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Analytical Methods

# Analysis of non-polar heterocyclic aromatic amines in beefburguers by using microwave-assisted extraction and dispersive liquid–ionic liquid microextraction

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ABSTRACT

A new sample preparation procedure to determine six heterocyclic aromatic amines (3-Amino-1,4-dimethyl-5H-pirido[4,3-b]indole, 3-Amino-1-methyl-5H-pirido[4,3-b]indole, 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine, 2-amino-9H-pyrido-[2,3-b] indole, 2-amino-3-methyl-9H-pyrido-[2,3-b] indole and 2-Amino-1,6-dimethylimidazo [4,5-b]-pyridine) in cooked beefburguers by using a combination of microwave-assisted solvent extraction and dispersive liquid–liquid microextraction with an ionic liquid generated *in situ* was used. The optimized microwave extraction procedure consisted of a clean-up step with *n*-heptane and a subsequent dissolution step in basic media to desorb the analytes from the matrix. Next, an aqueous solution of the ionic liquid 1-octyl-3-methylimidazolium tetrafluorborate and sodium hexafluorphosphate was added and a water-insoluble 1-octyl-3-methylimidazolium hexafluorphosphate was formed within the matrix sample. The amines were analyzed by liquid chromatography with fluorescence and diode-array detection by using a typical C18 column. Peak identities were confirmed by absorbance spectral matching. Repeatability (RSD%) between 5.4% and 10.9%, enrichment factors between 19 and 30, limits of detection between 0.35 and 2.4 ng $mL^{-1}$ and recoveries between 69% and 100% were achieved. The extraction methodology is simple, rapid (about 40 min/sample) cheap and green since small amounts of non-toxic solvents are necessary.

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## 1. Introduction

Heterocyclic aromatic amines (HAAs) are compounds with high carcinogenic potential formed during the cooking process at high temperatures of protein-rich foods. This is possible due to the simultaneous presence of creatine, creatinine, sugars and amino acids (Jägerstad, Skog, Grivas, & Olsson, 1991; Sugimura, 1997). The content of HAAs depends on several conditions such as temperature, additives, pH and cooking time. At temperatures higher than 150 °C the non-polar HAAs begin to appear and their concentrations increase with cooking temperature (Adamson et al., 1990; Dooley, Von Tungeln, Bucci, Fu, & Kadlubar, 1992; Nagao, 1999; Sugimura, 1997; Wakabayashi et al., 1997). Although there is no up today enough scientific evidence to demonstrate carcinogenicity in humans, several studies have attributed colorectal, pancreatic and breast cancer to HAAs (Alaejos, González, & Afonso, 2008; Felton et al., 2004; Sugimura, 2002). However, these compounds have proven to be carcinogenic in rodents and non-human primates (Adamson et al., 1990; Dooley et al., 1992; Nagao, 1999; Wakabayashi et al., 1993).

Since the matrices where HAAs are present (foods, plasma, urine) are very complex and the concentrations are in the ng $g^{-1}$ levels, the analytical methodologies to determine these compounds must be sensitive, selective and precise to establish a reliable relationship between intake and effects in human health. Previous to the analysis step, a cleaning procedure is necessary to eliminate contaminants such as fat and proteins. Also, a preconcentration step is mandatory to reach the sensibility levels of the detectors usually used. Commonly, liquid–liquid extraction (Lee & Tsai, 1991) and solid phase extraction (SPE) (Toribio, Puignou, & Galceran, 1999) are used as clean-up and preconcentration steps and liquid chromatography with UV (Knize et al., 1995), fluorescence detection (Kanai, Wada, & Manabe, 1990) and mass spectrometry (Galceran, Moyano, Puignou, & Pais, 1996) or capillary electrophoresis (De Andrés, Zougagh, Castañeda, & Ríos, 2010) are used in the separation step.

Some years ago, sample pretreatment developed by Gross (1990) and Toribio et al. (1999) have been widely used as reference methods. Puignou et al. have reviewed several analytical strategies developed until year 2000 for the extraction and preconcentration

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of HAAs from several matrices, considering the solvents, sorbents and separation procedures used (Toribio, Galceran, & Puignou, 2000). However, such procedures usually require large amounts of organic solvents and involve tedious purification steps. In an attempt to overcome these limitations, Khan, Busquets, Santos, & Puignou (2008) have used the pressurized liquid extraction (PLE) technique to analyze HAAs in meat extracts. PLE is as effective as conventional extraction techniques but with the advantage of an important time reduction and solvent requirement.

Microwave-assisted solvent extraction (MASE) is another sample preparation method very suitable for routine analysis giving high efficiencies and allowing several simultaneous extractions within the microwave oven, producing less waste by sample than other conventional techniques; therefore, it is considered a clean technology (green chemistry) (Mester & Sturgeon, 2003). Also, the equipment is quite more cheap than the required for PLE. There are a large number of reports concerning application of this technique to the extraction of several compounds in matrices of diverse origin (Beyer & Biziuk, 2008; Chan, Yusoff, Ngoh, & Kung, 2011; Franke, Winek, & Kingston, 1996; Madej, 2009).

In 2006, Assadi and co-workers (Rezaee et al., 2006) developed a novel liquid phase microextraction technique, named dispersive liquid–liquid microextraction (DLLME). This method is based on a ternary solvent system, in which a solution of an extraction solvent (e.g. dichloromethane, toluene, *n*-octanol) in the disperser solvent (e.g. methanol, acetonitrile, isopropanol) is rapidly injected into the aqueous sample by a syringe. The disperser solvent must be miscible in both the aqueous and in the organic phase. During the dispersion process, very small droplets (high surface contact area) are formed which allows to increase mass transfer. After this, droplets collapse to form the organic layer containing the analytes in a very small volume obtaining high enrichment factors. This last step can be speed-up by centrifugation.

In 2009, Shemirani and Baghdadi developed the "ionic liquid-DLLME" (IL-DLLME) for determination of inorganic species in saline solutions in which the microdroplets of the extractant solvent were formed *in situ*, i.e., within the matrix of the analyte (Baghdadi & Shemirani, 2009). This was possible since microdroplets of the water insoluble ionic liquid (IL) 1-hexyl-3-methylimidazolium hexafluorphosphate, [HMIm][$PF_6$], have been generated within the matrix by mixing the water soluble 1-hexyl-3-methylimidazolium tetrafluorborate, [HMIm][$BF_4$] and $NaPF_6$. In this technique, there is no interface (infinite contact area) between water and the extractant phase at the moment of mixing the reactives. During the formation of the droplets, the extractant solvent (IL) collect the hydrophobic species. The mass transfer from aqueous phase into the IL phase has no significant effect on the extraction step. In comparison with the classical DLLME, in this technique no disperser solvent, which can reduce the extraction recovery, is used (Padró, Ponzinibbio, Agudelo Mesa, & Reta, 2011).

In this work, the MASE technique coupled to the *in situ* IL-DLLME technique was applied for the first time to the determination of five non-polar HAAs in cooked beefburguers. An optimized MASE procedure consisting of a clean-up step with *n*-heptane and a subsequent dissolution step in basic media to desorb the analytes from the matrix was performed. After that, an aqueous solution of the ionic liquid 1-octyl-3-methylimidazolium tetrafluorborate, [OMIm][$BF_4$], was mixed with an aqueous solution of $NaPF_6$ within the sample solution. Thus, the water-insoluble 1-octyl-3-methylimidazolium hexafluorphosphate, [OMIm][$PF_6$], was formed *in situ*. After fluidification of the IL phase, a fix volume was injected into the HPLC system. Fluorescence detection was used for quantitative analysis. Good reproducibility, sensitivity and recoveries were achieved.

## 2. Experimental

### 2.1. Chemicals and materials

3-Amino-1,4-dimethyl-5*H*-pirido[4,3-*b*]-indole (Trp-P-1), 3-amino-1-methyl-5*H*-pirido[4,3-*b*]-indole (Trp-P-2), 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP), 2-amino-9*H*-pyrido-[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido-[2,3-*b*]indole (MeAαC) and 2-amino-1,6-dimethylimidazo[4,5-*b*]-pyridine (DMIP) were obtained from Toronto Research Chemicals (North York, ON, Canada). Water was purified by means of a Milli-Q Purification System (Simplicity, Millipore, Massachusetts, USA). Methanol (J.T. Baker) was HPLC grade. Reagents were of analytical grade or better: sodium hexafluorphosphate ($NaPF_6$) 98.0% (Aldrich), *n*-heptane (Merck), hydrochloric acid, (Merck), phosphoric acid (Merck), sodium hydroxide (Analar, Poole, England). The IL 1-octyl-3-methylimidazolium tetrafluorborate ([OMIM][$BF_4$]) was synthesized in our laboratory (Padró et al., 2011). Myristic acid, palmitic acid and estearic acid were obtained from Analabs (West Virginia, USA).

All solutions were filtered through 0.22-μm membranes (Micron Separations, Westborough, MA, USA) before injection into the HPLC column.

### 2.2. Instrumentation

An HP 1100 liquid chromatograph equipped with a binary pump, degasser, a thermostat-controlled column compartment containing a Zorbax SB-C18 column (4.6 × 250 mm; 5 μm) connected to a guard column, and simultaneous diode-array detector (DAD) and fluorescence detector (FLD) connected to a Data Apex CSW (Data Apex, Czech Republic) workstation was used. Peak identities were confirmed by comparing the DAD spectra with those stored in the spectral library.

The microwave experiments were performed in a microwave oven (MARS-5, CEM Corporation, Matthews, NC, USA) equipped with 24 fluoropolymer-coated cells containing stirring bars and a programmable microcomputer controlling radiation power, time and temperature inside the cell through an IR probe. A culinary mini-pimer mixer for grinding the lyophilized cooked beefburger and homogenizing with *n*-heptane was used.

A Luguimac LC-20 (Luguimac S.R.L., Villa Lynch, Buenos Aires) and an Eppendorf 5417C/R centrifuges able to operate to 4200 and 14,000 rpm, respectively, were used. The first centrifuge was used for separation of the precipitated protein material after MASE experiments in polypropylene conical-bottom tubes of 15 mL. The second centrifuge was used with 1.5 mL Eppendorf polypropylene micro-tubes for phase separations after the IL-DLLME. For mixing the sample extracts with the water-soluble IL and the inorganic salt to generate the dispersion, a Vortex Genie 2 (Scientific Industries, USA) mixer was used. The temperature inside the beefburguer was measured with a flat profile thermometer (Cole Parmer). Water was purified with a Milli-Q system (Millipore Co.).

A gas chromatograph HP 6890 with a cyanopropylphenyl polydimethylsiloxane capillary column (30 m × 530 μm) and a FID detector for the cleaning studies of the fatty acids was used. The oven temperature was 200 °C and the injection volume was 1 μL.

### 2.3. Chromatographic conditions

Methanol-buffered phosphate (pH 3.50; 25 mM) was used as the mobile phase. Optimum separation was achieved with a binary mobile phase at a flow rate of 1 mL $min^{-1}$. Solvent A: methanol–buffer (10:90); solvent B: methanol–buffer, pH 3.5 (60:40). The

gradient elution program was: 20% B, 0 min; 45% B, 5–15 min; 60% B, 17 min; 90% B, 20 min; 93% B, 25 min; 100% B, 25–30 min. For fluorescence detection of HAAs, the optimum wavelengths were: excitation at 265 nm and emission at 380 nm. The sample injection volume was 10 μL. All mobile phases were filtered through 0.22-μm nylon membranes (Osmonics-Magna) for organic solvents and 0.45-μm cellulose-nitrate filters (Micron Separations) for aqueous phases.

### 2.4. Preparation of stock and standard solutions

Amine stock solutions (0.1 mg $mL^{-1}$) were prepared in methanol–water (50:50) and stored at 4 °C in darkness. Standard solutions for calibrations were prepared by the appropriate dilution of the stock solutions and filtered through 0.22-μm cellulose nitrate membranes before use. The state of conservation of the stock solution was checked by measuring the chromatographic peak areas immediately after the solution was prepared with the corresponding areas obtained at the moment of quantification.

### 2.5. Sample preparation

Beefburgers were obtained frozen from a local market. They were put immediately in a Teflon-coated ovenproof dish covered with a tiny layer of a commercial non-stick cooking spray and cooked for 14 min each side in a domestic oven at the maximum achievable temperature, 208 °C, measured with a digital contact thermometer. Before extraction, the crust (outer 3 mm from each side) of the burgers was removed with a scalpel, lyophilized and reserved in the freezer until use. By using a domestic mixer, the crust was homogenized during 5 min to obtain a powder and 3 g of this sample was mixed with *n*-heptane (9 mL in three steps of 3 mL each one) and put in the microwave oven. After application of an optimized temperature gradient in the microwave oven, the *n*-heptane containing the fat material and soluble organic matter was discarded. The absence of fat material after cleaning was confirmed by gas chromatography (see Section 3.1) by using as probes the three most common fatty acids present in foods: myristic, palmitic and stearic acids. Since the non-polar HAAs are not soluble in *n*-heptane at room temperature, loss of analytes in the cleaning process was minimized. After this, 12 mL of 0.5 M sodium hydroxide were added to the defatted sample, mixed in a vortex and centrifuged in a screw-cap conical-bottom polypropylene centrifuge tube for 6 min at 4200 rpm. In this step, the remaining lipid material (2.5%) is transformed into the respective saponification products and the proteins are precipitated. The obtained aqueous phase (1.6 mL) with the optimum pH was filtered with 0.2 μm nylon filters and it was ready for the IL-DLLME.

### 2.6. Dispersive liquid–ionic liquid microextraction (IL-DLLME)

200 μL of [OMIM][$BF_4$] (18 mg $mL^{-1}$) and 240 μL of $NaPF_6$ (120 mg $mL^{-1}$) are mixed with the aqueous sample containing the amines. Thus, the number of moles of the inorganic salt is 13 times higher than the number of moles of IL. This excess of salt is enough (see discussion Section 3.2) to assure displacement of the metathesis reaction toward the extracting product [OMIM][$PF_6$]. The solution is left to stand for 5 min for the tiny droplets to collapse, centrifuged (more than 9000 rpm to obtain a complete phase separation) and the obtained supernatant is discarded. An IL phase is formed (8 μL) at the bottom of the conical-bottom tube. Then, 50 μL of methanol (the minimum amount necessary to completely dissolve the IL phase) is added to decrease viscosity and 10 μL are injected into the HPLC column.

## 3. Results and discussion

In the experimental procedure, some important parameters that usually affect the extraction performance were optimized. As said before, in the first step a microwave procedure to remove the analyte from the matrix and clean the sample to remove the fat material was used. In a second step, the analytes in the aqueous solution were extracted by the IL-DLLME procedure.

### 3.1. Optimization of cleaning conditions and desorption of the solid matrix

The clean-up procedure started with the cooked, lyophilized and crushed beefburguer sample. As said before, in order to optimize the cleaning of the fat material, three fatty acids were used as probes. By using pure standards, the three compounds were identified in the sample and then quantified by an external standard procedure. *n*-Heptane as the extracting solvent for the fat material was used. Although *n*-heptane is a non-microwave absorbing solvent, the small water content of the sample and the small stirring magnet in the extraction vessel heats the sample which improves mass transfer into the organic solvent. Although the boiling point of *n*-heptane is 90 °C, higher pressures and, consequently, higher temperatures within the extraction vessel can be reached since it remains closed during the extraction process. If pressure inside the vessel exceeds a determined allowed limit, the safety rupture membrane breaks and the magnetron is automatically switched off.

Different MASE conditions for cleaning and desorption optimization were used. The microwave oven allows working at three different radiation powers: 1600, 800 and 400 W. Sample is burned at 1600 W in less than 2 min. In order to avoid burning the sample and loss of solvent through the vent tube, we decided to control the process by fixing the extraction temperature instead of the power supply. The vessels were heated by using different temperatures gradients at 800 W. By fixing the temperature the oven inject the necessary microwaves (% power) to reach the set value. If irradiation time is 1 min at each temperature, the fat material is not completely removed and when the irradiation time was 5 min the amount of extracted amine was the same as working at 3 min. Thus, we decided to work with stepped temperature gradients setting 800 W in the oven. Three gradients were assayed: 90–100–110, 70–80–90 and 50–60–70 °C, each step lasting 3 min. After these MASE programs were assayed, the best cleaning conditions were reached when the ramp of 90, 100 and 110 °C. As can be observed from Fig. 1, by using 3 g of sample, 9 mL of *n*-heptane are necessary to eliminate all the fatty acids. If no microwaves are used, the amount of solvent to extract all the lipid material for the same amount of sample is 42 mL, which takes about 42 min of experimental work. Thus, it is evident that microwaves speed up significantly the cleaning process and they simultaneously help to improve the extraction efficiency of the amines since they are removed from the solid matrix.

### 3.2. Optimization of the extraction conditions for the in situ IL-DLLME

In order to choose the best experimental conditions for the *in situ* IL-DLLME, a stepwise optimization procedure was chosen. A standard solution containing the five studied HAAs was prepared in water/methanol (70:30). A constant volume of this solution was used in all the optimization experiments, which were performed by triplicate. In the experimental procedure, some important parameters that would affect the extraction performance were optimized.

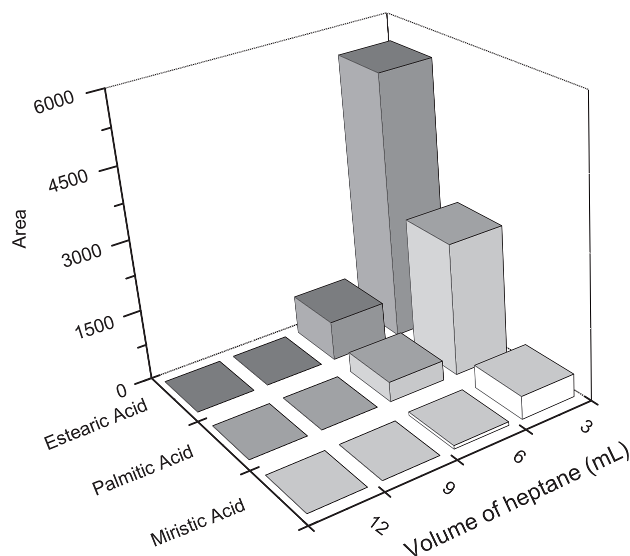


**Fig. 1.** Chromatographic areas of fatty acids after washing 3 g of beefburguer sample, in different steps, with *n*-heptane in the microwave oven.

*3.2.1. Selection of the water-soluble ionic liquid*

It is difficult to select a specific ionic liquid for a given extraction since several RTILs with very different chemical properties are commercially available or can be synthesized. For the *in situ* IL-DLMME, the obtained IL must be no miscible in water (or very little miscible), while the starting reactive must be water soluble. The formed IL must be liquid at room temperature and denser than the matrix solution so that the microdroplets can be settled down in the test tube. Finally, the formed IL should be able to be injected directly into the HPLC column. However, this last requirement is not usually fulfilled due to the high viscosity of the obtained product; thus, addition of an organic solvent to fluidify the phase is required. However, this last step will decrease the enrichment factor, EF. Considering all those requirements, the [OMIM][$BF_4$] soluble in water and $NaPF_6$ were chosen as the starting reactives for the *in situ* reaction. This ionic liquid was selected since it was synthesized in our laboratory (Padró et al., 2011).

*3.2.2. Effect of the $NaPF_6$ concentration*

The amount of $NaPF_6$ should be in excess as compared with the [OMIm][$BF_4$] in order to favor the formation of the desired water-immiscible extracting IL. For a fix number of millimoles of [OMIm][$BF_4$] (12.8 μmol obtained by taking 200 μL of a solution with a concentration of 18 mg $mL^{-1}$), the number of micromoles of $NaPF_6$ was varied from 35 to 285. As can be seen from Fig. 2A the amount of extracted amines increases with the amount of added inorganic salt up to 170 μmol (240 μL of $NaPF_6$, 120 mg $mL^{-1}$). Thus, according to this experiment the minimum number of moles of inorganic salt should be, at least, 13 times higher than the amount of IL.

*3.2.3. Effect of the amount of the water-soluble IL*

The volume of extraction solvent is an important parameter that affects the extraction performance in DLLME. Theoretically, larger volume of extraction solvent would result in higher extraction efficiency. However, the more IL is obtained the more organic solvent to decrease sample viscosity previous to the injection into the HPLC column is necessary, which decreases the enrichment factor, EF.

The effect of the RTIL volume prepared *in situ* was investigated by adding different amounts of the water-miscible [OMIm][$BF_4$] solution (18 mg $mL^{-1}$) to a constant amount of $NaPF_6$ (170 μmol). From Fig. 2B it can be observed that 12.8 μmol of the IL solution (200 μL) can be considered as an optimum amount to obtain better extraction efficiencies. Thus, the optimum relationship between the inorganic salt and the RTIL is 13 times, in agreement with the previous result.

*3.2.4. Effect of pH*

Sample pH determines the present state of analytes in aqueous solution, playing a determinant role in extraction efficiency. Since the HAAs are basic compounds, their p$K_a$'s have to be considered. Extraction into the IL phase could be increased by two types of intermolecular interactions: (i) the van der Waals interactions between the hydrocarbon moieties of the analyte with the hydrophobic chain of the RTIL and (ii) the electrostatic interactions between the charged analyte and the ions of the RTIL. However, these coulombic interactions could not contribute significantly in the extraction mechanism since van der Waals interactions seem to correlate with higher extraction efficiencies (Sanz Alaejos et al., 2008). The p$K_a$ values for the HAAs studied here are between 4.4 and 8.6 (Sanz Alaejos et al., 2008). Thus, in order to get all the amines non protonated, the pH effect in the range of 8.00–13.00 on the extraction efficiency was investigated. It can be observed from Fig. 2C that extraction increases up to pH 11. This result confirms that the non-charged state of the molecule favor the extraction mechanism. Thus, pH 11 can be selected as an optimum value for almost all the amines.

*3.2.5. Effect of extraction time*

We defined the extraction time as the interval between the instant when the water soluble IL is added to the $NaPF_6$ solution to form the water insoluble [OMIm][$PF_6$], until the mixture is put in the centrifuge. Thus, the extraction time was varied by let the mixture quiet from 1 to 15 min. From Fig. 2D it can be observed that signals increased up to 5 min and then remain constant up to 8 min. Then, they decrease a little because the analytes initially solubilized in the IL microdroplets are slightly solubilized into the aqueous phase. Thus, 5 min can be considered as an optimum extraction time.

*3.3. Quantitative performance of the procedure*

Important figures of merit such as limit of detection and quantification, linear range, inter-day reproducibility, recovery and enrichment factors to evaluate the performance of the methodology were obtained.

Inter-day reproducibility was calculated as the percentage relative standard deviation (RSD%) according to Eq. (1):

$$RSD\% = 100 \cdot SD/X_{ave} \quad (1)$$

where SD is the standard deviation and $X_{ave}$ is the average drug concentration (ng $mL^{-1}$). RSD% values assessed from six identical and independent experiments made during three different days were between 5.4% and 10.9%. These results are very acceptable considering the complexity of the sample and the whole sample preparation procedure.

In Table 1 calibration curves for the HAAs dissolved in methanol (external standard), by standard addition to the sample solution obtained after the MASE procedure ("MASE extract") and by standard addition to the sample matrix after IL-DLLME ("IL extract"), together with the linear ranges are shown. In all cases, six concentration levels by triplicate were used. Very good regression coefficients were obtained. Calibration curves in the different matrices were used in order to detect matrix effects (see next section).

Limits of detection (LOD) and quantification (LOQ) are shown in Table 2. LODs were evaluated as three times the signal to noise

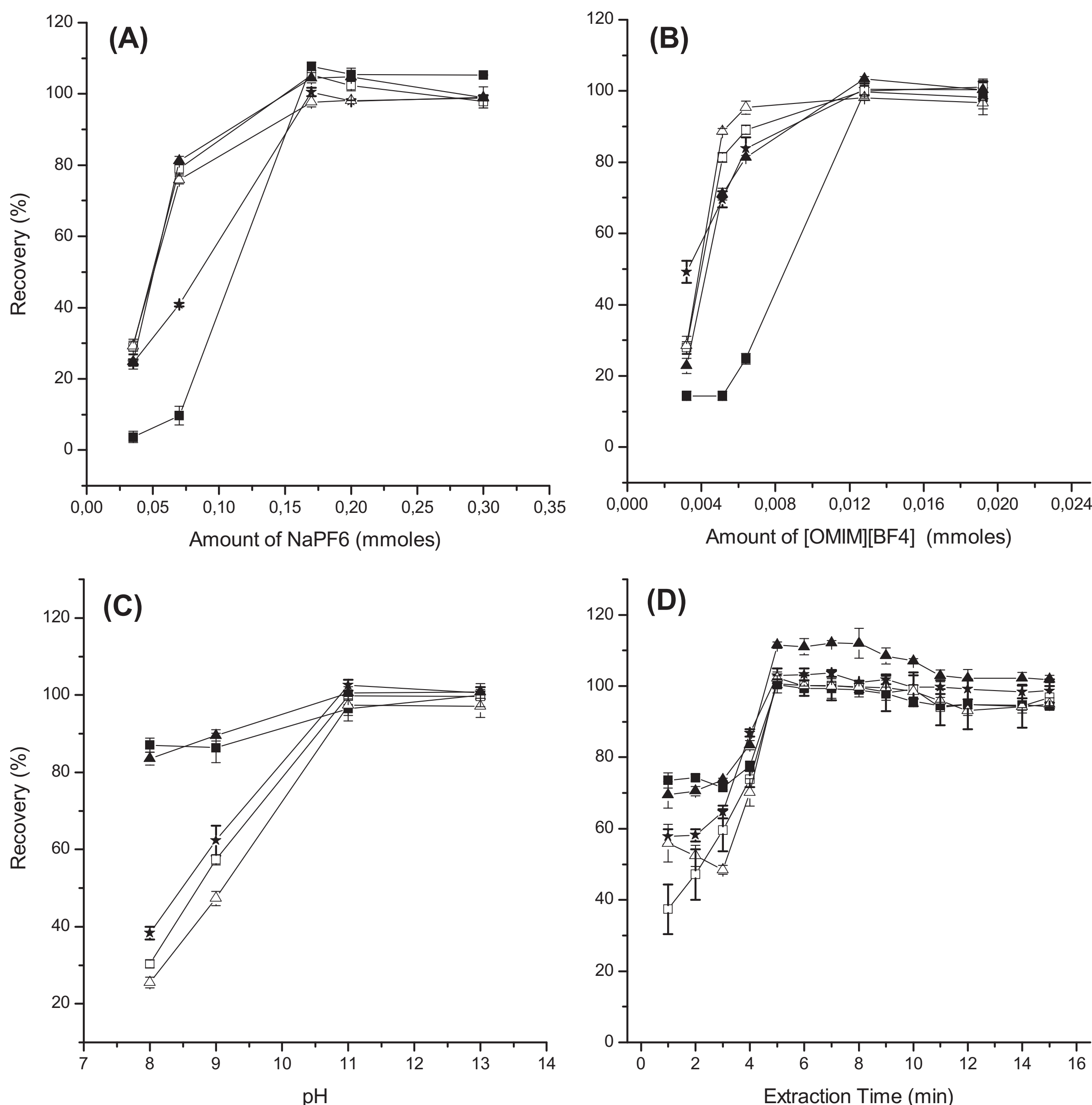


**Fig. 2.** Chromatographic peak areas of extracted amines *vs.* the different variables studied in the optimization of the IL-DLLME (□, TRP-P-1; ■, TRP-P2; ⁎, PhIP; △, AαC; ▲, MeAαC).

ratio (3S/N) in order to be compared with those reported in the literature. LOQs were evaluated as ten times the signal to noise ratio (10S/N). It can be seen that very low LODs (between 0.06 and 0.3 ng $g^{-1}$) were achieved. For TRP-P-1, the LOD is similar to those reported by other authors (Martin-, Pino, Ayala, Gonzales, & Afonso, 2007; Skog, Augusstson, Steineck, Stermberg, & Jägerstad, 1997), some higher for TRP-P-2 and PhIP and quite lower for AαC and MeAαC. The LODs obtained in this work are much lower than those obtained for the analysis of meat samples by supercritical fluid extraction (SFE) combined with capillary electrophoresis-fluorimetric detection (De Andrés et al., 2010). The LOQ for PhIP is very close to those found by Murray, Lynch, Knize, & Gooderham (1993), who used liquid–liquid extraction and capillary GC–MS to analyze patties and fried beef.

The recovery is defined according to Eq. (2). However, this should be considered a "surrogate recovery", and not the true recovery since it is assumed that the spiked analyte interact with matrix components as strong as the original analyte in the sample:

$$R\% = 100\frac{C_{IL}V_{IL}}{C_aV_a} = 100 \cdot EF\phi \qquad (2)$$

Here, $C_{IL}$ and $C_a$ are the concentrations of the spiked analyte in the extracting IL phase and initial aqueous phase, respectively. These concentrations were obtained from the calibration curves of Table 1 ("IL extract" and "MASE extract" respectively) for spiked samples which were left to stand for 30 min. $V_{IL}$ and $V_a$ are the volumes of the ionic liquid phase and aqueous phase, respectively; $\Phi$ is the phase ratio and EF the enrichment factor defined as:

$$EF = \frac{C_{IL}}{C_a} \qquad (3)$$

In Table 3 it can be seen that very good $R\%$ (between 69% and 100%) and EF values (between 19 and 30) were achieved.

**Table 1**
Calibration curves of the HAAs in the different studied matrixes, linear ranges and calculated $t$ values from Eqs. (4) and (5) for the statistical comparison of the slopes (see Section 3.4).

| Amine | Linear range (ng mL$^{-1}$) | Calibration curve ($n$ = 6) | $R^2$ | Matrix | $t$-values (Eq. (4)) | $t'$-values (Eq. (5)) |
|---|---|---|---|---|---|---|
| Trp-P-1 | 1.96–120 | $y$ = (22,863 ± 460)$x$ + (888 ± 84) | 0.993 | Methanol | – | – |
| | | $y$ = (11,710$x$ ± 141)$x$ – (84 ± 2) | 0.998 | MASE extract | 23.18 | 2.46 |
| | | $y$ = (11,813 ± 215)$x$ + (872 ± 12) | 0.995 | IL extract | 27.21 | 2.47 |
| PHIP | 2.04–200 | $y$ = (11,833 ± 165)$x$ + (258 ± 20) | 0.997 | Methanol | – | – |
| | | $y$ = (9482 ± 214)$x$ + (112 ± 23) | 0.995 | MASE extract | 8.70 | 2.62 |
| | | $y$ = (14,082 ± 115)$x$ + (47 ± 7) | 0.998 | IL extract | 10.6 | 2.92 |
| Trp-P-2 | 1.17–300 | $y$ = (30,327 ± 734)$x$ + (459 ± 50) | 0.994 | Methanol | – | – |
| | | $y$ = (41653 ± 3100)$x$ + (364 ± 188) | 0.950 | MASE extract | 3.55 | 2,86 |
| | | $y$ = (38421 ± 687)$x$ + (279 ± 37) | 0.994 | IL extract | 8.05 | 3.59 |
| AαC | 7.30–280 | $y$ = (21601 ± 608)$x$ + (332 ± 104) | 0.995 | Methanol | – | – |
| | | $y$ = (19,376 ± 423)$x$ – (621 ± 73) | 0.994 | MASE extract | 3.00 | 2.91 |
| | | $y$ = (29,374 ± 774)$x$ – (50 ± 37) | 0.991 | IL extract | 7.89 | 2.93 |
| MeAαC | 8.01–250 | $y$ = (15,718 ± 296)$x$ + (253 ± 47) | 0.999 | Methanol | – | – |
| | | $y$ = (15,857 ± 957)$x$ – (200 ± 80) | 0.995 | MASE extract | 0.13 | 2.70 |
| | | $y$ = (23,431 ± 488)$x$ – (6 ± 17) | 0.994 | IL extract | 13.51 | 3.89 |

**Table 2**
Limits of detection (LOD) from this work and from literature, quantification limits (LOQ) and concentrations found in the beefburguer sample.

| Analito | LOD[a] | | LOQ[b] | | LOD (ng/mL) | | Concentration in beefburguer | |
|---|---|---|---|---|---|---|---|---|
| | ng mL$^{-1}$ | ng g$^{-1}$ | ng mL$^{-1}$ | ng g$^{-1}$ | [1] | [2] | ng g$^{-1}$ | ng mL$^{-1}$ |
| TRPP2 | 0.58 | 0.06 | 1.90 | 0.21 | 0.3 | 0.41 | 4.3 ± 0.4 | 12.9 |
| PHIP | 0.61 | 0.07 | 2.04 | 0.23 | 0.4 | – | 1.9 ± 0.2 | 5.70 |
| TRPP1 | 0.35 | 0.04 | 1.17 | 0.13 | 0.3 | 0.16 | 0.42 ± 0.04 | 1.26 |
| AαC | 2 | 0.2 | 7.30 | 0.81 | 5 | 1.62 | NQ[c] | – |
| MeAαC | 2.4 | 0.3 | 8.01 | 0.89 | 5 | 2.14 | ND[d] | – |

[1] and [2] Martin-Calero, Pino, Ayala, Gonzales, and Afonso (2007) and Murray et al. (1993).
[a] 3 S/N.
[b] 10 S/N.
[c] NQ: non-quantifiable (bellow LOQ).
[d] ND: non-detected.

**Table 3**
Enrichment (EF) and recovery factors ($R$%) for different spiked amounts of HAAs after the IL-DLLME.

| Amine | Spiked amount (ng mL$^{-1}$) | Found amount (ng mL$^{-1}$) | EF[a] | $R$[a] (%) | RSD[b] |
|---|---|---|---|---|---|
| TRP-P-1 | 3 | 3 | 30 | 100 | 1.83 |
| | 1.5 | 1.47 | 29 | 98 | 1.37 |
| | 0.4 | 0.38 | 28 | 96 | 1.67 |
| PHIP | 2 | 1.58 | 21 | 79 | 1.07 |
| | 1 | 0.76 | 22 | 76 | 0.65 |
| | 0.5 | 0.38 | 22 | 77 | 1.6 |
| TRP-P-2 | 12 | 8.04 | 19 | 67 | 0.06 |
| | 3 | 2.01 | 19 | 67 | 1.34 |
| AαC | 7 | 4.9 | 20 | 70 | 0.3 |
| | 3.5 | 2.55 | 21 | 73 | 0.29 |
| | 1.5 | 1.06 | 21 | 71 | 1.57 |
| MeAαC | 5 | 4.2 | 24 | 84 | 0.81 |
| | 3.5 | 2.94 | 24 | 84 | 1.7 |
| | 1.5 | 1.26 | 24 | 84 | 0.38 |

[a] Averages of three different samples.
[b] Corresponding to the $R$% values.

### 3.4. Matrix effect studies

In order to detect matrix effects, calibration curves were performed: (i) in methanol (external-standard method); (ii) by an standard addition to the aqueous matrix after MASE and (iii) by an standard addition to the RTIL extract. The slopes obtained from these calibration curves were statistically compared by using a $t$-test according to Eq. (4) (Vandeginste et al., 1998):

$$t = \frac{b_1 - b_2}{\sqrt{s_{b_1}^2 + s_{b2}^2}} \quad (4)$$

where $b_1$ and $b_2$ are the slopes of the regression equations to be compared and $s_{b_1}$ and $s_{b_2}$ are the respective standard deviations. If the residual variances $s_e^2$ for both set of data are equal (according to an $F$-test), a "combined standard deviation" can be calculated to obtain a $t$ value which is compared with tabulated values for

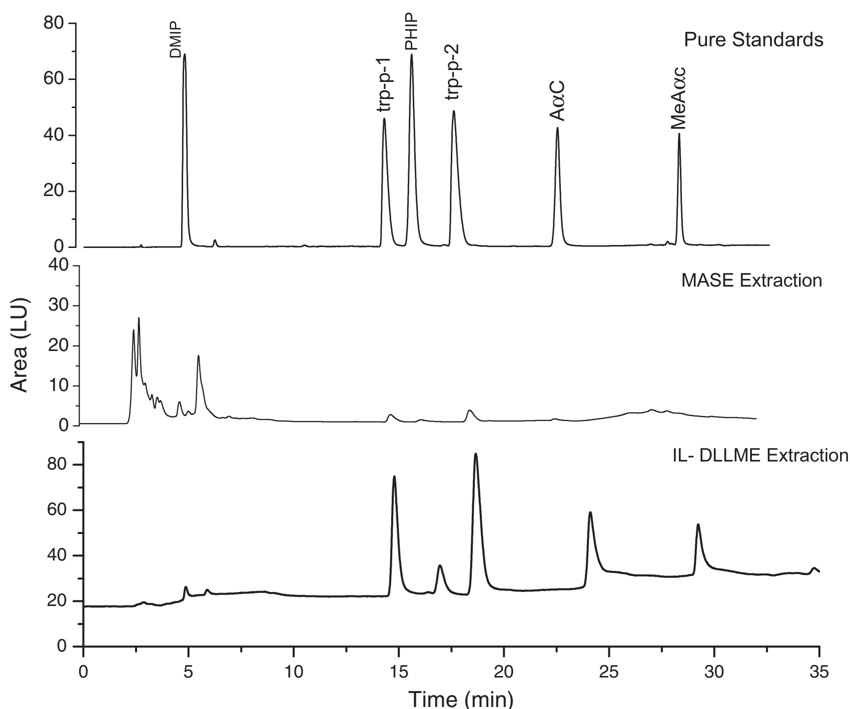


**Fig. 3.** Fluorescence chromatographic signals for the pure standards, for the solution after MASE extraction and for the beefburger extract after IL-DLLME (peak identity was confirmed by DAD absorbance spectral matching).

$n_1 + n_2 - 4$ degrees of freedom. However, if the residual variances are not equal, the Cochran test for the comparison of two slopes with unequal variances must be used. Thus, if $s_{b_1}^2 \neq s_{b_2}^2$, theoretical $t$ values, $t_1$ and $t_2$, at the chosen level of significance and $n_1 - 2$ and $n_2 - 2$ degrees of freedom, respectively, can be obtained from a $t$-table. An "combined $t'$ value" can be calculated by Eq. (5), which can then be compared with the calculated $t$ values by using Eq. (4):

$$t' = \frac{t_1 s_{b_1}^2 + t_2 s_{b2}^2}{s_{b_1}^2 + s_{b2}^2} \quad (5)$$

If the number of data points were the same for both regression lines, it is not necessary to calculate $t'$ ($n_1 = n_2$), in which case $t' = t_1 = t_2$ (Vandeginste et al., 1998). It is observed from Table 1 that the slopes of the calibration curves are very different in these matrixes. In fact, the calculated $t$ values are higher than the $t'$ values and, as a consequence, matrix effects are present. Thus, the standard addition method to correct for matrix effects for quantification of HAAs in beefburguer samples and for the experiments made in the previous Section to calculate $C_{IL}$ and $C_a$ must be used.

### 3.5. Sample analysis and comparison with other studies from the literature

The sample was analyzed according to Section 2 and the optimized MASE conditions from Section 3. After the $n$-heptane is discarded from the MASE extraction vessel, a solution of sodium hydroxide is added to precipitate the proteins and, after filtration, let the sample with the optimum pH ready for the IL-DLLME extraction/preconcentration step. In Fig. 3 the chromatograms for the pure standards, for the aqueous sample after MASE procedure and for the IL phase after IL-DLLME are shown. The first peak in the chromatogram corresponds to the polar amine DMIP, which is detected in the beefburguer sample also (the other polar HAAS were not analyzed). This observation could indicate that MASE procedure could be effective to determine polar heterocyclic amines, which are not extracted into the RTIL phase. It is also observed in the chromatogram that only TRP-P-1 and TRP-P-2 are detected as very small peaks before the IL-DLLME procedure but after the preconcentration step, the peaks corresponding to the five non-polar HAAs clearly appear in the chromatogram.

The amounts of TRP-P1, TRP-P2 and PhIP found in the beefburguer sample are shown in Table 3. They are expressed as ng $mL^{-1}$ and ng $g^{-1}$ in order to compare with results from the literature. The amount of PHIP found in this paper is very similar to those reported previously for beefburgers cooked in a similar fashion (Felton, Fultz, Dolbeare, & Knize, 1994; Johansson, Fredholm, Bjerne, & Jägerstad, 1995; Knize & Felton, 1998; Richling, Decker, Haring, Herderich, & Schreier, 1997). Also, similar concentrations were found for roast beef loin and grilled beef fillet samples (Khan et al., 2008). AαC is present bellow the LOQ (non-quantifiable) and MeAαC was not detected, in agreement with the previous cited works. The amounts of TRP-P-1 and TRP-P-2 detected in this work are much higher (about 10 times) than those found in loin and fillet samples, which is probably due to the higher fat content of the beefburguer which favors the formation of the amines (Skog, Johansson, & Jägerstad, 1998).

## 4. Conclusions

The MASE procedure combined with DLLME using an IL generated *in situ* was proven to be an efficient, fast, reproducible and sensitive methodology to analyze non-polar HAAs in cooked beefburguers. The cleaning of the sample and the desorption of the analytes from the solid matrix were simultaneously done in the microwave oven. Both MASE and IL-DLLME techniques were optimized by using a stepwise procedure. The methodology has the advantage of eliminate the extensive clean-up procedure used in the traditional solid-phase extraction technique, reducing sample manipulation. Considering the complexity of the matrix sample, the extraction methodology is reproducible and fast (about 40 min/sample). Also, the technique is simple, cheap and green since very small amounts of non-toxic solvents are used. Matrix ef-

fects were detected and, as a consequence, the standard addition method must be applied for the analysis of this type of sample.

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