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# Optimization of a clean-up procedure for the determination of heterocyclic aromatic amines in urine by field-amplified sample injection–capillary electrophoresis–mass spectrometry

Sònia Sentellas, Encarnación Moyano, Lluís Puignou, M. Teresa Galceran*

*Department of Analytical Chemistry, University of Barcelona, Diagonal 647, 08028 Barcelona, Spain*

**Abstract**

Heterocyclic amines (HAs), generated when proteinaceous food is cooked, are of special interest since they can be carcinogenic for humans. In this paper, the optimization of a clean-up procedure for the isolation and preconcentration of 15 heterocyclic amines in urine samples is described. The method proposed combines liquid extraction on a solid support of diatomaceous earth with solid-phase extraction in cartridges. Tests were performed on several cartridges containing graphitic carbon or mixed phases, i.e., combining reversed-phase and cation-exchange mechanism, and the best results were obtained with Oasis MCX. The optimized purification method was applied to the quantification of heterocyclic amines in hydrolyzed spiked human urine. The method was carried out by capillary electrophoresis (CE) coupled to mass spectrometry (MS) and applying field-amplified sample injection (FASI) as in-line preconcentration procedure. We obtained detection limits down to 0.3 ng/ml of urine and errors lower than 17%.


*Keywords:* Sample preparation; Field-enhanced sample stacking; Amines, heterocyclic aromatic

## 1. Introduction

Heterocyclic amines (HAs) are a group of compounds that have shown mutagenic activity in numerous in vitro and in vivo test systems. A great variability of mutagenicity has been found for these compounds using the Ames test [1]. Moreover, the specific mutagenic activities of some of HAs are much higher than those of typical mutagens/carcinogens, such as aflatoxin $B_1$, 4-nitroquinoline 1-oxide and benzo[*a*]pyrene. These amines are formed from free amino acids, creatine/creatinine, and carbohydrates [2,3] when food is cooked, particularly when proteinaceous food is heated at moderate to high temperatures. To evaluate the intake of amines, their determination in cooked food is usually performed. However, as the content of HAs varies greatly with cooking conditions, it is difficult to obtain an accurate estimation of the exposure. Thus, methods have been developed to determine not only the parent compounds but also their metabolites in biological samples (mainly urine samples) [4–8] to reflect recent HAs exposure. Such methods of analysis are principally based on liquid chromatography (LC) [9–13], but gas chromatography (GC) [5,14,15] and capillary electrophoresis (CE) [16–22] have also been used. Nevertheless, due to the complexity of such matrices, clean-up and preconcentration procedures are usually needed in order to carry out the amine analysis. These purification methods are based on liquid–liquid extraction [23], extraction with blue cotton [24], solid-phase extraction (SPE) with disposable columns [9,25] or immunoaffinity purification [26]. More specifically, urine samples are principally purified with liquid–liquid extraction and often a double extraction in basic and acid media is reported [5,27,28]. Moreover, as considerable amounts of HAs are conjugated to glucuronic acid and sulphate, an initial step of hydrolysation is usually performed to break up such conjugates. Alkali [7], acid [5] and enzymatic [27,29,30] hydrolysis have been reported to carry out the analysis of HA conjugates in urine.

The present paper describes the optimization of a clean-up and preconcentration procedure for the determination of fifteen heterocyclic amines in human urine samples. The compounds selected are those more frequently analysed in foods and biological fluids. The aim of the study is to develop a simple procedure to obtain a clean extract for

* Corresponding author. Tel.: +34-934021100; fax: +34-934021233.
*E-mail address:* galceran@apolo.qui.ub.es (M.T. Galceran).

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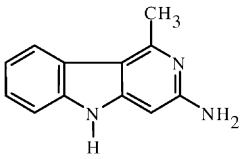
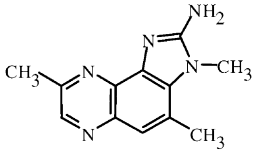
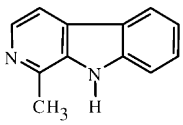
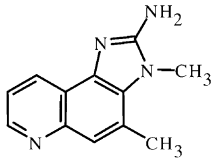
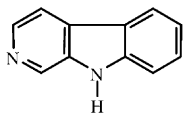
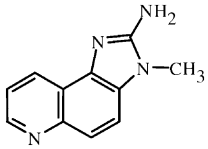
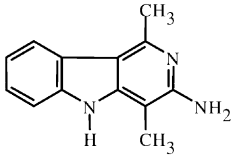
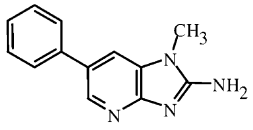
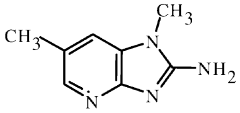
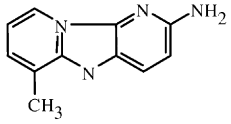
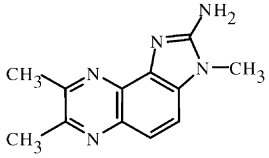
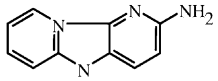
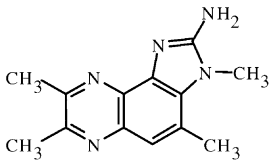
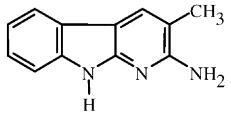
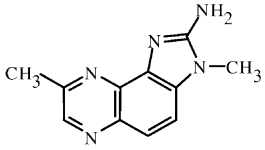
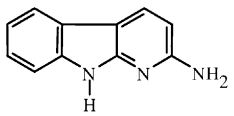
the determination of HAs by field-amplified sample injection (FASI)–CE–MS. To determine total amine content, an acid hydrolysis of glucuronide and sulphate conjugates were needed.

## 2. Experimental

### 2.1. Chemicals

The heterocyclic aromatic amines (see Table 1 for their structures) 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3-trideuteromethylimidazo[4,5-*f*]quinoline ($D_3$-IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-8-methyl-3-trideuteromethylimidazo[4,5-*f*]quinoxaline ($D_3$-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), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-1-trideuteromethyl-6-phenylimidazo[4,5-*b*]pyridine ($D_3$-PhIP), 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine (DMIP), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-

Table 1
Structures of the heterocyclic aromatic amines

| Amine | Structure | Amine | Structure |
|---|---|---|---|
| Trp-P-2 |  | 4,8-DiMeIQx |  |
| H |  | MeIQ |  |
| NH |  | IQ |  |
| Trp-P-1 |  | PhIP |  |
| DMIP |  | Glu-P-1 |  |
| 7,8-DiMeIQx |  | Glu-P-2 |  |
| TriMeIQx |  | MeAαC |  |
| MeIQx |  | AαC |  |

9*H*-pyrido[2,3-*b*]indole (MeAαC), 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) and 2-aminodipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) were 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) were from Sigma (Steinheim, Germany). Individual stock standard solutions between 110 and 215 μg/g in methanol (Merck, Darmstadt, Germany) were prepared and used for further dilution. Deuterated amines and TriMeIQx were used as internal standards.

Empty Extrelut-20 extraction cartridges were provided by Merck, and Isolute diatomaceous earth refill material was obtained from IST (Hengoed, UK). LiChrolut TSC (300 mg), Oasis MCX (500 and 30 mg) and Supelclean Envi-Carb (200 mg) cartridges were from Merck, Waters (Milford, MA, USA) and Supelco (Bellefonte, PA, USA), respectively. Coupling pieces and stopcocks were purchased from Varian (Harbor, City, USA).

Other reagents were analytical grade: ammonium formate (Fluka, Buchs, Switzerland), 98–100% (w/w) formic acid, 25% ammonia solution, 25% hydrochloric acid, ethyl acetate, dichloromethane and methanol (Merck). Water purified with an Elix/Milli-Q system (Millipore, Bedford, MA, USA) was used.

### 2.2. Instrumentation

A Beckman (Fullerton, CA, USA) P/ACE System 5500 equipped with a diode array detector and modified for coupling to an LCQ-ThermoFinnigan (San Jose, CA, USA) ion trap mass spectrometer was used. A pneumatically-assisted electrospray ionization (ESI) source and a sheath liquid flow device were employed for this coupling. A fused silica capillary (Beckman, Fullerton, CA, USA) of 80 cm total length and 50 μm i.d. was used. The CE voltage and temperature were set at 28 kV and 25 °C, respectively. UV detection was performed at 213 nm. In MS detection, the CE capillary protrudes from the electrospray needle 0.2 mm, and the distance to the heated capillary was 1.5 cm. The optimum sheath liquid was methanol–20 mM formic acid (75:25) and it was introduced at a flow-rate of 5 μl/min by a syringe pump (Shorewood, IL, USA). The nitrogen sheath gas flow-rate was set to 13.5 l/h (corresponding to 15 arbitrary units). A voltage of 3.5 kV was applied to the ESI needle and the heated capillary temperature was kept at 175 °C. Full scan mode, from *m*/*z* 100 to 300 was used with 5 microscans and 150 ms as maximum injection time. For CE–MS–MS experiments product ion scan has been performed using as parent ion the protonated molecular ion. Collision energies between 39 and 45% and an activation Q of 0.45 were used [31].

For sample preparation a Supelco Visiprep and Visidry SPE vacuum (Supelco, Gland, Switzerland) were used to manipulate the solid-phase extraction cartridges and solvent evaporation, respectively.

### 2.3. Analytical procedure

To optimize the clean-up procedure heterocyclic amines were determined using CE–MS or CE–UV. Good separation can be achieved using as optimum running electrolyte 16 mM formic acid/ammonium formate buffer at pH 4.5 containing 60% of methanol, as reported previously [32]. Hydrodynamic injection (0.5 psi; 1 psi = 6894.76 Pa) or, when a higher sensitivity was needed, FASI were applied. The FASI method consists of an in-line preconcentration method where a methanol–5 mM formic acid (50:50) solution was used as sample solvent, a methanol plug was injected by hydrodynamic injection (0.5 psi) during 3 s and then the injection of the sample was performed by of electrokinetic injection (10 kV) during 25 s [32].

Acid hydrolysis of urine samples was carried out adding 660 μl hydrochloric acid 25% (w/w) to 5 ml of urine and heating at 100 °C for 2 h [5]. After hydrolysis was completed, 6 M sodium hydroxide was added to obtain a basic medium and then the sample was processed using the optimized clean-up procedure.

## 3. Results and discussion

### 3.1. Clean-up procedure

Three different cartridges (LiChrolut TSC (300 mg), Oasis MCX (500 mg) and Supelclean Envi-Carb (200 mg)) were studied to find the most suitable and simple conditions for the clean-up and preconcentration of heterocyclic amines in urine samples. The Oasis MCX and the LiChrolut TSC cartridges contain mixed phases, i.e. they combine reversed-phase and cation-exchange mechanisms. In contrast, Supelclean Envi-Carb cartridges contain porous graphitic carbon that interact by hydrophobic, electronic and ion-exchange mechanisms. As our studies were focused on finding a simple clean-up procedure that allowed the extraction and preconcentration of heterocyclic amines, the urine samples were directly introduced into the cartridge without any pre-treatment. For graphitic carbon, extractions under two conditions, with and without addition of NaOH (0.5 ml 6 M NaOH) to the urine samples, were tested. To precondition the cartridge 2 ml of methanol, 2 ml of methanol–water (50:50), 2 ml of water and, when extraction at basic conditions was performed, an additional step of 2 ml of water with 200 μl of 6 M NaOH were used. A volume of 5 ml of urine (or basified urine) were passed through the cartridge. Then the cartridge was washed with 2 ml of water and amines eluted with 2 ml of methanol–HCl (95:5, v/v). Finally the extract was evaporated to dryness under a stream of nitrogen and reconstituted with 100 μl of a solution of methanol–5 mM formic acid (50:50) containing the internal standard. As no heterocyclic amines were recovered in any working conditions, further studies using Envi-Carb cartridges were not performed. As regards mixed phase

cartridges, the sample was acidified (100 μl of 5 M HCl in 10 ml of urine) and directly introduced in the cartridge, which was then cleaned with 5 ml of 0.1 M HCl and 5 ml of methanol as it is recommended for basic drugs. Finally, the amines were eluted with 5 ml of 5% $NH_3$ in methanol, evaporated to dryness under a stream of nitrogen and re-dissolved in 100 μl of MeOH–5 mM formic acid (50:50) containing the internal standard. This procedure was applied to an aqueous mixture (Milli-Q water) and to urine samples (spiked and non spiked). Fig. 1 shows the electropherograms obtained after clean-up with both Oasis MCX and LiChrolut TSC cartridges. As can be seen, peak intensities in the chromatograms obtained with both sorbents were similar for most of the amines, although H, NH and MeIQx showed slightly higher recoveries with the Oasis MCX cartridge. Conversely, amines such as Trp-P-1, Trp-P-2, MeAαC and AαC gave slightly better recovery with the LiChrolut TSC cartridge. Nevertheless, DMIP, Glu-P-1 and Glu-P-2 showed an important loss of peak intensity when LiChrolut TSC cartridges were used. Taking into account these facts, Oasis MCX cartridges were chosen for further studies.

When urine samples were analyzed, interferences that prevented HA identification were observed with both cartridges. In order to avoid these interferences, a liquid–liquid extraction was performed before the SPE step; however, problems due to emulsions and manipulation of the sample occurred. In order to avoid these problems, the contact was increased between both liquid phases and macromolecules were eliminated by the addition of a solid support of diatomaceous earth. Two organic solvents (25 ml), ethyl acetate and dichlormethane, were tested. Fig. 2 shows the electropherograms of a blank of urine obtained with (b) and without (a) ethyl acetate extraction prior to the clean-up with Oasis MCX. Matrix components were efficiently eliminated and only some interferences remained in the final extract when this extraction was performed. However, a large peak appeared at ∼22 min that prevented the analysis of PhIP, Glu-P-1 and Glu-P-2. Moreover, some other

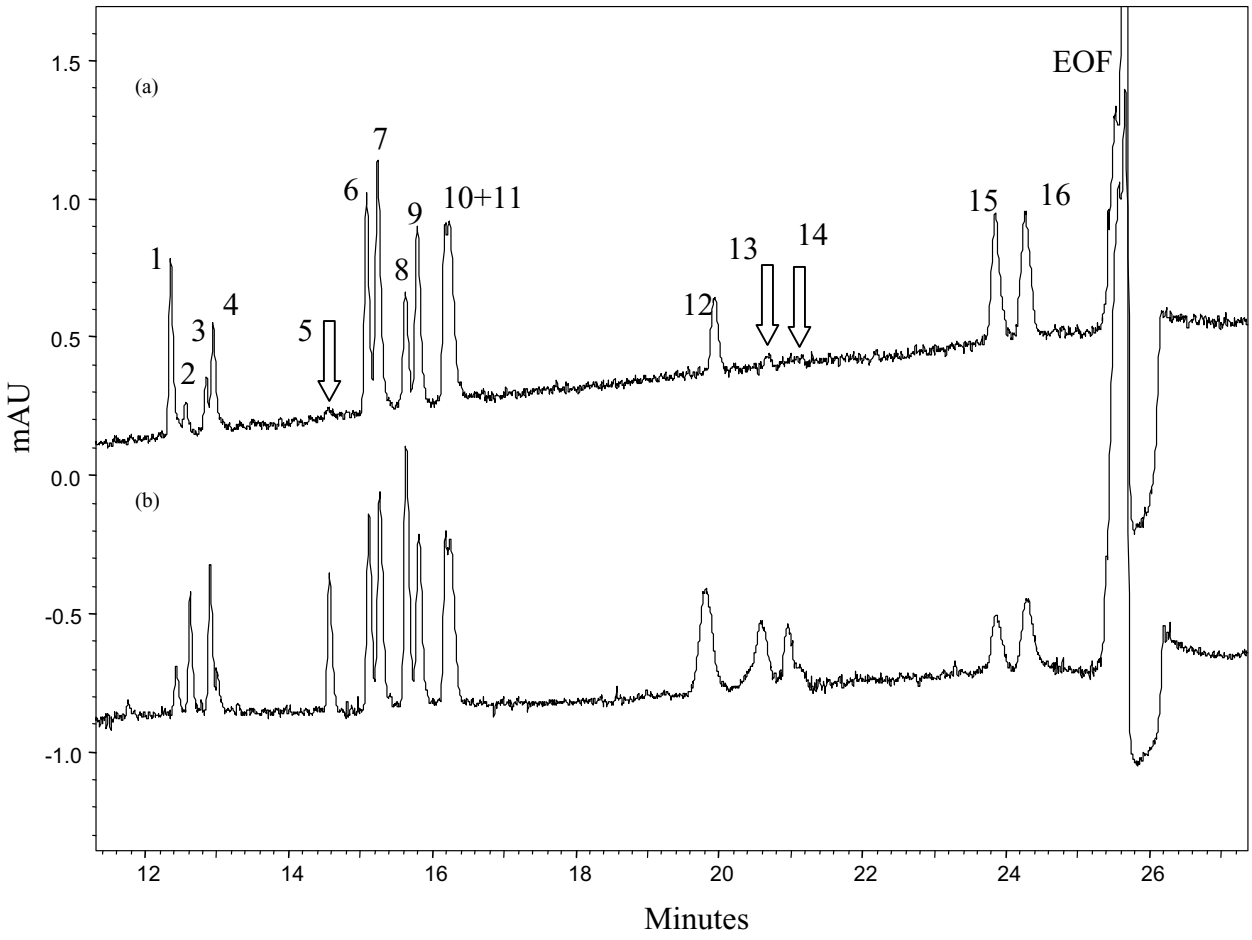



Fig. 1. CE–UV electropherograms of a HA mixture in Milli-Q water (∼60 ng/ml) treated with (a) LiChrolut TSC (300 mg) and (b) Oasis MCX (500 mg) cartridges using the clean-up procedure. Capillary: fused silica, 80 cm (effective length 70 cm) × 50 μm i.d. Applied voltage: 28 kV. Running electrolyte: formic acid/ammonium formate 40 mM pH 4.5, 60% MeOH. Detection: 213 nm. Assignation: (1) Trp-P-2, (2) NH, (3) H, (4) Trp-P-1, (5) DMIP, (6) 7,8-DiMeIQx, (7) TriMeIQx, (8) MeIQx, (9) 4,8-DiMeIQx, (10) MeIQ, (11) IQ, (12) PhIP, (13) Glu-P-1, (14) Glu-P-2, (15) MeAαC and (16) AαC.

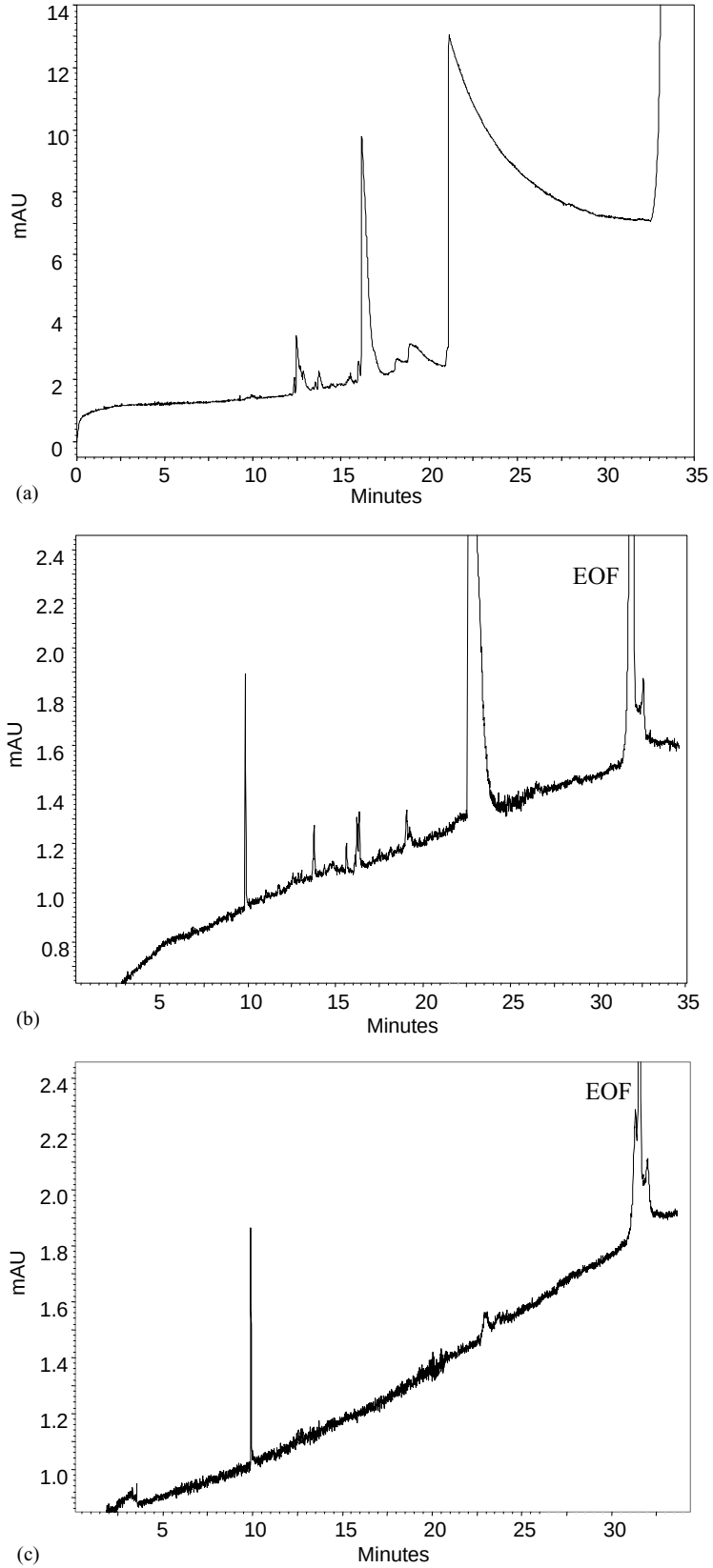



Fig. 2. Comparison of the CE–UV electropherograms obtained for a blank of urine using different clean-up conditions in a Oasis MCX cartridge. (a) Direct solid-phase extraction, (b) liquid extraction (ethyl acetate) and solid-phase extraction and (c) liquid extraction (DCM) and solid-phase extraction. CE conditions as Fig. 1.

interferences appeared at migration times of Trp-P-2, NH and DMIP. When using dichloromethane (DCM) an almost total elimination of matrix components was obtained (see Fig. 2c). In addition, all the compounds were recovered when DCM was used as extractant solvent. In contrast ethyl acetate did not allow the extraction of carbolines. So, dichloromethane was chosen as organic solvent for this extraction step. In summary, the proposed method consists of two steps, a liquid extraction with DCM of the HAs adsorbed on a diatomaceous earth cartridge and a solid-phase extraction on a mixed phase sorbent Oasis MCX.

In order to optimize the method, the effect of the volume of both the elution solvent in the SPE step and DCM in the recovery of HAs was studied. In order to optimize the minimum elution volume, fractions of 1 ml of the elution solvent (5% ammonia in methanol) were collected and analyzed individually to check in which volume the elution of HAs was completed. The results of this study showed that a strong interaction between amines and the cartridge sorbent occurred, especially for some of the compounds. As an example, Fig. 3 shows a plot of the relative response versus the fraction collected for amines Trp-P-1, MeAαC, DMIP and 7,8-DiMeIQx. The maximum elution for most of the compounds was observed between fractions 3–5 although some of them were detected until fraction 7 or 8, for instance MeAαC and 7,8-DiMeIQx (Fig. 3). For other components, DMIP and IQ, the maximum elution was observed at the third fraction and was not detected in the following ones. Finally, the most retained amines were Trp-P-1 and Trp-P-2, which were not eluted before fraction 4 and were still detected at fraction 10. Probably due to the high ionizable capacity of these compounds (the most basic between the amines studied), this strengthened their interaction with the sulphonic groups of the Oasis MCX cartridge. So, more than 10 ml was needed to recover all amines. In order to elute the amines in a smaller volume the percentage of ammonia was increased to 10% but the volume needed was not reduced. As shown in Fig. 3, the behavior of the different compounds at the two percentages of ammonia was very similar. Then, a reduction of the amount of the sorbent (30 mg cartridges) was considered. In this case, a significant decrease of the volume needed to elute the amines from the cartridge was observed for all the compounds. The maximum elution was obtained in the first fraction and no elution was observed over the third fraction except for Trp-P-1 and Trp-P-2 that needed 5 ml. So, 5 ml of the eluent (10% $NH_3$ in methanol) was chosen as minimum volume to elute the compounds under study.

For the elution of the amines from the diatomaceous earth cartridge a volume of DCM higher than 20 ml was needed to ensure that the solid support is completely wetted by the extractant. Obviously, higher volumes could improve the

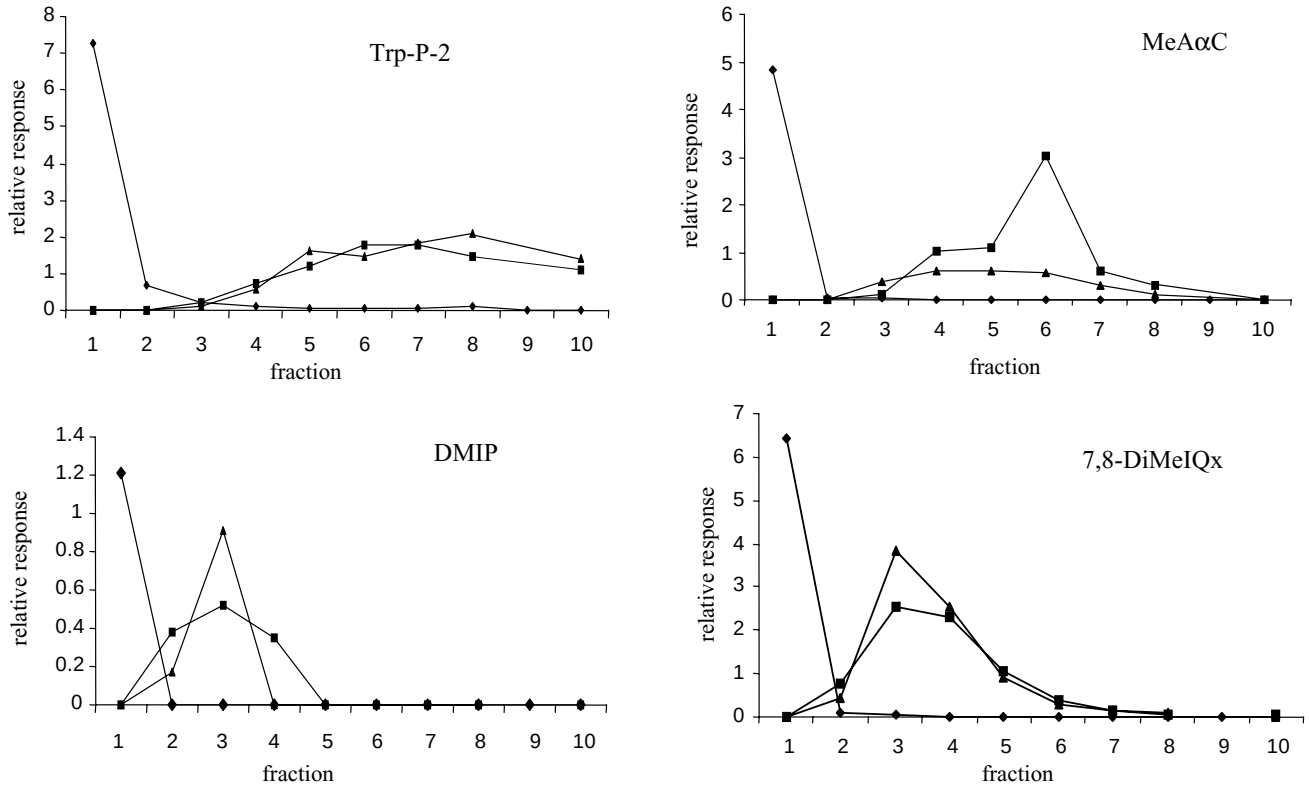



Fig. 3. Plot of the response relative to the I.S. (TriMeIQX) vs. fraction collected for Trp-P-2, MeAαC, DMIP and 7,8-DiMeIQx. (▲) Oasis MCX (500 mg) cartridge, elution solvent: 5% $NH_3$ in methanol; (■) Oasis MCX (500 mg) cartridge, elution solvent: 10% $NH_3$ in methanol; (◆) Oasis MCX (30 mg) cartridge, elution solvent: 10% $NH_3$ in methanol.

Table 2
Comparison optained of the response for each amine using different volumes of DCM

| Amine | Volume of DCM | | | |
|---|---|---|---|---|
| | 25 ml | 40 ml | 55 ml | 70 ml |
| NH | **100** | 51 | 15 | 15 |
| H | **100** | 67 | 38 | 37 |
| Trp-P-2 | 98 | 84 | 69 | **100** |
| MeAαC | **100** | 27 | 3 | 0 |
| DMIP | 52 | 75 | 86 | **100** |
| Trp-P-1 | **100** | 94 | 84 | 95 |
| IQ | 94 | **100** | 86 | 85 |
| Glu-P-1 | **100** | 39 | 22 | 0 |
| MeIQ | **100** | 96 | 85 | 78 |
| MeIQx | **100** | 92 | 60 | 45 |
| 7,8-DiMeIQx | **100** | 88 | 73 | 50 |
| 4,8-DiMeIQx | **100** | 69 | 45 | 30 |
| Glu-P-2 | **100** | 47 | 26 | 0 |
| PhIP | **100** | 87 | 74 | 53 |
| AαC | **100** | 40 | 9 | 0 |

Responses normalized to the maximum one.

extraction of amines, nevertheless, as the sorbent content of the Oasis MCX cartridges was low (30 mg), high volumes of solvent could surpass the breakthrough volume and as a consequence the compounds retained in the solid phase could be eluted. Volumes of dichloromethane from 25 to 70 ml were tested in order to increase the recovery. In Table 2 , the relative responses obtained for each HAs are given. As can be seen in general a decrease on the response was obtained at higher volumes probably because HAs were eluted from the Oasis MCX cartridge by DCM. For some compounds this effect was compensated by the highest extraction from the urine and, as a consequence, similar recoveries using different DCM volumes were obtained. As a compromise between maximum extraction and minimum DCM volume 25 ml of DCM were proposed. The optimized clean-up procedure can be summarized as follows: 0.5 ml of 6 M NaOH were added to 5 ml of urine and mixed with 5 g of diatomaceous earth. The mixture was placed into a empty cartridge and extracted with 25 ml of DCM. The eluate was directly passed through and Oasis MCX (30 mg) cartridge pre-conditioned with 1 ml of DCM. Then, after washing the cartridge with 1 ml of 0.1 M HCl and 1 ml of methanol, amines were eluted with 5 ml of 10% $NH_3$ in methanol. Finally, the extract was evaporated to dryness under a stream of nitrogen and redissolved in 100 μl of a solution of methanol–5 mM formic acid (50:50) containing the internal standard.

### 3.2. Urine samples analysis

Using the optimized clean-up procedure and the FASI–CE–MS method previously published [32], electropherograms with no interferences were obtained in the analysis of HAs in urine samples. In order to determine free HAs and also glucoronide and sulphate conjugates, acid hydrolysis was performed as indicated in the Section 2. A spiked urine sample and an urine blank were processed to determine whether hydrolysation generates any compound that could interfere with the analysis of amines. Only for Trp-P-2 a co-migrating substance was observed which could cause an error in its determination especially at low concentrations. As is known, MS–MS experiments could be very useful for the unambiguous identification of analytes, and can prevent false positives in the analysis of real samples. When standards were analyzed any increase in the sensitivity was observed because the decrease in the noise was compensated by the decrease in the signal. Nevertheless, when samples in complex matrices, such as biological fluids, were analyzed, an important reduction on the baseline noise was observed and an increase on the sensitivity was obtained. As an example, Fig. 4 shows the electropherogram obtained for the hydrolyzed spiked urine, where the potential biomarkers of amine exposure (MeIQx, PhIP and carbolines), Trp-P-1 and Trp-P-2 were also analyzed by CE–MS–MS (product ion scan) in order to increase sensitivity and prevent interferences.

At these conditions, detection limits based on a signal-to-noise ratio of 3 were determined (Table 3), obtaining values between 0.3 and 45 ng/ml of urine. Taking into account these values and data reported in the literature [5,7,8], this method can be proposed for the determination of some of the parent amines in human urine samples after the consumption of thermal processed food containing HAs. For instance, the LOD value obtained for PhIP using MS/MS would allow its detection in urine samples at the levels found by Friesen et al. [7].

Table 3
Heterocyclic aromatic amines detection limits in urine samples

| HA | LOD (ng/ml urine) | HA | LOD (ng/ml urine) | HA | LOD (ng/ml urine) |
|---|---|---|---|---|---|
| Trp-P-2 | [a] | MeIQx | 2 | MeAαC | 7 |
| Trp-P-2[b] | 0.6 | MeIQx[b] | 0.3 | MeAαC[b] | 3 |
| H | 2 | 7,8-DiMeIQx | 3 | Glu-P-1 | 16 |
| NH | 2 | 4,8-DiMeIQx | 2 | Glu-P-2 | 22 |
| Trp-P-1 | 2 | PhIP | 2 | AαC | 45 |
| Trp-P-1[b] | 0.3 | PhIP[b] | 0.3 | AαC[b] | 3 |
| DMIP | 12 | IQ | 3 | MeIQ | 1 |

[a] An interference prevented LOD calculation.
[b] LODs in MS–MS.

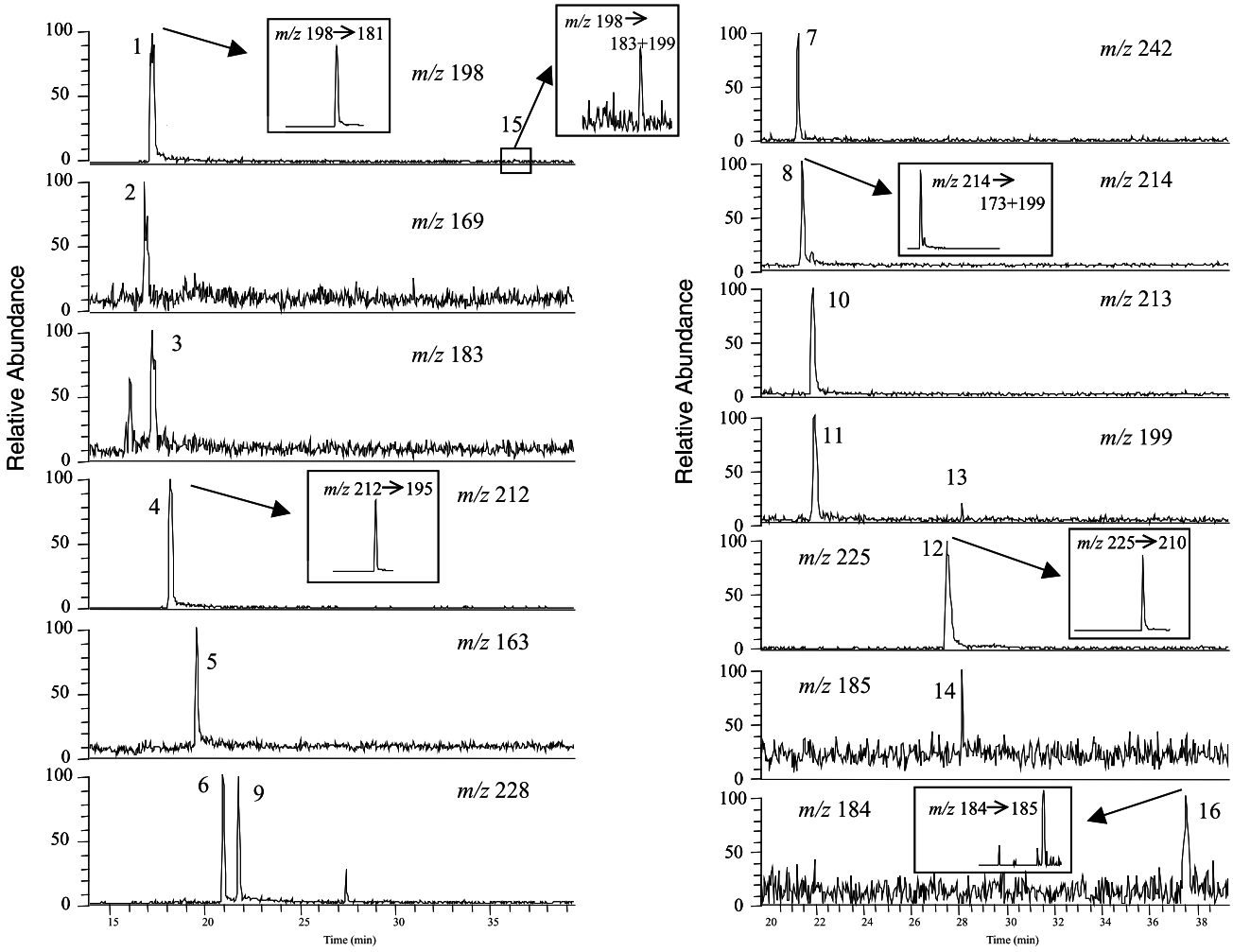



Fig. 4. CE–MS (full scan) and CE–MS–MS (product ion scan) electropherograms of an acid hydrolysed spiked human urine (~45 ng/ml). Capillary: silica, 86 cm × 50 μm i.d. Assignation: (1) Trp-P-2, (2) NH, (3) H, (4) Trp-P-1, (5) DMIP, (6) 7,8-DiMeIQx, (7) TriMeIQx, (8) MeIQx, (9) 4,8-DiMeIQx, (10) MeIQ, (11) IQ, (12) PhIP, (13) Glu-P-1, (14) Glu-P-2, (15) MeAαC and (16) AαC. Other CE conditions as in Fig. 1.

Table 4
Heterocyclic aromatic amines determination in urine samples

| HA[a] | Target value | Calculated conc. (ng/ml) | HA | Target value | Calculated conc. (ng/ml) |
|---|---|---|---|---|---|
| Trp-P-2(1) | 42.6 | 45.6 ± 4.6 | 4,8-DiMeIQx(3) | 41.1 | 38.0 ± 6.0 |
| H(2) | 42.6 | 35.8 ± 7.4 | PhIP(4) | 43.9 | 37.9 ± 5.3 |
| NH(1) | 38.8 | 37.0 ± 6.0 | IQ(2) | 39.1 | 37.5 ± 3.9 |
| Trp-P-1(2) | 43.3 | 40.4 ± 9.7 | MeAαC(4) | 39.5 | 32.0 ± 6.9 |
| DMIP(3) | 42.6 | 35.0 ± 1.9 | Glu-P-1(4) | 39.1 | 39.2 ± 1.4 |
| MeIQx(3) | 45.7 | 39.5 ± 7.4 | Glu-P-2(4) | 44.6 | 36.9 ± 1.4 |
| 7,8-DiMeIQx(3) | 41.1 | 38.5 ± 2.9 | AαC(4) | 45.2 | 28.3 ± 3.1 |
| MeIQ(2) | 44.0 | 41.1 ± 6.3 | | | |

(1) TriMeIQx, (2) IQ-3D, (3) MeIQx-3D, (4) PhIP-3D.
[a] Internal standard used in the quantification.

Finally, in order to study the applicability of the method, the analysis of a spiked urine was performed and results are given in Table 4. Due to the high matrix effects observed using FASI–CE–MS, the quantification was performed using standard addition. Results obtained were in agreement with the spiked level in the original samples with errors lower than 17% except for AαC that showed higher error.

## 4. Conclusions

A fast and simple clean-up procedure has been optimized to extract HAs from urine samples. The proposed method allows the effective recuperation in one single extract of 15 HAs, such as quinolines (IQ and MeIQ), quinoxalines (MeIQx, 7,8-DiMeIQx and 4,8-DiMeIQx), pyridines (PhIP

and DMIP) and carbolines (AαC, MeAαC, H, NH, Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2). For such a purpose, three different cartridges were studied and the best results were obtained using Oasis MCX cartridges, which contain mixed phases. However, it was necessary to include a previous liquid–liquid extraction in order to prevent matrix components from interfering in the analysis. This liquid extraction was more effective and simple when it was carried out on a solid support of diatomaceous earth.

The procedure developed allows detection of amines at 1 ng/ml of urine or even lower. Moreover, the method was applied to the analysis of spiked human urine giving good qualitative and quantitative results. So, this clean-up procedure can be proposed for the analysis of urine samples in the study of human exposure to HAs.

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