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Journal of Chromatography A, 775 (1997) 125–136

JOURNAL OF CHROMATOGRAPHY A

Liquid chromatography–electrospray mass spectrometry with in-source fragmentation for the identification and quantification of fourteen mutagenic amines in beef extracts

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Received 5 November 1996; revised 3 March 1997; accepted 6 March 1997

Abstract

Conditions for the liquid chromatography–electrospray mass spectrometric (LC–ES-MS) determination of the fourteen most abundant heterocyclic amines and related compounds that can be produced in thermally processed foods were established. The simultaneous separation of all HAs on an octadecylsilane microbore column using a gradient elution with 5 m*M* ammonium acetate, pH 4.0, and acetonitrile was optimized. The mass spectra obtained at different extraction voltages were studied. The highest extraction voltages provided structural information for the characterization of these amines. Good detection limits comparable with those of LC and electrochemical detection were obtained. The method was applied to the analysis of these mutagens in beef extracts after a solid-phase extraction clean-up and in-source fragmentation had been applied for confirmation purposes. The levels of several heterocyclic amines measured were estimated to be in the range of 7.8–135.2 ng g^{-1}. © 1997 Elsevier Science B.V.

Keywords: Liquid chromatography–mass spectrometry; Amines; Heterocyclic amines

1. Introduction

The formation of mutagenic compounds (aromatic hydrocarbons) during the cooking of foods, primarily meat and fish products, was reported by Lijinsky and Shubick in 1964 [1]. The development of the Ames assay in 1975 [2] made it possible to test food products for mutagenic activity and then to isolate new mutagenic compounds formed in these products. Several years later, Sugimura [3] reported that the mutagenic activity of cooked fish and beef products could not be accounted for by aromatic hydrocarbons alone. This resulted in the discovery of the heterocyclic amines (HAs) as the group of compounds responsible for this additional mutagenic activity [4]; they were found to be carcinogenic in long-term animal studies [5]. These compounds are thought to be formed from amino acids, creatine, creatinine and sugars on heating rich-proteinaceous food and can be found in the low parts per billion range. These findings substantiate the need to develop sensitive analytical techniques to detect these compounds in processed foods.

Although high-performance liquid chromatography (HPLC) has been the most commonly used technique for the determination of HAs with a variety of detectors, mainly UV and fluorescence spectrophotometry [6,7], but also electrochemical

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PII S0021-9673(97)00274-4

[8,9], the use of mass spectrometry (MS) in conjunction with LC may be a key element for the future of the analysis of these compounds. The confirmation of analytes is an important requisite when analyzing HAs in processed foods because co-eluting interferences are always present in chromatograms. The confirmation of the analyte, in this case, is as important as its quantification, and LC–MS provides the best on-line identification, due to its selectivity and high specificity. Nowadays, LC–MS is a reliable and robust technique that can be routinely applied in many areas of analytical chemistry and its fields of application. The analysis of the HAs by LC–MS has been applied using a thermospray interface [10–12], but it has been restricted to the detection of some HAs. Electrospray (ES) ionization is opening new avenues to the analysis of low molecular mass trace constituents in complex matrices. In a previous study, we have described an LC–ES-MS method for the determination of seven HAs in beef extracts [13]. In a recent study, Richling et al. [14] have studied up to sixteen HAs and related compounds by LC–ES-MS–MS using two different chromatographic separations, but the applicability of the method to real samples has not yet been demonstrated. In this work, LC–MS with pneumatically assisted electrospray as the interface was used for the simultaneous determination of the fourteen most abundant HAs and related compounds in beef extracts. A microbore octadecylsilane column was used to separate all of the compounds, elution being carried out in gradient mode. An ES interface provides soft ionisation in which little structural information is directly obtained, but in-source fragmentation can be applied for this purpose, as we did in the present work, obtaining structural information without using an MS–MS technique. The method developed has been applied to the determination of HAs in commercial beef extracts, a solid-phase extraction clean-up procedure being performed prior to the chromatographic analysis. The presence of 2-amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole (Glu-P-1), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), 1-methyl-9H-pyrido[4,3-b]indole (Harman) and 9H-pyrido[4,3-b]indole (Norharman) was determined in the beef extract samples and their quantification was performed as well.

2. Experimental

2.1. Chemicals

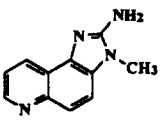
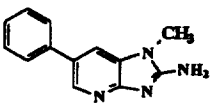
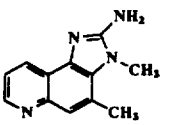
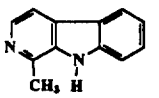
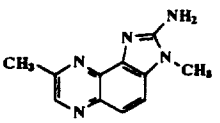
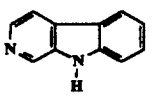
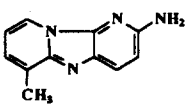
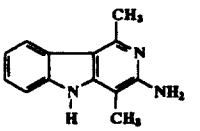
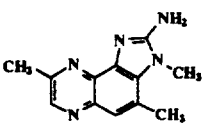
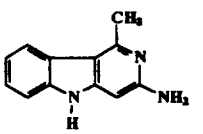
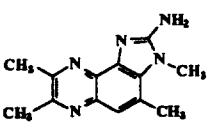
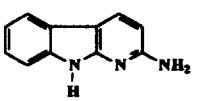
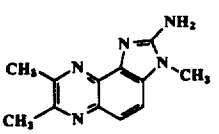
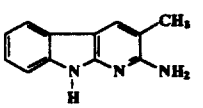
The compounds studied (Table 1) were 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,-8-DiMeIQx), 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido-[4,3-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-amino-9H-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), purchased from Toronto Research Chemicals (Toronto, Canada); and 1-methyl-9H-pyrido[4,3-*b*]indole (Harman) and 9H-pyrido[4,3-*b*]indole (Norharman), which were from Aldrich (Steinheim, Germany). Stock standard solutions of 100 μg ml^{-1} in methanol were prepared and used for further dilutions. 2-Amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) and 2-amino-3,4,7,8-tetramethyl-imidazo[4,5-*f*]quinoxaline (TriMeIQx) (Toronto Research Chemicals) were used as possible internal standards (solutions of 1 μg ml^{-1} in methanol). Diatomaceous earth extraction cartridges (Extrelut; 20 ml) were provided by Merck (Darmstadt, Germany). Bond Elut propylsulfonyl silica gel (PRS; 500 mg) and octadecylsilane (C_{18}; 500 and 100 mg) cartridges, as well as coupling pieces and stopcocks, were from Analytichem (ICT AG, Basle, Switzerland). These cartridges were preconditioned with dichloromethane (4 ml) for PRS and methanol (10 ml) and water (10 ml) in the case of C_{18}.

Solvents and chemicals used were of HPLC or analytical grade, and the water was purified in a Culligan system (Barcelona, Spain). All of the solutions were passed through a 0.45-μm filter before being injected into the LC system.

2.2. Instruments

MS was performed using a VG Quattro (Fisons Instruments, VG Biotech, Altrincham, UK) triple quadrupole mass spectrometer equipped with an electrospray interface, which was assisted pneumati-

Table 1
Structures of the heterocyclic amines studied

Abbreviation	Structure	Mr	Abbreviation	Structure	Mr
IQ		198	PhIP		224
MeIQ		212	Harman		182
MeIQx		213	Norharman		168
Glu-P-1		198	Trp-P-1		211
4,8-DiMeIQx		227	Trp-P-2		197
TriMeIQx		241	AαC		183
7,8-DiMeIQx		227	MeAαC		197

cally with nitrogen at a flow-rate of 10 l h^{-1}. Drying nitrogen was heated to 90°C and introduced into the capillary region at a flow-rate of 300 l h^{-1}. The electrospray needle was held at a potential of +3.2 kV relative to the potential at the counter-electrode for the positive mode. The focus potential was 70–150 V.

For data acquisition, the mass spectrometer operated over a mass range of m/z 40–300 in the centroid mode at a cycle time of 1.00 s and an inter-scan time of 0.10 s. Ion intensity was optimized using mobile phase ion clusters, and calibration was performed with these clusters. A drying nitrogen flow of 50 l h^{-1}, a focus potential of 80 V and a flow-rate of mobile phase of 50 μl min^{-1} were used in the calibration, in order to improve cluster formation. The verification of resolution (1 Da) in the working mass range was obtained by measuring the mobile phase ions as we have described previously [13].

Liquid chromatography was performed using two Phoenix 20 (Carlo Erba, Milan, Italy) syringe pumps; a master (A) and a slave (B) pump. A Valco (Houston, TX) injector equipped with a 200-nl loop

was used to introduce the sample. Separations were performed using a Hypersyl DBS C_{18} (5 μm particle size, 25.0 cm×1 mm I.D.) reversed-phase column (Shandon Scientific, Cheshire, UK). A mixture of 5 m*M* ammonium acetate at different pH values and acetonitrile was used as the mobile phase at a flow-rate of 50 μl min^{-1}. Separations were carried out at room temperature. Optimization of the separation between the fourteen HAs was performed using a Micro UVIS 20 UV detector (Carlo Erba).

2.3. Analytical procedure

Sample preparation and clean-up were performed following the method previously reported [15]. The first step used a diatomaceous earth cartridge (Extrelut) after the sample had been homogenised in a sodium hydroxide solution. In the second step, the analytes are eluted directly to a PRS cartridge using dichloromethane. The PRS was then washed with three different solvents: 0.01 *M* HCl solution, MeOH–0.1 *M* HCl mixture, and H_2O. These fractions, which contained the imidazopyridine and indolpyridine derivatives (Trp-P-1, Trp-P-2, PhIP, Harman, Norharman, AαC, MeAαC and Glu-P-1), were collected and concentrated using a C_{18} cartridge (500 mg) (extract B). The amino-imidazoquinoxalines and -quinolines (IQ, MeIQ, MeIQx and 4,8-DiMeIQx) retained in the PRS cartridge were eluted using ammonium acetate (0.5 *M*, pH 8.0) directly into another C_{18} cartridge (100 mg) (extract A). Finally, the HAs retained in the C_{18} cartridges were eluted (methanol/ammonia) to give two final extracts. Each extract was evaporated to dryness under a stream of nitrogen and redissolved in a methanolic internal standard (I.S.) solution, 25 μl for the unspiked samples and 100 μl for the spiked ones. The two extracts obtained were analyzed by LC–MS under the final working conditions. The analytes in the beef extract samples were quantified by external calibration and the results were corrected by the recoveries for each amine calculated by the standard addition method, adding to the samples accurately measured amounts of each standard at the beginning of the clean-up procedure.

The LC–MS measurements were performed by multiple ion detection (MID) of the most important masses for each mutagen, using a dwell time of 100 ms. The *m/z* of the $[M+H]^+$ ions selected when quantifying the beef extracts samples were: IQ, *m/z* 199; MeIQ, *m/z* 213; MeIQx, *m/z* 214; 4,8-DiMeIQx, *m/z* 228; PhIP, *m/z* 225; Glu-P-1, *m/z* 199; AαC, *m/z* 184; MeAαC, *m/z* 198; Trp-P-1, *m/z* 212; Trp-P-2, *m/z* 198; Harman, *m/z* 183; Norharman, *m/z* 168, and the internal standard TriMeIQx, *m/z* 242.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The separation of the fourteen HAs was performed using a microbore C_{18} reversed-phase column in order to avoid a post-column split to adjust the LC flow-rate to the optimum ES flow-rate in this instrument (50 μg l^{-1}). To establish the chromatographic conditions for this separation, variables such as pH and composition of the mobile phase were studied. A suitable mobile phase was chosen to achieve good separation between all compounds in accordance with the ES limitations. Different binary phases of 5 m*M* ammonium acetate (solvent A) and acetonitrile (solvent B) were tested, based on the results of a previous study on a conventional C_{18} column [8] and those obtained with a 10 cm×1 mm I.D. microbore C_{18} column [13]. Values lower than pH 4.0 must be used, otherwise the ionization of the HAs, and therefore the signal on the mass spectrometer will decrease. The mobile phases tested were ammonium acetate at pH values of 4.0 and 3.5 and different percentages of acetonitrile (between 10 and 20%). At pH 4, capacity factors (k') were higher and an increase in acetonitrile caused a reduction in the k' values for all compounds. Aminoimidazoazaarenes (IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx and TriMeIQx) and Glu-P-1 present different chromatographic behaviours from indolpyridine derivatives (Trp-P-1, Trp-P-2, AαC, MeAαC, Harman and Norharman) and PhIP. For the first group, low percentages of acetonitrile allowed good separations, but for the second group, higher percentages of acetonitrile were needed. From these results, it can be concluded that is not possible to achieve good separation between the fourteen HAs without apply-

ing a gradient elution mode. The pH chosen was 3.5 because this value provided optimum separations between aminoimidazoazaarenes when low percentages of acetonitrile were used. The optimized mobile phase was 5 m*M* ammonium acetate at pH 3.5 (solvent A) and acetonitrile (solvent B) using a gradient elution programme as follows: 0–11 min, 88–84% A; 11–20 min, 84–45%A; 20–25 min, 45% A; re-equilibration; initial conditions for 15 min. The internal standard was TriMeIQx, because 7,8-DiMeIQx was not sufficiently separated from 4,8-DiMeIQx, which has the same *m/z* value. In Fig. 1, a chromatogram of a standard solution of HAs using UV detection is shown.

3.2. Electrospray mass spectra

HAs gave protonated molecules $[M+H]^+$ as the base peak in the positive-ion mode, as has been reported previously [13]. Both the combination of ES with MS–MS [14] and the less expensive way using in-source fragmentation can provide structural information. In this paper, the infusion mode, using the solvent corresponding to the gradient for each amine, was used to inject analytes (200 nl of a 10 mg l^{-1} standard solution of HAs) at extraction voltages ranging from 70 to 150 V and the mass spectra in full-scan were obtained, in order to characterise the HAs by ES-MS and study the behaviour of these compounds under in-source fragmentation conditions. Table 2 shows the main ions obtained at two extraction voltages (100 and 150 V), with their corresponding tentative assignations and relative abundances. The same results were obtained when on-line LC–MS was used.

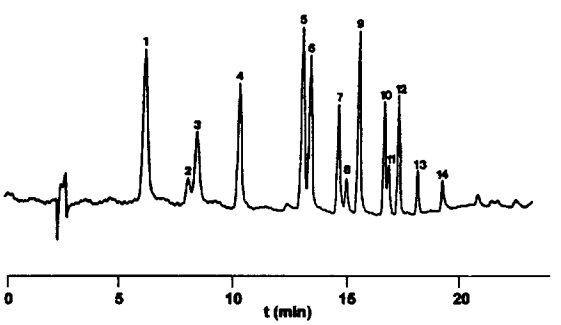


Fig. 1. HPLC–UV chromatogram. Mobile phase: 5 m*M* ammonium acetate, pH 3.5 (solvent A) and acetonitrile (solvent B) using a gradient elution programme: 0–11 min, 88–84% A; 11–20 min, 84–45% A; 20–25 min, 45% A, at λ=263 nm. Peaks: 1=IQ; 2=Glu-P-1; 3=MeIQ; 4=MeIQx; 5=7,8-DiMeIQx; 6=4,8-DiMeIQx; 7=Norharman; 8=TriMeIQx; 9=Harman; 10=PhIP; 11=Trp-P-2; 12=Trp-P-1; 13=AαC and 14=MeAαC.

When high extraction voltages were used, more fragmentation was observed and a decrease in the intensity of the protonated molecule $[M+H]^+$ occurred, as can be seen in Fig. 2, where the responses for $[M+H]^+$ ion of the HAs at different potentials are given. This figure show that the highest response was obtained at 100 V, so it was used for quantification purposes when coupling LC to MS.

As can be seen in Table 2, where the spectra at 100 and 150 V are summarized, aminoimidazoazarenes exhibited similar fragmentation patterns. The loss of CH_3 from protonated molecules and the loss of the aminoimidazyl moiety ($-CH_3$-HCN and $-C_3H_4N_2$) are the common route of fragmentation for these compounds. As a representative example, Fig. 3A shows the mass spectrum of TriMeIQx. The base peak corresponds to the fragment at *m/z* 227, which originated by the loss of CH_3 from the protonated molecule (*m/z* 242). Additional fragment ions (*m/z* 201 and 174) may result from the loss of the aminoimidazyl moiety. In this spectrum, *m/z* 264 corresponds to the $[M+Na]^+$ ion.

Trp-P-1, Trp-P-2, AαC and MeAαC gave almost identical fragmentation patterns. The spectra were dominated by the loss of the primary amino group, except for Trp-P-2 (Fig. 3B), where the relative abundance for *m/z* 181 ($-NH_3$) is 79% and the base peak corresponded to the fragment *m/z* 154 (−44 u) that may be assigned to the elimination of one molecule of HCN from fragment *m/z* 181.

Simpler spectra were observed for the other HAs studied. The spectra at 150 V of Harman and Norharman were characterized by the cleavage of the methylpyridyl and pyridyl moieties, respectively (*m/z* 115). Moreover, Harman gave the fragment at *m/z* 168 as a result of the loss of CH_3 from the protonated molecule $[M+H]^+$ (Fig. 3C). For Glu-P-1, besides the fragment ion *m/z* 182, which originated from the protonated molecular ion $[M+H]^+$ by the loss of the amino group, the stepwise elimination of two molecules of HCN from $[M+H]^+$ (*m/z* 172 and *m/z* 145) was observed (Fig. 3D). Finally, only

Table 2
Main ions obtained at extraction voltages of 100 and 150 V with their tentative assignations and relative abundances

Compound	M_r	m/z	Tentative assignation	Relative intensity (%)	
				100 V	150 V
IQ	198	199	$[M+H]^+$	100.0	44.7
		184	$[M+H-CH_3]^+\cdot$	–	100.0
		157	$[M+H-CH_3-HCN]^+\cdot$	–	10.5
MeIQ	212	213	$[M+H]^+$	100.0	37.1
		198	$[M+H-CH_3]^+\cdot$	–	100.0
		197	$[M+H-CH_4]^+\cdot$	–	27.1
		172	$[M+H-CH_3-CN]^+$	–	8.4
		145	$[M+H-CH_3-HCN-CN]^+$	–	5.6
MeIQx	213	214	$[M+H]^+$	100.0	47.5
		199	$[M+H-CH3]^+\cdot$	–	100.0
		173	$[M+H-CH_3-CN]^+$	–	22.1
		172	$[M+H-CH_3-HCN]^+$	–	21.0
Glu-P-1	198	199	$[M+H]^+$	100.0	100.0
		184	$[M+H-CH_3]^+\cdot$	–	32.2
		182	$[M+H-NH_3]^+$	–	53.1
		172	$[M+H-NCN]^+$	–	30.1
		145	$[M+H-(HCN)_2]^+$	–	31.0
4,8-DiMeIQx	227	228	$[M+H]^+$	100.0	99.3
		213	$[M+H-CH_3]^+\cdot$	–	100.0
		160	$[M+H-C_3H_4N_2]^+$	–	39.2
TriMeIQx	241	264	$[M+Na]^+$	–	12.0
		242	$[M+H]^+$	100.0	90.0
		227	$[M+H-CH_3]^+$	–	100.0
		201	$[M+H-CH_3-CN]^+$	–	32.1
		174	$[M+H-CH_3-HCN-CN]^+$	–	25.4
7,8-DoMeIQx	227	228	$[M+H]^+$	100.0	100.0
		213	$[M+H-CH_3]^+\cdot$	–	90.0
		187	$[M+H-CH_3-CN]^+$	–	32.0
		131	$[M+H-C_4H_7N_3]^+$	–	21.2
PhIP	224	225	$[M+H]^+$	100.0	97.2
		210	$[M+H-CH_3]^+\cdot$	–	100.0
Harman	182	183	$[M+H]^+$	100.0	100.0
		168	$[M+H-CH_3]^+\cdot$	–	32.9
		115	$[M+H-C_4H_6N]^+\cdot$	–	62.9
Norharman	168	169	$[M+H]^+$	100.0	100.0
		115	$[M+H-C_3H_4N]^+\cdot$	–	42.7
Trp-P-1	211	212	$[M+H]^+$	100.0	46.0
		195	$[M+H-NH_3]^+$	–	100.0
		168	$[M+H-NH_3-HCN]^+$	–	72.0
Trp-P-2	197	198	$[M+H]^+$	100.0	52.4
		181	$[M+H-NH_3]^+$	–	79.0
		154	$[M+H-NH_3-HCN]^+\cdot$	–	100.0
AαC	183	184	$[M+H]^+$	100.0	32.2
		167	$[M+H-NH_3]^+$	–	100.0
		140	$[M+H-NH_3-HCN]^+\cdot$	–	79.0
MeAαC	197	198	$[M+H]^+$	100.0	31.5
		183	$[M+H-CH_3]^+\cdot$	–	51.7
		181	$[M+H-NH_3]^+\cdot$	–	100.0
		154	$[M+H-NH_3-HCN]^+$	–	52.4
		129	$[M+H-C_3H_5N_2]^+\cdot$	–	29.4

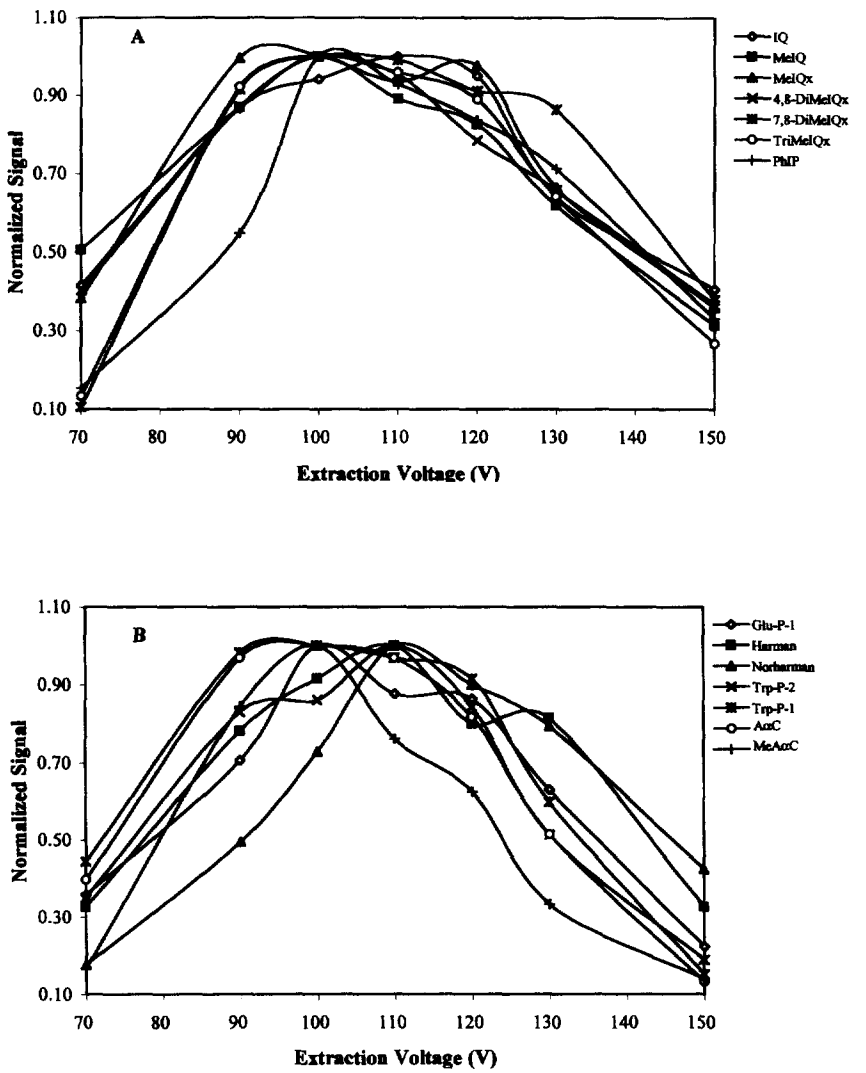


Fig. 2. Response of the molecular protonated ion of each HA vs. extraction voltage. (A) IQ, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx and PhIP; (B) Glu-P-1, Harman, Norharman, Trp-P-2, Trp-P-1, AαC and MeAαC.

the ion fragment at m/z 210, due to the loss of CH_3 from the $[M+H]^+$, was observed for PhIP (Fig. 3E).

The spectra obtained in this paper showed the same fragmentation patterns, with only small differences in ion abundances from those published by Richling et al. [14] using LC–ES-MS–MS.

3.3. LC–MS

A chromatogram for each mass and the total ion chromatogram (TIC) at the final working conditions for a standard solution of HAs are shown in Fig. 4. It can be observed that the resolution is poor and that Glu-P-1 and MeIQ co-eluted, but mass spectrometry is a selective technique and the poor resolution is compensated by selecting unambiguous masses to monitor. The $[M+H]^+$ ion for each HA was used when the chromatogram data for beef extract samples were registered with MID.

The quality parameters of the LC–MS system were studied and intervals of linearity and detection limits were established. Calibrations for HAs were

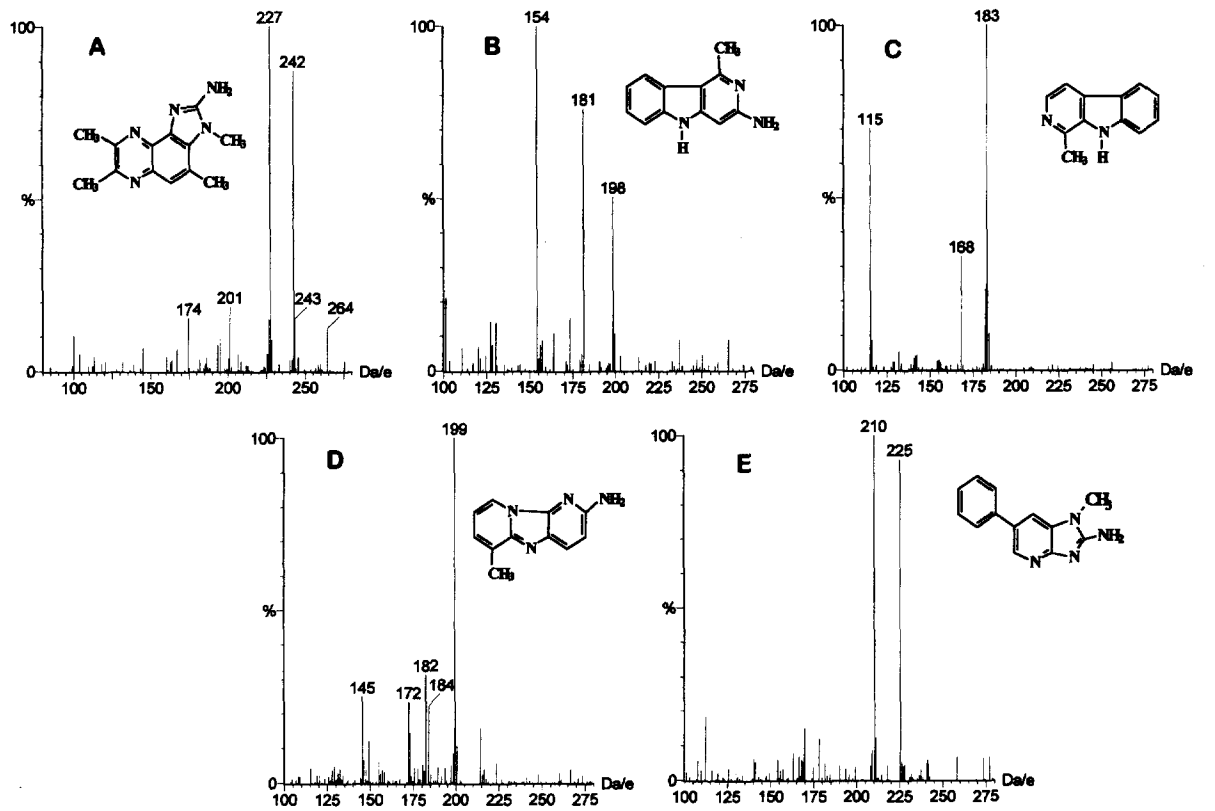


Fig. 3. ES mass spectra obtained at 150 V of (A) TriMeIQx, (B) Trp-P-2, (C) Harman, (D) Glu-P-1 and (E) PhIP.

carried out using the optimum separation conditions for each compound, with concentrations in the range of 0.01–2 μg ml^{-1}. Peak area was used as the response and the intervals of linearity corresponding to each amine are indicated in Table 3. The correlation coefficients of calibration functions in the interval of linearity were better than 0.995 for all of the HAs studied.

Detection limits in MID, based on a signal-to-noise ratio of 3:1, were investigated for each HA and ranged between 0.53 and 21.4 pg injected. The values corresponding to full-scan are between 15 and 50 times higher than these results, as we have reported previously [13]. The detections limits, expressed as the amount injected, and the concentrations are given in Table 3.

Detection limits in the infusion mode were also investigated in order to compare the effect that the LC column produced in the detection of these compounds. The values obtained in this case were similar to those obtained in the chromatographic separation, showing that it does not influence the sensitivity. Comparing these results with those obtained in a previous work using LC–ES-MS [13] and another column, we can observe an important improvement in the detection limits, which are lower by a factor of more than ten in some cases. This is due to the high efficiency of the chromatographic column used in this work. The values obtained are comparable with those obtained with electrochemical detection [8,9,16,17], except for MeIQx and Glu-P-1, which are higher, and with those obtained by LC–ES-MS–MS [14].

Detection limits for real samples were established by spiking beef extracts with standards at low concentration levels (0.05–4 ng g^{-1}) and applying the clean-up procedure. Detection limits of the HAs already present in the samples were estimated from

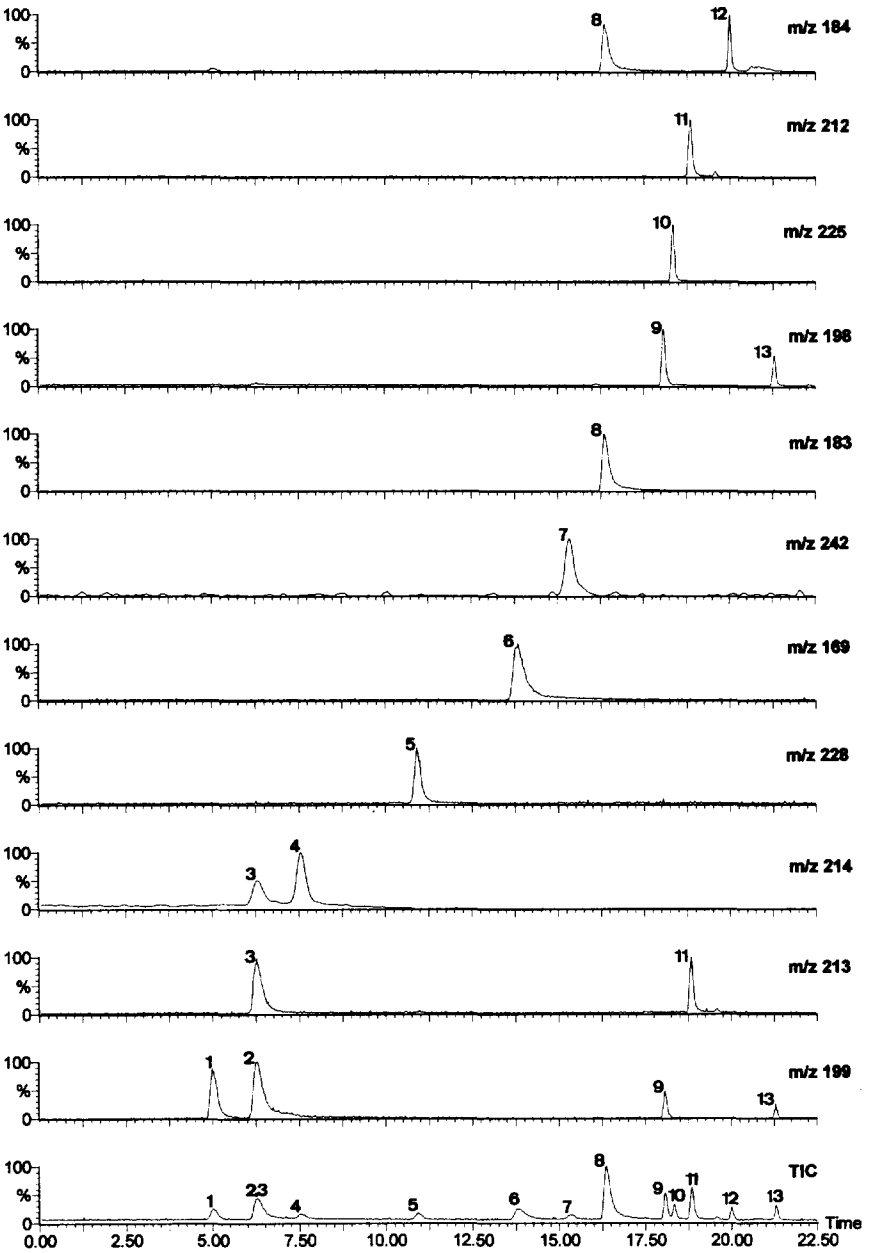


Fig. 4. LC–MS with positive ES ionization at 100 V of HAs. The bottom trace is the TIC obtained by summing all of the ions above. Peaks: 1=IQ; 2=Glu-P-1; 3=MeIQ; 4=MeIQx; 5=4,8-DiMeIQx; 6=Norharman; 7=TriMeIQx; 8=Harman; 9=PhIP; 10=Trp-P-2; 11=Trp-P-1; 12=AαC and 13=MeAαC.

calibration curves, taking into account the recovery values. The results are in agreement with those obtained using standard solutions. The values, expressed in ng of amine per gram of beef extract, are shown in Table 3 and are lower than those previously reported [13] with LC–ES-MS and are compar-

Table 3
Quality parameters of the HAs

Heterocyclic amine	Interval of linearity (ng)	Detection limits		
		Standard solution		Beef extract
		μg ml^{-1} [a]	pg injected	ng g^{-1}
IQ	0.099–22.9	6	1	0.2
MeIQ	0.096–22.2	6	1	0.3
MeIQx	0.089–20.5	94	19	1.1
Glu-P-1	0.094–21.8	107	21	2.3[b]
4,8-DiMeIQx	0.080–18.5	8	5	0.2
PhIP	0.086–19.9	8	2	0.3[b]
Harman	0.11–26.0	4	0.7	0.2
Norharman	0.099–22.9	12	2	0.2[b]
Trp-P-1	0.092–21.1	5	1	0.2[b]
Trp-P-2	0.080–18.4	9	2	0.2
AαC	0.084–19.3	3	0.5	0.1
MeAαC	0.081–18.6	8	2	0.2

[a]200 nl injected.
[b]Value estimated from calibration curve.

able with those obtained in beef extracts using HPLC with electrochemical detection [18].

3.4. Application

The LC–ES-MS method studied in this paper was mainly developed to determine HAs and related compounds in processed food samples. Consequently, after optimization of the chromatographic separation and the mass spectrometry conditions, the method was applied to the determination of HAs in a commercial beef extract. Purification of the sample was performed using the method previously published [15], as described in Section 2. The extracts obtained after the clean-up procedure were analyzed by LC–ES-MS.

The amines PhIP, Trp-P-1 and Glu-P-1, and the co-mutagens Harman and Norharman, were identified in the beef samples (extract B) using an extraction voltage of 100 V and by monitoring the protonated molecules $[M+H]^+$. Chromatograms corresponding to the two extracts obtained in the clean-up procedure are shown in Fig. 5, where it can be observed that in extract A, only the internal standard was identified. Confirmation of the identity of the compounds was carried out by applying a different extraction voltage (150 V) to induce fragmentation and by monitoring the most important fragments for each analyte that was tentatively identified according to Table 2. In Fig. 6, the ion chromatogram corresponding to the confirmation of the five compounds identified is shown.

Good recoveries according to the usual ranges in this kind of sample were obtained for all of the HAs (Table 4), where the amounts found for the identified compounds, ranging from 7.8 to 135.2 ng g^{-1}, are also given.

4. Conclusions

An LC–ES-MS method for the determination of HAs and related compounds in beef extracts has been developed. The method is more sensitive than the usually used HPLC–UV method, and gives results similar to those obtained using HPLC with electrochemical detection, however, it has the advantage of being more stable than the latter. Furthermore, it is a selective and highly specific technique and can be considered as the best on-line identification method, which is an important requisite when working in the analysis of HAs in processed food samples. The use of in-source fragmentation provided an easier and less expensive technique than MS–MS for obtaining structural information. The method allowed the simultaneous determination of

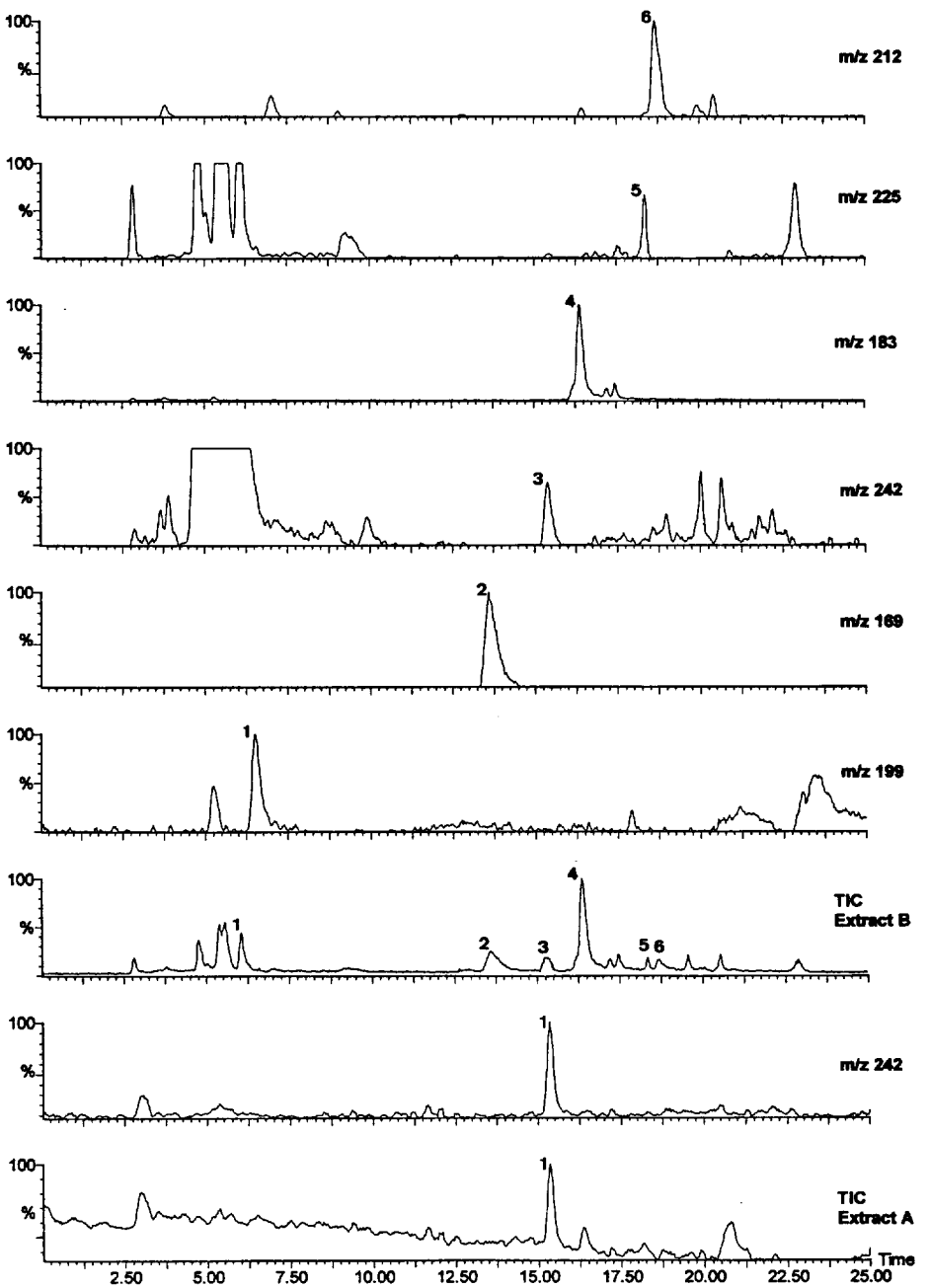


Fig. 5. LC–MS analysis of a beef extract sample. The bottom trace of each extract is the TIC obtained by summing all of the ions above. Peaks: Extract A: 1=TriMeIQx; Extract B: 1=Glu-P-1; 2=Norharman; 3=TriMeIQx; 4=Harman; 5=PhIP and 6=AαC.

fourteen HAs and related compounds in highly complex matrices such as the beef extract analyzed, giving chromatograms that were almost free from interferences, providing the identification and quantification of HAs and related compounds at 0.1 ng g^{-1} levels. The method is judged to be generally applic-

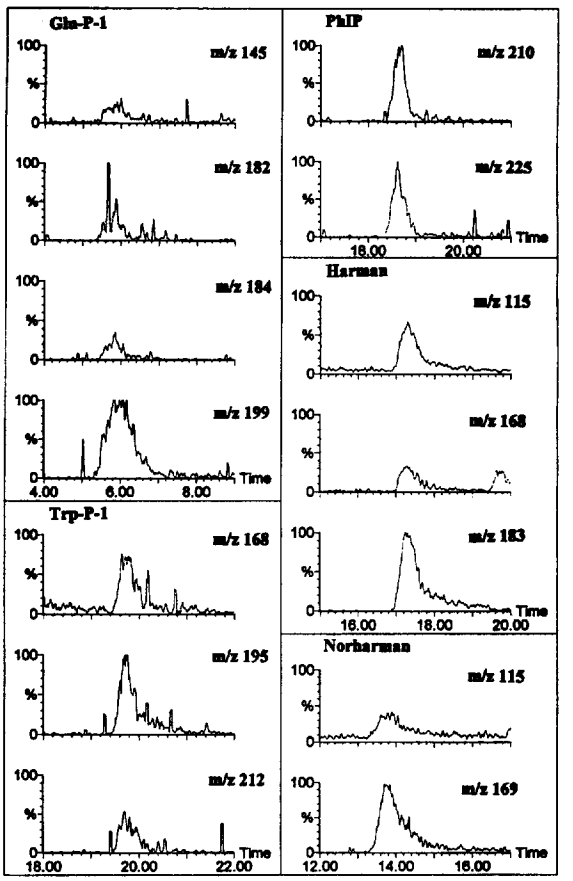


Fig. 6. LC–MS confirmation of a beef extract sample (Extract B) using 150 V as the extraction voltage.

Table 4
Analysis of a beef extract

Analyte	Recovery (%)	Concentration (ng g^{-1})
IQ	74±3	ND
MeIQ	80±8	ND
MeIQx	82±9	ND
4,8-DiMeIQx	89±4	ND
Glu-P-1	79±13	8±1
Trp-P-1	76±12	13±2
Trp-P-2	61±7	ND
PhIP	54±5	10±1
Harman	61±9	135±12
Norharman	105±9	61±7
AαC	60±7	ND
MeAαC	59±8	ND

ND: Not detected.

able after the usual sample treatment for the detection of mutagens at the low parts per billion in cooked foods.

Acknowledgments

Dra. Irene Fernández from the Servei d'Espectrometria de Masses of the University of Barcelona is gratefully acknowledged for LC–MS technical support and laboratory assistance. This work was financed by the C.I.C.Y.T. to research project number ALI96-0863.

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