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Ionic liquid based dispersive liquid–liquid microextraction for the extraction of pesticides from bananas

Lidia M. Ravelo-Pérez, Javier Hernández-Borges, María Asensio-Ramos, Miguel Ángel Rodríguez-Delgado*

Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Química, Universidad de La Laguna (ULL), Avenida Astrofísico Francisco Sánchez s/n, 38206 La Laguna, Tenerife, Spain

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ABSTRACT

This paper describes a dispersive liquid–liquid microextraction (DLLME) procedure using room temperature ionic liquids (RTILs) coupled to high-performance liquid chromatography with diode array detection capable of quantifying trace amounts of eight pesticides (i.e. thiophanate-methyl, carbofuran, carbaryl, tebuconazole, iprodione, oxyfluorfen, hexythiazox and fenazaquin) in bananas. Fruit samples were first homogenized and extracted (1 g) with acetonitrile and after suitable evaporation and reconstitution of the extract in 10 mL of water, a DLLME procedure using 1-hexyl-3-methylimidazolium hexafluorophosphate ($[C_6MIM][PF_6]$) as extraction solvent was used. Experimental conditions affecting the DLLME procedure (sample pH, sodium chloride percentage, ionic liquid amount and volume of disperser solvent) were optimized by means of an experimental design. In order to determine the presence of a matrix effect, calibration curves for standards and fortified banana extracts (matrix matched calibration) were studied. Mean recovery values of the extraction of the pesticides from banana samples were in the range of 69–97% (except for thiophanate-methyl and carbofuran, which were 53–63%) with a relative standard deviation lower than 8.7% in all cases. Limits of detection achieved (0.320–4.66 μg/kg) were below the harmonized maximum residue limits established by the European Union (EU). The proposed method, was also applied to the analysis of this group of pesticides in nine banana samples taken from the local markets of the Canary Islands (Spain). To the best of our knowledge, this is the first application of RTILs as extraction solvents for DLLME of pesticides from samples different than water.

1. Introduction

In recent years there has been a growing interest in simplifying and miniaturizing sample pretreatment steps, the introduction of alternative non-contaminant solvents and in decreasing the high quantities of organic solvents used. In this sense, different microextraction techniques have been explored as alternatives to conventional sample preparation procedures, such as solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE), single-drop microextraction (SDME), etc. Among them, dispersive liquid–liquid microextraction (DLLME) firstly developed by Rezaee et al. in 2006 [1], has been reported as a useful sample pretreatment procedure due to its main advantages: simplicity of operation, low time and cost, high recoveries and enrichment factors, low consumption of organic solvents, etc. Since its introduction, DLLME has been applied for the extraction of several organic and inorganic compounds mainly from water samples [1–9].

Concerning pesticide analysis, DLLME has been mainly used for the extraction of these compounds from waters [10–12] and in a much lower extent for their analysis in foods. In fact, to the best of our knowledge there only exist five works in the literature concerning the DLLME of pesticides from food matrices [13–17]. In these studies, 2 insecticides (carbaryl and triazophos) have been determined in peach, grape and apple juices [16], 10 insecticides (phorate, diazinon, disolfotane, parathion-methyl, sumithion, malathion, fenthion, profenophos, ethion and phosalone) in tea [17], 3 fungicides (captan, folpet and captafol) in apples [15], 6 insecticides (malathion, chlorpyrifos, buprofezin, triazophos, carbosulfan and pyridaben) in tea [14] and other 6 insecticides (ethoprophos, parathion-methyl, fenitrothion, malation, chlorpyrifos and profenophos) in watermelon and cucumber [13]. In all these works, conventional solvents, in general, chlorinated solvents (tetrachloroethane [16], chlorobenzene [13,15], carbon tetrachloride [14] and n-hexane [17]) were used.

In the last decade, the use of room temperature ionic liquids (RTILs) as extractants has been found to be especially important

* Corresponding author. Tel.: +34 922 31 80 46; fax: +34 922 31 80 03.
E-mail address: mrguez@ull.es (M.Á. Rodríguez-Delgado).

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in analytical chemistry (in order to replace the volatile ones used during sample preparation procedures) because of their negligible vapor pressure, good solubility for organic and inorganic compounds, non-flammability, high thermal stability, wide temperature range as a liquid phase, etc. [18,19]. They are therefore useful in liquid–liquid extraction (LLE) applications, although in this case a large volume of RTILs is required making it tedious and costly. Thus, microextraction techniques like SDME, SPME or DLLME based on RTILs are preferred. With respect to DLLME, the use of RTILs can replace the use of highly toxic chlorinated solvents, usually employed as extractants, with a simple injection into HPLC systems after dilution [20].

To date, IL-DLLME has hardly been explored for extraction purposes. In fact, only six applications have been proposed [20–25], the majority of them dealing, once more, with the extraction of water samples [20–24]. Only in three of these works, IL-DLLME was used for the extraction of pesticides. This is the case of the work of Liu et al. [20] in which four insecticides (fipronil, chlorfenapyr, buprofezin and hexythiazox) were determined in tap, lake and fountain waters, or the work of Zhou et al. [21] in which two insecticides (parathion-methyl and phoxim) were analyzed in rain, ground, reservoir and river waters and the work of Zhou et al. [22] who analyzed five insecticides (cyhalothrin, deltamethrin, fenvalerate, taufluvalinate and biphenthrin) in tap, ground, river and reservoir waters. Up to now, other samples different than waters have not been extracted by IL-DLLME for the analysis of pesticides, probably, because of the complexity of the matrices and also, because of the characteristics of the ILs required: extremely low solubility in the sample and compatibility with the subsequent separation technique. Therefore, extending the application of IL-DLLME for the extraction and preconcentration of pesticides from non-aqueous matrices is of great interest in order to fully demonstrate the potential of this technique.

The aim of this work is the development of a selective IL-DLLME method for the determination of trace levels of eight pesticides in bananas using HPLC with diode array detection (DAD). These pesticides (i.e. thiophanate-methyl, carbofuran, carbaryl, tebuconazole, iprodione, oxyfluorfen, hexythiazox and fenazaquin), which belong to different chemical families, have been selected because of their widespread use in the treatment of banana pests. Experimental design methodology was used for the optimization of the extraction parameters (sample pH, sodium chloride percentage, ionic liquid amount and volume of disperser solvent). The application of the method for the analysis of these pesticides in different commercial banana samples from the Canary Islands (first banana producer of the EU production regions) was also carried out. To the best of our knowledge, this is the first application of RTILs as extraction solvents in the DLLME of pesticides from matrices different than waters (fruits) and also the first application of IL-DLLME in which the highest number of pesticides has been extracted.

2. Experimental

2.1. Chemicals and reagents

Pesticide analytical standards of thiophanate-methyl, carbofuran, carbaryl, iprodione, hexythiazox and fenazaquin were from Riedel-de-Haën (Sigma–Aldrich, Madrid, Spain); purity was higher than 98.0%. Tebuconazole and oxyfluorfen were from Fluka (Sigma–Aldrich, Madrid, Spain); purity was higher than 99.6%. Individual stock solutions of each pesticide of approximately 500 mg/L were prepared by dissolving each compound in acetonitrile (ACN) and stored at 4 °C. Mixtures of appropriate concentration were prepared by appropriate combination and dilution with ACN. The working solutions were prepared daily by dilution of these mixtures with ACN.

Methanol and ACN of HPLC grade were from Scharlau (Barcelona, Spain). Acetone of analytical reagent grade was from Merck (Darmstadt, Germany). Anhydrous magnesium sulphate, sodium chloride, sodium hydrogencitrate sesquihydrate and sodium citrate tribasic dihydrate were for Sigma–Aldrich while hydrochloric acid was from Merck. The ionic liquids 1-hexyl-3-methylimidazolium hexafluorophosphate ($[C_6MIM][PF_6]$) and 1-butyl-3-methylimidazolium hexafluorophosphate ($[C_4MIM][PF_6]$) were provided by Fluka; purity was higher than 97.0%.

2.2. Apparatus and software

HPLC analyses were performed on a Waters Alliance HPLC system (Milford, MA, USA), equipped with two pumps (model 1525), an autosampler (model 717 plus) and a DAD (model 2996). For data storage and evaluation Empower 2 software from Waters and a personal computer were used. Separations were carried out using a Nova-Pak C_{18} column (150 mm × 3.9 mm, 4 μm) and a Guard-Pak C_{18} pre-column (4 μm), both from Waters. Gradient HPLC elution was performed with 100% Milli-Q water as mobile phase A and 100% ACN as mobile phase B. The initial mobile phase gradient condition was 75:25 of solvents A and B, respectively. The elution was isocratic for the first 3 min and was altered gradually to 45:55 over 4 min (curve 3). Then, the eluent composition was changed to 40:60 over 5 min (curve 7) and later to 100% B for 10 min (curve 5). Then, the elution was isocratic for 5 min and finally, the initial eluent composition was restored in 5 min (curve 6) and maintained for 5 min more. The flow rate was set at 1.0 mL/min and the injection volume always was 20 μL. The working wavelengths were 205, 215 and 220 nm. Milli-Q water was obtained from a Milli-Q gradient system A10 from Millipore (Bedford, MA, USA).

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

2.3. Banana samples

Banana samples (ecological and non-ecological) were bought in local markets and supermarkets of Tenerife (regional production). One gram of homogenized bananas (1 kg) was weighed into the 50 mL centrifuge tube and spiked with a small volume of an appropriate standard mixture solution. Samples and standards were carefully mixed (with the help of ultrasounds) and left at room temperature for at least 30 min before the extraction procedure. Then, 5 mL of ACN were added and the tube was closed and shaken vigorously by hand for 1 min. To induce phase separation and pesticide partitioning, a buffer-salt mixture (consisting of 2 g of anhydrous magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of sodium citrate tribasic dehydrate and 0.25 g of sodium hydrogencitrate sesquihydrate) was added. The tube was closed and immediately shaken vigorously on a Vortex mixer for 1 min. Then, the mixture was sonicated for 5 min and centrifuged at 4000 rpm for 10 min. The supernatant was filtered through a Chromafil Xtra PET-45/25 filter (pore size 0.45 μm, Macherey-Nagel, Düren, Germany) and evaporated at 40 °C and 205 mbar using a Rotavapor R-200 (Büchi Labortechnik, Flawil, Switzerland). The residue was dissolved with 10 mL of Milli-Q water at pH 2.7 (adjusted with 1.0 M HCl) containing 28.9% NaCl (w/v) and subjected to DLLME, as described below.

2.4. DLLME procedure

The solution previously obtained was placed in a 15 mL glass centrifuge tube. A mixture of 88 mg of $[C_6MIM][PF_6]$ (extraction solvent) and 714 μL of methanol (disperser solvent) was immediately injected into the sample solution in order to induce the

formation of a cloudy solution, which consisted in fine droplets of IL dispersed in the aqueous sample. The mixture was subsequently centrifuged for 20 min at 4000 rpm and the upper aqueous phase was removed with a syringe (dispersive particles of IL were sedimented at the bottom of the centrifuge tube). After this process, the IL phase (20 μL) was dissolved in ACN (the final volume was 125 μL) and 20 μL was injected into the HPLC system for analysis.

3. Results and discussion

3.1. HPLC–DAD method

The group of pesticides selected in this work consists of eight pesticides (thiophanate-methyl, carbofuran, carbaryl, tebuconazole, iprodione, oxyfluorfen, hexythiazox and fenazaquin) that have been widely used in the Canary Islands (Spain), especially for banana production. Bananas represent nearly the 30% of the regional agricultural production, being the most important crop of the islands in economic terms and the second in cultivated area (after wine grapes). It should be mentioned that very recently (at the end of 2008) the use of both carbofuran and carbaryl was forbidden in Europe for agricultural purposes since they were excluded from Annex I of Commission Directive 91/414/CEE (EU Pesticides Database, 2009. Available at http://ec.europa.eu/sanco_pesticides/public/index.cfm?event=activesubstance.selection&a=1 April). Even though, their analysis is of special interest in order to corroborate the presence of their residues in bananas in subsequent months.

In order to optimize the HPLC–DAD separation of the eight pesticides, several experiments with isocratic or gradient elution were carried out. For this purpose, 100% Milli-Q water (as mobile phase A) and 100% ACN (as mobile phase B) were used. The results of these experiments showed that only the use of gradient elution provided appropriate peak resolution. Moreover, the best results in terms of separation capacity and retention time were obtained using the gradient program shown in Section 2.2. On the other hand, the study of the DAD spectra revealed that, among the different wavelengths examined, 205, 220 and 215 nm were the ones of maximum UV absorbance (205 nm for all pesticides except for carbaryl and tebuconazole, 220 nm, and fenazaquin, 215 nm) and thus, further experiments were developed at these values. Fig. 1 shows the optimum separation of the selected group of pesticides by HPLC–DAD at their maximum UV absorbance wavelengths. As it can be seen in the figure, some chromatographic peaks were also obtained. They are assigned to the solvents used as mobile phases which highly absorb at these extremely low UV wavelengths (they all disappear at high wavelength values). Although we tried hard to eliminate them by suitable changes of the quality of the solvents or mobile phase composition, changes in the gradient program, etc. these chromatographic peaks could not be completely eliminated. Even though, they do not have at all any influence in the correct detection and quantification of the selected pesticides.

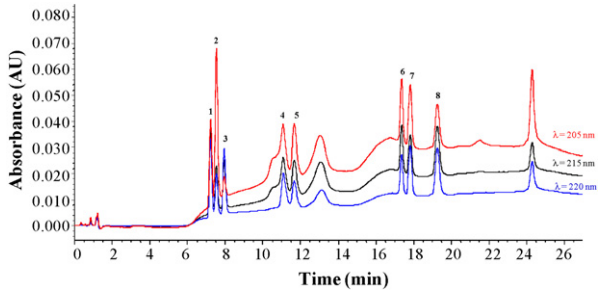

Fig. 1. HPLC–DAD chromatograms of the selected pesticides at their maximum absorbance wavelengths. Mobile phase A: 100% Milli-Q water. Mobile phase B: 100% ACN. Flow rate: 1.0 mL/min. Gradient program shown in Section 2.2. Injection volume: 20 μL. Sample dissolved in ACN: (1) thiophanate-methyl (3.4 mg/L), (2) carbofuran (5.4 mg/L), (3) carbaryl (0.57 mg/L), (4) tebuconazole (6.8 mg/L), (5) iprodione (2.3 mg/L), (6) oxyfluorfen (2.2 mg/L), (7) hexythiazox (5.0 mg/L) and (8) fenazaquin (2.4 mg/L).

Under the selected separation conditions of Fig. 1, a repeatability study at three levels of concentration (0.50, 1.0 and 2.5 mg/L) with three consecutive injections for the same day ($n = 3$) in five different days ($n = 15$) was carried out. Table 1 shows the RSD values obtained for both retention times and peak areas for a concentration of 1.0 mg/L for all the pesticides. As it can be observed, acceptable precision was obtained in all cases: intraday RSD values were below 4.7% for peak areas and below 0.9% for retention times, while interday RSD values were below 4.8% and 0.8% for peak areas and retention times, respectively. Once the repeatability study was carried out, regression equations (based on the peak areas) were obtained by injecting seven different concentrations of each standard in triplicate. Table 1 also shows the calibration parameters. As it can be seen, R^2 values were higher than 0.996 for all cases. LODs were in the range of 1.81 μg/L for carbaryl (peak 3) and 34.1 μg/L for tebuconazole (peak 4).

3.2. IL and experimental parameters selection

Preliminary experiments were carried out with the aim of selecting the best IL for the extraction of the analytes. The extraction solvent in DLLME has to meet several requirements: low water solubility, good chromatographic behavior, low volatility, higher density than water and high extraction capability of organic compounds [26]. The few works that have used IL-DLLME showed that $[C_4MIM][PF_6]$ and $[C_6MIM][PF_6]$ meet most of these requirements [20,27]. Moreover, they are relatively inexpensive [19,20,28] and thus, their use was tested in the present study. Initially and in order to select the best IL, 10 mL of spiked Milli-Q water containing 22 μg/L of each pesticide were extracted by using different volumes (200–800 μL) of methanol or acetone (disperser solvents) containing different quantities (50–80 mg) of each IL. It was observed that when using $[C_4MIM][PF_6]$ the solution was always transparent and no sedimented phase appeared at the bottom of the tube after centrifugation (not even with the addition of sodium chloride, which is frequently used to provide a salting-out effect). On the contrary, this was not observed when $[C_6MIM][PF_6]$ was tested (a sedimented phase was clearly obtained). The main reason is the higher solubility of $[C_4MIM][PF_6]$ in water than that of $[C_6MIM][PF_6]$ (1.88 and 0.75 g/100 mL, respectively) [29,30] and also its lower density (450 and 586 cP at 25 °C, respectively) [27,30]. Therefore, $[C_6MIM][PF_6]$ was selected as extraction solvent.

Further preliminary experiments were carried out to evaluate the influence of the amount of IL, type and volume of disperser solvent, time and temperature of the extraction, centrifugation time, sample pH and salt addition and to select the levels of the factors used in the experimental design. These experiments were carried out in duplicate with 10 mL of spiked Milli-Q water (22 μg/L of each pesticide). Firstly, extraction of spiked Milli-Q water samples of different pH values (3, 6 and 8) was developed. In this case, a solution of 52 mg of $[C_6MIM][PF_6]$ (extraction solvent) and 500 μL of methanol (disperser solvent) was injected into the sample solution. Then, the mixture was centrifuged for 15 min at 4000 rpm and the IL phase (20 μL) was dissolved in ACN (final volume: 125 μL). Fig. 2A shows the efficiency of sample pH in the extraction of the eight pesticides. The use of high pH values provided low recovery values (specially for thiophanate-methyl, carbofuran and carbaryl), due to the increase of the solubility of $[C_6MIM][PF_6]$ in the water sample. As it can be seen in the figure, the use of pH 6 provided better results for all the pesticides (recovery values between 30% and 92%) and therefore it was used in subsequent experiments. Regard-

Table 1
Results of the repeatability study (expressed as %RSD) obtained for the HPLC–DAD procedure (data given for 1.0 mg/L) and calibration data for the selected pesticides.

Peak	Pesticide	Intraday precision ($n=3$)		Interday precision ($n=15$)		Calibration data ($n=7$)				LOD (μg/L)[a]
		t_R	Area	t_R	Area	Range of concentration tested (mg/L)	b (S_b)	a (S_a)	R^2	
1	Thiophanate-methyl	0.8	4.0	0.8	1.6	0.061–3.42	8.81×10^4 (1419)	−1796 (2621)	0.996	17.6
2	Carbofuran	0.9	1.8	0.4	2.3	0.097–5.44	1.02×10^5 (936)	−1111 (2749)	0.998	15.5
3	Carbaryl	0.9	2.9	0.8	3.2	0.010–1.02	4.58×10^5 (4061)	−2882 (1980)	0.998	1.81
4	Tebuconazole	0.4	0.5	0.2	3.1	0.122–6.83	2.40×10^4 (452)	−2235 (1667)	0.996	34.1
5	Iprodione	0.7	1.6	0.4	4.8	0.041–2.31	1.29×10^5 (1340)	−4600 (2049)	0.998	13.6
6	Oxyfluorfen	0.1	2.3	0.1	4.4	0.040–2.24	1.12×10^5 (1596)	−1804 (1928)	0.998	10.3
7	Hexythiazox	0.2	4.7	0.1	3.5	0.089–5.01	5.71×10^4 (825)	−148 (2232)	0.998	24.3
8	Fenazaquin	0.7	2.5	0.3	3.6	0.043–2.42	1.30×10^5 (1979)	−3307 (2583)	0.996	15.4

b, slope; S_b, SD of the slope; a, intercept; S_a, SD of the intercept; R^2, determination coefficient.
[a] Calculated as three times the S/N.

ing the influence of salt addition, experiments with 0%, 15% and 25% NaCl (w/v) were carried out to induce a salting-out effect. Results showed that in general, an increase of the NaCl percentage provided greater recovery values, especially for thiophanate-methyl, carbofuran, carbaryl and tebuconazole (Fig. 2B). Therefore, the subsequent experiments were carried out using 25% NaCl (w/v) because it provided recoveries in the range 48–99%.

Extraction time, centrifugation time and extraction temperature were also investigated, with the previous selected conditions. Regarding extraction time, this parameter was increased up to 25 min. However, all these experiments showed no significant differences in the recovery values for all pesticides and thus, subsequent experiments were carried out centrifuging the mixture immediately after injecting the combination of dispersive and extraction solvents in the sample, which has also been observed by other authors [31,32]. Centrifugation time was modified between 10 and 25 min, finding that in general, the recoveries of all the analytes were higher at 20 min. Concerning extraction temperature, several experiments at 30, 40 and 60 °C were carried out. However, high temperatures provided higher solubility of the IL in the aqueous phase and therefore lower recovery values were obtained in these cases. Therefore, subsequent experiments were performed at room temperature (25 °C approx.).

Amount of IL and type and volume of disperser solvent were also evaluated in these preliminary experiments. The effect of IL amount was investigated dissolving 52, 65 and 78 mg of [C_6MIM][PF_6] in 500 μL of methanol. In general, recovery values increased as the IL amount increased. Regarding type of disperser solvent, methanol and acetone were tested because they are very common in DLLME together with ACN [20,33] and they had provided satisfactory results in previous works for the determination of insecticides in water samples [10,20]. However, in this case acetone provided lower recoveries (between 22% and 64%). ACN was not used because it forms a miscible system with the IL and the aqueous sample [20]. Therefore, methanol was used as disperser solvent. The amount of methanol was evaluated using values of 300, 500, 650, 800 and 1000 μL with a constant amount of IL (78 mg). As it can clearly be seen in Fig. 2C, satisfactory recoveries (between 60% and 102%) could be achieved with intermediate volumes of methanol.

3.3. Experimental design

Taking into account the results obtained in the preliminary studies described above, a central composite design (2^4 full factorial design + star with three central points, with an axial distance equal to 2) was selected with the aim of appropriately optimizing the

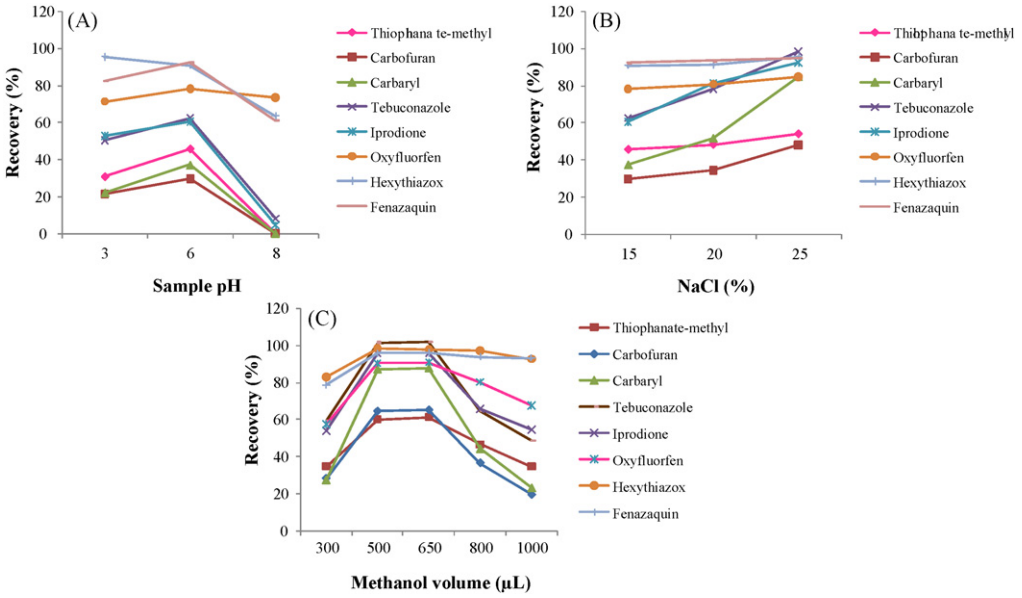

Fig. 2. Effect of sample pH (A), percentage of NaCl (B) and methanol volume (C) on extraction efficiency. Extraction conditions: (A) 10 mL of spiked Milli-Q water (22 μg/L of each pesticide) with 0% NaCl, 52 mg of [C_6MIM][PF_6], 500 μL of methanol, 15 min of centrifugation at 4000 rpm. (B) 10 mL of spiked Milli-Q water (22 μg/L of each pesticide) at pH 6.0, 52 mg of [C_6MIM][PF_6], 500 μL of methanol, 15 min of centrifugation at 4000 rpm. (C) 10 mL of spiked Milli-Q water (22 μg/L of each pesticide) at pH 6.0, with 25% NaCl, 78 mg of [C_6MIM][PF_6], 20 min of centrifugation at 4000 rpm.

Table 2
Factors levels used in the central composite design.

Factor	Central composite design			
	Full factorial design levels		Star design levels	
	Low	High	Low	High
pH	3.5	6.5	2.7	7.3
Percentage of NaCl (%)	6.0	24.0	1.1	28.9
$[C_6MIm][PF_6]$ amount (mg)	52	85	43	93
Methanol volume (μL)	400	800	291	909

main factors affecting the DLLME extraction yield (i.e. sample pH, NaCl percentage, IL amount and methanol volume). Table 2 shows the levels of the factors in both full factorial and star designs. These levels were selected according to preliminary experiments (Section 3.2) and on the base of previous works in the literature regarding the extraction of pesticides in waters by DLLME [20,21]. The resulting 27 experiments were carried out randomly, using 10 mL of spiked Milli-Q water samples (22 μg/L of each pesticide), at room temperature, 20 min of centrifugation, $[C_6MIM][PF_6]$ as extraction solvent and methanol as disperser solvent.

Individual peak areas of the eight analytes and the sum of the peaks areas of all the pesticides were introduced separately as the response in the statistical program. The results of the experimental design were firstly analyzed for each pesticide individually. According to this optimization study, in general, higher NaCl percentages provided high recovery values. However, concerning sample pH, higher recoveries were obtained when this parameter decreased. Regarding the amount of $[C_6MIM][PF_6]$, the extraction of all pesticides (except for oxyfluorfen, hexythiazox and fenazaquin) increased when this parameter increased. On the other hand, the recoveries of all analytes increased as methanol volume increased before a maximum value from which recoveries decreased. These results agreed with the observations obtained from preliminary experiments. However, the complexity of developing the simultaneous extraction of pesticides of different properties requires a compromise between each individual extraction optimum condition. That is why the mean recovery percentage was chosen as a good "compromise" response. Fig. 3 shows the response surfaces of the extraction of the selected pesticides choosing mean recovery percentage as response. In general, higher IL amounts and NaCl percentages as well as lower pH values and intermediate-high methanol volumes provided the highest extraction of these compounds. Overall, the following optimum experimental conditions were obtained: pH 2.7, 28.9% NaCl (w/v), 88 mg of $[C_6MIM][PF_6]$ and 714 μL of methanol. When these optimum conditions were tested it was found that they effectively provided the highest extraction for all the pesticides (recovery values in the range 66–101%). Despite these results, other experiments in which the values of the factors were slightly changed near the optimum ones were carried out. Specifically, pH and NaCl percentage were modified (pH: 2 and 3, NaCl percentage: 30%) and it was observed that recoveries did not increase.

Finally, in order to test the repeatability of this procedure, five extractions of 10 mL of spiked Milli-Q water at three different concentration levels were developed (results and concentration levels are shown in Table 3). Mean recovery values ranged between 66% (thiophanate-methyl) and 101% (hexythiazox) for all the pesticides. LODs, which were calculated as three times the S/N, ranged between 0.250 μg/L (carbaryl) and 3.86 μg/L (tebuconazole).

3.4. IL-DLLME-HPLC–DAD of banana samples

In order to apply the optimized IL-DLLME-HPLC–DAD procedure to the analysis of banana samples, an ultrasound-assisted extraction of the pesticides from homogenized bananas with an organic solvent was developed. For this purpose, preliminary tests were carried out twice with different volumes (5 and 10 mL) of ACN and acetone as extraction solvents, since they have good solubility in them and they are among the most commonly used for their extraction from fruits [34–36]. In each case, 5 g of spiked homogenized bananas (50 μg/kg of each pesticide) were ultrasound-assisted extracted for 5 min. After centrifugation for 5 min at 4000 rpm, the extracts were filtered using 0.45 μm filters, evaporated to dryness using a rotavapor at 40 °C (205 and 500 mbar for ACN and acetone, respectively) and reconstructed in 10 mL of Milli-Q water with 28.9% NaCl (w/v) at pH 2.7 (see Section 2 for details). Then, the optimized IL-DLLME procedure was applied. Fig. 4A shows the influence of the type and volume of organic solvent (expressed as recovery percentages). From the figure it can clearly be observed that the conditions tested provided low recoveries (19–48% with

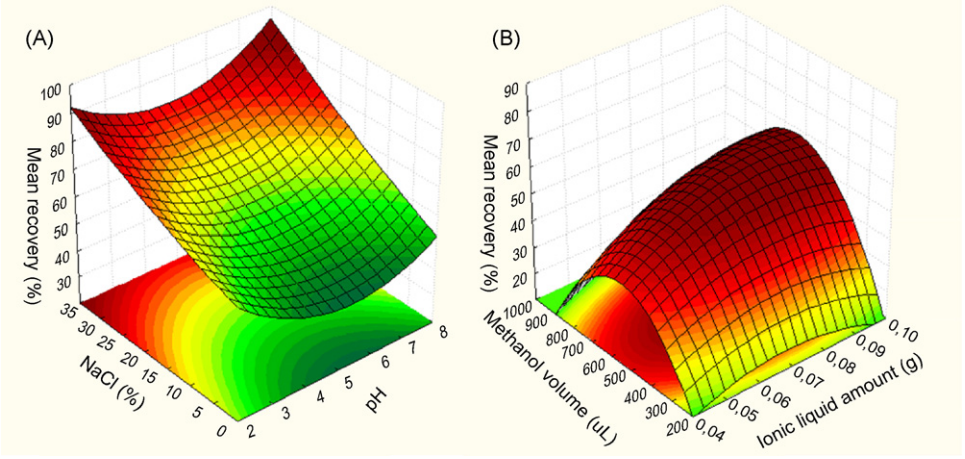

Fig. 3. Response surface estimated for the central composite design of the IL-DLLME optimization. (A) Mean recovery percentage, NaCl percentage and pH. (B) Mean recovery percentage, methanol volume and IL amount.

Table 3
Mean recoveries, RSD and LODs values of the selected pesticides in Milli-Q water samples after the IL-DLLME-HPLC–DAD method.

Peak	Pesticide	Spiked level (μg/L)	Mean recovery (%)	RSD (%)	LOD (μg/L)[a]	Peak	Pesticide	Spiked level (μg/L)	Mean recovery (%)	RSD (%)	LOD (μg/L)[a]
1	Thiophanate-methyl	3.05	66	3.2	2.20	5	Iprodione	2.07	78	5.2	1.99
		76.3	68	7.3				51.6	88	2.2	
		171	70	7.7				116	84	6.6	
2	Carbofuran	4.86	70	2.1	3.08	6	Oxyfluorfen	2.00	85	3.5	1.15
		121	71	3.2				50.0	92	3.6	
		272	73	3.7				112	90	3.8	
3	Carbaryl	0.508	71	3.3	0.250	7	Hexythiazox	4.48	98	1.3	2.48
		12.7	78	3.0				112	101	2.5	
		28.5	73	1.6				251	98	3.5	
4	Tebuconazole	6.10	93	2.5	3.86	8	Fenazaquin	2.16	90	2.3	1.79
		152	98	5.0				53.9	93	4.3	
		341	97	5.0				121	90	1.8	

[a] Calculated as three times the S/N.

ACN and 8–28% for acetone). As a result, several modifications of the extraction procedure prior to DLLME (salt addition) were studied using ACN in order to improve the recoveries. These modifications consisted in the addition of $MgSO_4$, NaCl, sodium hydrogencitrate sesquihydrate and sodium citrate tribasic dehydrate in the sample, because these salts enable its warming and the salting-out effect. These considerations had been studied by several authors as part of other extraction procedures of pesticides from fruits and vegetables [37–40]. Overall, in these works, the use of 10 g of sample, 10 mL of ACN and suitable amounts of these salts (4 g of $MgSO_4$, 1 g of NaCl, 0.5 g of sodium hydrogencitrate sesquihydrate and 1 g of sodium citrate tribasic dehydrate) increased phase separation and the extraction of the pesticides. In fact, NaCl enables the salting-out effect, $MgSO_4$ reduces the aqueous phase (enabling the liquid–liquid partition and therefore increasing the recoveries of the analytes) [41] while citrates provide a suitable buffer medium (pH between 5 and 5.5, values in which pesticides are more stable) which induces the partitioning of all the analytes into the ACN phase [42].

Taking into account these considerations, different experiments with the same proportions of salts and ACN volume from the previous studies were carried out [37–40] considering different quantities of sample (1–5 g). When 5 g of homogenized sample were extracted it was observed that recovery values were similar to those obtained without the addition of salts (see Fig. 4B). However, when 1 g was extracted with the same amounts (as well as half amounts) of salts and ACN described in previous works (10 mL of ACN, 4 g of $MgSO_4$, 1 g of NaCl, 0.5 g of sodium hydrogencitrate sesquihydrate and 1 g of sodium citrate tribasic dehydrate) [37–40] recovery values obtained in both cases (see Fig. 4B) were similar and both greater than those obtained without salts (recoveries between 56% and 94%). Moreover, in order to increase the preconcentration factor, 2 g of sample were tested, but the recovery values decreased (see Fig. 4B). The use of acetone instead of ACN was also tested in

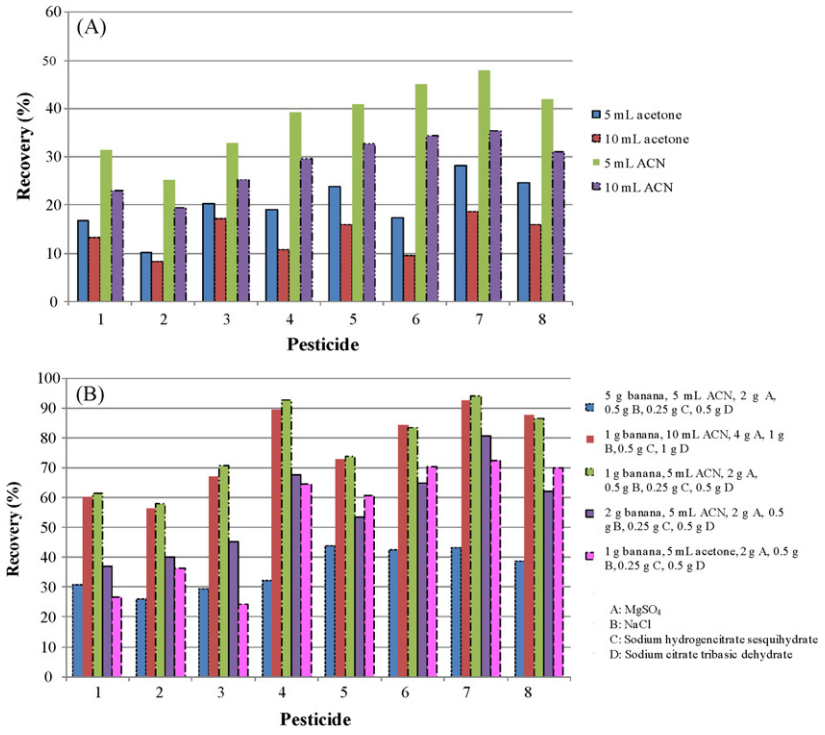

Fig. 4. (A) Effect of the type and the volume of the organic solvent, (ACN and acetone) and (B) effect of the salt addition ($MgSO_4$, NaCl, sodium hydrogencitrate sesquihydrate and sodium citrate tribasic dehydrate) on the extraction of the eight pesticides from banana samples (5 g of sample in (A)). Ultrasound-assisted extraction prior to DLLME for 5 min. Sample: 50 μg/kg of each pesticide. Identification: see Fig. 1.

Table 4
Calibration data from standards prepared in banana extracts after IL-DLLME-HPLC–DAD and assessment of the matrix effect.

Pesticide	Range of concentration tested (mg/L)	b (S_b)	a (S_a)	R^2	Matrix effect[a]
Thiophanate-methyl	0.061–3.42	7.91×10^4 (1336)	962 (2469)	0.998	Yes
Carbofuran	0.097–5.44	9.76×10^4 (1819)	2788 (5343)	0.994	Yes
Carbaryl	0.010–1.02	4.28×10^5 (6520)	−1997 (3179)	0.998	Yes
Tebuconazole	0.122–6.83	2.14×10^4 (376)	−1096 (1386)	0.998	Yes
Iprodione	0.041–2.31	1.15×10^5 (2031)	−1906 (2537)	0.996	Yes
Oxyfluorfen	0.040–2.24	1.20×10^5 (1603)	788 (1938)	0.994	Yes
Hexythiazox	0.089–5.01	5.61×10^4 (1401)	979 (3793)	0.996	No
Fenazaquin	0.043–2.42	1.17×10^5 (1692)	1409 (2209)	0.998	Yes

b, slope; S_b, SD of the slope; a, intercept; S_a, SD of the intercept; R^2, determination coefficient.
[a] Statistical difference is considered when p-values for the comparison of the slopes or intercepts are ≤0.1 (see text for further explanation).

the same way. However, the extraction of the analytes under these conditions were worse than those previously obtained, due to the fact that acetone is more miscible with water and also enables, at a higher extent than ACN, the coextraction of lipids, sugars and other compounds from the banana matrix [37,43]. As it can clearly be seen in Fig. 4B, the best extraction conditions were the following: 1 g of homogenized bananas, 5 mL of ACN, 2 g of $MgSO_4$, 0.5 g of NaCl, 0.25 g of sodium hydrogencitrate sesquihydrate and 0.5 g of sodium citrate tribasic dehydrate.

3.4.1. Method validation

For method validation, matrix matched calibration, recovery and accuracy studies were developed. Firstly, with the aim of checking the absence of the selected pesticides, extractions from ecological non-spiked banana samples were carried out. No pesticides in these samples and also no chromatographic interferences that difficulted the correct detection and quantification of the target compounds were found. Therefore, these samples could be used for the method validation.

Due to the presence of many compounds in the banana samples that could influence (increase/decrease) the chromatographic signal of the selected pesticides, it is of great interest to perform a statistical comparison between the calibration equations obtained from standards dissolved in ACN and in spiked sample extracts (matrix matched calibration). This assessment can clearly show/demonstrate if there exists a strong matrix effect for the selected pesticide (changes in the slope and intercept of the calibration curve) and if suitable calibration in the sample matrix should be developed. For this purpose, banana extracts free of pesticides were spiked at different concentration levels ($n = 7$). Each concentration level was injected in triplicate. Statistic parameters calculated from the least-square regression are presented in Table 4. In all cases, determination coefficients (R^2) higher than 0.994 were obtained. In order to clearly evaluate the matrix effect, matrix matched calibration graphs were statistically compared with calibration curves from standards, using a statistical program that calculates F- and p-values for the comparison of the slopes and the intercepts. As it can be seen in Table 4, for all pesticides (except for hexythiazox), statistical differences were observed (p-values for the comparison of the slopes or intercepts were ≤0.1) and as a result, quantification should be developed using the calibration curves obtained with the banana samples.

The accuracy and repeatability of the whole method were evaluated by the development of a recovery study ($n = 5$) carried out at three concentration levels (one level at the MRLs of the pesticides established for bananas). Mean recovery values in the range 53–97% (for carbofuran and hexythiazox, respectively) were obtained (RSD < 8.7% in all cases, see Table 5). As a result, LODs values (Table 5) ranged between 0.320 μg/kg (carbaryl) and 4.66 μg/kg (tebuconazole), which are well below the harmonized EU MRLs established for bananas (see Table 6). Verification of the LODs was also carried out experimentally.

Regarding the analysis of bananas by HPLC, it has only been applied in few works [44–48] although it should be mentioned than in none of these studies the simultaneous determination of this group of pesticides has been developed. Only in three of these works, one of the selected pesticides (thiophanate-methyl [44,47] or hexythiazox [48]) was analyzed and recoveries and LODs similar to the ones obtained in the present work were achieved.

In order to verify the accuracy of the developed method, a Student's t-test [49] was used. For this purpose, five consecutive extractions of spiked banana samples at three levels of concentration (equivalent or very near to those of the UE MRLs established for these samples) were carried out. Table 6 shows the results of this study (all calculations were developed taking into account the recovery factors). As it can clearly be seen in the table, t values

Table 5
Mean recoveries, RSD values and LODs of the selected pesticides in banana samples after IL-DLLME-HPLC–DAD.

Peak	Pesticide	EU's MRL[a] (mg/kg)	Spiked level (μg/kg)	Mean recovery (%)	RSD (%)	LOD (μg/kg)[b]	Peak	Pesticide	EU's MRL[a] (mg/kg)	Spiked level (μg/kg)	Mean recovery (%)	RSD (%)	LOD (μg/kg)[b]
1	Thiophanate-methyl	0.1	7.63	59	7.9	3.92	5	Iprodione	0.02	5.16	71	2.9	2.20
			100	61	7.3					20.0	75	3.9	
			150	63	5.1					30.0	78	8.7	
2	Carbofuran	0.02	12.1	53	2.6	4.03	6	Oxyfluorfen	0.05	5.00	82	3.4	1.35
			20.0	56	3.2					50.0	84	6.9	
			30.0	62	6.6					75.0	85	2.9	
3	Carbaryl	0.05	1.27	69	2.7	0.320	7	Hexythiazox	0.5	11.2	92	5.1	2.98
			50.0	70	2.8					500	94	4.7	
			75.0	71	5.7					625	97	3.7	
4	Tebuconazole	0.05	15.2	89	8.6	4.66	8	Fenazaquin	0.2	5.39	83	2.6	2.14
			50.0	93	5.0					200	86	4.3	
			75.0	96	3.1					300	89	2.8	

[a] Taken from EU MRLs sorted by pesticide. Available at <http://www.ec.europa.eu/food/plant/protection/pesticides/index_en.htm>.
[b] Calculated as three times the S/N.

Table 6
Results of assays to check the accuracy of the proposed method for the selected pesticides in banana samples.

Pesticide	Spiked level (μg/kg)	Found (μg/kg)[a]	Accuracy (%)	*t*
Thiophanate-methyl	50.0	48.9 ± 1.6	98	0.13
	100	92.4 ± 3.8	92	1.64
	125	117 ± 22	93	1.22
Carbofuran	16.0	15.0 ± 0.9	94	1.51
	20.0	19.6 ± 1.7	98	0.75
	25.0	24.0 ± 1.5	96	1.26
Carbaryl	24.0	23.9 ± 2.1	100	0.09
	50.0	49.0 ± 6.3	98	0.12
	62.0	60.0 ± 7.4	97	1.13
Tebuconazole	30.0	29.8 ± 3.6	99	0.18
	50.0	48.8 ± 5.5	98	1.15
	62.0	61.1 ± 4.8	98	0.62
Iprodione	10.0	10.3 ± 1.1	103	2.33
	20.0	20.1 ± 2.6	101	0.19
	25.0	24.1 ± 3.2	96	1.29
Oxyfluorfen	22.0	22.4 ± 2.8	102	1.63
	50.0	49.2 ± 4.9	98	1.42
	62.0	61.2 ± 3.8	99	0.96
Hexythiazox	250	253 ± 14	101	2.50
	500	481 ± 29	96	2.72
	575	608 ± 41	106	2.49
Fenazaquin	100	98.3 ± 9.9	98	0.65
	200	206 ± 17	103	2.05
	250	263 ± 20	105	2.29

t: experimental *t* value.
[a] Average value ± standard deviation of five determinations (95% confidence level).

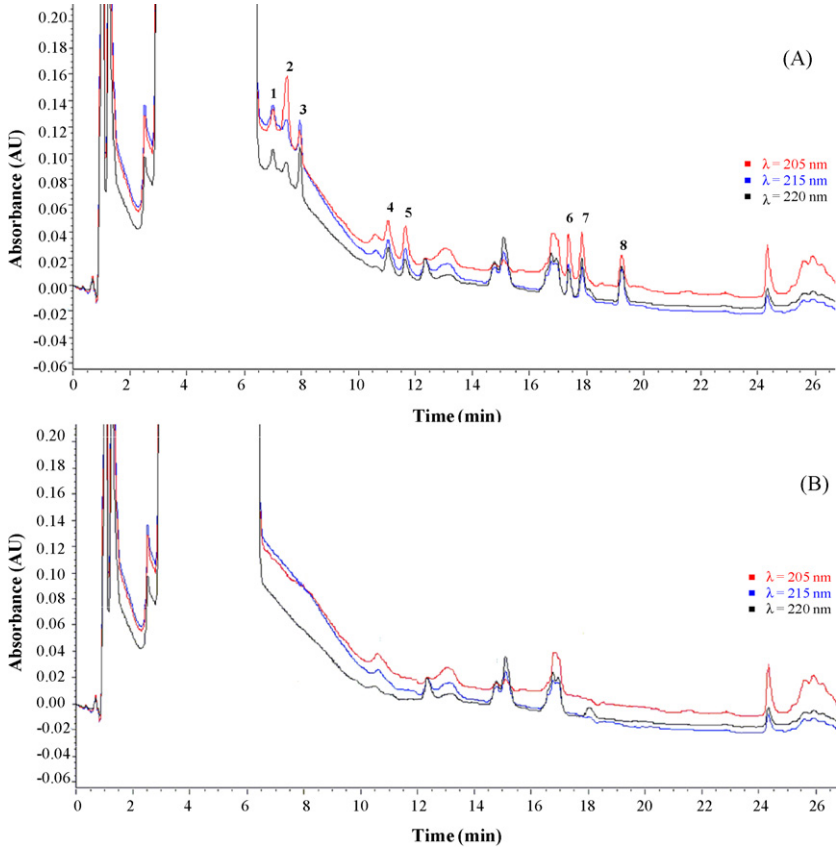

Fig. 5. HPLC–DAD chromatograms of (A) spiked and (B) non-spiked banana sample after optimum IL-DLLME conditions. Peak identification: (1) thiophanate-methyl (100 μg/kg), (2) carbofuran (20 μg/kg), (3) carbaryl (50 μg/kg), (4) tebuconazole (50 μg/kg), (5) iprodione (20 μg/kg), (6) oxyfluorfen (50 μg/kg), (7) hexythiazox (500 μg/kg) and (8) fenazaquin (200 μg/kg).

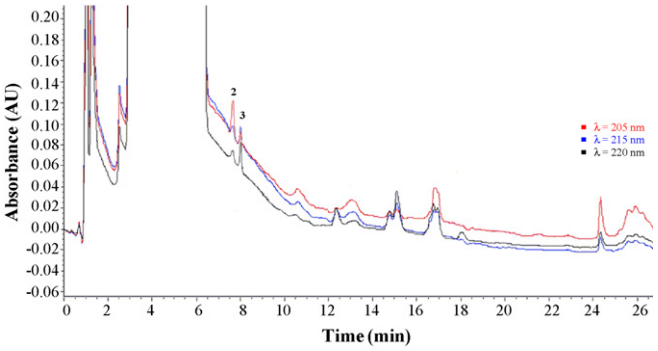

Fig. 6. Chromatograms of non-spiked banana sample after optimum IL-DLLME-HPLC–DAD procedure. Peak identification: (2) carbofuran and (3) carbaryl.

were lower than the tabulated one (2.78 for $n = 5$) and thus the null hypothesis might be accepted (no significant differences were observed between the real and the experimental value). Accuracy percentages ranged between 92% and 106%.

Fig. 5 shows the HPLC–DAD chromatograms of a spiked (A) and non-spiked (B) banana sample. As it can clearly be seen, no residues of the selected pesticides were found in the samples. A wide peak associated to the IL and IL impurities, which has also been observed by other authors using IL-DLLME [20,24] was also observed. However it was possible to correctly identify and quantify the selected pesticides without any problem at their maximum UV absorbance wavelengths.

3.5. Analysis of banana samples

With the aim of demonstrating the potential of the proposed methodology for the monitoring of these pesticide residues in bananas, nine commercial samples (three ecological and six non-ecological) bought in local markets (regional agricultural production) were analyzed. After the homogenization of 10 individual bananas (approx. 1 kg) as indicated by the Spanish legislation [50], 1 g of the homogenate was taken as analytical sample. In the three ecological samples, no residues of pesticides were found. However, concerning the non-ecological samples, in two of them both carbofuran and carbaryl appeared, while in three of the samples only one pesticide was present (either carbofuran or carbaryl). Pesticide concentration in the samples ranged between 14 μg/kg (carbofuran) and 23 μg/kg (carbaryl), although in two of them, concentrations of these pesticides were below the LOQ of the method. Fig. 6 shows the chromatograms of one of the analyzed samples that contained both carbofuran (peak 2) and carbaryl (peak 3). Identification of the pesticides was carried out by fortifying the samples with the mixtures of the pesticides and also by comparison of the DAD spectra of both samples and standards. Although both compounds appeared in some of the samples their concentration was below the UE MRLs. Therefore, in general, the levels of these residues cannot be considered a serious public health problem. The presence of these pesticides (currently forbidden) is associated to the fact that some of these products remained in stock in the last months (carbofuran and carbaryl could not be used after the end of the year 2008).

4. Conclusions

This paper proposes a new method for the analysis of a group of 8 multi-class pesticides in banana samples by IL-DLLME-HPLC–DAD. The use of the ionic liquid [C_6MIM][PF_6] as extraction solvent in combination with DLLME includes several advantages: reduction of the exposure danger to toxic solvents used in the conventional extraction procedures, sensitivity enhancement, rapidity, ruggedness and simplicity. The validation of the optimized method in terms of linearity, precision, recovery, accuracy and selectivity showed that the proposed procedure is highly sensitive, precise and repeatable (mean recoveries were between 53% and 97%), with LODs in the range 0.320–4.66 μg/kg (values well below the EU MRLs established for these compounds in bananas). The comparison of the calibration equations of standards and banana extracts showed the existence of a strong matrix effect for all the pesticides (except for hexythiazox). The applicability of the whole method was tested by analyzing nine commercial banana samples (three ecological and six non-ecological). Only in four of them no residues of the selected pesticides were found, while the rest showed the presence of carbofuran and carbaryl below MRLs established for bananas. The work represents the first application of ILs as extraction solvents in DLLME for the extraction of pesticides from non-aqueous samples.

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