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Solid-phase microextraction coupled with high-performance liquid chromatography for the analysis of heterocyclic aromatic amines

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Abstract

Solid-phase microextraction (SPME) coupled with high-performance liquid chromatography (HPLC) with UV diode array detection (DAD) for the analysis of heterocyclic aromatic amines (HAs) is described. Four kinds of fiber coatings: Carbowax–templated resin (CW–TPR), Carbowax–divinylbenzene (CW–DVB), poly(dimethylsiloxane)–divinylbenzene (PDMS–DVB) and polyacrylate (PA) were evaluated for extraction of nine most biologically active heterocyclic aromatic amines. Different parameters affecting to the microextraction and determination of HAs were studied, such as absorption and desorption time, desorption mode, composition of the solvent for desorption, pH, ionic strength, and percentage of methanol in the sample. To determine these amines in food samples a new simplified procedure is proposed, consisting of treatment of the sample with methanolic NaOH prior microextraction by CW–TPR fiber coating and HPLC–DAD determination. The advantages of this new method are the reduced amounts of time and organic solvents required.

Keywords: Solid-phase microextraction; Food analysis; Amines, heterocyclic aromatic

1. Introduction

Heterocyclic amines (HAs) are considered the main food mutagens in cooked meat products [1], they are formed by pyrolysis of proteins, amino sugars and amino acids [2–6]. The formation mechanism probably involves Maillard reactions and many factors such as temperature and time affecting HA production [7,8].

They are usually divided into two main classes: the pyrolytic amines and the aminoimido-azaarenes. The first group is formed at high temperature, above 300 °C, and includes the amines: 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), AαC, MeAαC, Glu-P-1 and Glu-P-2; the compounds harman and norharman are not primary amines nor mutagenic in the Ames test, but have been shown as co-mutagenic [9] and are frequently included in this class of compounds. The aminoimidazo-azaarenes are formed at the ordinary household cooking temperatures of 100–225 °C and are sometimes termed thermic mutagens. The aminoimido-azaarenes commonly reported in cooked foods are: 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), IQx, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,4-dimethylimidazo[4,5-*b*]quinoxaline (MeIQx), 4,8-DiMeIQx and 7,8-DiMeIQx and PhIP. According to the chemical behaviour of these compounds, they are sometimes grouped as polar (aminoimidazo-azaarenes together with Glu-P-1 and Glu-P-2) and nonpolar (all others) amines.

HAs are present at low levels in a complex sample matrix, there is a need for an effective purification method and a sensitive and selective analytical method. Several methods have been used for the extraction and purification of HAs based on liquid–liquid extraction (LLE), extraction with blue cotton, solid-phase extraction (SPE) or immunoaffinity purification [10–13]. Frequently, reversed-phase high-performance liquid chromatography (HPLC) with different types of detection (diode array, fluorescence, electrochemical or mass spectrometry) is used for the determination of HAs [14].

Traditional extraction techniques such as liquid–liquid extraction and, in particular, solid-phase extraction are, however, characterized by intrinsic disadvantages like the use of toxic solvents and plugging of the cartridges. These drawbacks can be avoided by the use of solid-phase

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microextraction (SPME), a quite efficient extraction technique introduced by Arthur and Pawliszyn in 1990 [15]. It enables simultaneous extraction and pre-concentration of analytes from gaseous, aqueous, and solid matrices. The principle of SPME is equilibration of the analytes between the sample matrix and an organic polymeric phase usually coating a fused-silica fiber; the amount of the analyte absorbed by the fiber is proportional to the initial concentration. It is also possible to obtain good extraction yields and reliable analysis under non-equilibrium conditions [16], if the extraction conditions are held constant.

Until recently, the extensive applications of SPME have been performed in combination with gas chromatography. However, many classes of organic compounds are semi- or nonvolatile and are best analyzed by HPLC. SPME coupled with HPLC was reported by Chen and Pauliszyn [17]. The difference between SPME–GC and SPME–HPLC is the desorption step. In HPLC analysis, desorption is performed in an appropriate interface consisting of a six-port HPLC injector with a special fiber-desorption chamber, installed in place of the sample loop. Desorption is performed by placing an organic solvent in the desorption chamber (static desorption) during desorption time, or by passing mobile phase through the desorption chamber (dynamic desorption). The number of SPME–HPLC applications is substantially less than for SPME–CG, despite its potential. Most of these applications have been, however, developed in the last 3 years [18–22], clearly indicating an increasing interest in the technique. Moreover, the in-tube SPME method coupled with liquid chromatography has been developed for the analysis of mutagenic heterocyclic amines [23].

In this paper, we present the application of SPME–HPLC to the analysis of aromatic heterocyclic amines. Four kinds of fiber coatings: Carbowax–templated resin (CW–TPR), Carbowax–divinylbenzene (CW–DVB), poly(dimethylsiloxane)–divinylbenzene (PDMS–DVB) and polyacrylate (PA) were compared for the extraction efficiency of the heterocyclic amines. The parameters of the desorption procedure are optimized. The effects of the properties of the analytes and fiber coatings, duration of absorption, pH, ionic strength, and percentage of methanol in the sample solution were also investigated. The developed method was applied to study the recoveries of heterocyclic amines in a commercial beef extract.

2. Experimental

2.1. Chemicals

The compounds studied were as follows: 2-amino-3-methylimidazo[4,5-*f*]quinoline, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline, 2-amino-3,4-dimethylimidazo[4,5-*b*]quinoxaline, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 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. Stock standard solutions of 80 μg/ml in methanol were prepared and used for further dilutions.

All chemicals and solvents used were of HPLC or analytical-reagent grade, and water was purified using Milli-Q gradient A10 system (Millipore, UK). All the solutions were filtered through a 0.45 μm filter before being injected into the LC system.

2.2. Instruments

The SPME fiber assembly and SPME–HPLC interface with Rheodyne valve (Supelco, USA) were used to perform the microextraction.

The microextraction fibers (Supelco, USA) studied were coated with CW–TPR (50 μm), CW–DVB (65 μm), PDMS–DVB (60 μm), PA (85 μm). The fibers were conditioned with the mobile phase until stable baseline was obtained.

The HPLC equipment used was a liquid chromatograph consisting of a delivery solvent ProStar 230 and a photodiode array detector ProStar 330 (Varian, USA). Data were acquired and evaluated by the Star 5.51 chromatography workstation (Varian, USA).

2.3. Chromatographic conditions

The amines were separated by means of reversed-phase LC using a TSK-Gel ODS-80T_M column (5 μm, 150 mm × 4.6 mm i.d.) (Tosoh Biosep, Germany), and a Pelliguard LC-18 guard column (Supelco, USA).

Optimal separation was achieved with a ternary mobile phase at flow-rate 1 ml/min, triethylamine phosphate 0.01 M, pH 3.2 (A solvent), acetonitrile (B solvent) and triethylamine phosphate 0.01 M, pH 3.6 (C solvent). The gradient program was: 5–14% B in A, from 0 to 13.5 min; 14–25% B in C, 13.5–19 min; 25–50% B in C, 19–31.3 min; returned to initial conditions in 5 min and 5 min of post-run delay. UV detection was performed with a time program: 0–10.2 min, 253 nm; 10.2–17.5 min, 263 nm; 17.5–21.5 min, 253 nm; 21.5–25.0 min, 263 nm; 25.0–30.0 min, 228 nm. UV spectra from 220 to 400 nm were also recorded for peak identification.

2.4. Solid-phase microextraction procedure

Aliquots of 4 ml of standard solutions in water–methanol (95:5, v/v) or samples are placed in 4 ml glass vials which are sealed with a septum-type cap and the fiber is directly immersed in the solutions for 30 min at room temperature. Agitation of the sample is carried out (800 cycles min^{-1}) with a small stirring bar to increase the rate of equilibration.

Then, the fiber is withdrawn into the needle, the needle is removed from the septum and is inserted in the desorption chamber of the interface SPME–HPLC that is off-line under ambient pressure when the injection valve is in load position. The chamber (60 μl) was previously filled with initial mobile phase (A–B, 95:5). After 10 min, the valve was switched to inject position and integration was begun with a flow-rate set to 1 ml/min (no band broadening or peak tailing were observed). The valve was returned to load position after 1 min, then the chamber was flushed three times with 500 μl portions of mobile phase to minimize the possibility of analyte carry-over, and then the SPME fiber was removed.

3. Results and discussion

3.1. Fiber evaluation

A preliminary and qualitative assay was performed in order to evaluate the available fibers, CW–TPR (50 μm), CW–DVB (65 μm), PDMS–DVB (60 μm), PA (85 μm). To select the best fiber coating, the extraction was performed in 60 min and desorption was carried out in static mode, the fiber was soaked into the chamber for a period of 10 min. The relative extracting efficiencies of the heterocyclic aromatic amines (HAs) (expressed by peak areas in the chromatograms) with four fiber coatings are shown in Table 1. The fiber coatings studied show the following polarity order: CW–TPR > CW–DVB > PDMS–DVB > PA. We found that the most polar CW–TPR fiber studied exhibited better extracting efficiency except for norharman. On the other hand, the less polar PA fiber studied showed the lowest extracting efficiency for the greater part of analytes. The PDMS–DVB fiber exhibited better or equal extracting efficiency as compared to the PA fiber.

The 65 μm CW–DVB fiber provided good extraction efficiencies for all analytes. However, this fiber was not suitable for SPME–HPLC when methanol is present in the sample, even at 5% (v/v). After a few analysis, the coating was stripped off the fiber due to swelling of the phase. Other authors [24,25] also observed swelling of the CW–DVB phase in the water–methanol mixture or acetatonitrile/acetate buffer mixture. CW–TPR, PDMS–DVB and PA fibers were therefore used for further investigation.

Table 1
Extracting efficiencies (expressed by peak areas) obtained with four fiber coatings

Compound	CW–TPR	CW–DVB	PDMS–DVB	PA
IQ	60.9	50.9	13.8	14.5
MeIQ	99.4	74.0	29.5	24.1
MeIQx	29.0	19.3	–	11.2
Norharman	409	509	491	104
Harman	323	285	224	50.3
Trp-P-2	3022	2141	318	635
Trp-P-1	3333	2845	486	1050
AαC	1241	1222	372	299
MeAαC	2128	1844	846	540

3.2. Absorption and desorption conditions

To ensure extraction efficiency of analytes from a sample, one of the important steps in the development of a SPME method is to determine the time needed when an extraction process reaches equilibrium between sample matrix and coating of a fiber. The amount of analyte extracted onto the fiber depends not only on the polarity and thickness of the stationary phase, but also the extraction time and the concentration of the analyte in the sample [26]. Fig. 1 shows the extraction time profiles of four amines chosen to illustrate the different chromatographic behaviours of HAs studied (520 ng/ml for each amine) with each fiber coating used, for extraction times of 10, 30, 45 and 60 min.

For PA fiber, all the amines, except harman, reached the equilibrium in10 min. However, for CW–TPR fiber, it is necessary higher extraction times for non-polar amines, so for Trp-P-1, after 60 min equilibrium had not been reached yet, as shown in Fig. 1, since for polar amines 10 min was enough. For PDMS–DVB fiber 30 min were needed in all cases except for harman.

We found that peak areas corresponding to some amines decrease when absorption times are longer than 10 min, this is probably caused by over absorption due to stronger interaction force between the analyte and fiber.

An extraction period of 30 min was chosen for subsequent experiments. Since this time was approximately equivalent to the time required to run the HPLC chromatogram. It is not essential for equilibrium to be reached, shorter times can be used as long as the extractions are timed carefully and the mixing conditions remain constant.

After the immersion of the fiber in the sample solutions for 30 min, the fiber was introduced into the SPME–HPLC interface for desorption. We have studied the effect of the desorption mode and the composition of the solvent used for static desorption.

To compare both the dynamic and static modes of desorption, we used aqueous samples with a concentration of 520 ng/ml of each amine. In the dynamic mode, the fiber was placed in the desorption chamber and the valve was immediately switched from the load to the inject position. After 5 min, the valve returned to the load position and the fiber was removed from the SPME–HPLC interface. In the static mode, the fiber was placed in the desorption chamber full of methanol for 10 min. Then, the valve was switched from the load to the inject position and the analytes were transferred to the chromatographic column and after 1 min, the valve returned to the load position and the fiber was removed from the SPME–HPLC interface. Table 2 shows the peak areas obtained with both desorption modes. In general, the results were best in the static mode for all the compounds, so this was the mode of desorption used in further experiments.

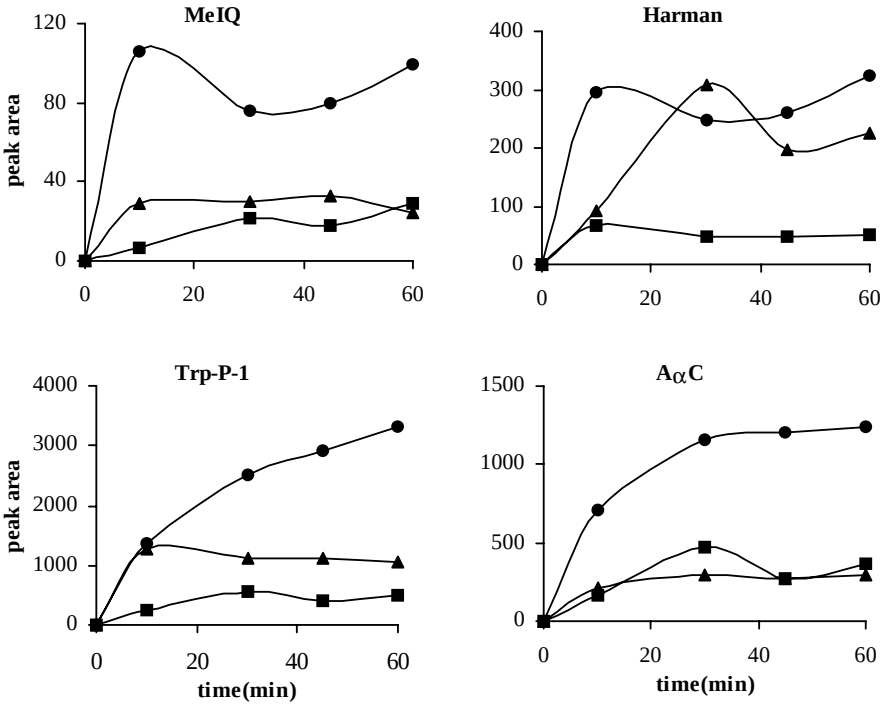

Fig. 1. Absorption time profile for CW–TPR (●), PDMS–DVB (■) and PA (▲) fibers for MeIQ, harman, Trp-P-1 and AαC. Concentration, 520 ng/ml; desorption mode, static 10 min.

Methanol and the initial composition of mobile phase (triethylamine phosphate 0.01 M, pH 3.2–acetonitrile, 95:5) have been tested as solvent for static desorption for CW–TPR fiber, Fig. 2 shows the effect of both solvents for different periods of time. The peak areas of the analytes increased with increasing desorption period, specially when the solvent is methanol and the period of desorption is increased from 0 to 3 min, this increasing is more marked for less polar analytes. However, when mobile phase is used as desorption solvent, times must be longer and 10 min are needed. After this time, the recoveries did not increase significantly for the greater part of the analytes studied. Due to the easier performance and the effect of methanol on some coatings, as we have described above, mobile phase was chosen as desorption solvent, therefore, a desorption period of 10 min was selected as the optimum.

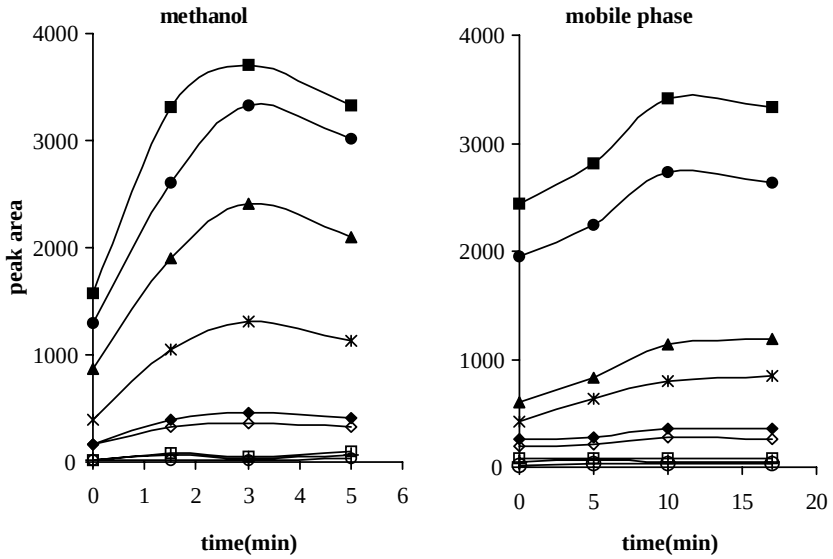

Fig. 2. Effect of desorption period using methanol and mobile phase (triethylamine phosphate (0.01 M, pH 3.2–acetonitrile, 95:5) as solvent for static desorption for CW–TPR fiber.(■) Trp-P-1, (●) Trp-P-2, (▲) MeAαC, (◆) norharman, (✱) AαC, (◇) harman, (- -) MeIQx, MeIQ, IQ. Concentration, 520 ng/ml.

Table 2
Peak areas obtained with the three fiber coatings in both elution mode

Compound	CW–TPR		PDMS–DVB		PA	
	Dynamic	Static	Dynamic	Static	Dynamic	Static
IQ	46.7	50	0	31.5	0	19
MeIQ	78.2	81.8	0	28.3	21.1	29.8
MeIQx	22.2	30.7	0	12	0	7
Norharman	258	363	292	533	65.1	103.8
Harman	197	271	116	291	34.8	46.3
Trp-P-2	1948	2732	167	384	527	676
Trp-P-1	2435	3416	264	617	784	1102
AαC	417	791	287	248	129	290
MeAαC	600	1141	625	390	276	544

3.3. Effect of other parameters on efficiency extraction

The effect of methanol content of the sample on absorption was studied by preparing a set of samples that contained methanol at concentrations from 0 to 20% (v/v). The results obtained, Fig. 3, reveal that an increase in methanol concentration involves a diminishing in the extracting efficiency. This effect, found for all analytes, decreases with the polarity of the fiber coating used. An increasing proportion of methanol in aqueous solution decreases the polarity of the aqueous sample, so the distribution constant decreases [27].

The extraction efficiency is highly affected by the pH of the sample. The amount of amines absorbed increased with increasing of the pH in all the fiber coatings studied. An increase in the pH produced an enhancement due to a diminishing in the amine ionization. In Fig. 4 it can be observed how the extraction efficiency expressed as peak area increases considerably for pH higher than 5.

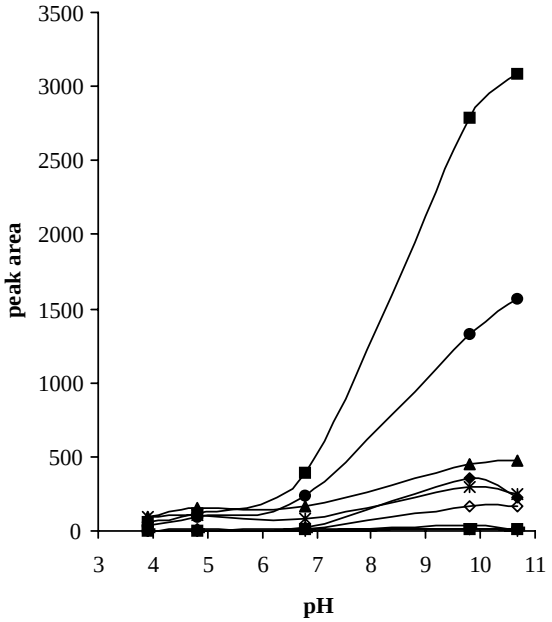

Fig. 4. pH profile for PA fiber. (■) Trp-P-1, (●) Trp-P-2, (▲) MeAαC, (◆) norharman, (✱) AαC, (◇) harman, (--) MeIQX, MeIQ, IQ. Concentration, 520 ng/ml.

The effect of ionic strength on the absorption of heterocyclic amines was studied by preparing standards with Na_2SO_4 concentrations ranging from 0 to 5% (w/v). It was found that the increase of ionic strength has a negative effect in the extraction of the amines. Area peaks decrease dramatically when Na_2SO_4 is added to the standard solution, even at 0.2% (w/v), except for AαC and MeAαC which are not affected for this fact.

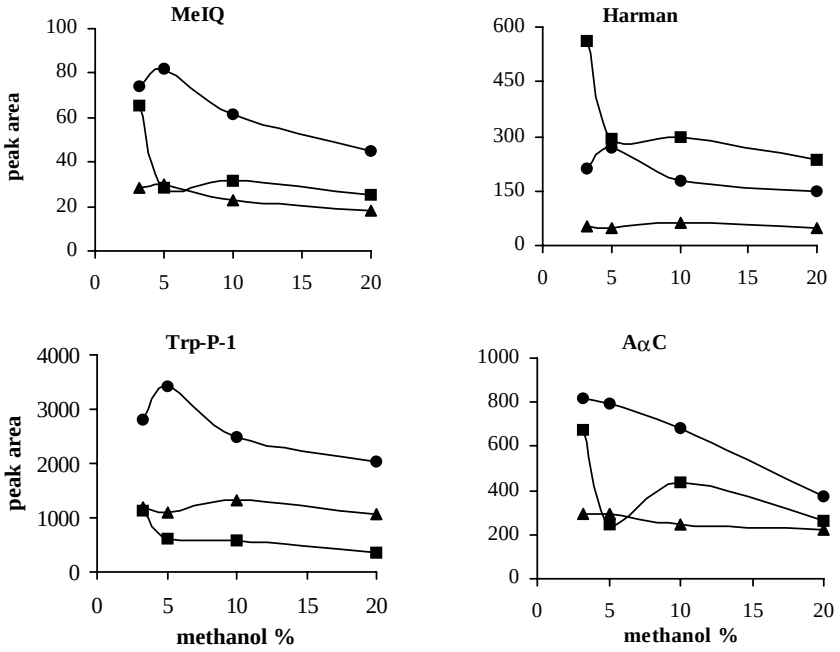

Fig. 3. Effect of concentration of methanol in standards solutions for (●) CW–TPR, (■) PDMS–DVB and (▲) PA fibers. Concentration, 520 ng/ml.

Table 3
The linearity, precision and LOD for the determination of heterocyclic amines with three fiber coatings

Compound	CW–TPR				PDMS–DVB				PA			
	Slope	r^2	LOD	R.S.D. (%)	Slope	r^2	LOD	R.S.D. (%)	Slope	r^2	LOD	R.S.D. (%)
IQ	0.129	0.941	2.6	16								
MeIQ	0.160	0.98	2.2	22					0.071	0.999	5	3.9
MeIQx	0.039	0.927	14	11					0.153	0.951	3.6	3.1
Norharman	0.707	0.996	0.6	2.4	0.898	0.999	0.5	6	0.43	0.997	0.9	3.3
Harman	0.533	0.991	0.9	1.3	0.453	0.997	1.1	6	0.114	0.999	4.1	6.8
Trp-P-2	6.99	0.986	0.1	7.9	0.371	0.996	1.1	6	1.273	0.945	0.3	3.9
Trp-P-1	8.58	0.997	0.1	9.6	0.793	0.999	1	8.2	2.067	0.951	0.3	5.3
AαC	2.19	0.994	0.8	6.7	0.5813	0.989	3.1	7.3	0.536	0.988	3.3	7.8
MeAαC	3.52	0.992	0.5	7.1	0.9776	0.992	1.9	11.6	0.929	0.986	2.2	9.2

3.4. Analytical performance of the SPME–HPLC method

The main analytical characteristics of the methods for analysis of HAs have been established. Table 3 illustrates the linearity, precision and detection limits with CW–TPR, PDMS–DVB and PA fibers.

The linearity has been investigated over the range 0.4–280 ng/ml, 52–300 ng/ml and 52–400 ng/ml for CW–TPR, PDMS–DVB and PA fiber, respectively. The correlation coefficients were better than 0.941.

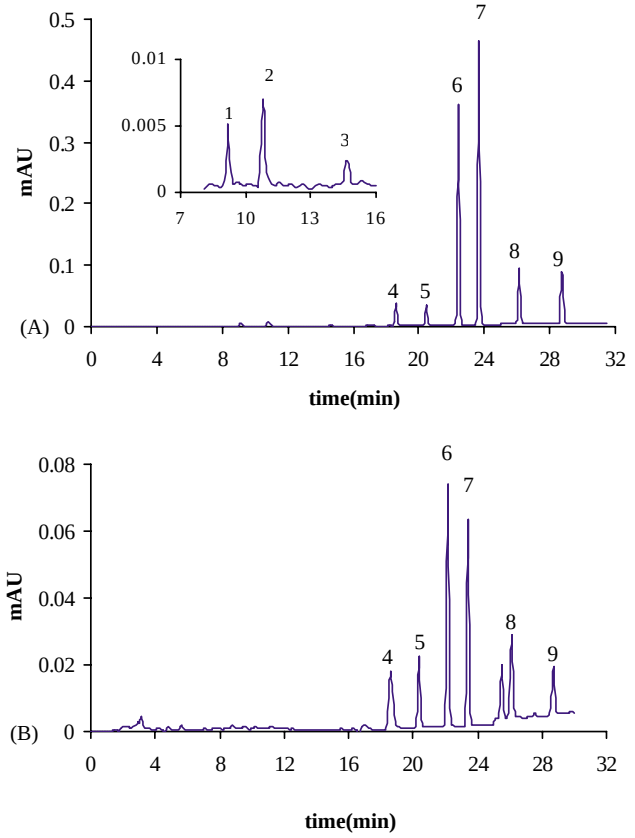

Fig. 5. (A) Chromatogram of standard solution (520 ng/ml). (B) Chromatogram of the spiked sample (100 ng/ml). Peaks: (1) IQ, (2) MeIQ, (3) MeIQx, (4) norharman, (5) harman, (6) Trp-P-2, (7) Trp-P-1, (8) MeAαC, (9) AαC. Microextraction was carried out with a CW–TPR fiber coating; absorption time, 30 min, static desorption time, 10 min.

The precision was determined by performing six consecutive extractions with HAs concentrations of 100, 200 and 300 ng/ml for the CW–TPR, PDMS–DVB and PA fiber, respectively. The R.S.D. values obtained using the CW–TPR fiber ranged from 1.3 to 22%, being this coating the only one that allows the detection of all the analytes at the studied concentration levels. Fig. 5 shows a typical chromatogram obtained. The detection limits based on a signal-to-noise ratio the 3:1 ranged from 14 to 0.1 ng/ml for MeIQx and Trp-P-2, respectively, for the three fiber coatings studied.

3.5. Test on food sample

When the extraction method had been established for pure references, we attempted to determine heterocyclic amines in processed-food samples. To carry out this analysis 1 g of commercial beef extract (Starlux) was spiked with standard compounds (400 ng/g). Then the sample was extracted with 2 ml of methanolic NaOH (1.4 ml 1 M NaOH and 0.6 ml methanol) in a centrifuge tube (4000 rpm, 20 min). Aliquot of 1 ml of supernatant was diluted to 4 ml with deionized water and analysed according to the procedure described above using CW–TPR as fiber coating. Fig. 5B, shows the chromatogram obtained.

Percentage recovery for each compound is indicated in Table 4. Amines such as MeIQ, MeIQx, Trp-P-1 and Trp-P-2 gave low recovery factor and IQ was not recovered by this

Table 4
Recoveries of HAs in a spiked commercial meat extract

Compound	Recovery (%)[a]
IQ	ND
MeIQ	19.1
MeIQx	28.5
Norharman	74.9
Harman	82.4
Trp-P-2	17.8
Trp-P-1	19
AαC	67.9
MeAαC	57.4

[a] Recoveries referred to those obtained in aqueous medium.

method. However, the other amines presented recoveries ranging between 57.4 and 82.4%. The optimization of this sample pretreatment is currently being studied.

4. Conclusions

SPME coupled with HPLC–diode array detection (DAD) was successfully applied to determine a group of heterocyclic amines at low ng/ml levels. The various parameters that affect both absorption and desorption in SPME–HPLC were optimized for different coatings. CW–TPR fiber is recommended for the determination of these amines.

The first results obtained by applying the developed methodology to the HAs analysis in meat extract samples, show that this method would allow to simplify the clean-up steps and eliminate different solid-phase extraction stages required in the analysis of these amines with the reduced amounts of time and organic solvents required.

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