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Determination of heterocyclic amines by pneumatically assisted electrospray liquid chromatography–mass spectrometry

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Abstract

Electrospray ionization mass spectrometry was applied to the study of the amines IQ, Trp-P-1, Trp-P-2, PhIP and AαC and the co-mutagens harman and norharman. The results obtained on a triple quadrupole mass spectrometer equipped with a pneumatically assisted electrospray source are reported. The chromatographic conditions were optimized with a reversed-phase column (1 mm I.D.) using acetonitrile–5 m*M* ammonium acetate (pH 6.7) (50:50) as the mobile phase at a flow-rate of 50 μl min^{-1}. Different parameters influencing the mass spectra were investigated. For these compounds $[M + H]^+$ in the positive-ion mode and also some fragments produced through collisionally activated decomposition in the interface were observed. Detection limits of 5.4–44 pg were obtained for standard solutions of these amines. Analysis of a meat extract was performed by HPLC–MS using single-ion monitoring after a solid-phase extraction clean-up.

Keywords: Liquid chromatography–mass spectrometry; Carcinogens; Mutagens; Interfaces, LC–MS; Electrospray ionization; Amines, heterocyclic

1. Introduction

Major causative factors in the development of human cancer are chemicals, ionizing radiation, viruses and oxygen radicals formed in situ. Epidemiological studies have revealed that many cases of cancer depend on lifestyle [1]. Dietary habits and cigarette smoking are two dominant factors and together could cause about two-thirds of human cancer. It has also been pointed out that diet and nutrition are closely related to cancer development [2]. Various types of carcinogens are present in foods as minor contaminating components, most of which show mutagenic activity using the Ames test [3]. Much effort has been expended on the identification and measurement of these mutagenic compounds in foods. Some of them occur naturally or as a result of the action of microorganisms and others, which have been found in fish and meat products, are believed to be formed during cooking processes by pyrolysis of amino acids and proteins. These mutagens are identified as heterocyclic amines (HAs) and are present at ng g^{-1} concentration levels. The isolation of these HAs from highly complex fractions containing many pyrolysis products requires efficient clean-up procedures.

The determination of HAs is performed using different techniques, mainly chromatographic:

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HPLC–UV and HPLC–fluorescence [4,5], HPLC–electrochemical detection [6–8], HPLC–MS [9–11] or GC–MS [12,13]. The sample matrix greatly influences the clean-up procedures and many peaks with the same retention times as those of HAs are often present in the chromatograms of real samples. Hence, peak confirmation is an important requisite in order to rule out co-eluting interferences, which is difficult when working with low levels of compounds such as those of HAs. The most accessible and widely used procedure to identify HAs in food samples is photodiode-array UV detection, which efficiently prevents false peak identification. Moreover, mass spectrometry in conjunction with chromatographic techniques is one of the best on-line identification systems, because of its selectivity and relatively high sensitivity. GC–MS has been used to identify and quantify some heterocyclic amines [12,13] but it requires derivatization before the GC analysis.

During the last decade, improvements in LC–MS coupling have made it possible to apply this technique to the detection and quantification of amines. Few data are available on the LC–MS analysis of HAs. Some workers have determined IQ, MeIQ, MeIQx, DiMeIQx and Trp-P compounds in different cooked foods using the thermospray interface (TSP) [9–11]. In this paper, we discuss the applicability of electrospray (ES) with positive ionization to the simultaneous determination of HAs, developing MS conditions suitable for the identification of these compounds. The method developed was applied to the determination of IQ, PhIP, Trp-P-1, Trp-P-2, AαC, harman and norharman in meat extracts.

2. Experimental

2.1. Chemicals

The compounds studied (Table 1) were as follows: 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 3-amino-1,4-dimethyl-5H-pyridol[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), purchased from Toronto Research Chemicals (Toronto, Canada), and 1-methyl-9*H*-pyrido[4,3-*b*]indole (harman) and 9*H*-pyrido[4,3-*b*]indole (norharman), purchased from Aldrich (Steinheim, Germany). Stock standard solutions of 100 μg ml^{-1} in the mobile phase were prepared and used for further dilutions. 2-Amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline (TriMeIQx) was used as an internal standard (5 μg ml^{-1} solution in methanol).

Diatomaceous earth extraction cartridges (Extrelut; 20 ml) were provided by Merck (Darmstadt, Germany). Bond Elut propylsulphonyl silica gel (PRS; 500 mg) and octadecylsilane (C_{18}; 500 and 100 mg) cartridges, and coupling pieces and stopcocks were obtained from Analytichem International (ICT, Basle, Switzerland). These cartridges were preconditioned with dichloromethane (4 ml) for PRS and methanol (10 ml) and water (10 ml) for C_{18}.

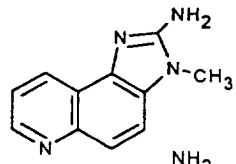
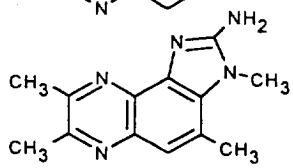
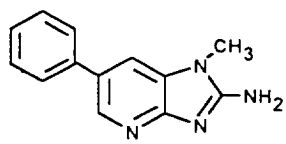
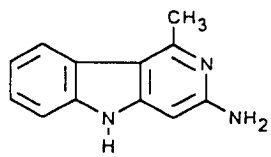
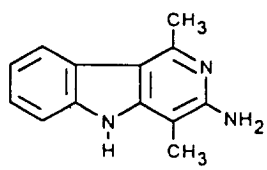
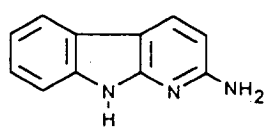
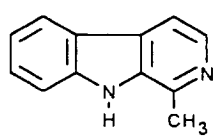
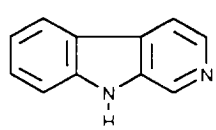
Solvents and chemicals used were of HPLC or analytical-reagent grade; water was purified using a Culligan (Barcelona, Spain) system. All the solutions were passed through a 0.45-μm filter before injection into the LC system. A Visiprep and a Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used for sample preparation by means of solid-phase extraction cartridges.

2.2. Instrumentation

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 pneumatically with nitrogen at a flow-rate of 10 l h^{-1}. Drying nitrogen was heated to 80°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-ion mode. The focus potential was 100 V.

For data acquisition, the mass spectrometer operated over a mass range of m/z 10–300 in the centroid mode with a cycle time of 1.00 s and an interscan time of 0.10 s. Ion intensity was opti-

Table 1
Structures of the heterocyclic amines studied

Compound	Structure	Abbreviation	M_r
2-Amino-3-methylimidazo[4,5-*f*]-quinoline		IQ	198.0905
2-Amino-3,4,7,8-tetramethylimidazo-[4,5-*f*]quinoxaline		TriMeIQx (I.S.)	241.1327
2-Amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine		PhIP	224.1062
3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole		Trp-P-2	197.0953
3-Amino-1,4-dimethyl-5*H*-pyrido-[4,3-*b*]indole		Trp-P-1	211.1109
2-Amino-9*H*-pyrido[2,3-*b*]indole		AαC	183.0796
1-Methyl-9*H*-pyrido[4,3-*b*]indole		Harman	182.0844
9H-Pyrido[4,3-*b*]indole		Norharman	168.0687

mized using the 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 the mobile phase of 50 μl min^{-1} were used in the calibration in order to improve cluster formation.

Flow injection analysis (FIA) using acetonitrile–5 m*M* ammonium acetate (pH 6.7) (50:50) at 50 μl min^{-1} was carried out in an HPLC system with two Phoenix 20 (Carlo Erba, Milan, Italy) syringe pumps, a master (A) and a slave (B) pump. An ODS-Hypersil C_{18} (5 μm particle size, 100 × 1 mm I.D.) reversed-phase column (Shandon Scientific, Cheshire, UK) was used for the LC separation of heterocyclic amines. Mixtures of standards were prepared in mobile phase and 10-μl aliquots were injected in the FIA mode and 200-nl aliquots in the chromatographic mode.

2.3. Analytical procedure

Sample preparation and clean-up were performed following the method reported previously [14], which includes different solid-phase extraction stages. The sample was homogenized in sodium hydroxide solution and passed through a diatomaceous earth cartridge (Extrelut). The analytes were eluted directly to a propylsulphonyl cartridge (PRS) using dichloromethane as eluent. The PRS was washed in 0.01 *M* HCl, MeOH–0.1 *M* HCl (60:40) and water. These fractions, which contained the imidazopyridine and indolpyridine derivatives, were collected and concentrated using a C_{18} cartridge (500 mg). The aminoimidazoquinolines retained in the PRS cartridge were eluted using 0.5 *M* ammonium acetate (pH 8.0) directly into another C_{18} cartridge (100 mg). Finally, the HAs retained in the C_{18} cartridges were eluted (methanol–ammonia) to give two final extracts. Each extract was evaporated to dryness using nitrogen and the residue was dissolved in a methanolic internal standard (I.S.) solution, 25 μl for the unspiked samples and 100 μl for the spiked samples. Two different extracts were obtained: extract A, which contained IQ and PhIP; and extract B, which contained PhIP, Trp-P-1, Trp-P-2, harman, norharman and AαC. The extracts were analysed by HPLC–MS using the previously optimized conditions. The analytes in beef extract samples were quantified by the standard additions method, adding to the samples accurately measured amounts of each standard at the beginning of the clean-up procedure. The HPLC–MS measurements were performed by single-ion monitoring (SIM) of the protonated molecular ions for each mutagen, using a dwell time of 100 ms: IQ, *m/z* 199; PhIP, *m/z* 225; AαC, *m/z* 184; Trp-P-1, *m/z* 212; Trp-P-2, *m/z* 198; harman, *m/z* 183; norharman, *m/z* 169; TriMeIQx, *m/z* 242.

3. Results and discussion

3.1. Calibration

One of the problems associated with "soft" ionization techniques such as electrospray is that an MS reference compound, e.g., perfluorokerosene, cannot be used to calibrate the mass spectrometer. The use of poly(ethylene glycol)s and poly(propylene glycol)s [15–18] as reference standards has been studied, but these mixtures are not suitable as reference compounds, since they cause rapid contamination of the ion source because of the deposition of non-volatile material. Cesium iodide cluster ions and other alkali metal halide salts [19,20] are used as mass calibration standards in fast atom bombardment and liquid secondary ion MS, but they also provide a source contamination in electrospray. An alternative approach to calibration for HPLC–MS is to use the mobile phase itself. Therefore, the calibration was performed using the mobile phase ions to prevent contamination of the ion source by deposition of non-volatile material. The mobile phase spectrum obtained under calibration conditions is given in Fig. 1. The spectrum showed a series of cluster ion triplets which contained different proportions of ammonium, water and acetonitrile such as $[NH_4(H_2O)_n]^+$, $[NH_4(H_2O)_{n-2}(CH_3CN)]^+$ and $[NH_4(H_2O)_{n-4}(CH_3CN)_2]^+$. These clusters allowed the calibration in the *m/z* range 18–300.

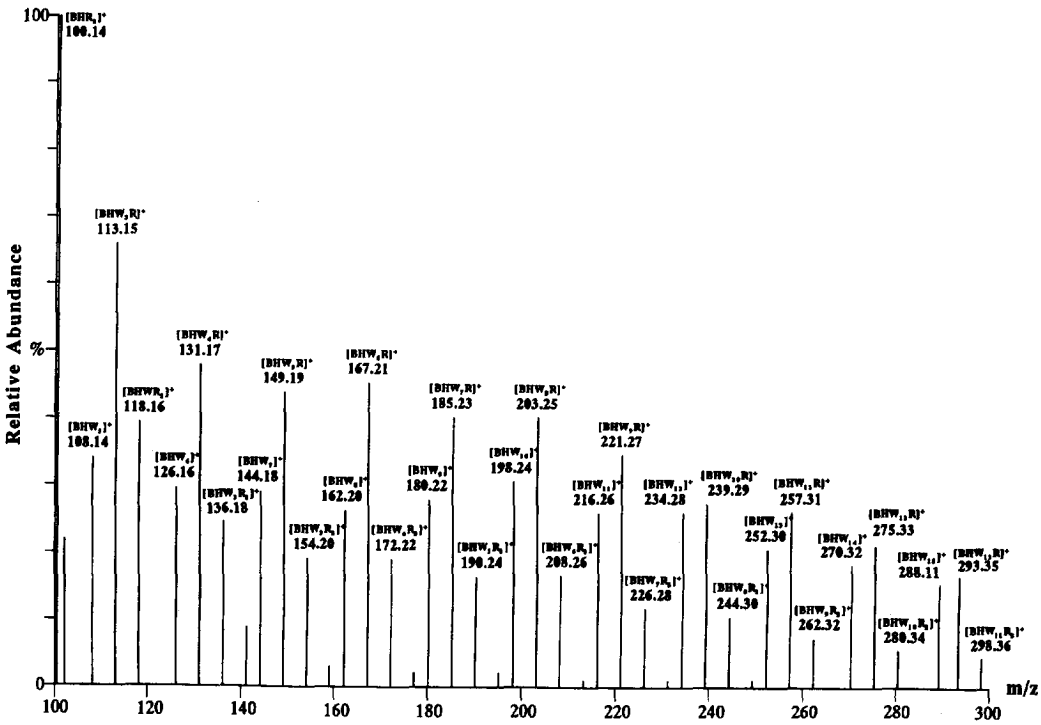


Fig. 1. ES mass spectrum of the mobile phase with positive ionization. B = NH_3; W = H_2O; R = CH_3CN.

3.2. Electrospray mass spectra

Heterocyclic amines are stronger bases than the components of the mobile phase, so proton addition to form $[M + H]^+$ is the common route of ionization for these compounds, as can be seen in Figs. 2 and 3, where ES mass spectra in the positive-ion mode for these heterocyclic amines are shown. Most of them gave a simple mass spectrum in which the only peak is due to $[M + H]^+$. These compounds were stable towards the ionization process and did not undergo significant fragmentation; only IQ and TriMeIQx showed the $[MH - 15]^+$ fragment. In this case, the focus potential might be sufficient to cause the loss of the methyl group linked to N − 3. Moreover, low-intensity water and acetonitrile adducts of the protonated analyte ($[MH(H_2O)_n]^+$ and $[MH(CH_3CN)_n]^+$) were also observed in the spectra of some amines, as happens when a thermospray interface is used [9–11]. As a consequence of these features, and the fact that the ion current derived from each component is mainly due to a single ion species, the sensitivity of detection of HAs by HPLC–MS is relatively high.

3.3. HPLC–MS

A synthetic mixture of the seven HAs was used for the optimization of the separation on the microbore C_{18} reversed-phase column. In order to establish the chromatographic conditions, the mobile phase was chosen according to a previous study performed on a conventional C_{18} column [7]. Different binary phases of acetonitrile–5 m*M* ammonium acetate at different pHs were tested. The best separations were obtained at low percentages of organic solvent in the mobile phase and at high pH. It was observed that ionization in the mass spectrometer improved when the content of acetonitrile in the mobile phase was higher. Taking into account the HPLC resolution and MS sensitivity, the mobile phase chosen was acetonitrile–5 m*M* ammonium acetate (pH 6.7) (50:50), although

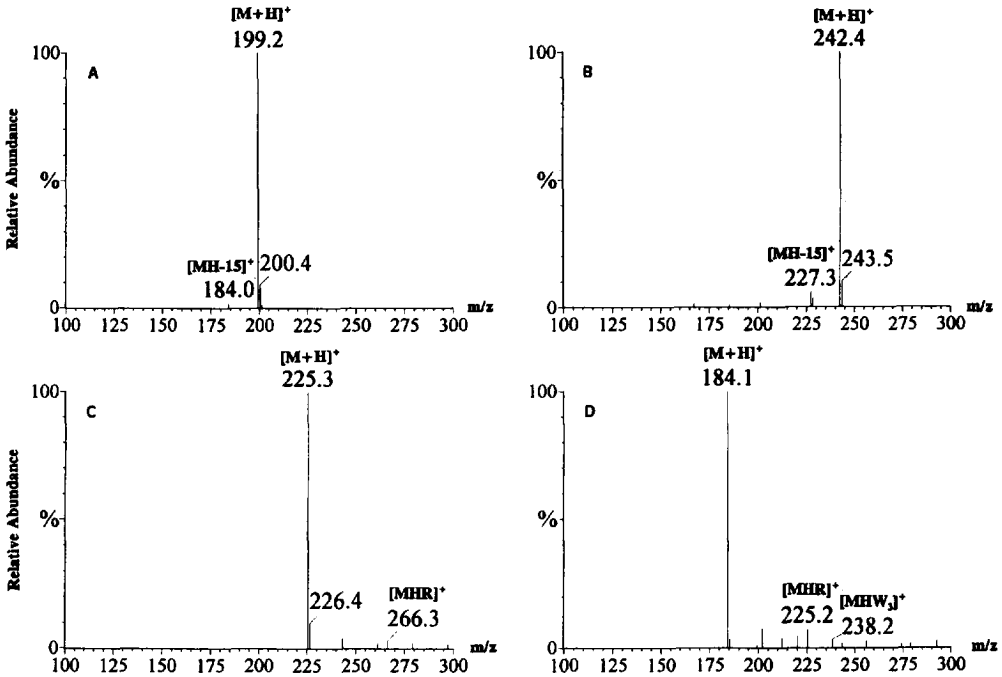


Fig. 2. ES mass spectra of (A) IQ ($M_r = 198$), (B) TriMeIQx ($M_r = 241$), (C) PhIP ($M_r = 224$) and (D) AαC ($M_r = 183$). W = H_2O; R = CH_3CN.

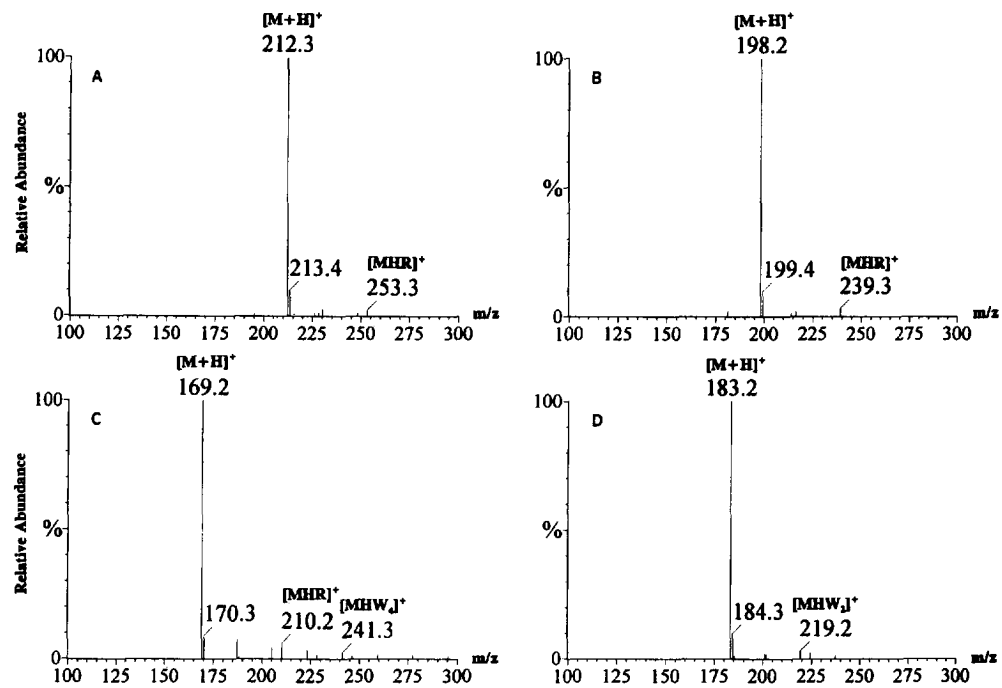


Fig. 3. ES mass spectra of (A) Trp-P-1 ($M_r = 211$), (B) Trp-P-2 ($M_r = 197$), (C) norharman ($M_r = 168$) and (D) harman ($M_r = 182$). W = H_2O; R = CH_3CN.

low resolution between IQ and PhIP was observed and co-elution of IQ and Aα-C occurred. Mass spectrometry is a selective technique and the poor resolution obtained in the separation and also the co-elution of IQ and AαC can be compensated for by selecting unambiguous masses to monitor. The $[M+H]^+$ ion for each heterocyclic amine was used when SIM was employed to register the chromatogram data. The reconstructed ion liquid chromatogram for each mass and the total ion chromatogram (TIC) are given in Fig. 4.

3.4. Detection limits

The detection limits in the full-scan and SIM modes based on a signal-to-noise ratio of 3:1 were calculated for the seven compounds, and ranged from 0.12 to 2.2 ng and from 5.4 to 44 pg, respectively. The results for each compound are given in Table 2, where the concentration of the solution injected and the amount injected are given. In the full-scan mode the detection limits were higher than in SIM, as expected. Trp-P-1 and Trp-P-2 gave the highest values, which may be related to their long retention times under these LC conditions.

The detection limits of the heterocyclic amines using HPLC and electrochemical detection are at ppb (ng ml^{-1}) levels. Billedeau et al. [6] gave values between 5.4 and 24 ng ml^{-1} for IQ, Trp-P-1 and Trp-P-2, Schwarzenbach and Gubler [8] reported values of 5–20 ng ml^{-1} for quinoline and quinoxaline derivatives and Galceran et al. [7] obtained data ranging from 9.5 to 37 ng ml^{-1} for aminoimidazoazarenes and aminocarbolines. Using UV detection, the detection limits are 2–3 times higher than with electrochemical detection [21]. The sensitivity data obtained in the present study for HPLC–ES-MS are comparable to the detection limits using HPLC with UV detection, and lower than values reported using HPLC–TSP-MS [10], which make this technique suitable for the determination of HAs in real samples.

The detection limits for real samples after a preconcentration step are always higher than those obtained for standard solutions. The values for beef extract samples were calculated using low-level spiking of reference standards. Samples were spiked with small amounts of each HA (0.5–5 ng g^{-1}), extracted using the Extrelut–PRS–C_{18} tandem method described under Ex-

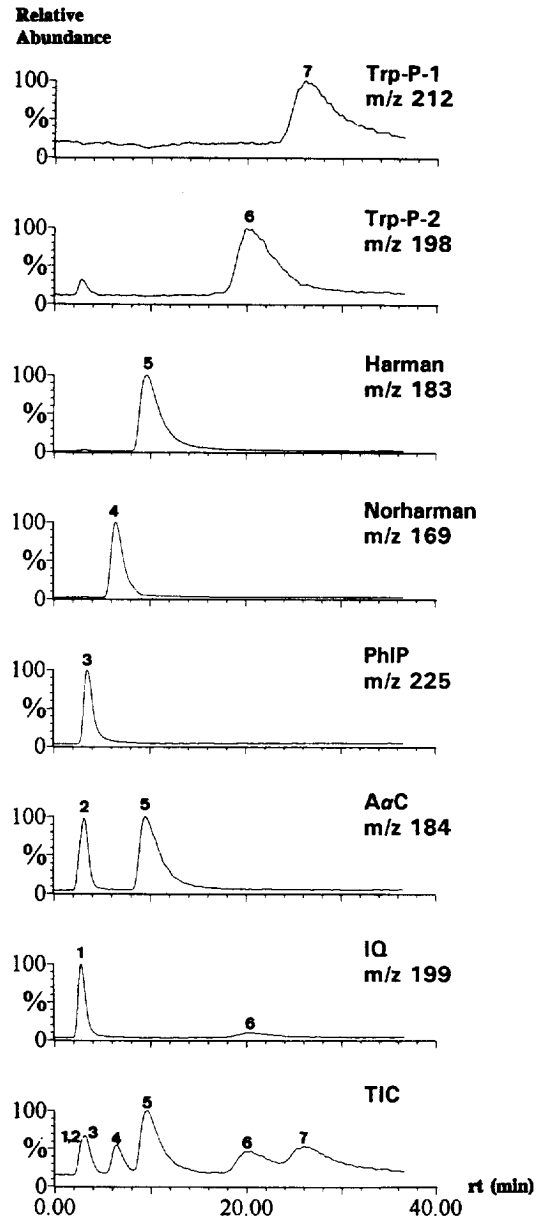


Fig. 4. HPLC–MS with positive electrospray ionization of HAs. The bottom trace is the TIC obtained by summing all ions above. Peaks: 1 = IQ; 2 = AαC; 3 = PhIP; 4 = norharman; 5 = harman; 6 = Trp-P-2; 7 = Trp-P-1.

Table 2
Detection limits for heterocyclic amines

Compound	Retention time (min)	Detection limits				
		Standard solutions				Beef extracts: SIM (ng g^{-1})
		Scan		SIM		
		$\mu g\ ml^{-1}$	ng injected	ng ml^{-1}	pg injected	
IQ	2.7	0.78	0.16	39.0	7.8	3[a]
PhIP	3.4	0.59	0.12	40.0	7.9	3
Trp-P-2	20.0	5.5	2.2	222.0	44.0	5
Trp-P-1	25.9	4.8	1.9	194.0	39.0	4[a]
AαC	2.9	0.97	0.19	39.0	7.8	6
Harman	9.8	0.82	0.16	27.0	5.4	1[a]
Norharman	6.4	0.60	0.12	30.0	6.0	1[a]

[a] Values calculated from the calibration graph.

perimental and analysed by HPLC–MS. Detection limits for the HAs already present in the beef samples were calculated from the calibration graph taking into account the recovery values previously reported [14] between 55–91% [14]. The detection limits obtained ranged from 1 to 6 ng g^{-1}, and are given in Table 2. In the analysis of meat extracts, the detection limits obtained by different workers using UV detection ranged from 1 ng g^{-1} [22] to 2–5 ng g^{-1} [5], lower values have been obtained with fluorescence detection [22], and values of about 0.2 ng g^{-1} have been reported for the determination of quinoline and quinoxaline derivatives in beef extracts using electrochemical detection [23]. For the determination of MeIQx, DiMeIQx and PhIP in meat products using GC–MS, values of 0.05–0.2 ng g^{-1} were obtained [13].

Generally, when samples are processed at high temperatures, the chromatograms become more complex and the detection limits for the HAs increase. For instance, Schwarzenbach and Gubler [8] were not able to achieve a detection limit of less than 50 ng g^{-1} for processed flavours with UV detection. The values obtained in this work by HPLC–MS with an ES interface are comparable to the UV detection limits when the samples are not subject to much interference. Moreover, in LC–MS analysis the presence of interferences may not significantly affect the detection limits owing to the selectivity of the MS detection.

3.5. Application

Commercial beef extract samples were purified using the solid-phase extraction procedure described under Experimental, which provided a suitable clean-up of the HAs to permit their detection and quantification by HPLC–MS. The two purified extracts obtained were analysed by HPLC–MS with SIM. The amines IQ, Trp-P-1, harman and norharman were identified in the samples. Figs. 5 and 6 show the chromatograms of both extracts. PhIP was evaluated in extract A because of the interference that appeared in extract B. The quantification of the samples was performed by the standard additions method (see Experimental) and gave values of 15.0, 5.5, 110.3 and 64.1 ng g^{-1} for IQ, Trp-P-1, harman and norharman, respectively.

4. Conclusions

The ES mass spectra obtained for the eight HAs showed that proton addition giving $[M+H]^+$ was the common route of ionization. The LC separation conditions and MS detection pa-

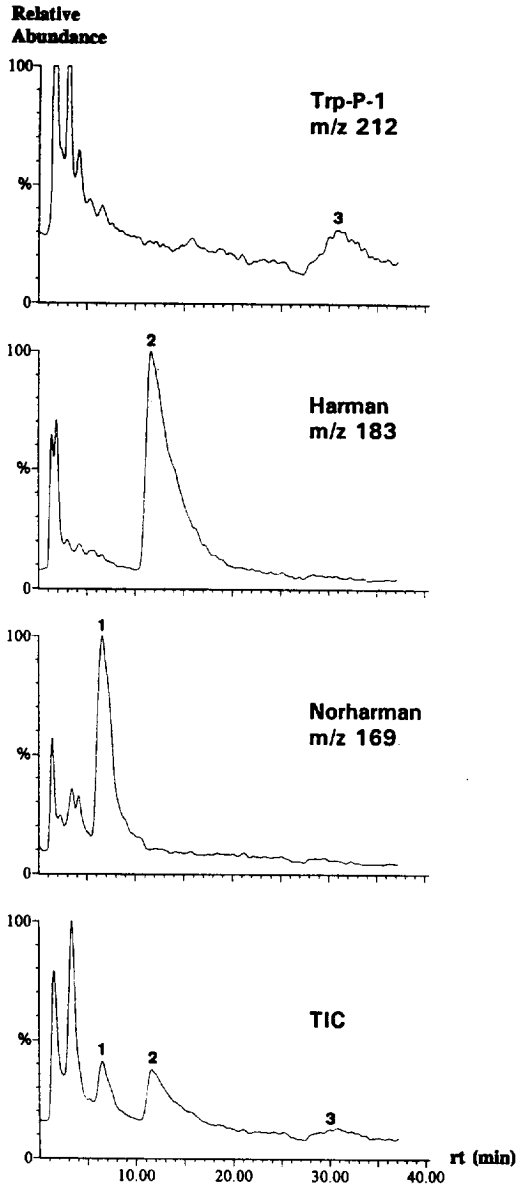


Fig. 5. HPLC–MS analysis of extract B of beef extract sample. Mobile phase: acetonitrile–5 m*M* ammonium acetate (pH 6.7) (50:50). The bottom trace is the TIC obtained by summing all ions above. Identified peaks: 1 = norharman; 2 = harman; 3 = Trp-P-1.

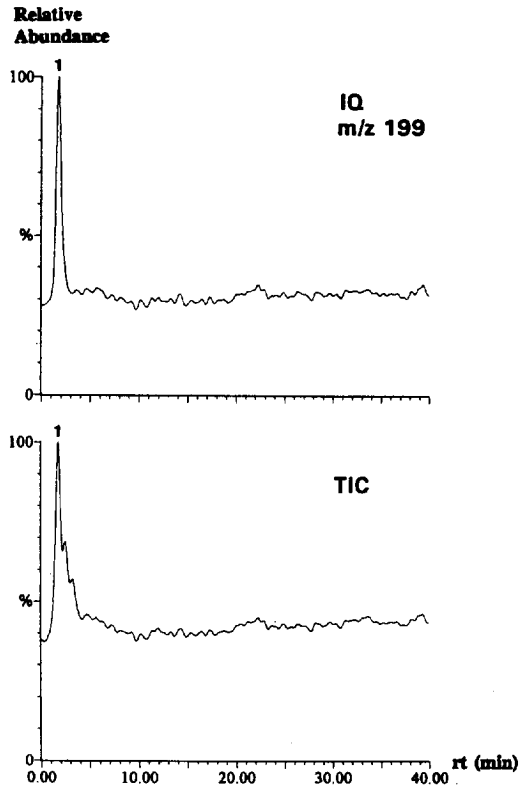


Fig. 6. HPLC–MS analysis of extract A of beef extract sample. Mobile phase: acetonitrile–5 m*M* ammonium acetate (pH 6.7) (50:50). The bottom trace is the TIC obtained by summing all ions above. Identified peak: 1 = IQ.

rameters were established for the determination of these amines by HPLC–MS. The applicability, selectivity and sensitivity of the method were studied. The procedure was applied to the determination of HAs in beef extract samples after a clean-up process. Generally, the clean-up of these biological samples produces highly complex fractions containing a high number of Maillard reaction products and methods for the detection and determination of HAs are usually difficult. Peak confirmation is an important requirement in order to rule out co-eluting interferences and MS would be the best choice for the identification and confirmation of these compounds. GC–MS is possible when volatile derivatives of the HAs are obtained, but only some mutagenic amines have been derivatized successfully and a general method does not exist. Furthermore, incomplete derivatization leads to low sensitivity and irreproducible results. The use of MS in conjunction with HPLC does not require any derivatization step and would be the preferred method for the on-line identification of

HAs. Some workers have applied TSP to the determination of some HAs in different cooked foods but the detection limits reported are slightly higher than those obtained in this work using ES. LC–ES-MS, because of its selectivity and relatively high sensitivity, provided chromatograms free from interfering peaks, allowing the identification and quantification of several HAs and related compounds at concentration levels between 5.5 and 110.3 ng g^{-1}.

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