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A novel *in situ* preconcentration method with ionic liquid-based surfactants resulting in enhanced sensitivity for the extraction of polycyclic aromatic hydrocarbons from toasted cereals

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ABSTRACT

A preconcentration procedure utilizing IL-based surfactants it is described for the first time. The procedure is based on transforming a water-soluble IL-based surfactant, 1-hexadecyl-3-butylimidazolium bromide ($C_{16}C_4$Im-Br), into a water-insoluble IL, 1-hexadecyl-3-butylimidazolium bis[(trifluoromethane)sulfonyl]imide ($C_{16}C_4$Im-NTf_2), *via* a simple metathesis reaction. The preconcentration procedure is used in combination with a micellar microwave-assisted extraction (MMAE) method for the analysis of sixteen polycyclic aromatic hydrocarbons (PAHs) from toasted cereals. The obtained microdroplet of $C_{16}C_4$Im-NTf_2 containing the extracted PAHs is then diluted with acetonitrile and injected into a high-performance liquid chromatograph (HPLC) employing UV–vis and fluorescence detection. This *in situ* preconcentration step highly improves the sensitivity of the MMAE despite the complexity of the solid matrix. The developed *in situ* preconcentration method exhibited almost quantitative extraction efficiencies (80–95% in average) for the PAHs studied, and good precision values (lower than 14%). The overall MMAE + *in situ* preconcentration method presented limits of detection down to 0.03 μg kg^{-1}.
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1. Introduction

The determination of trace analytes in complex solid matrixes is a serious challenge for analytical chemists. This becomes more difficult when the elimination (or minimization) of hazardous substances such as organic solvents in the analytical extraction step is intended. New trends are focused on utilizing more benign extraction solvents as well as microextraction procedures [1,2].

Surfactants have been widely used in the analytical extraction of hydrophobic analytes contained in solids as they provide an alternative to organic solvents. They are often coupled with microwaves in micellar microwave-assisted extraction (MMAE) to increase the extraction kinetics [3,4]. The micellar extract containing the analytes of interest can normally be subjected to high-performance liquid chromatographic (HPLC) separation without the need of additional clean-up steps. However, this approach lacks sensitivity due to the fact that there is no preconcentration step.

Several strategies have been carried out to improve the detection limits of MMAE. For example, the preconcentration of non-ionic micellar extracts can be conducted by cloud-point extraction (CPE) [5]. In this approach, aqueous solutions of non-ionic surfactants (at concentrations above the critical micelle concentration) become turbid at a temperature known as the cloud point, above which there is a separation of the solution into two phases; namely, a small volume of a concentrated phase which contains the majority of the surfactant (V_s), and a high volume of a diluted phase which contains low amounts of surfactant (V_{aq}). Analytes that are preconcentrated in the surfactant-rich phase (V_s) can be injected directly into HPLC.

Cationic surfactants do not exhibit cloud-point behavior unless high acidic conditions or high ionic strengths are employed [6,7]. The development of preconcentration methods employing cationic surfactants without the requirement of such extreme conditions is of interest, and in this sense, several authors have proposed the mixing of cationic surfactants with nonionic or anionic surfactants to favor their cloud-point [8,9].

Ionic liquids (ILs) are non-molecular solvents possessing low melting points and a wide variety of interesting properties [10]. Their use and application in separation science has increased

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rapidly [11–14]. A new group of ILs able to form micellar aggregates in aqueous solution have been described [15,16]. These IL-based surfactants, which can be classified as cationic surfactants, constitute a new area of surfactant development; especially considering the limited number of traditional cationic surfactants. It is important to highlight the ease of tuning IL-based surfactants by simple chemical modifications to the cation/anion pair. Thus, up to 48 IL-based surfactants have been reported and their colloidal and interfacial behavior studied [17]. Many IL-based surfactants also possess low critical micelle concentration (CMC) values which permit the formation of organized media when small amounts of the surfactant are employed. Furthermore, they have been successfully used in the extraction of organic analytes from environmental and food samples, in combination with microwaves or ultrasounds [18–23]. The extraction methods developed with IL-based surfactants have exhibited superior performance to those carried out with conventional cationic surfactants and are comparable to that of organic solvents. Given the increasing utilization of IL-based surfactants as extraction media, it is of high interest to develop preconcentration procedures in order to attain adequate levels of sensitivity.

The utilization of a metathesis reaction to transform a water soluble IL into a water insoluble IL is a strategy that has been used in a *in situ* solvent formation microextraction method by Shemirani and co-workers to determine metals [24–26]. A similar approach, which was termed *in situ* dispersive liquid–liquid microextraction by Anderson et al., was used to determine several organic compounds [27,28], and has been employed by López-Darias et al. to determine endocrine disrupting chemicals [29]. In all cases, a water soluble IL containing the chloride (Cl^-) or tetrafluoroborate ($BF_4{}^-$) anion, is transformed into a water insoluble IL containing the bis[(trifluoromethane)sulfonyl]imide ($NTf_2{}^-$) or hexafluorophosphate ($PF_6{}^-$) anion, by reaction with $LiNTf_2$ [27–29] and $NaPF_6$ [24–26], respectively. The best performance of the reaction is accomplished when the water soluble IL and the ion-exchange reagent are mixed in a 1:1 molar ratio. It is therefore desirable to evaluate if preconcentration achieved with pure ILs is also possible when employing IL-based surfactants.

This work reports, for the first time, the development of an *in situ* preconcentration procedure using IL-based surfactants. The extraction of organic compounds from solid samples can be achieved by employing MMAE with IL-based surfactants followed by *in situ* preconcentration and subsequent HPLC analysis. The developed method is fast, takes place in a single vessel, is free of organic solvent (only requires few μL of acetonitrile), and does not require heating of the sample solution. Moreover, this preconcentration step is able to enormously improve the limits of detection of analytical extraction methods for solid samples which use IL-based surfactants [17–23]. The IL-based surfactant 1-hexadecyl-3-butylimidazolium bromide ($C_{16}C_4$Im-Br) has been selected in this work due to its proven performance in analytical extractions, being superior to the comparable cationic surfactant cetyltrimethylammonium bromide (CTAB) [19,20]. The analytical application selected is the extraction/preconcentration of sixteen polycyclic aromatic hydrocarbons (PAHs) from toasted cereals.

2. Experimental

2.1. Reagents and materials

The polycyclic aromatic hydrocarbons used in this study were Benzo[*c*]fluorene (BcL), Cyclopenta[*c,d*]pyrene (CPP), Benz[*a*]anthracene (BaA), Chrysene (CHR), 5-Methylchrysene (5MC), Benzo[*j*]fluoranthene (BjF), Benzo[*b*]fluoranthene (BbF), Benzo[*k*]fluoranthene (BkF), Benzo[*a*]pyrene (BaP), Dibenzo[*a,l*]pyrene (DlP), Dibenz[*a,h*]anthracene (DhA), Benzo[*g,h,i*]perylene (BgP), Indeno[*1,2,3-cd*]pyrene (IcP), Dibenzo[*a,e*]pyrene (DeP), Dibenzo[*a,i*]pyrene (DiP), Dibenzo[*a,h*]pyrene (DhP), supplied as individual stock solutions by Dr. Ehrenstorfer (Reference Materials, Augsburg, Germany), at a concentration of 10 μg mL^{-1} in acetonitrile; except for BkF (>99% purity), which was supplied by Fluka (Buch, Switzerland); and DhA (97%), BaP (98%), BgP (98%), and CHR (98%), which were all supplied by Aldrich-Chemie (Beerse, Belgium).

Acetonitrile of HPLC gradient grade for liquid chromatography (Merck, Darmstadt, Germany) was used to prepare working standard solutions of PAHs. Deionized water was obtained from a Milli-Q gradient A10 system (Millipore, Watford, UK). Acetonitrile of HPLC gradient grade and deionized water were used for HPLC analysis. All solvents were filtered through a 0.45 μm Durapore® membrane filter (Millipore) before use in the chromatographic system. Stir bars (10 mm × 3 mm) were supplied by Aldrich (Milwaukee, WI, USA) and were used to favor the mixing in the microwave tube.

The ionic liquid 1-hexadecyl-3-butylimidazolium bromide ($C_{16}C_4$Im-Br) was synthesized and fully characterized using previously published methods [30]. The lithium bis[(trifluoromethane)sulfonyl]imide ($LiNTf_2$) salt was supplied by Sigma–Aldrich GmbH (Steinheim, Germany).

Several toasted cereals of different nature (wheat, barley, rye and maize corn) were acquired from two local mills (Barrio de La Salud and El Sauzal, both in Tenerife, Spain). Wheat toasted cereals samples, which were used in the optimization study, presented the following characteristics: humidity content of 3.17%, ash content of 1.54% (w/w), total content in proteins of 10.6% (w/w), and fiber content of 1.55 (g/100 g). Humidity contents for barley, rye and maize corn were 2.35, 3.07 and 2.74%, respectively. Ash contents for barley, rye and maize corn were 2.16, 2.09 and 0.79% (w/w), respectively. Total content of proteins for barley, rye and maize corn were 11.4, 10.1 and 8.5% (w/w), respectively. Fiber contents for barley, rye and maize corn were 4.02, 4.88 and 1.37 (g/100 g), respectively. Spiking of the toasted cereal sample was performed as follows: 1 g of cereal sample was mixed with 1 mL of acetonitrile containing known concentrations of each PAH and then stirred in a vortex for several minutes. The samples were then stored in the dark (for 24 h) and allowed to dry. The spiked concentration of PAHs during the study varied from 9 to 450 μg kg^{-1} for all PAHs except for the non-fluorescent CPP, which varied from 0.22 to 4.5 mg kg^{-1}, on dry-weight basis. Non-spiked samples were also stored in the dark until the extraction was conducted. 0.1 g of the dry (spiked or not) cereal sample was further extracted by microwaves.

2.2. Instrumentation

The HPLC equipment consisted of a delivery solvent ProStar 230 SDM (Varian, Palo Alto, CA, USA). The detection of PAHs was carried out using a ProStar 325 UV–vis Detector operating at 375 nm to analyze CPP, and a Waters 474 Scanning Fluorescence Detector (Milford, MA, USA) for all other PAHs. The analytical column was a Vydac 201TP54 reversed-phase C18, 5 μm, 300 Å (250 mm × 4.6 mm ID) supplied by Waters, and protected by a Pelliguard LC-18 guard column (Supelco, Bellefonte, PA, USA). The temperature of the column was kept at 32 °C using the column oven. Data were acquired with the Star 6.41 LC chromatography workstation software (Varian).

Focused microwave-assisted extractions were carried out using a CEM Focused Microwave™ Synthesis System apparatus model Discover (CEM, Matthews, NC, USA) with stirring and cooling options. This microwave is equipped with an infrared temperature control system. Data were acquired with the ChemDriver™ software (CEM).

A vortex model reax-control from Heidolph Instruments GMBH (Schwabach, Germany), and an Eppendorf Centrifuge 5702 (Hamburg, Germany) were used in the studies. A 100 μL HPLC syringe from Hamilton (Reno, NV, USA) was used to sample the PAHs after the *in situ* preconcentration method.

2.3. Microwave-assisted extraction procedure

The optimum microwave conditions were optimized in a previous study by our group [20]. Briefly, 0.1 g of cereal sample (free or contaminated with PAHs) was placed in a 25 mL glass Pyrex® tube. A volume of 4.5 mL of 40 mM $C_{16}C_4$Im-Br aqueous solution was added and the extraction tube introduced into the microwave cavity after ensuring that a stir bar was placed into the tube. Then, a microwave power of 50 W was applied to reach a maximum temperature of 80 °C (reached in about 4 min), followed by a hold time of 10 min. Afterwards, the tube was allowed to cool to room temperature. The obtained extract was then centrifuged for 5 min at 4000 rpm, followed by filtration using 0.45 μm filters of Chromafil Xtra PET-45/25 supplied by Panreac (Barcelona, Spain). 1.5 mL of the remaining filtrate (~1.9 mL) was then transferred to a 2 mL vial and subjected to direct HPLC injection (for obtaining the chromatographic calibrations without preconcentration) or to the *in situ* ionic liquid preconcentration (by applying conditions of Section 2.4), depending on the experiment.

2.4. In situ ionic liquid preconcentration procedure

During the optimization study of the *in situ* ionic liquid preconcentration procedure, different concentrations of the IL-based surfactant $C_{16}C_4$Im-Br (dissolved in deionized water) were mixed with different aliquots of aqueous solutions of $LiNTf_2$. The mixtures were then subjected to vortex, centrifugation, and freezing, using times dependant on the specific experiment being performed. The specific volume of the formed IL microdroplet was measured and subjected to HPLC quantification if PAHs were present in the initial solution.

Under optimum conditions, 1.5 mL of filtrate ($C_{16}C_4$Im-Br containing PAHs, which originated from the micellar microwave-assisted extraction step of toasted cereals described in Section 2.3), were mixed with 34 μL of $LiNTf_2$ with a concentration of 0.5 g mL^{-1}, and a turbid solution appeared. Afterwards, this turbid solution was vortexed during 2.5 min, followed by centrifugation during 4 min at 3400 rpm. The tube was then placed in the freezer for one hour (at −8 °C), and a microdroplet of ~65 μL was formed ($C_{16}C_4$Im-NTf_2 containing PAHs) at the bottom of the tube. The microdroplet was withdrawn with a Hamilton 100 μL syringe, and diluted up to 95 μL with acetonitrile to decrease its viscosity and to favor the HPLC compatibility (while avoiding excessive dilution), and the mixture vortexed for 1 min. The diluted microdroplet was then injected in the HPLC without any additional clean-up step.

2.5. Chromatographic procedure

The chromatographic procedure for the 15 + 1 EU PAHs has been optimized in a previous work of our group [20]. Briefly, the HPLC method used for the separation and determination of the 15 + 1 priority EU PAHs consisted of a gradient elution procedure with UV–vis and fluorescence detectors (FLD). The optimal chromatographic method utilized a mixture of acetonitrile–water with a linear gradient elution, with the conditions listed in Table S1. The wavelength program of the fluorescence detector can be also observed in Table S1, using an excitation split of 18 nm and an emission split of 10 nm.

3. Results and discussion

3.1. In situ preconcentration with the IL-based surfactant $C_{16}C_4$Im-Br

The selected IL-based surfactant for this study was 1-hexadecyl-3-butyl-imidazolium bromide ($C_{16}C_4$Im-Br). Preliminary experiments were conducted by mixing 1:1 molar proportions of $C_{16}C_4$Im-Br and $LiNTf_2$, however, no appreciable microdroplet was observed even after vigorously mixing and centrifuging. A microdroplet did form when the experiment was left to settle overnight. Hence, it was decided to apply freezing to the tube after vigorously mixing $C_{16}C_4$Im-Br and $LiNTf_2$ to improve the microdrop settling, due to lower solubility of the IL under lower temperatures. However, the possibility of applying this preconcentration step to IL-based surfactants, which can be used to extract solid samples, would enormously improve the sensitivity of those extraction methods. It must be noted that pure ILs are rarely used in extraction methods of solid samples [14].

3.2. Study of the variables influencing the in situ preconcentration with $C_{16}C_4$Im-Br

Taking into account that the IL-based surfactant ($C_{16}C_4$Im-Br) and the ion-exchange reagent ($LiNTf_2$) must be mixed into a 1:1 molar ratio to promote the formation of a water insoluble IL, specifically $C_{16}C_4$Im-NTf_2, the main variables investigated in the preconcentration step were: the agitation time after mixing (using vortex), the centrifugation time after stirring, the freezing time, the IL-based surfactant concentration, and the total volume of the IL solution. All experiments were carried out using 5 mL of an aqueous solution of $C_{16}C_4$Im-Br and $LiNTf_2$ with a concentration of 0.5 g mL^{-1} (aqueous solution).

3.2.1. Effect of IL-based surfactant concentration

The effect of $C_{16}C_4$Im-Br concentration was evaluated within the range 10–40 mM. The critical micelle concentration (CMC) value for this IL-based surfactant has been previously determined to be 0.1 mM in deionized water [30], and ranged from 0.23 to 0.46 mM when the acetonitrile content in solution varied between 1 and 20% (v/v) [31]. The minimum value selected (10 mM) corresponds to 100 times its CMC in pure water, and ~20 times when organic solvent is present in the aqueous solution. It is always desirable to work well above the CMC if a surfactant-based extraction procedure is applied towards complex hydrophobic analytes [32]. The maximum value selected (40 mM) is near the solubility of $C_{16}C_4$Im-Br in water at room temperature (~46 mM). The *in situ* preconcentration was carried out by mixing $C_{16}C_4$Im-Br at different concentrations with the corresponding volumes of $LiNTf_2$ to ensure a 1:1 molar ratio. The following experimental conditions were kept fixed: 4 min for the vortex time after mixing $C_{16}C_4$Im-Br and $LiNTf_2$, followed by 4 min of centrifugation (at 3400 rpm) and 1 h in the freezer. Fig. 1 shows the obtained volume values for the microdrop of $C_{16}C_4$Im-NTf_2, as well as the calculated enrichment factor values (E_F). Enrichment factors were determined based on the ratio of the initial sample volume to the final microdroplet volume. Experiments were carried out by triplicate. It can be observed that increases in the $C_{16}C_4$Im-Br concentration are accompanied by almost linear increases in the obtained $C_{16}C_4$Im-NTf_2 microdroplet, with values ranging from 20.0 ± 1.5 to 90.0 ± 2.5 μL. Accordingly, enrichment factors varied from 250 ± 3 to 56 ± 2. When the microdroplet volume was larger, a lower enrichment factor was obtained. Although high enrichment factors are desired, a compromise must be found between an adequate enrichment factor and a corresponding microdroplet who size allows it to be easily manipulated.

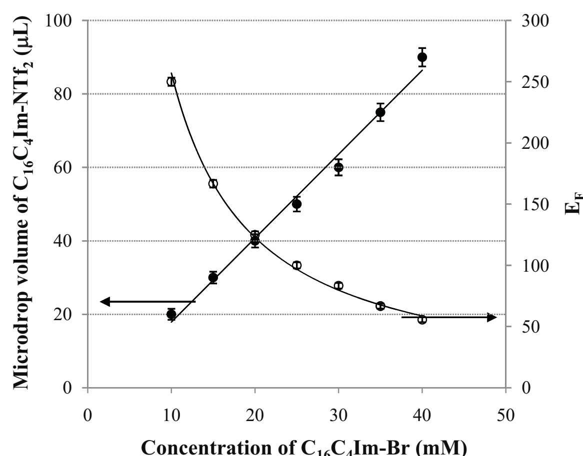

Fig. 1. Effect of the initial concentration of aqueous solutions of the IL-based surfactant $C_{16}C_4$Im-Br on the volume of the microdroplet obtained of $C_{16}C_4$Im-NTf_2 (left side of the plot) and on the enrichment factor (right side of the plot), for the *in situ* preconcentration procedure developed.

The selected IL-based surfactant concentration is dependent on the preconcentration required.

3.2.2. Effect of the stirring time

It has been described that the utilization of vortex in DLLME methods that use low density extraction solvents improve the performance of the method [33,34]. Given the singularities of this *in situ* preconcentration method with the IL-based surfactant $C_{16}C_4$Im-Br, it was decided to use vortex to improve the mixing of the surfactant with the ion-exchange reagent. The following experimental conditions were kept fixed: 4 min of centrifugation time and 1 h in the freezer after centrifugation. It was observed that the optimum stirring-vortex time to ensure an adequate final microdroplet was dependent on the IL concentration. Thus, when using 40 mM of $C_{16}C_4$Im-Br, a vortex time of 30 s was sufficient. It was necessary to use 1.5 min for 20 mM of $C_{16}C_4$Im-Br. For the lowest concentration tested, 10 mM of $C_{16}C_4$Im-Br, it was necessary to use 2.5 min of vortex. Hence, it is desirable a vortex time of 2.5 minutes in order to traverse the entire range of IL concentrations examined.

3.2.3. Centrifugation time

The criteria for choosing the best centrifugation time was dependant on the formation of a microdroplet that could be easily manipulated for subsequent HPLC analysis. Experiments were conducted with 10 and 40 mM of $C_{16}C_4$Im-Br, 2.5 min of vortex, and 1 h storage in the freezer. In both cases, it was observed that 4 min was sufficient to reach these requirements. Longer times did not effect the microdroplet size.

3.2.4. Freezing time

The freezing time, which is an important parameter for this *in situ* preconcentration with the IL-based surfactant $C_{16}C_4$Im-Br, was also studied with the minimum and the maximum IL concentrations tested (10 and 40 mM). The remaining experimental conditions included 2.5 min of vortex and 4 min of centrifugation. It was observed that one hour was the minimum time required to achieve the maximum microdroplet volume of $C_{16}C_4$Im-NTf_2. It is also evident that lower times were not sufficient to ensure efficient settling of the IL to form a stable microdroplet.

3.2.5. Initial volume of $C_{16}C_4$Im-Br

The effect of the initial volume of the IL-based surfactant $C_{16}C_4$Im-Br on the preconcentration procedure must be studied, particularly since the main utility of the preconcentration step lies

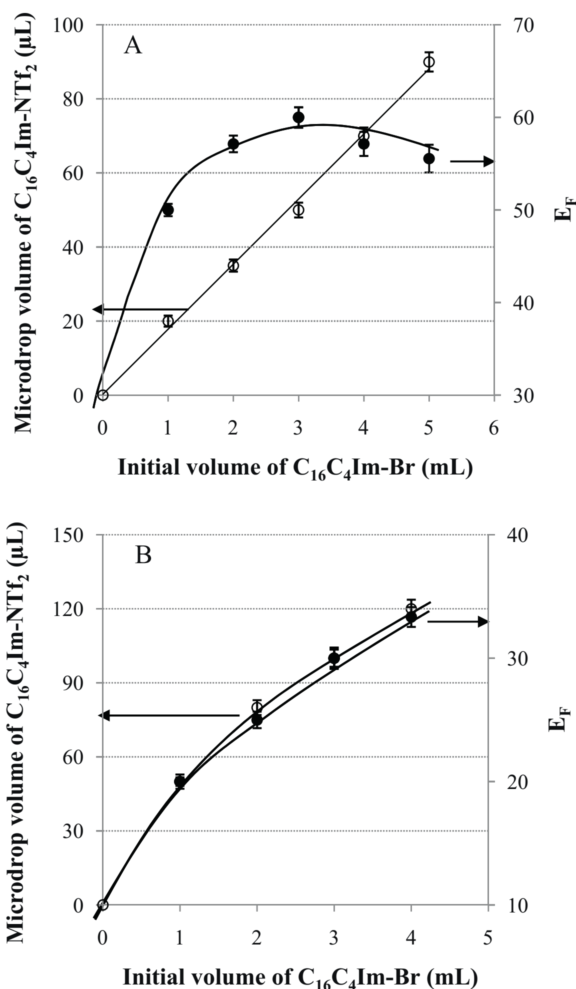

Fig. 2. Effect of the initial volume of the IL-based surfactant $C_{16}C_4$Im-Br on the volume of the microdroplet obtained of $C_{16}C_4$Im-NTf_2 (left side of the plot) and on the enrichment factor (right side of the plot), for the *in situ* preconcentration procedure developed. The method is conducted with (A) aqueous solutions of $C_{16}C_4$Im-Br, and (B) $C_{16}C_4$Im-Br in the wheat cereal extracts obtained after micellar microwave-assisted extraction.

in its application towards extracts originating from MMAE of solid samples. The study was carried out by fixing the aqueous $C_{16}C_4$Im-Br concentration to 40 mM (the maximum tested). Fig. 2(A) shows the variation in the microdroplet volume (V_s) and the enrichment factor values when volumes of $C_{16}C_4$Im-Br were varied from 1 to 5 mL while maintaining a vortex time of 2.5 min, centrifugation time of 4 min, and freezing time of one hour. Again, increases in the initial volume of $C_{16}C_4$Im-Br (40 mM) were accompanied by an almost linear increase in the obtained $C_{16}C_4$Im-NTf_2 microdroplet with values ranging from 20 ± 2 to 90 ± 3 μL, and E_F values between 50 ± 2 and 60 ± 2. The initial volume of the IL-based surfactant to be used is often governed by the initial MMAE procedure, which is a step that must be optimized for any specific application of organic compounds in solid samples.

3.3. In situ preconcentration method with real extracts from toasted wheat cereals

Previous sections have been devoted towards evaluating and optimizing the *in situ* preconcentration method for $C_{16}C_4$Im-Br, but using the IL-based surfactant dissolved in deionized water. It was decided to evaluate the feasibility of this *in situ* IL preconcentration method in an application with real solid samples extracted with

IL-based surfactants. The $C_{16}C_4$Im-Br-based extraction method of PAHs from toasted cereals using MMAE was selected as it has been previously optimized and validated by our group [20]. Given the fact that the IL-micellar extracts are going to contain not only PAHs but also matrix components of toasted cereals, it is necessary to evaluate the performance of the *in situ* preconcentration method in such a matrix. It must be considered that for this specific application, several variables are already fixed, such as the IL concentration of the extract which is 40 mM [20]. Hence, the influence of the volume of $C_{16}C_4$Im-Br (40 mM) was studied. This volume was obtained as the final extract of wheat toasted cereals samples by MMAE in the preconcentration step. The rest of the optimized variables of the in the *in situ* preconcentration step were maintained as follows: addition of $LiNTf_2$ (0.5 g mL^{-1}) to ensure a 1:1 molar ratio with $C_{16}C_4$Im-Br, 2.5 min vortex, 4 min centrifugation time, and 1 h in the freezer. The V_s and E_F values obtained in the *in situ* preconcentration method with the real matrix are shown in Fig. 2(B). In this case, it results evident that there is a modification in the obtained V_s, which now vary from 50 ± 3 to 120 ± 4 μL for initial volumes between 1 and 4 mL of real extract of the matrix. V_s volumes from deionized water varied between ~20 and ~70 μL for the same range of volume (Fig. 2(A)). The obtained E_F values in real wheat toasted cereals extracts changed accordingly. Clearly, the nature of the matrix significantly affects the preconcentration procedure and is an important variable that must be taken into account in future applications of the method.

3.4. *Calibration performance of the in situ preconcentration method with toasted wheat cereal extracts*

Calibrations of the *in situ* preconcentration method were conducted in toasted cereals extracts by extracting non-spiked toasted wheat cereal samples with $C_{16}C_4$Im-Br by MMAE according to our previous work [20]. The extracts were then used as the solvent medium to carry out the *in situ* preconcentration of PAHs. Thus, different concentrations of PAHs were spiked in toasted cereal extracts, and such standards were subjected to the optimized *in situ* preconcentration procedure. The selected optimum conditions were an initial volume of 1.5 mL of $C_{16}C_4$Im-Br (the toasted wheat cereal extract coming from MMAE), 34 μL of $LiNTf_2$ (0.5 g mL^{-1}), 2.5 min vortex, 4 min centrifugation, 1 h in the freezer, and dilution of the obtained $C_{16}C_4$Im-NTf_2 microdroplet containing the PAHs to 95 μL with acetonitrile. Calibration curves for the overall method were constructed by plotting the peak-area of the PAHs *versus* the initial concentration of PAHs in the cereal extracts. The European Food Safety Authority (EFSA) recommends the monitoring of 16 PAHs in foods [35,36], and these have been the analytes selected in this study. This group of PAHs only has 8 PAHs in common with the 16 PAHs regulated by the United States Environmental Protection Agency (US-EPA).

Table 1 shows several quality analytical parameters obtained for the calibrations of the *in situ* preconcentration method. It can be observed that there is no calibration data reported for BcL. It was not possible to obtain a clear peak for BcL due to cereal matrix components that were also preconcentrated with the *in situ* approach resulting in fluorescence interference with BcL. It was verified that the peak originated from the cereal extract once it was subjected to *in situ* preconcentration, and was not an interfering signal from the $C_{16}C_4$Im-NTf_2 IL formed (Fig. S1 of the Supplementary Material). Therefore, it was not possible to quantify BcL from cereal samples using the preconcentration procedure developed. For the remaining PAHs, linear calibrations were obtained for the *in situ* preconcentration method in wheat cereal extracts, with R values ranging from 0.993 to 0.999. The reproducibility of the retention times was acceptable, with relative standard deviation (RSD) values ranging from 0.32 to 2.13%. Detection limits were calculated from a signal to noise ratio of three, and verified by preparing the cereal extracts spiked at calculated levels, followed by the *in situ* preconcentration procedure. They have been expressed in the cereal extract and range from 2 ng L^{-1} for BkF and BgP to 0.65 ng mL^{-1} for BjF as well as 5.0 ng mL^{-1} for CPP. It must be highlighted that for nine out of the sixteen EU PAHs, the detection limits are in the low nanograms per liter region. In the literature, few reports have demonstrated extraction methods for all EU PAHs in foods [37,38] with the majority of studies being related to the US-EPA PAHs. Ciecierska and Obiedzinski carried out conventional extraction and purification methods for milk, followed by HPLC with FD detection, and reported LODs ranging from 0.05 to 0.47 μg kg^{-1} for the 16 EU PAHs [37]. Hollosi and Wenzl reported LODs for the 16 EU PAHs in edible oils ranging from 0.19 to 0.36 μg kg^{-1} using LC–MS [38].

For comparative purposes, Table 2 includes the same quality parameters, but in this case obtained for PAHs spiked in aqueous extracts of 40 mM $C_{16}C_4$Im-Br coming from MMAE (without *in situ* preconcentration) of non-spiked wheat cereals, and directly injected into HPLC. The calibrations conducted in MMAE extracts without preconcentration were also linear, with R values ranging from 0.990 to 0.998. In this case, BcL could be adequately quantified, because the matrix components extracted by MMAE (but not preconcentrated) are not at sufficient concentration levels to interfere with BcL. The limits of detection of the MMAE procedure were calculated based on a signal to noise ratio of three, and verified by spiking blank cereal extracts obtained by MMAE at such levels and subjecting them to HPLC. They oscillate between 0.02 ng mL^{-1} for BkF to 4.0 ng mL^{-1} for BbF, and 22 ng mL^{-1} for the non-fluorescent PAH CPP. Detection limits for the 15 + 1 EU PAHs using HPLC with fluorescence detection (also with no preconcentration step) have been reported to vary between 1.4 and 12 ng mL^{-1} [39], and between 0.02 and 2.7 ng mL^{-1} (equivalent to 0.5–54 pg injected) [40]. It can be observed that the detection limits obtained by *in situ* preconcentration are around 10–100 times lower than those obtained by MMAE without preconcentration (depending on the PAH), with an average sensitivity increase in the detection limits of 30. A sensitivity gain is also observed when comparing the ratio of the slopes of the calibration with and without preconcentration. The average increase in the slopes was 8 ± 2.

3.5. *Extraction efficiency, precision, and enrichment factors of the in situ preconcentration method*

The *in situ* IL preconcentration method has been evaluated by spiking wheat toasted cereals at different levels of concentration and subjecting them to MMAE, *in situ* preconcentration, and HPLC determination. The selected wheat toasted cereal samples were previously subjected to the overall method to verify the absence of PAHs so that they could be used as blanks in the spiking procedure. The precision (as RSD, in %), relative recovery (RR, in %), enrichment factor (E_F), and the extraction efficiency (E_R, in %) of the *in situ* preconcentration method were determined by performing six non-consecutive extractions of wheat cereals at each spiked level, and applying the overall procedure. The RR of the *in situ* preconcentration method was calculated according to:

$$RR(\%) = 100 \times \frac{C_{found}}{C_{initial}}$$

where C_{found} is the obtained concentration in the cereal extract after MMAE and is determined from the overall *in situ* preconcentration calibration method (Table 1), and $C_{initial}$ is the theoretical concentration in the cereal extract after MMAE, which is calculated from the initial spiked concentration in the wheat toasted cereal and taking into account the extraction efficiency of the MMAE method [20].

Table 1
Calibrations obtained for the *in situ* preconcentration method with $C_{16}C_4$Im-Br (1.5 mL, 40 mM), conducted in toasted wheat cereal extracts obtained after MMAE. The optimized conditions were: 34 μL of $LiNTf_2$ (0.5 g mL^{-1}), 2.5 min vortex, 4 min centrifugation, 1 h in the freezer, and dilution of the obtained $C_{16}C_4$Im-NTf_2 microdroplet containing the PAHs to 95 μL with acetonitrile.

PAH	Calibration range (ng mL^{-1})[a]	(Slope ± SD[b]) × 10^{-6}	Error of the estimate[c]	R	LOD[d] (ng L^{-1})
BcL	–	–	–	–	–
CPP	15–200	0.0044 ± 0.0001	47,254	0.995	5.0 ng mL^{-1}
BaA	0.2–4	2.48 ± 0.07	324,123	0.994	8
CHR	0.2–4	1.00 ± 0.03	124,253	0.994	30
5MC	0.2–4	1.87 ± 0.04	194,768	0.996	5
BjF	2–40	0.12 ± 0.01	190,488	0.993	0.65 ng mL^{-1}
BbF	2–40	0.37 ± 0.01	386,181	0.996	0.25 ng mL^{-1}
BkF	0.2–3	5.54 ± 0.10	261,385	0.999	2
BaP	0.2–4	2.72 ± 0.05	246,958	0.997	4
DlP	0.2–4	1.17 ± 0.02	112,312	0.997	6
DhA	0.2–4	0.80 ± 0.16	764,478	0.996	5
BgP	0.2–4	0.26 ± 0.01	11,670	0.999	2
IcP	0.2–4	0.37 ± 0.01	35,558	0.997	42
DeP	0.2–4	1.09 ± 0.03	129,883	0.995	10
DiP	0.2–4	0.58 ± 0.01	47,196	0.998	20
DhP	0.2–4	1.91 ± 0.04	165,560	0.998	6

[a] Concentration in the extract (the toasted wheat cereal extract obtained after MMAE is used as solvent to carry out the *in situ* preconcentration method).
[b] Error of the slope for $n = 6$ calibration levels.
[c] Standard deviation of the regression.
[d] Detection limits calculated as three times the signal to noise ratio, and verified by preparation of a blank cereal extract spiked at such levels and subjected to the *in situ* preconcentration and HPLC injection.

The E_F of the *in situ* preconcentration method is calculated by:

$$E_F = \frac{C_{drop}}{C_{initial}}$$

where C_{drop} is the concentration of the final IL microdroplet obtained by the *in situ* preconcentration and taking into account its dilution with acetonitrile before HPLC injection, and is obtained from the chromatographic calibration (Table 2).

The E_R of the *in situ* preconcentration method, also known as relative enrichment factor or real extraction recovery, is calculated from:

$$E_R(\%) = RE_F(\%) = 100 \times \frac{E_F}{E_{F\,max}}$$

where E_{Fmax} is the maximum preconcentration achieved if all analytes are effectively concentrated in the final IL microdroplet of the *in situ* preconcentration method. E_{Fmax} can be calculated from the ratio $V_{initial}/V_{drop}$, being $V_{initial}$ the cereal extract volume (1.5 mL), and V_{drop} the microdroplet volume including its dilution factor with acetonitrile. Therefore, E_R (%) is a measure of the real extraction efficiency of the *in situ* preconcentration method.

Table 3 shows the obtained results at the spiked levels tested. The lower spiked levels were 220 μg kg^{-1} for the non-fluorescent PAH CPP, 90 μg kg^{-1} for BjF and BbF, and 9 μg kg^{-1} for the remaining PAHs. It must be noted that low amounts were used in the spiking procedure at this level. The higher spiked levels were 4500 μg kg^{-1}, 450 μg kg^{-1}, and 45 μg kg^{-1}, respectively.

Average relative recoveries (in %) of 114 ± 8 and 118 ± 13 were obtained at the highest and lowest level tested, respectively. Relative recoveries approaching 100% are commonly described in DLLME applications [33,34].

The RSD values ranged from 5.6 to 12% at the highest level spiked and from 5.5 to 22% at the lowest level spiked; with average RSD values of 8.3 and 12%, respectively. Higher RSD values are obtained when lower amounts of PAHs are spiked in the toasted cereal.

The E_F values are listed in Table 3. The average E_F values were 11 ± 2 and 9 ± 2 at the lowest and at the highest spiked level, respectively. This is in agreement with the aforementioned sensitivity

Table 2
Calibrations obtained by direct injection of toasted wheat cereals extract obtained by MMAE (without *in situ* IL preconcentration).

PAH	Calibration range (ng mL^{-1})[a]	(Slope ± SD[b]) × 10^{-5}	Error of the estimate[c]	R	LOD[d] (ng mL^{-1})
BcL	0.5–10	0.69 ± 0.03	11,912	0.996	0.22
CPP	50–250	0.004 ± 0.001	3208	0.993	22
BaA	0.5–10	3.15 ± 0.29	97,041	0.991	0.07
CHR	0.5–10	3.17 ± 0.23	91,435	0.995	0.16
5MC	0.5–10	2.17 ± 0.15	34,970	0.995	0.16
BjF	20–150	0.13 ± 0.01	98,213	0.992	3.1
BbF	20–150	0.38 ± 0.02	196,242	0.995	4.0
BkF	0.5–10	7.82 ± 0.28	127,750	0.997	0.02
BaP	0.5–10	3.55 ± 0.16	71,214	0.996	0.04
DlP	0.5–10	1.49 ± 0.06	27,376	0.997	0.18
DhA	0.5–10	1.02 ± 0.04	18,634	0.997	0.17
BgP	0.5–10	0.27 ± 0.04	38,658	0.990	0.22
IcP	2–50	0.45 ± 0.04	10,207	0.995	0.25
DeP	0.5–10	1.10 ± 0.04	15,941	0.998	0.38
DiP	0.5–10	0.78 ± 0.03	13,903	0.997	0.27
DhP	0.5–10	2.47 ± 0.10	43,265	0.997	0.26

[a] Concentration in the toasted wheat cereal extract obtained by MMAE, which is then directly injected in the HPLC.
[b] Error of the slope for $n = 6$ calibration levels.
[c] Standard deviation of the regression.
[d] Detection limits in the microwave extract, directly injected in the HPLC, calculated as three times the signal to noise ratio and verified by preparing a blank cereal extract spiked at such levels and injecting it in the HPLC.

Table 3
Performance of the *in situ* preconcentration method (RR, RSD, E_R and E_F) for toasted wheat cereals spiked, and subjected to the overall procedure including MMAE. The limits of detection correspond to the overall method: MMAE + *in situ* preconcentration + HPLC determination.

PAH	Spiked level ($\mu g\,kg^{-1}$)	RR (%)	RSD (%)	E_R (%)	E_F	Spiked level ($\mu g\,kg^{-1}$)	RR (%)	RSD (%)	E_R (%)	E_F	LOD[a] ($\mu g\,kg^{-1}$)
BcL	45	–	–	–	–	9	–	–	–	–	–
CPP	4500	124	11	92.5	11	220	127	5.5	119	13	83
BaA	45	106	7.1	76.3	9.8	9	125	11	98.1	11	0.14
CHR	45	106	8.4	68.0	5.6	9	126	10	82.2	8.0	0.55
5MC	45	108	11	65.3	8.8	9	128	12	98.2	11	0.08
BjF	450	103	12	68.8	9.7	90	115	22	79.9	10	11
BbF	450	118	8.2	72.1	9.3	90	128	8.7	68.4	6.9	3.9
BkF	45	126	8.6	78.5	8.2	9	126	12	112	12	0.03
BaP	45	121	7.0	72.9	8.4	9	123	12	99.9	11	0.07
DlP	45	119	6.7	93.9	8.4	9	128	11	122	10	0.12
DhA	45	118	6.6	93.3	8.3	9	95.0	11	76.3	9.6	0.10
BgP	45	107	5.6	105	12	9	87.9	13	110	14	0.04
IcP	45	116	9.0	75.7	8.4	9	127	12	92.1	9.4	0.75
DeP	45	118	7.0	79.0	9.4	9	116	10	115	14	0.17
DiP	45	111	6.4	79.2	11	9	109	11	76.6	10	0.32
DhP	45	102	9.9	75.8	13	9	107	13	75.4	12	0.08

[a] Limits of detection of the overall MMAE + *in situ* preconcentration + HPLC method, calculated considering the preconcentration achieved in the *in situ* step altogether with the efficiency obtained by MMAE, and verified by spiking toasted wheat cereals at such levels and performing the overall procedure.

increase in the slopes before and after *in situ* preconcentration: 8 ± 2. Literature E_F values for the 16 EPA PAHs using IL-DLLME and HPLC have been reported to be ~332 [41], for aqueous samples of 10 mL that were preconcentrated to a drop of ~27 μL, which was further diluted with methanol to 0.5 mL and injected in the HPLC (E_{Fmax} = 370 and theoretical E_{Fmax} including dilution = 20). The *in situ* preconcentration procedure developed in this work begins with 1.5 mL of extract, originating from MMAE and filtration, and is preconcentrated to ~65 μL and later diluted with acetonitrile to ~95 μL (E_{Fmax} = 23 and theoretical E_{Fmax} including dilution = 16).

As it has been observed, the enrichment factor is not an adequate parameter to compare the performance of the *in situ* preconcentration method with other preconcentration procedures due to the fact that it is a function of the final microdroplet volume obtained. A more reliable approach is to compare the relative enrichment factor (RE_F) (or real extraction efficiency E_R). Calculated average E_R values were of 95 ± 18 in % at the lowest level spiked, 80 ± 11 in % at the highest spiked level, as observed from Table 3. It must be highlighted that extraction efficiencies close to 100% are obtained in this *in situ* preconcentration procedure, and is not common in preconcentration procedures. Indeed, in microextraction procedures such as solid-phase microextraction (SPME), the E_R values are rarely 100%, but the method is still valid because huge enrichment factors with adequate precision values are obtained. Few studies have reported real extraction efficiencies in DLLME methods as it is more common to report relative recoveries (RR). E_R values of 29–91% [42] or 55–74% [43] have been reported in DLLME applications for various classes of analytes.

The limits of detection of the overall method were calculated taking into account the limits of detection of the *in situ* preconcentration procedure (included in Table 1), and the extraction efficiency of the MMAE method, reported in a previous study by our group [20]. They were verified by spiking wheat toasted cereals at such levels and carrying out the whole method. They oscillated between 0.03 $\mu g\,kg^{-1}$ for BkF to 11 $\mu g\,kg^{-1}$ for BjF, being of 83 $\mu g\,kg^{-1}$ for CPP. In the literature, limits of detection for 11 of the 15 + 1 EU PAHs, calculated on a signal-to-noise ratio of three, have been described to range from 0.33 to 5.8 $\mu g\,kg^{-1}$ in bread ash samples [44], and from 0.01 to 0.70 $\mu g\,kg^{-1}$ in infant cereal samples [45], using in both cases an UAE-SPE-HPLC-FLD approach. With regards to PAH content in cereals, the EU has established a maximum level (ML) for BaP in processed cereal-based foods for infants and young children of 1 $\mu g\,kg^{-1}$ (humid weight basis, and referred to final product) [35,36]. The *in situ* preconcentration method developed in this study clearly fulfills the required limits. A wide survey in the EU has shown content in cereals for 8 of the 15 + 1 EU PAHs to be around 2–5 $\mu g\,kg^{-1}$. It is true that this content is lower than that found in barbecue meats (around 10 $\mu g\,kg^{-1}$). However, the high consumption of bread and cereals may increase the risks due to PAH intake. Again, the developed method is adequate for estimating the reported content in cereals.

Fig. 3(A) includes a chromatogram obtained when applying the overall method (MMAE, *in situ* preconcentration, and HPLC determination) for a toasted wheat cereal spiked at 3.0 $\mu g\,g^{-1}$ for CPP, 0.30 $\mu g\,g^{-1}$ for BjF and BbF, and 30 $\mu g\,kg^{-1}$ for the remaining PAHs.

3.6. Extraction performance of the in situ preconcentration method for various toasted cereals

The performance of the *in situ* preconcentration method was evaluated with various toasted cereals, such as barley, maize and rye, in order to evaluate the influence of the cereal nature on its performance. The selected cereal samples were examined to be free of PAHs before being used as blank samples. Thus, toasted cereals were spiked at 4.5 $\mu g\,g^{-1}$ for CPP, 0.45 $\mu g\,g^{-1}$ for BjF and BbF, and 4.5 $\mu g\,kg^{-1}$ for the remaining PAHs. The toasted cereal samples were then extracted by MMAE, preconcentrated using the developed *in situ* approach, and injected into HPLC. This procedure was carried out six times with each cereal sample. The data obtained was evaluated using the calibrations for the chromatographic method (Table 2), and the calibrations for the overall method which were obtained with toasted wheat cereals (Table 1). Table 4 shows the performance of the *in situ* preconcentration method evaluated by RR (%), RSD (%), E_F and E_R (%) when analyzing cereals of different nature.

Average relative recoveries of 118, 120 and 145% were obtained for barley, maize and rye, respectively. It can be observed that barley and maize toasted cereals behave quite similar to wheat cereals, and so the calibration method is valid for them. However, there is a quite important matrix effect for rye cereals (average RR of 145%), and so the overall calibration obtained with wheat cereals (Table 1) is not valid. Therefore, toasted rye cereals would require a specific overall calibration method in order to properly account for efficiency performance. Average enrichment factors were 10 ± 2, 9 ± 2, and 8 ± 1, for barley, maize, and rye, respectively. Average E_F was 9 ± 2 for wheat cereals. With regards to the precision of the method, average RSD values were 7.7, 9.7, and 16%, for barley, maize, and rye, respectively. It can be also observed that the worse precision

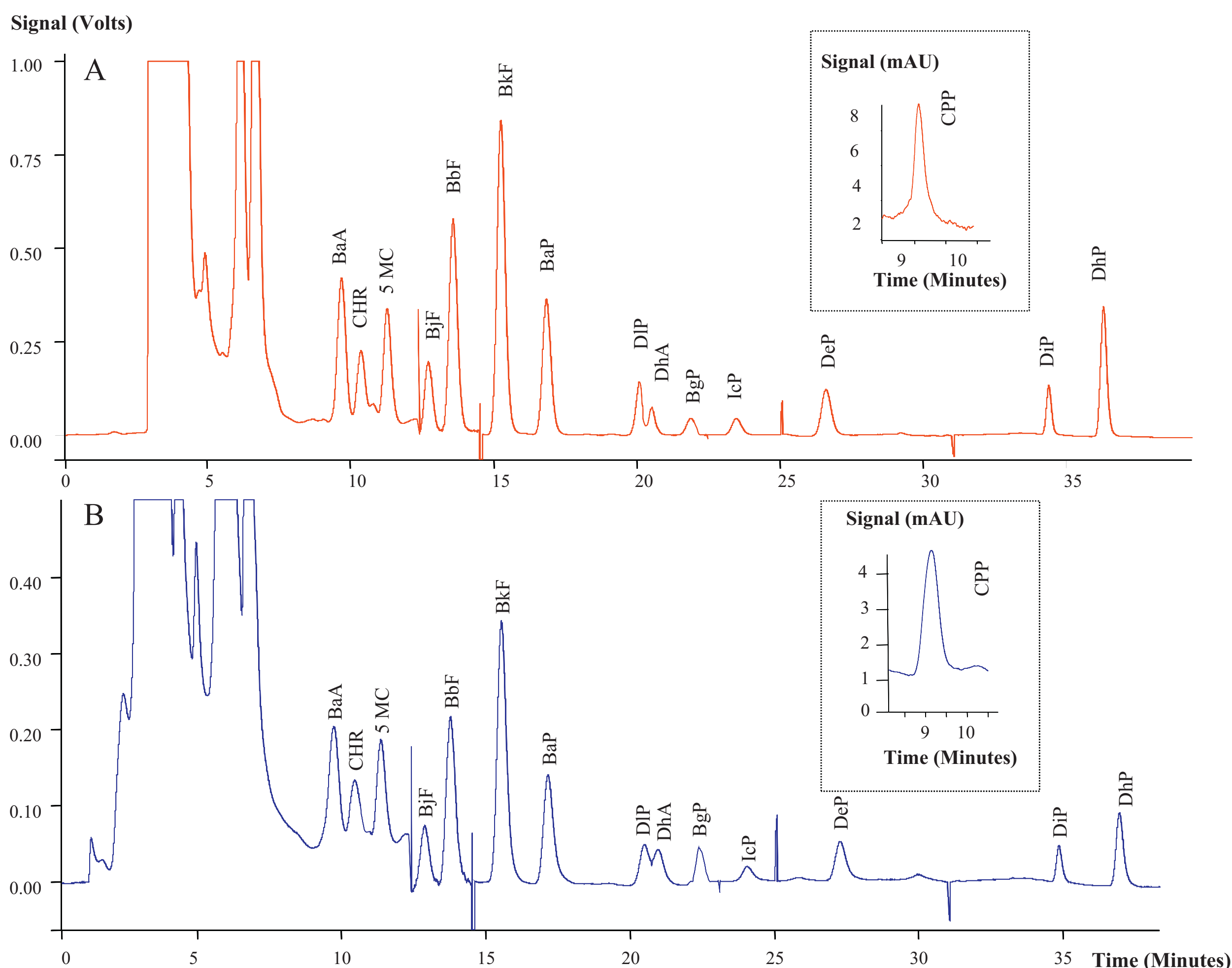

Fig. 3. Chromatograms obtained for toasted cereals spiked with PAHs and subjected to the overall MMA + *in situ* preconcentration method, followed by HPLC determination, with (A) spiked toasted wheat cereals, and (B) spiked toasted barley cereals. The main chromatograms correspond to FD detection, and the chromatograms for CPP were obtained using UV–vis detection. The spiked concentrations of PAHs are cited within the manuscript.

Table 4
Performance of the *in situ* preconcentration method (RR, RSD, E_R and E_F) with cereals samples of different nature. Cereals samples were spiked, and subjected to the overall method: MMAE, *in situ* preconcentration, and HPLC determination.

PAH	Barley[a]				Maize[a]				Rye[a]			
	RR (%)	RSD (%)	E_R (%)	E_F	RR (%)	RSD (%)	E_R (%)	E_F	RR (%)	RSD (%)	E_R (%)	E_F
BcL	–	–	–	–	–	–	–	–	–	–	–	–
CPP	129	6.4	85.8	10	124	4.9	85.5	10	140	16	95.0	10
BaA	110	6.9	70.4	9.7	111	11	82.6	9.7	132	16	91.7	9.4
CHR	113	6.8	45.2	5.6	109	6.2	46.5	5.6	139	17	62.8	5.7
5MC	124	8.3	75.9	8.7	128	9.9	79.1	8.7	150	18	90.9	8.7
BjF	104	14	61.1	9.7	108	14	67.2	9.7	125	18	79.0	9.7
BbF	124	10	74.1	9.3	120	3.6	66.7	9.3	151	14	107	9.5
BkF	127	5.4	76.8	8.7	129	10	81.4	8.6	153	12	113	8.4
BaP	128	3.8	80.9	8.7	125	10	75.4	8.8	155	12	117	8.1
DlP	123	6.7	104	9.8	121	10	100	9.8	148	19	120	6.5
DhA	122	6.6	108	10	120	9.9	105	10	151	18	121	6.7
BgP	116	9.9	100	11	129	16	106	8.1	150	13	102	7.8
IcP	118	7.4	79.0	8.4	128	10	82.4	8.4	143	11	108	8.4
DeP	115	6.0	72.0	9.4	127	9.8	82.6	9.5	147	19	123	9.6
DiP	113	8.5	83.1	11	121	9.9	81.8	11	142	17	105	7.7
DhP	101	8.6	81.1	13	103	9.9	92.8	13	150	19	94.0	8.1

[a] The spiked level was 4.5 μg g^{-1} for CPP, 450 μg kg^{-1} for BjF and BkF, and 4.5 μg kg^{-1} for the rest of PAHs.

was obtained with rye cereals, however, the value is still acceptable (<19%). With regards to the extraction efficiency, average E_R values of 80, 82 and 102% were obtained for barley, maize, and rye, respectively; with 80% for wheat cereals when working at the same level spiked.

In summary, it can be observed that the developed method works extremely well for quite different samples such as barley and maize cereals, and works moderately well for rye, in which another calibration would be necessary in order to account for matrix effects.

Fig. 3(B) includes a chromatogram obtained when applying the overall method (MMAE, *in situ* preconcentration, and HPLC determination) for a toasted barley cereal spiked at 1.5 $\mu g\,g^{-1}$ for CPP, 0.15 $\mu g\,g^{-1}$ for BjF and BbF, and 15 $\mu g\,kg^{-1}$ for the remaining PAHs.

4. Conclusions

It has been developed for the first time a preconcentration step using IL-based surfactants. The procedure is simple and consists of a water-soluble IL-based surfactant containing the Br^- anion undergoing a metathesis reaction with $LiNTf_2$ to form a water-insoluble IL. The preconcentration method has been optimized to minimize the final volume of the IL containing the NTf_2^- anion (microdroplet volume of a few μL) to ensure adequate preconcentration. The final microdroplet can be directly injected into HPLC by simply diluting it with acetonitrile. IL-based surfactants are comparable to cationic surfactants but differ in that their structural tuneability can be designed to increase the overall sensitivity of the method. The main advantage of this *in situ* approach is its application to the extraction of organic molecules contained in solid samples, which are often extracted with IL-based surfactants using MMAE (or even an ultrasound-assisted micellar extraction method). Another important feature of the developed extraction/preconcentration method is that it requires practically no organic solvent (~30 μL of acetonitrile) and very little of the IL-based surfactant.

The *in situ* preconcentration method was carried out with the IL-based surfactant $C_{16}C_4$Im-Br, and the application was in the extraction of the 15 + 1 EU PAHs from toasted cereals. Cereals were extracted with $C_{16}C_4$Im-Br using MMAE, and preconcentrated with the *in situ* approach in the same extraction vessel, followed by separation of the $C_{16}C_4$Im-NTf_2 microdroplet with a syringe. The extraction efficiency of the *in situ* preconcentration method was quite high, with average efficiencies ranging between 80 and 95%, and precision values lower than 14%. Furthermore, the overall method is quite sensitive, with detection limits ranging from 0.03 $\mu g\,kg^{-1}$ for BkF to 11 $\mu g\,kg^{-1}$ for BjF, and 83 $\mu g\,kg^{-1}$ for CPP. The obtained detection limits meet current EU regulations that have been imposed for these matrices. In addition, when the method was evaluated using various types of toasted cereals, satisfactory results were obtained.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.097.

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