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Use of ionic liquid aggregates of 1-hexadecyl-3-butyl imidazolium bromide in a focused-microwave assisted extraction method followed by high-performance liquid chromatography with ultraviolet and fluorescence detection to determine the 15 + 1 EU priority PAHs in toasted cereals ("gofios")

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ABSTRACT

A focused-microwave assisted extraction method using aggregates of the ionic liquid (IL) 1-hexadecyl-3-butylimidazolium bromide (HDBIm-Br) followed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection and single-channel fluorescence detection (FLD) has been developed for the determination of polycyclic aromatic hydrocarbons (PAHs) in toasted cereals ("gofios") of different nature (wheat, barley, rye, and maize corn) from the Canary Islands, Spain. The optimized HPLC–UV–vis/single-channel FLD method takes 40 min for the chromatographic run with limits of detection varying between 0.02 and 4.01 ng mL^{-1} for the fluorescent PAHs from the European Union (EU) priority list in foods, and 20.5 ng mL^{-1} for the non-fluorescent PAH cyclopenta[*c*,*d*]pyrene (CPP). The optimized microwave step presented extractions recoveries ranging from 70.1 to 109% and precision values lower than 12.6% (as relative standard deviation), using an extraction time of 14 min. The extraction method also utilizes low amounts of sample (0.1 g), and low amounts of IL (77 mg), avoiding completely the use of organic solvents.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are well known compounds for their mutagenic and carcinogenic effects [1]. They are formed at high temperatures in natural processes (fires, volcanic eruptions, etc.) and in anthropogenic processes (burning of fossil fuels, vehicles emissions, plants of petroleum processing, etc.) due to the incomplete combustion of organic matter. PAHs are largely known as ubiquitous environmental contaminants due to their ability to sorbtion onto atmospheric particulate matter and become transported all over the planet [2]. Soils, surface waters, and sediments may be contaminated by PAHs due to atmospheric fallout, urban runoff, deposition from sewage, and by oil or gasoline spills. Hence, there is a potential for food crops to become environmentally contaminated as a result [3]. Furthermore, PAHs can be introduced in food during processing and food preparation, such as drying, roasting, frying, smoking, barbecuing, baking, or grilling [4–6]. Contamination of food due to food cans is also possible. The presence of PAHs in foods is particularly important because the epidemiological studies reveal that around 40% of human cancers are related with diet [7,8].

The European Food Safety Authority (EFSA) recommends the monitoring of 15 PAHs in foods, in addition to benzo[*c*]fluorene, instead of the traditional exclusive monitoring of benzo[*a*]pyrene as PAH marker in foods [9]. These 16 EU PAHs only have 8 PAHs in common with the sixteen hydrocarbons traditionally monitored in environmental samples according to the US Environmental Protection Agency (EPA), which are referred as the sixteen EPA priority pollutants PAHs.

Many gas chromatography (GC) capillary columns efficiently separate all 16 EPA priority PAHs. However, the same situation does not occur with the 15 + 1 EU PAHs. There has been a trend to develop successful GC stationary phases in an effort to improve such separations [10–13]. High-performance liquid chromatography (HPLC) with fluorescence detection (FLD) has been also widely employed for the analysis of the 16 EPA PAHs. However, there are not many works that report the use of this technique for the analysis of the 15 + 1 EU PAHs [14–16], and all of them use a multi-channel FLD. It is important to develop methods to determine the 15 + 1 EU PAHs using HPLC–FLD (but not necessarily multichannel) to enable laboratories without GC–MS to accomplish their determination.

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Cereals contribute in a large extent to PAH intake in diet because of higher intake rates of grain-based products. Among them, wheat grain is one of the major food crops consumed internationally, including infants and children. In spite of this, few reports have examined the determination of the overall 15 + 1 EU PAHs in cereals. Works have been mainly conducted with several PAHs (from 8 to 11) of the 15 + 1 EU PAHs [4,17,18], or with the overall 16 EPA PAHs [19–21]. Dietary exposure to PAHs in Spain has mainly been conducted with the 16 EPA PAHs or with several of the 15 + 1 EU PAHs [22–24]. In the Canary Islands (Spain), there is a high consumption of toasted cereals, so called "gofios", mainly for infant diet.

Extraction of 11 EU PAHs from cereals has been carried out using ultrasonic-assisted extraction (UAE) followed by solid-phase extraction (SPE) [4,17]. The extraction of eight EU PAHs from cookies has been carried out by combining microwave-assisted extraction (MAE) with oil saponification and SPE [18]. The extraction of the 16 EPA PAHs from bread has been done by saponification followed by further clean-ups steps using columns and microcolumns of alumina and silica [21], and from wheat grain using UAE and several clean-up steps including gel-permeation chromatography (GPC) [20].

Conventional extraction techniques present an important disadvantage from an environmental point of view, primarily, due to their high consumption of toxic organic solvents. It is contradictory to develop analytical methods to monitor toxic compounds while the method being employed generates toxic waste. Thus, there has been an important trend in analytical chemistry to develop environmentally friendly methods. Among them, solid-phase microextraction (SPME) [25] and liquid-phase microextraction (LPME) [26] can be highlighted. Another important trend in analytical chemistry is the utilization of less toxic reagents. Ionic liquids (ILs) are nonmolecular solvents that have gained significant attention recently as a newer class of designer solvents [27,28]. Most ILs possess negligible vapor pressure, high thermal stability, and unique catalytic properties. ILs possessing long aliphatic substituents have been reported to undergo micellization in aqueous solutions [29–31]. Recently, several works have reported the utilization of aqueous IL-aggregates as extractant solvents in extraction procedures [32–36].

The main aim of the present work is to develop an extraction scheme to analyze the 15 + 1 EU PAHs in "gofios". PAHs can be present in "gofios" due to environmental contamination and/or the own toasting procedure. To the best of our knowledge, there are no studies related to PAHs in "gofios", and none which monitor the overall 15 + 1 EU PAHs in cereal foods. The further determination of the 15 + 1 EU PAHs is accomplished by HPLC–FLD. The FLD used in this work is single-channel, instead of the multi-channel fluorescence detector that has been always reported for the 15 + 1 EU PAHs [14–16]. Moreover, the extraction procedure is conducted utilizing aggregates of the IL 1-hexadecyl-3-butylimidazolium bromide (HDBIm-Br) in a microwave-assisted extraction approach, with the intention to expand the use of these reagents in analytical chemistry.

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), ndeno[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.

The ionic liquid 1-hexadecyl-3-butylimidazolium bromide (HDBIm-Br) was synthesized and fully characterized according to a previous work [37]. The critical micelle concentration (CMC) value for this IL has been previously determined to be 0.1 mM in deionized water [37], and ranged from 0.23 to 0.46 mM when the acetonitrile content in solution varied between 1 and 20% (v/v) [38]. The preparation of HDBIm-Br aggregates simply implies the dissolution of the IL in water.

Several "gofio" samples of different nature (wheat, barley, rye and maize corn) were acquired in two local mills (Barrio de La Salud and El Sauzal, both in Tenerife, Spain). Wheat "gofio" 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 contents in 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.

Amber vials of two milliliters with screw top caps were used for the chromatographic determination (Supelco, Bellefonte, PA, USA). Stir bars (10 mm × 3 mm) were supplied by Aldrich (Milwaukee, WI, USA) and were used to increase the equilibrium rate in the microwaves.

2.2. Instrumentation

The HPLC equipment consisted of a delivery solvent ProStar 230 SDM (Varian, Palo Alto, CA, USA) and an Autosampler Varian Prostar 410 with a 20 μL loop. The autosampler also incorporates a column oven. 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). The temperature of the column was kept at 32 °C. Data were acquired with the Star 6.41 LC chromatography workstation software (Varian). The Statgraphic (Statistical Graphics, Rockville, USA) software package Version 5.1 was used for the statistical treatment.

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).

An Eppendorf Centrifuge 5702 (Hamburg, Germany) was also used in the extraction studies.

Table 1
Optimum HPLC conditions to achieve the separation of the 15 + 1 priority EU PAHs.

Time (min)	Acetonitrile:water (% v/v)	Flow (mL min^{-1})
0	70:30	1
28	90:10	1
29	90:10	2
32	100:0	2
40	100:0	2
45	70:30	1

2.3. Procedures

An amount of 0.1 g of "gofio" (free or contaminated with PAHs) was placed in a glass Pyrex® tube of 25 mL. A volume of 4.5 mL of the HDBIm-Br aggregate aqueous solution at the optimum concentration (40 mM) was added, and the extraction tube is then introduced into the microwave cavity after ensuring that a stir bar was placed into the tube.

The optimum microwave extraction conditions were: microwave power of 50 W to reach a maximum temperature of 80 °C in 4 min, and a hold time of 10 min. Once the optimum extraction time was achieved, the tube was allowed to cool at room temperature.

The obtained extract was then centrifuged for 5 min at 4000 rpm. The supernatant was transferred to a 2 mL vial, and the vial was finally placed in the HPLC autosampler to carry out the chromatographic analysis without any further clean-up step.

Spiking of the "gofio" sample was performed as follows: 1 g of "gofio" 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 optimization study was 1.35 mg kg^{-1} for all PAHs except for the non-fluorescent CPP, which was 6.75 mg kg^{-1}, on dry-weight basis. Other amounts were spiked depending on the specific experiment. 0.1 g of the dry spiked sediment was analyzed as aforementioned. Non-spiked samples were also stored in the dark until the extraction was conducted.

The HPLC method used for the separation and determination of the 15 + 1 priority EU PAHs consists of a gradient elution procedure with UV–vis and fluorescence detector (FLD). The optimal chromatographic method utilizes a mixture of acetonitrile–water with a linear gradient elution, with the conditions listed in Table 1. The wavelength program of the fluorescence detector can be observed in Table 2, using an excitation split of 18 nm and an emission split of 10 nm.

3. Results and discussion

3.1. HPLC–UV–vis/FLD determination of the 15 + 1 EU PAHs

HPLC with FLD has been widely used for the analysis of the 16 EPA PAHs [19]. However, its utilization for the separation of the 15 + 1 EU PAHs has not been widely reported [14–16]. Simon et al. [14] utilized a fluorescence detector which permits the simultaneous acquisition of the fluorescence data at four different emission wavelengths (370, 420, 470 and 500 nm) and a common excitation wavelength of 270 nm. The gradient program of mobile phase consisted of water and acetonitrile, and the total chromatographic run time was 50 min. Brasseur et al. [15] used a multi-wavelength fluorescence detector with several channels. The gradient program of mobile phase utilized a ternary mixture of water, methanol and acetonitrile, being 40 min the total run time. Windal et al. [16] also used a multi-wavelength fluorescence detector with four channels (280/438, 300/512, 304/360 and 350/397 nm), and a ternary mobile phase (water, acetonitrile and methanol) to accomplish an efficient separation. Multi-wavelength fluorescence detectors are undoubtedly more expensive than traditional fluorescence detector with variable wavelength program (one channel).

Table 2
Optimum wavelength fluorescence detector program, using an excitation split of 18 nm and an emission split of 10 nm.

Time (min)	λex/λem (nm)
0	273/384
14.5	240/505
17.2	289/422
26.5	297/496
28.5	280/404
33	292/440

The present work has optimized the HPLC separation of the 15 + 1 EU PAHs in order to obtain an efficient separation using a regular fluorescence detector (not multichannel) with variable wavelength program, without the necessity of simultaneous acquisition of fluorescence data. This would expand the analytical determination of the 15 + 1 EU PAHs in analytical laboratories possessing a conventional FLD. It is important to note that the program is conducted using an excitation split of 18 nm and an emission split of 10 nm of the fluorescence detector. Tables 1 and 2 show the optimized chromatographic separation conditions, and the wavelength program of the fluorescence detector. CPP, the non-fluorescence PAH, was determined with UV detection at 375 nm. Under these conditions, PAHs can be efficiently separated in less than 40 min. Fig. 1(A) shows a chromatogram of a standard mixture of the 15 + 1 EU PAHs at a concentration of 25 ng mL^{-1} in acetonitrile, being 150 ng mL^{-1} the concentration of CPP in acetonitrile.

3.2. Quality parameters of the HPLC–UV–vis/FLD method for the 15 + 1 EU PAHs

Under the optimized separation and detection programs, the 15 + 1 EU PAHs gave satisfactory retention times with relative standard deviation (RSD) values ranging from 0.76 to 2.02%. Calibration curves were constructed by plotting the chromatographic peak-area *versus* the PAH concentration (using standards of PAHs in acetonitrile). The quality parameters of the analytical calibration plots can be observed in Table 3. Linear relationships with R^2 values varying between 0.993 and 0.999 were obtained. The precision was calculated injecting in the HPLC system a standard (corresponding to 150 μg L^{-1} for CPP, 50 μg L^{-1} for BbF and BjF, and 5 μg L^{-1} for the rest of fluorescent PAHs) dissolved in acetonitrile four non-consecutive times. The calculated relative standard deviation (RSD, %) values oscillated between 0.02 and 6.60%. Detection limits for the studied PAHs were obtained from the calibration plots and ranged from 0.02 ng mL^{-1} for BkF to 4.01 ng mL^{-1} for BbF, being of 20.5 ng mL^{-1} for CPP (the non fluorescent PAH). They were calculated as three times the signal to noise ratio, and were verified by injection of standards prepared at such concentration values. Detection limits for the 15 + 1 EU PAHs using HPLC with fluorescence detection have been reported to vary between 1.4 and 12 ng mL^{-1} [14], and between 0.02 and 2.7 ng mL^{-1} (equivalent to 0.5–54 pg injected) [16]. It can be observed that the performance of the proposed HPLC–FLD method for the 15 + 1 EU PAHs using a single-channel FLD is comparable to that of multi-channels detectors. To the best of our knowledge, this is the first time that HPLC with single-channel FD detector has been successfully applied to the determination of the 15 + 1 EU PAHs (different from the 16 EPA priority pollutants PAHs).

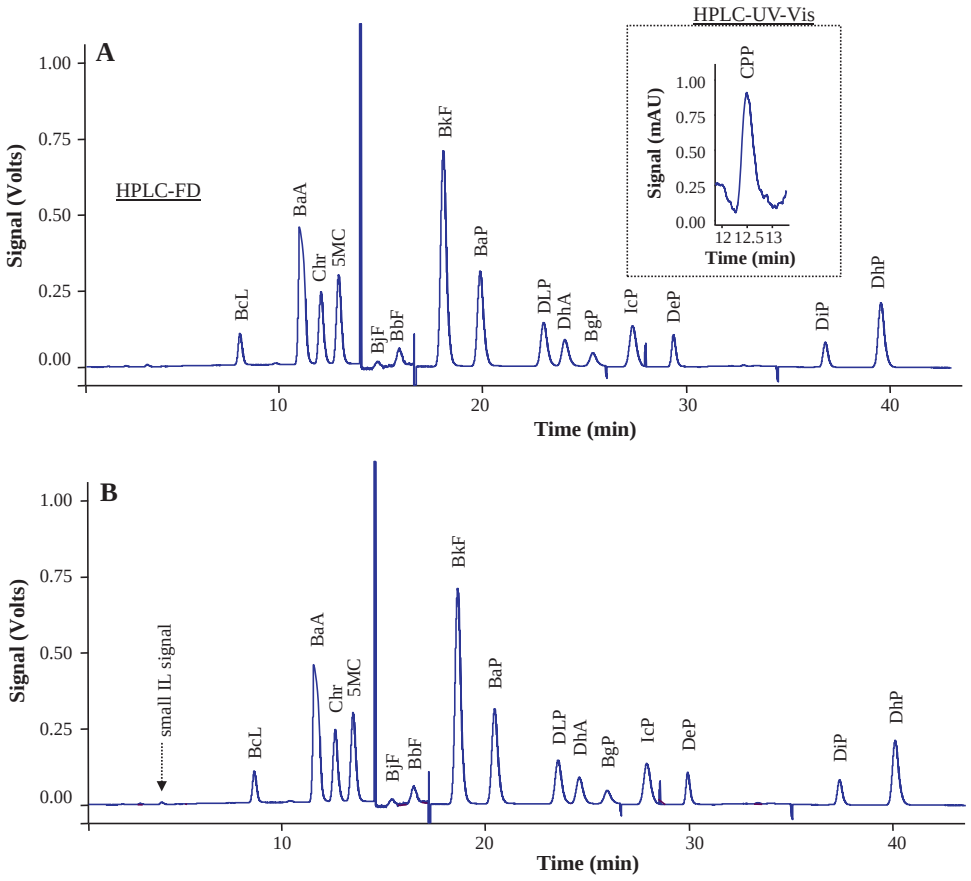

Fig. 1. HPLC–FLD chromatograms corresponding to the 15 EU fluorescent PAHs, and HPLC–UV–vis chromatogram corresponding to CPP (the non-fluorescent PAH) obtained using the optimized separation and detection conditions described in Tables 1 and 2 and: (A) standards of PAHs dissolved in acetonitrile, and (B) standards of PAHs dissolved in HDBIm-Br 40 mM. The concentrations of the fluorescent PAHs were 25 ng mL^{-1} and the concentration of CPP was 150 ng mL^{-1}.

3.3. Utilization of HDBIm-Br aggregates in HPLC–UV–vis/FLD

The utilization of micellar media as alternatives to traditional organic solvents in analytical extractions is of interest from an environmental point of view. Surfactants are generally less toxic than chlorinated organic solvents and are used in lower amounts in extraction schemes [39–41]. One of the problems often encountered when using traditional surfactant systems and HPLC is that the chromatographic signal from the surfactant itself can disrupt the quantification of the analytes of interest [42,43]. This is especially significant when using nonionic surfactants containing alkylphenolic-polyoxyethylated chains, which produce a high fluorescence or absorbance signal [42]. Recently, ionic liquid aggregates have been proposed as an alternative to traditional surfactant

Table 3
Quality parameters of the calibration method by HPLC–UV–vis/FLD for the 15 + 1 EU PAHs.

PAH	Calibration range (ng mL^{-1})	Slope ± SD[a] (×10^{-4})	Intercept ± SD[b] (×10^{-4})	Error of the estimate[c]	R^2	RSD%[d] (5 ng mL^{-1})	LOD (ng mL^{-1})
BcL	0.5–20	6.52 ± 0.09	−1.88 ± 0.16	16,726	0.999	1.70	0.19
CPP	50–250	0.04 ± 0.002	0.66 ± 0.20	3047	0.994	0.79	20.5
BaA	0.5–20	30.3 ± 0.5	−0.53 ± 0.83	85,628	0.999	0.02	0.06
CHR	0.5–20	16.8 ± 0.1	−4.13 ± 0.21	21,638	0.999	0.71	0.17
5MC	0.5–20	22.1 ± 0.4	−6.74 ± 0.58	59,649	0.999	0.22	0.15
BjF	10–100	1.74 ± 0.04	−12.6 ± 1.0	32,282	0.998	6.60	3.02
BbF	10–100	4.46 ± 0.15	−5.56 ± 3.65	11,3465	0.996	5.18	4.01
BkF	0.5–20	69.4 ± 1.3	−14.5 ± 2.1	215,614	0.999	0.20	0.02
BaP	0.5–20	31.6 ± 0.6	−9.43 ± 0.92	94,778	0.999	0.17	0.03
DlP	0.5–20	12.1 ± 0.5	−5.55 ± 0.81	82,977	0.993	3.25	0.16
DhA	0.5–20	7.89 ± 0.20	−2.47 ± 0.33	33,487	0.997	0.95	0.17
BgP	0.5–20	4.11 ± 0.13	−1.82 ± 0.20	20,321	0.997	1.24	0.20
IcP	1–100	4.35 ± 0.03	0.53 ± 0.23	25,234	0.999	0.62	0.24
DeP	0.5–20	6.04 ± 0.25	−3.15 ± 0.40	41,637	0.993	1.08	0.31
DiP	0.5–20	5.21 ± 0.05	−0.98 ± 0.08	7723	0.999	0.25	0.23
DhP	0.5–20	14.6 ± 0.5	−5.54 ± 0.79	81,063	0.996	0.18	0.22

[a] Error of the slope for $n = 6$ calibration levels.
[b] Error of the intercept for $n = 6$ calibration levels.
[c] Standard deviation of the regression.
[d] 150 ng mL^{-1} for CPP and 50 ng mL^{-1} for BbF and BjF.

systems in extraction schemes [32–36]. It is therefore necessary to study the effect of the utilization of HDBIm-Br on the chromatographic signal considering that this IL has not been studied previously as an extractant solvent in combination with HPLC. The utilization of the IL 1-hexadecyl-3-methylimidazolium bromide (HDMIm-Br) in HPLC with UV and FLD has been reported to present a small FLD interference at 275/374 (λex/λem), and a larger UV interference at 254 nm [32]. In both cases, the HDMIm-Br eluted during the first minutes of the chromatogram (band between 3 and 6 min) and thus it did not interfere with the analytes studied [32]. In another work, the same IL (HDMIm-Br) has been reported to present a much lower UV absorption interference when working at 270 nm [35].

PAHs were dissolved in HDBIm-Br aqueous solutions at three different concentrations of IL (10, 25 and 40 mM). These concentrations were above the critical micelle concentration to ensure the existence of aggregates of HDBIm-Br in solution. The acetonitrile content in the IL solutions was always lower than 4.5% (v/v). The PAHs concentration was 25 ng mL^{-1} in acetonitrile. Fig. 1(B) shows the obtained chromatogram when using the 40 mM IL concentration and fluorescence detection. A small fluorescence signal of the HDBIm-Br IL appears at around 4 min (273 nm as λex and 384 nm as λem) for all the studied concentrations of IL, and it does not interfere with the PAHs. A much higher HDBIm-Br absorbance signal appears in the UV chromatograms (375 nm) between 3 and 6 min, and therefore, there were no problems for the CPP quantification (~12.6 min). In this sense, the utilization of HDBIm-Br aggregates resulted quite convenient for the monitoring of the 15 +1 PAHs. Moreover, the obtained peak-areas (and peak-heights) for the PAHs dissolved in HDBIm-Br are not different from those obtained for the PAHs dissolved in acetonitrile. In fact, calibrations obtained with PAHs dissolved in HDBIm-Br (40 mM) were not statistically different to those obtained with PAHs dissolved in acetonitrile (which were included in Table 3) at the 95% confidence level. Another important result is that no changes on the C18 column efficiency were observed after repeated injections of the IL.

3.4. Optimization of a focused microwave-assisted extraction method with aggregates of HDBIm-Br

The extraction of organic analytes contained in a solid matrix by means of micellar media of traditional surfactant systems [39–41] or ionic liquid aggregates [32–36] has been conducted with the aid of microwaves or ultrasounds in order to accelerate the extraction step. The utilization of microwaves normally requires lower concentrations of extractant agents when compared to ultrasounds [40], which results very convenient from an environmental point of view. The focused microwave system (using micellar solutions of HDBIm-Br as the extracting media) was selected for this study due to its advantages over the conventional microwaves: mainly, the safety due to operation at atmospheric pressure.

The optimization of the focused microwave system was carried out by means of an experimental design. Several factors were fixed before carrying out the optimization study, namely, the microwave power (50 W), the amount of wheat "gofio" (0.1 g), the spiked amount of PAHs (1.35 mg kg^{-1}, being 6.75 mg kg^{-1} for CPP), and the extractant volume (4.5 mL of the HDBIm-Br aqueous solution). Our experience using the focused microwaves dictates that microwave powers higher than 50 W are not necessary. Several previous experiments pointed out that the best extractant volume/amount of wheat "gofio" ratio to work with was 4.5 mL/0.1 g. Higher ratios conducted to a non-desirable dilution of the extract, altogether with further problems in the centrifugation step. Lower ratios presented problems in the centrifugation step. Hence, the studied variables in the optimization study were the extraction temperature and the extraction time in the microwave, and the HDBIm-Br IL concentration. The selected levels (minimum and maximum) of these variables were: 40 and 80 °C for the microwaves extraction temperature, 2 and 10 min for the extraction time (hold time), and 10 and 40 mM for the HDBIm-Br aqueous concentration. Temperatures higher than 80 °C were not examined to avoid water evaporation and possible losses of PAHs. The purpose of utilizing a focused microwave system was to minimize the overall extraction time, and so a hold time of 10 min was fixed as the maximum. The hold time in the microwaves is defined as the time at which the fixed temperature is kept constant. The run time is defined as the time necessary to reach the selected temperature. For the maximum temperature (80 °C), the run time was 4 minutes. With respect to the HDBIm-Br concentration, the minimum concentration selected was 10 mM to ensure working well above the critical micelle concentration. Its CMC is 0.1 mM in absence of acetonitrile, and around 0.3 mM when using an acetonitrile content of 4%. The maximum HDBIm-Br concentration was 40 mM to avoid solubility problems of the IL in water. In these conditions, a 2^3 factorial design with 2 central points was used. The peak area of each PAH was selected as the response variable. This design involved a total of 10 randomized runs. The matrix of the design is shown in Table 4. The order of the runs in the table is only referred to the randomized order in which experiments were undertaken.

Table 4
Experimental design matrix used in the optimization study.

Run	T (°C)	HDBIm-Br (mM)	Hold time (min)
1	80	40	2
2	80	40	10
3	80	10	10
4	40	40	10
5	80	10	2
6	40	40	2
7	40	10	2
8	60	25	6
9	40	10	10
10	60	25	6

All PAHs presented a similar behavior with respect to three experimental variables considered, as it can be observed in the examples included in Fig. 2. Fig. 3(A) shows the effects of the fac-

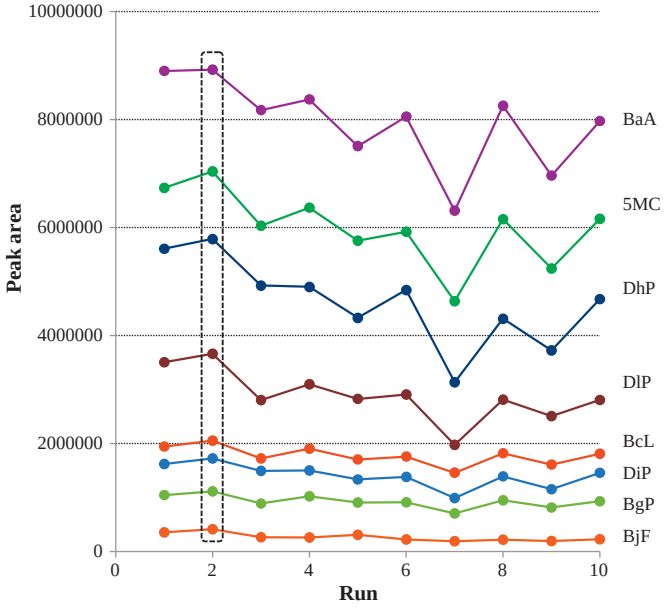

Fig. 2. Results obtained in each run of the factorial design for several of the PAHs studied. Dashed lines and lines connecting-points are only plotted with the purpose to facilitate the interpretation of the figure.

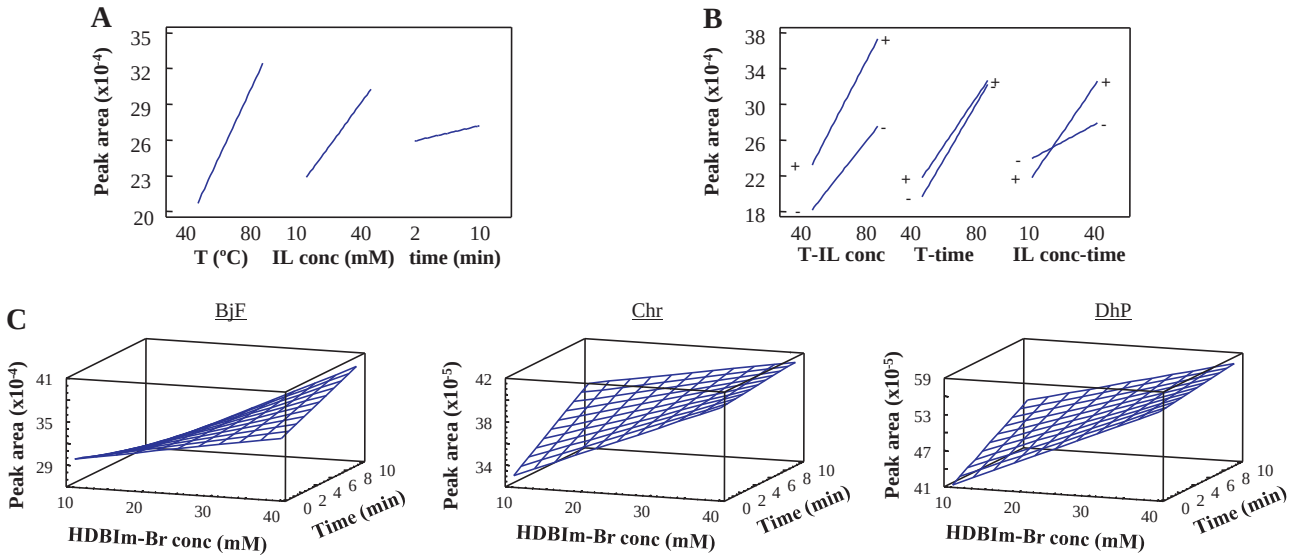

Fig. 3. Plots obtained in the interpretation of the design: (A) effects of the main factors for BjF, (B) interaction among factors for BjF, and (C) responses surfaces for BjF, CHR and DhP (extraction temperature fixed at 80 °C).

tors for BjF, a representative PAH. This PAH was selected as example due to its lowest sensitivity in FLD. It can be observed that all factors have a positive effect on the extraction efficiency, especially the microwave extraction temperature. The study of factor interactions, Fig. 3(B), reveals that there is an interaction between the concentration of HDBIm-Br and the hold time, reaching the maximum efficiency when both the concentration of HDBIm-Br and the extraction time are at the maximum value. Several response surfaces are shown in Fig. 3(C). None of the obtained response surface showed a maximum in the experimental region covered by the design. Nevertheless, maximum extraction efficiencies are obtained using high extraction times, and high concentration values of HDBIm-Br. Attending to the obtained results, the following conditions were selected as optimum: 80 °C for the microwaves extraction temperature, which implies a ramp time of 4 minutes to reach such temperature; 40 mM of the aqueous solution of HDBIm-Br; and 10 min for the hold time. The overall microwave extraction time is 14 min. In fact, these values correspond to run 2 of the design (highlighted with a dotted circle line in Fig. 2). Fig. 4 shows the chromatogram corresponding to the best extraction conditions obtained with spiked wheat "gofio" samples.

3.5. Quality parameters of the extraction method with toasted cereals ("gofios")

The overall focused-microwave extraction method followed by HPLC–UV–vis/FLD was carried out under the optimized conditions using spiked wheat "gofio" samples at two different levels in order to evaluate the performance of the method in terms of extraction efficiency and precision. The wheat "gofio" samples were previously subjected to the optimized method and were shown to be free of the 15 + 1 EU PAHs. The precision and the extraction efficiency of the method were then determined by performing five non-consecutive extractions at each spiked level. The obtained results are shown in Table 5, including the specific spiked amounts for each PAH at each spiked level. Extraction recoveries ranging from 70.1 to 107% at the highest spiked level, and ranging from 66.8 to 109% at the lowest spiked level, were obtained; being the

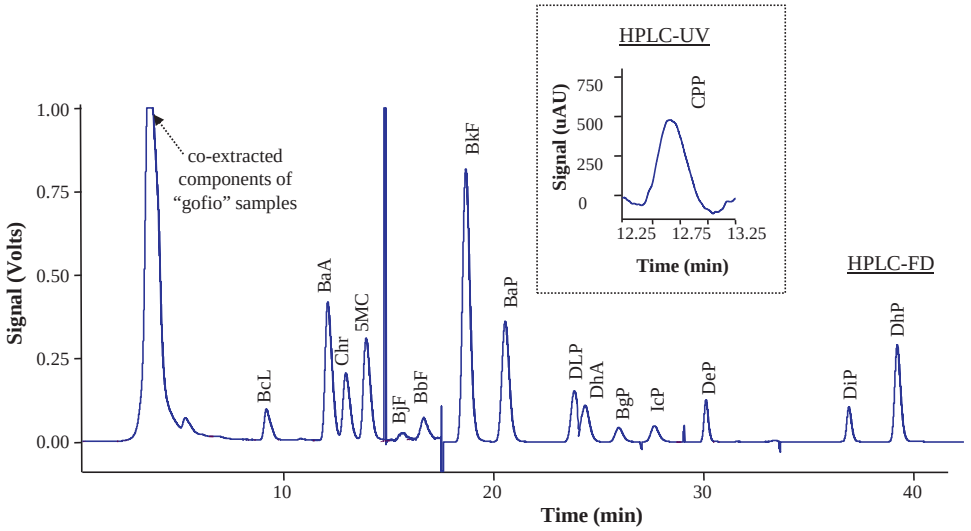

Fig. 4. Chromatogram of an extract obtained from a spiked wheat "gofio" sample subjected to the overall optimized procedure, being 30 ng mL^{-1} the concentration of fluorescent PAHs in the final extract, and 250 ng mL^{-1} for CPP.

Table 5
Quality parameters of the optimized overall focused microwave-assisted extraction method in combination with HPLC–UV–vis/FLD.

PAH	Average extraction efficiency at spiked level 1[a] (mg kg^{-1})	RSD (%)[a] at spiked level 1	Average extraction efficiency at spiked level 2[a] (mg kg^{-1})	RSD (%)[a] at spiked level 2	LOD (mg kg^{-1})
BcL	94.7 (0.04)	6.94	92.5 (0.90)	5.67	0.009
CPP	89.0 (4.50)	2.27	93.8 (11.2)	1.71	1.037
BaA	85.9 (0.04)	1.02	90.1 (0.90)	7.08	0.003
CHR	77.5 (0.04)	8.00	73.0 (0.90)	4.02	0.010
5MC	91.0 (0.04)	5.96	88.4 (0.90)	5.74	0.007
BjF	92.0 (0.90)	7.82	97.9 (4.50)	1.74	0.148
BbF	95.3 (0.90)	5.43	97.7 (4.50)	0.77	0.189
BkF	86.1 (0.04)	3.11	86.0 (0.90)	10.8	0.001
BaP	87.5 (0.04)	3.71	87.1 (0.90)	10.1	0.002
DLP	66.8 (0.04)	7.02	70.1 (0.90)	2.85	0.011
DhA	67.2 (0.04)	6.11	70.3 (0.90)	4.38	0.011
BgP	74.7 (0.04)	2.32	77.1 (0.90)	7.23	0.012
IcP	81.2 (0.04)	12.1	81.5 (0.90)	10.5	0.013
DeP	88.8 (0.04)	2.49	89.6 (0.90)	0.52	0.016
DiP	94.0 (0.04)	3.24	92.5 (0.90)	3.80	0.011
DhP	109 (0.04)	1.04	107 (0.90)	12.6	0.009

[a] Five consecutive extractions.

average recoveries 86.3 and 87.2%, respectively. It is important to note that the extraction procedure is quantitative, being conducted in one-single step followed by direct injection in the HPLC without any further clean-up or preconcentration step. The utilization of the IL HDMIm-Br has reported to be almost quantitative for 6 PAHs (P, BaA, BaP, BbF, BkF and BaP), but not for IcP [32].

The obtained precision presented relative standard deviation values (RSD, %) varying between 1.02 and 12.1% at the lowest spiked level, and between 0.52 and 12.6% at the highest spiked level. It should be noted that RSD values lower than 12.6% were obtained in all cases.

The limits of detection of the overall method were calculated considering the detection limits of the HPLC–UV–vis/FLD method (Table 3), and the microwave extraction–preconcentration, and also considering the extraction efficiency obtained at the lowest spiked level. These calculated values were verified by spiking wheat "gofio" samples at such levels and subjecting the samples to the whole procedure. The obtained LODs have also been included in Table 5. For the majority of PAHs studied (up to 13 PAHs), they oscillate between 1 ng g^{-1} for BkF and 16 ng g^{-1} for DeP, being 0.148 mg kg^{-1} for BjF and 0.189 mg kg^{-1} for BbF, and of 1.037 mg kg^{-1} for CPP, the non-fluorescent PAH. 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 ng g^{-1} in bread ash samples [4], and from 0.01 to 0.70 ng g^{-1} in infant cereal samples [17], using in both cases an UAE-SPE–HPLC–FLD approach, that is, incorporating a preconcentration step after the ultrasonic extraction. In this sense, it should be highlighted the LODs obtained with this work because the proposed method does not utilize an extra preconcentration step after the microwave-assisted extraction. It would be certainly of interest to increase the sensitivity of the proposed method by incorporating a preconcentration step; ongoing work aimed at this objective is being conducted in our laboratory. The ionic liquid HDBIm-Br can be classified as a cationic surfactant. Cloud-point extraction (CPE) is a valuable preconcentration step for nonionic surfactants, but its validity with cationic surfactants is not wide. Indeed, CPE for cationic surfactants is only possible under extreme acidic and/or salty conditions, with avoids the applicability of the technique.

The influence of the nature of the "gofio" on the extraction efficiency of the optimized method was assessed. Three "gofio" samples of barley, rye and maize corn, were analyzed in the same conditions as wheat "gofios", but PAHs were not detected in the samples. Hence, the samples were spiked at the lowest level (4.5 mg kg^{-1} for CPP, 0.90 mg kg^{-1} for BjF and BbF, and 0.04 mg kg^{-1} for the rest of fluorescent PAHs) to evaluate the influence of the matrix in the relative recoveries and precision of the method. The lowest spiked level was used in this study to evaluate the

Table 6
Analytical performance of the optimized method with different "gofio" samples.

PAH	Barley		Rye		Maize corn	
	Average extraction efficiency (spiked level in mg kg^{-1})[a]	RSD[a] (%)	Average extraction efficiency (spiked level in mg kg^{-1})[a]	RSD[a] (%)	Average extraction efficiency (spiked level in mg kg^{-1})[a]	RSD[a] (%)
BcL	102 (0.04)	6.5	107 (0.04)	7.8	95.2 (0.04)	8.4
CPP	99.4 (4.5)	1.3	98.9 (4.5)	1.1	96.0 (4.5)	1.6
BaA	95.9 (0.04)	10	85.5 (0.04)	11	82.2 (0.04)	5.2
CHR	87.8 (0.04)	9.5	79.3 (0.04)	13	82.6 (0.04)	8.6
5MC	89.9 (0.04)	5.2	90.8 (0.04)	4.7	91.1 (0.04)	13
BjF	104 (0.90)	13	96.5 (0.90)	16	98.9 (0.90)	11
BbF	97.9 (0.90)	2.9	96.9 (0.90)	4.1	105 (0.90)	10
BkF	90.2 (0.04)	7.0	91.4 (0.04)	9.4	89.2 (0.04)	14
BaP	87.8 (0.04)	12	92.5 (0.04)	8.6	91.5 (0.04)	3.6
DLP	73.0 (0.04)	9.6	70.1 (0.04)	6.5	74.3 (0.04)	7.4
DhA	72.1 (0.04)	3.7	71.2 (0.04)	13	72.8 (0.04)	3.8
BgP	83.2 (0.04)	5.7	89.9 (0.04)	8.0	70.9 (0.04)	12
IcP	79.1 (0.04)	9.8	76.9 (0.04)	6.1	84.5 (0.04)	7.7
DeP	95.1 (0.04)	8.6	91.2 (0.04)	8.7	91.7 (0.04)	13
DiP	91.5 (0.04)	8.6	91.5 (0.04)	13	99.3 (0.04)	7.9
DhP	101 (0.04)	7.9	95.9 (0.04)	7.1	89.9 (0.04)	5.6

[a] Five consecutive extractions.

method under extreme conditions. The extraction performance is always more difficult when dealing with low contaminated samples. Table 6 shows the results obtained when carrying out the optimized method with five samples for each "gofio". It can be observed good extraction performance for all PAHs in all the studied samples, between 70.1 and 107%. The average relative recoveries were 90.6, 89.1, and 88.4% for barley, rye, and maize corn, respectively, showing the robustness of the method. The obtained precision was always lower than 13% as relative standard deviation.

4. Conclusions

A focused microwave-assisted extraction method using the IL-aggregate HDBIm-Br as the extraction agent has been successfully carried out for the determination of the 15 + 1 EU PAHs contained in "gofio" samples. The optimization was carried out by means of an experimental design approach. The method was further accomplished by HPLC with FLD single-channel, which is first applied for the analysis of the 15 + 1 EU PAHs. Under the optimized conditions, the HPLC–FLD method is characteristic for presenting LODs ranging from 0.02 ng mL^{-1} for BkF to 4.01 ng mL^{-1} for BbF, being of 20.5 ng mL^{-1} for CPP (the non fluorescent PAH).

The overall microwave extraction-HPLC determination is characteristic for presenting short extraction times: 14 min for the extraction step followed by 40 min for the chromatographic time, and for using small amounts of sample: 0.1 g. Under optimized conditions, extraction recoveries ranging from 70.1 to 109% and precision values lower than 12.6% were obtained. Furthermore, the method requires a small amount of IL to generate an adequate extractant solution: 77 mg in each extraction. These low amounts must be highlighted, especially when considering the high volumes of organic solvents required in conventional extraction methods. The proposed method does not require clean-up steps because the IL-extract can be directly injected in a HPLC. The developed method was not affected by the nature of the "gofio" matrix, and presented average relative recoveries of 90.6, 89.1, and 88.4% for barley, rye, and maize corn "gofios", respectively.

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