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Review

Separation of heteroaromatic amines in food products

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Abstract

In recent years, many studies have dealt with the role of certain heteroaromatic amines (HAs) as mutagenic compounds, and their occurrence in foodstuffs. Here we examine the determination of HAs, focusing on the analytical strategies for their extraction and preconcentration from several matrices. We summarise the properties of heteroaromatic amines and the main drawbacks involved in their analysis, and then concentrate on the separation procedures, sorbents and solvents used in the sample treatment. We discuss the requirements of the analytical techniques and the strategies most frequently followed to achieve accurate results.

Keywords: Reviews; Heteroaromatic amines

Contents

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1. Introduction

1.1. Heterocyclic amines: formation, structures and toxicity

As many epidemiological reports have shown, diet is a key factor in cancer development [1]. Food can positively contribute to human carcinogenesis in two ways: the first is related to the presence of genotoxic chemicals as food contaminants, which can form DNA adducts, and the second involves natural food components, such as water or fat content, which can indirectly enhance the formation of mutagenic compounds in the thermal processing of foods.

In the mid 1970s, the development of a short-term assay for the determination of mutagenic activity allowed Nagao et al. to detect a high level of mutagenicity in the charred parts of grilled beef and fish and in the smoke produced while broiling sardines [2]. After further studies, some of the compounds responsible of the mutagenic activity, identified as heterocyclic amines (HAs), were isolated from pyrolysed amino acids and proteins (amino-carbolines, named as pyrolytic HAs) [3] and from a variety of protein-rich foods, such as meat or fish, cooked by ordinary household methods (amino-imidazoazaarenes, AIAs, also named thermic HAs) [4].

To date, more than 20 compounds included in this family have been isolated from different food samples, and most of their structures have been elucidated [5]. The corresponding names and abbreviations are given in Table 1, and structures are shown in Figs. 1 and 2.

Table 1
Heterocyclic amines found in model systems or in cooked foods

Name and classification	Abbreviation
I. Aminoimidazo azaarenes	
2-Amino-3-methylimidazo[4,5-*f*]quinoline	IQ[a]
2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline	MeIQ[a]
2-Amino-3-methylimidazo[4,5-*f*]quinoxaline	IQx
2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline	4-MeIQx
2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline	8-MeIQx[a]
2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline	4,8-DiMeIQx
2-Amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline	7,8-DiMeIQx
2-Amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline	TriMeIQx
2-Amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]quinoxaline	4-CH_2OH-8-MeIQx
2-Amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline	7,9-DiMeIgQx
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine	PhIP[a]
2-Amino-1,6-dimethylimidazo[4,5-*b*]pyridine	DMIP
2-Amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine	TMIP
2-Amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine	4′-OH-PhIP
2-Amino-1,6-dimethylfuro[3,2,*e*]imidazo[4,5,*b*]pyridine	IFP
I. Amino-carbolines	
2-Amino-9*H* -pyrido[2,3-*b*]indole	AαC[a]
2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole	MeAαC[a]
1-Methyl-9*H*-pyrido[4,3-*b*]indole	H
9*H*-Pyrido[4,3-*b*]indole	NH
3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole	Trp-P-1[a]
3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole	Trp-P-2[a]
2-Amino-6-methyldipyrido[1,2-a:3′,2′-*d*]imidazole	Glu-P-1[a]
2-Aminodipyrido[1,2-a:3′,2′-*d*]imidazole	Glu-P-2[a]
2-Amino-5-phenylpyridine	Phe-P-1
4-Amino-6-methyl-1*H*-2,5,10,10*b*-tetraazafluoranthene	Orn-P-1
4-Amino-1,6-dimethyl-2-methylamino-1*H*,6*H*-pyrrolo[3,4-*f*]benzimidazole-5,7-dione	Cre-P-1
3,4-Cyclopentenopyrido[3,2-*a*]carbazole	Lys-P-1

[a] Mutagenic and carcinogenic heterocyclic amines.

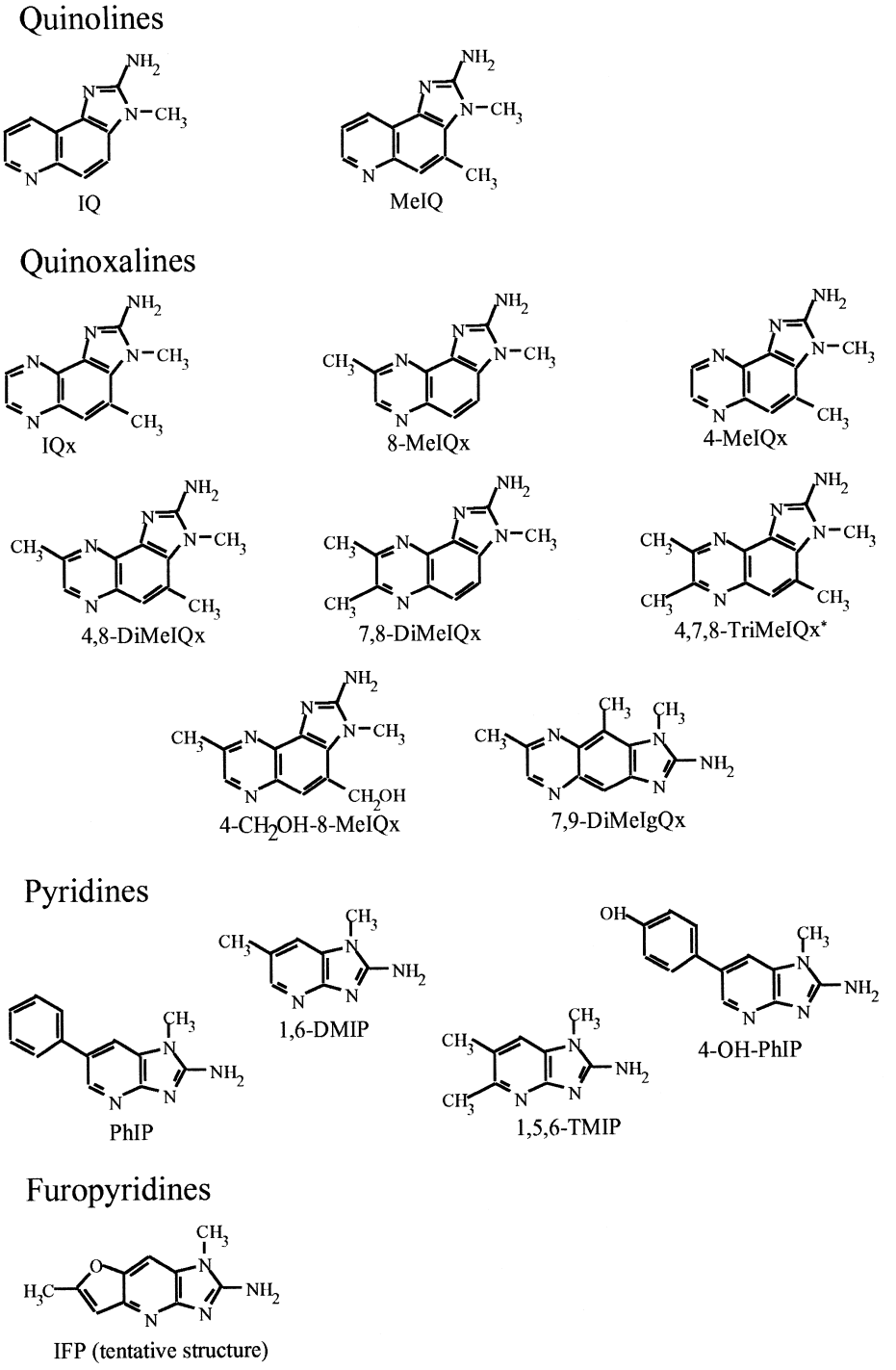

Fig. 1. Structures of the AIAs or thermic mutagens. Not reported in foods.

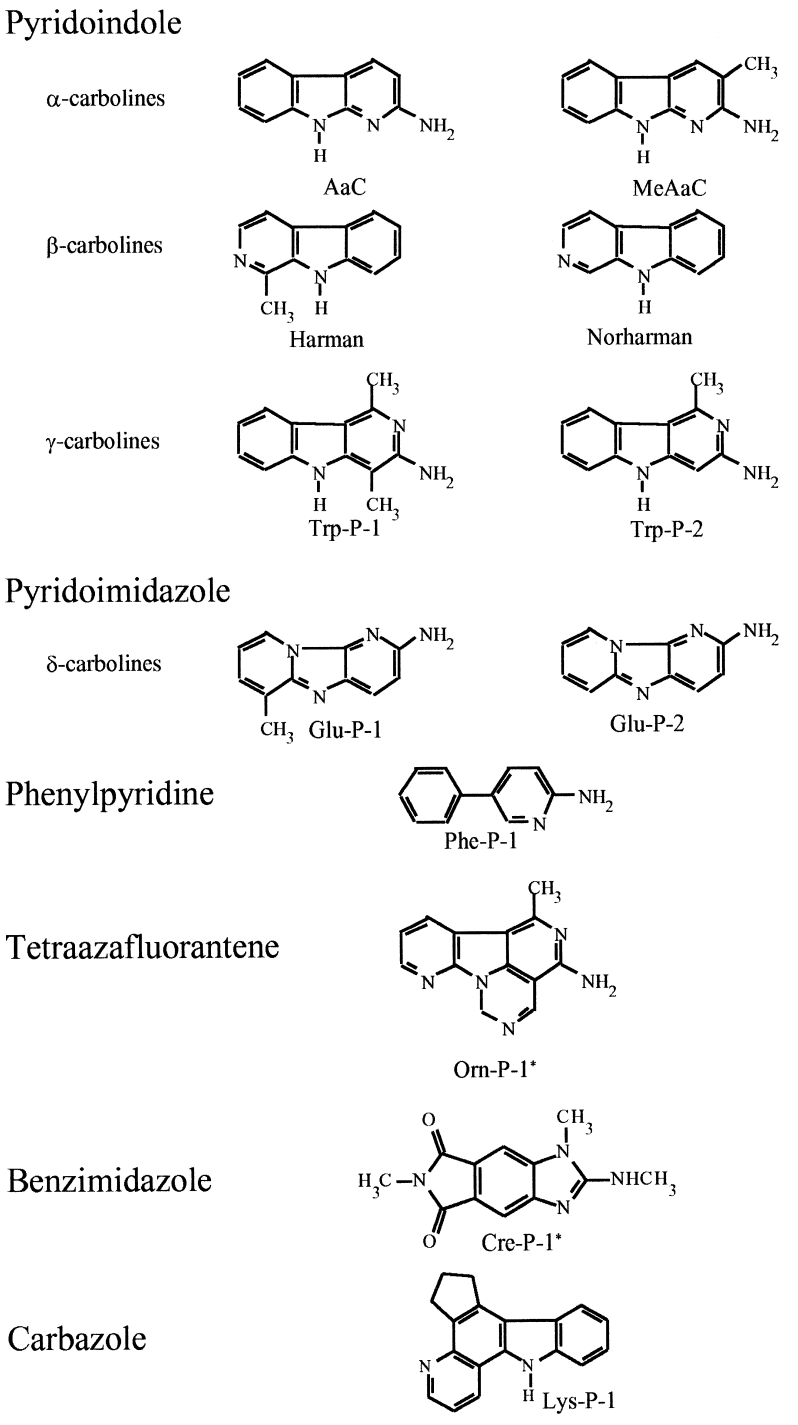

Fig. 2. Structures of the amino-carbolines or pyrolytic mutagens. Not reported in foods.

HAs have a planar, multiring aromatic structure with one or more nitrogen atoms in their ring system and an exocyclic amino group, except in the case of harman, norharman and Lys-P-1. Their formation is greatly dependent on several factors including cooking temperature and duration, concentration of precursors, presence of enhancers or inhibitors, amount of lipids or water and pH [6]. Chemical modelling has shown that, while amino-carbolines are generated via free radical reaction at temperatures generally above 300°C, AIAs are more easily formed during ordinary cooking by means of the reaction between creatinine, sugars and free amino acids, with the Maillard reaction playing an important role [7,8].

Heterocyclic amines are potent mutagens after metabolic activation when tested both in vivo and in vitro [9,10], except harman and norharman, which are not mutagenic but enhance the mutagenicity of the rest of HAs. Moreover, the 10 HAs bioassayed to date (Table 1) with long-term animal studies have been shown to be carcinogenic in rats, mice or even monkeys [11,12].

These results, together with epidemiological studies which show a certain correlation between consumption of meat and increased risk of tumour generation in humans [13,14], suggest that HAs are a potential risk factor in the aetiology of human cancer. In order to evaluate the significance of these HAs in human cancer, research is focusing on their formation, bioavailability, bio-transformation, carcinogenicity and occurrence in food [15–18]. One of the most relevant aspects is the accurate determination of the dietary intake of HAs, which requires the establishment of highly selective and sensitive analytical methodology.

The determination of HAs in foods is hindered by several factors such as their very low concentration level (ppb). Moreover, the considerable complexity of the matrix prevents an efficient clean-up and, thus, several interfering substances are present in the final extracts. Therefore, many aspects of the analysis, such as extraction, purification, chromatographic separation and detection need to be optimised.

1.2. Scope of the review

In recent years a number of studies have shown several aspects concerning heterocyclic amines namely, their occurrence in food and in a general environment, their chemistry and formation of new mutagenic heteroaromatic amines and both their biological activity and potential human toxicity. Moreover, great efforts have been made to establish or improve analytical tools for the reliable determination of HAs in a variety of food samples, and the establishment standard methodology for their analysis has been attempted by means interlaboratory exercises. Many of these aspects have been summarised [11,17,19–24]. However, actual status on levels of human exposure to HAs is not well known, and reliable knowledge about these levels is mandatory for designing epidemiological studies and future risk assessments. Therefore, this review deals with some aspects of the analytical methodology necessary for the determination of HAs in foods. Primarily attention is paid on strategies for sample preparation to achieve both sample clean-up and sample concentration. Finally, procedures for the accurate quantification of HAs are surveyed in relation to the difficulties of obtaining reliable recoveries in the sample preparation.

2. Analytical strategies

Heteroaromatic amines can occur in a wide variety of samples, but they have been mainly found in proteinaceous foods, including cooked meat and fish and industrial origin samples like bouillon concentrates, process food flavours or meat extracts [25–27]. Furthermore, simulation systems have been developed as models for studying the mechanisms by which mutagenic amines could be generated when food is thermally processed, and also for determining the influence of various parameters in the formation of HAs [28,29]. The analytical procedures for monitoring these mutagens in both matrices, food or model systems, always require consideration of the sample matrix composition, which entails laborious analytical approaches including several steps to obtain extracts clean enough for quantification purposes.

Besides proteinaceous foods, other sources of human exposure to HAs have been studied. Thus, wine and beer have been successfully analysed in order to determine the presence of these mutagenic

substances [30,31]. In addition, some of these analytes have been detected in several environmental samples such as outdoor and indoor air, cigarette smoke, cooking fumes, rain water and incineration ash [22,32,33].

On the other hand, metabolism studies involve the analysis of biological matrices including plasma, urine, faeces or bile [34,35] in order to establish the biotransformation pathways and determine the ability of humans to metabolically activate or detoxify the procarcinogenic amines. In addition, the analysis of HAs in human urine or plasma can be used as an indicator of dietary exposure to these mutagenic compounds in daily life.

The origin of the sample to be analysed and the analytical technique selected for the determination, greatly influence the degree of purification required, and therefore the complexity of the sample preparation process, which is one of the most important steps in the analysis of heterocyclic amines. Other essential aspects of the chemical analysis are both the unequivocal identification and the accurate quantitative determination of the HAs. Before 1983, the only method applied to determine the presence and amount of mutagenic amines was the Ames/*Salmonella* test, consisting of the assay of the genotoxicity of the compounds studied in front of bacteria. In some cases, total mutagenic response was measured [36,37], while in others individual responses of various analytes were obtained [4,38]. In order to measure the mutagenicity of a single compound, it was necessary to purify the sample exhaustively and to isolate each mutagen in independent fractions by means of liquid chromatography (LC) or high-performance liquid chromatography (HPLC). Quantitative results were not accurate because the estimation of the content was based on highly purified fractions with low percentages of the initial quantity of the mutagenic substance, with a large variation in the recovery of the analyte. Therefore, this methodology was mostly applied as a preparative step for the isolation of unknown mutagens, with subsequent characterisation of the compounds by more selective techniques.

Nowadays, the determination of the HAs is commonly carried out by means of chromatographic or electrophoretic techniques using different detection systems. Gas chromatography (GC) is used for the analysis of heterocyclic amines because of its simplicity, high resolving power and low cost. Moreover, the use of nitrogen–phosphorus detection (NPD) [39] or mass spectrometry (MS) [40,41] allows highly sensitive detection. Some of the heterocyclic amines, namely, Trp-P-1, Trp-P-2, AαC, MeAαC, H and NH, due to their low polarity can be directly analysed with GC without previous derivatisation [42]. However, most HAs are polar and non-volatile, and tend to elute as tailing peaks due to their strong adsorption to the column and injector during GC analysis. Therefore, an appropriate derivatisation procedure is required for the detection of low concentration levels.

HPLC is used to analyse HAs, because the derivatisation step required in GC is avoided and several detection systems can be used. The chemical structure of HAs provides a characteristic UV spectrum with high extinction coefficients, some amines exhibit fluorescence, and most of them can be oxidised electrochemically. Therefore, these compounds can be measured with UV detection [43,44], electrochemical detection (ED) [45,46] and fluorescence detection [29,47]. The detection method most commonly used is diode array detection (DAD) [48,49], which allows on-line identification of the analytes by spectral library matching and has a low cost. Usually fluorescence detection is used as a complement to DAD, because unavoidable interferences are frequently produced when using UV detection.

On the other hand, the improvements introduced during the last decade in the coupling of LC with MS, especially the development of modern atmospheric pressure ionisation (API) sources based on electrospray ionisation (ESI) [50,51] and atmospheric pressure chemical ionisation (APCI) [52,53], have allowed this technique to be successfully applied to the analysis of HAs. The mass spectrometer, a high selective, sensitive detection system, behaves essentially as a mass selector. Therefore, interference levels for a complex sample matrix are reduced when comparing with more universal detectors such as UV, and laborious isolation procedures can be reduced. In addition, a more selective detection can be carried out by means of MS–MS [54].

The separation technique most recently proposed for the analysis of HAs is capillary electrophoresis

(CE), which has higher separation efficiency and requires lower volume of solvents and sample than HPLC. Analytical methodology based on CE with UV [55], DAD [56], ED [57] or MS [58] has been successfully developed.

Other analytical procedures for the determination of HAs are based on the ELISA (enzyme-linked immunosorbent assay) methods. Because of their high sensitivity, high selectivity and low sample preparation requirements, immunoassays attract attention of some scientists, and some monoclonal antibodies (MAbs) have been developed to carry out the analysis. However, since this method is highly specific and only a few MAbs have been synthesised, only some of the mutagenic amines, including IQ, MeIQ, MeIQx, 4,8-DiMeIQx and PhIP, have been analysed by immunoassay [59,60].

3. Sample preparation for the analysis of HAs in foods

To assess the risk to human health derived from the daily consumption of foods containing heterocyclic amines, an accurate quantification of the amount of potential carcinogens to which man is chronically exposed is essential.

There are several factors that hinder the analysis of heterocyclic amines in foods. These analytes are present at part-per-billion levels (ng/g), which requires an optimisation of chromatographic efficiency and both detector sensitivity and selectivity. Moreover, many foods are often a complex heterogeneous mixture composed by a high number of chemical substances, therefore the accurate quantification of individual chemical compounds in this kind of samples is difficult.

3.1. The search for new mutagenic HAs

As shown in Table 2, where the sample treatment for mutagenicity and characterisation studies are related in chronological order, earlier studies focused on the determination of mutagenic activity in food [61–64]. The aim of later studies was the isolation and characterisation of the compounds responsible for the mutagenicity, which were mainly present in the basic fractions [4,65–71].

In general, the procedures detailed in Table 2 have in common an initial homogenisation step, mainly carried out by adding hydrochloric acid to the sample [4,37,61,63,65,68–73]. Other solvents used are acetone [62,64], water [66,74], methanol [36] and a water–acetonitrile mixture [67].

When total mutagenic activity is measured, the procedures are very simple, based mainly on successive liquid–liquid extractions at different pH after protein precipitation [36,37,61,62,64]. A purification step with Amberlite XAD-2 is described only in two of the reviewed cases [63,73]. However, when the objective is the mutagenicity or the characterisation of a single compound, extensive fractionation is required. Thus, highly laborious procedures, which require large amounts of starting material (10–100 kg) to obtain enough mass for the analyses, have been developed. After the initial homogenisation, further purification is carried out by acid–base partition [66], LC using different sorbents [70,71,74,75] or combinations of both methodologies [4,65,67–69]. Final purification is attained by means of one or more HPLC steps, which also provide the analytes isolated in different fractions, whose genotoxicity is tested with the Ames/*Salmonella* test. The isolated mutagens are then characterised using more selective methodologies, including UV and fluorescence spectrophotometry, high-resolution MS or nuclear magnetic resonance (NMR).

3.2. Separation and quantification of HAs in foods

Once a mutagenic compound has been identified and standard solutions are available, analytical-scale purification procedures and chromatographic methods for the accurate quantification of this analyte can be developed (Tables 3–5).

Sample preparation procedures before the identification and quantification of mutagenic amines have several steps. As mentioned above, the first consists of a solution step, where the sample is homogenised and dispersed using different solvents. In the cases compiled in Table 3, the solvents used are organic, such as methanol [38,76–78], acetone [9,79], ethyl acetate [3], hydro–alcoholic mixtures [80–83], or aqueous, like hydrochloric acid [30,40,41,45,59, 60,84–86], water [87,88] or sodium hydroxide [89,90]. In all the procedures (Tables 3 and 4),

Table 2
Sample preparation methodology for mutagenic studies and isolation of new compounds

Processed sample	Compounds	Sample preparation	Analysis	Year	Ref.
Ground beef		1. HCl, pH 2.0 2. Add ammonium sulfate, filter 3. LLE: wash with DCM 4. LLE: adjust to pH 10, extract with DCM 5. Evaporate, dissolve in DMSO	Mutagenicity assay	1978	[61]
Ground beef		1. Acetone 2. Filter, −15°C, filter 3. Evaporate, dilute with 0.01 *M* HCl 4. LLE: wash with DCM 5. LLE: adjust to pH 12, extract with DCM 6. Evaporate, redissolve in DMSO	Mutagenicity assay	1981	[62]
Ground beef		1. HCl, pH 2 2. Centrifuge, neutralise 3. LC: Amberlite XAD-2, elute with acetone 4. Evaporate, dissolve in DMSO	Mutagenicity assay	1982	[63]
Beef extract	IQ, MeIQ, MeIQx	1. 0.1 *M* HCl, pH 2 saturated with NaCl 2. Filter 3. LLE: wash with DCM 4. LLE: adjust to pH 12, extract with DCM 5. LC: Silica gel+Adsorbosil-5, elute with MeOH–DCM 6. LC: Sephadex LH-20, elute with hexane–chloroform–MeOH 7. HPLC–UV: Ultrasil-NH_2, elute with 8. HPLC: Supelcosil LC-18, elute with MeOH–TEA phosphate (40:60) 9. HPLC: Ultrasil-NH_2, elute with CH_3COOH–PrOH–hexane	Mutagenicity assay MS	1983	[4]
Smoked mackerel, breaded cod sticks, fermented trout, cheese, chips, herring tid-bits, fish cake and pudding, anchovy, bread, caramel, wheatmeal buns		Same as Ref. [62]	Mutagenicity assay	1984	[64]
Ground beef	IQ, MeIQx, $C_9H_{12}N_4$, $C_{13}H_{11}N_3$, $C_{12}H_{13}N_5$	1. HCl, pH 2 2. Centrifuge, neutralise, centrifuge 3. LC: Amberlite XAD-2, elute with acetone 4. LLE: adjust to pH 2, wash with DCM 5. LLE: adjust to pH 12, extract with DCM (apolar bases) 6. Neutralise aqueous phase 7. LC: Amberlite XAD-2, elute with acetone (polar bases) 8. Recombine 5 and 7 9. HPLC: PRP-1 styrene–divinylbenzene, elute with 1% TEA in water–ACN 10. HPLC–UV: Spherisorb-NH_2, elute with 0.1% CH_3COOH in hexane–PrOH 11. HPLC–UV: LiChrosorb RP-18, elute with water–MeOH	Mutagenicity assay MS	1984	[65]

Beef	IQ, Trp-P-2, Trp-P-1	1. Boiling water 2. LLE: adjust to pH 12, extract with DCM–MeOH (3:1) 3. HPLC–UV: PRP-1 styrene–divinylbenzene, elute with ACN–water 4. Ascending paper chromatography: Whatman 3MM, elute with water–BuOH–PrOH–HAc (100:100:70:1) 5. Ascending paper chromatography: Whatman 3MM, elute with BuOH–HAc–water (12:3:5) 6. Paper strip electrophoresis, 10% HCOOH, pH 1.5 7. HPLC–UV: Nucleosil C_{18}, elute with MeOH–water	MS UV spectra	1985	[66]
Ground beef	PhIP	1–9: same as Ref. [65] 10. HPLC: PRP-1, elute with 0.1% DEA in water–MeOH 11. HPLC: Nucleosil C_{18}, elute with MeOH–water 12. HPLC–UV: Lichrosorb RP-18, elute with MeOH–water 13. HPLC–UV: Econsphere CN, elute with MeOH–water	NMR MS UV spectra	1986	[68]
Egg patties	IQ	1. ACN–water (10:1) 2. Filter and evaporate, dissolve in 0.01 *M* HCl 3. LLE: wash with ethyl ether 4. Neutralise 5. LC: Amberlite XAD-2, elute with acetone 6. LC: Sephadex LH-20, elute with MeOH–DCM (3:2) 7. HPLC: μ-Spherogel, elute with ACN 8. HPLC: PRP-1, elute with ACN–water 9. HPLC: Spherisorb 5-NH_2, elute with PrOH–hexane	UV MS	1986	[67]
Beef		1. Water 2. Centrifuge 3. LC: blue cotton, filter and elute with MeOH–NH_3 (50:1) 4. LC: Carboxymethyl cellulose column, elute with formic acid, water and MeOH–NH_3 (1:1) 5. HPLC: Nucleosil C_{18}, elute with MeOH–$HCOONH_4$	Mutagenicity assay	1986	[74]
Beef patties, hamburgers, sausages		Same as Ref. [65]	Mutagenicity assay	1988	[72]
Norwegian meat product	IQ, IQx, MeIQx, 4,8-DiMeIQx, DMIP, TMIP, PhIP	1. HCl, pH 2 2. Centrifuge, neutralise 3. LC: Amberlite XAD-2, elute with acetone 4. Evaporate, dissolve in dilute HCl, pH 2 5. LLE: extract with DCM 6. Aqueous phase: – Neutralise – LC: Amberlite XAD-2, elute with acetone 7. DCM extracts: – LLE: extract with acetone – Evaporate, dissolve in dilute acid, pH 2 – LLE: wash with DCM – Neutralise – LC: Amberlite XAD-2, elute with acetone	Mutagenicity assay MS	1988	[69]

Table 2. Continued

Processed sample	Compounds	Sample preparation	Analysis	Year	Ref.
		8. Acetone eluates from 6 and 7: – HPLC: PRP-1, elute with ACN–water – HPLC: Nucleosil NH_2, elute with PrOH–hexane – HPLC–UV: Nucleosil C_{18}, elute with MeOH–water – HPLC–DAD: LiChrosorb CN, elute with MeOH–water			
Beef and lamb		1. MeOH 2. Filter, acidify 3. LLE: wash with diethyl ether 4. LLE: alkalinise, extract with DCM 5. Evaporate to dryness and redissolve in 4 ml EtOH	Mutagenicity assay	1990	[36]
Heated reaction mixture	MeIQx, 4,8-DiMeIQx, TriMeIQx	1. LC: Amberlite XAD-2, elute with acetone 2. HPLC: PRP-1, elute with ACN–water 3. HPLC: Nucleosil C_{18}, elute with 0.1% DEA in MEOH–water 4. HPLC: Nucleosil CN, elute with MeOH–water	Mutagenicity assay MS NMR	1992	[70]
Beef patties		1. 0.01 *M* HCl 2. Centrifuge, neutralise 3. LC: Amberlite XAD-2, elute with acetone 4. Evaporate, dissolve in DMSO 5. HPLC: PRP-1 PS–DVB, elute with ACN–water	Mutagenicity assay	1992	[75]
Beefburgers		Same as Ref. [63]	Mutagenicity assay	1993	[73]
Hamburgers, hot dogs		1. 0.2 *M* HCl 2. LLE: wash with DCM 3. LLE: alkalinise, extract with DCM 4. LLE: extract with 0.2 *M* HCl 5. LLE: alkalinise, extract with DCM 6. Evaporate, dissolve in DMSO	Mutagenicity assay	1995	[37]
Beef	4′-OH-PhIP, 4-CH_2OH-8-MeIQx, 7,9-DiMeIgQx	1. 0.1 *M* HCl 2. LC: neutralise, blue cotton, elute with NH_3–MeOH (1:50) 3. Evaporate, dissolve in MeOH 4. LC: TIN-100 H05E, elute with MeOH–NH_3 (10:1) 5. HPLC: TSKgel ODS-120A 6. HPLC: TSKgel SP-2SW 7. HPLC: YMC A303 ODS	UV spectra Fluorescence spectra MS	1995	[71]

except in Refs. [85,91], where hydrochloric acid is used, sodium hydroxide is the solvent of choice, and mild heating is sometimes proposed [43,92]. In all cases, the sample treatment after the solution step often involves separation techniques such as centrifugation or filtration after protein precipitation. Further purification is carried out by one or various separation procedures including liquid–liquid extraction, column liquid chromatography, and solid-phase extraction. Moreover, a preconcentration stage is required to achieve low detection limits. This is commonly performed by evaporating the final extract to dryness and redissolving the residue in a small volume of the appropriate solvent for the determination procedure.

3.2.1. Liquid–liquid extraction

Liquid–liquid extraction is the separation method preferred by most of the authors for the first step in the isolation of the analytes from the food matrix. In some of the procedures summarised in Table 3, after homogenisation of the sample, elimination of the solids and change of the solvent if necessary, an acid–base partition is performed. The acidic solution obtained is directly extracted with an organic solvent, which can be DCM [9,38,40,41,77–79,84–86], diethyl ether [76] or ethyl acetate [80] in order to remove acidic or neutral interferences. If the solution obtained is basic, the analytes are directly extracted in their neutral form with DCM [30,31,45,89,90]. If the sample is homogenised in an organic solvent, the analytes are extracted with HCl [3]. In most cases, further purification is achieved by consecutive acid–base partition processes [3,9,38,40,41,76–80,84–86,89] or by combining this technique with the extraction using sorbents, which will be discussed in the following sections.

Liquid–liquid extraction can be also achieved by using inert solid materials such as diatomaceous earth, a sand-like porous material commercially available as Kieselguhr, Extrelut NT or Hydromatrix. These materials can be added to the liquid in the batch mode or more frequently as a support in a chromatographic column. In Table 3, two examples of this method are shown [89,90]. The procedure which uses diatomaceous earth is generally referred to as liquid–liquid extraction, in contrast to the solid-phase extraction, which is usually coupled on-line to the column that contains the solid material, as described in Section 3.2.4 and Tables 4 and 5.

3.2.2. Column liquid chromatography

Liquid chromatography is based on a physical separation process that involves a stationary phase and a liquid mobile phase. Both the liquid solution containing the analytes and the stationary phase disposed in an adequate recipient, usually a column, are placed in contact. In general, the stationary phase interacts with the analytes allowing their selective separation and, depending on the elution conditions, liquid chromatography can be used as a semipreparative technique to collect fractions with the different compounds (Table 3). For example, in earlier works, reversed-phase HPLC was used to isolate the fraction corresponding to the analytes before quantitative analysis by HPLC–UV [38,76,77] or HPLC–fluorescence [30]. In a different case, preparative LC was used as a fractionation step, by means of an open column filled with Sephasorb HP [89]. Another chromatographic technique used for the separation of the analytes in a preparative step is thin layer chromatography, which has been applied to the isolation of IQ from ground beef [77].

When the objective is not the fractionation but the purification of the analytes, liquid chromatography is used as a clean-up step. Considering an ideal case, the compounds of interest are completely retained on the surface of the solid, the interferences are eliminated by washing the sorbent, and finally the analytes are eluted using the most appropriate solvent. In most cases, this process is performed using open columns, but sometimes the two phases are mixed in a batch with mechanical stirring, and separated by filtration after distribution equilibria are achieved.

Adsorption in resins was one of the first chromatographic mechanisms used to purify HAs. Among them, Amberlite XAD-2, a non-ionic polymeric adsorbent based on polystyrene, was the most popular (Table 3). After the corresponding treatment, the aqueous phase is neutralised and passed through the sorbent in order to concentrate the relatively non-polar chemicals. The analytes are then eluted with acetone, combined with methanol in some cases. This procedure was used in the sample treatment of beef or beef extracts to analyse some imidoazamines [81,83], or prior to immunoassay analysis [59,60].

Table 3
Sample preparation methodology based on LLE or LC for the quantification of HAs

Processed sample	Compounds	Sample preparation	Analysis	Ref.
Using classic LLE as purification and concentration technique				
Beef extract	IQ, MeIQx	1. Acetone 2. Filter, −15°C, filter 3. Evaporate, dilute with 0.01 *M* HCl 4. LLE: wash with DCM 5. LLE: adjust to pH 12, extract with DCM 6. Evaporate, dissolve in DMSO	GC–MS Mutagenicity assay	[9]
Chicken, pork, Baltic herring, meat patties	IQ, MeIQ, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP	Same as Ref. [9]	Mutagenicity assay GC–MS	[79]
Meat foodstuff	MeIQx, 4,8-DiMeIQx, PhIP	1. HCl 2. LLE: wash with DCM 3. LLE: alkalinise, extract with ethyl acetate	GC–MS	[84]
Chicken	MeIQx, 4,8-DiMeIQx, TriMeIQx, PhIP	1. 0.25 *M* HCl 2. Centrifuge 3. LLE: wash with DCM 4. LLE: alkalinise, extract with ethyl acetate	GC–MS	[41]
Hamburger, chicken breast	IQ, MeIQx, PhIP, AαC	1. MeOH–NH_3 (50:1) 2. Centrifuge, evaporate, dissolve in H_3PO_4 3. LLE: wash with ethyl acetate 4. LLE: adjust to pH 9.0, extract with ethyl acetate 5. Evaporate, dissolve in mobile phase	HPLC–APCI-MS–MS	[80]
Using LC as purification and concentration technique				
Beef	IQ, MeIQ, 8-MeIQx, 4,8-DiMeIQx, PhIP	1. 0.01 *M* HCl 2. Neutralise 2. LC: Amberlite XAD-2, elute with acetone and MeOH 3. Dilute with water, neutralise	Competition ELISA	[59]
Beef	IQ, MeIQx	1. MeOH–water 2. Centrifuge, adjust to pH 8.5 3. LC: Amberlite XAD-2, elute with acetone and MeOH 4. Evaporate, dissolve in phosphate buffer 5. Immunoaffinity purification: monoclonal antibody column, elute with MeOH 6. Evaporate, dissolve in buffer	Mutagenicity assay HPLC–UV	[81]
Beef	PhIP	1. HCl 2. LC: Amberlite XAD-2	Competition ELISA	[60]

Beef, chicken, pork, lamb	PhIP, IQ, IQx, MeIQx, 4,8-DiMeIQx, DMIP, 1,5,6-TMIP, 3,5,6-TMIP	SCX method: 1. 0.1 *M* HCl–MeOH (3:2) 2.Centrifuge, acidify 3. SPE: clean with Isolute C_{18} 4. SPE: Bond Elut SCX, elute with 1 *M* $AcONH_4$, pH 8–MeOH (1:1) 5. SPE: Bond Elut C_{18}, elute with MeOH–NH_3 (9:1) 6. Evaporate, dissolve in mobile phase	HPLC–DAD HPLC–fluorescence	[82]
Using a combination of classic LLE and LC as purification and concentration technique				
Sardines	Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2, AαC, MeAαC, Lys-P-1	1. MeOH 2. Centrifuge and evaporate to dryness, redissolve in 1 *M* HCl 3. LLE: wash with diethyl ether 4. LLE: adjust to pH 10, extract with diethyl ether 5. Evaporate, dissolve in ethyl acetate 6. LC: silica gel, elute with ethyl acetate–MeOH (70:30) 7. Suspend in MeOH–HCOOH (10:90), centrifuge 8. HPLC–UV: μBondapak C_{18}, elute with MeOH–HCOOH	Mutagenicity assay HPLC–UV	[76]
Ground beef	IQ	1. MeOH 2. Evaporate to residual water, filter 3. LLE: adjust to pH 1.5, wash with DCM 4. LLE: adjust to pH 10, extract with DCM 5. LLE: wash with 0.05 *M* $NaHCO_3$ 6. TLC: silica gel in MeOH–$CHCl_3$ (20:80), elute with MeOH–$CHCl_3$ (1:1) 7. HPLC–UV: LiChrosorb C_{18}+Partisil PXS 10/25 ODS 3, elute with MeOH–0.02 *M* TEA, pH 3 (40:60)	HPLC–UV MS	[77]
Beef extract	IQ, MeIQx	1. Water 2. LC: blue cotton, filter and elute with MeOH–NH_3 (50:1) 3. Evaporate, dissolve in 0.1 *M* HCl 4. LLE: wash with DCM 5. Evaporate aqueous layer, dissolve in MeOH–chloroform (3:7) 6. SPE: SepPak SI, elute with MeOH–chloroform 7. Evaporate, dissolve in MeOH	HPLC–ED	[87]
Beef extract	4,8-DiMeIQx	Same as Ref. [87]	HPLC–ED HPLC–DAD	[88]
Meat and fish	IQ, MeIQ	1. MeOH 2. Filter and evaporate, dissolve in 1 *M* HCl 3. LLE: wash with DCM 4. LLE: adjust to pH 10, extract with DCM 5. Evaporate, dissolve in water 6. LC: blue-cotton, filter and elute with MeOH–NH_3 (50:1)	HPLC–UV LC–TSI-MS	[78]

Table 3. Continued

Processed sample	Compounds	Sample preparation	Analysis	Ref.
Trp pyrolysate	Trp-P-1, Trp-P-2	1. Ethyl acetate 2. LLE: extract with HCl 3. LLE: adjust to pH 10, extract with ethyl acetate 4. LC: Silica gel, elute with ethyl acetate–MeOH–NH_3 5. Evaporate, dissolve in 1 *M* HCl 6. LC: blue cotton, elute with MeOH–NH_3 (49:1)	LC–TSI-MS	[3]
Beef, beef extract	IQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx	1. MeOH–water 2. Centrifuge, adjust to pH 8.5 3. LC: Amberlite XAD-2, elute with acetone and MeOH 4. Evaporate, dissolve in HCl, pH 2 5. LLE: wash with ethyl acetate 6. LLE: adjust to pH 12, extract with ethyl acetate 7. Evaporate, dissolve in water 8. LC: blue cotton, elute with MeOH–NH_3 (50:1) 9. Evaporate, dissolve in MeOH:water (1:1)	LC–TSI-MS	[83]
Beef, beef extract	IQ, MeIQx, Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP	1. 0.4 *M* NaOH 2. LLE: Extrelut, elute with DCM 3. LLE: extract with 0.1 *M* HCl 4. LLE: wash aqueous phase with DCM 5. LLE: adjust to pH 11, extract with DCM 6. Evaporate, dissolve in MeOH. 7. LC: Sephasorb HP, elute with MeOH–water 8. Evaporate, redissolve in MeOH	HPLC–UV UV spectra	[89]
Beef, beef extract	IQ, MeIQ, MeIQx, TriMeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, Trp-P-1, Trp-P-2, H, NH, PhIP, AαC	CPC method: 1. 1 *M* NaOH 2. LLE: Extrelut, elute with DCM 3. LC: CPC Sephasorb, elute with DCM–MeOH–NH_3 4. Evaporate, redissolve in MeOH–water 5. LC: Sephasorb HP, elute with MeOH–NH_3 (9:1) 6. Evaporate, dissolve in MeOH	HPLC–DAD	[90]
Canned roasted eel	IQ, MeIQx, MeIQ, 4,8-DiMeIQx, 7,8-DiMeIQx	1. MeOH 2. Filter, adjust to pH 2.5 3. LLE: wash with DCM 4. LLE: adjust to pH 10, extract with DCM 5. HPLC–UV: μBondapack C_{18}, elute with MeOH–phosphate 6. Evaporate, dissolve in MeOH	HPLC–DAD Mutagenicity assay	[38]

Beer, wine	PhIP	1. 1 *M* HCl 2. LLE: adjust to pH 10, extract with DCM 3. SPE: Bond Elut SI, elute with MeOH–0.1 *M* HCl 4. HPLC–fluorescence: Asahipack ES-502C, elute with ammonium phosphate–ACN	HPLC–fluorescence MS	[30]
Poultry meat	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP	1. 0.5 *M* HCl 2. LLE: adjust to 9.0, extract with DCM 3. LC: blue cotton, elute with MeOH–NH_3 (50:1) 4. LLE: alkalinise, extract with DCM 5. Evaporate, dissolve in MeOH	HPLC–ED HPLC–fluorescence	[45]
Lean beef	MeIQx, 4,8-DiMeIQx, PhIP, 4-OH-PhIP	1. 0.25 *M* HCl 2. LLE: wash with DCM 3. Centrifuge, alkalinise 4. LLE: extract with ethyl acetate 5. Evaporate, dissolve in water 6. LC: blue cotton, elute with MeOH–NH_3 (99:1) 7. Evaporate, dissolve in ethyl acetate	GC–MS	[40]
Wine	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP, Glu-P-1, Glu-P-2, Trp-P-1, Trp-P-2, AαC, MeAαC	1. LLE: alkalinise, extract with DCM 2. SPE: Isolute PRS, elute with MeOH–NH_3 (9:1) 3. Evaporate, dissolve in water–MeOH–ACN	HPLC–ESI-MS–MS	[31]
Flavours	IQ, MeIQ, MeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP,Trp-P-1, Trp-P-2	1. 0.2 *M* HCl 2. LLE: wash with DCM 3. LLE: alkalinise, extract with DCM 4. LLE: extract with 0.2 *M* HCl 5. LLE: alkalinise, extract with DCM 6. Evaporate, dissolve in 0.06% HCOOH–ACN (80:20) 7. SPE: Maxi-Clean sulfobutyl-HEMA, wash with 0.1 *M* HCl–MeOH (4:1), elute with ACN–$AcONH_4$, pH 9.4 (2:3) 8. Evaporate, dissolve in mobile phase	HPLC–APCI-MS	[85]
Model system	PhIP	1. 0.1 *M* HCl 2. LLE: wash with DCM 3. LLE: alkalinise, extract with DCM 4. Evaporate, dissolve in MeOH–water 5. LC: blue cotton, elute with MeOH–NH_3 (50:1) 6. Evaporate, dissolve in MeOH	HPLC–DAD HPLC–fluorescence	[86]

Table 4
Procedures based on LLE and SPE yielding a single extract

Processed sample	Compounds	Sample preparation	Analysis	Ref.
Beef, beef extract	IQ, MeIQ, MeIQx, TriMeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, Glu-P-1, Glu-P-2, PhIP	PRS method: 1. 1 *M* NaOH 2. LLE: Extrelut, elute with DCM 3. SPE: Bond Elut PRS: – Wash with 0.01 *M* HCl and MeOH–0.1 *M* HCl (6:4) – Elute with 0.5 *M* $AcONH_4$, pH 8 4. SPE: Bond Elut C_{18}, elute with MeOH–NH_3 (9:1) 5. Evaporate, dissolve in MeOH	HPLC–DAD	[90]
Beef extract	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, Glu-P-1	Same as PRS method [90] washing PRS cartridge with MeOH–0.1 *M* HCl (4:6)	HPLC–ED	[94]
Model system	IQ, MeIQ, IQx, MeIQx, 4,7-DiMeIQx, 5,7-DiMeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, 4,7,8-TriMeIQx	Same as Ref. [94]	Mutagenicity assay HPLC-UV HPLC–DAD MS	[95]
Beef extract	IQ, MeIQ, MeIQx	Same as Ref. [94]	HPLC–ED	[96]
Meat model system	IQ, MeIQ, IQx, MeIQx, 7,8-DiMeIQx, 4,8-DiMeIQx, PhIP	Same as Ref. [94]	HPLC–UV	[44]
Meat extract	MeIQ, MeIQx, PhIP, Trp-P-2, Glu-P-1	Same as Ref. [94]	CZE–UV	[55]
Minced lean pork	IQ, MeIQ, MeIQx, Glu-P-1, Glu-P-2, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx	Same as PRS method [90], washing PRS cartridge with 0.1 *M* HCl	MEKC–ED	[57]
Ground beef	IQ, MeIQ, 4,8-DiMeIQx, PhIP	Same as Ref. [57]	HPLC–DAD HPLC–fluorescence	[49]
Beef flavours	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, Glu-P-1, Glu-P-2, PhIP	Same as PRS method [90] washing PRS cartridge with 0.1 *M* HCl and MeOH–0.1 *M* HCl (4:6)	HPLC–DAD HPLC–fluorescence	[97]
Chicken, ox, pork, duck, Döner Kebab, Currywurst, chicken, Bratwurst, Schnitzel, meatball, nuggets, shish-kebab, seatrout, hamburger	IQ, MeIQ, IQx, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP, Glu-P-1, Glu-P-2	Same as Ref. [97]	HPLC–ESI-MS–MS	[98]
Beefsteak, meat extract, salmon	IQ, MeIQx, 4,8-DiMeIQx, PhIP	Same as Ref. [97] eluting PRS cartridge with $AcONH_4$, pH 8.5	CZE–DAD	[99]

Fast-food meat products: hamburgers, chicken, chicken breast sandwiches, fish sandwiches, breakfast sausages	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP	Same as Ref. [97], eluting Extrelut with DCM–toluene (95:5)	HPLC–DAD HPLC–fluorescence	[118]
Chicken	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP	Same as Ref. [118]	HPLC–DAD HPLC–fluorescence	[119]
Chicken, ground beef	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP	Same as Ref. [118]	HPLC–DAD HPLC–fluorescence	[120]
Chicken	IQ, MeIQx, 4,8-DiMeIQx, PhIP	Same as Ref. [118]	HPLC–DAD HPLC–fluorescence HPLC-ESI-MS	[121]
Beef, chicken, pork, lamb	PhIP, IQ, IQx, MeIQx, 4,8-DiMeIQx, DMIP, 1,5,6-TMIP, 3,5,6-TMIP	PRS method: Same as Ref. [118]	HPLC-DAD HPLC-fluorescence	[82]
Beef hamburgers, beef steaks, pork ribs, chicken	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP	Same as Ref. [118]	HPLC-DAD HPLC-fluorescence	[47]
Beef products: hamburgers, beefsteak, gravy	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP	Same as Ref. [118]	HPLC-DAD HPLC-fluorescence	[48]
Pork products: bacon, sausages, hot dogs, pork chops, ham slices	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP	Same as Ref. [118]	HPLC-DAD HPLC-fluorescence	[122]
Round beef steak, model system	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP, DMIP, 1,5,6-TMIP, IFP	Meat: Same as Ref. [118] Model system: Same as Ref. [118], substitute DCM–toluene with ethyl acetate	HPLC-DAD HPLC-fluorescence	[23]
Meat extract	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx, PhIP, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC, MeAαC, H, NH	1-2. Same as PRS method [90] 3. SPE: Bond Elut PRS – Precondition with 0.1 *M* HCl – Wash with MeOH–water (4:6) – Elute with 0.5 *M* $AcONH_4$, pH 8 4. SPE: Bond Elut C_{18}, elute with MeOH–NH_3 (9:1) 5. Evaporate and dissolve with MeOH	HPLC–APCI-MS	[53]

Table 4. Continued

Processed sample	Compounds	Sample preparation	Analysis	Ref.
Flavours	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP	1. 1 *M* NaOH, 50°C 2. LLE: Extrelut, elute with DCM 3. SPE: Bond Elut PRS: – Wash with 0.1 *M* HCl and MeOH–0.1 *M* HCl (4:6) – Elute with 0.5 *M* $AcONH_4$, pH 8 4. SPE: Bond Elut C_{18}, elute with MeOH–NH_3 (9:1) 5. Evaporate, dissolve in MeOH–0.05 *M* K_2HPO_4 (1:1) 6. SPE: Bond Elut SCX, elute with MeOH–1 *M* $AcONH_4$, pH 8 (95:5) 7. Evaporate, dissolve in MeOH–0.05 *M* K_2HPO_4 (1:1)	HPLC–UV HPLC–fluorescence	[43]
Beef flavour	IQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx	1. 1 *M* NaOH, Kieselgur 2. Soxhlet extraction of fat with diethyl ether 3. LLE: elute from Kieselgur with DCM 4. SPE: Bond Elut PRS: – Wash with 0.1 *M* HCl and MeOH–0.1 *M* HCl (6:4) – Elute with 0.5 *M* NH_4AcO, pH 8 5. SPE: Bond Elut C_{18} 100 mg, elute with MeOH–NH_3 (9:1) 6. LC: blue cotton, elute with MeOH–NH_3 (50:1) 7. Evaporate and dissolve with MeOH	HPLC–UV HPLC–DAD HPLC–fluorescence HPLC–ED	[100]
Beef	IQ, MeIQ, MeIQx, Glu-P-1, PAHs, PANHs	1. 1 *M* NaOH 2. LLE: Extrelut, elute with DCM (HAs, PAHs, PANHs) 3. SPE: Bond Elut PRS PAHs: 4. Collect DCM, evaporate, dissolve in hexane 5. LC: Silica gel, elute with hexane–DCM (60:40) 6. Evaporate, dissolve in MeOH HAs: 4. Wash PRS with 0.1 *M* HCl, elute with 0.5 *M* $AcONH_4$, pH 8 5. SPE: Bond Elut C_{18}, elute with MeOH–NH_3 (9:1) 6. Evaporate, dissolve in MeOH PANHs: 4. Elute from PRS with MeOH–NH_3 (9:1) 5. Evaporate, dissolve in MeOH	HPLC–UV (PAHs, PANHs) GC–MS (PAHs, PANHs) HPLC–ED (HAs) HPLC–DAD (HAs)	[101]
Beef	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP	1. 1 *M* NaOH 2. LLE: Extrelut, extract with DCM 3. LC: blue cotton, elute with MeOH–NH_3 (50:1) 4. Evaporate, dissolve in MeOH–water 5. SPE: SepPak C_{18}, elute with MeOH–water (70:30) 6. Evaporate, dissolve in MeOH	HPLC–ED HPLC–DAD HPLC–fluorescence	[102]

Based on the observation that HAs have a planar structure and form complexes with hemin, Hayatsu et al. developed a specific sorbent named blue-cotton [93], a cellulose cotton bearing covalently linked to copper phthalocyanine trisulfonate, which is a blue pigment. This material adsorbs very efficiently HAs in aqueous solution by means of hydrophobic interactions between the copper-phtalocyanine nucleus and the aromatic substances. Afterwards, the mutagenic amines can be easily eluted with methanol–ammonia solution, as can be seen in Table 3. The first applications developed for the analysis of HAs in food matrices introduced blue cotton directly in the aqueous solution, and the material was filtered before the elution [74,78,87]. However, the sorbent can be placed in preparative columns for the same treatment [3,40,45,83,86]. Some modified versions can be found in the literature. For example, a product called CPC Sephasorb, which consists of copper phthalocyanine bound to the dextran polymer Sephasorb HP, was developed and successfully applied to the analysis of meat extracts by Gross [90].

Other sorbents less currently used are summarised as follows. For example, Sephasorb HP, which fractionates by size exclusion and gel adsorption, was used by Gross [90] in the mentioned CPC method. As has been pointed out, this sorbent was also used by the same author to obtain different fractions by means of a preparative LC column.

Silica gel is also applied for the extraction of HAs from foods in some of the methods in Table 3. After a liquid–liquid extraction treatment, the neutralised analytes are extracted from the ethyl acetate solution by means of a column filled with the sorbent. The analytes are then eluted using ethyl acetate–MeOH [76] or ethyl acetate–MeOH–NH_3 [3].

The use of specific sorbents containing MAbs for the purification of heterocyclic amines provides a simple and rapid sample preparation. However, specific antibodies are needed for each compound, the synthesis of MAbs is highly complex and MAbs are not commercially available, which makes impractical this methodology. Therefore, few papers using this methodology for the extraction of HAs from food samples have been published. Among them, we find that by Turesky et al. who analysed IQ and MeIQx in beef [81] (see Table 3).

3.2.3. Solid-phase extraction

Solid-phase extraction (SPE) can be considered a special case of liquid chromatography, where the extraction of the analytes is performed using disposable commercial cartridges, which typically contain from 100 mg to 500 mg of a solid sorbent as stationary phase. In general, the solid phase shows a greater attraction for the analytes than the solvent in which the analytes are dissolved. As mentioned in the column liquid chromatography section, in an ideal case the compounds studied are retained in the surface of the solid, then the interferences are eliminated by washing the column and finally the analytes are eluted. Most of the solid-phase extraction procedures allow to work at microanalytical scale. Moreover, analytical sensitivity and selectivity can be optimised with the use of different sorbents and eluents and, in some cases, by coupling different sorbents in tandem. Thus, most of the sample preparation procedures apply this separation technique for the analysis of HAs, which allows one to obtain extracts purified enough to prevent interferences, and a high throughput analysis.

Some of the extraction methods described in Table 3, include isolated steps of SPE using sorbents in disposable formats such as SepPak SI [87], Bond Elut SI [30], Isolute PRS [31] or Maxi-Clean sulfobutyl-HEMA [85]. In another work [82], a coupling of three different cartridges, Isolute C_{18}/Bond Elut SCX/Bond Elut C_{18}, is described. Another important aspect is the possibility of coupling on-line the liquid–liquid extraction using diatomaceous earth with several SPE steps, which allows the development of tandem extraction procedures. Most of the SPE techniques are integrated in these tandem extraction procedures, which are discussed in the next section.

3.2.4. On-line coupling of liquid–liquid extraction and solid-phase extraction

As has been mentioned above, when liquid–liquid extraction using diatomaceous earth and solid-phase extraction are coupled, the result is a time saving and practical method suitable for multiple analyses, because few sample transfer and evaporation steps are required during the work-up. This is beneficial not only for sample handling, but also ensures high

Table 5
Procedures based on LLE and SPE yielding two extracts

Processed sample	Compounds	Sample preparation	Analysis	Ref.
Meat extract, salmon	Polar amines: MeIQx, TriMeIQx, IQ, MeIQ, PhIP, Glu-P-1, Glu-P-2, H, NH, 4,8-DiMeIQx, 7,8-DiMeIQx, IQx Non-polar amines: AαC, Trp-P-1, Trp-P-2	1. 1 *M* NaOH 2. LLE: Extrelut, elute with DCM 3. SPE: Bond Elut PRS cartridge, elute with: – Non-polar amines: 0.1 *M* HCl and MeOH–0.1 *M* HCl (4:6) – Polar amines: 0.5 *M* NH_4AcO, pH 8 Non-polar amines: 4. Neutralise with NH_3, dilute with water to <20% MeOH 5. SPE: Bond Elut C_{18} 500 mg, elute with MeOH–NH_3 (9:1) 6. Evaporate and dissolve with MeOH Polar amines: 4. SPE: Bond Elut C_{18} 100 mg, elute with MeOH–NH_3 (9:1) 5. Evaporate and dissolve with MeOH	HPLC–DAD HPLC–fluorescence	[103]
Amino acids mixture	Polar amines: IQ, MeIQx, 4,8-DiMeIQx, PhIP Non-polar amines: AαC, Trp-P-1, Trp-P-2	Same as Ref. [103]	HPLC–DAD HPLC–fluorescence	[104]
Beef patties	Polar amines: IQ, MeIQx, 4,8-DiMeIQx, PhIP Non-polar amines: AαC, Trp-P-1, Trp-P-2	Same as Ref. [103]	HPLC–DAD HPLC–fluorescence	[105] [106]
Meat extract, beef, Merguez sausage, chicken flavour paste, peanut butter	Polar amines: IQ, IQx, MeIQ, MeIQx, Glu-P-1, Glu-P-2, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP. Non-polar amines: H, NH, Trp-P-1, Trp-P-2, AαC, MeAαC	Same as Ref. [103]	HPLC–ESI-MS	[50]
Beef extract	Polar amines: IQ, MeIQ, MeIQx, 4,8-DiMeIQx Non-polar amines: PhIP, Trp-P-1, Trp-P-2, Glu-P-1, AαC, MeAαC, H, NH	Same as Ref. [103], but using 0.01 *M* HCl and MeOH–0.1 *M* HCl (6:4) to elute apolar amines	HPLC–ED HPLC–fluorescence HPLC–DAD	[46]
Beef extract	Polar amines: IQ, PhIP Non-polar amines: H, NH, Trp-P-1, Trp-P-2, AαC, PhIP	Same as Ref. [46]	HPLC–ESI-MS	[107]
Beef extract	Polar amines: IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx Non-polar amines: H, NH, Glu-P-1, Trp-P-1, Trp-P-2, AαC, MeAαC, PhIP	Same as Ref. [46]	HPLC–ESI-MS	[51]
Beef extract	Polar amines: IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx Non-polar amines: H, NH, Glu-P-1, Trp-P-1, Trp-P-2, AαC, MeAαC, PhIP	Same as Ref. [46]	HPLC–APCI-MS	[52]

Bologna, summer sausage, ham, bratwurst, fresh pork sausage, Italian sausage, ground beef, eye round steak	Polar amines: IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP Non-polar amines: H, NH, Trp-P-1, Trp-P-2	Same as Ref. [103] eluting non-polar amines with MeOH–0.1 *M* HCl (45:55)	HPLC–DAD HPLC–fluorescence	[26]
Flavour	Polar amines: MeIQx, IQ, MeIQ, 4,8-DiMeIQx, PhIP, Glu-P-1, Glu-P-2 Non-polar amines: Trp-P-1, Trp-P-2, AαC, H, NH	Non-polar amines: 1–5. Same as Ref. [103] 6. LC: Fractogel TSK CM, elute with MeOH–NH_3 (9:1) 7. Evaporate and dissolve with MeOH Polar amines: 1–4. Same as Ref. [103] 5. LC: Fractogel TSK CM, elute with MeOH–NH_3 (9:1) 6. Evaporate and dissolve with MeOH	HPLC–DAD HPLC–fluorescence	[108]
Grain-food products	Polar amines: IQ, MeIQx, 4,8-DiMeIQx, PhIP Non-polar amines: AαC, Trp-P-1, Trp-P-2	Same as Ref. [108]	HPLC–DAD HPLC–fluorescence	[109]
Turkey or chicken breast	Polar amines: MeIQx, 4,8-DiMeIQx, PhIP Non-polar amines: NH, H, Trp-P-2, Trp-P-1, AαC, MeAαC	Same as Ref. [108]	HPLC–DAD HPLC–fluorescence	[110]
Pork chop, sliced pork belly, bacon, minute steak, sirloin steak, Falusausage, ground lean bovine meat	Polar amines: IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 4,7,8-TriMeIQx, Glu-P-1, Glu-P-2, PhIP. Non-polar amines: AαC, MeAαC, Trp-P-1, Trp-P-2, H, NH	Meat: Same as Ref. [103] Pan residue: Same as Ref. [108], but adding 5% phenol to the 1 *M* NaOH and heating to 50°C	HPLC–DAD HPLC–fluorescence	[92]
Cod fillet, Baltic herring, chicken breast, reindeer meat, lamb chops, fillet of pork, pork stewing meat, minced beef, Prince sausage, Falu sausage, black pudding, egg	Polar amines: IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP, Glu-P-1, Glu-P-2. Non-polar amines: H, NH, Trp-P-1, Trp-P-2, AαC, MeAαC	Same as Ref. [92]	HPLC–DAD HPLC–fluorescence	[111]
Reindeer, pork fillet, pork chops, pan residues, meat extracts	Trp-P-1, Trp-P-2, AαC, MeAαC, H, NH	Same as Ref. [92], only non-polar fraction	GC–MS	[42]
Beef steaks, beef patties, shark, bacon	Polar amines: IQ, MeIQ, MeIQx, Glu-P-1, Glu-P-2, PhIP, 4,8-DiMeIQx, 7,8-DiMeIQx. Non-polar amines: AαC, MeAαC, Trp-P-1, Trp-P-2, H, NH	Cooked samples: Same as Ref. [103], eluting Extrelut with DCM–toluene (95:5) Grill scrapings: Same as Ref. [108], eluting Extrelut with DCM–toluene (95:5)	HPLC–DAD HPLC–fluorescence HPLC–TSI-MS	[114]
Ground beef	Polar amines: IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP Non-polar amines: AαC, Trp-P-2,	Same as Ref. [114] for grill scrapings	HPLC–DAD HPLC–fluorescence	[115]
Beef, pork and a soy-based food	Polar amines: IQ, MeIQx, 4,8-DiMeIQx, PhIP Non-polar amines: AαC, Trp-P-1, Trp-P-2	Same as Ref. [115]	HPLC–DAD HPLC–fluorescence	[116]

Table 5. Continued

Processed sample	Compounds	Sample preparation	Analysis	Ref.
Beefburgers, pan residues	IQ, MeIQ, MeIQx, 4,8-DiMeIQx, PhIP, Glu-P-1, Glu-P-2, H, NH	Same as Ref. [115], only polar extract, heating polar solution to 50°C For pan residues, add 5% phenol to the DCM	HPLC–DAD HPLC–fluorescence	[117]
Beef, chicken breast, turkey breast, pork chops, cod fish, model systems	Polar amines: IQ, MeIQ, IQx, 8-MeIQx, 4-MeIQx, 4,8-DiMeIQx, DMIP, TMIP, PhIP, Glu-P-1, Glu-P-2, IFP. Non-polar amines: H, NH, Trp-P-1, Trp-P-2, AαC, MeAαC	1. 1 *M* NaOH 2. LLE: Extrelut, elute with DCM–toluene (95:5) or ethyl acetate 3. SPE: PRS cartridge, elute with – Non-polar amines: MeOH–HCl (6:4) – Polar amines: 0.5 *M* NH_4AcO, pH 8 4. Non-polar amines: same as Ref. [103] 5. Polar amines: – SPE: Bond Elut C_{18}, elute with MeOH–NH_3 (9:1) – Evaporate, dissolve in MeOH–0.05 *M* K_2HPO_4 (1:1) – SPE: Bond Elut SCX, elute with MeOH–1 *M* $AcONH_4$, pH 8 (95:5) – Evaporate, dissolve in MeOH–0.05 *M* K_2HPO_4 (1:1)	HPLC–DAD HPLC–fluorescence	[29]
Flavours	Polar amines: IQ, MeIQ, MeIQx, 7,8-DiMeIQx, TriMeIQx Non-polar amines: PhIP, Trp-P-1, Trp-P-2	1. 0.2 *M* HCl 2. LLE: wash with DCM 3. LLE: alkalinise, extract with DCM 4. LLE: extract with 0.2 *M* HCl 5. LLE: alkalinise, extract with DCM 6. Evaporate, dissolve in 0.1 *M* HCl–MeOH (80:20) 7. SPE: Bond Elut PRS, elute with – Polar amines: 0.1 *M* HCl and MeOH–0.1 *M* HCl (20:80) – Non-polar amines: ACN–0.5 *M* $AcONH_4$, pH 8.5 (40:60) 8. Non-polar amines: – Neutralise with NH_3, dilute with water to <20% MeOH – SPE: Bond Elut C_{18} 500 mg, elute with MeOH–NH_3 (9:1) – Evaporate, dissolve in mobile phase 9. Polar amines: – SPE: Bond Elut C_{18}, elute with MeOH–NH_3 (9:1) – Evaporate, dissolve in mobile phase	HPLC–APCI-MS	[85]
Pre-processed meat cuts	Polar amines: IQ, MeIQ, MeIQx, 7,8-DiMeIQx, TriMeIQx Non-polar amines: PhIP, Trp-P-1, Trp-P-2	Same as Ref. [85]	HPLC–APCI-MS	[91]
Chicken legs	IQ, IQx, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP, Glu-P-1, Glu-P-2, AαC, MeAαC, TriMeIQx, H, NH, Trp-P-1, Trp-P-2	1. 1 *M* NaOH 2. LLE: Extrelut, extract with DCM centrifuging 3. SPE: Bond Elut PRS, elute with – Non-polar amines: 0.1 *M* HCl and MeOH–HCl (45:55) – Polar amines: 0.5 *M* NH_4AcO, pH 8 4. Non-polar amines: – Neutralise with NH_3, dilute with water to <20% MeOH – SPE: Bond Elut C_{18} 500 mg, elute with MeOH–NH_3 (9:1) 5. Polar amines: – SPE: Bond Elut C_{18} 100 mg, elute with MeOH–NH_3 (9:1) 6. Recombine polar and apolar extracts, evaporate and dissolve with MeOH	HPLC–DAD HPLC–fluorescence	[112]

Flavours, flavour ingredients, bouillon concentrates, pan residue	Polar amines: IQ, IQx, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP. Non-polar amines: H, NH, Trp-P-1, Trp-P-2, AαC, MeAαC	Mode A 1. 1 *M* NaOH 2. LLE: Extrelut, extract with DCM–toluene (95:5) 3. SPE: Bond Elut PRS, elute with: – Non-polar amines: 0.1 *M* HCl and MeOH–0.1 *M* HCl (40:60) – Polar amines: 0.5 *M* NH_4AcO, pH 8 4. Non-polar amines: – Neutralise with NH_3, dilute with water to <20% MeOH – SPE: Bond Elut C_{18} 500 mg, elute with MeOH–NH_3 (9:1) 5. Polar amines: – SPE: Bond Elut C_{18} 100 mg, elute with MeOH–NH_3 (9:1) 6. Recombine polar and apolar extracts, evaporate and dissolve with MeOH–$AcONH_4$, pH 6 7. SPE: Isolute CBA, elute with MeOH–NH_3 8. Evaporate and dissolve with MeOH Mode B (more complex samples) Same as Mode A, treating per separate polar and apolar extracts with CBA cartridge	HPLC–DAD HPLC–fluorescence GC–MS	[27]
Meat juice model system	Polar amines: IQ, MeIQ, IQx, MeIQx, 7,8-DiMeIQx, 4,8-DiMeIQx, PhIP. Non-polar amines: H, NH, Trp-P-1, Trp-P-2, AαC, MeAαC	Normal samples: 1. 4 *M* urea, 1 *M* NaOH 2. LLE: Extrelut, elute with DCM 3. SPE: Bond Elut PRS, elute with: – Non-polar amines: 0.01 *M* HCl and MeOH–0.1 *M* HCl (4:6) – Polar amines: 0.5 *M* NH_4AcO, pH 8 4. Non-polar amines: same as Ref. [103] 5. Polar amines: same as Ref. [103] More complex samples: Same as Ref. [27]	HPLC–DAD HPLC–fluorescence	[28]

analyte recovery. Moreover, the high number of commercial stationary phases and the possible optimisation of each step by changing the working solvents, greatly enhances both the selectivity and the quantativity of these tandem methods. Therefore, they can be regarded as standard procedures, although their suitability for the chromatographic analysis of a given sample depends on both the selectivity of the detection technique and the sample matrix. Some examples of the methods based on LLE–SPE tandem proposed in the bibliography are summarised in Tables 4 and 5. Table 4 includes sample preparation procedures that lead to a single extract, while Table 5 refers to the procedures that yield two extracts.

Diatomaceous earth are coupled to the SPE by disposing the solid material in an empty preparative column, which is also commercially available. The use of diatomaceous earth as solid support for liquid–liquid extraction is recommended for the sample preparation of aqueous samples, and can be used within the pH range 1–13. When this material is mixed with the sample, which has been previously homogenised in sodium hydroxide solution, the aqueous phase is distributed itself in the form of a thin film over the chemically inert matrix. Subsequently, HAs are eluted using organic solvents that are non-miscible with water, therefore this process could be considered as a liquid–liquid chromatography. When diatomaceous earth are used for the liquid–liquid extraction, lipophilic substances are extracted from the aqueous into the organic phase, and macromolecules like proteins and carbohydrates remain adsorbed on the inert material. In comparison with the classic liquid–liquid extractions, the advantages of this methodology are that emulsions are avoided, the process is faster and less solvent is required.

Currently, the solvents most commonly used as mobile phase are dichloromethane (DCM) [26,28,42–44,46,49–53,55,57,90,92,94–112] and ethyl acetate [23,29]. Moreover, Gross and Fay [113] observed that the elution from diatomaceous earth was improved by adding 5% toluene or phenol to dichloromethane. Several studies have applied this improvement [23,27–29,47,48,82,114–122].

Hitherto, the most popular tandem method is the proposed by Gross in 1990 [90], and consists of the combination of diatomaceous earth with propylsulfonate silica (PRS cartridge). The sample homogenised with 1 *M* sodium hydroxide is loaded in an Extrelut column, which is coupled to a PRS cartridge. The analytes are then transferred from the diatomaceous earth to the cationic exchanger sorbent by passing DCM, DCM with additives or ethyl acetate through the tandem. To activate the ion-exchange process, 0.01 *M* hydrochloric acid is passed by the PRS sorbent, and the cartridge is then washed with a methanol–hydrochloric acid (MeOH–0.1 *M* HCl, 6:4) solution. The analytes are then eluted with ammonium acetate, pH 8 and retained in a C_{18} cartridge, in order to achieve a preconcentration prior to chromatography. The scheme of this method is shown in Table 4, together with the rest of procedures which also obtain a single extract after the sample preparation. The main modifications of the original method are the increase in the hydrochloric acid concentration from 0.01 *M* to 0.1 *M* [49,57], in order to raise the ionic activation, or the change in the MeOH–water ratio from (6:4) to (4:6) [44,55,94–96], to minimise the elution of the analytes during the washing step. Some methods [23,47,48,82,97–99,118–122] include both modifications. Other methods change the ammonium acetate [99]. Thus, in this work the eluting solvent is adjusted to pH 8.5. Moreover, in order to avoid the losses of the analytes during the washing step of PRS with hydrochloric acid, acidic preconditioning before the sample treatment has been proposed [53]. This same group of scientist made some minor modifications to the tandem proposed by Gross and Grüter for analysing different families of compounds, namely HAs, PAHs and PANHs [101]. The PRS extraction method shows its limitations when more complex samples, such as process flavours, bouillon concentrates or pan residues, are analysed by UV detection. Therefore, additional clean-up steps should be used to purify more efficiently these complex samples, in order to improve chromatographic efficiency and to obtain detection sensitivities similar to those obtained with heated meat products. Some examples described in Table 4 include an additional purification step using a Bond Elut SCX cartridge [43], which is a strong cation exchanger, or a blue-cotton column [100]. Finally, an alternative to the PRS method is proposed in [102], where the dichlorome-

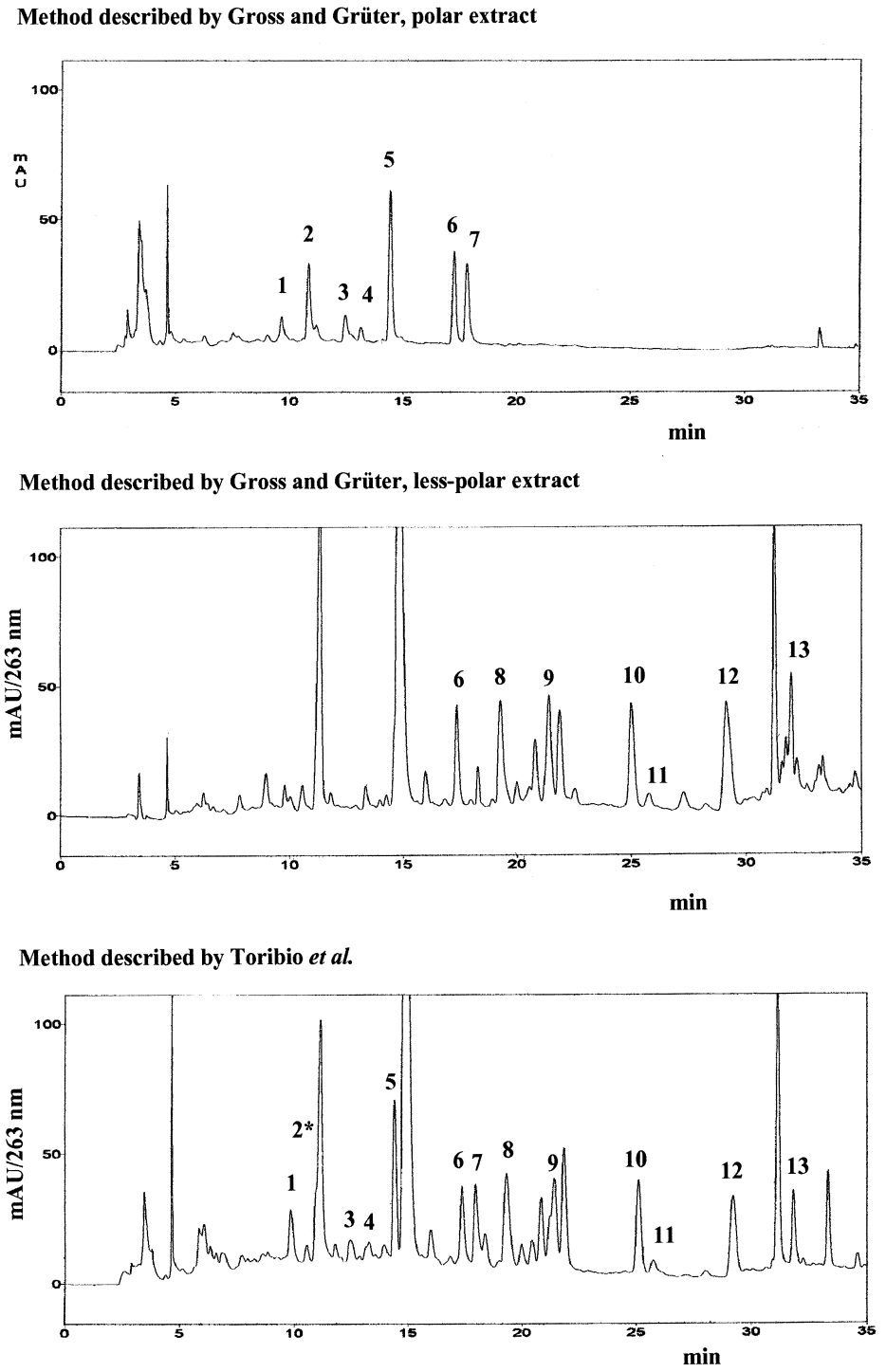

Fig. 3. Chromatograms obtained with the method described in Refs. [103] and [123]. The sample was a meat extract spiked with 100 ng/g of each amine. Peak identification: 1=Glu-P-2; 2=IQ; 3=MeIQ; 4=Glu-P-1; 5=MeIQx; 6=7,8-DiMeIQx (I.S.); 7=4,8-DiMeIQx; 8=norharman; 9=harman; 10=Trp-P-2; 11=PhIP; 12=Trp-P-1; 13=AαC. * Non pure peak. Reproduced with permission from Ref. [123].

thane is directly introduced into a blue cotton column.

As has been stated, the acidic washing of the PRS sorbent results not only in the activation of the ionic exchange, but also in the elution of the less polar analytes (PhIP, carbolines). Another tandem was thus developed [103]. In this work, the effect of the ratio MeOH–HCl in the washing solution was tested, and it was observed that the best compromise between selectivity and recovery was obtained when 0.1 *M* hydrochloric acid containing 40–50% of methanol was used, achieving selective desorption of the less polar amines. As shown in Table 5, this tandem extraction system allowed the simultaneous analysis of most HAs present in heated meat and fish and in meat extracts [50,103–106]. On the other hand, Galceran et al. [46] studied the influence of the concentration of HCl in the rinsing step, and better results were obtained when 0.01 *M* HCl and MeOH–0.1 *M* HCl (6:4) were used in the first elution of PRS sorbent. The increase of MeOH in the solution allows the collection of PhIP in the less-polar extract [46,51,52,107]. The ratio MeOH–HCl is also changed in Refs. [26–28,85,91,112], and, in some cases, the modification implies the use of ammonium acetate pH 8.5 [85,91]. Further changes are aimed at the analysis of more complex samples, and consist of the addition of a clean-up step. In some cases, this additional step is carried out using a Fractogel TSK CM column, a weak cation-exchange gel [42,92,108–111,114–117]. By using a polymeric sorbent, the irreversible adsorption produced when silica gel is used is avoided. In other cases, Bond Elut SCX [29] or Isolute CBA [27,28], which is a weak cation exchanger consistent of propylcarboxylic acid, are used for the additional clean-up step. Other possible modifications are the recombination of the final extracts obtained [112], and the substitution of the LLE using Extrelut by a classic LLE process [85,91].

The tandem extraction procedures discussed in this section, which have been classified in two groups according to the number of extracts yielded, are the most applied procedures for the sample preparation in the quantitative analysis of HAs. In general, a compromise between high recovery and clean-up efficiency must be achieved. The choice of some of these methods for a particular analysis will depend on the matrix sample, sensitivity required and detection technique. As an example, Fig. 3 shows the chromatograms obtained using two of the most significant methods in Tables 4 and 5, respectively. As can be seen, for polar amines a cleaner extract is obtained using the Gross and Grüter method [103] modified by Galceran et al. [46], which provides two extracts. However, the method of Toribio et al. [123] only provides a single extract, therefore this method is faster and seems suitable for the screening of unknown samples.

4. Strategies for the correction of analytical results

Since analyte extraction during the sample treatment is not complete, a correction taking into account the recovery values has to be done in order to accurately quantify HAs. One option is to add an exact amount of a substance not present in the sample before the extraction, and to extrapolate the recovery of this compound to the analytes studied [41,90,95]. However, the use of a single standard is not suitable, because HAs include several classes of compounds, and significant differences in recovery values have been observed even between substances belonging to the same family. Moreover, clean-up efficiency is greatly influenced by the sample matrix and so to predict recoveries is very difficult. In this case, the most suitable method to calculate recoveries is the multiple standard addition, which is able to determine the recovery of each analyte individually. Triplicate or quadruplicate extractions with one or two samples spiked with a reference solution of standards are performed to obtain sets of concentration data for each analyte. Using the slope obtained from linear regression analysis, using the added concentration of standards (x) as independent variable and the measured concentration (y) as dependent variable, extraction efficiencies are calculated. Uncorrected results can be calculated as the y-axis intercept of the same linear regression, and corrected results are obtained by dividing the intercept by the slope. The analytical precision can be estimated through the standard errors of slope and intercept [103]. Some authors suggest the addition of an amount similar to the amount of analyte found in

foods, i.e., additions of 50 ng [112], 100 ng [27,90] or 250 ng of each analyte [49,103,118]. Nevertheless, in order to detect the effect of spiking level on recoveries, standard addition methods with several spiking levels, ranging from 0 to 50 ng [99,108], from 0 to 320 ng [53] and from 0 to 500 ng [46,94] have been described. When addition standard method is applied as quantification model, the risk to obtain inexact results should be considered because of an overestimation of recovery, which is more significant when the interaction between sample matrix and analyte is strong. In these cases, it is recommended to optimise the contact time between the sample and the amount spiked. On the other hand, a drawback when using the standard addition method to quantify HAs is that replicate extractions have to be performed.

When LC–MS is used, stable isotope dilution quantification, using analytes marked with [^{2}H] or [^{13}C] as internal standards, reduces the number of samples to be extracted per determination. This quantification method consists of the enrichment of the sample with the labelled compound before the extraction procedure, and the subsequent quantification by means of a calibration curve, using the same labelled analyte as internal standard. Thus, deuterated analytes [50,80,83,84,91,98] or compounds marked with [^{13}C] [80] have been used for the quantitative determination of the respective non-marked analytes. The use of labelled internal standards for isotope dilution reduces the variability due to extraction efficiencies or changes in instrument performance and ensures the accuracy of quantification. In some cases, when not all the labelled analytes are available, one of the marked compounds can be added as internal standard for more than one analyte. For example, [^{13}C,^{15}N]MeIQx is used for the quantification of MeIQx and 4,8-DiMeIQx [40,84], [$^{2}H_{3}$]PhIP can act as internal standard for PhIP and 4-OH-PhIP [40] or even for all the analytes collected in the polar fraction obtained after a clean-up procedure, while [$^{2}H_{4}$]NH can be used for the non-polar compounds [31]. Recovery of the analytes can be measured by comparing labelled internal standard peak areas in extracted samples with those in non-extracted standards [84] or by spiking the sample with non-marked analytes [31,80,91,98].

Another option for determining the extraction efficiency consists of the addition of radiolabelled analytes. By this procedure, the measurement of the radioactivity provides the recovery of the native analyte. For example, [^{14}C]IQ and [^{14}C]MeIQx were used to determine the recovery of IQ and MeIQx when some food samples were analysed [81,83,87].

The recoveries obtained using some of the tandem extraction procedures described in the bibliography are given in Table 6, following the same citation order as in Tables 4 and 5. As previously described, these methods are based on the coupling of diatomaceous earth with an SPE cartridge. When the tandem proposed by Gross [90] or some of the modifications which yield a single extract are applied, only the most polar compounds are recovered, except in Ref. [53], where all the HAs analysed are recovered. In contrast, when the washing solutions from PRS cartridge are collected [46,51,52,91, 103,105,112] and two extracts are obtained, recoveries in general higher than 60% are achieved for both polar and non-polar analytes, even with an additional clean-up step using a CBA cartridge [27] or a Fractogel TSK CM column [108]. However, low recoveries have been observed for some carbolines [26,94,106,111]. In the case of PhIP, the extraction efficiency is low in most cases, and the recovery is hardly ever higher than 70% [43,44,53,91]. The wide range of recoveries obtained with the same method, even within laboratories [23,97,121], can result from the marked influence of sample matrix on the clean-up efficiency.

5. Conclusions and remarks

The extraction of heteroaromatic amines from foods is a complex task, especially when the quantification of the amines is concerned. Most of the procedures for the concentration and clean-up of the sample combine LLE and LC techniques but, so far, have not provided suitable reproducibility or accuracy for quantitative purposes. One of the main drawbacks is the unequivocal assessment of the identity of the analyte, which is hindered due to the great number of interferences present in the food sample. Furthermore, the recovery of the analytes greatly depends on the matrix of sample, which prevents the establishment of a general procedure for

Table 6
Recoveries of different LLE–SPE sample treatment procedures

Ref.	Recovery (%)																	
	IQ	MeIQ	IQx	MeIQx	4,8-DiMeIQx	7,8-DiMeIQx	4,7,8-TriMeIQx	PhIP	Glu-P-1	Glu-P-2	Trp-P-1	Trp-P-2	AαC	MeAαC	H	NH	DMIP	1,5,6-TMIP	3,5,6-TMIP
Refs. from Table 4																			
[90] PRS method	83	68		78	81	78	75	t.r.	51	70	n.r.	n.r.	n.r.		n.r.	n.r.			
[94]	68	77		63	66				18										
[96]	36	51		28															
[44]			88	92	89	88		79											
[55]		78		67															
[49]	75	75		84	73			65											
[97]	55–83	55–83		44–72	44–72			33–56	55–80	55–80									
[98]	51	19	43	80	59	79	48	22	93	138									
[99]	55–90			67–80	62–78			6–12											
[118]	55	47		68	61			30											
[121]	35–98			35–98	35–98			9–63											
[82] PRS method	45	38	95	67	72			30									6	n.r.	70
[23]				41–83				14–67											
[53]	70	73		70	52	75		74	57	58	46	50	49	51	50	54			
[43]	90	88		83	87			88[a]											
[101]	54	80		60					60										
[102]	77	69		70	61			69											
Refs. from Table 5																			
[103]	70	66		73	84			38	70	70									
[105]	62			71	59			46			65	69	52						
[106]	26–80			26–80	26–80			30–57			7–37	7–37	7–37						
[46]	82	99		87	78			55	81		91	74	68	70	70	73			
[51]	74	80		82	89			54	79		76	61	60	59	61	105			
[52]	72	68		83	85			50	74		64	73	58	62	68	83			
[26]	67	48		73	42			46				31			70	77			
[108]	>75	>75		>75	>75			>41	t.r.	t.r.	>75	>75	>53		>75	>75			
[92]	60			70	66			40											
[111]	66	63		68	72			20			45	37	7	25	66	57			
[42]											42	44	52	58					
[117]				71	63			66											
[91]	91	86		93		89	90	77			86	83							
[112]	74	72	73	89	69	73		52	71	74	80	68	69	69	72	75			
[27]	>70	>70	>55	>70	>70	>70		>65			>55	>65	>55	>65	>70	>70			
[28]	53–69	50–61	54–58	80–86	74–84	75–84		49–80			33–54	60–70	35–39	47–55	97–100	83–89			

[a] Determined before SCX cartridge.

the analysis of HAs. To date, the methods developed as tandem extraction procedures, coupling on-line several clean-up steps, have been found to be the most appropriate procedures for many samples, but could still be improved.

Since the evaluation of daily intakes of HAs requires reliable analytical methods to determine these compounds in foods cooked in the laboratory, many efforts to improve the analytical methodology should be done. Intercomparison exercises within both national or international programs should be promoted, and the preparation of certified materials would be welcome by the scientific community. Furthermore, once a standard procedure would be properly validated, representative food products should be analysed to assess the daily exposure to HAs. Regarding the standard methodology, it is important to consider the necessary compromise between high recovery, clean-up efficiency and throughput analyses, which should also be considered at the same time as the detection system is selected. If a sensitive and selective technique such as MS is available, the use of a tandem procedure including diatomaceous earth–PRS–C_{18} allows to analyse a large group of amines in a single extract [53]. This method can be a useful choice method for screening new samples, although in some cases poorer detection limits than those obtained with standard solutions can be achieved. However, this procedure allows to perform the purification and preconcentration step with a considerable reduction in analysis time and materials. When UV detection is used, the CPC method described by Gross is also able to provide a single extract for a complete analysis of HAs, although the SPE steps are not coupled on line, and the copper phthalocyanine (CPC) sephasorb sorbent is not found as a commercial cartridge. On the other hand, the PRS tandem method previously described can be modified to yield two extracts clean enough for the determination of polar and less-polar amines groups [46,103], providing more sensitive and accurate results. In this case, UV detection, as well as other techniques such as electrochemical, fluorescence, or MS detection, can be used [46,50,51,104,106].

Another quantitation aspect of the HAs analysis, is concerned with the strategies used for the correction of analytical results by means an accurate estimation of the recovery. The standard addition method with several spiking levels (at least three) is the most recommended procedure, although if the sample is well known, and a high throughput analysis is required, the Gross model procedure with a single spiking level (by duplicate) can be applied. The most reliable results would be obtained if the corresponding isotopic labelled compounds were available, and the isotopic dilution procedure was applied.

Undoubtedly, the analysis would be less complex if the analysis of only a few HAs was required, so that the extractions could be optimised for just a single group of amines. Thus, further studies must be carried out in order to establish occurrence, bioavailability, genotoxicity and carcinogenicity as a way to determine the amines with most significant role in human cancer development. To date, there is considerable evidence that the major subclass of HAs found in the human diet comprises the aminoimidazoazaarenes, including some of the polar amines and PhIP as well [17]. Therefore, this reduced group of amines could be analysed preferably, and the sample preparation could be more efficient and less time-consuming than that for the complete HA analysis.

6. Abbreviations

ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionisation
BCR	Community Bureau of Reference
CZE	Capillary zone electrophoresis
DAD	Diode array detection
DCM	Dichloromethane
DEA	Diethylamine
DMSO	Dimethylsulfoxide
ED	Electrochemical detection
ESI	Electrospray ionisation
HAs	Heteroaromatic amines
LLE	Liquid–liquid extraction
MEKC	Micellar electrokinetic chromatography
NPD	Nitrogen–phosphorus detection
PAHs	Polycyclic aromatic hydrocarbons
PANHs	Nitrogen-containing polycyclic aromatic hydrocarbons
PS–DVB	Polystyrene–divinylbenzene

Ref.	Reference
SPE	Solid-phase extraction
TEA	Triethylamine
TLC	Thin-layer chromatography
TSI	Thermospray ionisation

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