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Solid-phase extraction for the selective isolation of polycyclic aromatic hydrocarbons, azaarenes and heterocyclic aromatic amines in charcoal-grilled meat

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Abstract

A method for the simultaneous analysis of 12 mutagenic and/or carcinogenic compounds is described; these substances belong to three different chemical groups: polycyclic aromatic hydrocarbons (PAHs), azaarenes, i.e., nitrogen-containing polycyclic aromatic hydrocarbons (PANHs), and heterocyclic aromatic amines (HAAs). The selective enrichment procedure includes coupling of solid-phase extraction (SPE) steps using diatomaceous earth, propylsulfonic acid, silica gel and octadecylsilane columns. The eluted fractions were analyzed by high-performance liquid chromatography with UV and electrochemical detection. Levels measured were estimated to be 4–19 ng g^{-1}. Peak confirmation was carried out by GC–MS for both PAHs and PANHs, and by LC with a photodiode array detector for HAAs. The method was applied to the analysis of charcoal-grilled meat and was judged to be generally applicable for detection of these mutagens at the ppb level in processed foods.

Keywords: Food analysis; Sample handling; Polynuclear aromatic hydrocarbons; Azaarenes; Amines, heterocyclic aromatic

1. Introduction

For a long time, the diet has been associated with varying cancer rates in human populations, yet the causes of the observed variation in cancer patterns have not been adequately explained [1]. As early as 1964 polycyclic aromatic hydrocarbons known to be carcinogenic, such as benzo[*a*]pyrene, were detected in broiled meat [2]. Numerous papers have been published about PAHs found in smoked and thermally-treated foods as a result of pyrolysis or incomplete combustion of organic matter [3–7]. Less information is available on their nitrogen analogues, the basic azaarenes PANHs, but they have been shown to be present in association with PAHs in various samples that contain nitrogen, such as processed foods [8–10].

The introduction of the Ames test in 1975 [11] provided a rapid method of isolating potential carcinogens in food on the basis of their mutagenic activity. Over the past 10 years a number of potent bacterial mutagens, all belonging to the class of heterocyclic aromatic amines (HAAs), have been purified from pyrolyzed amino acids and proteins, cooked, protein-rich foods such as, beef, chicken and fish, cooked by typical household methods [12–16] and also in beef extracts [17]. The overlap of mutagenicity and carcinogenicity, although contro-

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versial, is now widely accepted [18]. Bacterial mutagenicity assays have shown most of these compounds to be powerful mutagens and several of them induce cancer at multiple sites in rodents and may be potential human carcinogens [19,20].

The assessment of mutagenic activity in cooked foods requires tedious extraction work in order to isolate and quantify the chemicals responsible at the ng level. Efforts have been made to develop a rapid and efficient method to obtain chromatograms free of interfering material. Co-extracted matrix components influence analyte detection limits more than does the absolute detector sensitivity [21]. The sample work-up, therefore, is the most critical part of the analysis [22]. Solid-phase extraction (SPE) with different coupled columns provides an improvement [14] over liquid–liquid extraction and the use of large columns filled with XAD resin. The determination of PAHs and PANHs in foods has been carried out by different chromatographic techniques, including liquid chromatography (LC) with fluorescence [4,5,23] and/or UV detection [9,24], and gas chromatography (GC) with flame ionization detection [6,24] or mass spectrometry [25]. Recommended analytical procedures for the determination of PAHs in environmental samples are documented or proposed in several European and USA guidelines including the US Environmental Protection Agency (EPA) methods [26,27], International Standard Organization (ISO) method [28] and German Standard (DIN) method [29]. All of these methods specify reversed-phase chromatography using octadecyl (C_{18}) bonded phases in combination with either fixed or wavelength-programmed UV and fluorescence detection techniques. Ultimately, major efforts to develop coupled chromatographic techniques have been performed to alleviate the problem of manual sample pretreatment and to enhance the sensitivity and selectivity in the analysis of PAHs in foodstuffs [30] and environmental samples [31,32]. Liquid chromatography-mass spectrometry [33,34], gas chromatography-mass spectrometry [13], HPLC with UV absorbance and fluorescence detection [15], electrochemical detection (ED) [17] and ELISA immunoassay [35] have been successfully used for the determination of HAAs.

In this paper an analytical method for the simultaneous determination of PAHs, HAAs and PANHs, such as those listed in Table 1, is established. The method for purification of these compounds reported here involves coupling of different solid-phase extraction (SPE) steps. The final eluted fractions were analyzed by LC, with UV detection for PAHs and PANHs [8] and electrochemical detection for HAAs [17]. Peak confirmation was carried out by GC–MS for both PAHs and PANHs [8], and by HPLC with a photodiode array detector for HAAs [14]. The method developed was applied to the determination of these mutagenic compounds in charcoal-grilled meat.

2. Experimental

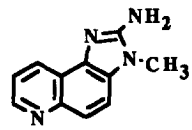
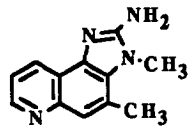
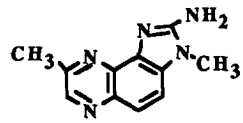
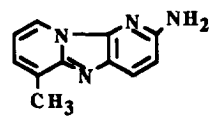
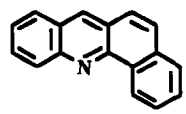
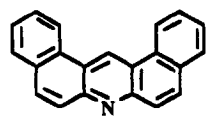
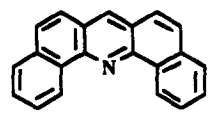
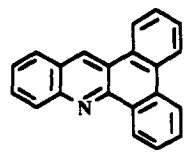
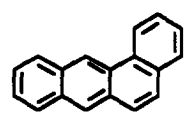
2.1. Chemicals

Heterocyclic amines were provided by Toronto Research Chemicals (Toronto, Canada), acridines were obtained from the Commission of the European Communities, Bureau of Reference (BCR) (Brussels, Belgium) and polycyclic aromatic hydrocarbons were obtained from Fluka (Buchs, Switzerland); standard stock solutions of 100 $\mu g\ ml^{-1}$ in methanol for HAAs and PANHs, and in isooctane for PAHs were prepared and used for further dilutions. Aniline (Carlo Erba, Milano, Italy) and coronene (Fluka) were used as internal standard (solutions of 1 $\mu g\ ml^{-1}$ in methanol and acetonitrile, respectively).

Diatomaceous earth extraction columns (Extrelut; 20 ml) were provided by Merck (Darmstadt, Germany). Bond-Elut propylsulfonic acid (PRS; 500 mg) and octadecyl–silane columns (C_{18}; 100 mg) as well as coupling pieces and stopcocks were from Analytichem International (ICT, Basel, Switzerland). These columns were preconditioned with dichloromethane (4 ml) for PRS and methanol (10 ml) and water (10 ml) for C_{18}, respectively. Silica gel (70–230 mesh) was provided by Merck and was activated at 200°C for 12 h and preconditioned with hexane.

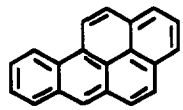
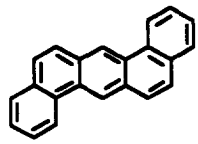
Other solvents and chemicals were HPLC or analytical grade, and the water was purified using a Culligan (Barcelona, Spain) system. All the solutions were passed through a 0.45-μm filter before injection onto the HPLC system.

Table 1
Identification, abbreviations and structures of the compounds studied in this work

	Name	Structure	Abbreviation
1	2-Amino-3-methylimidazo[4,5-*f*]quinoline		IQ
2	2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline		MeIQ
3	2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline		MeIQx
4	2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole		Glu-P-1
5	Benzo[*c*]acridine		B(c)Ac
6	Dibenzo[*a*,*j*]acridine		Db(a,j)Ac
7	Dibenzo[*a*,*c*]acridine		Db(a,c)Ac
8	Dibenzo[*c*,*h*]acridine		Db(c,h)Ac
9	Pyrene		PYR
10	Benzo[*a*]anthracene		B(a)A

(Continued on p. 88)

Table 1
Continued

	Name	Structure	Abbreviation
11	Benzo[*a*]pyrene		B(a)P
12	Dibenzo[*a*,*h*]anthracene		Db(a,h)A

2.2. Instruments

HPLC with electrochemical detection was carried out with a Gilson Model 302 pump with an 802 manometric module (Gilson, Villier-le-Bel, France) and a Metrohm wall jet electrochemical detector, Model 656, equipped with a working electrode (Glassy Carbon Electrode, Model 6.0805.010), a reference electrode (Ag/AgCl/KCl 3 *M*) and an auxiliary electrode (Glassy Carbon Electrode, Model 6.0805.010) (Metrohm, Herisau, Switzerland). An Applied Biosystem (Foster City, CA, USA) model 1000s photodiode array UV detector was used for the confirmation of the HAA peaks of the samples. A data processor, Chromatopac C-R3A (Shimadzu, Kyoto, Japan), was used.

HPLC with UV-spectrophotometric detection was carried out with a Hewlett-Packard (Waldbronn, Germany) Series 1050 pump. Chromatographic data were recorded with a Hewlett-Packard Vectra QS/16S data system. A Rheodyne 7125 injector equipped with a loop of 20 μl was used.

GC–MS analyses were performed by electron impact (EI) (70 eV) in a Hewlett-Packard 5988A MS quadrupole coupled to a 5890 GC interfaced to a 9825A data system. Transfer line, ion source and analyzer temperatures were held at 280, 250 and 250°C, respectively. Samples were injected in the splitless injection mode.

A Supelco Visiprep and Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used for the solid-phase extraction steps in the clean-up procedure.

2.3. Analytical procedure

Beef samples were thermically processed as described previously [8]. The charcoal grilled samples obtained were purified according to the procedure schematically presented in Fig. 1. After saponificating the sample with 12 ml of 1 *M* NaOH and shaking until homogenisation during 6 h, the first step used a diatomaceous earth column (Extrelut 20) coupled to a propylsulfonic column (PRS). The alkaline solution was mixed with Extrelut refill material and used to fill an Extrelut column that was coupled to a Bond-Elut PRS column. The extraction was performed with 45 ml dichloromethane (DCM). The DCM fraction eluted, which contained the PAHs, was evaporated to dryness, redissolved in 1 ml hexane and applied to the top of a 10-g deactivated silica column, 25 ml hexane were added and discarded, then the PAHs were eluted with 25 ml hexane–DCM (60:40), eliminating the fat content of the final extract. The solvent of the eluate was evaporated and the residue was dissolved in methanol (250 μl) for the HPLC-UV analysis.

The Extrelut column was discarded, and the PRS column was successively rinsed with 6 ml 0.1 *M* HCl and 2 ml water. The PRS column was then coupled to C_{18} column (100 mg), previously conditioned as described before. This tandem was first eluted with 20 ml 0.5 *M* ammonium acetate at pH 8.0 in order to pass the HAAs onto the C_{18} column, whereas the PANHs remained in the PRS. This coupling was removed and both columns were rinsed with 10 ml water. The adsorbed HAAs and PANHs

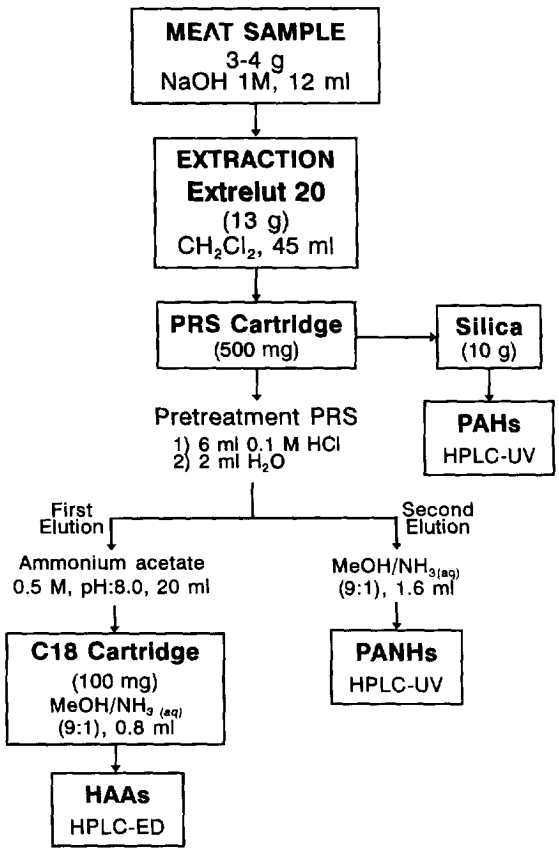


Fig. 1. Clean-up procedure scheme.

were eluted with 0.8 and 2 ml methanol–ammonia (9:1), respectively. The solvent was evaporated under a stream of nitrogen and the analytes were dissolved in the internal standard solution (50 μl for HAAs and 250 μl for PANHs).

Chromatographic analysis was performed using HPLC under the conditions previously described [8,17] and given in the legend to Fig. 2. Fig. 2 shows chromatograms of standard solution mixtures (1 $\mu g\ ml^{-1}$) under the working conditions. Peak confirmation for the PAHs and PANHs fractions was performed using GC–MS on a DB-5 fused-capillary column (30 m×0.25 mm I.D., 0.25 μm film thickness) (J&W Scientific, Folsom, CA, USA) with helium (30 cm s^{-1}) as carrier gas. Peak confirmation of the HAAs fraction was carried out by HPLC with photodiode array detection. In this case the sepa-

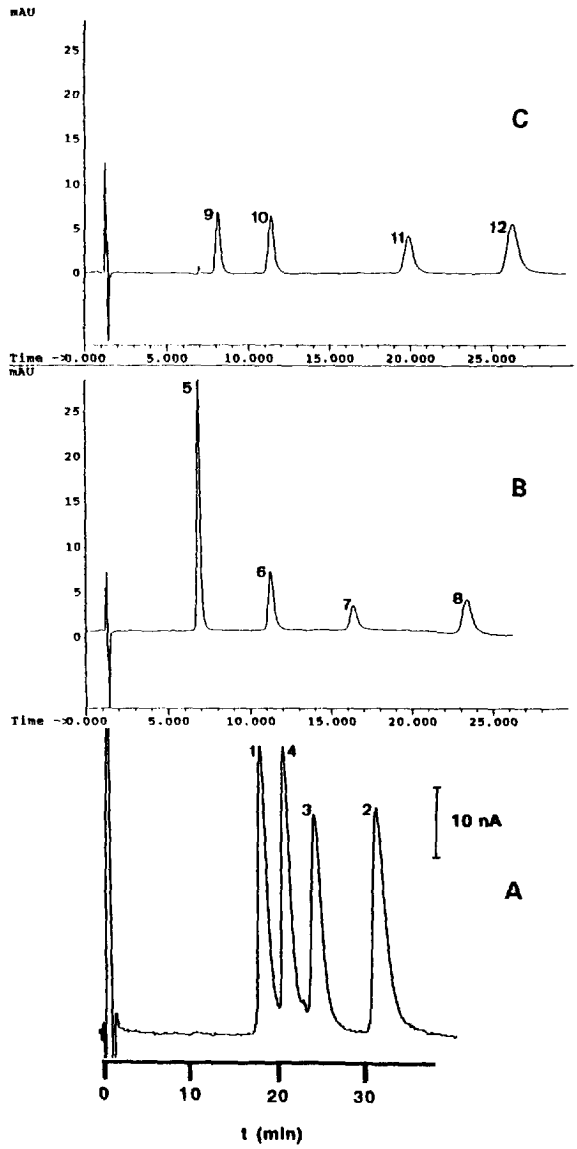


Fig. 2. Chromatogram of standard solutions. (A) HAAs, (B) PANHs and (C) PAHs. Peaks: 1=IQ; 2=MeIQ; 3=MeIQx; 4=Glu-P-1; 5=B(c)Ac; 6=Db(a,j)Ac; 7=Db(a,c)Ac; 8=Db(c,h)Ac; 9=PYR; 10=B(a)A; 11=B(a)P; 12=Db(a,h)A. (A) HPLC-ED: column, TSK-Gel ODS 80T C_{18} (5 μm, 25.0 cm×4.6 mm); mobile phase, acetonitrile–50 m*M* ammonium acetate (pH 4) (10:90); flow-rate, 1.0 ml min^{-1}; applied working potential, +1000 mV. (B) and (C) HPLC-UV: column, Nucleosil 120 C_{18} (5 μm, 15.0 cm×4.0 mm); mobile phase, methanol–water (84:16); flow-rate, 1.0 ml min^{-1}; wavelength, 280 nm.

ration was performed using the mobile phase described by Gross [14]. Peak identification was achieved by comparison of the retention time of both mobile phases, and by on-line recorded UV spectra with those corresponding to standards.

3. Results and discussion

3.1. Clean-up procedure study

The enrichment procedure applied in the sample preparation allowed to obtain three fractions corresponding to PAHs, PANHs and HAAs, with yielded recovery levels. Sample purification was based on the coupling of different solid-phase extraction steps, which had been successfully applied in the analysis of HAAs [14,36]. In this work, the procedure was extended for the determination of two more families of compounds, PAHs and PANHs, involving an additional SPE step using silica gel as stationary phase for PAHs and a selective elution of HAAs and PANHs from the PRS column using ammonium acetate and methanol–ammonia as described in Fig. 1.

In order to achieve good recovery values and to check the suitability of the clean-up procedure, the different steps were studied separately. This study was carried out with two compounds of each chemical group: MeIQx and IQ for HAAs, BaA and BaP for PAHs, and Db(a,c)Ac and Db(c,h)Ac for PANHs. The experiments were performed taking samples (10 ml) of a mixture of the reference standards in dichloromethane solution containing 50 ng ml^{-1} of each analyte, and the recovery values of each step were calculated by comparing the ratio of the peak areas (analyte/internal standard) with those of a control sample (which represents 100% recovery). The recovery values obtained for each step of the clean-up procedure are given in Table 2.

The SPE step using diatomaceaous earth as stationary phase and DCM as eluant was the first stage evaluated. The standard solution was mixed with the Extrelut packing and the elution was performed with 45 ml DCM. Irrespective of flow-rate (0.5–5 ml min^{-1}), high recovery values were obtained for each compound, i.e., about 100%.

The next stage consisted of coupling two SPE columns; thus, the solvent eluted from Extrelut was passed through a PRS column containing a strong cation exchanger as stationary phase (propylsulfonic acid on silica base). The retention and elution for each group of compounds in the PRS column was the second step studied. When DCM was passed through this coupling, the separation of the three types of compound into two groups was achieved: the HAAs and PANHs were completely retained, whereas the PAHs were eluted. Different flow-rates were tested; at high values (i.e., 5 ml min^{-1}) acridines were not retained sufficiently, thus, Db(c,h)Ac was collected with the PAH fraction and Db(a,c)Ac had a low recovery (42%). The best separation with good recovery values was achieved at lower flow-rates, and further experiments were performed at flow-rates of 0.5 ml min^{-1}.

In the analysis of real samples the extract that contained the PAHs was further purified in a silica column in order to remove the fat content; the efficiency of this step was also checked. A standard solution in DCM was passed through an active silica column, where PAHs were retained, and further elution was performed with a mixture of hexane–

Table 2
Percentage of recovery and relative standard deviations for a standard solution in the different clean-up steps ($n=4$)

SPE	BaA	BaP	Db(ac)Ac	Db(ch)Ac	IQ	MeIQx
Extrelut	96±2	94±2	95±1	94±1	93±2	95±1
PRS	102±1	105±6	92±6	95±4	90±6[a]	92±7[a]
Silica	95±2	100±1	–	–	–	–
C_{18}	–	–	–	–	100±2	98±3

[a]Tandem PRS-C_{18}.

DCM (60:40). Recoveries higher than 90% were obtained in this final step.

The HAAs and the PANHs were selectively eluted using 0.5 *M* ammonium acetate solution (pH 8.0) as first eluant in the PRS column. Then, the HAAs were eluted into a second column with octadecylsilane as stationary phase, and both the PANHs retained in the PRS and the HAAs retained in the C_{18} were finally removed using methanol–ammonia (90:10). Recovery values of this step for all the compounds ranged from 62 to 103%.

As the clean-up optimization of each step gave satisfactory results, the total procedure was tested for two more compounds of each family before applying it to the analysis of real samples. Figures of merit for the proposed method are given in Table 3. The recovery values and the relative standard deviations obtained for five replicate analyses of each compound were obtained in order to establish the run-to-run reproducibility of the overall method. Duplicate analyses were carried out at four different days in order to determinate the day-to-day reproducibility of the described method. The correlation coefficients of calibration functions in the intervals of linearity were higher than 0.999 for all the compounds. High recoveries [>80%, except for B(c)Ac (62%)] and good reproducibility (day-to-day) with low relative standard deviations (R.S.D. between 4 and 11%) were obtained, showing the suitability of the method for the simultaneous analysis of these compounds in complex samples.

3.2. Analysis of charcoal-grilled meat sample

The method was applied to the determine these analytes in charcoal-grilled beef. To prevent matrix effects on the extraction efficiency, the compounds were quantified by the standard addition method, performing duplicate analysis of one unspiked sample and three samples spiked at three different concentration levels. These spiked samples were prepared by addition of accurately measured amounts of each standard at the beginning of the clean-up process, when 12 ml NaOH was added. Recoveries of these compounds were estimated from the slope of the regression line performed with the added amount versus the measured amount. The percentage of recovery and the relative standard deviations for four replicates for each level are given in Table 4. Compared with the results in Table 3, most of the compounds gave lower recovery values, probably due to the matrix effect. The detection limits of these compounds in real samples, based on a signal-to-noise ratio of 3, are given in Table 4. The values

Table 3
Figures of merit of the clean-up procedure

Analyte	Interval of linearity (ng)	Run-to-run ($n=5$)		Day-to-day ($n=4$)	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
PYR	1.3–24.0	102	3	96	7
B(a)A	0.6–21.3	99	3	100	8
B(a)P	2.1–34.2	101	3	100	5
Db(a,h)A	5.2–62.4	101	2	98	4
IQ	6.5–65.0	103	4	99	6
Glu-P-1	5.6–56.5	88	5	85	7
MeI Qx	7.2–72.1	96	8	97	11
MeIQ	11.5–115.0	103	4	98	7
B(c)Ac	1.1–45.2	62	3	65	5
Db(a,j)Ac	1.2–49.2	87	4	80	8
Db(a,c)Ac	0.5–18.1	92	5	93	7
Db(c,h)Ac	1.1–42.8	93	5	95	8

Table 4
Analysis of charcoal-grilled beef

Analyte	Recovery (%)	Concentration[a] (ng g^{-1})	Limits of detection (ng g^{-1})[d]
PYR	103±8	15±3[b]	0.3
B(a)A	91±6	19±3[b]	0.6
B(a)P	47±6	6±3[b]	1.2
Db(a,h)A	64±8	ND	2.6
IQ	54±2	7±2	4.8
Glu-P-1	60±5	ND	4.2
MeIQx	60±4	4±2[c]	4.6
MeIQ	80±1	8±2[c]	8.4
B(c)Ac	24±1	ND	1.5
Db(a,j)Ac	49±1	ND	1.6
Db(a,c)Ac	95±3	ND	0.8
Db(c,h)Ac	34±2	ND	3.0

ND, not detected. Confidence intervals are expressed as R.S.D.
[a]Value corrected by % recovery.
[b]Peak confirmed by GC–MS.
[c]Peak confirmed by HPLC with photodiode array detection.
[d]Values obtained from the charcoal-grilled meat sample.

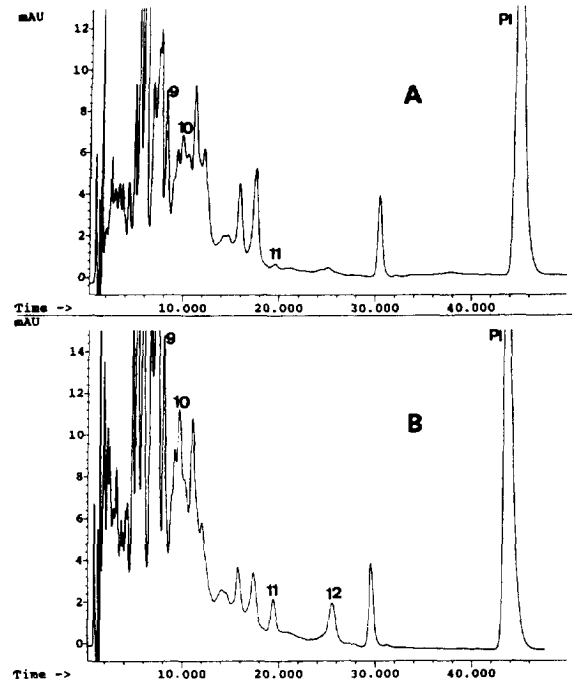


Fig. 3. HPLC-UV chromatogram of a processed meat sample. (A) unspiked charcoal grilled meat and (B) spiked sample at ppb levels of PAHs (0.2–1.5 ng g^{-1}). Peaks: 9=PYR; 10=B(a)A; 11=B(a)P; 12=Db(a,h)A; I.S.=coronene.

were calculated using low level spiking of reference standards. Samples were spiked with low amounts of each compound, extracted using the clean-up method described in the Experimental section and analyzed by HPLC. Detection limits for the analytes already present in the samples were calculated from the calibration curve, taking into account the recovery values for each compound.

In the sample preparation all the PAHs were extracted, yielding good recoveries [>65%, except for B(a)P (47%)]. HAAs recoveries were in agreement with the data previously reported [17] except for Glu-P-1, which was higher, 60%, due to changes in the pretreatment of the PRS before the selective elution. In the third fraction, which contained PANHs, only Db(a,c)Ac was extracted at a high level (95%).

PYR, B(a)A and B(a)P were identified and quantified at relatively high levels. Fig. 3 shows the chromatograms of an unspiked and spiked sample which confirm their presence. IQ, MeIQ and MeIQx were identified in the charcoal-grilled beef at ppb levels, as can be seen in Fig. 4 where chromatograms obtained with HPLC–ED are shown. In the third extract, B(c)Ac was identified with the HPLC–UV method and was found to be present at 4 ppb. Fig. 5 shows chromatograms of an unspiked and a spiked sample and confirms the presence of this compound. The remaining compounds were below the detection limits of our analytical system (Table 4).

The identification of the compounds was performed comparing the retention times of the standards and the peaks of the sample using the corresponding mobile phases. However, in the analysis of real samples peak confirmation is necessary because the chromatograms generally present peaks that elute at the same retention times as the analytes. All PAHs were confirmed by GC–MS. The mass spectrum of the B(c)Ac in the sample did not correspond to that of the standard. In order to confirm the identification of the HAAs, the mobile phase in gradient mode proposed by Gross [14] was used. The retention times for IQ, MeIQ and MeIQx agreed with the standards in both mobile phases. Furthermore, the identity of each suspected HAA was checked by comparing the UV spectrum, obtained with a photo-

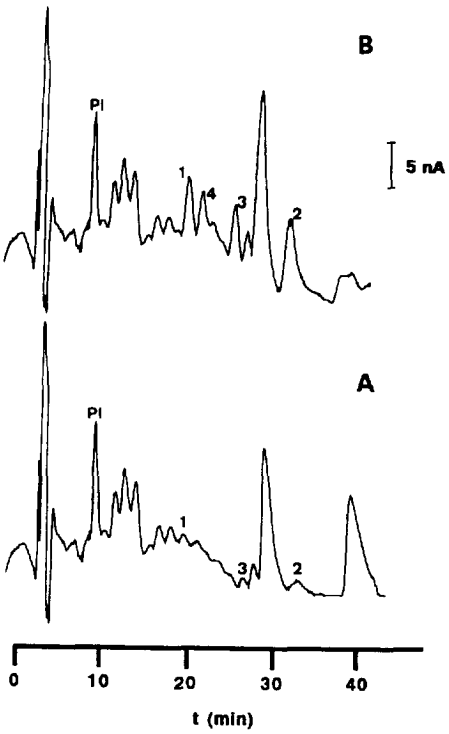


Fig. 4. HPLC–ED chromatogram of a processed meat sample. (A) Unspiked charcoal grilled meat and (B) spiked sample at ppb levels of HAAs (1.7–3.5 ng g^{-1}). Peaks: I.S.=aniline; 1=IQ; 2=MeIQ; 3=MeIQx; 4=Glu-P-1.

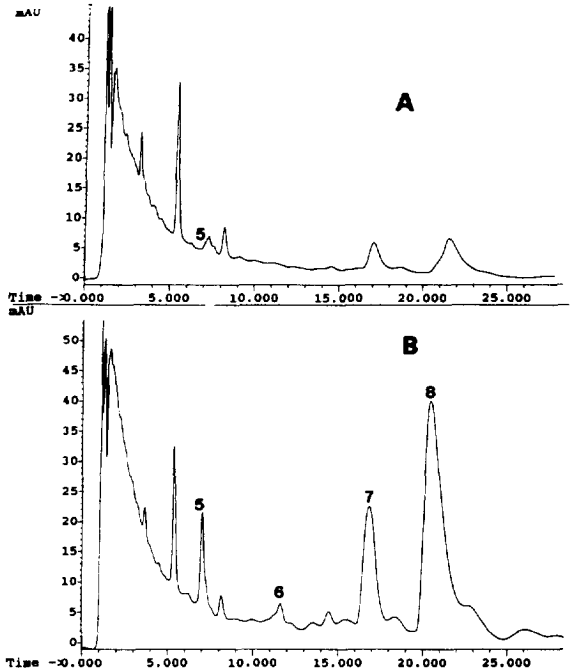


Fig. 5. HPLC–UV chromatogram of a processed meat sample. (A) Unspiked charcoal-grilled meat and (B) spiked sample at ppb levels of PANHs (0.3–1.0 ng g^{-1}). Peaks: 5=B(c)Ac; 6=Db(a,j)Ac; 7=Db(a,c)Ac; 8=Db(c,h)Ac. Peak 5 of the unspiked sample (A), evaluated by GC–MS, did not correspond to B(c)Ac.

diode array detector, with those of the standards. The presence of MeIQ and MeIQx was clearly confirmed using this detection. IQ was not confirmed because an interfering peak with a spectrum that did not correspond to that of the standard co-eluted when the UV detection conditions were used.

4. Conclusions

The extraction technique using solid-phase (SPE) coupling columns with different adsorbents, ion exchange and reversed-phases allowed us to describe a clean-up procedure for the simultaneous analysis of PAHs, PANHs and HAs from the same sample. The recoveries obtained from standard solutions have shown the suitability of the procedure developed, and were higher than 87% for all the compounds, except for B(c)Ac (62%). The sample matrix had a marked influence on the analysis, by decreasing the recoveries. Therefore, it is necessary to use the standard addition method for quantification. The sample treatment before analysis yielded a significant amount of almost all the compounds at levels between 15 and 4 ng g^{-1}. The presence of PYR, B(a)A, B(a)P, MeIQ and MeIQx at concentration levels of 15, 19, 6, 8 and 4 ng g^{-1}, respectively, was confirmed by GC–MS or HPLC–photodiode array detection.

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