0 ± 13	74.7 ± 5.0	83.8 ± 3.6		46.6 ± 8.8	26.2 ± 3.8	
HPLC–DAD	D.L. (ng)					0.21			0.12	[96]
	Recovery (%)	92.1 ± 5.2	85.7 ± 3.0	87.4 ± 1.8	90.3 ± 3.2	74.8 ± 6.1	73.2 ± 2.5	84.0 ± 3.1	70.9 ± 3.6	
HPLC–FD-DAD	D.L. (ng)			0.1	0.1	0.1	0.3			[125]
	Recovery (%)			82.3 ± 5.1	85.7 ± 3.2	73.6 ± 2.8	75.4 ± 3.6			
HPLC–DAD SPME (PDMS-DVB fiber)	D.L. (ng/mL)			1.1	0.5	3.1	1.9	1.1	1	[117]
	RSD (%)			6	6	7.3	11.6	6	8.2	
HPLC–DAD SPME (PA fiber)	D.L. (ng/mL)			4.1	0.9	3.3	2.2	0.3	0.3	[117]
	RSD (%)			6.8	3.3	7.8	9.2	3.9	5.3	
HPLC–DAD SPME (CW-TPR fiber)	D.L. (ng/mL)			0.9	0.6	0.8	0.5	0.1	0.1	[117]
	RSD (%)			1.3	2.4	6.7	7.1	7.9	9.6	
	Recovery (%)			82.4	74.9	67.9	57.4	17.8	19	
HPLC–DAD SPME (CW-TPR fiber)	D.L. (ng/mL)			5.07	7.47	3.24	13.1	1.58	3.43	[118]
	RSD (%)			6.38	6.34	8.65	8.21	2.21	5.84	
	Recovery (%)			111.7 ± 26.8	81.7 ± 1.0	64.1 ± 3.7	70.9 ± 3.3	48.9 ± 3.2	41.7 ± 3.7	
	Linearity (ng/mL)			19.0–72.9	21.8–83.9	20.0–76.8	20.0–76.8	10.0–38.4	10.1–38.9	

Table 5 (*Continued*)

Method	Figure of merit	Glu-P-2	Glu-P-1	Harman	Norharman	AαC	MeAαC	Trp-P-2	Trp-P-1	Ref.
HPLC–ECD, extraction with focused microwave system	D.L. (ng)	1.98		1.13	2.68	1.62	2.14	0.41	0.16	[120]
	RSD (%)	7.4		8.2	10.4	12.7	8.4	12.5	7.7	
	Recovery (%)	64.1–77.6		47.6–92.2	65.4–100.0	49.9–92.9	43.5–76.9	50.8–57.5	70.7–109.5	
HPLC–ECD	D.L. (ng)		0.51	0.26	0.26		0.74	0.19		[67]
	RSD (%)		3.50	3.95	1.68		1.61	2.33		
	Recovery (%)		17.66							
	Linearity (ng)		3.42–171.2	2.61–260.8	1.60–160.0		1.55–155.2	1.62–161.6		
HPLC–ECD/FD	Recovery (%)		81 ± 4	70 ± 7	73 ± 9	68 ± 4	70 ± 7	74 ± 8	91 ± 9	[72]
HPLC–ECD	D.L. (ng/g) fried chicken	1.5	1.8	0.4	0.4	2.4	1.2	2	1	[108]
	D.L. (ng/g) beef extract	nq	nq	4	2	6	nq	5	nq	
	RSD (%)	5.2	4.8	2.3	2.7	3.3	3.5	0.9	3.4	
HPLC–ECD	D.L. (ng/g)		4.2							[74]
	Recovery (%)		60 ± 5							
	Linearity (ng)		5.6–56.5							

metric array detection system coupled to HPLC proved to be a powerful confirmation system [14,104,112]. This technique offers the possibility to detect compounds at various potentials simultaneously. The electrode array detector containing eight coulometric cells, and eight working electrodes, each of them was adjusted at different potential. The chromatograms, one from each electrode, were obtained simultaneously.

3.4. Liquid chromatography–mass spectrometry (LC–MS)

For identification purposes, mass spectrometry in conjunction with chromatographic techniques is a good on-line system due to its high selectivity and specificity. HPLC-MS, capable of simultaneously measuring retention times and molecular mass, is a powerful technique comparable to GC–MS and can identify and quantify HAAs in complex samples without derivatization. Some disadvantages of the HPLC-MS are the high quantities of mobile phase and the low sample concentration. Other conditions needed are: high vacuum in the ion source, and that buffers and other additives required for chromatographic separation be volatile. A review has recently been published [126]. Three ionization techniques have been used: thermospray (TSI), electrospray (ESI) and atmospheric pressure chemical ionization (APCI). The sensitivity of MS can be increased if only a few selected ions are monitored instead of full spectra, as it occurs when single-ion monitoring (SIM) technique is applied [51,68,71,127]. Another procedure that allows to achieve high selectivity and extreme sensitivity is the use of selected reaction monitoring (SRM) mode. Analytical properties for the determination of thermic and pyrolytic HAAs by HPLC-MS are collected in Tables 6 and 7.

The thermospray HPLC-MS can work with conventional-size HPLC columns and with reversed-phase columns. The ionization process for HAAs produces abundant pseudo-molecular ions and the base peaks in the mass spectra are detected as $[M+H]^+$. These amines are stable towards the ionization process and do not undergo notable fragmentation. Single-ion monitoring of the $[M+H]^+$ ion of the respective HAAs, such as Trp-P-1, Trp-P-2, IQ, MeIQ, and MeIQx can be used for analysis in complex matrices [68]. However, this technique has been replaced by APCI because of its higher sensitivity. HPLC-MS appears to be the main technique able to screen most of the known HAAs simultaneously, either using a thermospray [68], or an electrospray [127] interfaces [75].

Two powerful and promising interface methods based on atmospheric pressure ionization (API) sources are ESI and APCI. In ESI, droplet formation and charging take place simultaneously, while in APCI droplets are formed prior to ionization. Both API techniques involve mild ionization and, therefore, the unfragmented ions obtained, quasi-molecular ions, provide information on molecular mass, but little structural information. The application of higher voltage difference between different regions of an API source generally induces more fragmentation of the formed ions. This procedure is designed as in-source fragmentation or pre-analyzer collision induced dissociation and allows to induce fragmentation before entering the quadrupole in HPLC-MS [71,77,78], or between the two quadrupoles when

Table 6
Figures of merit for the determination of thermic HAAs by liquid chromatography–mass spectrometry

Method	Figure of merit	DMIP	PhIP	IQ	MeIQ	8-MeIQx	7,8-DiMeIQx	4,8-DiMeIQx	Others	Ref.
LC-APCI-MS	D.L. (ng/g)		0.4	0.2	0.2	1.0		1.4		[78]
	RSD (%)		3.6	3.7	3.9	4.6		3.3		
	Recovery (%)		50.3 ± 6.8	72.2 ± 3.1	67.7 ± 7.5	83.1 ± 10.6		84.9 ± 9.7		
	Linearity (ng)		0.086–19.9	0.099–22.9	0.096–22.2	0.089–20.5		0.080–18.5		
LC-APCI-MS-SIM	D.L. (ppb)		0.6 ± 0.3	0.6 ± 0.3	0.6 ± 0.3	0.6 ± 0.3	1.1 ± 0.4		TriMeIQx: 1.1 ± 0.4	[60,61]
LC-APCI-IT-MS	D.L. (ng/g)		1.5	4.9	10.1	5.3	2.9	2.7		[93]
	RSD (%)		2.9	2.7	4.2	3.1	3.3	4.8		
LC-APCI-IT-MS	D.L. (ng/g)		1.7–2.4	3.9–4.3	6.5–10.2	2.9–5.2	3.1–4.5	3.2–4.4		[94]
	RSD (%)		2.0–3.9	2.0–4.1	2.0–7.2	1.1–3.9	1.6–2.9	0.8–2.9		
	Recovery (%)		74.3–88.3	69.5–93.4	73.2–94.3	70.3–84.0	75.2–84.6	52.3–63.3		
LC-AP-ESI–MS-SIM	D.L. (ng/mL)		0.21	0.39	0.55	0.53	0.92	1.33		[58]
LC-ESI-MS	D.L. (ng/g)		0.3	0.2	0.3	1.1		0.2		[77]
	Recovery (%)		54 ± 5	74 ± 3	80 ± 8	82 ± 9		89 ± 4		
	Linearity (ng)		0.086–19.9	0.099–22.9	0.096–22.9	0.089–20.5		0.080–18.5		
HPLC–ESI-MS-SIM	D.L. (ng/g)		3	3						[71]
LC-ESI-IT-MS	D.L. (pg)	5	13	2	5	6	3	5		[100]
HPLC–ESI-IT-MS-SIM	Recovery (%)	14 ± 4	53 ± 7	76 ± 4	82 ± 4	52 ± 4	39 ± 6	28 ± 6	IQx: 51 ± 6	[4]
HPLC–ESI-IT–MS-SIM	D.L. (pg)		1							[20]
	Recovery (%)		92 ± 6							
LC-APCI-IT–MS–MS (clean-up A)	D.L. (ng/g)	10.3	0.7	1.0	1.1	1.2	0.9	1.0		[97]
	RSD (%)	4	3	3	3	4	5	4		
	Recovery (%)	14	87	87	93	81	78	87		
LC-APCI-IT-MS–MS (clean-up B)	D.L. (ng/g)	4.9	1.6	2.3	2.4	1.6	1.5	1.7		[97]
	RSD (%)	8	6	6	4	5	5	6		
	Recovery (%)	35	67	72	65	80	75	69		
LC-APCI-IT–MS–MS (single extract method)	Recovery (%)	25.4	47.9	26.3	24.8	43.0	45.5	51.3		[111]
LC-APCI-IT–MS–MS (two extract method)	Recovery (%)	5.5	54.8	30.0	26.6	31.8	52.7	47.5		
HPLC-ESI–MS–MS-SRM	Recovery (%)		78 ± 1	109 ± 11	85 ± 11	90 ± 10			TriMeIQx: 80 ± 8	[12]
HPLC-ESI–MS–MS-SRM	RSD (%)		2.3–15	3.1–8.5		2.7–4.5		4.2–6.5	IQ[4,5-*b*]: 5.1, IQx: 8.3–30, 7,9-DiMeIgQx: 10.2–15	[4]
	Recovery (%)		31 ± 15	>54 ± 24		50 ± 16		>43 ± 12	IQ[4,5-*b*]: >20 ± 4, IQx: 7,9-DiMeIgQx	
HPLC-ESI-MS–MS-SRM	D.L. (pg/mL)		0.4			3.1		1.1		[29]
	Quantif. Lim. (pg/mL)		0.7			5.2		2.7		
	Recovery (%)		14.3 ± 10.3			7.1 ± 5.1		8.4 ± 6.0		

Table 6 (*Continued*)

Method	Figure of merit	DMIP	PhIP	IQ	MeIQ	8-MeIQx	7,8-DiMeIQx	4,8-DiMeIQx	Others	Ref.
UPLC-ESI–MS–MS-SRM	D.L. (pg injected)	0.07	0.08	0.06	0.08	0.10	0.06	0.09		[113]
	D.L. (ng/g)	0.042	0.015	0.025	0.017	0.009	0.034	0.024		
	RSD (%)	4.4	6.5	3.9	7.2	7.9	6.5	7.6		
LC-ESI-IT-MS–MS	D.L. (ng/g)	3.6	1.3	0.6	0.7	2.9	1.1	1.2		[99]
	Recovery (%)	6	8	5	8	9	7	7		
LC-ESI–MS-SIM single quadrupole	D.L. (ng/g)	0.5	0.8	0.5	1.2	1.7	0.1	0.2		[99]
	Recovery (%)	5	3	8	5	6	7	7		
LC-ESI–MS–MS-SIM triple quadrupole	D.L. (ng/g)	0.4	0.4	0.7	0.4	1.1	0.5	0.5		[99]
	Recovery (%)	6	2	8	5	7	6	7		
LC-ESI–MS–MS-MRM triple quadrupole	D.L. (ng/g)	0.1	0.01	0.04	0.04	0.1	0.1	0.5		[99]
	Recovery (%)	0.4	3	3	2	3	5	4		
Py-MAB–ToF-MS[a]	D.L. (ng)		0.9		0.3	0.15		1.5	IQx: 0.9	[114]
	RSD (%)		11.8	22.6	23.1	17.1		12.2	IQx: 20.7	
	Recovery (%)		100.3 ± 2.8		96.3 ± 3.3	85.6 ± 2.3		85.6 ± 2.9	IQx: 81.3 ± 2.5	

[a] Pyrolysis-metastable atom bombardment-time of flight-mass spectrometry.

HPLC-MS–MS is used [128,129]. The application of in-source fragmentation provides an easier and less expensive method than tandem-MS for confirmation of the HAAs. In this way, the lowest extraction potential applied (+100 V) is used for quantification purposes, because higher responses are obtained. The highest potential (+150 V) induces fragmentation of the primarily formed ions $[M+H]^+$, and allows the confirmation of the detected peaks [77,78]. It is a selective and highly specific technique and can be considered as one of the best on-line identification method, which is an important requisite when working in the analysis of HAAs in complex matrices, such as processed food samples [77]. Both methods (ESI and APCI) are more sensitive than the usually used HPLC-UV method, and give similar results to those obtained using HPLC with electrochemical detection, and they have the advantage of being more stable than the latter [77,78]. The chromatograms are almost free of interfering peaks due to the high selectivity and specificity of this technique. Triethylamine is not used in API-MS due to the strong ionization suppression allowing tailing peaks to decrease the chromatographic efficiency. Therefore, the detection limits increase [36]. Galcerán et al. have determined simultaneously harman, norharman and other HAAs in processed food samples, with a triple quadrupole-mass spectrometer using APCI [78] or electrospray HPLC-MS [71,77] pneumatically assisted as the interface, with positive ionization. Measurements were performed by single-ion monitoring (SIM) of the protonated molecular ions [71]. In this most sensitive single-ion mode frequently no abundant secondary ions are present for confirmation of the base peak [52,77]. Also, the HPLC-MS measurements were performed by multiple ion detection (MID) of the most important masses for each HAAs [77,78]. The potential and the limitations of HPLC-APCI-MS–MS and of HPLC-ESI-MS–MS techniques applied to HAAs have been discussed [130].

As low-flow rates are usually needed for the electrospray HPLC-MS technique, microbore or semi-microbore columns must be used. Columns with smaller diameter have the advantages of low solvent consumption, higher sensitivity, and good separation at low flow rates. This last characteristic makes microcolumns and capillary columns suitable for HPLC-ESI-MS techniques. The sensitivity increases because microcolumns elute analytes at higher concentrations than conventional columns. Different narrow-bore reversed-phase columns employed in HPLC-ESI-MS were studied [100]. These columns were “Discovery”, “Purospher”, “Symmetry”, “Synergi”, “TSK Gel ODS”, and “Zorbax”, The strong adsorption of AαC and MeAαC on the “Discovery” column, giving very wide peaks that prevent their detection, caused that this column was rejected. The “Zorbax” column was also rejected because of the low values of peak symmetry and peak height, probably due to the absence of endcapped treatment in its stationary phase. Among the rest of columns, the TSK Gel ODS column provides the best separation for HAAs determination by HPLC-ESI-MS in combination with the best values of peak height, peak symmetry, and number of theoretical plates, as well as highest injection volume and lowest limits of detection. Moreover, a low equilibration time was needed [100]. Therefore, TSK gel ODS column is frequently used [51,52,68,92–94,105,111,115]. The

Table 7
Figures of merit for the determination of pyrolytic HAAs by liquid chromatography–mass spectrometry

Method	Figure of merit	Glu-P-2	Glu-P-1	Harman	Norharman	AαC	MeAαC	Trp-P-2	Trp-P-1	Ref.
LC-APCI-MS	D.L. (ng/g)		0.4	0.08	0.1	0.3	0.4	0.8	0.4	[78]
	RSD (%)		4.2	3.3	3.3	4.0	3.9	4.1	4.1	
	Recovery (%)		74.0 ± 7.0	68.2 ± 8.8	82.5 ± 5.9	58.4 ± 2.9	61.5 ± 7.7	73.4 ± 3.4	64.6 ± 6.6	
	Linearity (ng)		0.094–21.8	0.11–26.0	0.099–22.9	0.084–19.3	0.081–18.6	0.080–18.4	0.092–21.1	
LC-APCI-MS-SIM	D.L. (ppb)							0.6 ± 0.3	1.1 ± 0.4	[60,61]
LC-APCI-IT-MS	D.L. (ng/g)	7.9	9.0	2.7	2.3	0.8	1.0	1.9	1.7	[93]
	RSD (%)	2.2	3.8	3.5	2.1	5.1	4.7	2.5	2.2	
LC-APCI-IT-MS	D.L. (ng/g)	3.7–5.4	4.7–9.1	3.0–5.2	2.8–3.6	0.4–1.2	0.7–1.3	2.0–11.7	2.4–15.0	[94]
	RSD (%)	0.5–2.1	0.8–4.0	2.1–8.6	1.6–4.4	0.5–2.1	1.1–1.7	1.0–3.3	0.8–4.8	
	Recovery (%)	57.6–70.4	53.4–58.0	23.5–60.0	54.1–67.7	49.4–61.6	51.1–61.1	10.1–59.7	2.4–61.6	
LC-AP-ESI–MS-SIM	D.L. (ng/mL)	2.71	3.13			1.57		0.95	1.45	[58]
LC-ESI-MS	D.L. (ng/g)		2.3	0.2	0.2	0.1	0.2	0.2	0.2	[77]
	Recovery (%)		79 ± 13	61 ± 9	105 ± 9	60 ± 7	59 ± 8	61 ± 7	76 ± 12	
	Linearity (ng)		0.094–21.8	0.11–26.0	0.099–22.9	0.084–19.3	0.081–18.6	0.080–18.4	0.092–21.1	
LC-ESI–MS-SIM	D.L. (ng/g)			1	1	6		5	4	[71]
LC-ESI-IT-MS	D.L. (pg)	3	2	3	3	8	8	4	3	[100]
HPLC-ESI-IT–MS-SIM	Recovery (%)			70 ± 8	63 ± 10	57 ± 8	45 ± 14	72 ± 9	72 ± 6	[4]
LC-APCI-IT-MS–MS (clean-up A)	D.L. (ng/g)	1.2	1.7	3.0	1.8	0.4	0.4	0.8	0.8	[97]
	RSD (%)	3	5	10	7	2	5	3	3	
	Recovery (%)	87	82	87	89	63	75	90	98	
LC-APCI-IT-MS-MS (clean-up B)	D.L. (ng/g)	2.7	4.6	2.5	1.6	1.0	0.8	1.7	3.1	[97]
	RSD (%)	5	7	16	8	5	5	4	9	
	Recovery (%)	83	74	50	58	46	61	63	72	
LC-APCI-IT-MS-MS (single extract method)	Recovery (%)	59.6	51.5	53.3	48.1	54.3	57.0	40.2	36.7	[111]
LC-APCI-IT-MS-MS (two extract method)	Recovery (%)	32.1	29.4	58.3	49.1	60.9	61.9	50.9	40.9	
HPLC-ESI-MS–MS-SRM	Recovery (%)	88 ± 5	98 ± 33				51 ± 9			[12]
HPLC-ESI-MS–MS-SRM	RSD (%)					16.5	10			[4]
	Recovery (%)					20 ± 12	–			
HPLC-ESI-MS–MS-SRM	D.L. (pg/mL)					1.1				[29]
	Quantif. Lim. (pg/mL)					3.4				
	Recovery (%)					6.9 ± 5.4				
UPLC-ESI-MS–MS-SRM	D.L. (pg injected)	0.09	0.08	0.18	0.19	0.11	0.17	0.23	0.16	[113]
	D.L. (ng/g)	0.009	0.017	0.045	0.051	0.023	0.014	0.028	0.005	
	RSD (%)	4.9	6.8	8.1	7.5	5.6	3.8	9.1	8.2	
LC-ESI-IT-MS-MS	D.L. (ng/g)	1.9	1.4	0.6	0.3	1.7	1.7	0.6	0.1	[99]
	Recovery (%)	2	8	5	8	7	7	7	9	
LC-ESI-MS-SIM single quadrupole	D.L. (ng/g)	0.6	0.3	0.6	0.4	0.5	0.5	0.9	0.3	[99]
	Recovery (%)	7	7	6	7	5	5	5	2	
LC-ESI-MS–MS-SIM triple quadrupole	D.L. (ng/g)	0.8	0.6	0.4	0.2	0.2	0.2	0.2	0.04	[99]
	Recovery (%)	6	8	6	6	5	6	5	2	
LC-ESI-MS–MS-MRM triple quadrupole	D.L. (ng/g)	0.1	0.1	0.1	0.04	0.02	0.02	0.02	0.02	[99]
	Recovery (%)	3	4	4	3	3	3	1	1	
Py-MAB–ToF-MS[a]	D.L. (ng)	0.5				0.6		1.2	0.3	[114]
	RSD (%)	18.1				15.0	10.5	17.7	21.6	
	Recovery (%)	82.5 ± 7.5				88.5 ± 1.8		78.3 ± 2.6	94.8 ± 3.7	

[a] Pyrolysis-metastable atom bombardment-time of flight-mass spectrometry.

use of a TSK ODS-Super column was particularly advantageous, since this allowed to cut the analysis time by half without loss of chromatographic resolution, and the increase in MS sensitivity due to the very sharp peaks obtained [75,92,127]. Also Gross et al. [68] achieved a sensitive detection of these compounds using a narrow-bore Vydac column. MeIQx, 4,8-DiMeIQx, PhIP and AαC were confirmed by HPLC-ESI-MS in these complex samples that are difficult to confirm by DAD [68]. However, it is possible to work in ESI with high flow-rates by directing a gas flow into the effluent stream. This is the called ionspray or pneumatically assisted ESI [36]. To prevent mass spectrometer contamination when running, a divert valve was used for a few minutes at the beginning of the chromatogram [20,91,93,94,97].

The electrospray HPLC-MS using soft ionization interface is a powerful technique for the analysis of low molecular weight trace constituents in complex matrices. ESI source requires analytes to be ionized in the liquid phase, so for HAAs analysis the pH of the mobile phase should be lower than pK_a of the HAAs to protonate the amino group. As HAAs are stronger bases than the components of the mobile phase, this ionization process can transform the HAAs from solution to protonated ions in the gas phase. As a result, the HAAs give a simple mass spectrum in which the only peak is due to $[M+H]^+$, the abundant protonated molecular ion. These compounds are stable towards the ionization process and do not undergo notable fragmentation except for IQ and 4,7,8-TriMeIQx, which show the $[MH-15]^+$ fragment [71,77]. When higher extraction voltages were used, more fragmentation was observed and a decrease in the intensity of the protonated molecule $[M+H]^+$ occurred [58,77]. $[M+NH_4\text{-}H_2O]^+$ ions were observed from Glu-P-1 and Glu-P-2 [58]. The loss of CH_3 from protonated molecules and the loss of the aminoimidazyl moiety ($-CH_3$–HCN and $-C_3H_4N_2$) are the common route of fragmentation for these compounds.

Stavric et al. [51,52] applied HPLC-APCI-MS to the determination of thermic and pyrolytic HAAs. A dual channel with UV detector was installed after the HPLC column but before the LC/MS interface, which was attached to the APCI source of the triple quadrupole-MS, operated in the single quadrupole mode. The mass spectrometer was operated in SIM mode and the resolution was set at around 1–1.2 mass units at the base line. Although additional clean-up procedures were used, interferences were still observed even with trideuterated standards. Therefore, for samples where some interference was observed a second HPLC column, TSK gel ODS, was used. All the studied HAAs were quantified and the minimum detection limits were 1–3 ppb [51–52].

The problems derived from a less exhaustive purification of the extract have been resolved by using HPLC-APCI provided of an ion-trap (IT) mass analyzer [93,94], but with this simplification of the clean-up, detection limits in the meat extract analyzed were higher than expected [93]. Comparison of different commercial SPE cartridges to extract HAAs was made [94] by this simplified purification procedure. A liquid chromatography-electrospray ionization-ion trap mass spectrometry (HPLC-ESI-IT-MS) method has been developed [20] to study the metabolism of PhIP by the human liver microsomes and prostate tissue. A mixture of ammonium acetate buffer and acetonitrile was used for elution from the SPE cartridges. To improve the recovery, dimethyl sulfoxide was added because it is a very good solvent for PhIP and its metabolite 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. Although the recovery was better, the evaporation of dimethyl sulfoxide was difficult and therefore unsuitable for larger sample volumes or greater number of samples [20].

The electrospray ionization can also be used in combination with tandem mass spectrometry (MS–MS) to enhance the sensitivity of the detection. The tandem mass spectrometry technique provides a high degree of selectivity, leading to chromatograms that are almost free of interfering peaks. Moreover, false peak identification was avoided by comparing the product ion full scan mass spectra of the sample with those of the standards [97]. Richling et al. [12,85,128,129] developed a sensitive and selective method for the simultaneous analysis of the 10–16 most abundant HAAs in several food samples by HPLC-electrospray interface (ESI) MS–MS using triple quadropole in combination with SRM. The ionization of analytes in HPLC-ESI-MS–MS is influenced by different factors, in particular, the sample matrix, thus requiring the use of deuterated standards. Separation of the polar and non-polar compounds was achieved by means of two different HPLC gradients [12] with trifluoroacetic/H_2O and CH_3OH/acetonitrile as solvents.

As the solvent composition affects the HPLC-ESI-MS systems, the influence of the concentration of a volatile ion-pairing reagent has been studied [131]. The chromatographic behaviour of the HAAs using a formic acid/ammonium formate (pH 2.8, 3.7 and 4.7) was compared with that observed using acetic acid/ammonium acetate buffer (pH 4.0) in the mobile phase. Reversed phase ion-pair chromatography (IP-LC–ESI-MS–MS-SRM) was carried out with formate, or acetate, as counter ion in an aqueous eluent with acetonitrile as organic modifier. Higher detectability was obtained with formate buffer at pH 2.8 [115,131]. It was observed that under isocratic conditions pH values higher than 3.7 produced broad peaks of all the HAAs [131].

An ion-pair liquid chromatography-electrospray tandem mass spectrometry with SRM for identification is reported for determining HAAs in meat-based infant foods. Mean recoveries ranged between $78 \pm 4\%$ and $98 \pm 2\%$ for IQ, MeIQ, MeIQx, PhIP, AαC, harman and norharman. Limits of quantification generally are lower than 8 ng/g. Some factors are identified as statistically significant in influencing chromatographic separation and response: the mobile-phase pH resulted to be a critical parameter for the capacity factor (k') of IQ, MeIQ, and norharman, whereas the mobile-phase flow rate was statistically significant for k' values of all analytes, except AαC peak [115].

Holland et al. [3] discovered, by LC/MS analysis, an isomer of 8-MeIQx in the urine of meat eaters. The compound has currently been isolated and identified in pan-fried scrapings of cooked beef, by UV and MS [5]. The pan-fried meat scrapings were spiked with comparable amounts of the synthetic 6-MeIgQx and 7-MeIgQx isomers, which have different t_R. 7-MeIgQx was found to coelute with the analyte in cooked meat. The use of the characteristic t_R as a means of identification of the analyte was crucial for identification purposes because the

product ion spectra of the analyte and the synthetic 6-MeIgQx and 7-MeIgQx isomers are identical. Also UV spectroscopy was used to corroborate the identity of the analyte as 7-MeIgQx. The UV spectra of the synthetic 6-MeIgQx and 7-MeIgQx isomers are very similar; however, subtle differences are discernible in the absorbance maxima centered about 260 and 360 nm. Again, the UV spectrum of the analyte purified from the beef is a perfect match to the spectrum of 7-MeIgQx. The spectral data were compared to those of several synthesized angular and linear tricyclic isomers of 8-MeIQx. The product ion spectrum of the novel analyte is similar to the spectrum of 8-MeIQx, except that the fragment ion at *m/z* 131 is more abundant, suggesting that the analyte contains an *N*-methylimidazo[4,5-*g*]quinoxaline ring system and not a *N*-methylimidazole[4,5-*b*]quinoxaline skeleton; the latter ring structure would not be expected to undergo fragmentation to produce the fragment ion at *m/z* 131 as the base peak in the spectrum. The spectral data support the assigned structure of the molecule as 7-MeIgQx. This newly identified HAA is one of the most abundant HAAs formed at 165 °C for 10 min per side, in cooked groundbeef and pan-fried scrapings [5].

Three HPLC-ESI-MS systems equipped with an electrospray as ionization source and different analyzers, using the same chromatographic conditions, were evaluated for the determination of 16 HAAs [99]. The analyzers were: (a) an ion trap, (b) a single quadrupole, and (c) a triple quadrupole. The (b) and (c) systems were equipped with a Turbo Ionspray as ionization source. Selected ion monitoring was used as data acquisition mode for the systems (b) and (c). The systems (c) and (a) used a multiple reaction monitoring (MRM) and a product ion scanning, respectively, using as precursor ion the protonated molecular ions $[M+H]^+$. Post-column addition of formic acid-acetonitrile was needed to increase ionization efficiency when using the ion trap analyzer. In contrast with the observed when using the ionization source of the ion trap instrument, no post-column addition was needed in either single or triple quadrupole instruments. This fact can be explained by the higher electrospray ionization efficiency that provides the Turbo Ionspray compared with the ion trap system. The best RSD values were obtained when using triple quadrupole with MRM acquisition. In addition, triple quadrupole provided lower limits of quantification than the other systems. Because of this, its linearity range was generally two orders of magnitude larger. The results obtained with all the instruments and acquisition modes are in agreement, although the most precise ones were obtained with the triple quadrupole instrument. Nevertheless, the results achieved with the ion trap were also good and has the additional advantage of providing spectral information for false positive peak identification [99].

HPLC-APCI-MS–MS has also been applied [47,91]. To improve the detection and quantification limits, a 2-mm internal diameter HPLC column was used instead of the conventional 4.6-mm one [12,91]. When IQ, 8-MeIQx, 4,8-DiMeIQx and 7,8-DiMeIQx were treated under higher collision-induced dissociation conditions, the found data imply that the pyrazine moiety has been lost, with retention of the charge on the benzoimidazole-2-yl-amine moiety of 8-MeIQx and its homologues. Thus, MS–MS analysis of $[M+H-15]^+$ in the constant neutral acquisition mode enabled the identification of two other HAAs (IQx and 7,9-DiMeIgQx), which have rarely been reported in cooked meats [91]. Recently, two unidentified chromatographic peaks with product ion spectra and retention time very similar to those of 8-MeIQx and 4,8-DiMeIQx, have been found in the griddled beef samples. Turesky et al. [4] have recently mentioned the presence of these chromatographic peaks among others in fried or barbecued chicken and beef, and have proposed them to be isomers of 8-MeIQx and DiMeIQx [111]. HPLC-MS–MS is used as quantification technique, and product ion scan mass spectra provided by the ion trap mass analyser is used to confirm the identity of the analytes [111].

The addition of protons to form $[M+H]^+$ ions, and for some HAAs, sodium addition to form lower abundant ions of $[M+Na]^+$ were the major route of fragmentation of the HAAs using APCI-MS [78]. Several fragments produced in the source were also observed in the mass spectra by applying different extraction voltages and the fragmentation was always higher than in ESI [71,77]. An increase of $[M+H]^+$ ions abundance at higher voltages was found [78]. In order to explain this fact, the presence of adducts has to be considered. The gas phase in APCI can contain clusters ions from the interaction of the analyte with the mobile phase (H_2O, CH_3CN, NH_4^+ and CH_3COO^-). An extraction voltage of 40 V did not produce fragments of $[M+H]^+$ but it seemed to be high enough to dissociate the adducts with mobile phase ($[M+H+(H_2O)_m]^+$, $[M+H+(CH_3CN)_n]^+$. These dissociations may provide the increase of the $[M+H]^+$ ion abundance at 50 V [78].

A method based on HPLC-APCI-ion-trap (IT) MS–MS for the analysis of 16 HAAs is described [97]. The fragmentation patterns of the aminoimidazoazarenes observed are consistent with those obtained by other authors [47,91,128] using triple quadrupole instruments [97]. In addition to that, some ion-molecule reactions were observed into the trap according to Galcerán et al. [71,78] using HPLC-APCI-MS–MS [99,100]. These reactions occurred only for carbolines (Trp-P-1, Trp-P-2, AαC, MeAαC, norharman, harman, Glu-P-1 and Glu-P-2) by recombination of the product ion $[M+H-NH_3]^+$ with neutral molecules present in the ion-trap, such as water or acetonitrile. Also, adducts of *m/z* higher than parent ion were obtained [71,97,99,100]. The abundance of these product ions is highly dependent on small changes of experimental conditions. As these ions had a very high signal, they must be added to the base peak to carry out the quantification of carbolines by MS–MS in order to obtain reproducible results [99,100]. But these adducts have not been observed by other authors [12,128] working with triple quadrupole instruments, with the exception of Galcerán et al. [71,78]. On the other hand, the MS–MS spectra obtained with the ion trap and the triple quadrupole systems, were very similar in both fragment ions and relative abundances, except for carbolines that showed adduct formation in the ion trap [99]. These adducts observed in the ion trap spectra were not present in the MS–MS spectra obtained with the triple quadrupole instrument. This fact can be explained by the absence of neutral molecules from the mobile phase inside the collision cell [99].

A new ultra-performance liquid chromatography method (UPLC-ESI-MS–MS-SRM) was developed to allow the determination of 16 HAAs in less than 2 min [113]. UPLC operates at much higher pressure, and to address the very narrow peaks produced, a high data capture rate detector is necessary. Argon was used as collision gas instead of N_2, because Ar seems to need relatively low collision energy for fragmentation. The linearity range was established over three orders of magnitude. In addition to the reduction in analysis time, the detection limits obtained are up to 10-fold lower than those obtained using similar triple quadrupole instruments but with a conventional HPLC system [113].

Time required for extraction with SPE cartridges, chromatographic separation and HPLC-MS–MS determinations are too long. Therefore, a method using selective ionization of metastable atom bombardment (MAB) has been developed in order to detect HAAs in non-purified meat extracts, thus avoiding purification and concentration steps and reducing analysis time around 18-fold [114]. MAB ionization forms radical ions by electron transfer from a molecule to a species (noble gas or nitrogen) excited in a metastable state. By selecting metastable gas for ionization, it is possible to precisely control the available ionization energy in the gas phase. This allows one to control fragmentation extent of the studied species and to selectively ionize some molecules in a mixture depending on their ionization potentials. Metastable nitrogen was selected as the best MAB gas for the analysis of HAAs. The MAB ionization source was coupled to a pyrolyser, which allows analysis by direct introduction of the sample into the mass spectrometer, therefore constituting a fast analytical technique. The pyrolysis probe was not designed to achieve a real pyrolysis process involving thermal degradation of molecules, but was rather used to rapidly transfer molecules into the gas phase. Detection of HAAs is completed in 27 s. However, RSDs are quite large and are due to the manual introduction of the sample into the pyrolysis probe. This Pyrolysis-metastable atom bombardment ionization-time of flight-mass spectrometry (Py-MAB–ToF-MS) method was in good agreement with a HPLC–APCI-MS–MS-MRM method [114].

3.5. Planar chromatographic methods

HPTLC is a versatile offline method and offers multiple detection possibilities due to the local fixation of separated substances.

A rapid HPTLC method has been applied to the separation and quantification of apolar, pyrolytic HAAs. The amines were separated on silica gel HPTLC plates by a multiple development with diethyl ether. Quantification by fluorescence measurement at 366 nm was performed immediately after development. Limits of detection were in the low-nanogram range [132]. Recently, other method in which all the HPTLC steps are performed automatically has been proposed [133]. After preconditioning the HPTLC silica gel layer with ammonia vapour, the plate was developed with methanol–chloroform. The ammonia vapour had a decisive effect on separation efficiency, because if the preconditioning step is not carried out, separation was not achieved. Besides, if preconditioning and development were performed in the same chamber the ammonia vapour had a negative effect on chromatography. Migration time was 30 min at room temperature and 34% relative humidity. To confirm the absence of potentially coeluting minor HAAs, mass spectra were recorded by online HPTLC-FD-ESI–MS-SIM [133]. A harman sample was separated on silica gel 60 HPTLC plates at pH 10.4, with mixtures of diethyl ether and methanol as mobile phases. By use of a newly developed device the spot was extracted from the TLC plate and transferred to ESI-MS or ESI-MS–MS. As extraction solvent, methanol/formate buffer (pH 4.0) was used. LOQ/LOD obtained were of similar magnitude as reported for HPLC–MS methods. Mass spectrometric signal can be obtained within 1 min. An advantage of coupling HPTLC with MS is the minimal employment of the MS equipment due to the local fixation of separated substance zones on the planar chromatogram [134]. Figures of merit are shown in Table 8.

3.6. Capillary electrophoresis

Compared with HPLC, capillary zone electrophoresis is capable of achieving higher separation efficiency, uses lower volume of organic solvents, and requires small amounts of samples. However, the migration behaviour of ionized compounds is less well characterized than their retention behaviour in HPLC. Analytical methods with ultraviolet (CE-UV) [79,135], diode array detection [18,49,109,135–137] or electrochemical (CE-ECD) detection [48] have been proposed although high detection limits have been obtained. For routine use, optical detectors are more preferred over the expensive and complicated MS system. Fluorescence detection is limited to apolar HAAs, in spite of its higher sensitivity and selectivity. UV and UV-DAD are most used because all HAAs can be detected, although sometimes the

Table 8
Figures of merit for the determination of HAAs by high-performance thin-layer chromatography

Method	Figure of merit	PhIP	MeIQx	4,8-DiMeIQx	Norharman	Harman	Ref.
HPTLC-FD-ESI–MS-SIM	D.L. (ng)	3.7	4.7	3.9	0.4	0.4	[133]
	Repeatability (%)	1.5–2.3	2.0–3.6	2.2–4.2	2.1–4.1	2.5–4.4	
	Reproducibility (%)	0.4	0.6	0.7	0.9	1.1	
HPTLC-FD-ESI–MS-SIM	L.O.Q. (pg)					2.3–35.6	[134]
	Repeatability (%)					12.5 ± 4.3 (4.27–19.31)	
HPTLC-FD-ESI-MS–MS-SRM	L.O.Q. (pg)					>20	[134]

low sensitivity of the UV-DAD does not allow the detection of ng/g levels of HAAs in real samples.

To increase the sensitivity of UV-DAD, preconcentration methods that can be combined with CE are required. The on-line preconcentration methods utilize the differences in mobilities and conductivities to preconcentrate the analytes. The field-amplified sample injection (FASI) procedure consists in to dissolve the sample in a solvent of lower conductivity than that of the running electrolyte; then the sample is injected in electrokinetic mode; when applying of the voltage, the electric field strength of the low-conductivity zone is increased, producing an increase of electrophoretic velocities and a narrower analyte zone. That is, the focusing process occurs during the electrokinetic injection of the sample [109]. Tables 9 and 10 show the figures of merit for the determination of HAAs.

Micellar electrokinetic chromatography with amperometric detection (MEKC-ECD) allows that HAAs with a wide range of polarities can be rapidly and efficiently separated. Injection of samples were made hydrodynamically [48]. Amperometric detection was performed in the end-column mode using a three-electrode configuration. The electrolyte consisted of borax–H_3PO_4 buffer (pH 9.1) and cetyl-trimethylammonium bromide (CTAB). To minimize the required oxidation potential, a high pH is favourable. However, at pH 9.1 HAAs are uncharged and as a consequence the CE-separation requires the addition of micelles to the electrolyte. The charged micelles serve as a dynamic stationary phase, and separation is governed by differing solubility of the analytes in the micellar phase. The more apolar amines (with more methyl groups) increasing their solubility in the micelles, migrate slower than the corresponding amines without methyl groups. If additional nitrogens are present in the aromatic structures, the hydrophobicity decreases, and faster migration times result. CTAB also prevents wall adsorption, since it forms a positively charged layer on the capillary wall, efficiently covering the negative silanol groups [48].

Likewise, MS detection can be coupled. This method offers high separation efficiency with low operation cost, but sample preparation with high enrichment is needed due to its high detection limits. For example, detection limits ranging from 18 to 360 ng/g, and precisions up to 1.4% and 12% for migration time and concentration, respectively, were obtained [138]. In order to improve sensitivity, FASI was applied as an in-line preconcentration method. Methanol/5 mM formic acid (50/50) as a sample solvent, 3 s hydrodynamic injection of a methanol plug, and 25 s of electrokinetic injection of the sample were found to be the optimum conditions. Detection limits up to 25 times lower and similar precisions than those reported for hydrodynamic injection were obtained [138].

It is known that electrophoretic mobility depends strongly on the pH. A lot of parameters (e.g. pH, applied voltage, temperature, concentrations, etc.) need to be incorporated in the optimization strategy to achieve an adequate separation of complex mixtures [136]. Strategies for the systematic optimization of capillary electrophoresis have proved to be ineffective in locating the true optimum and are time-consuming. The advantages of combining the orthogonal array design (OAD) with the overlapping resolution mapping (ORM) scheme in optimizing the separation of eleven HAAs and two carbolines by capillary zone electrophoresis are demonstrated. The OAD method was used to perform preliminary screening to identify the important factors affecting resolution. The ORM scheme was used to determine the global optimum conditions within the experimental ranges of the variables under consideration. The combination of the two methods overcomes the disadvantages of each individual method when used alone, and provides a powerful approach which can be utilized for the optimization of separation of complex mixtures. For example, within the selected range, buffer pH is the most important factor, and pH 2.5 was better than pH 3.5. The concentration of organic modifier (methanol) was the next important factor, in this case, 15–35%, and thus for other parameters. Interaction between factors are also determined [136]. However, when other test was applied for other authors [49], the main influencing parameters found were pH (2.00–3.20) and temperature (20–24 °C). Concentration of methanol (30–40%) had less influence [49]. These finds are explainable: HAAs are a group of weak bases, hence they are converted to protonated species at low pH (pH 2.5 is better). Methanol improved the solubility of HAAs in the buffer, interacted strongly with the capillary wall, and therefore reduced the chance of interaction between solutes and the wall. Also, methanol has a poor conductivity. Increasing the MeOH content of the buffer solution increased the migration times of HAAs. Temperature affects viscosity and, therefore, electrophoretic and electroosmotic flows.

More than 12 HAAs were separated using uncoated silica capillary (25 °C) within 12–15 min [135,136]. Ultraviolet with diode array detection system was used. The buffer system was Na_2HPO_4–NaCl–citric acid, pH 2.1. Addition of NaCl reduces the electroosmotic flow by decreasing the thickness of the double-layer, but in the experimental conditions of these investigations, no effect was detected. The sum of resolutions of a buffer with NaCl was even smaller than that one without NaCl. It is likely that the effect was masked by relatively high concentrations of other electrolytes, such as Na_2HPO_4 in the system. But the addition of NaCl resulted in a competition between Na^+ and amines for cation-exchange sites on the silica surface and therefore reduced the adsorption of HAAs on the wall [136].

More than 25 peaks have been resolved in a very short time, but only MeIQ and MeIQx have been quantified [79]. Methanol was not added to the buffer solution that is composed by KCl–HCl at pH 2.20. Selective and sensitive detectors must be used to confirm the identities of resolved peaks. As usual, higher responses were achieved for the electrokinetic injection mode, but the hydrodynamic (pressure injection) mode gave higher run times and better resolutions [79]. The hydrodynamic injection provided better reproducibilities than the electrokinetic one [49,79].

The effects of buffer pH and the concentration of the organic modifier (methanol and acetonitrile) on the separation and elution order of HAAs were studied and compared with those found in the literature [137]. The KCl–HCl system [79] had several advantages over the phosphate buffer [135,136], such as lower detection limits, better resolution, and lower background noise. In addition, phosphate buffer produced very high currents that

Table 9
Figures of merit for the determination of thermic HAAs by electrophoretic methods

Method	Figure of merit	DMIP	PhIP	IQ	MeIQ	MeIQx	7,8-DiMeIQx	4,8-DiMeIQx	Others	Ref.
MEKC-ECD[a]	D.L. (μg/L)			4.0	9.6	7.4	9.4	12	TriMeIQx: 21	[48]
	RSD (%)			2.2	2.3	3.5	3.3	3.4	4.5	
	Migration time (min)			5.21	6.00	5.07	5.59	5.90	6.69	
CZE-UV-DAD (electrokinetic injection)	D.L. (mg/L)		0.16	0.13		0.12		0.08		[49]
	RSD (%)		11.2	20.7		16.4		8.6		
CZE-UV-DAD (hydrodynamic injection)	D.L. (mg/L)		0.22	0.20		0.05		0.14		[49]
	RSD (%)		4.7	7.3		5.5		7.9		
	Extraction recovery (%)		6–12	55–90		67–80		62–91		
CZE-UV-DAD (hydrodynamic injection)	D.L. (μg/g)		1.05		0.674	0.542			Iso-IQ: 0.596	[109]
	RSD (%)		3.38		2.88	3.07			4.18	
	Linear range (μM)		7.5–100		5.0–100	5.0–100			5.0–50	
	Linear function, y		$0.103x \pm 0.171$		$0.130x \pm 0.229$	$0.107x \pm 0.356$			$0.121x \pm 0.273$	
	r		0.9997		0.9996	0.9979			0.9988	
	Response time		1.0		1.0	1.0			1.0	
CZE-UV-DAD (electrokinetic injection)	Improved response time		23.0		30.5	20.6			31.8	[109]
CZE-UV-DAD (field-amplified sample injection, FASI)	Improved response time		24.0		35.6	22.8			2.6	[109]
	D.L. (ng/g)		7.96		7.25	3.14			1.33	
	RSD (%)		4.95		5.95	3.58			9.38	
	Recovery (%)		69.1		51.6	54.1			35.0	
CZE-UV	Recovery (%)				66.6	77.7				[79]

[a] MEKC-ECD: micellar electrokinetic chromatography with amperometric detection.

Table 10
Figures of merit for the determination of pyrolytic HAAs by electrophoretic methods

Method	Figure of merit	Glu-P-2	Glu-P-1	Harman	Norharman	AαC	MeAαC	Trp-P-2	Trp-P-1	Ref.
MEKC-ECD[a]	D.L. (μg/L)	8.5	7.0							[48]
	RSD (%)	6.2	5.4							
	Migration time (min)	4.97	6.16							
CZE-UV-DAD (hydrodynamic injection)	D.L. (μg/g)			1.40	1.78	1.06	0.971			[109]
	RSD (%)			3.95	7.47	4.28	6.20			
	Linear range (μM)			10–100	25–200	7.5–100	7.5–100			
	Linear function, y			$0.0521x \pm 0.242$	$0.0408x \pm 0.212$	$0.106x \pm 0.0502$	$0.0743x \pm 0.272$			
	r			0.9965	0.9983	0.9996	0.9987			
	Response time			1.0	1.0	1.0	1.0			
CZE-UV-DAD (electrokinetic injection)	Improved response time			32.8	43.6	25.2	13.8			[109]
CZE-UV-DAD (field-amplified sample injection, FASI)	Improved response time			34.1	48.8	23.9	34.1			[109]
	D.L. (ng/g)			3.99	3.51	18.3	19.4			
	RSD (%)			4.36	3.58	6.43	11.1			
	Recovery (%)			49.1	64.5	78.0	64.0			

[a] MEKC-ECD: micellar electrokinetic chromatography with amperometric detection.

might produce a breakdown of current in the instrument. Also, the running buffer was modified with α-, β-, and γ-cyclodextrin. The overall separation was improved with β-cyclodextrin [137]. Other authors [109] have chosen formic acid-ammonium formate solution, 5 mmol/L, at pH 2.20 as the running electrolyte. At higher concentrations of buffer, the baseline became fluctuant. Methanol–water (1:1) was applied as the sample solvent. A voltage of 18 kV was chosen to allow both low analysis time, background noise and band dispersion. To improve sensitivity, FASI was used with 3 s hydrodynamic injection of a water plug and 25 s electrokinetic injection of the sample [109].

A miniaturized technique to analyze and detect HAAs using micro solid-phase extraction (SPE) coupled on-line (in-capillary) to capillar electrophoresis (CE) separation with nanospray (nESI) mass spectrometry detection has been developed [139]. The on-line coupling of SPE, CE and n-ESI-MS reduced the time of extraction and identification to less than half an hour. This technique provides short analysis time, low sample and solvent consumption, and HAAs in standard solution were easily detected at 12–17 fmol injections, and in spiked urine samples at 750–810 fmol injections [139].

CZE is also a convenient technique for the determination of ionization constants. Thus, constants for eight HAAs have been determined and their values were confirmed with UV-spectroscopy [140]. The technique is rapid, precise, uses small quantities of solute, the exact concentration of the compounds is not needed, and can be automated. However, the source of error lies in the measurement of buffer pH.

On the other hand, capillary electrochromatography (CEC) is a separation technique that has recently been drawing increasing attention due to its analytical potential. CEC is a hybrid technique that combines the selectivity of LC and the separation efficiency of CE. Galcerán et al. [141] have evaluated the applicability of CEC for the separation of HAAs. A new methacrylate-based monolithic column that contained a *N*,*N*-dimethylamino ethyl acrylate group was used. Cathodic polarity and counter-directional mode were employed, and good performance was obtained in terms of resolution, efficiency and asymmetry factors. Moreover, the method showed an acceptable sensitivity (detection limits were 0.1–1.2 ppm) and good column-to-column reproducibility (5–10%) [141].

4. Conclusions

The accurate determination of HAAs is a difficult analytical task since traces of these compounds have to be determined in highly complex food matrices. Some HAAs or HAAs derivatives can bind with other food components. All of these formed compounds cannot be extracted from food by the usual extraction methods. Therefore, different extraction procedures have to be applied to cooked and uncooked meat, before and after enzymatic proteolysis. This problem can only be solved by combining both elaborate sample preparation steps with selective separation steps, and then followed by sensitive detection methods to quantify low levels of HAAs. Tedious clean-up procedures that include extraction, purification, and pre-concentration steps, followed by a separation technique, such as liquid or gas chro-

matography and capillary electrophoresis are usually used. The main detection systems used are UV, fluorescence, electrochemical and MS. Really, recent advances in the analytical instrumentation, concretely in LC–MS and GC–MS, have greatly facilitated the ability to measure HAAs in foods. Nevertheless, some problems in extraction recoveries must also be taken into account.

Arvidsson et al. [86] found that average recoveries from the purification stage differed depending on the temperature and duration of heating applied through the cooking of food. At 100–125 °C, recoveries were time dependent and started at low values, increasing to plateau values that were about the same as recoveries at 150–225 °C. In addition, recovery of HAAs has been found to be greatly dependent on the sample matrix [103,106]. For example, relatively high recoveries were obtained for the analysis of grilled sausage, minced meat (>75%), whereas the analysis of goose and rabbit resulted in lower recoveries (≈40%) [106]. Similar percentages were found for 4,8-DiMeIQx in griddled chicken breast and in griddled beef steak, respectively [103]. Consequently, the use of absolute or relative values of HAAs content in food can lead to erroneous conclusions when possible cancer risks are established for the intake of these foods.

To quantify HAAs accurately, an internal standard must be used since analyte extraction efficiency is not 100%, and amounts of HAAs have to be corrected for incomplete recoveries. As the sample matrix influences the extraction efficiency, the multiple standard addition quantification method is the best way to quantify the HAAs. In addition, internal standards are used to control the final volume obtained from the purification and preconcentration steps. As examples of internal standards used are 7,8-DiMeIQx, TriMeIQx, caffeine, and several labelled standards of HAAs, with ^{2}H, ^{13}C, ^{15}N.

Two interlaboratory exercises on the determination of selected HAAs in beef extract, organised in the framework of an European project are presented [106,142]. The aim of these exercises was to improve the quality of the laboratories and to evaluate the performance of a standardised analytical method and also the methods currently used by each one of the participants for the analysis of these compounds. For it, a beef extract was prepared as a laboratory reference material. Homogeneity and stability studies were performed at different temperatures and times [143]. For these reasons, "method B" (Fig. 1) for clean-up and LC–MS or LC–MS–MS for identification and quantification are the most recommended methods, especially when the concentrations of HAAs in the samples are very low [142]. Also, HPLC with fluorescence detector leads to similar results [106].

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