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Evaluation of different clean-up procedures for the analysis of heterocyclic aromatic amines in a lyophilized meat extract

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Abstract

Along with other mutagenic and carcinogenic contaminants in foods such as aflatoxins and polycyclic aromatic hydrocarbons, heterocyclic aromatic amines (HAAs) have received considerable attention in recent years. A major drawback in the analysis of HAAs in foods is their very low level of concentration (0.1–50 ng g^{-1}) as well as matrix interferences. Solid-phase extraction (SPE), forming an integral part of chromatographic analysis, is one of the procedures currently used for the extraction and purification of HAAs in food samples. In this paper a comparative study of several SPE procedures for HAAs determination was performed. Recoveries of the heterocyclic amines in the analysis of both a simple matrix such as a standard methanolic solution and a contaminated meat extract were established. HAAs were determined by HPLC analysis with photodiode-array detection (DAD) of the purified extracts, and the adequacy of different clean-up procedures for the analysis of a contaminated meat extract was discussed.

Keywords: Clean-up methods; Solid-phase extraction; Meat; Amines, heterocyclic

1. Introduction

Heterocyclic aromatic amines (HAAs) comprise a variety of basic compounds belonging to two main chemical classes, aminocarbolines and amino-imidazoazaarenes, to which humans are regularly exposed [1]. These chemicals constitute a major health risk due to their potent mutagenic activity [2,3]. Most HAAs are produced at trace quantities (ng g^{-1} level) from protein rich foods, such as meat and fish, when they are processed by thermal treatments, that is, typical cooking practices [4]. Previous studies have shown that meat extracts, some beef flavours and other kinds of matrices including wine, beer and environmental samples also contain potent mutagenic HAAs [5–8]. To date, more than 20 HAAs have been isolated as mutagens, and the structures of 19 of them have been elucidated [9] and are shown in Fig. 1. All 10 HAAs so far examined have proved to be carcinogenic in experimental animals with target organs including the lung, liver, mammary gland, colon and skin [10,11]. In addition, several epidemiological studies have revealed a positive association between consumption of cooked meat and fish and development of tumours [12,13], thus, it has been demonstrated that people who eat heavily browned meat are at 2.0–6.0-fold higher risk of colorectal cancer development [14].

A major drawback in the analysis of HAAs from foods is their very low level of concentration (0.1–50 ng g^{-1}) and the high number of matrix interferences. For the analysis of these compounds, a variety

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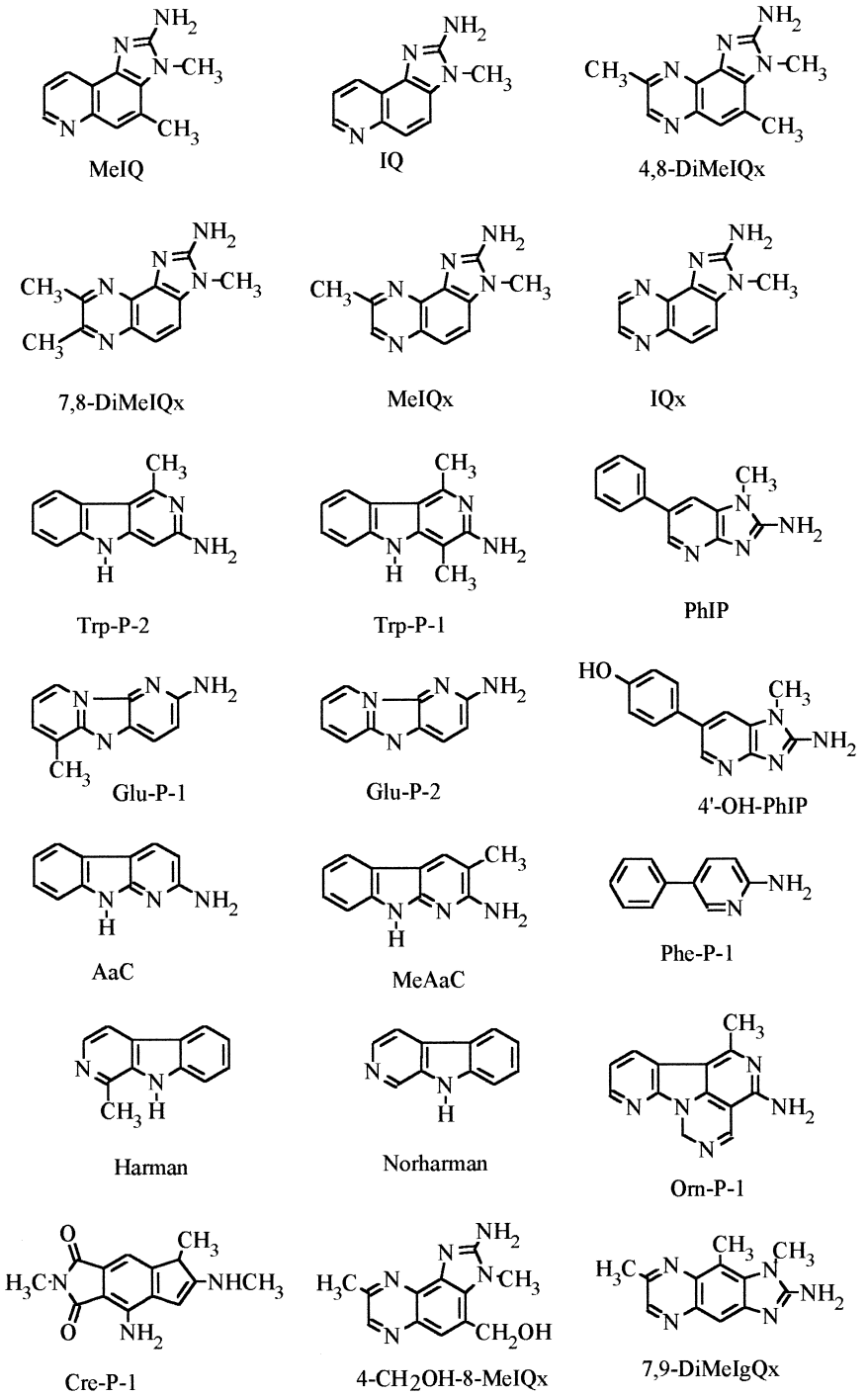

Fig. 1. Structures of mutagenic amines and comutagens Harman and Norharman.

of liquid–liquid [15,16] or solid-phase purification techniques can be found in the literature [5,17,18], followed mainly by chromatographic techniques [high-performance liquid chromatography (HPLC)–UV [19,20], HPLC–fluorescence [19,21], HPLC–electrochemical detection (ED) [22,23], HPLC–mass spectrometry (MS) [24,25], gas chromatography (GC)–MS [26]], capillary electrophoresis [27,28] or enzyme-linked immunosorbent assay (ELISA) [29]. The sample matrix greatly influences the clean-up efficiency and many peaks with the same retention times as those of HAAs are often present in the chromatograms of final extracts. Consequently, great efforts are needed to improve existing procedures of clean-up and preconcentration, which usually include extensive fractionations in multiple steps. As shown in Report EUR 17652 EN of BCR [30], an inter-comparison exercise on the determination of HAAs in a commercial beef extract, the complexity of extraction methods led to highly dispersed analytical results being obtained. This indicates a lack of accuracy, which could be attributable to both poor repeatability and the low level of recoveries. The study has also revealed that great discrepancies appeared between laboratories and also between-day within the same laboratory.

This study seeks to compare three liquid–solid extraction methods described for the analysis of conventional meat extracts [31–33] to establish the suitability of each method. Moreover, on the basis of existing solid-phase extraction (SPE) methods, different eluting solvents and cation exchangers were studied to improve extraction efficiencies and to achieve a high degree of accuracy and precision in the analysis of HAAs. Thus, an additional method was tested. These four methods were applied to a lyophilized meat extract that could be considered a candidate reference material. The analysis of the purified extracts was performed by HPLC with UV photodiode-array detection, and the results obtained from different clean-up procedures were discussed.

2. Experimental

2.1. Chemicals

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

The compounds studied 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-methyldipiryrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipiryrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-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[3,4-*b*]indole (Harman) and 9*H*-pyrido[3,4-*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. TriMeIQx and 7,8-DiMeIQx were used as internal standards (2 μg ml^{-1} methanolic solution).

Diatomaceous earth extraction cartridges (Extrelut-20) and refill material were provided by Merck (Darmstadt, Germany); PRS (500 mg), C_{18} (100 mg and 500 mg) and strong cation-exchange (SCX) (100 mg) Bond Elut cartridges, as well as coupling pieces and stopcocks were from Varian Associates (Harbor City, USA). These cartridges were preconditioned with dichloromethane (7 ml) for PRS and methanol (5 ml) and water (5 ml) for C_{18}. A commercial meat extract, Bovril (CPC, Esher, UK) was obtained from a local market.

2.2. Instruments

Reversed-phase HPLC analyses were performed by means of a Pharmacia LKB HPLC System (Uppsala, Sweden) equipped with a high pressure mixer and a Rheodyne 7125 Injector (Cotati, CA, USA). Detection and confirmation of the peaks in the sample were carried out with a Beckman System Gold 168 (Fullerton, CA, USA) photodiode-array

UV detector. Spectra of peaks were recorded from 200 to 300 nm. Peak identification was achieved by comparing the peaks' UV absorbance spectrum with library spectra acquired from standard solutions.

The amines were separated using a TSK-Gel ODS 80T column (5 μm, 25.0×4.6 mm I.D.) (Toso Haas, Stuttgart, Germany) and a Supelguard LC-8-DB pre-column (Supelco, Gland, Switzerland).

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

2.3. Chromatographic conditions

Binary mobile phase at a flow-rate of 1 ml min^{-1} was used to separate the analytes. Solvent A: 0.01 *M* triethylamine in water adjusted with phosphoric acid to pH 3.3; solvent B: acetonitrile. The gradient program was: 5–23% B in A, 0–18 min; 23% B in A, 18–25 min; 23–55% B in A, 25–33 min.

2.4. Sample preparation

A commercial meat extract (Bovril) was suspended in water and the mixture was homogenized by stirring with a peel mixer. Afterwards the material was spiked with measured amounts of IQ (30 μg kg^{-1}), MeIQx (50 μg kg^{-1}), 4,8-DiMeIQx (10 μg kg^{-1}) and PhIP (30 μg kg^{-1}) and the mixture was stirred for 2 h more. Finally, the extract was conveniently lyophilized, ground and sieved.

2.5. Solid-phase extraction procedures

Different SPE procedures were compared to establish the best conditions for the determination of HAAs in beef extracts. Most of the procedures were based on the method initially described by Gross [32] (method A), and later modified by several authors [31,33,34] (methods B and C). All include the use of various adsorbents to give a final extract containing the HAAs with minor interferences. In this paper another modification is proposed as method D. Fig. 2 outlines the different procedures. In some cases, with every new batch of solid adsorbent cartridges, specially PRS, the elution volume and/or solvent composition had to be adjusted for maximum recovery of HAAs.

2.5.1. Method A

A 2-g beef extract sample or an appropriate standard mixture aliquot was dissolved in 12 ml 1 *M* NaOH; after shaking until homogenization for 3 h, with sonication if necessary. The alkaline solution was mixed with Extrelut refill material (15 g) and used to fill an empty Extrelut column that was coupled to a Bond-Elut PRS column. The analytes adsorbed on the Extrelut packing were extracted using dichloromethane, which was introduced into the PRS (500 mg) column coupled on-line, and the extraction was stopped when 75 ml had passed through the coupling. The Extrelut column was discarded, and the PRS column was dried and successively rinsed with 6 ml 0.01 *M* HCl, 15 ml methanol–0.1 *M* HCl (4:6, v/v) and 2 ml water. The PRS column was then coupled to a C_{18} (100 mg) column. This tandem was eluted with 20 ml of 0.5 *M* ammonium acetate at pH 8.0. The adsorbed HAAs were then 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 analysed using the HPLC–DAD method described earlier.

2.5.2. Method B

This procedure was performed as in method A, but in this case the PRS column was rinsed with three different solvents: 6 ml 0.01 *M* HCl, 15 ml methanol–0.1 *M* HCl (6:4, v/v) and 2 ml water. These fractions, which contained the less polar HAAs, were collected and neutralized with 500 μl ammonia, the neutral solution was diluted with 25 ml water and passed through a C_{18} (500 mg) column. The amines retained were eluted, after rinsing with 2 ml water, using 1.4 ml of methanol–ammonia (9:1, v/v) providing the named *less-polar extract* (B1). The PRS column was then coupled to a C_{18} column and treated as in method A, which provided the named *polar extract* (B2). Finally the two extracts obtained were separately processed, as in method A, giving the methanolic solutions for analysis.

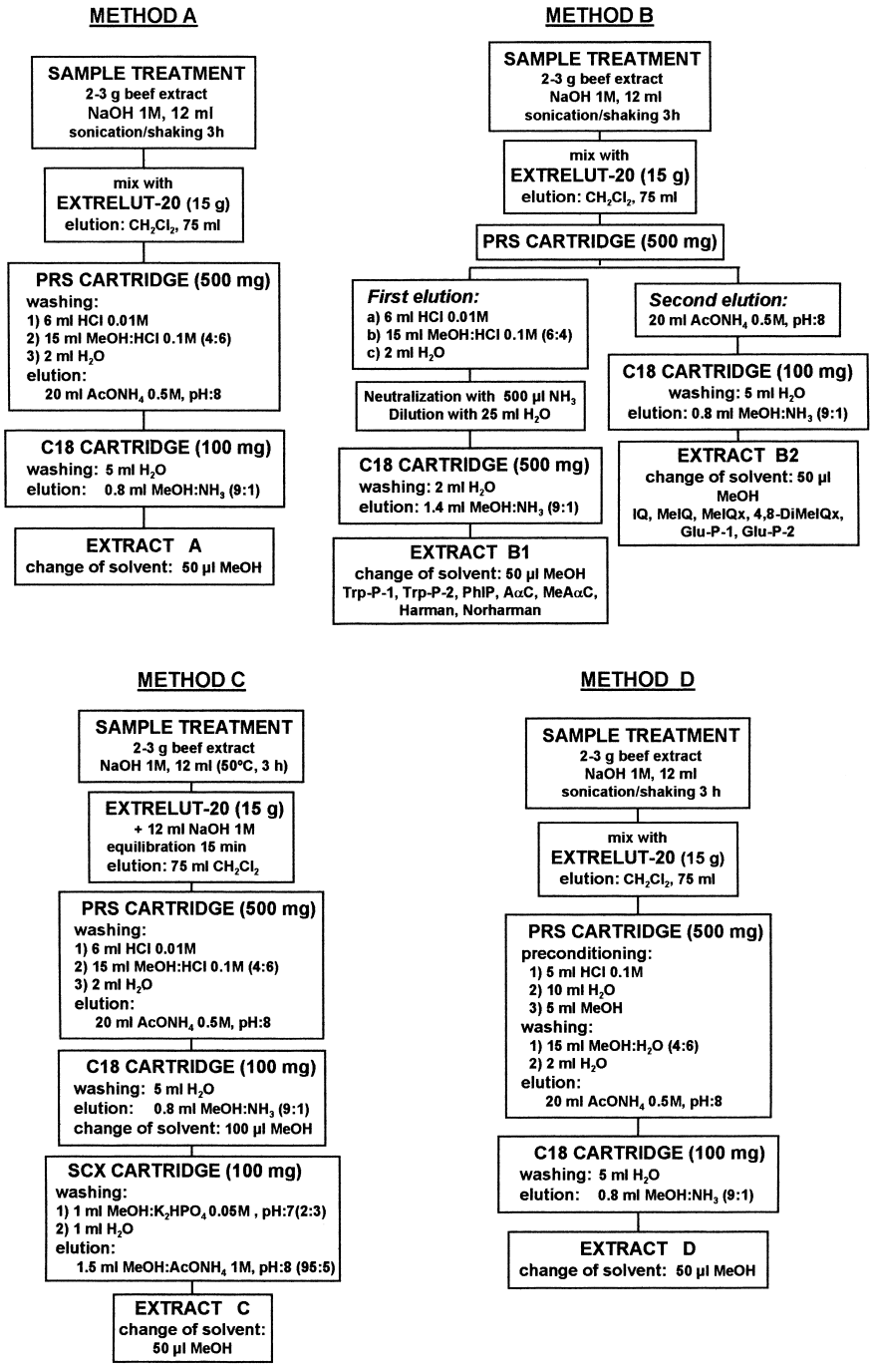

Fig. 2. Solid-phase extractions procedures.

2.5.3. Method C

In this procedure the alkaline solution was digested at 50°C for 90 min as the original reference recommended, and the option without heating was checked as well. Unlike method A, the final extract was additionally purified using a propylbenzylsulphonic column [strong cation-exchange (SCX)]. The methanolic solution (100 μl), without internal standard, was introduced into SCX column (100 mg). After rinsing with 1 ml methanol–0.05 *M* K_2HPO_4 at pH 7.0 (2:3, v/v) and 1 ml water, the adsorbed HAAs were eluted with 1.5 ml of methanol–1 *M* ammonium acetate at pH 8.0 (95:5, v/v). Finally, 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 analysed using the HPLC–DAD method described earlier.

2.5.4. Method D

In contrast with method A, the PRS column was preconditioned with 5 ml 0.1 *M* HCl, 10 ml water and 5 ml methanol, moreover, this column was rinsed with 15 ml methanol–water (4:6, v/v) instead of the methanol–0.1 *M* HCl (4:6, v/v) solution.

3. Results and discussion

The aim of this study was to compare different solid phase extraction methods for the determination of heterocyclic aromatic amines in complex matrices such as meat extracts. Recoveries of HAAs using A, B, C and D clean-up procedures were obtained by processing both a standard mixture solution of HAAs in methanol and the contaminated meat extract described above.

The HAAs were arranged in two groups, the polar and less-polar amines, on the basis of their behaviour in the chromatographic separation. The polar amines group included to Glu-P-1, Glu-P-2, IQ, MeIQ, MeIQx and 4,8-DiMeIQx, and the less-polar amines were Trp-P-1, Trp-P-2, PhIP, AαC, H and NH. The recoveries corresponding to each clean-up procedure were calculated using the standard addition method performed in duplicate at three addition levels. The spiked samples were prepared by addition of a standard mixture solution containing about 80, 160 and 320 ng of each HAA at the beginning of the process, when 10 ml of NaOH was added. Recoveries were estimated from these additions with HPLC–DAD system. These values were calculated from the slope of the regression line performed for the amount added versus the amount measured. The recovery values for the most polar of the amines, obtained using a standard mixture solution, are given in Table 1; methods A, B and D provided similar recovery values ranging between 60% to 90% with confidence intervals of about 10%. Method C, proposed by Perfetti, gave the lowest recoveries especially for Glu-P-1 and Glu-P-2, the latter not being detected in the final extract.

In relation to the less polar amines, a different behaviour was observed between methods (Table 1). They can be paired in two groups; methods B and D gave similar recoveries that were good enough for quantification, while methods A, Gross and C, Perfetti, gave poor results for all less-polar amines because none of them could be detected in the final extracts except PhIP when using method A, where the recovery was 15%.

When the SPE procedures were applied to a complex matrix, the meat extract, the polar amines presented a similar behaviour as with methanolic standard mixture solution. Methods A, B and D gave the best recoveries for all amines except IQ for method D, in this case a matrix interference coeluted with the analyte impeded quantification. In method C, two temperatures (room temperature and 50°C) were used for solving the sample in sodium hydroxide solution. In both cases, at 25 and at 50°C, recoveries were lower than for other procedures, and Glu-P-1 and Glu-P-2 were lost as had occurred with methanolic solutions. Nevertheless, extract C provided the more efficient clean up as can be seen in Fig. 3, where the chromatograms corresponding to extracts A and C are given. Comparing both chromatograms, extract C was cleaner, in particular in the zone where IQ appears. This could be due to the additional clean-up step using SCX cartridges, in fact, several peaks in extract A approach to that of the analyte, reducing the robustness of the separation.

The recoveries of the less-polar amines in a complex matrix, the meat extract, agreed with those obtained for a single matrix, the methanol solution,

Table 1
Analyte recoveries obtained by processing a standard mixture solution of HAAs in methanol[a]

Compound	Method A	Method B	Method C	Method D
Glu-P-2	83.5 (4.2)	75.5 (5.0)	n.r.	87.2 (3.4)
IQ	71.8 (2.7)	69.7 (5.7)	28 (12)	74.89 (0.74)
MeIQ	63 (10)	56.4 (8.6)	47 (11)	70.3 (1.3)
Glu-P-1	87.0 (4.6)	84.3 (8.2)	3.38 (0.67)	90.6 (4.4)
MeIQx	87.17 (0.21)	77.2 (4.8)	37.9 (6.3)	85.6 (3.4)
7,8-DiMeIQx	82.3 (2.3)	71.0 (4.6)	49.7 (9.6)	82.5 (2.5)
4,8-DiMeIQx	82.1 (1.9)	72.3 (2.9)	53 (10)	82.0 (1.5)
Norharman	n.r.[b]	75.2 (7.6)	n.r.	74.7 (5.0)
Harman	n.r.	83.2 (4.4)	n.r.	55 (13)
Trp-P-2	n.r.	72.5 (8.2)	n.r.	46.6 (8.8)
PhIP	16.9 (3.2)	70.0 (4.4)	n.r.	70.1 (2.7)
Trp-P-1	n.r.	72.8 (6.3)	n.r.	26.2 (3.8)
AαC	n.r.	57.9 (2.3)	n.r.	83.8 (3.6)

[a] Confidence intervals ($n=8$, $\alpha=0.05$) are expressed in parentheses.
[b] n.r.=Not recovered.

though only methods B and D provided good results. Recovery values ranged between 45 and 90%, and their confidence intervals were less than 10%. When using methods A and C the less-polar amines were completely lost in the clean-up process. Fig. 4 shows the chromatograms obtained for extracts B and D. Method B provided two final extracts containing polar and less-polar amines respectively. In contrast, method D provided only a single extract. For polar amines, a cleaner extract was obtained using method B and, as can be seen, two major interferences that appeared in the less polar extract were eliminated in the clean-up procedure. One of these interferences prevented the determination of IQ with method D. For the less-polar amines a similar clean-up was achieved with both methods except for AαC for which higher purification was obtained with method D due to the washing of the PRS cartridge before extraction. This method had the advantage of being faster (analysis time was reduced by 40%) and would seem suitable for the screening of unknown samples.

In Table 2 the results obtained in the analysis of the meat extract using methods A, B, C and D are shown. For comparative purposes, the results obtained using method B and MS-electrospray ionization (ESI) [24] and MS-atmospheric pressure chemical ionization (APCI) [35] and our results from the intercomparison exercise [30] using ED are also included. As can be seen, the results were in good agreement for all the compounds and methods used for clean-up, in addition the results agreed with those obtained using method B with different LC procedure and operator. Nevertheless, an interference prevented the analysis of IQ using method D, PhIP was not detected when methods A and C were used, and method C (Perfetti) gave higher standard deviations which might be related to the lower recoveries.

4. Conclusions

To date, procedures based on the Gross method are the most commonly applied to the sample treatment for HAA analysis. Most of these procedures lack in both the clean-up efficiency and the reproducibility of the results, therefore, much efforts are still necessary to improve them. Furthermore, parameters such as recovery depend, in a great extent, on the matrix of sample, thus, is very difficult to establish a general procedure for the analysis of HAAs. For these reasons, MS becomes a valuable tool being the most suitable to obtain reliable results in the analysis of complex matrices. However, in the routine analysis of known samples, the use of detection methods such as DAD can produce results good enough for quantitative purposes, provided that the method is previously validated.

In general, a compromise needs to be achieved between high recovery and clean-up efficiency. This depends greatly on the complexity of the sample

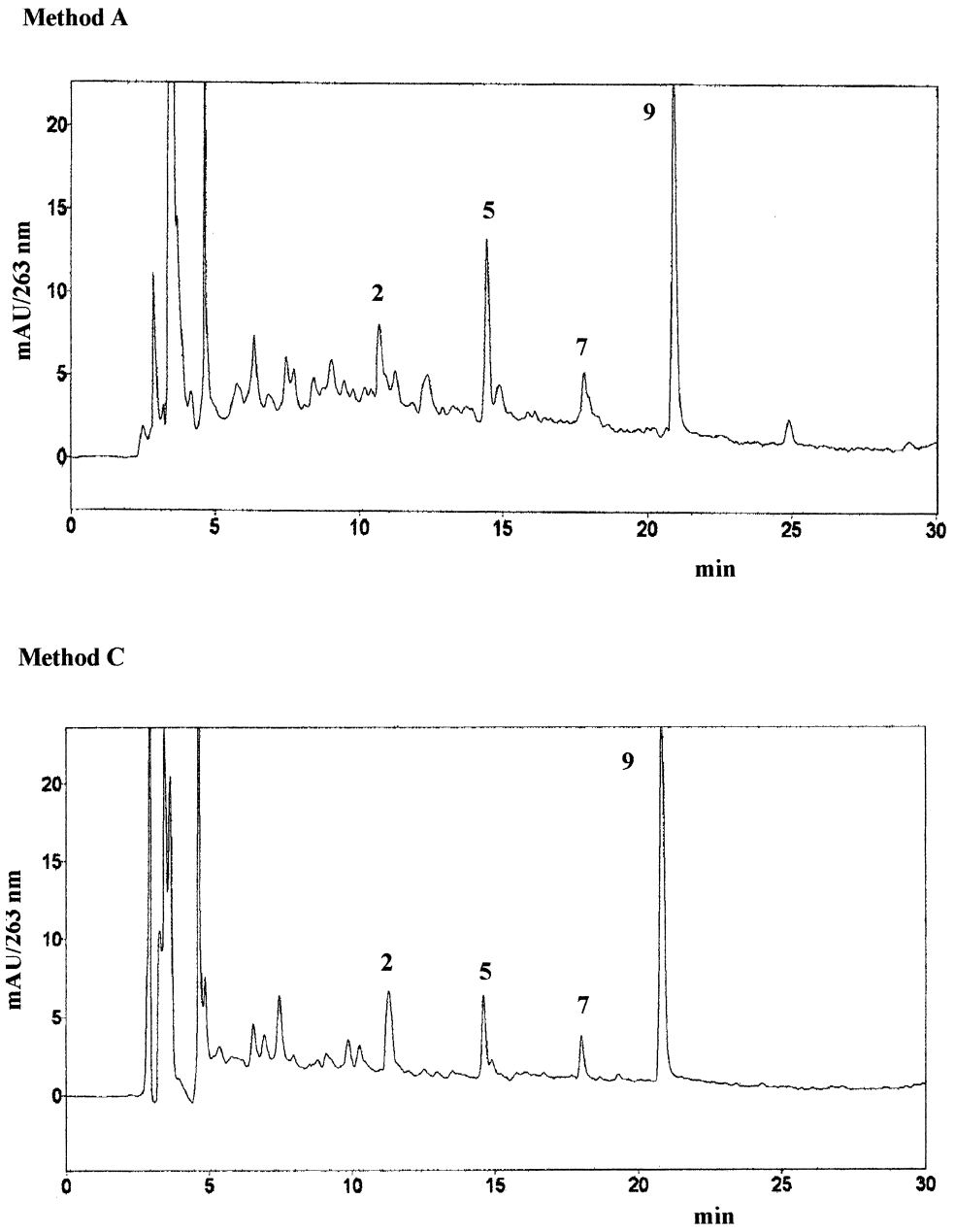

Fig. 3. Chromatograms of the meat extract obtained with methods A and C. Peaks: 2=IQ; 5=MeIQx; 7=4,8-DiMeIQx; 9=TriMeIQx (I.S.).

matrix. In our tests, the best performance was obtained when using a clean-up procedure that combined Extrelut, PRS and C_{18} cartridges, a selective elution of polar and less-polar HAAs from PRS cartridge, as described in method B. This provided both high recoveries (60–90%) and a high degree of accuracy in the results, although this time-consuming procedure was not suitable for screening analysis.

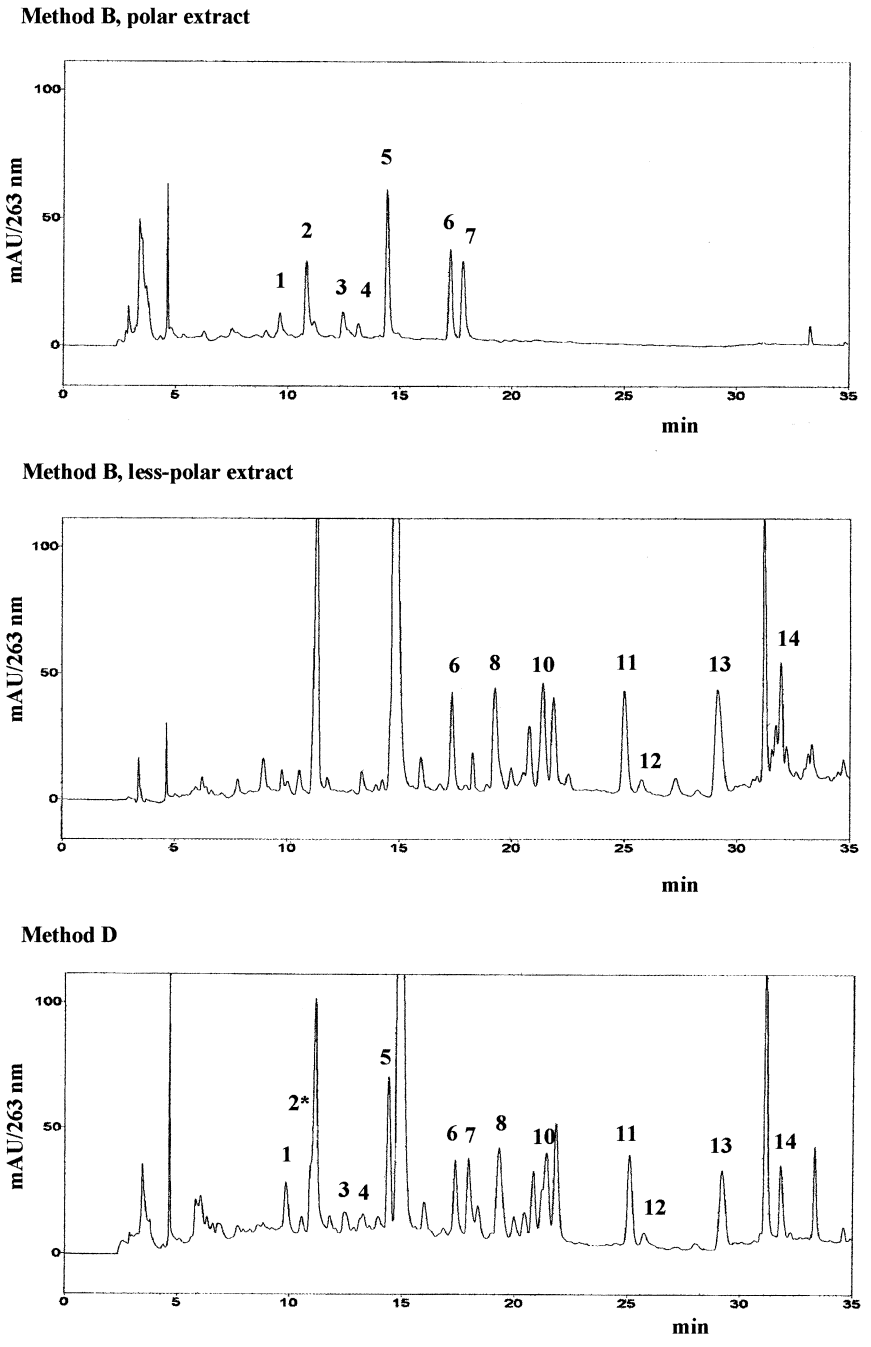

Fig. 4. Chromatograms of the meat extract spiked at 100 ng/g of each amine obtained with methods B and D. Peaks: 1=Glu-P-2; 2=IQ; 3=MeIQ; 4=Glu-P-1; 5=MeIQx; 6=7,8-DiMeIQx (I.S.); 7=4,8-DiMeIQx; 8=Norharman; 10=Harman; 11=Trp-P-2; 12=PhIP; 13=Trp-P-1; 14=AαC. *=Non pure peak.

Table 2
Analytical results (ng g^{-1}) for the contaminated meat extract

Method	Compound			
	IQ	MeIQx	4,8-DiMeIQx	PhIP
A	29.6±9.9	23.5±10	11.1±6.9	–
B	29.7±8.8	41±14	14±6.1	38±12
B[a]	36±4.9	38±4.4	10±2.2	30±4.3
B[b]	40±4.8	41±3.6	12±2.1	29±5.0
B[c]	43±2.0	40±1.0	13±2.0	32±2.7
C, 50°C	40.5±6.4	18.4±8.6	6.7±5.2	–
D	–	33.4±2.5	8.9±5.8	28.8±9.2

[a] LC–ES-MS.
[b] LC–APCI-ES.
[c] LC–ED.

The method proposed by Perfetti, method C, was the most efficient clean-up procedure for polar amines, although lower recoveries and higher standard deviations were obtained, moreover, Glu-P-1, Glu-P-2, and less-polar amines were not recovered. Method D gave acceptable results but some interferences might prevent quantitative determinations as was the case with IQ. Method B appeared to be the most consistent when analysing materials containing both polar and less-polar amines; nevertheless, method D proved to be a relatively faster procedure suitable for screening unknown samples. It should be emphasized that complex matrices such as processed flavors containing meat extracts may need further clean-up for a successful determination of HAAs. Furthermore, the reliability of different clean-up methods should also be considered in relation to the HPLC–detection system. Thus, for UV detection and in the determination of polar amines method C provided the cleanest extract; nevertheless, when more selective detection methods are used, such as mass spectrometry and electrochemical detection, procedures A, D and of course, B could be applied to obtain results good enough for quantitative purposes.

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