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Liquid chromatography–atmospheric-pressure chemical ionization mass spectrometry as a routine method for the analysis of mutagenic amines in beef extracts

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Abstract

A liquid chromatography–mass spectrometry (LC–MS) method using atmospheric-pressure chemical ionisation as interface was developed for the simultaneous determination of 14 heterocyclic aromatic amines and related compounds in beef extracts. The separation was performed on a conventional C_{18} column using a binary mobile phase composed of acetonitrile and 50 m*M* ammonium acetate at pH 5.7, and elution was carried out in gradient mode. Several parameters influencing the mass spectra were optimized, and the effect of the variation of cone voltage on the mass spectra was studied. The $[M+H]^+$ ions and some fragments produced in the source were observed in the mass spectra when several extraction voltages were applied. Quality parameters (run-to-run and day-to-day reproducibility, intervals of linearity, and limits of detection) were studied in the optimum working conditions. The method was used to analyze the heterocyclic amines present in a commercial beef extract. Therefore, a solid-phase extraction clean-up procedure was performed prior the LC–MS analysis due to the complexity of the sample and the compounds Glu-P-1, Harman, Norharman and AαC were identified in the samples at ppb levels and successfully confirmed using in-source fragmentation. © 1997 Elsevier Science B.V.

Keywords: Food analysis; Amines; Heterocyclic amines

1. Introduction

There is now extensive evidence linking dietary factors to the occurrence of certain forms of human cancer [1]. For that reason, much effort has been devoted to the development of analytical methods to identify and measure mutagenic compounds in foods. Over the past 15 years a number of potent bacterial mutagens and/or carcinogens, all belonging to the class of heterocyclic amines (HAs) have been obtained from pyrolyzed amino acids, proteins, and cooked protein-rich foods. These compounds have been found in meats such as beef and chicken, and fish cooked by typical domestic methods [2].

The determination of HAs in processed foods at ppb levels has been carried out using chromatographic techniques, and because of the complexity of the matrices analyzed, all the procedures include several clean-up steps before chromatographic analysis. High-performance liquid chromatography (HPLC) is the most common method of analysis [3–6], nevertheless, peak confirmation is a crucial problem when working with such low levels of HAs since co-elution with other co-extracted compounds occurs. The most widely used instrument to rule-out co-eluting interference is the UV photodiode array detector, which efficiently prevents false peak identification. For identification purposes, mass spectrometry (MS) in conjunction with chromatographic techniques is a good on-line system due to its high

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selectivity and specificity. Gas chromatography–mass spectrometry (GC–MS) has been used in the analysis of some HAs in processed foods [7,8] but it requires a derivatization step and it has been applied for the determination of only four HAs. Liquid chromatography (LC)–MS has developed into a mature technique, which is routinely applied in a large number of areas, e.g., environmental and bioanalysis, natural products and biochemistry-related applications. At present, the most powerful and promising interfaces are those that can be used in combination with atmospheric-pressure ionisation, i.e., electrospray (ES) and atmospheric-pressure chemical ionisation (APCI). The ES interface has been applied to the analysis of HAs using microbore chromatographic columns [9–11] and good results have been achieved. In a recent work, Richling et al. have studied up to sixteen HAs and related compounds by LC–ES-MS–MS [10]. The thermospray (TS) interface has also been applied for the determination of five HAs in processed foods [12–14] but it has been replaced by APCI because it is more sensitive [15], suggesting that LC–APCI-MS may be an excellent method for the determination of HAs.

In the present study, LC–APCI-MS has been used for the determination of the fourteen most abundant HAs in processed foods. The separation of the analytes has been performed in a C_{18} column using a suitable mobile phase compatible with the mass spectrometer. The mass spectra have been studied, optimizing all the parameters influencing the ion formation. Reproducibility, repeatibility, linearity, and limits of detection were studied in order to establish the quality parameters of the method. Another objective of the study was to demonstrate the applicability of LC–APCI-MS as a routine method for the determination of HAs in processed foods, so the procedure was applied to the determination of HAs in a commercial beef extract.

2. Experimental

2.1. Chemicals

The amines studied were obtained from the following sources: 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), from Toronto Research Chemicals (Toronto, Canada); 1-methyl-9H-pyrido[4,3-*b*]indole (Harman) and 9H-pyrido[4,3-*b*]indole (Norharman) from Aldrich (Steinheim, Germany); and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) and 2-amino-3,4,7,8-tetramethylimidazo [4,5-*f*]quinoxaline (TriMeIQx) from Toronto Research Chemicals; they were used as possible internal standards. Stock standard solutions of 100 $\mu g\ ml^{-1}$ in methanol were prepared and used for further dilutions. Solutions of internal standard of 1 $\mu g\ ml^{-1}$ in methanol were used to dissolve sample extracts. 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 International (ICT, Basle, Switzerland).

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

2.2. Instruments

MS was performed using a VG Platform (Fisons Instruments, VG Biotech, Altrincham, UK) quadrupole mass spectrometer equipped with an APCI interface, which was assisted pneumatically with nitrogen at a flow-rate of 50–250 $l\ h^{-1}$. Drying nitrogen was heated to 80–150°C and introduced into the capillary region at a flow-rate of 100–500 $l\ h^{-1}$. The capillary was heated to 100–500°C. The corone voltage was held at +2.5–3.5 kV for the positive

mode. The cone voltage was applied, ranging from 10 V to 70 V.

For data acquisition in full-scan mode, the mass spectrometer operated over a range of *m/z* 50–300 in the centroid mode at a cycle time of 1.00 s and at interscan time of 0.10 s. In multiple ion detection (MID), the mass corresponding to $[M+H]^+$ ions were used. The acquisition and data procesing were performed with MassLynx software. The calibration of the mass spectrometer was carried out using a standard solution of HAs in mobile phase.

LC was performed using a quaternary pump Hewlett–Packard (Palo Alto, CA, USA) Series 1050. An automatic injector Hewlett–Packard Series 1050 was used to introduce 15 μl of sample. Separations were performed using a TSK-Gel ODS 80T (5-mm particle size, 25.0-cm×4.6-mm I.D.) column (Toso Haas, Stuttgart, Germany) and a Supelguard LC-8-DB precolumn (Supelco, Bellefonte, PA, USA). A mixture of 50 m*M* ammonium acetate at pH 5.7 and acetonitrile was used as mobile phase at a flow-rate of 1 ml/min in gradient mode. Separations were carried out at room temperature. The optimization of the separation of the HAs was performed using a Hewlett–Packard UV detector Series 1050 (263 nm as operating wavelength).

2.3. Analytical procedure

Sample preparation and clean-up was performed following the procedure previously reported [16]. The sample dissolved in sodium hydroxide was introduced in a diatomaceous earth cartridge (Extrelut) and eluted with dichloromethane by coupling to a PRS cartridge. After washing the PRS, two fractions were obtained: the fraction eluted in the washing step, which contained the imidazopyridine and indolpyridine derivatives, and that retained in the PRS containing the aminoimidazoquinoxalines and quinolines. Both fractions were collected and concentrated using C_{18} cartridges. Finally, the HAs retained in both C_{18} cartridges were eluted (methanol–ammonia) to give two final extracts: extract A containing the aminoimidazoquinoxalines and quinolines (IQ, MeIQ, MeIQx and 4,8-DiMeIQx), and extract B the imidazopyridine and indolpyridine derivatives (Glu-P-1, PhIP, Trp-P-1, Trp-P-2, Harman, Norharman, AαC and MeAαC). Each extract was evaporated to dryness under a stream of nitrogen and redissolved in a methanolic internal standard (I.S.) solution. Both extracts were analyzed by LC–MS a gradient elution using 50 m*M* ammonium acetate pH 5.7–acetonitrile (80:20, v/v) (solvent A) and acetonitrile (solvent B) was applied: 10 min isocratic conditions: 100% A; 10–30 min: gradient elution to 60% B; re-equilibration for 15 min: 100% A. Quantification of HAs in the beef extract was carried out by the standard addition method. The LC–MS measurements were performed by MID of the protonated molecular ions for each mutagen, using a dwell time of 100 ms: 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*; 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. LC–MS

The chromatographic separation of the HAs was performed in a C_{18} column using a mobile phase chosen according to the restrictions of APCI interface, which only tolerates volatile salts.

APCI involves a mild ionisation process that readily provides unfragmented quasi-molecular ions, which give information about the molecular mass. The information obtained from in-source fragmentation made it possible to use APCI-MS for confirmation purposes. HAs gave proton addition to form $[M+H]^+$ as the base peak in the positive-ion mode, the same as in ES ionisation [9–11]. In order to optimize the MS response several extraction voltatges were tested.

Various parameters influencing mass spectra were investigated: the drying and auxiliary nitrogen flow-rates, source and capillary temperature, and focus and corone voltages in order to study the ionisation and characterization of the HAs. The optimal results were obtained at a sheath gas (N_2) flow-rate of 100 $l\,h^{-1}$. Drying nitrogen was heated to 100°C and introduced into the capillary region at a flow-rate of 400 $l\,h^{-1}$. The capillary was heated to 450°C, and

the corone voltage was held at +2.5 kV for the positive mode.

In order to characterise these compounds by APCI-MS standard solutions of 10 μg ml^{-1} of the 14 HAs were injected at different extraction voltages under the LC final working conditions. Fig. 1 shows the relative intensity of $[M+H]^+$ vs. extraction voltage, the highest responses for most of these compounds were obtained at 30 V. So, for maximum sensitivity, 30 V was chosen for quantification purposes. Proton addition and sodium addition were the common route of ionisation at low extraction voltages (Table 1). More fragmentation was observed (Table 1) when the extraction voltage was increased from 10 to 70 V, and the fragmentation was always higher than in ES [9–11]. For almost all the compounds, a minimum was observed at 40 V, followed by an increase at 50 V. At this point fragmentation of

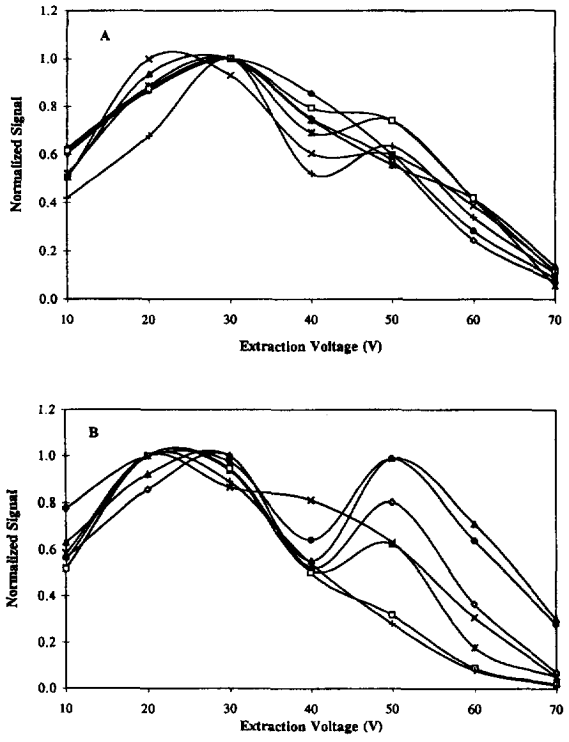

Fig. 1. Variation of the response of the molecular protonated ion of each HA vs. the extraction voltage. (A) (◇) IQ, (●) MeIQ, (▲) MeIQx, (×) 4,8-DiMeIQx, (*) 7,8-DiMeIQx, (□) TriMeIQx and (+) PhIP; (B) (◇) Glu-P-1, (●) harman, (▲) norharman, (×) Trp-P-2, (*) Trp-P-1, (□) AαC and (+) MeAαC.

$[M+H]^+$ ions began, reducing its abundance. The $[M+Na]^+$ ions gave only a maximum at 30 V, as can be seen in Fig. 2 where as an example, the responses for PhIP and 7,8-DiMeIQx are given. These profiles can be attributed to focalization and dissociation effects. $[M+H]^+$ ions suffered also focalization effects which would explain the profile observed at potentials lower than 40 V. In order to explain the increase in $[M+H]^+$ abundance at higher voltages the presence of adducts has to be considered. The gas phase in APCI can contain cluster ions from the interaction of the analyte with the mobile phase (H_2O, CH_3CN, NH_4^+ and CH_3COO^-). As can be seen in Table 1, several ions with acetonitrile are clearly observed in the mass scan tested. An extraction voltage of 40 V did not produce fragments of $[M+H]^+$, but it seemed to be high enough to dissociate the adducts with mobile phase ($[M+H+(CH_3CN)_n]^+$, $[M+H+(H_2O)_m]^+$). These dissociations may provide the increase of the $[M+H]^+$ ion abundance at 50 V.

The aminoimidazoquinaxolines (MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, TriMeIQx) gave sodium addition. Moreover, clusters with acetonitrile were also present in most of the spectra. As a representative example, Fig. 3 shows the spectra for MeIQx and TriMeIQx at 30 V and 70 V. These compounds and aminoimidazoquinolines (IQ and MeIQ) presented a similar pattern; the loss of CH_3 and the fragmentation of the aminoimidazyl group [–HCN and $-CH_3-(HCN)_2$] gave the main fragments. Moreover, the aminoimidazoquinoxalines gave additional fragments by breaking the quinoxaline group; for instance MeIQx gave *m/z* 131 [$-CH_3-(HCN)_2-N$] as can be seen in Fig. 4.

The aminopyridoindoles (Trp-P-1, Trp-P-2, AαC and MeAαC) gave the loss of the amino and the methyl groups as main fragmentation. Moreover, the stepwise elimination of different units of HCN from $[M+H]^+$, $[M+H\text{-}NH_3]^+$ or $[M+H\text{-}CH_3]^{+\cdot}$ ions were also present in their spectra. For these compounds, except for Trp-P-1, the loss of three hydrogens was observed, for example, for AαC this fragment (*m/z* 181) was the base peak as can be seen in Fig. 4A.

Besides the typical fragment ion at *m/z* 115 due to the pyridyl group, harman and norharman also lost one unit of HCN. In addition, harman gave a

Table 1
Main ions obtained at extraction voltages of 30 V and 70 V with their tentative assignations and relative abundance

Compound	M_r	m/z	Tentative assignation	Relative intensity (%)	
				30 V	70 V
IQ	198	225	$[M+H-CH_3+CH_3CN]^{+\cdot}$	–	19.6
		199	$[M+H]^+$	100.0	7.2
		184	$[M+H-CH_3]^{+\cdot}$	–	100.0
		157	$[M+H-CH_3HCN]^{+\cdot}$	–	34.8
		131	$[M+H-CH_3HCN-CN]^+$	–	8.0
MeIQ	212	213	$[M+H]^+$	100.0	21.4
		198	$[M+H-CH_3]^{+\cdot}$	–	100.0
		197	$[M+H-CH_4]^+$	–	27.1
		171	$[M+H-CH_3-HCN]^+$	–	5.0
		145	$[M+H-CH_3-HCN-CN]^+$	–	8.6
MeIQx	213	277	$[M+Na+CH_3CN]^+$	27.5	–
		255	$[M+H+CH_3CN]^+$	2.9	–
		240	$[M+H-CH_3+CH_3CN]^{+\cdot}$	–	10.2
		236	$[M+Na]^+$	19.6	–
		214	$[M+H]^+$	100.0	50.0
		199	$[M+H-CH_3]^{+\cdot}$	–	100.0
		172	$[M+H-CH_3-HCN]^+$	–	32.1
		146	$[M+H-CH_3-HCN-CN]^+$	–	28.6
		131	$[M+M-CH_3-HCN-CN-HN]^+$	–	14.3
Glu-P-1	198	199	$[M+H]^+$	100.0	96.4
		184	$[M+H-CH_3]^{+\cdot}$	–	45.0
		182	$[M+H-NH_3]^+$	–	37.1
		172	$[M+H-HCN]^+$	–	58.6
		155	$[M+H-NH_3-HCN]^+$	–	17.6
		145	$[M+H-(HCN)_2]^+$	–	53.6
		110	$[C_5H_8N_3]^+$	–	100.0
		92	$[C_6H_6N]^+$	–	50.0
4,8-DiMeIQx	227	291	$[M+Na+CH_3CN]^+$	30.0	–
		269	$[M+H+CH_3CN]^+$	2.9	–
		250	$[M+Na]^+$	22.8	–
		228	$[M+H]^+$	100.0	63.6
		213	$[M+H-CH_3]^{+\cdot}$	–	100.0
		212	$[M+H-CH_4]^+$	–	60.0
		201	$[M+H-HCN]^+$	–	14.3
		187	$[M+H-CH_3-CN]^+$	–	50.0
		160	$[M+H-CH_3-CN-HCN]^+$	–	57.8
TriMeIQx	241	264	$[M+Na]^+$	20	4.3
		242	$[M+H]^+$	100	12.1
		227	$[M+H-CH_3]^+$	–	100.0
		201	$[M+H-CH_3-CN]^+$	–	30.0
		185	$[M+H-(CH_3)_2-HCN]^+$	–	20.0
		174	$[M+H-CH_3-HCN-CN]^+$	–	31.4
		160	$[M+H-C_3H_4N_3]^{+\cdot}$	–	6.4
		145	$[M+H-C_5H_9N_2]^{+\cdot}$	–	16.4
7,8-DiMeIQx	227	291	$[M+Na+CH_3CN]^+$	20.0	12.1
		250	$[M+Na]^+$	17.1	17.1
		228	$[M+H]^+$	100.0	100
		199	$[M+H-CH_3-N]^+$	–	12.1

(continued on p. 212)

Table 1 (*continued*)

Compound	M_r	*m/z*	Tentative assignation	Relative intensity (%)	
				30 V	70 V
PhIP	224	225	$[M+H]^+$	100.0	43.6
		210	$[M+H\text{-}CH_3]^{+\cdot}$	–	100.0
		183	$[M+H\text{-}CH_3\text{-}HCN]^{+\cdot}$	–	10.7
		168	$[M+H\text{-}NH_3\text{-}CN_2]^{+\cdot}$	–	10.8
		157	$[M+H\text{-}CH_3\text{-}HCN\text{-}CN]^+$	–	9.3
		143	$[M+H\text{-}C_3H_4N_3]^{+\cdot}$	–	7.1
		130	$[M+H\text{-}C_4H_5N_3]^+$	–	6.4
		115	$[M+H\text{-}C_4H_6N_4]^+$	–	5.0
Harman	182	224	$[M+H+CH_3CN]^+$	2.9	–
		183	$[M+H]^+$	100.0	71.4
		168	$[M+H\text{-}CH_3]^{+\cdot}$	–	21.4
		156	$[M+H\text{-}HCN]^+$ or $[M+H+CH_3CN\text{-}C_4H_6N]^{+\cdot}$	–	26.4
		142	$[M+H\text{-}CH_3\text{-}CN]^+$	–	12.8
		115	$[M+H\text{-}C_4H_6N]^{+\cdot}$	–	100.0
Norharman	168	210	$[M+H+CH_3CN]^+$	6.5	–
		169	$[M+H]^+$	100.0	100.0
		156	$[M+H\text{-}C_3H_4N+CH_3CN]^{+\cdot}$	–	25.7
		142	$[M+H\text{-}HCN]^+$	–	14.3
		115	$[M+H\text{-}C_3H_4N]^{+\cdot}$	95.7	
Trp-P-1	211	212	$[M+H]^+$	100.0	18.6
		195	$[M+H\text{-}NH_3]^+$	–	77.8
		180	$[M+H\text{-}NH_3\text{-}CH_3]^{+\cdot}$	–	23.6
		168	$[M+H\text{-}NH_3\text{-}HCN]^+$	–	95.7
		167	$[M+H\text{-}NH_3\text{-}H_2CN]^{+\cdot}$	–	100.0
		141	$[M+H\text{-}C_3H_7N_2]^{+\cdot}$	–	22.1
		115	$[M+H\text{-}C_5H_9N_2]^{+\cdot}$	–	7.1
Trp-P-2	197	198	$[M+H]^+$	100.0	15.7
		195	$[M+H\text{-}H_3]^{+\cdot}$	–	18.6
		181	$[M+H\text{-}NH_3]^+$	–	45.0
		154	$[M+H\text{-}NH_3\text{-}HCN]^{+\cdot}$	–	100.0
		130	$[M+H\text{-}CH_3\text{-}HCN\text{-}CN]^{+\cdot}$	–	17.8
		117	$[M+H\text{-}C_4H_5N_2]^{+\cdot}$	–	7.9
AαC	183	225	$[M+H+CH_3CN]^+$	22.8	5.7
		208	$[M+H+CH_3CN\text{-}NH_3]^+$	–	30.7
		184	$[M+H]^+$	100.0	12.9
		181	$[M+H\text{-}H_3]^{+\cdot}$	–	100.0
		167	$[M+H\text{-}NH_3]^+$	–	57.1
		140	$[M+H\text{-}CH_4N_2]^{+\cdot}$	–	82.5
MeAαC	197	239	$[M+H+CH_3CN]^+$	21.4	–
		198	$[M+H]^+$	100.0	11.6
		195	$[M+H\text{-}H_3]^{+\cdot}$	–	11.5
		183	$[M+H\text{-}CH_3]^{+\cdot}$	–	63.0
		181	$[M+H\text{-}NH_3]^{+\cdot}$	–	97.1
		168	$[M+H\text{-}CH_4N]^{+\cdot}$	–	49.3
		154	$[M+H\text{-}CH_4N_2]^+$	–	99.8
		129	$[M+H\text{-}CH_3\text{-}(HCN)_2]^{+\cdot}$	–	99.3
		128	$[M+H\text{-}CH_4\text{-}(HCN)_2]^+$	–	100.0
		117	$[M+H\text{-}C_4H_5N_2]^{+\cdot}$	–	8.7

Fragment ions corresponding to relative intensity>5%.

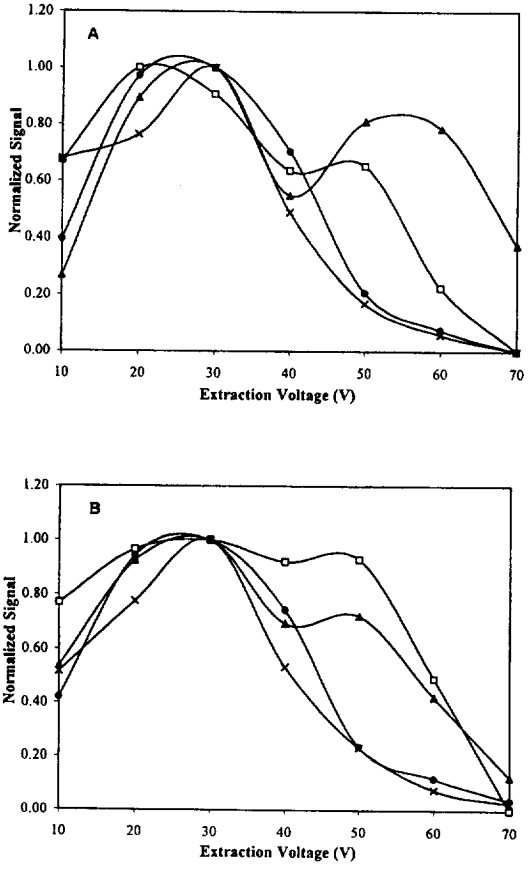

Fig. 2. Variation of the response of the (▲) $[M+H]^+$, (●) $[M+Na]^+$, (□) $[M+H+CH_3CN]^+$ and (×) $[M+Na+CH_3CN]^+$ ion of PhIP (A) and 7,8-DiMeIQx (B) vs. the extraction voltage.

fragment ion at *m/z* 168 due to the loss of CH_3 as can be seen in Fig. 4B.

The APCI spectra at 70 V of PhIP (Fig. 4C) showed the protonated molecular ion $[M+H]^+$ at *m/z* 225 and the loss of CH_3 at *m/z* 210. Also fragment ions originated by losses of the amino group and different units of HCN were observed. For Glu-P-1 (Fig. 4D) the losses of the CH_3, NH_3 and some stepwise elimination of HCN were given at 70 V. Moreover, the most important ions were $[M+H]^+$ and those corresponding to the breaking of the imidazole ring (*m/z* 110 and *m/z* 92).

A chromatogram for each mass and the total ion chromatogram (TIC) performed with the final gradient elution is given in Fig. 5. This mobile phase separated 4,8-DiMeIQx and MeIQ, Norharman and AαC, and TriMeIQx and Glu-P-1 with a low resolution, and IQ and MeIQx co-eluted, but due to the high selectivity and specifity of MS, the low resolution and co-elution of HAs is compensated by selecting unambiguous masses to monitor.

3.2. Quality parameters

Precision, linearity and limits of detection were determined with standard solutions in mobile phase. Calibrations for the HAs were performed with concentrations in the range of 0.01 and 2 mg ml^{-1}. The calibration curves were calculated from the representation of the peak area of the HAs in relation to the peak area of the internal standard (TriMeIQx) vs. the content of each HA (Ci) in relation to the content of internal standard (Cis). The correlation coefficients of these calibrations in the intervals of linearity were better than 0.995 for all the HAs. In Table 2 the values corresponding to the intervals of linearity are given.

Ten replicate determinations on the same day of 0.5 mg ml^{-1} of each HAs in mobile phase solution were carried out under the optimal conditions to the determine the run-to-run precision of the LC–MS analysis. Moreover, five injections performed on three different days of that solution were carried out in order to establish the day-to-day precision of the chromatographic analysis. Relative standard deviations (Table 2) based on Ci/Cis ratios for the run-to-run precision ranged between 3.3 and 4.6, and for day-to-day precision between 5.6 and 7.9. Values corresponding to peak area are comparable with those obtained from peak height; therefore, for further studies the response will be corresponded to the peak area.

Detection limits for the HAs in MID for standard solutions based on a signal-to-noise ration of 3:1 ranged from 10.7 to 671 pg, and are listed in Table 2. The values corresponding to full-scan were at least 20 times higher. Detection limits in MID are similar to those obtained in an ES interface [10,11], except for harman and norharman, which are 5 and 16 times lower, respectively. The values are comparable to those obtained by HPLC with electrochemical detection for the determination of HAs [5,6,17,18], and more sensitive than the HPLC–UV methods [19]. Moreover, detection limits without the chromato-

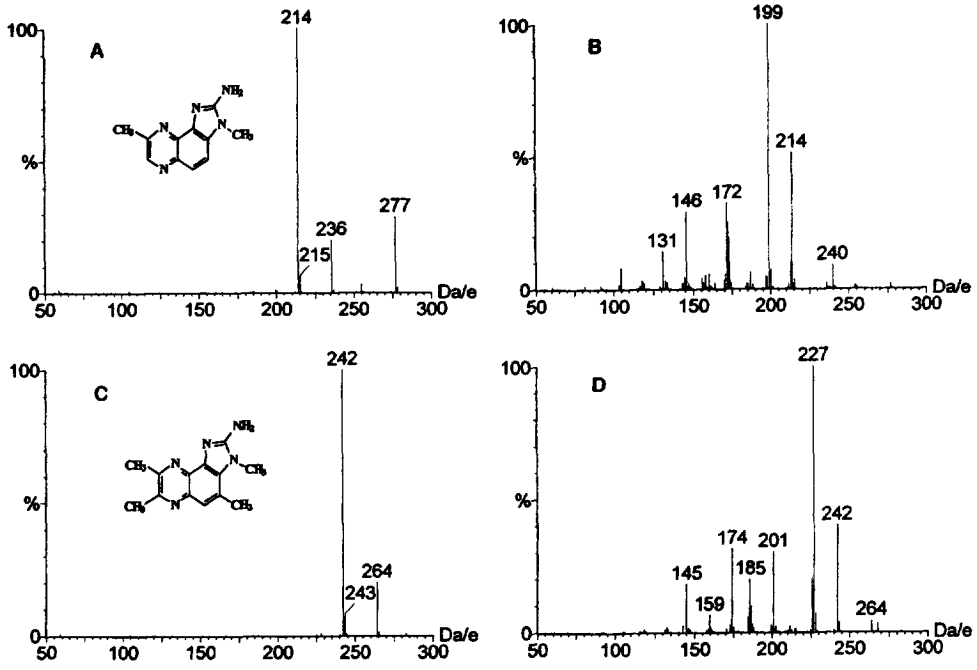

Fig. 3. APCI mass spectra obtained at 30 and 70 V of (A) MeIQx and (B) TriMeIQx.

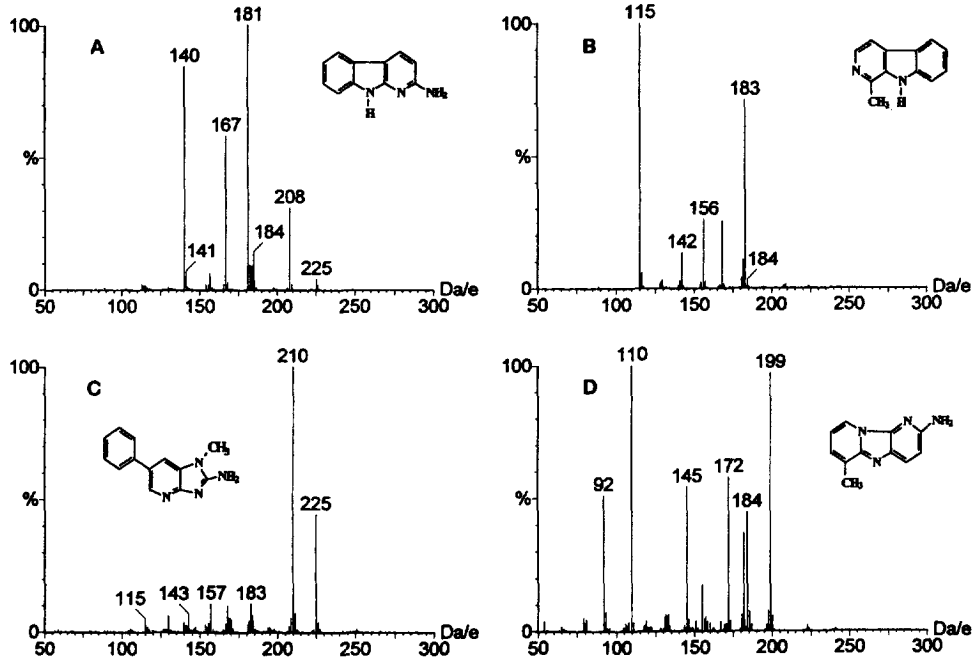

Fig. 4. APCI mass spectra obtained at 70 V of (A) AαC, (B) harman, (C) PhIP and (D) Glu-P-1.

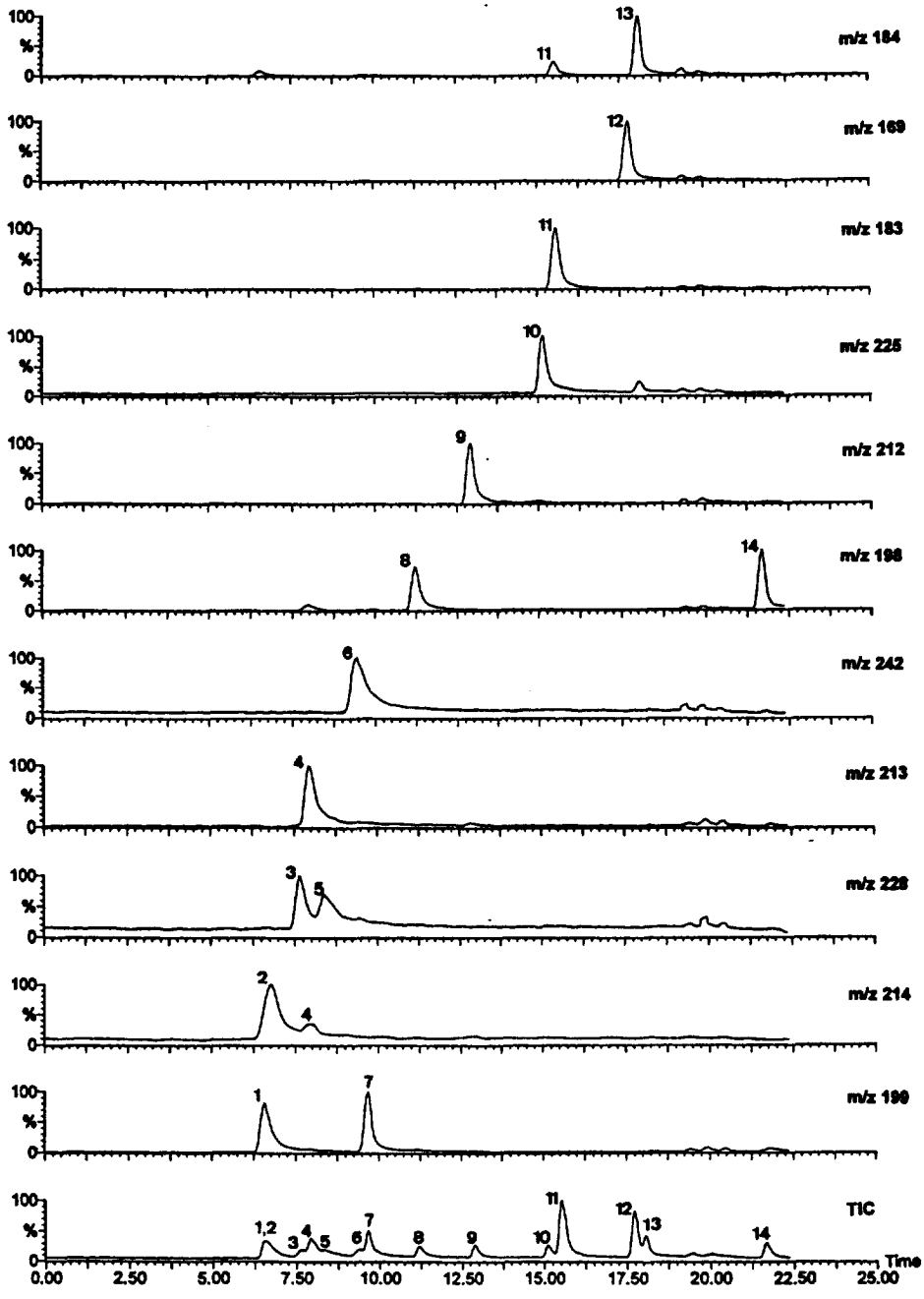

Fig. 5. LC–MS with positive APCI ionization at 30 V of HAs. The bottom trace is the TIC obtained by summing all ions above. Peaks: 1. IQ; 2. MeIQx; 3. 7,8-DiMeIQx; 4. MeIQ; 5. 4,8-DiMeIQx; 6. TriMeIQx; 7. Glu-P-1; 8. Trp-P-2; 9. Trp-P-1; 10. PhIP; 11. harman; 12. norharman; 13. AαC; 14. MeAαC.

Table 2
Quality parameters

Heterocyclic amine	Linearity interval (ng)	Precision				Detection limits				
		Run-to-run		Day-to-day		Standard solution		Standard solution		Beef extract ng g^{-1}
		R.S.D. (%)		R.S.D. (%)		With column		Without column		
		Area	Height	Area	Height	ng ml^{-1}	pg	ng ml^{-1}	pg	
IQ	0.099–22.9	3.7	3.5	6.0	6.1	6.9	103.7	0.17	2.5	0.2
MeIQ	0.096–22.2	3.9	4.4	7.0	7.1	6.7	100.5	0.61	9.1	0.2
MeIQx	0.089–20.5	4.6	4.5	6.7	6.5	18.6	279.1	1.1	16.9	1.0
Glu-P-1	0.094–21.8	4.2	4.1	7.1	7.4	2.6	39.5	0.071	1.1	0.4[a]
4,8-DiMeIQx	0.080–18.5	3.3	3.9	7.2	7.9	44.7	671.0	6.7	100.6	1.4
PhIP	0.086–19.9	3.6	3.5	6.6	6.5	12.0	180.1	1.1	16.4	0.4
Harman	0.11–26.0	3.3	3.6	6.0	6.1	0.71	10.7	0.71	10.7	0.08[a]
Norharman	0.099–22.9	3.3	3.9	5.6	5.7	0.74	11.2	0.74	11.2	0.1[a]
Trp-P-1	0.092–21.1	4.1	4.1	6.6	6.3	2.5	38.2	0.58	8.7	0.4
Trp-P-2	0.080–18.4	4.1	4.0	6.8	7.3	5.5	83.2	0.65	9.7	0.8
AαC	0.084–19.3	4.0	4.0	5.8	5.9	5.8	87.7	0.53	8.0	0.3[a]
MeAαC	0.081–18.6	3.9	4.4	7.0	7.1	5.6	84.3	0.67	10.1	0.4

[a] Value obtained from the calibration curve.

graphic column were investigated by flow injection analysis (FIA) (Table 2), and found to be between 5 and 40 times lower than those obtained with LC column, except for harman and norharman, which gave the same value. The decrease in sensitivity for the amines compared with the co-mutagens harman and norharman can be related to the decrease in HAs chromatographic efficiency due to interactions between the amino groups and silanol groups of the stationary phase.

Detection limits in real samples are always higher than those obtained for standard solutions, because of the influence of the matrix of the sample. Detection limits based on a signal-to-noise ratio of 3:1 were calculated by spiking the beef extract at low concentration levels (0.02–5 n g^{-1}) with reference standards. Detection limits of the HAs already present in the samples were estimated from calibration curves taking into account the recovery values. The values obtained are given in Table 2 and ranged from 0.08 to 1.4 ng g^{-1}. These results are comparable with those obtained by HPLC with electrochemical detection for the determination of aminoimidazoquinoxalines and quinolines in beef extracts [20].

3.3. Analysis of beef extract

After optimizing the LC–APCI-MS conditions and studying the figures of merit, the second objective of this work was demonstrating the applicability of the method to the analysis of processed food samples. Consequently, a commercial beef extract was analyzed by LC–APCI-MS method after applying the clean-up procedure described in the Section 2. A chromatogram corresponding to extract A, which could contain the amines IQ, MeIQ, MeIQx and 4,8-DiMeIQx, and another of extract B, which could contain Trp-P-1, Trp-P-2, Glu-P-1, PhIP, harman, norharman, AαC and MeAαC are given in Fig. 6. The chromatograms are almost free of interfering peaks due to the high selectivity and specificity of the mass spectrometry technique.

The compounds Glu-P-1, harman, norharman and AαC were detected and quantified by the standard addition method. The results are presented in Table 3, together with the recovery values for each amine. These recoveries are similar to those obtained previously [16] for a commercial beef extract. These peaks were confirmed by applying a higher extraction voltage (70 V) to induce fragmentation and

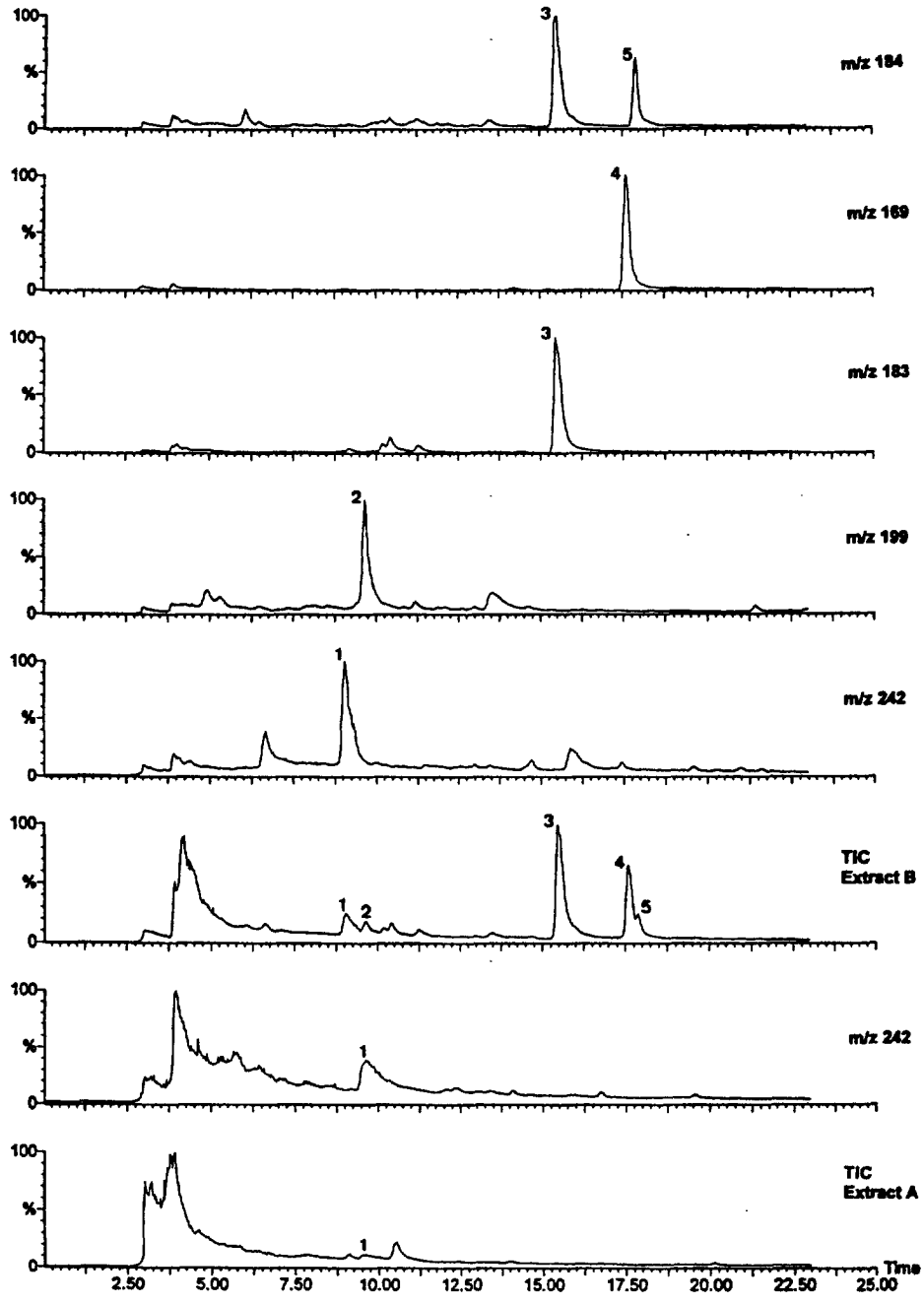

Fig. 6. LC–MS analysis of a beef extract sample. The bottom trace of each extract is the TIC obtained by summing all ions above. Peaks: Extract A: 1. TriMeIQx; Extract B: 1. TriMeIQx; 2. Glu-P-1; 3. harman; 4. norharman; 5. AαC.

Table 3
Analysis of a beef extract

Analyte	Recovery (%)	Concentration (ng g^{-1})
IQ	72.2±3.1	ND
MeIQ	67.7±7.5	ND
MeIQx	83.1±10.6	ND
4,8-DiMeIQx	84.9±9.7	ND
Glu-P-1	74.0±7.0	10.1±1.1
Trp-P-1	64.4±6.6	ND
Trp-P-2	73.4±3.4	ND
PhIP	50.3±6.8	ND
Harman	68.2±8.8	129.5±16.8
Norharman	82.5±5.9	74.0±7.4
AαC	58.4±2.9	2.8±0.38
MeAαC	61.5±7.7	ND

ND: Not detected.

monitoring the most important fragments for each analite according with Table 1.

4. Conclusions

The addition of protons to form $[M+H]^+$ ions, and for some HAs, sodium addition to form lower abundant ions of $[M+Na]^+$ were the major route of fragmentation of the HAs using APCI-MS. Furthermore, several fragments produced in the source were observed in the mass spectra by applying different extraction voltages. The determination of mutagenic heterocyclic amines using an LC–APCI-MS technique has been successfully achieved with detection limits comparable to those obtained with electochemical detection. The sensitivity, selectivity and specificity of the method offers a new tool for the analysis of HAs in complex matrices of processed food samples using a benchtop instrument and allowing the use of conventional LC columns. The use of in-source fragmentation provided an easier and less expensive technique than MS–MS for the confirmation of the identity of the compounds.

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