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Determination of heterocyclic aromatic amines in meat extracts by liquid chromatography–ion-trap atmospheric pressure chemical ionization mass spectrometry

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Abstract

When protein-rich foods are processed under normal cooking conditions, heterocyclic aromatic amines (HAAs) can be generated at a few parts per billion level. In this work, we have analyzed the HAAs present in a lyophilized meat extract by means of a simplified solid-phase extraction procedure. All the analytes were collected in a single extract with recoveries in the range of 45.6–75.2%, so the analysis time has been greatly reduced. Problems derived from the less exhaustive purification of the extract have been solved by using MS(ion trap) detection. The RSD for quantification ranged from 2.1% to 5.1% for run-to-run precision and from 5.2% to 11% for day-to-day precision. The limits of detection for standard solutions ranged from 20 to 150 pg injected. For the meat extract analyzed limits of detection from 0.9 to 11.2 ng g^{-1} were obtained. Results of the quantification are in agreement with those obtained using different clean-up procedures. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Food analysis; Amines; Heterocyclic aromatic compounds

1. Introduction

Heterocyclic aromatic amines (HAAs) are a group of basic compounds to which humans are regularly exposed from diet, since they are produced in trace quantities (ng g^{-1} level) when proteinaceous foods, such as meat and fish, are processed by typical cooking practices [1–3]. Previous studies have shown that meat extracts, some beef flavours [4–6] and other matrices including wine, beer and environmental samples also contain HAAs [7–9].

These chemicals constitute a major health risk due to their potent mutagenic activity [10,11]. To date, more than 20 HAAs have been isolated as mutagens, and the structure of 19 of them have been elucidated [12]. The ten HAAs so far examined in animals have proved to be tumourigenic in standard animal experiments, with target organs including lung, liver, mammary gland, colon and skin [13,14]. In addition, several epidemiological studies have revealed a positive association between consumption of cooked meat and fish and risk of colorectal cancer development [15,16], and they also suggest a relationship between methods of cooking meat and various cancers [17,18].

To assess potential health risks associated with the consumption of HAAs, it is of vital importance that their occurrence should be monitored by reliable quantitative methods. A major drawback in the analysis of these mutagens from foods is their very low level of concentration (0.1–50 ng g^{-1}) and the high number of matrix interferences. So, many purification techniques based on liquid–liquid

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[19,20] or solid-phase extraction [21,22] can be found in the literature, mainly followed by different separation techniques: liquid chromatography (LC) [23–25], gas chromatography (GC) [26–28] or capillary electrophoresis (CE) [29–31]. The degree of selectivity in the detection depends on the efficiency of the clean-up procedure. The use of poor specific detectors such as UV detectors [32,33] requires exhaustive purification processes, but this often leads to a decrease in analyte recovery and therefore in the accuracy of the results. This is the reason why some authors use more specific detectors like diode array detection (DAD) [34,35], fluorescence [36,37], electrochemical detection (ED) [38,39], mass spectrometry (MS) [40,41] or even tandem mass spectrometry (MS–MS) [42,43]. Since MS offers very selective detection and on-line identification, in this work we used this technique to analyze the heterocyclic aromatic amines present in a lyophilized meat extract in order to prove the applicability of a clean-up procedure described in a previous work [44], which is easier than the usual ones [45,46]. In addition, since all the amines are recovered in a single extract, the analysis time is reduced by 40%. The determination of the amines was achieved by means of liquid chromatography coupled to mass spectrometry via an atmospheric pressure chemical ionization source and an ion trap as analyzer (LC–APCI-MS(IT)). The separation of the analytes was performed in a C_{18} column using a suitable mobile phase compatible with mass spectrometry. The parameters that influence the ion formation and detection were optimized, and repeatability, medium term precision and limits of detection have been studied in order to establish the quality parameters of the system. The proposed method was applied to the determination of heterocyclic amines in a lyophilized meat extract, proposed as a reference material [47].

2. Experimental

2.1. Chemicals

Methanol and acetonitrile were gradient grade (Merck, Darmstadt, Germany), water was purified in an Elix-Milli Q system (Millipore, Bedford, MA, USA) and dichloromethane was HPLC grade (Fisher, Leicestershire, UK). Both ammonia solution and formic acid were analytical grade (Merck), as was ammonium acetate (Fluka, Buchs, Switzerland). Caffeine, MRFA (L–Met–Arg–Phe–Ala acetate·H_2O) and Ultramark 1621 (polyethylene glycol mixture) were purchased from Sigma (Steinheim, Germany). He and N_2 were N50 quality, and all the solutions were passed through a 0.45 μm filter before injection into the LC system.

The compounds studied, which are shown in Fig. 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), 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), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-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), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), purchased from Toronto Research Chemicals Inc. (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. Stock standard solutions of 100 $\mu g\ ml^{-1}$ in methanol were prepared and used for further dilutions. TriMeIQx was used as internal standard (2 $\mu g\ ml^{-1}$ methanolic solution).

Diatomaceous earth extraction cartridges (Extrelut-20) and refill material were provided by Merck; PRS sodium form (500 mg) and endcapped C_{18} (100 mg) Bond-Elut cartridges, as well as coupling pieces and stopcocks were from Varian Associates (Harbor City, CA, USA). These cartridges were preconditioned with dichloromethane (7 ml) for PRS and methanol (5 ml) and water (5 ml) for C_{18}. A lyophilized meat extract [47] was used for the analysis.

2.2. Instruments

The separation of the amines was optimized using a Beckman System Gold 168 (Fullerton, CA, USA)

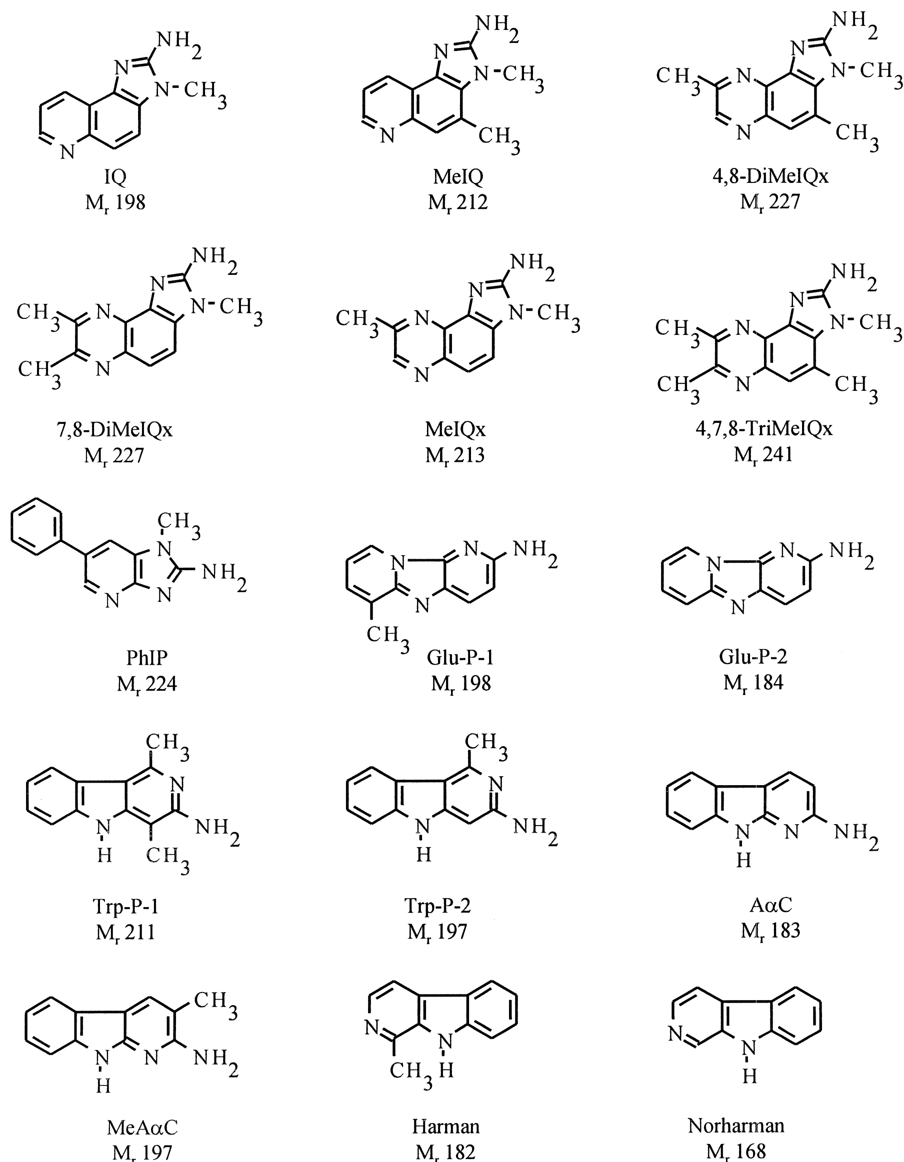

Fig. 1. Structures of the studied aromatic amines together with their abbreviation and isotopical molecular mass.

photodiode-array UV detector, which acquired spectra of peaks from 200 to 300 nm. In this case, the pumping system was a Pharmacia LKB HPLC system (Uppsala, Sweden) equipped with a high-pressure mixer, a low-pressure mixer and a Rheodyne 7125 injector (Cotati, CA, USA).

For mass spectrometry analysis, reversed-phase LC analyses were performed by means of a Waters 2690 Separations Module (Milford, MA, USA), and determination and identification of the peaks in the sample were carried out with an LCQ (Finnigan MAT, San Jose, CA, USA) provided with an APCI interface and an ion trap mass analyzer. Source working conditions to record positive ions were optimized by varying the parameters influencing the ionization. Discharge voltage was varied between 3 and 7 kV; heated capillary and vaporizer temperatures were tested in the range of 100–250°C and

300–500°C respectively, and nitrogen was used as drying gas (0–144 l h^{-1}) and as nebulizing gas (270–450 l h^{-1}). For data acquisition in full scan mode, the mass spectrometer operated over a range of m/z 150–250 in the centroid mode with a maximum injection time, which was varied from 200 to 1000 ms, 1 microscan, automatic gain control (AGC) ON and inject waveform off. Efficiency of ion transference from source to the ion trap was automatically optimized by infusing methanolic solutions of IQ, 4,8-DiMeIQx and Trp-P-1. To prevent MS contamination when running LC–MS, a divert valve was used. MS calibration was carried out with the infusion of a mixture containing caffeine, MRFA and Ultramark 1621 into the APCI source.

A Supelco Visiprep and a Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used for manipulations with solid-phase extraction cartridges and solvent evaporation, respectively.

2.3. Chromatographic conditions

In all cases, the amines were separated 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 with ammonia solution to pH 3.25; solvent B: 30 m*M* formic acid in water adjusted with ammonia solution to pH 3.7; solvent C: acetonitrile. For LC–MS analysis, the gradient 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 post-run delay. For UV detection 7 m*M* triethylamine was used, but with MS detection it was discarded due to the strong ionization suppression observed. In all cases the amount injected was 15 μl.

2.4. Sample analysis

To extract the analytes from a lyophilized meat extract a previously described purification method [44] was used. Briefly, 1 g beef extract sample was dissolved in 12 ml 1 *M* NaOH with sonication and shaking until homogenization for 3 h. The alkaline solution was mixed with Extrelut refill material (12.9 g) and used to fill an empty Extrelut column. A Bond-Elut PRS (500 mg) column was preconditioned with 5 ml 0.1 *M* HCl, 10 ml water and 5 ml methanol. After drying the cartridge under vacuum, 7 ml dichloromethane were passed, and then the PRS column was coupled on-line with the Extrelut column. To extract the analytes from diatomaceous earth, 75 ml dichloromethane were passed through the tandem, and 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 a preconditioned C_{18} (100 mg) column, and this tandem was eluted with 20 ml of 0.5 *M* ammonium acetate at pH 8.0. The adsorbed HAAs were finally eluted from C_{18}, after rinsing with 5 ml water, using 0.8 ml of methanol–ammonia (9:1, v/v). The solvent was evaporated with a stream of nitrogen and the analytes were redissolved with 50 μl of the internal standard in methanol. The final extract was analyzed using the LC–MS method described above.

Quantification and recovery calculation of the amines in the beef extract was carried out by standard addition method. The meat extract was spiked with all the analyzed compounds at three levels (80, 160 and 320 ng g^{-1}) by adding different volumes of a methanolic solution of the analytes to the sample. The solvent was allowed to evaporate before the addition of NaOH.

3. Results and discussion

3.1. LC–MS

As it was described in the experimental section, the chromatographic separation of the HAAs was performed in a C_{18} column with a volatile mobile phase, based in ammonium formate and acetonitrile, to be compatible with the mass spectrometric system. APCI involves a soft ionization process, therefore studied HAAs readily provide unfragmented protonated-molecular ions $[M+H]^+$ as the base peak. In order to optimize the ionization, various parameters were studied, and the best results were obtained with the following conditions: discharge voltage and

current 5 kV and 5 μA respectively; the capillary was heated to 150°C, and the vaporizer temperature was 450°C; nitrogen was introduced as drying gas at a flow-rate of 72 l h^{-1}, and used for nebulization at a flow-rate of 360 l h^{-1}.

The chromatogram (Fig. 2) can be divided in three regions where the amines 1 to 5, 6 to 10 and 11 to 15 were eluted. These regions corresponded to different mobile phase composition according to the gradient program mentioned in the experimental section.

The parameters which influence ionization, desolvatation and ion transference from source to analyzer, including capillary voltage, tube lens voltage and optics, were automatically optimized for each segment using a methanolic solution of the amines (1 μg ml^{-1} concentration level) chosen as model. For the first segment (0–18 min) IQ was used, segment two (18–24.7 min) was tuned using 4,8-DiMeIQx and for segment three (24.7–40 min) Trp-P-1 was chosen. The amine solutions were introduced into the system by flow injection analysis (FIA) using the corresponding mobile phase composition for each amine by means a T-piece at a flow-rate of 9 μl min^{-1}.

Fig. 2 shows the total ion chromatogram (TIC) and the traces for each m/z corresponding to $[M+H]^+$ for a standard solution of 3.7 μg ml^{-1}. This chromatogram, where it can be seen that resolution between all the traces is acceptable, was acquired under the optimal conditions.

3.2. Quality parameters

Calibration curves for the amines were performed at six concentration levels in the range of 0.15–7.30 μg ml^{-1}. Calibration curves were calculated daily from the representation of the peak area of the analytes in relation to the peak area of the internal standard (TriMeIQx) vs. the concentration of each compound. The curves were fitted to a quadratic function using a $1/x^2$ weighting, which gave regression coefficients better than 0.994 for all the analytes.

The quality parameters repeatability or run-to-run precision, medium term or day-to-day precision and limit of detection were calculated. To determine both repeatability and medium term precision, five daily replicate injections of a methanolic solution of all the analytes at an approximate level of 0.8 μg ml^{-1} were carried out on three successive days. A study of the variance of one factor for both concentration and retention time was then performed [48]. The target value for the concentration of each analyte and the mean values for retention time and concentration are shown in Table 1, together with the relative standard deviations for run-to-run and day-to-day precision obtained with the variance analysis study. It can be observed that the range of variability for concentration is 2.1–5.1% for run-to-run precision, and 4.6–11.0 for day-to-day precision. For retention times, run-to-run precision is comprised between 0.04 and 0.4, and the day-to-day between 0.07 and 0.7. The good figures of merit obtained are better than those calculated when the conventional phosphoric acid/dihydrogenphosphate is used as mobile phase with UV detection [49].

Detection limits for standard solutions, which are based on a signal-to-noise ratio of 3:1, ranged from 27 pg to 150 pg injected (1.8 ng ml^{-1}–10 ng ml^{-1}), as it can be seen in Table 1. These full scan values are comparable to those obtained using electrospray [40] and APCI sources [41] with selected ion monitoring (SIM). In addition, they are similar to the limits of detection obtained with electrochemical detection [38,39] and at least 10 times lower than those obtained with UV detection [4,50].

3.3. Analysis of a beef extract

The main objective of this work was to demonstrate the applicability of the simplified SPE method, using MS detection, to the analysis of a real sample. For this reason it has been applied to a lyophilized meat extract proposed as a reference material [47]. As it can be seen in Fig. 3, the quantification with UV detection is difficult due to the complexity of the matrix. For instance, IQ coeluted with an interfering compound, and the internal standard TriMeIQx had to be replaced by 7,8-DiMeIQx due to matrix interferences. By using MS detection and due to its high selectivity and specificity, the low resolution and the interferences coextracted from the matrix can be compensated by selecting adequate masses to monitor. In Fig. 4 the ion trace chromatogram for each amine is given and the compounds IQ, MeIQx, 4,8-DiMeIQx, norharman, harman and PhIP were

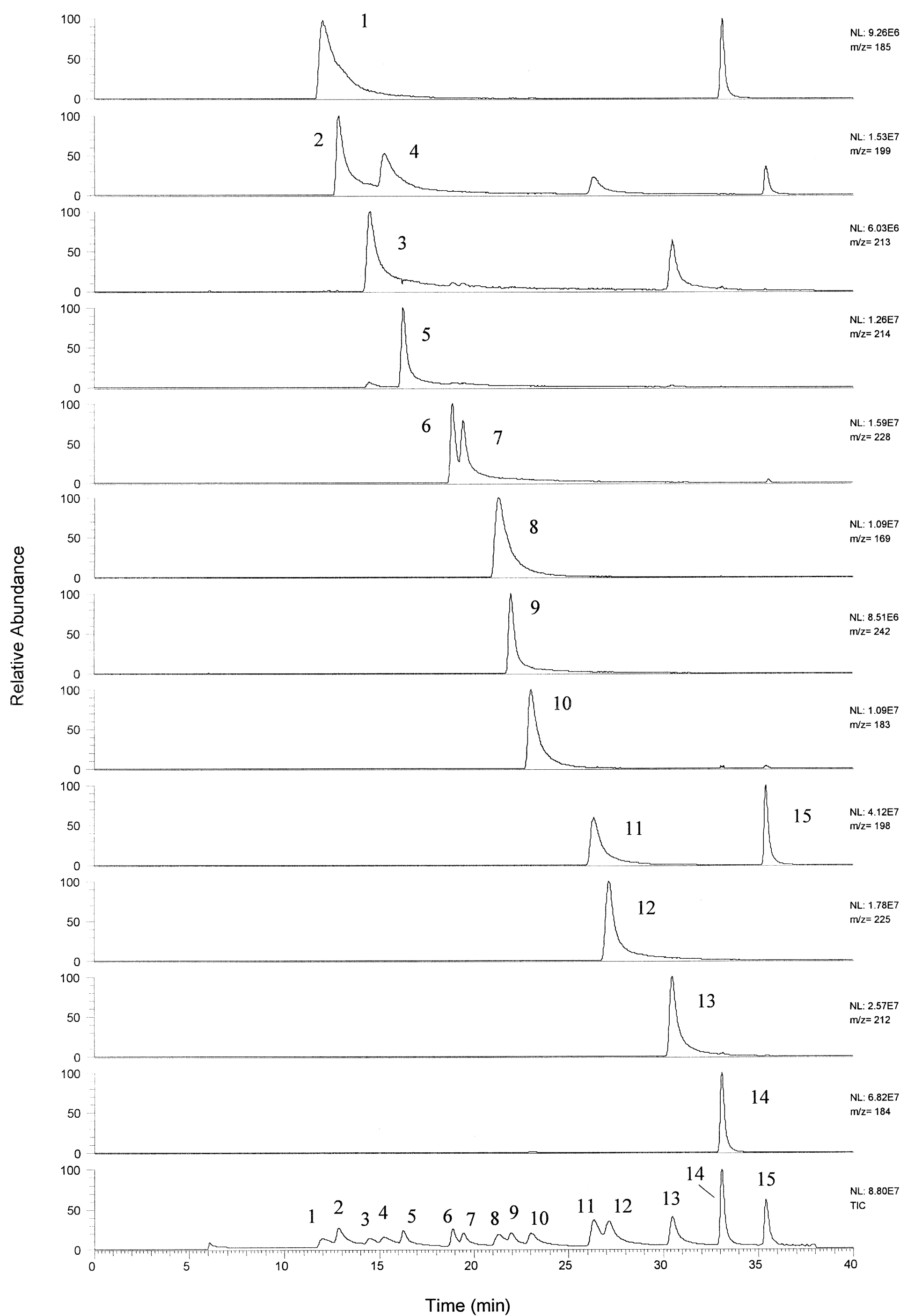

Fig. 2. Total ion chromatogram and chromatogram for each mass of a standard solution (3.7 μg ml^{-1}). Peak identification: 1. Glu-P-2; 2. IQ; 3. MeIQ; 4. Glu-P-1; 5. MeIQx; 6. 7,8-DiMeIQx; 7. 4,8-DiMeIQx; 8. Norharman; 9. TriMeIQx (IS); 10. Harman; 11. Trp-P-2; 12. PhIP; 13. Trp-P-1; 14. AαC; 15. MeAαC. Chromatographic conditions as given in experimental section.

Table 1
Quality parameters: run-to-run precision, day-to-day precision and limit of detection

Compound	Target value (μg ml^{-1})	Mean values (n=15)		Precision RSD% (n=15, α=0.05)				Limit of detection			
		Conc. (μg ml^{-1})	t_R (min)	Conc.		t_R		Standards		Sample	
				run-to-run	day-to-day	run-to-run	day-to-day	ng ml^{-1} [a]	pg injected	pg injected	ng g^{-1}
Glu-P-2	0.78	0.80	12.2	2.2	8.2	0.4	0.5	6.0	90	1300	7.9
IQ	1.16	1.14	13.1	2.7	7.1	0.3	0.6	6.2	93	1000	4.9
MeIQ	0.61	0.63	14.8	4.2	11.0	0.2	0.7	10.0	150	3100	10.1
Glu-P-1	0.70	0.67	15.4	3.8	7.3	0.2	0.5	4.8	72	1500	9.0
MeIQx	0.78	0.78	16.4	3.1	8.2	0.1	0.3	5.6	84	1100	5.3
7,8-DiMeIQx	0.85	0.84	19.0	3.3	4.6	0.1	0.4	6.9	103	710	2.9
4,8-DiMeIQx	0.62	0.63	19.6	4.8	10.1	0.2	0.3	6.1	91	400	2.7
Norharman	0.84	0.82	21.5	2.1	7.0	0.2	0.3	7.4	111	370	2.3
Harman	0.78	0.75	23.2	3.5	7.5	0.2	0.3	5.6	84	390	2.7
Trp-P-2	0.99	0.97	26.6	2.5	7.0	0.2	0.2	2.7	40	300	1.9
PhIP	0.82	0.83	27.4	2.9	5.2	0.1	0.3	6.0	90	380	1.5
Trp-P-1	0.82	0.81	30.6	2.2	6.8	0.08	0.1	1.8	27	175	1.7
AαC	1.27	1.22	33.2	5.1	8.1	0.04	0.1	2.0	30	100	0.8
MeAαC	0.82	0.78	35.5	4.7	5.8	0.04	0.07	3.0	45	200	1.0

[a] 15 μl were injected.

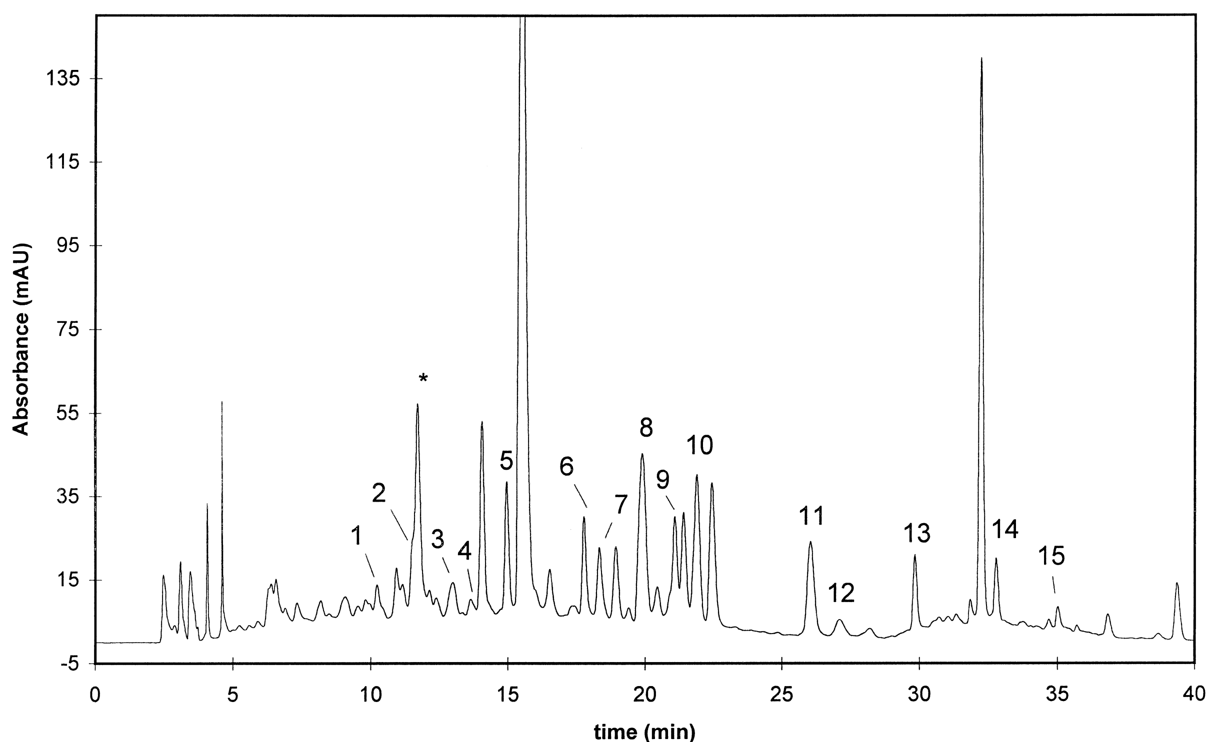

Fig. 3. Chromatogram of a meat extract spiked with 80 ng of each analyte (UV detection, at λ=263 nm). Peak identification: 1. Glu-P-2; 2. IQ; 3. MeIQ; 4. Glu-P-1; 5. MeIQx; 6. 7,8-DiMeIQx (IS_1); 7. 4,8-DiMeIQx; 8. Includes Norharman; 9. TriMeIQx (IS_2); 10. Harman; 11. Trp-P-2; 12. PhIP; 13. Trp-P-1; 14. AαC; 15. MeAαC; * interfering compound. Chromatographic conditions as given in experimental section.

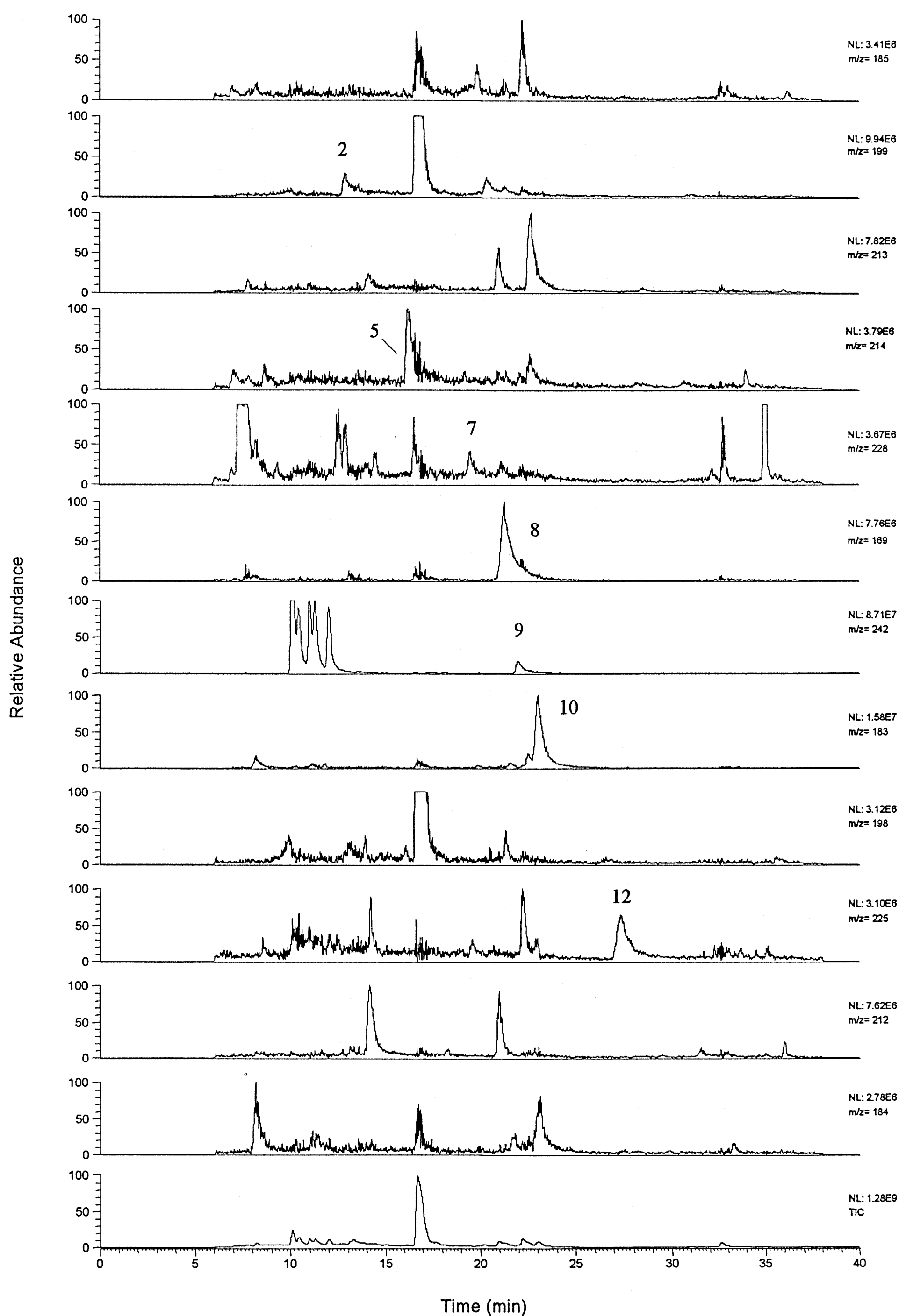

Fig. 4. Chromatogram of the non-spiked meat extract, which includes the signal obtained in the mass correspondent to each analyte and the total ion current (TIC). Identification of the peaks: 2. IQ; 5. MeIQx; 7. 4,8-DiMeIQx; 8. Norharman; 9. TriMeIQx (IS); 10. Harman; 12. PhIP. Chromatographic conditions as in Fig. 2.

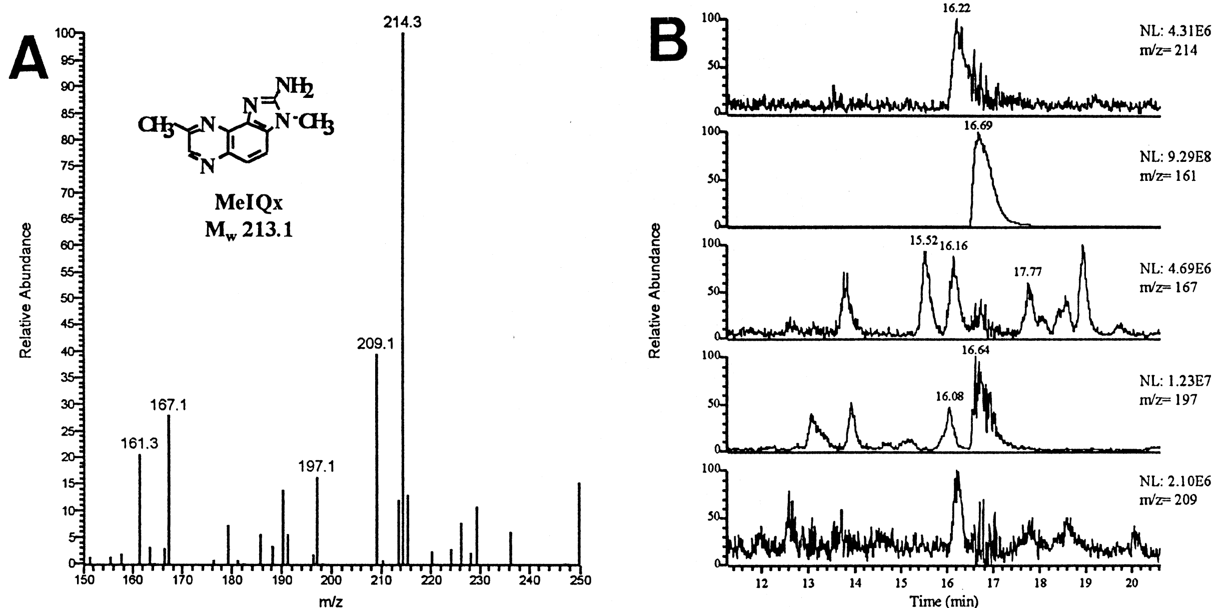

Fig. 5. (A) Full scan mass spectra of the peak eluted at the retention time of MeIQx. (B) Ion trace chromatograms of the most intense m/z shown in (A). Chromatographic conditions as in Fig. 2.

clearly identified. Nevertheless an important noise was observed for some of the compounds, for instance MeIQx. The Fig. 5A shows the full scan spectra of the eluted peak corresponding to this analyte, there it can be seen that an important number of interfering ions in addition to the molecular protonated ion were present. From the trace ion chromatogram (Fig. 5B) it can be deduced that the noise in the target compound is due to the coelution of the interferences. Moreover, when this coelution occurred the ionization of the target compounds may be affected by a suppression phenomenon, giving a lower signal-to-noise ratio. As a consequence, detection limits in the meat extract were higher than expected, as it can be seen in Table 1. This effect was more pronounced for the compounds eluted in the first zone of the chromatogram which is the less exhaustively purified.

Nevertheless, as it can be seen in Table 2, the results obtained in the analysis of the lyophilized meat extract are in agreement with those when UV detection was used for the analysis of the meat extract [44], avoiding the problems originated in the UV detection. For instance, it was impossible to quantify IQ using UV–DAD and, in addition, the identity of some peaks could not be confirmed using UV spectra, as in the case of MeIQ, Glu-P-2 or Trp-P-1. Selectivity of MS detection has permitted more reliable quantification, providing results similar to those obtained previously with other clean-up procedures [47,51].

Furthermore, it must be mentioned that some differences were observed between recoveries obtained with both LC–MS and LC–UV, as it can be seen in Table 2, that can be attributed to differences in cartridges batches or to the matrix interferences which occur when UV detection was used. This suggested that standard addition is mandatory to guarantee accurate quantification of the analytes, but if the analytes were strongly retained by the matrix components, an overestimation of the recoveries could be introduced.

4. Conclusions

The SPE method applied in this work has been shown to be suitable for the analysis of heterocyclic aromatic amines in proteinaceous matrices when it is used in conjunction with a sensitive, specific and

Table 2
Analysis of a lyophilized meat extract

Analytes	UV detection				MS detection			
	Recovery (%)	SD	ng g^{-1}	RSD (%)	Recovery (%)	SD	ng g^{-1}	RSD (%)
Glu-P-2	67.9	7.8	37[a]	27.0	57.6	1.9	n.d.	–
IQ	[b]	–	[b]	–	69.5	3.2	32.5	22.4
MeIQ	79.31	3.1	17.3[a]	55.5	73.2	4.4	n.d.	–
Glu-P-1	78.1	2.5	16.6[a]	27.1	57.2	1.1	n.d.	–
MeIQx	80.8	2.0	33.4	7.5	70.3	3.9	41.4	6.3
7,8-DiMeIQx	IS	–	IS	–	75.2	1.8	n.d.	–
4,8-DiMeIQx	80.1	8.0	12.4	24.2	52.3	1.9	9.7	17.5
Norharman	51.0	5.7	177	6.2	54.1	3.3	146	6.8
Harman	67.2	4.8	234	7.3	49.6	5.2	263	15.2
Trp-P-2	43.6	2	n.d.	–	49.8	1.0	n.d.	–
PhIP	57.2	3	28.8	31.9	74.3	2.7	27.1	3.7
Trp-P-1	50.8	3	9.0[a]	90.0	45.6	2.0	n.d.	–
AαC	65.8	3.7	6.4[a]	135	49.4	1.5	n.q.	–
MeAαC	–	–	–	–	51.1	1.4	n.q.	–

[a] Identity not confirmed with UV spectra.
[b] Coelution with a major interference prevented quantification.
n.d.: non detected compounds in the meat extract.
n.q.: analyte nearly its limit of detection.

selective LC detection technique, such as MS. This purification and preconcentration method allowed a reduction in analysis time and in materials, therefore it can be recommended for the analysis of heterocyclic amines in different matrices. However, in the case of complex samples such as meat extracts, where a high number of coeluting compounds are present, detection limits were found to be higher than those obtained with standard solutions. It was observed that an increase between 3 and 20 times occurred, showing that a compromise has to be achieved between limits of detection and clean-up efficiency, that depends on the food sample to be analyzed and the HAAs concentration levels.

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