

## RESEARCH ARTICLE

# Hollow fibre-supported liquid membrane extraction and LC-MS/MS detection for the analysis of heterocyclic amines in urine samples

Rosa Busquets<sup>1</sup>, Jan Åke Jönsson<sup>2</sup>, Henrik Frandsen<sup>3</sup>, Lluís Puignou<sup>1</sup>, Maria Teresa Galceran<sup>1</sup> and Kerstin Skog<sup>4</sup>

<sup>1</sup>Department of Analytical Chemistry, University of Barcelona, Barcelona, Spain

<sup>2</sup>Department of Analytical Chemistry, Lund University, Lund, Sweden

<sup>3</sup>National Food Institute, Technical University of Denmark, Søborg, Denmark

<sup>4</sup>Department of Food Technology, Engineering and Nutrition, Lund University, Lund, Sweden

Heterocyclic amines (HCAs) are potent mutagens/carcinogens to which humans are frequently exposed through the consumption of cooked meat and fish food. The effect of normal intake of HCAs and their role in the aetiology of human cancer is unknown. To some extent, limitations of the existing analytical methods in monitoring the low levels of HCAs in biological samples have hindered obtaining conclusive results. In this study, a method for the analysis of HCAs in human urine has been studied to detect HCAs and metabolites at levels resulting from consumption of food cooked at ordinary conditions. The analytical method consisted of extraction and clean-up by the novel technique liquid-phase microextraction combined with LC-MS/MS. The effect of pH during the extraction and hydrolysis step was examined. High sensitivity was achieved when the extraction was performed in raw urine adjusted to pH 5.5, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine being detected from 2 pg/g urine, levels comparable with a normal exposure. Good reproducibility and repeatability was obtained for 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, below 9% using isotopic dilution. The performance of the method on 9*H*-pyrido[3,4-*b*]indole, 2-amino-1-methyl-6-(4'-hydroxyphenyl)imidazo[4,5-*b*]pyridine and 2-amino-1-methyl-6-(5-hydroxy)phenylimidazo[4,5-*b*]pyridine was also studied.

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## 1 Introduction

The presence of mutagenic/carcinogenic heterocyclic amines (HCAs) in cooked meat and fish has been extensively studied since the discovery of mutagenic activity in smoke particles

from broiled fish [1]. HCAs are formed during heating of meat and fish *via* the Maillard reaction, which involves the naturally occurring precursors creatine/creatinine, sugars and amino acids [2]. Among the HCAs identified in cooked foods, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyri-

**Correspondence:** Dr. Rosa Busquets, Department of Analytical Chemistry, Chemistry Faculty, University of Barcelona, c/Martí Franquès 1-11, Barcelona 08028, Spain

**E-mail:** rosabs5@gmail.com

**Fax:** +34-93-402-12-33

**Abbreviations:** D<sub>3</sub>-PhIP, 2-amino-1-trideuteromethyl-6-phenylimi-

dazo[4,5-*b*]pyridine; **HCA**, heterocyclic amine; **LPME**, liquid-phase microextraction; **MelQx**, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; **norharman**, 9*H*-pyrido[3,4-*b*]indole; **4'-OH-PhIP**, 2-amino-1-methyl-6-(4'-hydroxyphenyl)imidazo[4,5-*b*]pyridine; **5-OH-PhIP**, 2-amino-1-methyl-6-(5-hydroxy)phenylimidazo[4,5-*b*]pyridine; **PhIP**, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; **TriMelQx**, 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline

dine (PhIP) is the most abundant [3]. In structural terms, HCAs can be regarded as pyridines, quinoxalines, quinolines and pyridoindoles. The International Agency for Research on Cancer has classified eight HCAs, including PhIP, as possible human carcinogens (class 2B) and one (IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline) as a probable human carcinogen (class 2A) [4]. So far, some epidemiological studies show a correlation between intake of HCAs and increased risk of different types of human cancer [5–7], whereas other studies do not find such a correlation [8, 9].

There are several problems associated with the estimation of the intake of HCAs by means of dietary assessments, for example difficulties in getting accurate dietary information [10, 11], and lacking data on HCA concentrations in representative food samples [12]. HCAs are absorbed and metabolized by humans, and have a half-life of less than 12 h [13]. One way of estimating the recent exposure is by analysing HCAs and their metabolites in human urine samples to obtain data on the capacity of individuals to bioactivate and detoxify HCAs [14–16]. Urinary 2-amino-1-methyl-6-(5-hydroxy)-phenylimidazo[4,5-*b*]pyridine (5-OH-PhIP) was found to be directly related to the ultimate electrophile nitrenium ion that form DNA-adducts at the guanine base, and suggested as a plausible biomarker of the genotoxic dose of PhIP [17–19].

Recently, a novel technique, liquid-phase microextraction (LPME), was applied for the analysis of HCAs in urine samples using fluorescence [20] and ultraviolet detection [21]. HCAs in diluted urine samples (donor phase) were extracted with an organic solvent in the pores of a polypropylene hollow fibre, and a simultaneous liquid–liquid extraction, where the analytes in the organic solvent were transferred and trapped in the aqueous solution in the lumen of the fibre (acceptor phase). The principles and applications of LPME were recently reviewed [22].

We report on an analytical method based on LPME and LC-MS/MS that has been established to detect HCAs and their metabolites in urine samples. The objective of this study was to obtain a high-throughput method capable of accurately quantifying PhIP at ordinary levels in urine samples, and detecting HCA metabolites such as 5-OH-PhIP, which is related to the active metabolite causing DNA damage.

## 2 Materials and methods

### 2.1 Chemicals and materials

2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-8-methyl-3-trideuteromethylimidazo[4,5-*f*]quinoxaline, PhIP, 2-amino-1-trideuteromethyl-6-phenylimidazo[4,5-*b*]pyridine (D<sub>3</sub>-PhIP) and 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline (TriMeIQx) were obtained from Toronto Research Chemicals (Ont., Canada). The chemical purity of these HCAs was >99%, according to the manufacturer. 9*H*-Pyrido[3,4-*b*]indole (norharman) was obtained

from Sigma-Aldrich (Steinheim, Germany) and their purity was >98%. Stock standard solutions of 150 µg/g in methanol were prepared and used for further dilution. The solutions were stored at +4°C.

5-OH-PhIP and 2-amino-1-methyl-6-(4'-hydroxyphenyl)imidazo[4,5-*b*]pyridine (4'-OH-PhIP) were obtained from incubations of PhIP with rat liver S9 fortified with NADP<sup>+</sup> and acetyl-coenzyme A as described previously [23]. 5-OH-PhIP and 4'-OH-PhIP were quantified by comparison of their chromatographic data with the data from incubations of [<sup>14</sup>C]-PhIP, where fractions were collected and analysed by liquid scintillation counting.

The ionization constants for some of the studied HCAs have been experimentally determined and are following given in brackets; MeIQx (5.95), TriMeIQx (6.00), PhIP (5.56) and norharman (6.76) [24].

3,4,5-Trihydroxybenzoic acid propyl ester (propyl gallate), EDTA disodium salt dihydrate and 1-octanol were obtained from Fluka (Sigma-Aldrich). All other chemicals and solvents were of HPLC or analytical purity and were supplied by Merck (Darmstadt, Germany).

Micro syringes of 100 µL volume equipped with 0.8 mm outer diameter needles were purchased from Hamilton Bondaluz AG (Switzerland). A 12-position magnetic stirrer model Multipoint HP 6 (Variomag, FL, USA) was used for stirring the samples during the extractions. Accurel® Q3/2 200/600 PP polypropylene hollow fibre membranes (200 µm wall thickness, 600 µm inner diameter and 0.2 µm pore size) were obtained from Membrana GmbH (Wuppertal, Germany). Centrifugation of urine samples in 0.5 L bottles was carried out in a Beckman J2-21 centrifuge with a Rotor JA-10 158 (Bromma, Sweden). A hot plate IKA Combimag Ret (IKA-Werke, Staufen, Germany) was used for the hydrolysis of samples.

### 2.2 Sample handling and storage

Blank urine samples were obtained from a volunteer, who refrained from eating fried foods for 4 days. To prevent oxidation of sensitive metabolites, propyl gallate (dissolved in ethanol) and EDTA (dissolved in water) were added to the urine bottle to give a final concentration of 2 mM [23]. The sample was centrifuged at 11 300 × *g* for 15 min, the pellet was discarded and the supernatant was distributed into 45 mL polypropylene vials and frozen at –18°C until analysis.

### 2.3 Extraction of HCAs and metabolites from urine samples

The previously developed hollow-fibre supported liquid membrane extraction method [20, 21] was applied with some modifications to increase the sensitivity. Briefly, polypropylene hollow fibres were cut into 4.3 cm pieces. Follow-

ing, one end of the fibres was sealed by heating and pressing the melted polymer with tweezers, the fibres were cleaned with acetone in an ultrasonic bath for 1 h and left to dry in the air. The acceptor phase, which was introduced into the fibre by a syringe, was 0.1 g 0.1 M  $\text{H}_2\text{SO}_4$ , and the extraction solvent was 1-octanol as optimized previously in our group [20, 21]. The unsealed end of the fibre was connected to a wire. Immobilized by the wire, the fibre was introduced into the 4 mL glass sample vial bending it in a U-form in a way that the fibre was completely immersed in the donor solution and above the magnetic stirring bar. In total, 3 mL of urine sample was placed in the vial and spiked with 0.025 mL of methanol standard of HCAs and both the volume and pH of the donor solution were adjusted as described as follows: three different extraction conditions were studied (i) Extraction at  $\text{pH} > 10$ , (ii) extraction at  $\text{pH} 5.5$  and (iii) hydrolysis at  $\text{pH} 1$  and extraction at  $\text{pH} > 10$ . *Extraction at  $\text{pH} > 10$* : Urine samples were spiked with 0.025 mL of methanol solution of internal standards (37.2 ng/g  $\text{D}_3$ -PhIP and 134.2 ng/g  $\text{D}_3$ -MeIQx). The samples were alkalized by 0.5 M NaOH (0.3 mL) and diluted with water (0.675 mL). *Extraction at  $\text{pH} 5.5$* : Urine samples were spiked with 0.025 mL of a methanol solution of internal standards (69.3 ng/g  $\text{D}_3$ -PhIP and 281.1 ng/g  $\text{D}_3$ -MeIQx). The pH was adjusted with 0.13 M ammonium acetate/acetic acid buffer to  $\text{pH} 5.5$  (1 mL). *Hydrolysis at  $\text{pH} 1$  and extraction at  $\text{pH} > 10$* : urine samples were spiked with 0.025 mL of a methanol solution of internal standards (69.3 ng/g  $\text{D}_3$ -PhIP and 281.1 ng/g  $\text{D}_3$ -MeIQx). The sample was acidified with 1 M HCl (0.35 mL). The hydrolysis was carried out in a water bath at 90–95°C for 1 h [16]. The volume of the solution that decreased because of evaporation during the hydrolysis was increased by adding 2 M NaOH (0.3 mL) and water (0.325 mL) to adjust the pH and keep the hollow fibre immersed during the subsequent extraction. A second urine sample was run in parallel to measure the pH at each step. Each sample vial was stirred at one of the 12-position magnetic stirring devices. The extraction was performed for 90 min with magnetic stirring (850  $\pm$  50 rpm) using 5  $\times$  2 mm magnetic stir bars. When the extraction finished, the fibre was removed from the vial, the unsealed end of the fibre was connected to the needle of a syringe and the edge of the sealed side of the fibre was cut. Then, the acceptor solution was introduced into a 1.1 mL conical vial by depressing the plunger of the syringe. The total acceptor solution from four extractions (40  $\mu\text{L}$ ), *i.e.* HCAs pre-concentrated from a total of 12 mL of urine, was collected into the same conical vial and injected into the LC-MS system. Water blanks, with and without internal standards, were extracted at each batch of extractions.

## 2.4 LC-MS/MS analysis of HCAs and metabolites

The chromatographic and MS conditions have been described elsewhere [25, 26]. The chromatograph was a Spectra Physics P2000 and the mass spectrometer an ion trap

LCQDeca equipped with an on-axis electrospray ionization source operating in the positive ionization mode (Thermo Finnigan, San Jose, CA, USA). A post-column addition of 0.1% formic acid in ACN at 0.1 mL/min was carried out. The injector of the chromatograph was automatically rinsed with 2 mL of 60% ACN in water after each injection to prevent both contamination and corrosion of the injector. After injection of each sample and standard, methanol was injected to verify that there was no cross-contamination of HCAs. Acquisition parameters for the determination of HCAs in MS/MS are summarized in Table 1.

## 2.5 Quality parameters in the analysis of HCAs in urine samples

LOD, repeatability, reproducibility, recovery and accuracy of the analytical method were determined by analysing different spiked blank urine samples. To determine repeatability, six replicate analyses of HCAs were carried out on the same day using the same urine. To determine reproducibility, two determinations were carried out along three non-consecutive days using three different spiked urine samples. RSD were calculated from concentrations measured. PhIP and MeIQx were quantified by isotopic dilution with  $\text{D}_3$ -PhIP and  $\text{D}_3$ -MeIQx, respectively, except when evaluating their recovery rates. Norharman, 4'-OH-PhIP and 5-OH-PhIP were estimated by external calibration without internal standard correction. Accuracy (%) was estimated as the agreement between the measured and the spiked concentration and was only evaluated for the analytes determined by isotopic dilution. A total of nine determinations performed along 3 days using three urine matrices spiked at a level of 0.12 ng/g were carried out for the evaluation of accuracy. Recovery of each analyte in the analytical process was estimated by standard addition at spiking levels of 0.05, 0.1, 0.2, 0.3 and 0.5 ng HCAs/g urine. LOD (signal-to-noise of 3) were assessed in spiked urine samples.

## 3 Results and discussion

Recently a simple, fast and high-throughput method based on LPME was developed in our group for the analysis of PhIP in urine and blood samples. However, the sensitivity and selectivity provided were still insufficient to quantify HCAs after a normal dietary exposure to the mutagens [20, 21]. The performance of the analytical method in both raw urine and hydrolysed urine was of our interest because of the different type of information that these matrices provide. Blank urine was obtained for the studied HCAs except for norharman, which is an ubiquitous amine that can be found in other food items than in cooked meat or fish, such as in coffee, vinegar or cheese, as reviewed by Pfau and Skog [27].

**Table 1.** Acquisition parameters in the MS

| Analyte               | Precursor ions ( <i>m/z</i> )<br>[M+H] <sup>+</sup> | Normalized collision<br>energy (%) | Product ions used for<br>quantification ( <i>m/z</i> ) | Product ion scan<br>range ( <i>m/z</i> ) |
|-----------------------|---|------------------------------------|--|--|
| <b>Pyridines</b>      |   |                                    |  |  |
| PhIP                  | 225   | 49                                 | 210<br>208   | [200–230]                                |
| 4'-OH-PhIP            | 241   | 49                                 | 226<br>213   | [200–250]                                |
| 5-OH-PhIP             | 241   | 49                                 | 223  | [200–250]                                |
| D <sub>3</sub> -PhIP  | 228   | 49                                 | 211<br>210   | [200–230]                                |
| <b>Quinoxalines</b>   |   |                                    |  |  |
| MeIQx                 | 214   | 45                                 | 199<br>197<br>187<br>173                               | [165–220]                                |
| D <sub>3</sub> -MeIQx | 217   | 45                                 | 200<br>190<br>173                                      | [165–220]                                |
| <b>Pyridoindole</b>   |   |                                    |  |  |
| Norharman             | 169   | 49                                 | 168<br>167<br>142<br>115                               | [110–175]                                |

### 3.1 Extraction conditions in raw urine

Different extraction conditions were tested for the analysis of HCA and metabolites from urine. The donor solution, constituted by diluted urine, was adjusted at two different extractions pHs: 5.5 and > 10. The effect of the urine matrix and the pH of the donor solution on the extraction and ion suppression in the detector were examined by comparing the signals obtained from spiked water and spiked urine samples with representative HCAs of different groups: quinoxalines (MeIQx), pyridines (PhIP) and pyridoindoles (norharman) at 0.12 ng/g. Average peak areas ( $n = 3$ ) from both 5-OH-PhIP and 4'-OH-PhIP extracted from spiked urine were significantly higher at pH 5.5 than at the most basic conditions ( $p = 0.05$ ). No significant differences were found for the other studied HCAs. The influence of the donor phase pH on the sensitivity can not only be caused by the extraction yield of the analytes but also by the extraction of matrix components affecting the ionization. The signal obtained for MeIQx and PhIP at both pH conditions in urine matrix was significantly lower than the response obtained in absence of matrix ( $p = 0.05$ ), indicating that the matrix interfered either in the extraction by LPME or in the electrospray ionization source causing signal suppression (data not shown). The matrix was not found to significantly decrease the response of the other HCAs studied here. However, ion suppression was shown to greatly affect the area of TriMeIQx, which had been added to the purified

extracts at a final concentration of 112 ng/g in preliminary studies as an internal standard (data not shown). High variations in the TriMeIQx area, up to 50%, lead us to discard its use for the correction of variations in the sample injection.

### 3.2 Extraction conditions in hydrolysed urine

The hydrolysis at pH 1 and the following extraction by LPME at pH > 10 caused a general increase of the background noise. Only a low number of samples were analysed under these conditions due to the possibility of losing peak efficiency and decreasing HPLC column lifetime. A marked decrease of the signal for both 4'-OH-PhIP and 5-OH-PhIP was observed in spiked urine. 4'-OH-PhIP was detected at levels below 2% of those obtained with unhydrolysed raw urine at the same pH, and 5-OH-PhIP was not detected, probably because of the lack of stability of this metabolite at these conditions. Substitution of the acid hydrolysis with enzymatic hydrolysis may result in lower background noise and reduce the loss of acid labile metabolites such as 4'-OH-PhIP and 5-OH-PhIP. The signal for norharman increased significantly after the hydrolysis ( $p = 0.05$ ). We found that the increase of the signal was caused by components inherent to the urine matrix, possibly as a consequence of the hydrolysis of norharman metabolites.

### 3.3 Quality parameters in the determination of HCAs in urine by LPME and LC-MS/MS

Quality parameters in the analysis of spiked urine at different extracting conditions by LPME and LC-MS/MS are summarized in Table 2. The LODs achieved ranged from 2 pg/g for PhIP up to 50 pg/g for norharman. Linearity ( $R^2$ ) range (LOD 3.3 ng/g urine) was  $>0.998$  for the HCAs and metabolites studied. The achieved sensitivity would allow detecting PhIP and 4'-OH-PhIP in urine after eating 100 g of fried chicken as reported in earlier works [26, 28], considering ordinary 12 h-urine volumes (0.3–1 L). This is the time period when most HCA metabolites are excreted [29], with excretion rates of 0.3–1.2% as reported for PhIP [30]. The sufficient sensitivity to detect ordinary levels of HCAs in urine was achieved by extracting HCAs from 12 mL urine samples and injecting the total amount of purified sample (40  $\mu$ L). The detection limit which was the only quality parameter estimated in hydrolysed urine, was 25 pg PhIP/g urine. The increase of detection limit in the hydrolysed urine samples compared with the analyses of raw urine may be due to a decrease in the extraction yield, an increase of the ion suppression in the electrospray ionization source due to the higher complexity of the sample, and in some urine samples by lack of resolution from co-extracted matrix substances.

The fact that PhIP presented the best LOD is partially due to the narrow chromatographic peak, of just 30 s at the limit of quantification, eluting at 60% of ACN. The lower sensitivity obtained for the other studied HCAs was in part caused by the higher peak width. Apart from chromatographic causes, the effect of co-extracted compounds from the urine matrix influenced the sensitivity, as was described for MeIQx and PhIP in Section 3.1. In fact, variations in sensitivity were observed in different spiked urine samples, although LODs were kept in the same order of magnitude as the values listed in Table 2. Differences in the sensitivity were attributed to different compositions of the urines, giving differences in ion suppression during the LC-MS/MS analysis and to the stability of 5'-OH-PhIP. Although pH 5.5

lead to better sensitivity for PhIP than the more basic extraction, possibly because of the most selective extraction of PhIP from urine, no big differences were found for the rest of the studied HCAs. The LODs achieved for PhIP and MeIQx in this study are comparable with the best sensitivities reported for the last decade (data reviewed in Table 3), with the advantage that the LPME method presented here implies lower cost, easier sample handling and use of less organic solvents than most of the SPE and LLE methods summarized in the table. In addition, the LC separation does not require of a derivatization step as for GC, with the subsequent increase of analysis time and decrease of the recovery, and it presents higher reproducibility and higher sensitivity than CE. Moreover, MS/MS provides more structural characterization for a proper identification of the analytes and it is more selective than MS, FD or UV detection.

Recovery of the analytical method that comprises the extraction by the LPME method and the ionization in the electrospray is a key factor influencing the minimum detectable levels. In principle, HCAs should be in neutral form for better recoveries in the extraction and the extraction would be favoured with the largest pH difference between the donor and the acceptor solution. However, recoveries for some of the studied HCAs were estimated to be higher at pH 5.5, probably due to less extraction of disturbing components from the matrix resulting in a decrease of the ion suppression effect in the ion source. At pH  $>10$ , 4'-OH-PhIP will partially be negatively charged, which may explain the lower recovery obtained at this pH than at pH 5.5. 5-OH-PhIP contains two functional groups ( $\text{NH}_2$  and OH) with small differences in  $\text{pK}_a$  values leaving a narrow pH range where the analyte is uncharged, which may explain the low recovery of this compound. In addition, 5-OH-PhIP is sensitive to oxidation, accordingly the lower recovery could partially be caused by loss of this analyte. This problem may be circumvented by addition of stabilizing agents to the acceptor solution or by using a different acceptor solution. Newer mass spectrometers than the one

**Table 2.** Quality parameters in the analysis of HCAs in urine by LPME and LC-MS/MS

|  | pH $>10$ |       |           |            |           | pH 5.5 |       |           |            |           |
|--|----------|-------|-----------|------------|-----------|--------|-------|-----------|------------|-----------|
|  | PhIP     | MeIQx | Norharman | 4'-OH-PhIP | 5-OH-PhIP | PhIP   | MeIQx | Norharman | 4'-OH-PhIP | 5-OH-PhIP |
| LOD (pg/g urine)                       | 9        | 23    | 40        | 11         | 13        | 2      | 28    | 50        | 5          | 9         |
| Repeatability (RSD, %) <sup>a)</sup>   | 9.8      | –     | –         | –          | –         | 7.0    | –     | –         | –          | –         |
| Reproducibility (RSD, %) <sup>a)</sup> | 12.6     | –     | –         | –          | –         | 9.1    | –     | –         | –          | –         |
| Repeatability (RSD, %) <sup>b)</sup>   | 2.2      | 1.1   | 17.5      | 15.1       | 8.2       | 5.3    | 5.0   | 13.0      | 10.3       | 3.9       |
| Reproducibility (RSD, %) <sup>b)</sup> | 2.8      | 1.9   | 13.0      | –          | –         | 5.1    | 8.5   | 23.9      | –          | –         |
| Recovery (%)                           | 50       | 64    | 27        | 17         | 7         | 59     | 56    | 28        | 58         | 10        |

a) Spiking level: 12 pg/g urine. At pH  $>10$ , precision was estimated for a concentration below the quantification limit.

b) Spiking level: 120 pg/g urine.

**Table 3.** Analytical method, sensitivity and amount of PhIP and MeIQx reported in raw and hydrolysed urine

| HCA          | Dose in urine (%) | Extraction and purification procedure | Determination                        | LOD (pg/mL urine) | Ref. |      |
|--------------|-------------------|---------------------------------------|--------------------------------------|-------------------|------|------|
| <b>Urine</b> |                   |                                       |                                      |                   |      |      |
| PhIP         | 0.7               | LLE                                   | GC-MS                                |                   | [31] |      |
|              |                   | LLE and SPE                           | GC-MS                                | 1                 | [16] |      |
|              | 0.5–2             | LLE-SPE (Blue cotton)                 | GC-MS                                | 2.5               | [33] |      |
|              |                   | μSPE                                  | CE-nESI-MS                           |                   | [34] |      |
|              |                   | μSPE                                  | CE-nESI-MS                           | 14                | [35] |      |
|              |                   | SPE (Blue chitin)                     | LC-MS                                |                   | [36] |      |
|              | 0.4–0.9           | Direct injection                      | LC-scintillation counting            |                   | [14] |      |
|              | 0.3–1.2           | Direct injection                      | LC-scintillation counting, μLC-MS/MS |                   | [30] |      |
|              | MeIQx             | 2.6                                   | LPME                                 | LC-FD             | 8    | [20] |
|              |                   |                                       | LPME                                 | LC-UV             | 500  | [21] |
| 1–6          |                   | LLE                                   | GC-MS                                |                   | [31] |      |
|              |                   | LLE-SPE (Blue cotton)                 | GC-MS                                | 10                | [33] |      |
|              |                   | μSPE                                  | CE-nESI-MS                           |                   | [34] |      |
|              |                   | SPE (Blue chitin)                     | LC-MS                                |                   | [36] |      |
|              |                   | LPME                                  | LC-UV                                | 100               | [21] |      |
|              |                   | <b>Hydrolysed urine</b>               |                                      |                   |      |      |
| PhIP         | 2–8.5             | LLE and SPE                           | GC-MS                                | 1                 | [32] |      |
|              |                   | LLE-SPE (Blue cotton)                 | GC-MS                                | 2.5               | [33] |      |
|              | 1.9–9.8           | LLE, immunoaffinity chromatography    | LC-MS/MS                             | 5–10              | [37] |      |
|              |                   | LLE, immunoaffinity chromatography    | LC-MS/MS                             | 4                 | [38] |      |
|              |                   | SPE                                   | LC-MS/MS                             |                   | [14] |      |
|              |                   | LLE and SPE                           | GC-MS                                | 1                 | [32] |      |
|              |                   | SPE, immunoaffinity chromatography    | GC-MS                                | 1                 | [16] |      |
|              |                   | SPE, immunoaffinity chromatography    | LC-FD, GC-MS                         | 50                | [16] |      |
|              |                   | SPE, MIP                              | LC-MS/MS                             |                   | [18] |      |
|              |                   | SPE                                   | FASI CE-MS/MS                        | 300               | [39] |      |
|              |                   | SPE, MIP                              | LC-MS                                |                   | [19] |      |
|              |                   | SPE, MIP                              | LC-MS <sup>3</sup>                   |                   | [23] |      |
| MeIQx        |                   | 20–63                                 | LLE-SPE (Blue cotton)                | GC-MS             | 10   | [33] |
|              | 13–32             | LLE, immunoaffinity chromatography    | LC-MS/MS                             | 5–10              | [37] |      |
|              | 3.2–22.7          | SPE                                   | FASI CE-MS/MS                        | 300               | [39] |      |

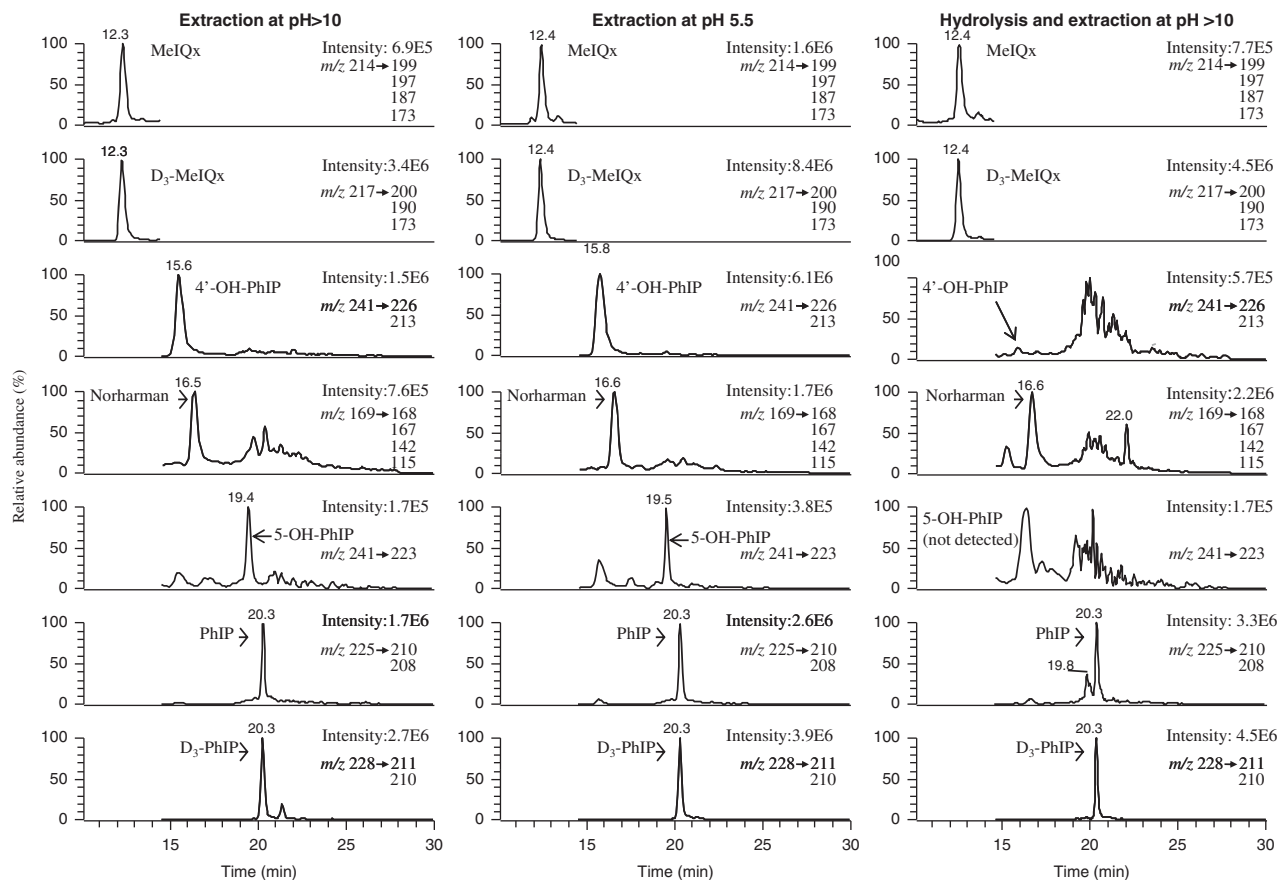
used in this study, equipped with new designs of ion sources [40] may improve the sensitivity.

Repeatability and reproducibility were evaluated at two different levels for PhIP, the lower concentration studied was very close to its detection limit because the expected level of PhIP in urine would be in that order according to data summarized in Table 2. For the rest of analytes, quality parameters were only evaluated at the higher spiking level studied because these compounds could not be quantified at the lower spiking level. The quantification by isotopic dilution provided high precision for both MeIQx and PhIP, showing that it is a good choice for the quantification. Repeatability and reproducibility for the rest of HCAs,

which were not corrected by any internal standard, showed that the performance of the method was suitable for the analysis of these amines as well.

Accuracy in the determination carried out in raw urine by isotopic dilution was not different from 100% ( $p = 0.01$ ). In some of the spiked and hydrolysed urines, co-extracted compounds were not sufficiently separated from PhIP but from D<sub>3</sub>-PhIP which may affect the accuracy of the quantification by isotopic dilution; however, accuracy was not evaluated for these samples.

Figure 1 shows the selectivity achieved in the detection of HCAs after the analysis of the same urine by the three different extraction conditions tested.



**Figure 1.** Comparison of the different assayed extraction conditions for the analysis of HCAs in urine by LPME and LC-MS/MS. Blank urine was spiked at 0.61 ng/g MelQx; 3.0 ng/g D<sub>3</sub>-MelQx; 1.9 ng/g 4'-OH-PhIP; 0.68 ng/g Norharman; 1.4 ng/g 5-OH-PhIP; 0.43 ng/g PhIP; 0.72 ng/g D<sub>3</sub>-PhIP.

#### 4 Concluding remarks

Most methodology published to date for the analysis of HCAs and their metabolites in urine involve several steps in the clean-up procedure in order to eliminate matrix components, to pre-concentrate the target compounds and are expensive in terms of analysis time and cost of materials and solvents. The simple and handy method proposed in this study based on LPME and LC-MS/MS is an improvement in the current analysis of HCAs in urine. Sensitivity capable of detecting HCAs in raw urine from a normal exposure has been achieved by means of an extraction at pH 5.5. The studied method has shown good precision and accuracy for the determination of PhIP and MelQx by isotopic dilution. The detection of other abundant HCAs in cooked meat, such as norharman and 4'-OH-PhIP, or 5-OH-PhIP, metabolites having potentials as human biomarkers of absorption and activation, makes it a method of choice for the determination of HCAs in routine analysis. The analysis of hydrolysed samples by the studied LPME conditions and LC-MS/MS did not offer any advantage with respect to the analysis of non-hydrolysed samples because of

the higher complexity of the matrix except for the increase of norharman.

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