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Ionic liquid-dispersive liquid–liquid microextraction for the simultaneous determination of pesticides and metabolites in soils using high-performance liquid chromatography and fluorescence detection

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ABSTRACT

In this work, an ionic liquid-dispersive liquid–liquid microextraction (IL-DLLME) procedure was developed for the extraction of a group of pesticides (carbendazim/benomyl, thiabendazole, fuberidazole, carbaryl and triazophos) and some of their key metabolites in soils (2-aminobenzimidazole, metabolite of carbendazim and 1-naphthol, metabolite of carbaryl) from aqueous soil extracts, using high performance liquid chromatography (HPLC) with fluorescence detection (FD). Analytes were previously extracted from four soils with different physicochemical properties (forestal, ornamental, garden and lapilli soils) by ultrasound-assisted extraction (USE). The IL 1-hexyl-3-methylimidazolium hexafluorophosphate ([HMIm][PF_6]) and methanol (MeOH) were used as extraction and dispersion solvent, respectively, for the DLLME procedure. Factors affecting IL-DLLME (sample pH, IL amount, volume of dispersion solvent and sodium chloride percentage) were optimized by means of an experimental design, obtaining the most favorable results when using 117.5 mg of IL and 418 μL of MeOH to extract the compounds from the aqueous soil extracts at pH 5.20 containing 30% (w/v) NaCl. Calibration of the USE–IL-DLLME–HPLC–FD method was carried out for every type of soil and accuracy and precision studies were developed at two levels of concentration, finding that no significant differences existed between real and spiked concentrations (Student's *t* test). LODs achieved were in the low ng/g range.

1. Introduction

Pests can affect, without distinction, crops, yards, home plants, forest trees, etc. Despite the fact that several alternatives can also be applied, the use of pesticides is still probably the most preferred due to their quick effectiveness. As a result, soils under such pesticide applications may receive an important and constant quantity of these compounds, which under the effects of climatologic phenomenons undergo transformations into more or less stable metabolites. Furthermore, pesticides and their degradation products can associate to organic matter of soils and might remain there for an extended time [1]. Additionally, pesticides can be bioavailable and it is widely demonstrated that they may transfer to other environmental compartments [2]. Thus, the determination of pesticides (as well as their metabolites) in soils is of basic importance.

Traditionally, analytical methodologies for their determination in soils require a previous solvent extraction (Soxhlet extraction, ultrasound-assisted extraction (USE), pressurized-liquid extraction (PLE), microwave-assisted extraction (MAE), etc.), normally followed by a clean-up solid-phase extraction (SPE) procedure [3,4]. In most of these works, gas chromatography (GC) and high performance liquid chromatography (HPLC) have been the techniques of choice, with electron capture detection (ECD), nitrogen phosphorus detection and mass spectrometry (MS) when using GC, or ultraviolet (UV), diode array detection (DAD) and also MS, when using HPLC.

Fluorescence detection in HPLC pesticide analysis is one of the most selective and sensitive detection systems but it has been classically limited by the fact that very few pesticides are fluorescent. This problem has been partially solved by carrying out a suitable derivatization; however, if possible, it is better to avoid this additional and, in some cases, tedious step. In this sense, only a small number of studies have been carried out and few fluorescent pesticides have been simultaneously determined. One of the chemical families of pesticides that show native fluorescence is that of benzimidazoles, some of which have been determined by means of a

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HPLC–FD method [5–11] in few occasions in soils [7,11]. Also other pesticides of different families have been recently analyzed by HPLC–FD, such as the carbamate carbaryl (CB) and the organophosphorus pesticide triazophos (TZ) [12,13] but only in one occasion in soils [13].

One of the most important tendencies in Analytical Chemistry is the miniaturization and simplification of the analytical process. In this sense, the so-called liquid-phase microextraction (LPME) techniques [14], which meet such requirements, have attracted much of recent attention. In particular, dispersive liquid–liquid microextraction (DLLME) is a relatively young LPME technique that is becoming more and more functional for the extraction of organic [15,16] and inorganic [16] analytes from diverse matrices. Like other LPME techniques, it is based on the extraction of the analytes by a water non-miscible solvent from an aqueous solution, but in the case of DLLME, a dispersive solvent is also added to the aqueous sample together with the extraction solvent, forming a cloudy solution which favors the extraction. It is a very simple and quick extraction procedure in which water samples have been the matrices most commonly selected, demonstrating a very good extraction capability.

Up to now, DLLME has been barely applied for organic analyte extraction from soils [11,13,17–19]. Among these works and, to the best of our knowledge, pesticide extraction has only been carried out in four occasions [11,13,17,18], using in all cases a step by step approach to optimize DLLME parameters. In the work of Xiong and Hu [17], for example, a group of six pesticides (malathion, chlorpyrifos, buprofezin, TZ, carbosulfan and pyridaben) were extracted from only one type of soil (also from waters and beverages), while in the work of Fu et al. [13] only two pesticides (CB and TZ) were determined in three of them. In both works, carbon tetrachloride and methanol (MeOH) were used as extraction and dispersion solvent, respectively, after a previous extraction of the soils. In the first case, water was used (which was later submitted to the DLLME procedure) while in the second, MeOH was selected (which was afterwards used as dispersion solvent). Another example is the work of Wu et al. [18], who used a mixture of acetone and $NaHCO_3$ solution to extract four sulfonylurea herbicides from one type of soil and then, after a suitable clean-up of the extract with C_{18}-disperive-SPE, chlorobenzene was added as extraction solvent (acetone acted as dispersive solvent). Finally, in the last of these works, Wu et al. [11], extracted carbendazim (MBC) and thiabendazole (TBZ) from two soil samples with a HCl solution which, after pH adjustment, was extracted using chloroform as extractant and tetrahydrofuran as dispersant.

Room temperature ionic liquids (ILs) are becoming more and more significant in Chemistry due to their advantages over conventional solvents (less toxic, less contaminating and less volatile) [20]. They have also been applied in DLLME as extraction solvents with good results, but the number of works in this field is still very low, especially the ones concerning the extraction of pesticides from other matrices different than waters (the most common), which are few [21–24] (two of them developed by our research group for the extraction of different pesticides from fruit samples [21,22]). Up to now, IL-DLLME has not been applied for the extraction of pesticides or metabolites from soils, not even for analytes different than pesticides, and therefore, there is a great interest on knowing if IL can be used for this purpose, taking into account the complexity of the sample.

In this work, IL-DLLME has been combined with HPLC–FD and applied for the extraction of six native-fluorescent pesticides and two of their key metabolites in soils {i.e. 2-aminobenzimidazole (2-AB), MBC/benomyl (BN), TBZ, fuberidazole (FBZ), CB, 1-naphthol (1-N) and TZ} from aqueous soil extracts using the IL 1-hexyl-3-methylimidazolium hexafluorophosphate ([HMIm][PF_6]) and MeOH as extraction and dispersion solvent, respectively. The group of compounds selected is formed by four of the most employed benzimidazolic pesticides (MBC/BN, TBZ and FBZ), which are anthelmintic products that have been used since the 60 s in the agricultural field as pre- or post-harvest fungicides, a carbamate (CB) that is an insecticide and a plant regulator, an organophosphorus pesticide (TZ) which can act as an insecticide, an acaricide or a nematicide, and two degradation products (2-AB, metabolite of MBC and 1-N, metabolite of CB). Factors affecting the DLLME procedure were optimized via an experimental design and the methodology was then validated through calibration, precision and accuracy studies in four different types of soils (garden, ornamental, forestal and lapilli). As far as we know and, as it has been previously commented, this is the first time that an IL-DLLME procedure is applied for the extraction of organic analytes from soil extracts. It is also the first time that this group of pesticides and their metabolites are simultaneously analyzed by HPLC–FD.

2. Materials and methods

2.1. Chemicals

Pesticide analytical standards of 2-AB, MBC, BN, FBZ, 1-N and TZ from Fluka (Sigma–Aldrich Chemie, Madrid, Spain) and CB and TBZ from Riedel-de Haën (Sigma–Aldrich Chemie) were used without further purification (purity >99.2% for all the pesticides except for TZ, 66.5%, which was the highest purity available). Table 1 shows some of the characteristics of these compounds. Stock solutions of each pesticide of approximately 500 mg/L were prepared in acetonitrile (ACN), except MBC/BN, that were prepared in MeOH due to their low solubility in ACN, at a concentration of 200 mg/L. They were all stored in the darkness at 4 °C and working analyte mixtures were prepared daily by dilution of these solutions with ACN.

All chemicals were of analytical reagent grade and used as received. Distilled water was deionized by a Milli-Q system from Millipore (Bedford, MA, USA). ACN of HPLC grade, di-sodium hydrogen phosphate dehydrate (purity >99.5%) and hydrochloric acid (25%, w/v) were from Merck (Darmstadt, Germany). Sodium chloride (purity >99.5%), magnesium sulfate monohydrated, sodium citrate tribasic dehydrate, sodium hydrogencitrate sesquihydrate and MeOH were from Sigma–Aldrich Chemie. Sodium hydroxide, sulfuric acid (96%, w/v) and *ortho*-phosphoric acid (85%, w/v) from Panreac Química (Barcelona, Spain). The IL [HMIm][PF_6] (purity >97%) was provided by Fluka.

2.2. Apparatus and software

HPLC analyses were performed with a Waters HPLC system (Milford, MA, USA) equipped with a binary pump (model 1525), an autosampler (model 717 plus) and a fluorescence detector (model 2475 Multi λ) with the Empower 2 software from Waters. Separations were carried out in a Nova-Pak C_{18} column (150 mm × 3.9 mm, 4 μm) using a Guard-Pak C_{18} pre-column (4 μm), both from Waters. Considering ACN as mobile phase A and 10 mM phosphate buffer at pH 8.70 as mobile phase B, the initial mobile phase was 22/78 (v/v) A/B. The elution was isocratic for the first 3 min, changed to 36/64 (v/v) A/B in 5 min (curve 10) and maintained for 2 min. Then, the composition was changed to 60/40 (v/v) A/B in 3 min (curve 6) and maintained for 1 min. Finally, it was changed to 100% of A, maintained for 6 min and returned to the initial composition in 5 min (curve 6). The flow rate was set at 1.0 mL/min and the injection volume was 20.0 μL. For detection, an adequate wavelength program was followed on the basis of the excitation and emission spectra obtained for each of the target analytes (see Table 1). Detector worked in multichannel mode to produce multiple chromatogram traces. Table 1 also

Table 1
Characteristics, chromatographic parameters and excitation and emission wavelengths of the compounds studied in this work.

Peak	Abbreviation	Characteristics					Chromatographic parameters and wavelengths			
		pK_a[a] (25 °C)	M_W[a]	Soil half live, DT_{50} (days, lab at 20 °C)[a]	K_{OW}[a] (log P, pH 7 at 20 °C)[b]	K_{OC}[a] (mL/g)[c]	t_R[d]	k[e]	λ_{ex} (nm)	λ_{em} (nm)
1	2-AB	7.39 (weak base)	133.15	–	0.91	22	2.29	0.68	275	310
2	MBC	4.20 (weak base)	191.21	260	1.48	223	3.45	1.53	280	300
	BN	4.48	290.32	0.8	1.4	1900				
3	TBZ	4.73 and 12.0	201.25	365	2.39	2500	4.65	2.41	305	335
4	FBZ	4.00 (weak base)	184.19	24.6	2.71	605	5.34	2.92	305	335
5	CB	10.4	201.22	16	2.36	211	12.80	8.39	280	320
6	1-N	9.34	144.17	0.53	2.85	245	13.30	8.76	285	460
7	TZ	–	313.30	44	3.55	358	17.20	11.62	250	305

(–) Data not available.
[a] Taken from Ref. [25].
[b] Octanol–water partition coefficient.
[c] Soil organic carbon sorption coefficient.
[d] Retention time.
[e] Retention factor.

shows the retention times and retention factors for each of the analytes.

pH values were measured with a Crison GLP 22 pH-meter (Barcelona, Spain), while for conductivity measurements a Crison CM 35 portable conductimeter with temperature measurement capability was used.

StatGraphics Plus Software Version 5.1 from Statistical Graphics (Rockville, MD, USA) was used for data processing and experimental design analysis.

2.3. Soil sample selection

In this work, four types of soils (forestal, garden, ornamental and lapilli) were collected in Tenerife (Canary Islands, Spain). Soil 1 (ornamental soil) was bought in a garden center (25 kg) while the other three samples were collected between 0 and 50 cm deep on the ground in different areas of the island in appropriate plastic bags (1 kg). Soil 2 (forestal soil) was collected in the forest of La Esperanza, La Laguna, soil 3 (garden soil) in a public backyard also in La Laguna, while soil 4 (lapilli) was collected in Santiago del Teide from a relatively recent volcanic eruption area. Table 2 shows some of the physicochemical properties of the samples, all of them determined in our laboratory. Organic matter was determined by means of the Walkley–Black method, according to the standard methods described by Page et al. [26]. The pH in distilled water, pH in KCl and conductivity were established in the same way as earlier reported [27,28]. pH values in water compiled in Table 2 show that forestal and ornamental soils are slightly and moderately acidic, respectively, the first probably having full nutrient availability, while the second had an adequate pH value for the development of any type of crop. However, the selected garden soil is slighly alkaline, which indicates the presence of $MgCO_3$ and the possibility of iron deficiency for plants. Finally, lapilli as expected, is a moderately basic soil, which is an evidence of the existence of $CaCO_3$ in it. Before use, soils were homogenized, sieved (2-mm mesh) and air-dried at room temperature. Three grams of soil were weighted in a 50 mL centrifuge tube and spiked at the desired concentration. Extractions of blank samples were also done in parallel to assure that no residues of the analytes were present in the spiked samples.

2.4. Soil sample extraction

A portion of 20 mL of MeOH containing 2.5% (w/v) of NaCl was added to the 50 mL centrifuge tube containing 3.0 g of spiked dried soil and the sample was strongly hand-shaken for 1 min. Then, extraction assisted by ultrasounds was carried out for 10 min in a Branson 3510 ultrasonic bath working at 42 kHz and 100 W from Branson Ultrasonic Corporation (Danbury, CT, USA). Centrifugation at 4400 rpm (3000 × g) for 5 min was carried out in a 5702 centrifuge from Eppendorf (Hamburg, Germany) and filtration through a 0.45 μm Chromafil® Xtra PET-45/25 filter from Macherey-Nagel (Düren, Germany) into a flask was performed. Extraction was repeated one more time in the same way and extracts were collected in the same flask, which were later evaporated to dryness at 40 °C and 235 mbar (23.5 kPa) using a Rotavapor R-200 equiped with a V-800 vacuum controller, both from Büchi Labortechnik (Flawil, Switzerland). The dry residue was then redissolved in 10 mL Milli-Q water and filtered through a 0.20 μm Chromafil® Xtra PET-20/25 filter.

2.5. IL-DLLME

Adjustment of the pH of the previously obtained solution (10 mL of aqueous extract) was performed to 5.20 (with 0.1 M HCl or NaOH). NaCl was added until a concentration of 30% (w/v) was reached and the solution was placed in a 15 mL centrifuge tube.

Table 2
Characteristics of the four soils studied.

	Soil 1	Soil 2	Soil 3	Soil 4
Origin or use	Forestal	Garden	Ornamental	Lapilli
pH (in water)	6.34	8.51	5.60	7.58
pH (in KCl 0.1 N)	5.26	7.64	5.03	6.19
DpH	1.08	0.87	0.57	1.39
Moisture[a] (%)	12.2	6.25	12.2	0
Organic carbon content (%)	2.78	2.66	2.27	0.16
Organic matter (%)	4.79	4.57	3.91	0.27
Conductivity (dS m^{-1}) 25 °C	0.63	2.38	3.67	0.181

[a] Air dried.

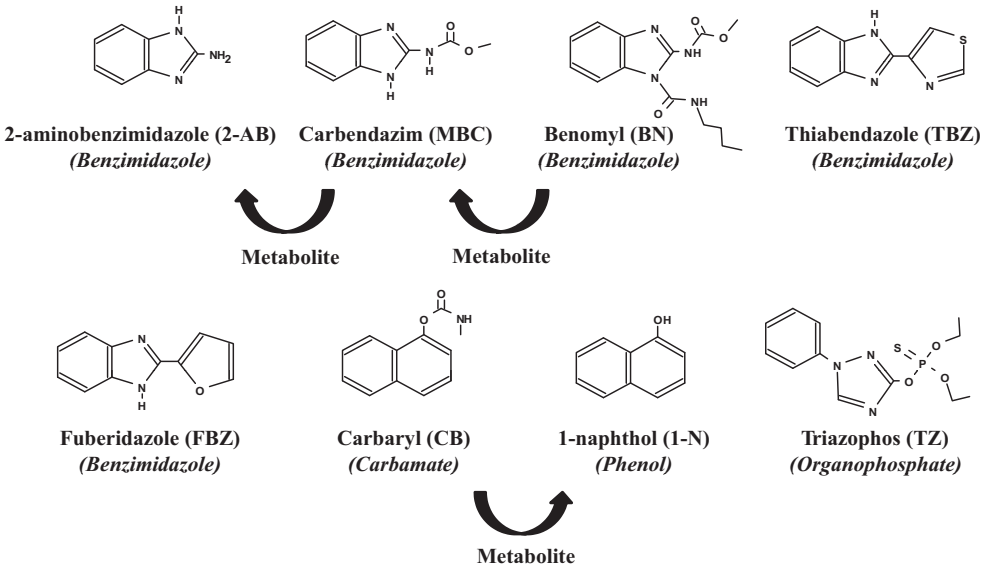

Fig. 1. Chemical structure and family of the selected pesticides and metabolites.

A mixture of 117.5 mg of the IL [HMIm][PF_6] and 418 μL of MeOH as extraction and dispersion solvents, respectively, was rapidly injected into the aqueous phase and the tube was vortex-shaken for 1 min. After 8 min of extraction time, the mixture was centrifuged at 4400 rpm (3000 × *g*) for 10 min and a settled phase consisting in a droplet of IL containing the target analytes was found at the bottom of the tube. The upper aqueous phase was removed by means of a syringe and the IL phase (80 μL) was collected and dissolved in 1120 μL of 59/41 (v/v) ACN/10 mM phosphate buffer at pH 8.70 (total final volume of 1200 μL) due to the insolubility of the IL in the initial mobile phase. Finally, 20 μL of this mixture was injected in the HPLC–FD system for analysis.

3. Results and discussion

3.1. HPLC–FD method

Among the different pesticides selected in this work, BN is very unstable in alkaline media and it is rapidly converted into MBC [29]. With this basis, it is preferred to determine them together as MBC/BN. Fig. 1 shows the chemical structure and family of these analytes. All of them show native fluorescence and therefore, FD without derivatization can be applied for a more sensitive and selective detection compared with typical UV detection. As can be seen in Table 1, some of them are slightly mobile in soils (those with high K_{OC} values) and moderately persistent (high DT_{50} values), properties that suggest the importance of their analysis in this type of matrices. It is also important to mention that BN, CB and TZ have been excluded in recent years from Annex I of Directive 91/414/EEC (available at http://ec.europa.eu/sanco_pesticides/public), so their use for agricultural purposes in EU is forbidden.

As can be seen in Table 1, pK_a values of the selected pesticides range between 4.00 and 12.0, so separation conditions must be carefully optimized. Complete separation with good efficiency and low analysis time was achieved using 100% ACN as mobile phase A and 100% of 10 mM phosphate at pH 8.70 as mobile phase B with the elution program described in Section 2.5. Isocratic and gradient elution were studied at different mobile phase composition and pH values. Since 2-AB has a pK_a value of 7.39 as a weak base, a higher pH was necessary to avoid adsorption by the stationary phase (neutral form). Also, isocratic elution was not found adequate at all due to the long retention times and poor efficiencies obtained especially for the last peaks.

On the other hand, a screening study was developed to determine the maximum fluorescence excitation and emission wavelengths for each analyte. The final selected program is shown in Table 1. Because changes in the wavelengths occasionally produced a slight jump in the baseline and due to the proximity of the peaks corresponding to CB and 1-N, detector was configured in multichannel mode to obtain a second chromatogram trace corresponding to the maximum excitation/emission wavelength of 1-N. Fig. 2 shows the separation of the seven analytes under these optimum conditions.

Afterwards, a repeatability study consisting in five consecutive injections ($n = 5$) in the same day of a standard mixture of the analytes at two levels of concentration (approximately 10 and 100 μg/L) in three different days ($n = 15$) was carried out. Good repeatability in the same day was obtained, with RSDs ranging 0.1–0.3% for retention time and 0.7–3.7% for peak area. Between days RSDs in the range 0.1–0.8% and 1.9–4.8%, were respectively obtained. Calibration curves based on peak areas were also obtained for each analyte injecting seven increasing levels of concentration in triplicate ($n = 7$), achieving determination coefficients (R^2) higher than 0.999 for all analytes (range of concentrations tested were 5.0–500 μg/L for most pesticides except for MBC/BN which

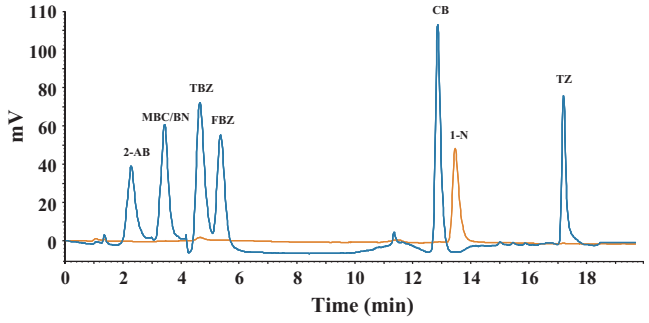

Fig. 2. HPLC–FD chromatograms of the target analytes at their maximum excitation/emission wavelengths. Flow rate: 1.0 mL/min (see gradient and wavelength programs in Section 2.2). Detector was configured in multichannel mode to obtain a second chromatogram trace corresponding to the maximum excitation/emission wavelengths of 1-N. Injection volume: 20 μL. Sample dissolved in 59/41 (v/v) ACN/10 mM phosphate buffer (pH 8.70). Analyte concentration: 2-AB (125 μg/L), MBC/BN (620 μg/L), TBZ (125 μg/L), FBZ (2.21 μg/L), CB (130 μg/L), 1-N (130 μg/L) and CB (125 μg/L).

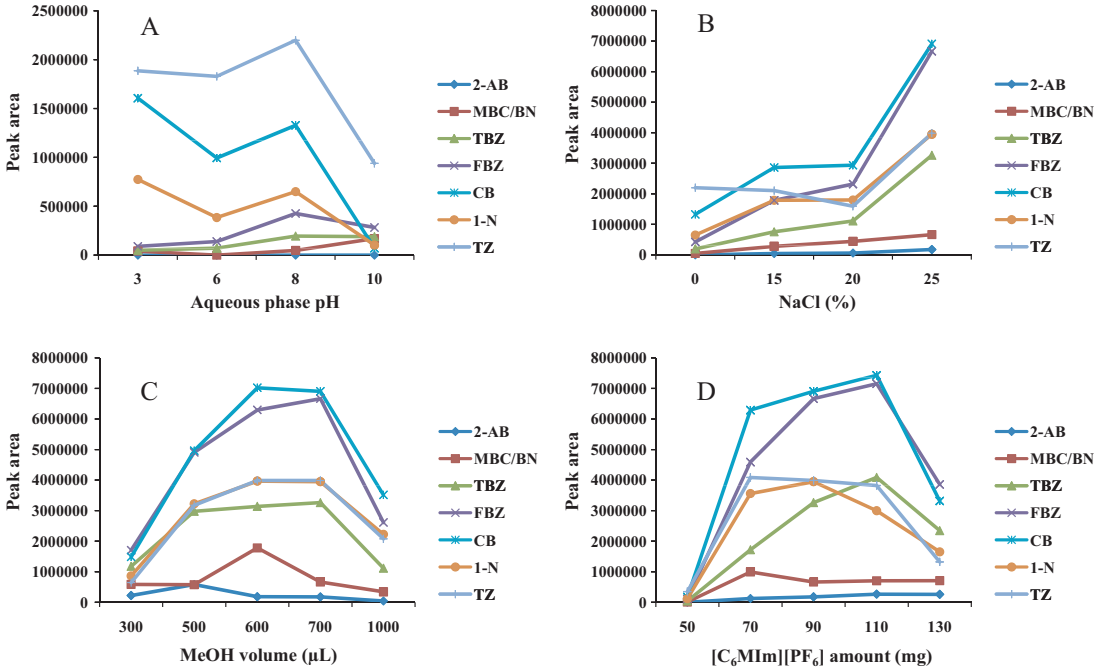

Fig. 3. Effect on peak areas of the variation in the DLLME procedure of: (A) aqueous phase pH, (B) NaCl percentage, (C) MeOH volume and (D) [HMIm][PF_6] amount. Common extraction conditions: 10 mL of spiked Milli-Q water (approximately 100 μg/L), centrifugation at 4400 rpm for 20 min. Other conditions: (A) 0% (w/v) NaCl, 700 μL MeOH and 90 mg [HMIm][PF_6]. (B) pH 8.0, 25% (w/v) NaCl and 90 mg [HMIm][PF_6]. (C) pH 8.0, 25% (w/v) NaCl and 90 mg [HMIm][PF_6]. (D) pH 8.0, 25% (w/v) NaCl and 600 μL MeOH.

was 25–5000 μg/L and FBZ 0.09–9.0 μg/L). Instrumental LODs were between 0.02 μg/L for FBZ and 6.53 μg/L for MBC/BN, while LOQs ranged from 0.08 μg/L and 21.8 μg/L for FBZ and MBC/BN, respectively.

3.2. DLLME optimization

From a deep revision of the literature, and concerning IL-DLLME applications, it is clear that the IL [HMIm][PF_6] is the one that has found a greater application in the pesticide analysis field [15,21,22,30–32] because it meets the requirements of low solubility in water and a higher density and, therefore, it has also been considered in the present study. On the other hand, the dispersion solvent must solubilize the extraction solvent and should also be miscible in water to allow the formation of the droplets of the extraction solvent in the aqueous sample. Based on previously works developed by our research group [21,22], MeOH was selected as dispersion solvent, instead of other common solvents like ACN that avoided the formation of the settled phase or acetone, which provided lower extraction efficiency.

3.2.1. Preliminary experiments

Once extraction and dispersion solvents had been selected, preliminary experiments were accomplished in duplicate to test the effect of the individual variation of sample pH, amount of NaCl added, volume of MeOH as dispersion solvent and amount of [HMIm][PF_6] in the DLLME procedure and to select the levels of the factors in the experimental design (see Fig. 3). Initially, 10 mL of Milli-Q water of different pH values (3, 6, 8 and 10) were extracted with a mixture of 90 mg of [HMIm][PF_6] and 700 μL of MeOH without the addition of NaCl. Judging from the figure, it is clear that the effect of pH is irregular. Because pH 8 provided a good increase of the recoveries for most of the pesticides, it was considered for subsequent preliminary experiments, although this effect would be studied in depth by the experimental design. Higher pH values clearly provided a drastic diminution of the extraction. Then, 10 mL of Milli-Q water at pH 8 containing different amounts of NaCl {0, 15, 20 and 25% (w/v)} to induce salting-out effect were extracted in the same way, observing a clear enhance of the extraction with increasing concentrations of salt for all pesticides. Therefore, a solution of 10 mL of Milli-Q water containing 25% (w/v) of NaCl at pH 8.0 was subsequently extracted with 90 mg [HMIm][PF_6] and different volumes of dispersion solvent (300, 500, 600, 700 and 1000 μL). For the majority of the analytes, the use of volumes between 500 and 700 μL of MeOH resulted in higher areas. The effect of different amounts of [HMIm][PF_6] (50, 70, 90, 110 and 130 mg) was initially studied considering 600 μL of MeOH to extract the analytes from the aqueous saline solution also at pH 8. As it can be seen, around 110 mg of the IL provided the best results.

Agitation during extraction, as well as centrifugation and extraction time was also studied in order to definitely fix these parameters for the experimental design. Duplicate extractions with previous considered conditions (pH 8, 25% (w/v) NaCl, 110 mg [HMIm][PF_6] and 600 μL MeOH) were made to examine these parameters in depth. Agitation to assist extraction was considered in three different ways as suggested in previous DLLME works [33,34]: 1 min with vortex, 1 min with ultrasounds and a double re-injection with a pipette of the triphasic system once the mixture of extraction and disperser solvent had been injected. The last two provided lower peak areas when compared with the absence of agitation, while 1 min of vortex led to a slight improvement in peak areas. Increasing the time of vortex agitation did not improve these results either. On the other hand, extraction time was also considered. Thus, different experiments were carried out waiting 5, 7.5, 10, 15 and 20 min before centrifugation. For all analytes, peak area improved to some extent when extraction time was increased from 0 to 5 min, but for MBC/BN this effect was much more remarkable, even up to 8 min of extraction time, so this value was finally fixed (higher extraction times provided similar results). Centrifugation time was studied at 4400 rpm ($3000 \times g$) between 5 and 20 min, achieving maximum extraction when centrifuging for 10 min, while higher times resulted in similar results.

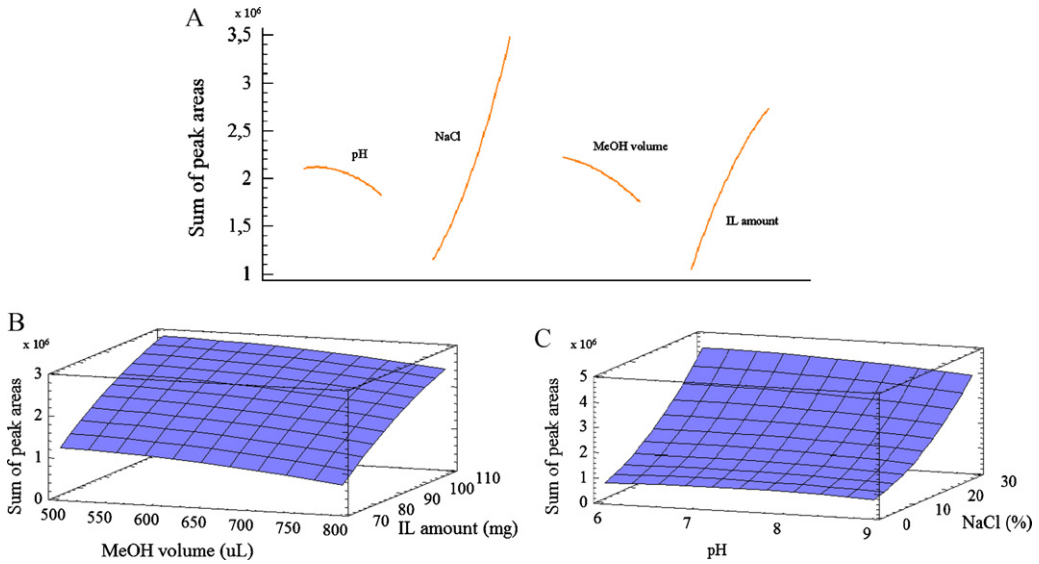

Fig. 4. (A) Graphic of the principal effects of the factors for the sum of mean peak areas and response surfaces estimated for the central composite design of the IL-DLLME optimization plotting (B) MeOH volume *vs.* IL amount (pH 7.5, 15.5% (w/v) NaCl) and (C) pH *vs.* NaCl percentage (90 mg IL, 650 μL of MeOH).

3.2.2. Experimental design

After these previous experiments, a central composite design was selected to optimize the experimental factors (sample pH, NaCl percentage, volume of MeOH and amount of IL) since interactions between them may also occur. Three replicates of the central point and an axial distance of 1.55 (orthogonal assay) were considered. pH was varied between 5.1 and 9.8, NaCl percentage between 0 and 30% (w/v), MeOH volume between 418 and 882 μL and IL amount between 59 and 121 mg, considering the sum of the mean peak areas as response. The levels of these factors were selected taking into account the results previously obtained and shown in Fig. 3. The resulting 27 experiments, in which 10 mL of water were spiked with the analytes and submitted to the DLLME procedure were randomly performed. As it has been commented before, the eight target analytes have different physicochemical properties (pK_a, K_{OW}, etc.), so one of the main problem comes out when trying to select optimum extraction conditions. Thus, results were analyzed for all analytes and for each individual compound. In general, when the response was studied for each separate compound, high NaCl percentages and amounts of IL as well as low volumes of MeOH led to larger peak areas. However and, as previously commented, the effect of pH was very uneven: while low pH values tended to improve peak areas for TBZ, FBZ, CB and TZ, intermediate values were better for 1-N and higher pH improved MBC/BN and 2-AB extraction. However, a compromise value must be fixed when simultaneously extracting a group of analytes, which is also the aim of the experimental design. Fig. 4A shows a graph of the individual effects of the factors for the sum of peak areas of all analytes. As can be seen, results agree with the previous tendency commented for individual pesticides and metabolites. Response surfaces estimated for the central composite design are also shown in Fig. 4 plotting MeOH volume *vs.* IL amount (Fig. 4B) and pH *vs.* NaCl percentage (Fig. 4C). From the figure, it can be clearly deduced that low volumes of MeOH as well as high amounts of IL and NaCl provided the highest response. In fact, the final optimum DLLME conditions predicted were: 30% (w/v) NaCl, 117.5 mg of [HMIm][PF_6], 418 μL of MeOH and pH 5.20. Several experiments were then developed under these optimum conditions, obtaining the highest sum of peak areas of all previous experiments. Furthermore, additional extractions were carried out by slightly varying each factor at its optimum value, but worse results were obtained in all cases.

3.3. IL-DLLME–HPLC–FD of Milli-Q water

With the aim of checking the repeatability of the previous developed IL-DLLME methodology, calibration as well as precision and

Table 3
Results of the precision and accuracy study of the IL-DLLME–HPLC–FD method for the selected compounds in Milli-Q water.

Peak	Analyte	Spiked level[a] (μg/L)	Found[b] (μg/L)	Accuracy	t^c
1	2-AB	30.00	30.36 ± 4.81	101	0.16
		375.00	424.00 ± 4.83	113	2.34
2	MBC/BN	30.00	35.61 ± 11.23	119	2.36
		374.00	345.75 ± 10.24	92	0.77
3	TBZ	2.00	1.93 ± 0.34	97	0.46
		25.00	22.60 ± 0.30	90	2.30
4	FBZ	0.03	0.03 ± 0.02	97	0.28
		0.38	0.36 ± 0.02	96	2.34
5	CB	2.00	1.89 ± 0.57	95	0.81
		25.00	25.82 ± 0.55	103	2.10
6	1-N	2.00	2.07 ± 1.24	104	0.52
		25.00	25.23 ± 1.09	101	0.28
7	TZ	2.00	2.15 ± 1.17	108	2.21
		25.00	25.97 ± 1.03	104	1.11

[a] $n = 5$.
[b] Average value ± standard deviation of five determinations (95% confidence value).
[c] $t_{tab} = 2.78$, $\alpha = 0.05$.

Table 4
Calibration data of the IL-DLLME–HPLC–FD procedure for the selected compounds in the four types of soils.

Peak	Analyte	Soil	Calibration data ($n = 6$)				LOD_{method} (ng/g)	LOQ_{method} (ng/g)
			Range of concentration tested (ng/g)	Slope	Intercept	R^2		
1	2-AB	Soil 1	50–3000	$0.67 \times 10^4 \pm 0.01 \times 10^4$	$19.61 \times 10^4 \pm 16.85 \times 10^4$	0.999	14.17	47.25
		Soil 2	50–3000	$0.56 \times 10^4 \pm 0.01 \times 10^4$	$33.42 \times 10^4 \pm 19.82 \times 10^4$	0.999	15.50	51.67
		Soil 3	50–3000	$0.88 \times 10^4 \pm 0.01 \times 10^4$	$26.83 \times 10^4 \pm 25.11 \times 10^4$	0.999	13.76	45.86
		Soil 4	50–3000	$1.10 \times 10^4 \pm 0.03 \times 10^4$	$20.29 \times 10^4 \pm 44.98 \times 10^4$	0.999	13.44	44.80
2	MBC/BN	Soil 1	50–3000	$0.93 \times 10^4 \pm 0.01 \times 10^4$	$9.29 \times 10^4 \pm 15.72 \times 10^4$	0.999	14.23	47.42
		Soil 2	50–3000	$0.91 \times 10^4 \pm 0.02 \times 10^4$	$-0.09 \times 10^4 \pm 35.51 \times 10^4$	0.999	13.92	46.41
		Soil 3	50–3000	$0.61 \times 10^4 \pm 0.03 \times 10^4$	$6.92 \times 10^4 \pm 50.73 \times 10^4$	0.998	14.85	49.52
		Soil 4	50–3000	$0.78 \times 10^4 \pm 0.03 \times 10^4$	$-13.53 \times 10^4 \pm 43.73 \times 10^4$	0.999	15.76	52.54
3	TBZ	Soil 1	4.5–270	$8.07 \times 10^4 \pm 0.53 \times 10^4$	$8.24 \times 10^4 \pm 62.14 \times 10^4$	0.997	1.21	4.03
		Soil 2	7–420	$7.97 \times 10^4 \pm 0.38 \times 10^4$	$-37.68 \times 10^4 \pm 82.83 \times 10^4$	0.998	2.05	6.85
		Soil 3	6–360	$6.34 \times 10^4 \pm 0.35 \times 10^4$	$51.68 \times 10^4 \pm 64.90 \times 10^4$	0.998	1.69	5.62
		Soil 4	10–600	$7.81 \times 10^4 \pm 0.31 \times 10^4$	$-53.89 \times 10^4 \pm 96.28 \times 10^4$	0.999	2.77	9.22
4	FBZ	Soil 1	0.07–4.2	$80.08 \times 10^5 \pm 45.49 \times 10^4$	$-6.61 \times 10^4 \pm 83.11 \times 10^4$	0.998	0.02	0.06
		Soil 2	0.07–4.2	$74.23 \times 10^5 \pm 13.38 \times 10^4$	$-14.25 \times 10^4 \pm 28.83 \times 10^4$	0.999	0.02	0.06
		Soil 3	0.07–4.2	$27.94 \times 10^5 \pm 21.23 \times 10^4$	$12.66 \times 10^4 \pm 48.78 \times 10^4$	0.996	0.02	0.06
		Soil 4	0.12–7.2	$63.30 \times 10^5 \pm 23.19 \times 10^4$	$-68.13 \times 10^4 \pm 74.81 \times 10^4$	0.999	0.03	0.11
5	CB	Soil 1	2.5–150	$23.27 \times 10^4 \pm 0.52 \times 10^4$	$-47.11 \times 10^4 \pm 33.60 \times 10^4$	0.999	0.64	2.14
		Soil 2	2.5–150	$18.47 \times 10^4 \pm 0.38 \times 10^4$	$-18.46 \times \times 10^4 \pm 29.10 \times 10^4$	0.999	0.63	2.09
		Soil 3	2.5–150	$17.87 \times 10^4 \pm 0.43 \times 10^4$	$-15.98 \times 10^4 \pm 32.81 \times 10^4$	0.999	0.63	2.11
		Soil 4	2.5–150	$15.51 \times 10^4 \pm 0.57 \times 10^4$	$11.69 \times 10^4 \pm 45.61 \times 10^4$	0.999	0.74	2.47
6	1-N	Soil 1	4–400	$6.66 \times 10^4 \pm 0.28 \times 10^4$	$-31.98 \times 10^4 \pm 53.77 \times 10^4$	0.999	1.12	3.73
		Soil 2	14–840	$0.98 \times 10^4 \pm 0.06 \times 10^4$	$38.54 \times 10^4 \pm 25.44 \times 10^4$	0.998	4.00	13.32
		Soil 3	10–600	$3.59 \times 10^4 \pm 0.34 \times 10^4$	$-61.80 \times 10^4 \pm 103.38 \times 10^4$	0.994	2.64	8.79
		Soil 4	8–700	$4.89 \times 10^4 \pm 0.27 \times 10^4$	$-89.69 \times 10^4 \pm 99.95 \times 10^4$	0.998	2.25	7.51
7	TZ	Soil 1	35–2000	$0.74 \times 10^4 \pm 0.03 \times 10^4$	$-14.02 \times 10^4 \pm 26.42 \times 10^4$	0.999	10.40	34.66
		Soil 2	35–2000	$0.58 \times 10^4 \pm 0.01 \times 10^4$	$26.33 \times 10^4 \pm 9.56 \times 10^4$	0.999	9.20	30.68
		Soil 3	30–1800	$0.54 \times 10^4 \pm 0.04 \times 10^4$	$8.78 \times 10^4 \pm 39.83 \times 10^4$	0.996	8.79	29.29
		Soil 4	92–5500	$0.40 \times 10^4 \pm 0.02 \times 10^4$	$-35.71 \times 10^4 \pm 58.76 \times 10^4$	0.998	27.07	90.25

accuracy studies in Milli-Q water were developed. For this purpose, 10 mL of Milli-Q water spiked at six increasing levels of concentration ($n = 6$) was subjected to the previously optimized IL-DLLME as described in Section 2.4 to obtain the calibration curves of the whole method. R^2 higher than 0.998 were obtained for all compounds. Low LODs and LOQs of the method (calculated as 3 and 10 times the signal-to-noise ratio (S/N), respectively) were obtained which ranged between 0.005 μg/L for FBZ and 5.01 μg/L for MBC/BN for the LODs and between 0.02 and 16.7 μg/L for the LOQs of the same compounds. These values were experimentally checked extracting Milli-Q water samples spiked at these concentrations and calculating the S/N. Then, Milli-Q water samples were spiked at two concentration levels in quintuplicate to assess the precision and accuracy of the method. Table 3 shows the results of this study, in which a statistical comparison between the spiked and the found concentrations was carried out using the Student's t test. As can be seen, experimental t values were equal or lower than the tabulated one (2.78 for $n = 5$), thus the null hypothesis can be accepted because there are not significant differences between the real and the found concentration, showing that the method is highly repeatable and accurate.

3.4. USE–IL-DLLME–HPLC–FD of soil samples

3.4.1. USE soil extraction optimization

The use of DLLME for solid or semisolid matrices, as shown in the literature [15,16] requires a previous extraction step and then suitable removal of the organic solvent and reconstitution in the aqueous extract. With this purpose, and based on our previous experience with the extraction of pesticides from solid or semisolid matrices [21,22,28], extraction of spiked and non-spiked soil samples (soil 3 was used for optimization purposes) was carried out using ACN together with different salts ($MgSO_4 \cdot H_2O$, NaCl, sodium citrate tribasic dehydrate and sodium hydrogencitrate sesquihydrate) with or without the addition of water, which have proven to be effective conditions for other pesticides and soils [28,35–37]. Extractions were done in duplicate and a blank soil extraction was always done in parallel. Soil and salt amounts were carefully changed, however, only CB and TZ, and TBZ and FBZ in a lesser extent, were extracted under the best performing conditions (5 g soil, 10 mL ACN, 4 g $MgSO_4 \cdot H_2O$, 1 g NaCl, 1 g sodium citrate tribasic dehydrate and 0.5 g sodium hydrogencitrate sesquihydrate), while the rest of compounds (2-AB, MBC/BN and 1-N) were not recovered at all.

Consequently, a brief screening was carried out to study the extraction capacity of several solvents or mixtures of them (ACN, MeOH, acetone, ethyl acetate, water, acidic (HCl) or basic (NaOH) water). In these cases, 10 min of ultrasounds was applied for the extraction of 5 g of soil with 10 mL of the considered solvent. When using ACN, relative unclean chromatograms were obtained, with several interfering peaks that overlapped with TBZ and FBZ. Furthermore, neither 2-AB nor MBC/BN were extracted, a fact that was also observed when using acetone. Ethyl acetate only extracted MBC/BN, CB, 1-N and TZ, but in a very small amount. Neutral, acidic or basic water had little capacity on the extraction of the analytes of interest and, also, extraction of humic acids took place when using basic water. In fact, when pH of the aqueous extract was adjusted to 5.20 with HCl 0.1 M and the DLLME was carried out, precipitation of these acids took place, making difficult the IL drop collection. MeOH was the solvent that provided higher extraction for all analytes (except 2-AB that was not extracted) as well as cleaner chromatograms, so after testing several mixtures of solvents without success, the amount of soil extracted (2.5–5.0 g), as well as the amount of MeOH (10–25 mL) and ultrasonic time (5–25 min) were varied. These studies demonstrated that the use of 20 mL of MeOH to extract 3.0 g of soil using 10 min of ultrasounds provided the best results in terms of extraction efficiency: higher amounts of MeOH gave similar recoveries and higher amounts of

Table 5
Results of the precision and accuracy study of the IL-DLLME–HPLC–FD method for the selected compounds in the four types of soils.

Peak	Analyte	Soil	Spiked level[a] (ng/g)	Found[b] (ng/g)	Accuracy	t^c	Peak	Analyte	Soil	Spiked level[a] (ng/g)	Found[b] (ng/g)	Accuracy	t^c
1	2-AB	Soil 1	120.00	134.54 ± 25.87	112	1.40	5	CB	Soil 1	6.25	6.72 ± 1.54	107	2.20
			768.00	782.93 ± 17.16	102	0.61				40.00	37.20 ± 1.41	93	2.76
		Soil 2	137.50	143.95 ± 35.41	105	1.03			Soil 2	6.25	6.80 ± 1.58	109	1.00
			880.00	932.59 ± 30.55	106	2.24				40.00	40.62 ± 1.37	102	0.36
		Soil 3	125.00	109.81 ± 28.98	88	2.73			Soil 3	6.25	5.31 ± 1.72	85	1.93
			800.00	731.71 ± 25.38	91	0.94				40.00	43.00 ± 1.48	108	1.64
		Soil 4	125.00	128.75 ± 41.69	103	0.50			Soil 4	7.50	7.75 ± 3.03	103	0.57
			800.00	805.77 ± 36.98	101	0.15				48.00	44.82 ± 2.68	93	0.85
2	MBC/BN	Soil 1	120.00	124.37 ± 18.67	104	1.01	6	1-N	Soil 1	20.00	19.32 ± 8.22	97	1.55
			768.00	686.67 ± 17.16	89	1.48				64.00	65.14 ± 7.82	102	0.56
		Soil 2	125.00	125.24 ± 39.42	100	0.04			Soil 2	88.25	105.34 ± 25.25	119	1.01
			800.00	776.95 ± 34.23	97	1.32				564.77	550.49 ± 25.19	97	0.43
		Soil 3	125.00	143.18 ± 79.54	115	2.04			Soil 3	88.25	75.71 ± 26.17	86	2.15
			800.00	850.58 ± 74.18	106	2.78				564.77	463.48 ± 30.73	82	2.74
		Soil 4	130.00	125.53 ± 58.01	97	0.76			Soil 4	88.25	94.95 ± 19.11	108	0.76
			832.00	796.59 ± 51.31	96	1.29				564.77	514.87 ± 21.48	91	1.64
3	TBZ	Soil 1	11.25	11.33 ± 8.20	101	0.04	7	TZ	Soil 1	87.50	101.99 ± 38.33	117	2.73
			72.00	68.44 ± 7.51	95	0.56				560.00	511.68 ± 35.55	91	1.40
		Soil 2	17.50	20.44 ± 10.38	117	2.75			Soil 2	80.00	75.85 ± 14.33	95	0.57
			112.00	101.01 ± 9.16	90	0.86				512.00	523.42 ± 12.97	102	0.72
		Soil 3	15.00	15.45 ± 10.29	103	0.19			Soil 3	75.00	82.27 ± 73.78	110	0.81
			96.00	87.97 ± 9.00	92	0.74				480.00	492.83 ± 64.25	103	0.75
		Soil 4	25.00	27.29 ± 12.36	109	1.32			Soil 4	230.00	270.11 ± 146.49	117	1.46
			160.00	128.77 ± 10.91	80	2.31				1472.00	1268.62 ± 129.28	86	2.63
4	FBZ	Soil 1	0.18	0.18 ± 0.11	100	0.06							
			1.12	0.99 ± 0.10	89	1.34							
		Soil 2	0.18	0.19 ± 0.04	107	1.27							
			1.12	1.10 ± 0.03	98	0.38							
		Soil 3	0.19	0.21 ± 0.13	111	1.16							
			1.20	1.39 ± 0.11	115	2.06							
		Soil 4	0.30	0.35 ± 0.12	115	2.71							
			1.92	1.68 ± 0.11	87	2.23							

[a] $n = 5$.
[b] Average value ± standard deviation of five determinations (95% confidence value).
[c] $t_{tab} = 2.78$, $\alpha = 0.05$.

sample led to lower recoveries, probably because some components of the soil matrix prevented the IL from extracting the target analytes. With the aim of improving the extraction efficiency, the addition of NaCl was tested between 0 and 10% (w/v), leading in general to a manifest increase of the peak areas and even to the extraction of 2-AB. Regarding the concentration of salt, only 2.5% (w/v) or higher percentages gave maximum extraction efficiency, so this value was set as the adequate salt percentage. Finally, a double extraction with the better conditions (USE with MeOH with 2.5% (w/v) for 10 min) was tested, obtaining enhanced results, therefore for further studies, the extraction procedure was repeated twice before the IL-DLLME.

3.4.2. USE–IL-DLLME–HPLC–FD method validation

Once the whole described method was optimized, it was applied to four different soil samples (soils 1, 2, 3 and 4, see Table 2 for their characteristics). For this purpose, calibration curves were obtained spiking soil samples at six different concentration levels ($n = 6$) and performing the extraction. Table 4 shows the calibration data obtained by plotting the peak area *vs.* analyte concentration. As can be seen, the detector response was linear in all the range tested with determination coefficients higher than 0.996 for all curves, except for 1-N in soil 3 which was 0.994. LODs and LOQs of the whole method for the four soils, calculated as 3 and 10 times the S/N, respectively, were between 0.02 and 0.06 ng/g for FBZ in soils 1, 2 and 3 and between 27.1 and 90.2 ng/g for TZ in soil 4. These LOD values are of the same order of magnitude (ng/g) as the ones frequently obtained for the analysis of pesticides in soils [3]. Slight differences among the LODs between one soil and another can be attributed to a matrix effect that directly affects the recovery of each analyte in each type of soil. In fact, statistical comparison between

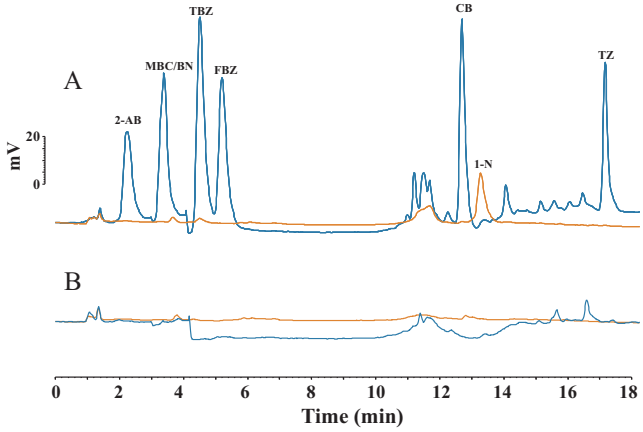

Fig. 5. HPLC–FD chromatograms after the complete USE–IL-DLLME methodology of (A) a spiked and (B) a non-spiked garden soil sample (blank sample). Analyte concentration: 2-AB (1100 ng/g), MBC/BN (1000 ng/g), TBZ (140 ng/g), FBZ (1.40 ng/g), CB (50.0 ng/g), 1-N (280 ng/g) and CB (640 ng/g). For the rest of conditions, see legend of Fig. 2.

the method calibration curves was carried out (data not shown) and it was observed that in all possible combinations (soil 1 with soil 2, soil 1 with soil 3, soil 1 with soil 4, etc.), significant differences between the curves for each compound occurred, except for MBC/BN curves in soils 1 and 2 and for TZB in soils 3 and 4. This clearly indicates the existence of a matrix effect for practically all pesticides and soils.

Fig. 5 shows the chromatograms after the application of the described methodology of a spiked (A) and a non-spiked (B) soil

sample (soil 3) and, as can be seen, there were no interfering peaks from the sample matrix. The chromatograms for the other three types of soils were very similar.

Considering those previous articles in which conventional DLLME has been applied for the extraction of pesticides from soils, comparison of LODs should be carefully made since few of the pesticides considered in our work were also analyzed and the characteristics of the analyzed soils are also different. In the work of Wu et al. [11], who applied a DLLME–HPLC–FD method to extract only two pesticides, MBC and TBZ, the LOD achieved for TBZ was in the same range (1.6 ng/g) as the ones obtained in this work (1.21–2.77 ng/g), while for MBC (1 ng/g) it was slightly lower (13.9–15.8 ng/g in our work), probably due to differences in the soils, which is a very complex matrix. In the work of Xiong and Hu [17], who analyzed six organosulfur pesticides by DLLME-GC-FPD, the LOD obtained for TZ (the only pesticide in common with this work) was approximately 23 ng/g (authors only provided LODs of the method for waters), which is also in accordance with the ones of our work. Results obtained in this work are also similar or even better to the ones obtained analyzing some of these compounds in soils using different techniques [7,38–44]. Only in two of them [41,42], a lower LOD than in the present work was obtained for CB.

With the aim of completing the validation of the method and of demonstrating its full potential for the analysis of soils, a precision and accuracy study was carried out. For this purpose, each type of soil was spiked at two different concentrations with the eight analytes and submitted to the developed USE–IL-DLLME–HPLC–FD method in quintuplicate ($n = 5$). As with Milli-Q water, Student's t test was used to compare the concentration found with the spiked one. Table 5 shows the results of this study as well as the spiking levels and, as can be seen, in all cases the experimental t value was below the tabulated one (2.78 for $n = 5$) with acceptable relative recovery percentages. These results show that the method is repeatable and accurate enough for all the pesticides and the four types of soils, all of them of different physicochemical properties.

4. Conclusions

In the present work, an IL-DLLME procedure was developed for the extraction of a group of eight fluorescent pesticides and metabolites from soil aqueous extracts of different physicochemical properties. Experimental design methodology was used for the optimization of extraction parameters. The method is very simple, quick, effective and repeatable, judging from validation data in terms of method calibration, precision and accuracy studies. LODs obtained were in the low ng/g range. The work represents the first application of ILs as extraction solvents in DLLME for the extraction of pesticides from soils.

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