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Ion-trap tandem mass spectrometry for the determination of heterocyclic amines in food

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Abstract

Heterocyclic amines (HAs) are mutagenic compounds to which humans are regularly exposed through diet. Due to the high complexity of the sample matrix and the low level of concentration of HAs, sensitive and selective analytical methodologies are required. Here we describe a methodology based on liquid chromatography–atmospheric pressure chemical ionisation tandem mass spectrometry using an ion-trap to analyse HAs. The collision-induced dissociation parameters for tandem ion-trap spectrometric analysis of these mutagenic compounds were optimised, and the full scan MS–MS spectra were used for unequivocal identification of the analytes. For aminoimidazoazaarenes, the most abundant ions were derived from the loss of a methyl group and the breaking of the aminoimidazole moiety, while for carbolines the major product ions arose from the loss of ammonia and HCN. Moreover, the performance of the LC–atmospheric pressure chemical ionisation MS–MS method was evaluated. The good precision (RSD lower than 11%) and the low detection limits achieved (10–60 pg injected) allow the determination of HAs at low part-per-billion level (0.4–5.0 ng g^{-1}) in a lyophilised meat extract.

Keywords: Food analysis; Amines, heterocyclic aromatic; Aminoimidazoazaarenes

1. Introduction

Diet is the main source of nutrients for humans; however, it can also contribute to the development of diseases [1]. As shown by several epidemiological studies, ~30–40% of cancers are related to diet [2,3]. Continuous exposure to mutagenic substances present in food, such as heterocyclic amines (HAs), can cause accumulated genetic alterations that can lead to the development of cancer [4]. Humans are regularly exposed to HAs through diet, since these compounds are produced during the cooking of meat and fish [5,6]. Depending on their chemical structure and their mechanism of formation, these xenobiotic genotoxic substances can be grouped into two main families. The first, named IQ type or amino-imidazoazaarenes (AIAs), includes mutagenic amines that have a 2-aminoimidazole group. These amines, also called thermic HAs, are generated from the reaction of free amino acids, creati(ni)ne and hexoses at ordinary cooking temperatures [7]. The other amines, called non-IQ type or pyrolytic HAs, are formed through the pyrolytic reaction of amino acids and proteins at temperatures above 300°C [8]. Some of these non-IQ type mutagens, the carbolines, contain a 2-aminopyridine moiety as a common structure.

To date, more than 25 HAs have been isolated from a number of food samples and model systems, and their structures elucidated. When tested both in

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vivo and in vitro [9], most of these compounds are potent mutagens after metabolic activation. Harman and norharman are not mutagenic, but are considered comutagenic substances because they enhance the genotoxicity of mutagenic HAs. Moreover, the ten HAs so far tested in long-term animal experiments are carcinogenic in mice, rats and non-human primates [10,11]. These results support the hypothesis that HAs are involved in the aetiology of cancer. To establish the role of HAs in human health, an accurate determination of their dietary intake is required, which can be accomplished by combining information about types and quantities of food consumed and amounts of HAs in food products [12]. Nevertheless, the quantitative determination of HAs in food samples is mainly hindered by the low level of concentration of these microcomponents and the high complexity of the matrix. Therefore, the development of sensitive and selective analytical methodology is mandatory.

Until now, laborious clean-up procedures based on liquid–liquid extraction (LLE) [13], preparative liquid chromatography (LC) using different adsorbents [14], solid-phase extraction (SPE) with disposable columns [15] or tandem extraction procedures consisting of the coupling of LLE and SPE [16,17], have been developed. The sample treatment procedures employed in the analysis of mutagenic heterocyclic amines are reviewed in Ref. [18].

In addition, identification and quantification of HAs has been commonly carried out by means of chromatographic or related techniques [19]. Thus, gas chromatography (GC) with nitrogen-phosphorus selective detection (GC–NPD) [20] and gas chromatography–mass spectrometry (GC–MS) [21] have been used to analyse HAs. However, most of these compounds are polar and non-volatile, and consequently a derivatisation step is needed. This step can be avoided using liquid chromatography with different detection systems such as ultraviolet [22,23], electrochemical (ED) [24] and fluorescence [25] detection. Nevertheless, an essential aspect in the analysis of such complex matrices is the unequivocal identification of HAs. This can be efficiently achieved by coupling liquid chromatography with mass spectrometry (LC–MS) [26,27], a highly selective and sensitive detection system. To enhance the selectivity of the detection, LC–MS–MS using triple quadrupole [15,28–33] or ion-trap [34] instruments has been used. Recently, capillary electrophoresis, either with mass spectrometry (CE–MS) [35], ultraviolet (CE–UV) [36] or electrochemical (CE–ED) detection [37], has also been proposed although high detection limits have been obtained.

Here we describe a method based on LC–atmospheric pressure chemical ionisation (APCI) ion-trap (IT) MS–MS for the analysis of 16 HAs. The characteristic MS–MS spectrum of each analyte was used for unequivocal identification, which is important when real food samples are analysed. The LC–APCI-MS–MS method was used to determine HAs in a lyophilised meat extract [38]. To purify the sample, two tandem clean-up procedures were tested, both based on the well-known Gross method [16,17] which uses the coupling of LLE with diatomaceous earth as solid support and two SPE steps with propylsulfonic acid (PRS) and C_{18}.

2. Experimental

2.1. Chemicals

The solvents and chemicals used were HPLC or analytical grade, and the water was purified in an Elix-Milli-Q system (Millipore, Bedford, MA, USA). All the solutions were passed through a 0.45-μm nylon filter (Whatman, Clifton, NJ, USA) before injection into the HPLC system.

The compounds studied (Fig. 1) were 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3-trideuteromethylimidazo[4,5-*f*]quinoline (D_3-IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-8-methyl-3-trideuteromethylimidazo[4,5-*f*]quinoxaline (D_3-MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx), 2-amino-3,4,7,8-tetramethylimidazo-[4,5-*f*]quinoxaline (TriMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-1-trideuteromethyl-6-phenylimidazo[4,5-*b*]pyridine (D_3-PhIP), 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-

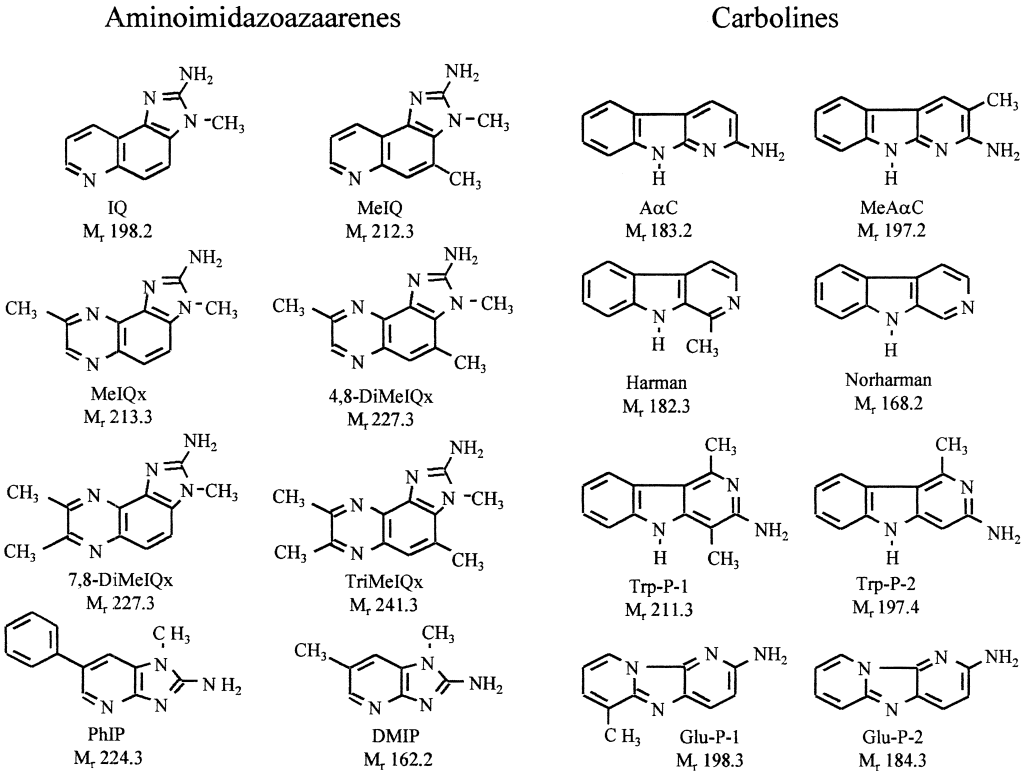

Fig. 1. Structure, abbreviated name and molecular mass of the compounds.

P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipyrido[1,2-*a*:3′,2′-*d*]-imidazole (Glu-P-2), purchased from Toronto Research Chemicals (Toronto, Canada), and 1-methyl-9*H*-pyrido[3,4-*b*]indole (harman) and 9*H*-pyrido[3,4-*b*]indole (norharman), which were from Sigma (Steinheim, Germany). Stock standard solutions of 130 μg g^{-1} in methanol were prepared and used for further dilution. TriMeIQx was used as internal standard (1.2 μg g^{-1} methanolic solution).

Empty Extrelut-20 extraction cartridges were provided by Merck (Darmstadt, Germany), and Isolute diatomaceous earth refill material was obtained from IST (Hengoed, UK). Bond Elut PRS (500 mg) and endcapped Bond Elut C_{18} (100 and 500 mg) cartridges were from Varian (Harbor City, USA), and Isolute PRS (200 mg) and endcapped tridimensional Isolute C_{18} (100 mg) cartridges were from IST. Coupling pieces and stopcocks were purchased from Varian. A lyophilised meat extract was prepared from a commercial meat extract (Bovril) as described in Ref. [38].

2.2. Sample analysis

Two previously described purification methods [27,39] with some minor modifications were used to extract analytes from a lyophilised meat extract. Sample preparation method A was as follows. A 1-g sample of beef extract was homogenised in 12 ml 1 *M* NaOH with sonication, and the suspension was then shaken for 3 h using a rotating shaker Rotary Mixer 34526 (Breda Scientific, Breda, The Netherlands). The alkaline solution was mixed with Extrelut refill material (14 g) and was used to fill an empty Extrelut column. After being preconditioned with 7 ml dichloromethane (DCM), an Isolute PRS column was coupled on-line to the Extrelut column. To extract the analytes from diatomaceous earth, 75 ml of dichloromethane were passed through the tandem. The PRS cartridge was then dried and successively

rinsed with 15 ml methanol–water (4:6, v/v) and 2 ml water. The cationic exchanger column was then coupled to an Isolute C_{18} column, previously conditioned with 5 ml MeOH and 5 ml water, and HAs were then eluted with 20 ml of 0.5 *M* ammonium acetate at pH 8.5. Finally, the C_{18} cartridge was rinsed with 5 ml water and the sorbed HAs were eluted using 0.8 ml of methanol–ammonia (9:1, v/v). The solvent was gently evaporated under a stream of nitrogen and the analytes were redissolved in 50 μl of the internal standard in methanol.

For method B, the cation-exchange column was a Bond Elut PRS column. The washing solutions arising from this cartridge, which consisted of 6 ml 0.01 *M* HCl, 15 ml MeOH–0.1 *M* HCl (6:4, v/v) and 2 ml of water, were collected for the analysis of the less polar compounds (PhIP; α-carbolines: AαC and MeAαC; β-carbolines: harman and norharman; γ-carbolines: Trp-P-1 and Trp-P-2). After lowering their organic solvent content by adding 25 ml of water, the acidic washing solutions were neutralised with 500 μl ammonia. Less polar HAs were preconcentrated in a 500-mg Bond Elut C_{18} column, which had previously been conditioned with 5 ml of MeOH and 5 ml of water. Finally, the reversed-phase minicolumn was washed with 5 ml water and the analytes were eluted with 1.4 ml MeOH–ammonia (9:1, v/v). On the other hand, a 100-mg Bond Elut C_{18} cartridge was conditioned with 5 ml MeOH and 5 ml water, and was then coupled on-line with the PRS cartridge. After that, the most polar amines (DMIP; δ-carbolines: Glu-P-1 and Glu-P-2; aminoimidazoquinolines: IQ and MeIQ; aminoimidazoquinoxalines: MeIQx, 4,8-DiMeIQx and 7,8-DiMeIQx) were eluted from the cationic exchanger with 20 ml of 0.5 *M* ammonium acetate at pH 8.5. Finally, the C_{18} cartridge containing the most polar analytes was rinsed with 5 ml water and the sorbed HAs were eluted using 0.8 ml of methanol–ammonia (9:1, v/v). The extracts containing either the most or least polar analytes were gently evaporated to dryness under a stream of nitrogen and were then redissolved in 50 μl of the internal standard in methanol.

A Supelco Visiprep and a Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used to manipulate the solid-phase extraction cartridges and solvent evaporation, respectively. The final extracts from both clean-up procedures were analysed using the LC–MS–MS method described in the next section.

Quantification and recovery calculation of the amines in the beef extract was carried out by standard addition. Before sample treatment, the meat extract was spiked at three different levels (50, 100 and 200 ng) by adding a methanolic solution of the analytes (4 μg g^{-1}). The solvent was allowed to evaporate for 30 min before applying the sample extraction procedure. Furthermore, D_3-IQ, D_3-MeIQx and D_3-PhIP were added to the meat extract at ~50 ng g^{-1}. Duplicate analyses of all the samples, including the fortified samples, were carried out.

2.3. Chromatographic conditions

LC was performed using a Waters 2690 Separations Module (Milford, MA, USA), equipped with a quaternary solvent delivery system and an autosampler. Amines were separated by reversed-phase LC using a TSK-Gel ODS 80T column (5 μm, 25.0×4.6 mm I.D.) (TosoHaas, Stuttgart, Germany) equipped with a Supelguard LC-8-DB precolumn (Supelco, Bellefonte, PA, USA).

Optimal separation was achieved with a ternary mobile phase at a flow-rate of 1 ml min^{-1}. Solvent A: 30 m*M* formic acid in water adjusted to pH 3.25 with a solution of ammonia; solvent B: 30 m*M* formic acid in water adjusted to pH 3.7 with a solution of ammonia; solvent C: acetonitrile. The gradient elution program was: 5–23% C in A, 0–18 min; 23% C in A, 18–21 min; 23% C in B, 21–25 min; 23–60% C in B, 25–33 min; 60% C in B, 33–40 min; return to the initial conditions, 40–50 min; 5 min equilibration. In all cases the amount injected was 15 μl.

2.4. Mass spectrometric conditions

MS analysis was carried out with an LCQ mass spectrometer (Finnigan MAT, San Jose, CA, USA) which operated using Excalibur 1.0 SR1 software. The mass spectrometer was provided with an atmospheric pressure chemical ionisation source and an ion trap as mass analyser. To prevent mass spectrometer contamination when running LC–MS, a divert valve

was used for a few minutes at the beginning of the chromatogram.

Optimal source working conditions for monitoring positive ions were as follows: spray current and discharge voltage were 5 μA and 5 kV, respectively; heated capillary temperature was 150°C, and that of the vaporiser 450°C; nitrogen was used as sheath gas at 72 l h^{-1} and as auxiliary gas at 360 l h^{-1}. The chromatographic separation was divided into three segments, corresponding to different eluting conditions (first segment: 0–18.5 min, second segment: 18.5–26 min, third segment: 26–40 min). Efficiency of ion transference from source to the ion trap was automatically optimised for each segment by infusing methanolic solutions of IQ, 4,8-DiMeIQx and Trp-P-1, respectively. Collision-induced dissociation (CID) conditions were optimised for each analyte as follows. Individual methanolic solutions (10 μg g^{-1}) were infused using a syringe pump (3 μl min^{-1}) and were mixed with the mobile phase corresponding to the eluting conditions of each HA by means a tee piece. Working activation Q (AQ) was 0.45, normalised collision energy (NCE) ranged from 36.3 to 43.6% and activation time (AT) was 30 ms. For data acquisition in full scan mode, the mass spectrometer operated over a range of m/z 150–250, and in full scan MS–MS the masses scanned varied from m/z 110 to m/z 250 (Table 1). In all cases, the acquisition of positive ions was performed in centroid mode, with a maximum injection time of 100 ms, three microscans, the automatic gain control activated and the inject waveform disconnected.

3. Results and discussion

3.1. Tandem mass spectrometry

In the positive mode, APCI provided only the peak corresponding to the protonated molecule ion $[M+H]^+$ in agreement with literature data [40,41]. Therefore, this ion was used as a precursor in MS–MS experiments. First of all, the effect of the value of isolation width (IW) on precursor ion intensity was studied. Maximum trapping efficiency, without interferences from isotopic species, was achieved using an IW of 1.5.

Fragmentation of precursor ions in an ion trap occurs by CID when a resonance excitation voltage is applied to the endcap electrodes. While the stability range of both precursor and product ions is defined by the magnitude of the trapping radio-frequency voltage (AQ) applied to the ring electrode, the energy applied is controlled by the amplitude (NCE) and the duration (AT) of the voltage applied to the endcap electrodes.

To determine the stability range of ions and to choose the optimum value for AQ, individual methanolic solutions (~10 μg g^{-1}) of HAs were infused at different AQ values ranging from 0 to 0.9. For precursor ions, NCE was zero, while for product ions an NCE value high enough to provide the major product ion as the base peak was used. AT was fixed at 30 ms for both precursor and product ions. As an example, Fig. 2 shows the AQ curves obtained for some of the compounds studied. Similar behavior was observed for all the HAs: while the precursor ions had a wide stability range, the product ions were stable in a narrower interval. An optimum AQ value of 0.45, which was inside the stability range of the precursor ions, was chosen to maximise the intensity of product ions.

The CID energy needed to fragment the precursor ion was optimised by studying the effect of NCE and AT on product ion intensity. For each compound, AQ was set at 0.45, AT was kept at 30 ms and NCE was varied from 0 to 70%. The precursor ions started to fragment at ~30% (Fig. 3). Beyond this value, an increase in NCE produced an enhancement of product ions intensity compared with the unfragmented precursor ion. At higher values, the abundance of product ions was generally constant and, in some cases, a decrease in the signal was produced because of further fragmentation. The optimum NCE value was selected to provide a maximum intensity of the product ion keeping a significant signal for the precursor ion. Finally, AQ and NCE were fixed to their selected values, and AT was studied between 20 and 40 ms, verifying that the optimum AT value was 30 ms. The final MS–MS working conditions are summarised in Table 1, together with a list of the main product ions for each compound and their tentative assignation.

In general, the most intense product ion in the MS–MS spectra of AIAs (aminoimidazoquinolines: IQ and MeIQ; aminoimidazoquinoxalines: MeIQx,

Table 1
Selected MS–MS conditions and product ions used for quantification of HAs

Analyte	MS spectra		MS–MS CID[a]	Product ions used for quantification		Full scan MS–MS range
	m/z (Rel.Ab.%)	Tentative Assign.	NCE (%)	*m/z* (Rel.Ab.%)	Tentative Assign.	
DMIP	163.2 (100)	$[M+H]^+$	40.7	148.2 (100)	$[M+H\text{-}CH_3]^{+\cdot}$	[140.0–170.0]
Glu-P-2	185.3 (100)	$[M+H]^+$	42.8	158.1 (100)	$[M+H–HCN]^+$	[150.0–190.0]
IQ	199.2 (100)	$[M+H]^+$	41.0	184.2 (100)	$[M+H–CH_3]^{+\cdot}$	[150.0–205.0]
MeIQ	213.3 (100)	$[M+H]^+$	40.3	198.2 (100)	$[M+H–CH_3]^{+\cdot}$	[165.0–220.0]
Glu-P-1	199.3 (100)	$[M+H]^+$	43.7	184.2 (89)	$[M+H–CH_3]^{+\cdot}$	[165.0–210.0]
				172.2 (100)	$[M+H–HCN]^+$	
MeIQx	214.3 (100)	$[M+H]^+$	41.3	199.2 (100)	$[M+H–CH_3]^{+\cdot}$	[165.0–220.0]
				173.2 (87)	$[M+H–C_2NH_3]^+$	
7,8-DiMeIQx	228.3 (100)	$[M+H]^+$	42.2	213.2 (89)	$[M+H–CH_3]^{+\cdot}$	[180.0–235.0]
				187.2 (100)	$[M+H–C_2NH_3]^+$	
4,8-DiMeIQx	228.3 (100)	$[M+H]^+$	41.1	213.3 (100)	$[M+H–CH_3]^{+\cdot}$	[180.0–235.0]
				187.2 (90)	$[M+H–C_2NH_3]^+$	
Norharman	169.2 (100)	$[M+H]^+$	44.6	167.2 (82)	$[M+H–2H]^+$	[110.0–175.0]
				142.1 (92)	$[M+H–HCN]^+$	
				115.1 (100)	$[M+H–2HCN]^+$	
TriMeIQx	242.3 (100)	$[M+H]^+$	41.3	227.2 (100)	$[M+H–CH_3]^{+\cdot}$	[195.0–250.0]
				201.2 (97)	$[M+H–C_2NH_3]^+$	
Harman	183.3 (100)	$[M+H]^+$	43.7	181.2 (40)	$[M+H–2H]^+$	[110.0–190.0]
				168.2 (100)	$[M+H–CH_3]^{+\cdot}$	
				115.1 (48)	$[M+H–CH_3CN–HCN]^+$	
Trp-P-2	198.4 (100)	$[M+H]^+$	40.3	222.1 (11)	$[M+H–NH_3+ACN]^+$	[175.0–225.0]
				199.2 (29)	$[M+H–NH_3+H_2O]^+$	
				181.1 (100)	$[M+H–NH_3]^+$	
PhIP	225.3 (100)	$[M+H]^+$	43.2	210.2 (100)	$[M+H–CH_3]^{+\cdot}$	[200.0–230.0]
Trp-P-1	212.3 (100)	$[M+H]^+$	40.1	236.0 (15)	$[M+H–NH_3+ACN]^+$	[190.0–240.0]
				213.2 (35)	$[M+H–NH_3+H_2O]^+$	
				195.2 (100)	$[M+H–NH_3]^+$	
AαC	184.2 (100)	$[M+H]^+$	38.5	208.1 (34)	$[M+H–NH_3+ACN]^+$	[165.0–215.0]
				185.2 (100)	$[M+H–NH_3+H_2O]^+$	
				167.1 (13)	$[M+H–NH_3]^+$	
MeAαC	198.2 (100)	$[M+H]^+$	37.2	222.0 (29)	$[M+H–NH_3+ACN]^+$	[175.0–225.0]
				199.2 (100)	$[M+H–NH_3+H_2O]^+$	
				183.2 (52)	$[M+H–CH_3]^{+\cdot}$	
				181.2 (20)	$[M+H–NH_3]^+$	

[a] In all cases, AQ value was 0.45 and AT was 30 ms.

7,8-DiMeIQx, 4,8-DiMeIQx and TriMeIQx; aminoimidazopyridines: DMIP, PhIP) arose from the loss of the 2-methyl group $[M+H–CH_3]^{+\cdot}$, as confirmed by the study of D_3-IQ, D_3-MeIQx and D_3-PhIP. The relative abundance of this fragment ion ranged from 89 to 100%. Moreover, aminoimidazoquinoxalines showed the cleavage of the aminoimidazole moiety $[M+H–C_2NH_3]^+$, with relative abundances from 87 to 100%. These fragmentation patterns are consistent with those obtained by other authors using triple quadrupole instruments [28,32,33].

In the case of carbolines (α-carbolines: AαC and MeAαC; β-carbolines: harman and norharman; γ-carbolines: Trp-P-1 and Trp-P-2; δ-carbolines: Glu-P-1 and Glu-P-2), the most abundant fragment ions were derived from the loss of a methyl $[M+H–CH_3]^{+\cdot}$ for the methylated carbolines (MeAαC, harman and Glu-P-1, relative abundances ranging

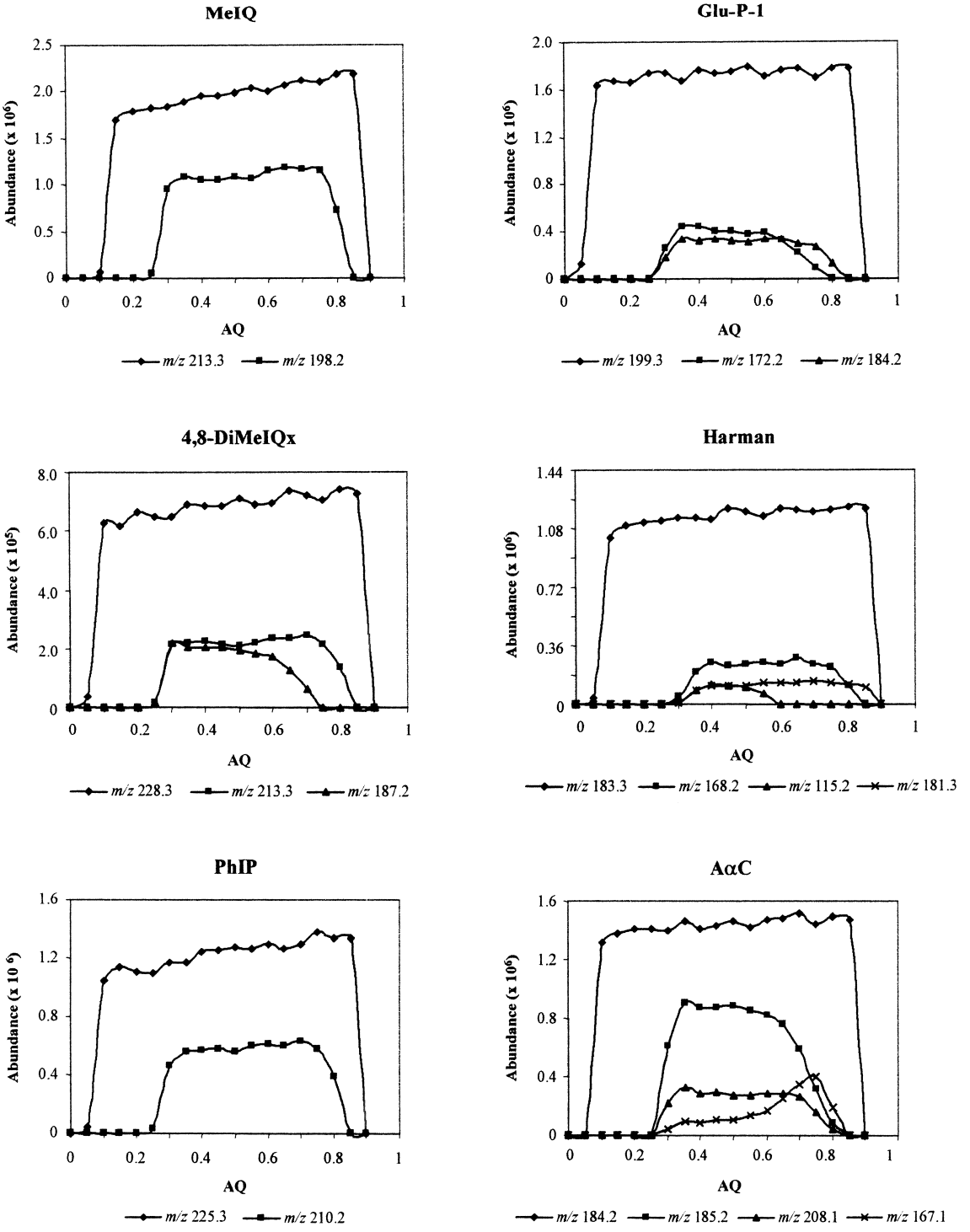

Fig. 2. Variation of precursor and product ion abundance as a function of the trapping radiofrequency voltage (AQ).

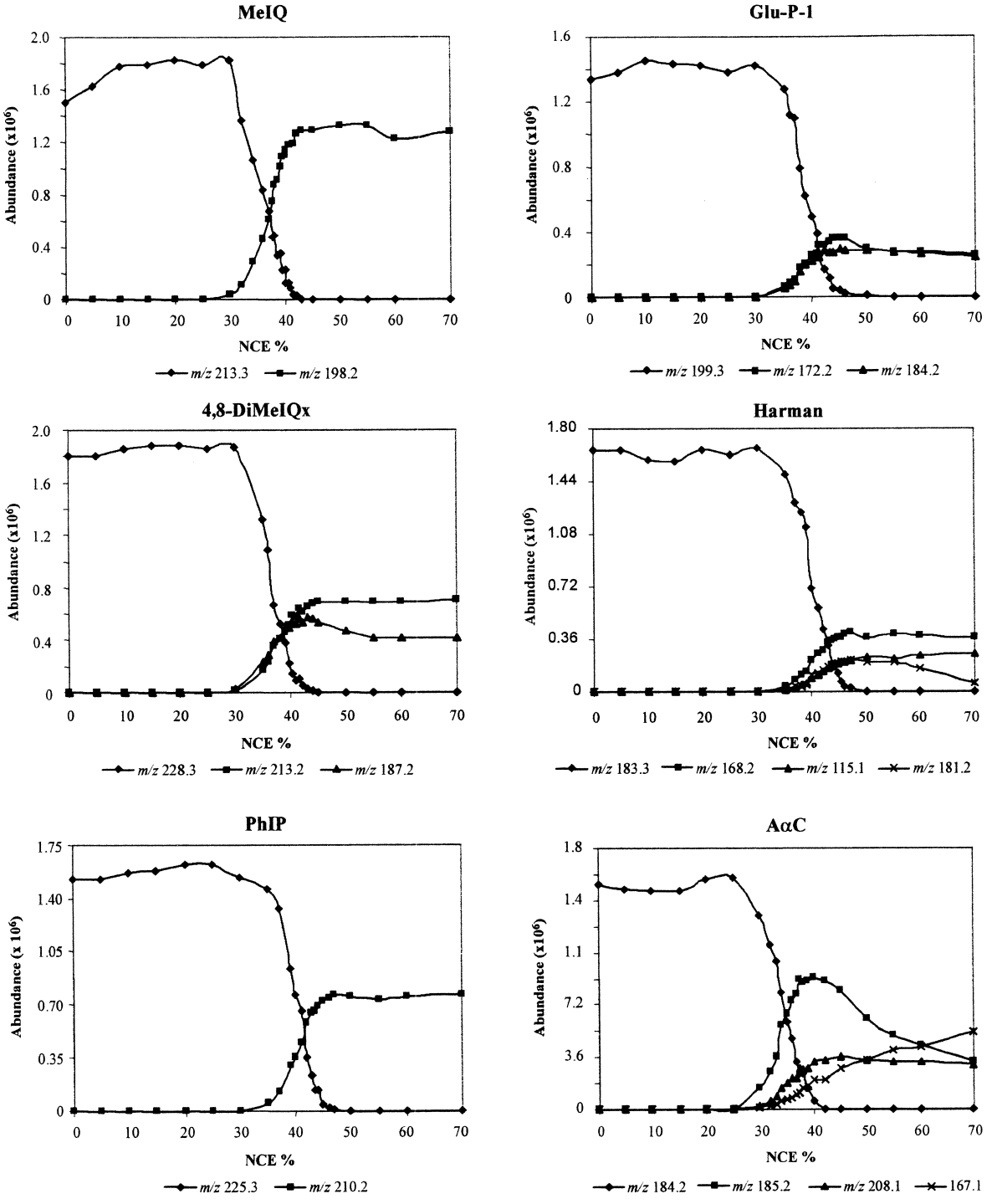

Fig. 3. Variation of precursor and product ion abundance as a function of the normalised collision energy (NCE).

from 52 to 100%), from the loss of ammonia $[M-NH_3]^+$ in the case of primary amines (100% of relative abundance for γ-carbolines, and in the range 13–20% for α-carbolines), and from the loss of HCN (100% for δ-carbolines and 92% for norharman). Moreover, other important fragment ions in the MS–MS spectra of harman and norharman corresponded to the loss of hydrogen atoms $[M-2H]^+$. In some cases, recombination of $[M+H-NH_3]^+$ with neutral molecules present in the ion trap, such as water or acetonitrile, was observed. These adducts are so abundant that, for instance, for α-carbolines they corresponded to the base peak, and for γ-carbolines they reached a relative abundance of 35%. The identity of these adducts was confirmed by changing the organic solvent of the mobile phase and also by carrying out higher-order multiple MS (MS^n) experiments. These adducts have not been observed by other authors working with triple quadrupole instruments [15,28].

Product ions with a relative abundance greater than 50% were chosen for the LC–MS–MS quantitative analysis. In the case of α-carbolines and γ-carbolines, the ion $[M-NH_3]^+$ and the adducts with water and acetonitrile were used to enhance the robustness of the quantitative analysis.

3.2. Performance of the LC–APCI-MS–MS method

Fig. 4 shows the chromatogram obtained after the injection of a standard solution (4 μg g^{-1}) at the selected conditions. A good resolution was obtained and the individual trace chromatograms were almost free of background noise.

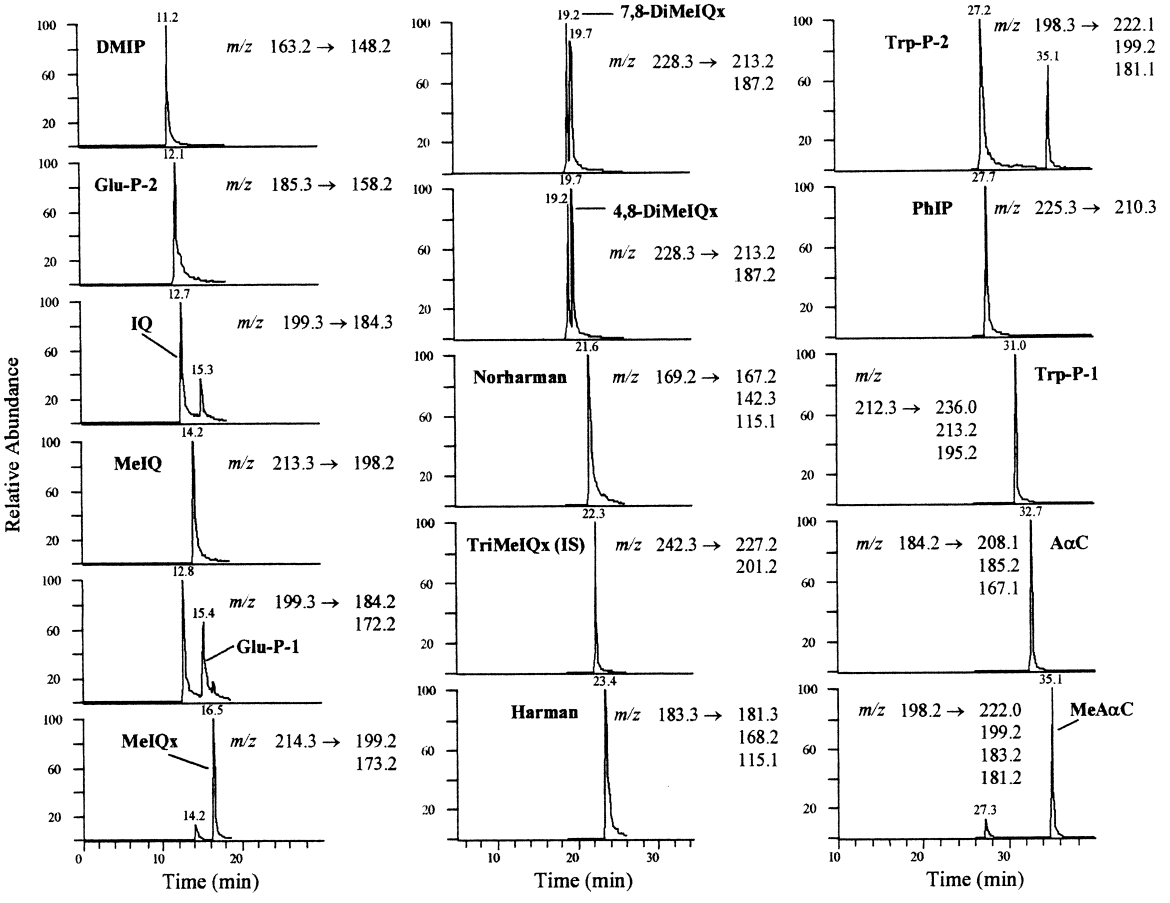

Fig. 4. LC–APCI-MS–MS chromatogram obtained for a standard solution (4 μg g^{-1}).

Calibration curves for the chromatographic method were performed at six concentration levels from 0.1 to 7.1 μg g^{-1} for each analyte. These curves were calculated daily from the representation of the ratio of the peak area of the analytes to the peak area of the internal standard (TriMeIQx) versus the ratio of the concentration of each compound to the concentration of the internal standard. Curves were fitted to a linear function, obtaining regression coefficients higher than 0.995 in all cases. To evaluate the analytical performance of the LC–MS–MS method, several quality parameters (repeatability or run-to-run precision, medium term or day-to-day precision and limit of detection) were determined. To calculate repeatability and medium term precision, five daily replicate analysis of a methanolic solution of all the analytes at ~1 μg g^{-1} were carried out on 3 successive days. A study of the variance of one factor for concentration and retention time was then performed. The RSD for concentration varied from 3.2 to 9.3% for run-to-run precision, and from 4.2 to 11.0% for day-to-day precision. For retention times, run-to-run precision, expressed as RSD, was between 0.1 and 0.5%, and day-to-day precision between 0.1 and 1.2% (Table 2).

Detection limits (LODs), based on a signal-to-noise ratio of 3:1, were determined in standard solutions and meat extracts. Full scan MS–MS LODs using standard solutions were from 2- to 8-fold lower than those obtained using full scan MS (Table 3). In the case of the meat extract, LODs were determined by fortifying blank samples at very low levels. For harman and norharman, which were present in the blank samples, LODs were obtained by extrapolating from a non-spiked sample. A comparison of LODs expressed as injected amount for standard solutions and meat extract (Table 3) shows that the LOD values were higher in the sample because of the effect of matrix on ionisation. Moreover, the improvement of LODs in MS–MS was greater in the sample than in the standard solutions, and this effect was more pronounced for the polar HAs using method A. This observation can be explained by the high selectivity of MS–MS, which allows reduction of background, thereby improving the signal-to-noise ratio.

In general and for both sample treatment procedures, limits of detection in the sample using full scan MS–MS were ~1 ng g^{-1}, which is a low value for such a complex sample. Except for DMIP,

Table 2
Run-to-run precision and day-to-day precision of the method for a standard solution

Analyte	Target conc. (μg g^{-1})	Mean value		Precision RSD % (n=15, α= 0.05)			
		Conc. (μg g^{-1})	t_r (min)	Conc.		t_r	
				Run-to-run	Day-to-day	Run-to-run	Day-to-day
DMIP	1.0502	1.078	11.2	5.7	7.7	0.1	1.0
Glu-P-2	1.0504	1.053	12.1	4.7	6.7	0.1	1.0
IQ	1.0445	1.058	12.8	4.9	5.5	0.5	1.0
MeIQ	1.0567	1.076	14.3	4.9	6.3	0.1	0.9
Glu-P-1	1.0447	1.049	15.4	5.0	10.1	0.1	0.9
MeIQx	1.0590	1.069	16.6	5.4	9.5	0.1	0.9
7,8-DiMeIQx	1.0690	1.096	19.2	6.4	10.6	0.1	0.8
4,8-DiMeIQx	1.0659	1.108	19.7	4.9	7.9	0.1	0.7
Norharman	1.0450	1.079	21.8	4.3	7.7	0.1	0.8
Harman	1.0278	1.093	23.6	4.6	4.2	0.1	0.8
Trp-P-2	1.0422	1.057	27.4	3.2	6.1	0.1	1.2
PhIP	1.0345	1.057	27.7	4.4	7.1	0.2	1.1
Trp-P-1	1.0378	1.139	31.0	6.3	10.5	0.1	0.6
AαC	1.0322	1.017	32.7	9.3	9.8	0.1	0.3
MeAαC	1.0401	1.026	35.1	7.7	11.0	0.1	0.1

Table 3
Limits of detection (LODs) in full scan MS and full scan MS–MS for a standard solution and a meat extract

Analyte	Standard solution				Meat extract							
	ng g^{-1}		Injected pg		Injected ng				ng g^{-1}			
	Full scan MS	Full scan MS–MS	Full scan MS	Full scan MS–MS	Full scan MS		Full scan MS–MS		Full scan MS		Full scan MS–MS	
					Clean-up A	Clean-up B	Clean-up A	Clean-up B	Clean-up A	Clean-up B	Clean-up A	Clean-up B
DMIP	18.8	2.3	358	44	6.7	3.3	0.7	0.5	101.0	32.2	10.3	4.9
Glu-P-2	14.8	2.4	283	45	5.0	2.9	0.5	0.7	11.9	11.5	1.2	2.7
IQ	12.3	2.0	235	38	4.8	3.2	0.5	0.5	10.6	14.7	1.0	2.3
MeIQ	10.6	2.0	202	39	4.7	3.3	0.5	0.5	10.4	16.6	1.1	2.4
Glu-P-1	13.6	2.1	259	41	5.4	3.3	0.7	1.0	13.5	14.7	1.7	4.6
MeIQx	10.2	1.2	196	24	3.9	2.2	0.5	0.4	10.0	9.2	1.2	1.6
7,8-DiMeIQx	4.1	1.7	79	33	2.6	2.0	0.4	0.4	6.7	9.1	0.9	1.5
4,8-DiMeIQx	3.4	1.8	66	34	2.7	1.9	0.4	0.3	6.4	9.4	1.0	1.7
Norharman	11.2	3.4	215	64	2.9[a]	3.0[a]	0.5[a]	0.5[a]	9.7[a]	10.1[a]	1.8[a]	1.6[a]
Harman	8.3	2.4	158	46	3.2[a]	3.5[a]	0.9[a]	0.8[a]	10.6[a]	11.5[a]	3.0[a]	2.5[a]
Trp-P-2	3.3	1.0	62	18	2.4	2.3	0.3	0.3	5.5	12.3	0.8	1.7
PhIP	2.1	0.8	41	16	2.8	2.7	0.3	0.3	6.5	13.5	0.7	1.6
Trp-P-1	2.8	0.6	53	12	2.0	2.0	0.4	0.7	4.3	9.2	0.8	3.1
AαC	2.1	0.5	40	10	0.6	0.7	0.1	0.1	2.0	4.8	0.4	1.0
MeAαC	2.4	0.6	46	11	0.6	0.5	0.1	0.2	1.8	2.9	0.4	0.8

[a] Extrapolated from the non-spiked meat extract.

slightly lower detection limits were achieved with clean-up method A, probably because of its higher extraction efficiency.

3.3. Determination of HAs in a meat extract

After optimisation of the chromatographic and spectrometric conditions, the LC–MS–MS method was used to analyse HAs in a lyophilised meat extract and the chromatogram is given in Fig. 5. The tandem mass spectrometry technique provided a high degree of selectivity, leading to chromatograms that were 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 standards. Thus, we confirmed the presence of nine HAs in the sample. As an example, Fig. 6 shows the MS–MS spectra of Trp-P-1, AαC and MeAαC, which were present in the meat extract at very low concentrations, between the detection and quantification limits. IQ, MeIQx, 4,8-DiMeIQx, norharman, harman and PhIP were quantified by the standard addition method. Method A showed extraction efficiencies that ranged from 75 to 98% for all the HAs except DMIP, whose recovery was only 14%. In the case of method B, the recovery of DMIP was 35%, and for the rest of the analytes recovery values fell between 50 and 83% (Table 4). These values are comparable to those obtained in previous studies [42,43]. Although the extraction efficiencies varied slightly for the two sample treatments, the amounts of HAs detected are consistent (Table 4). Nevertheless, clean-up A is less time consuming and requires fewer materials.

Furthermore, recovery values using D_3-IQ, D_3-MeIQx and D_3-PhIP were calculated and used to quantify the respective non-labelled HAs in the meat extract. This quantification method gave more precise data because a correction of extraction efficiency and changes in instrument performance was achieved using labelled compounds. For these three compounds, the percentage recovered after sample treatment was comparable with that obtained with the standard addition method (Table 5). The other HAs present in the meat extract at levels higher than their limit of detection, namely 4,8-DiMeIQx, norharman

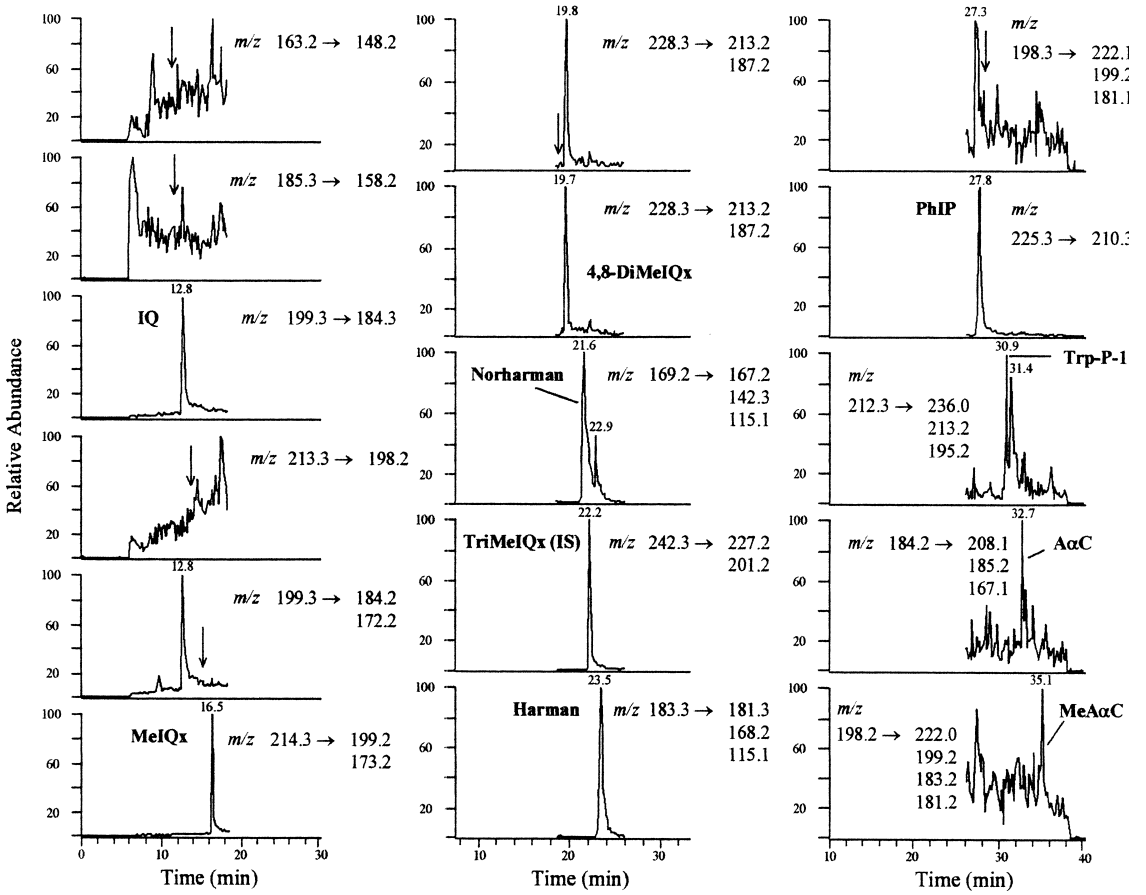

Fig. 5. LC–APCI-MS–MS chromatogram obtained for a meat extract purified with clean-up method A. Compounds identified: IQ, MeIQx, 4,8-DiMeIQx, norharman, harman, PhIP, Trp-P-1, AαC and MeAαC. The arrows indicate where the non-detected analytes would be expected.

and harman, were also quantified using the recovery values of the trideuterated HAs. In this case, higher standard deviations were obtained, because of differences in the extraction efficiency of the analytes. However, for clean-up A acceptable results were achieved because recovery values were very similar for most of the HAs.

4. Conclusions

The fragmentation of HAs in an ion trap was optimised to provide stable product ions for the analysis of these mutagenic amines by LC–APCI-MS–MS. For AIAs, the product ion derived from the loss of a methyl group was the base peak, while for carbolines the loss of ammonia and the corresponding adducts (water and acetonitrile) were the most relevant. The method was applied to the analysis of a lyophilised meat extract, and low LODs for such a complex matrix were found. The analytes present in this sample were determined, and reproducible and reliable data were obtained. False peak identification was prevented by matching the full scan MS–MS spectra of the sample with those of standards.

Moreover, although similar quantitative results

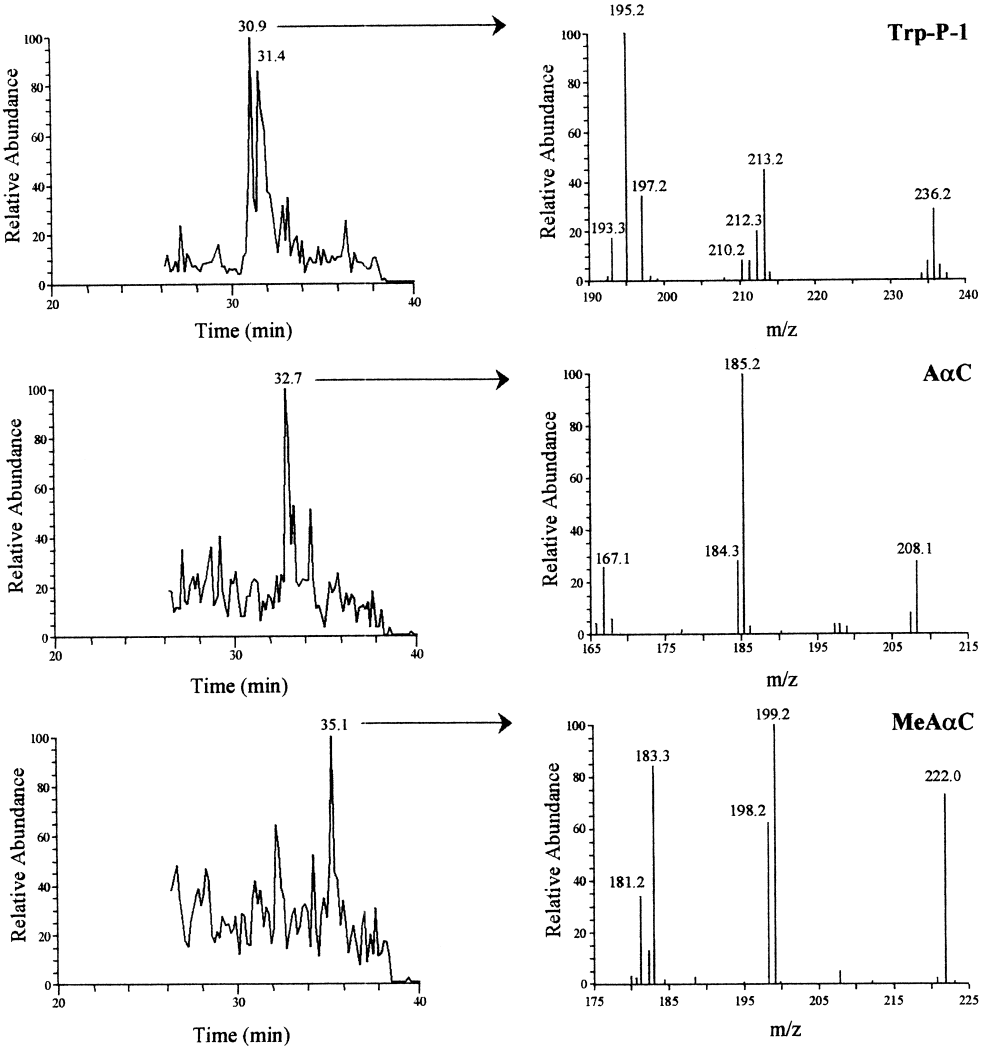

Fig. 6. Product ion scan confirmation of Trp-P-1, AαC and MeAαC in the meat extract.

were obtained using the two sample treatments, the clean-up that preconcentrates all the analytes in a single extract (method A) is less time consuming, requires the use of fewer materials and gives slightly higher recovery values. In addition, the use of deuterated analytes led to acquisition of more precise quantitative data for IQ, MeIQx and PhIP. Furthermore, for method A the use of labelled analytes also provided acceptable results for the other compounds analysed. Therefore this method could be used to estimate the content of HAs in food samples, reducing the number of spiked replicates in comparison with the classical standard addition method.

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Table 4
Amount of HAs found in the lyophilised meat extract and recovery values for the two clean-up procedures[a]

Analyte	Clean-up A				Clean-up B			
	Recovery %	RSD %	Amount found (ng g^{-1})	SD	Recovery %	RSD %	Amount found (ng g^{-1})	SD
DMIP	14	4	n.d.		35	8	n.d.	
Glu-P-2	87	3	n.d.		83	5	n.d.	
IQ	87	3	31.3	3.3	72	6	36.5	3.4
MeIQ	93	3	n.d.		65	4	n.d.	
Glu-P-1	82	5	n.d.		74	7	n.d.	
MeIQx	81	4	40.5	6.4	80	5	40.2	7.6
7,8-DiMeIQx	78	5	n.d.		75	5	n.d.	
4,8-DiMeIQx	87	4	16.4	1.2	69	6	18.1	1.2
Norharman	89	7	180.2	20.5	58	8	185.7	14.8
Harman	87	10	240.7	35.5	50	16	314.5	53.1
Trp-P-2	90	3	n.d.		63	4	n.d.	
PhIP	87	3	25.0	3.0	67	6	24.1	3.2
Trp-P-1	98	3	n.q.		72	9	n.q.	
AαC	63	2	n.q.		46	5	n.q.	
MeAαC	75	5	n.q.		61	5	n.q.	

[a] n.d., not detected; n.q., below the limit of quantification (signal-to-noise ratio 10:1).

Table 5
Comparison of the quantitative data obtained for IQ, MeIQx and PhIP using standard addition and isotopic surrogates

Analyte		Standard addition				Isotopic surrogates			
		Recovery %	RSD %	Amount found (ng g^{-1})	SD	Recovery %	RSD %	Amount found (ng g^{-1})	SD
Clean-up A	IQ	87	3	31.3	3.3	81	6	36.3	3.1
	MeIQx	81	4	40.5	6.4	86	7	37.4	1.7
	PhIP	87	3	25.0	3.0	78	7	27.8	0.9
Clean-up B	IQ	72	6	36.5	3.4	67	4	37.2	2.5
	MeIQx	80	5	40.2	7.6	84	3	39.5	0.5
	PhIP	67	6	24.1	3.2	63	6	28.0	1.5

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