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Hollow fiber-liquid-phase microextraction of fungicides from orange juices

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ABSTRACT

Liquid-phase microextraction (LPME) based on polypropylene hollow fibers was evaluated for the extraction of the post-harvest fungicides thiabendazole (TBZ), carbendazim (CBZ) and imazalil (IMZ) from orange juices. Direct LPME was performed without any sample pretreatment prior to the extraction, using a simple home-built equipment. A volume of 500 μL of 840 mM NaOH was added to 3 mL of orange juice in order to compensate the acidity of the samples and to adjust pH into the alkaline region. Analytes were extracted in their neutral state through a supported liquid membrane (SLM) of 2-octanone into 20 μL of a stagnant aqueous solution of 10 mM HCl inside the lumen of the hollow fiber. Subsequently, the acceptor solution was directly subjected to analysis. Capillary electrophoresis (CE) was used during the optimization of the extraction procedure. Working under the optimized extraction conditions, LPME effectively extracted the analytes from different orange juices, regardless of different pH or solid material (pulp) present in the sample, with recoveries that ranged between 17.0 and 33.7%. The analytical performance of the method was evaluated by liquid chromatography coupled with mass spectrometry (LC/MS). This technique provided better sensitivity than CE and permitted the detection below the $\mu g\,L^{-1}$ level. The relative standard deviations of the recoveries (RSDs) ranged between 3.4 and 10.6%, which are acceptable values for a manual microextraction technique without any previous sample treatment, using a home-built equipment and working under non-equilibrium conditions (30 min extraction). Linearity was obtained in the range 0.1–10.0 $\mu g\,L^{-1}$, with $r = 0.999$ and 0.998 for TBZ and IMZ, respectively. Limits of detection were below 0.1 $\mu g\,L^{-1}$ and are consistent with the maximum residue levels permitted for pesticides in drinking water, which is the most restrictive regulation applicable for these kinds of samples. It has been demonstrated the suitability of three-phase LPME for the extraction of pesticides from citrus juices, suppressing any pretreatment step such as filtration or removal of the solid material from the sample, that may potentially involve a loss of analyte.

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1. Introduction

In liquid-phase microextraction (LPME), a low polarity (water immiscible) organic solvent is immobilized as a thin supported liquid membrane (SLM) in the pores in the wall of a porous hollow fiber. The target analytes are extracted from an aqueous sample through the organic SLM and further into an acceptor solution inside the lumen of the hollow fiber [1,2]. This system offers greater stability than other LPME procedures such as single drop microextraction (SDME), where the hanging drop is often lost during extraction [3,4]. After the extraction, the acceptor solution is directly subjected to analysis. Regarding to the acceptor solution, it can be an organic solvent providing a two-phase LPME system, which is directly compatible with GC [5–7], or an aqueous solution providing a three-phase extraction system, compatible with HPLC or CE [8–12]. Preparation of the SLM may be accomplished by dipping the hollow fiber in a small vial containing the organic solvent for typically 5–10 s [13–16]. As a recent alternative, the organic solvent may be filled from the inside of the hollow fiber by injecting a known volume of organic phase with a microsyringe [17] simulating the operation of an automatic injector and providing a better repeatability.

In LPME, the volume of sample ranges between 50 μL and more than 1 L, whereas the volume of acceptor solution is lower than 30 μL in most cases. Two of the advantages of LPME are due to this sample-to-acceptor volume ratio that involves a reduction in the consumption of solvent and very high enrichments without evaporation of organic solvent. These features make LPME a very sensitive technique suitable for trace analysis. The low solvent consumption also makes LPME an environmental friendly extraction technique. Moreover, great selectivity can be achieved by methods using LPME, especially in the three-phase system, and further clean-up procedures are normally not required.

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Many applications of LPME have been performed in the bioanalytical and environmental fields, where efforts have been conducted to develop sample procedures that are simple, low-cost and capable of being performed at miniaturized scale. Bioanalytical methods focus on the determination of drugs and related substances in body fluids such as blood, plasma, serum, urine, breast milk or tissues [18–21]. In this area, some of the methods based on traditional liquid–liquid extraction have been easily transferred to LPME, improving their performance. On the other hand, various types of contaminants determined in different environmental matrices include, among others, pesticides, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and aromatic amines [22–28]. At this regard, LPME has shown to be a very useful and powerful analytical tool working in both two and three-phase mode [29].

Although LPME is very suited for the analysis of contaminants and toxic compounds, as shown by the numerous methods published in the environmental field, only a few papers have been published for LPME of food and beverages or foodstuff simulants [30–35]. In one publication, calibration was investigated for automated LPME and applied to the analysis of BTEX in orange juices [36]. However, to the best of our knowledge, there is not any publication dealing with the application of LPME for the analysis of fungicides in citrus juices. In the present work, thiabendazole (TBZ), carbendazim (CBZ) and imazalil (IMZ) are analyzed in commercially available orange juices.

Thiabendazole, carbendazim and imazalil are post-harvest systemic fungicides very commonly used to prevent vegetables and fruits, particularly citrus fruits, from deteriorating during storing and transportation. Thiabendazole, carbendazim and imazalil are three of the most widely extended, and it has been reported the presence of residual compounds in fruits, even in the edible part [37–40], as well as in other processed fruits, such as orange juices [41,42]. Various pesticides, including TBZ, CBZ and IMZ, have been also determined in fruit-based soft drinks [43]. There is not a specific regulation about these kinds of chemicals in drinks made from fruits and more than one regulation could be potentially applicable to this issue. Authorities have established the maximum residue levels (MRLs) for TBZ, CBZ and IMZ in fruits in the range from 0.05 to 15 mg kg^{-1}, depending upon the type of crop [44]. In the case of citrus fruits, the permitted levels have been established in 5 mg kg^{-1} for TBZ and IMZ, and 0.5 mg kg^{-1} for CBZ. However, and in order to protect the public health, the more restrictive regulation for drinking water [45] can be applied, establishing the maximum admissible concentrations for individual pesticides (and related products) in 0.1 and 0.5 μg L^{-1} for the total amount of pesticides.

2. Experimental LPME

2.1. Chemicals and samples

Thiabendazole (TBZ), carbendazim (CBZ), imazalil (IMZ), 2-propanol, 1-octanol, dodecylacetate, and sodium ocatonate were purchased from Sigma (St. Louis, MO, USA). Dihexyl ether, chloropentane, AS 4 silicone oil, 2-octanone, and formic acid were from Flucka (Buchs, Switzerland). Peppermint oil was obtained from Tamro (Oslo, Norway). Ethanol was from Arcus (Oslo, Norway). Methanol, sodium hydroxide, and hydrochloric acid were from Merck (Darmstadt, Germany). Orange juices samples were obtained from a local grocery in Oslo (Norway).

2.2. Standard solutions

Stock solutions containing 1000 mg L^{-1} of every analyte were prepared in ethanol and stored in darkness. Dilutions were made from the original solutions at the required concentration levels.

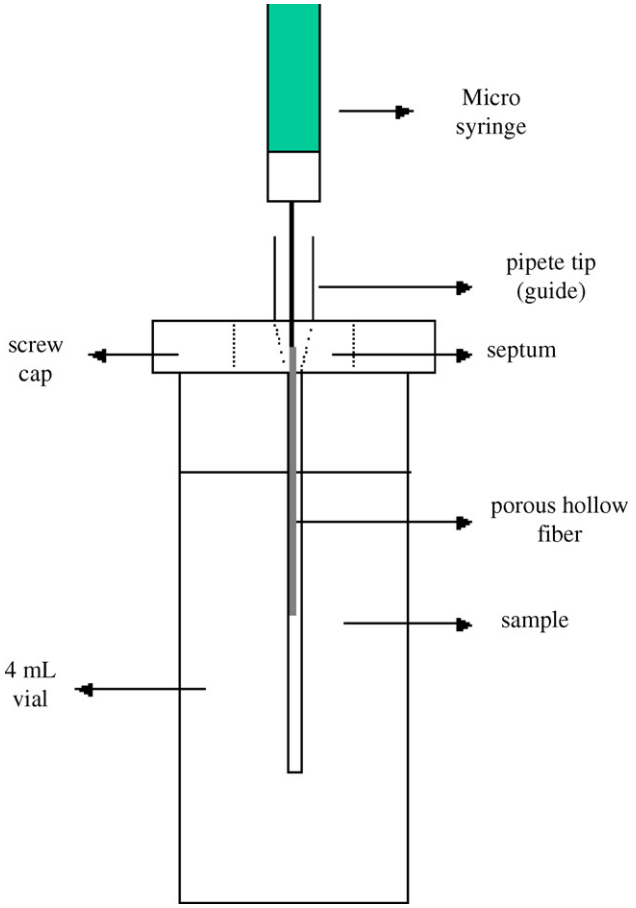

Fig. 1. Diagram of the hollow fiber-LPME device utilized.

When required, samples were spiked by addition of an appropriate amount of standard solution to the samples.

2.3. LPME device

The LPME device has been described in detail elsewhere [17]. In this work, LPME was carried out in 4 mL glass vials with a screw cap and a silicone septum from Supelco (Bellafonte, PA, USA). The porous hollow fiber used to support the organic phase and for containing the acceptor solution was a Q3/2 polypropylene hollow fiber (Membrana, Wupertal, Germany) with an internal diameter of 1200 μm, a 200 μm of wall thickness and 0.2 μm pores. A Model Finntip 200 Ext pipette tip (Thermo Scientific, Finland) was connected to a 2.2 cm piece of polypropylene hollow fiber. The lower end of the fiber was closed by mechanical pressure and the upper end was connected to the pipette tip that operated as guiding tube. This system consisting on the hollow fiber and the guiding tube was inserted through the silicone septum of a vial so that the fiber was immersed into the sample (Fig. 1).

2.4. LPME procedure

Both spiked and non-spiked samples (3 mL) were placed into the vials without any other pretreatment than the addition of 0.5 mL of an aqueous solution of 840 mM sodium hydroxide, in order to make the samples alkaline. A microsyringe (model # 805, Hamilton, Bonaduz, Switzerland) was used to inject the organic phase and the aqueous acceptor solution. A small quantity of organic solvent (20 μL) was injected into the lumen of the hollow fiber and immobilized from the inside of the fiber. After loading the SLM, the acceptor

solution (20 μL of 10 mM HCL) was filled into the lumen of the hollow fiber. After this, the hollow fiber was placed into the sample and the vial was agitated for an optimized time (30 min) at 1000 rpm using a Vibramax 100 agitator (Heidolph, Kelheim, Germany). After extraction, 15 μL of the acceptor solution was collected with a microsyringe, and finally transferred to a micro-insert for analysis by capillary electrophoresis or liquid chromatography–mass spectroscopy (LC–MS).

2.5. Capillary electrophoresis

Capillary electrophoresis was performed with a MDQ instrument (Beckman, Fullerton, CA, USA) coupled to a diode array detector (DAD). Separations were carried out using a 75-μm-I.D. fused silica capillary with an effective length of 50 cm (Beckman). The instrument was operated at 20 kV, generating a current level in the range of 45–55 μA. The running buffer was 25 mM phosphate adjusted to pH 2.7 with ortho-phosphoric acid. Activation protocol of the capillaries consisted on the introduction of NaOH 0.5 M at 20 psi for 30 min followed by H_2O for 2 min and buffer for 10 min. Capillaries were daily equilibrated passing NaOH for 10 min followed by H_2O for 2 min and buffer for 10 min. Samples were introduced by hydrodynamic pressure at 0.5 psi for 5 s. Capillary was rinsed with buffer for 1 min between runs. Detection was accomplished at 200 nm for CBZ and IMZ, and at 290 nm for the case of TBZ. At the end of the day, the capillary was rinsed passing buffer for 10 min, H_2O for 2 more min and finally, air for other 2 min.

2.6. LC/MS

The evaluation of LPME from orange juices was carried out by liquid chromatography and mass spectroscopy using a Shimadzu LC/MS-2010A system (Kyoto, Japan) equipped with a reverse phase C_8 analytical column of 50 mm × 1 mm and 5 μm particle size (Biobasic 8, Thermo Scientific, Norway). Mobile phase A was 95% of 20 mM formic acid and 5% of methanol. Mobile phase B was 5% of 20 mM formic acid and 95% of methanol. Extracts obtained from juices were diluted three times with mobile phase A and a volume of 30 μL was injected in each case. The chromatographic method was based on a linear gradient from 100% mobile phase A to 100% mobile phase B at 15 min. The composition was kept constant for 2 min at 100% B. The flow rate used was 0.05 mL min^{-1}. Finally, 100% mobile phase A was run for 7 min in order to re-equilibrate the column.

The LC system was connected to a mass spectrometer equipped with an electrospray interface operating in positive ion mode, using the following operating parameters: nitrogen at 1.5 L min^{-1} was used as drying gas, the block temperature was set at 200 °C, and the probe voltage was 4.5 kV.

2.7. Calculations

Recovery (R) was calculated according to the following equation for each analyte:

$$R = \frac{n_a \text{final}}{n_s \text{initial}} = \left(\frac{V_a}{V_s}\right)\left(\frac{c_a \text{final}}{c_s \text{initial}}\right) \times 100\%$$

where $n_{s\ \text{initial}}$ and $n_{a\ \text{final}}$ are the number of moles of analyte originally present in the sample and the number of analyte finally present in the collected acceptor solution, respectively; V_a is the volume of acceptor phase; V_s is the volume of sample; $c_{a\ \text{final}}$ is the final concentration of analyte in the acceptor solution; $c_{s\ \text{initial}}$ is the original analyte concentration within the sample.

3. Results and discussion

The post-harvest fungicides thiabendazole (TBZ), carbendazim (CBZ) and imazalil (IMZ) were selected due to their extensive use to prevent fruits form deterioration during transport and storage. Their structures are shown in Fig. 2, together with their pK_a and log P values (n-octanol/water partition coefficients). As seen from Fig. 2, log P varied significantly among the compounds, and one of the challenges of this work was to development a LPME method capable to handle this large log P window.

3.1. Initial experiences with water samples

In initial experiments, analytes were extracted from pure water samples spiked with a solution containing all the compounds. Due to the weak basic properties of the compounds, 0.5 mL of 70 mM sodium hydroxide was added to 3 mL of water sample in order to keep the species in their neutral state. The optimum agitation rate was set at 1000 rpm because worsening in the stability of the home-built extraction device can take place at higher speed. The rest of experimental conditions were set as described in the experimental section. A first screening of organic phases was performed to select the best candidates to be used in further optimizations. Seven different organic solvents with different chemistry were tested: 1-octanol, dihexylether, dodecylacetate, AS 4 silicon oil, 2-octanone, 1-chloropentane, and peppermint oil. All the solvents easily extracted IMZ, which is the most hydrophobic compound with log P = 3.8, and the recoveries of the extractions ranged approximately from 40 to 60%. Regarding to the recoveries of TBZ, with log P = 2.4, these ranged from less than 1% (in the case of the silicon oil) to 30% (when 2-octanone was used). Under such extraction conditions CBZ, with log P = 1.5, was very poorly extracted and only extractions using 1-octanol, peppermint oil, and 2-octanone showed any recovery, always below 5%.

These preliminary results were consistent with the conclusions obtained in previous studies [46], where it was demonstrated that basic compounds with log P below approximately 1.8 where ineffectively extracted by 3-phase LPME. Such kind of compounds remain in the donor phase because their high water solubility, and their hydrophilic nature prevented them from entering the organic phase. For these more polar compounds, in order to enhance the transport of the analytes through the supported organic liquid membrane, carrier-mediated LPME was recommended [19]. Fig. 3A shows an electropherogram of an extract obtained from a water sample using octanone as SLM. It can be observed two well-defined peaks corresponding to extracted TBZ and IMZ, whereas almost all CBZ remained in the sample. Electropherogram 3B corresponds to an extract from a sample solution containing 25 mM phosphate

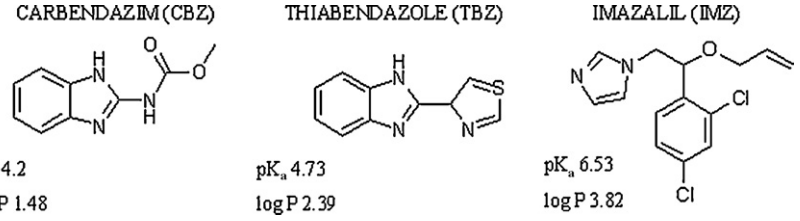

Fig. 2. Structures, pK_a and log P values of the target analytes.

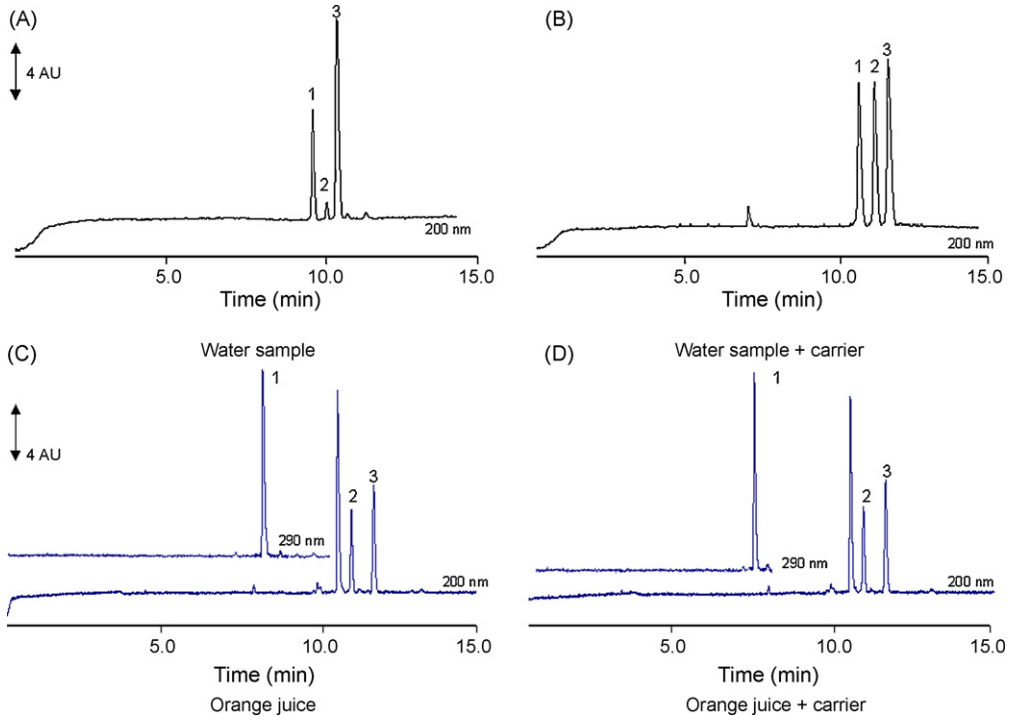

Fig. 3. Electropherograms of the extracts obtained after: (A) normal three-phase LPME from spiked water sample; (B) carrier-mediated LPME from spiked water sample; (C) normal three phase-LPME from spiked orange juice sample; (D) carrier-mediated LPME from spiked orange juice sample. All the samples were spiked at 0.2 μg mL^{-1}. Peak assignment: 1 TBZ; 2 CBZ; 3 IMZ.

buffer adjusted to pH 7.0 and octanoic acid as carrier to form ion-pair complexes. As suggested, the presence of the carrier clearly improved the extraction of CBZ.

3.2. LPME from real samples

As discussed briefly in Section 1, there are several parameters affecting to the efficiency of LPME. Among them, the composition of the matrix in the donor phase may be crucial. Taking this into account, it was decided to continue the optimization of LPME of TBZ, CBZ and IMZ using spiked orange juice samples.

First, extracts from spiked orange juices were obtained after LPME utilizing the same extraction conditions used for water samples (without carrier). As pH in the orange juices was lower than 4 in all the samples tested, 0.5 mL of 840 mM NaOH was added to 3 mL of spiked sample in order to achieve pH 10–11. Fig. 3C shows an electropherogram obtained under such extraction conditions. Whereas the addition of octanoic acid was needed to extract CBZ from water samples, all the three compounds were effectively extracted from different spiked orange juices without the addition of carrier into the sample. Obviously, some of the matrix constituents served as carrier when extractions were performed from the juice samples. The exact mechanism for this is currently unclear. In a separate experiment, carrier was added to the juice sample, but this system (Fig. 3D) provided similar results as the system without carrier, and therefore the remaining extractions in this work were carried out without any carrier added to the sample.

3.2.1. Selection of the organic phase

In a next series of experiments, the organic phase was optimized for extractions from orange juices (without carrier). As illustrated in Fig. 4A, both 1-octanol, 2-octanone, and peppermint oil worked as organic phase and were compatible with the juice samples. Superior results were obtained with 2-octanone, and this solvent was utilized for the rest of this work.

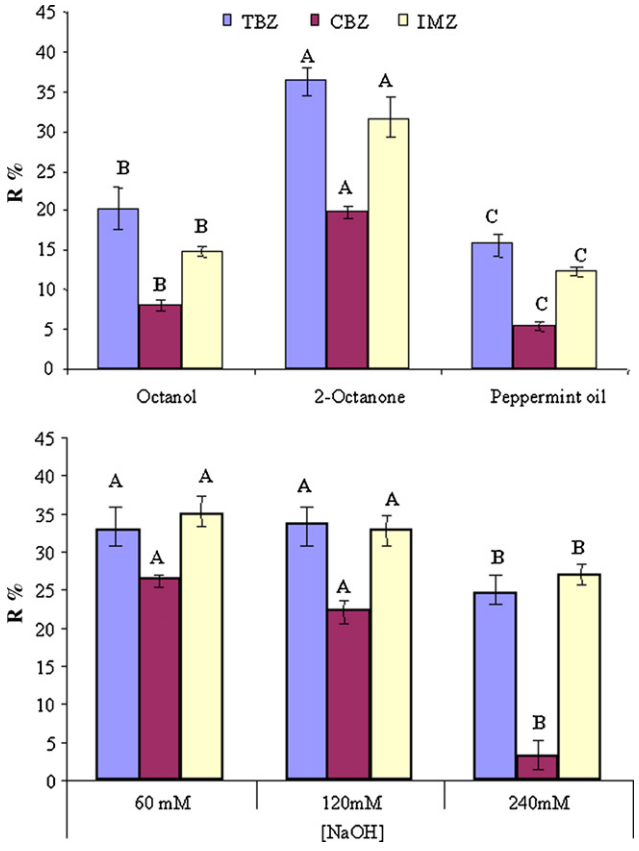

Fig. 4. Recoveries obtained after: (A) LPME from spiked orange juice using different organic phases; (B) LPME from spiked orange juice adding different concentrations of NaOH. All the samples were spiked at 0.2 μg mL^{-1}. A similar letter over the bars indicates that differences were not significative (according to ANOVA test).

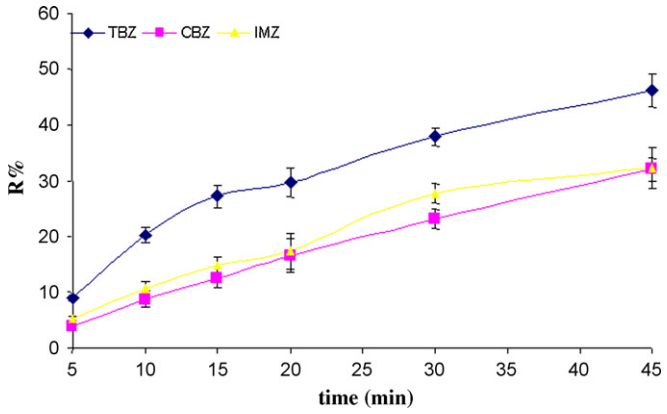

Fig. 5. Time curves of thiabendazole, carbendazim and imazalil obtained by representation of the recoveries *vs.* extraction time. All the samples were spiked at 0.2 μg mL^{-1}. Error bars correspond to four replicates on each measurement.

3.2.2. Selection of the pH

The composition and pH of the sample and the acceptor phase are also parameters affecting the efficiency of LPME. Different concentrations of hydrochloric acid and the more LC–MS friendly alternatives formic acid and trifluoroacetic acid were tested as acceptor solutions. Hydrochloric acid was found to be the most efficient acceptor phase. Although TBZ favored from increasing HCl concentration, a concentration level of 10 mM was utilized during the rest of the study. This was beneficial for CE analysis to avoid anti-stacking, and for LC–MS to maintain a reasonable compatibility with the HPLC column. To further improve the latter, the acceptor solutions were diluted with mobile phase prior to LC–MS.

Regarding to the pH of the sample, sodium hydroxide was needed in order to make the juices alkaline. Water solutions of 0.5 mL with different concentrations of sodium hydroxide were added to 3 mL of spiked juice sample. It was observed in Fig. 4B that the maximum recoveries were obtained for final concentrations of sodium hydroxide of 60–120 mM. At higher concentrations of NaOH, decreased recoveries of the analytes were observed, especially in the case of CBZ. Most probably, the highest pH partly suppressed the carrier-mediated transport of the analytes. For the rest of the study, 120 mM was used as the final concentration for NaOH in the juice samples. The reason for this decision was based on the variability of pH observed among the different orange juices tested. With the addition of 120 mM NaOH, alkaline conditions were guaranteed in all the kind of juices, even in the more acidic.

3.2.3. Selection of the extraction time

Recoveries of the analytes are highly dependent on the time that the sample is agitated favoring the transport through the organic phase to the acceptor solution. Consequently the extraction time was another important parameter to optimize for three-phase LPME. Several sets of extractions were performed using different extraction times. The agitation speed was 1000 rpm in all the cases. The results were displayed in Fig. 5. Recoveries increased continuously in the range up to 45 min, and even at this time equilibrium was not obtained. However, for this study, 30 min of extraction was used in order to maintain an acceptable time frame. In other words, extractions were accomplished under non-equilibrium conditions.

3.2.4. Applicability to different kinds of samples

According to the experiments discussed above, 2-octanone as the organic phase, an acceptor solution of 10 mM of hydrochloric acid, and 30 min of agitation at 1000 rpm were the conditions that provided the best performance. In order to check the robustness of the method, extractions were performed from six different orange juices purchased in local supermarkets. All samples were fortified to the same concentration level (0.2 μg mL^{-1}) with a solution containing the analytes before extraction. Table 1 shows the recoveries of each one of the analytes obtained after their extraction from the different samples. Although recoveries were below 34% for all the compounds, these results are consistent with values previously reported for liquid-phase microextraction of substances with similar log *P*. As seen from the results, only small variations were observed from sample-to-sample, which were within the experimental variations of the method. Thus, in spite of the fact that the six juices were from different producers, and their content of solid material (pulp) varied significantly, the extraction was not affected by the sample matrix. This was an important and interesting finding, especially taking into consideration that the extraction was promoted by natural carriers present in the sample (as discussed above).

Table 1
Recoveries and relative standard deviations (RSDs) obtained after liquid-phase microextraction of thiabendazole, carbendazim and imazalil from different kinds of orange juices spiked at 0.2 μg mL^{-1}.

	Recovery %		
	TBZ	CBZ	IMZ
Sample 1	33.7	22.3	27.9
Sample 2	29.3	21.4	24.6
Sample 3	31.2	19.0	28.2
Sample 4	26.6	17.0	19.4
Sample 5	29.9	20.1	23.1
Sample 6	30.1	19.9	24.6
RSD	8.6	10.4	14.8

3.3. Analytical performance

During the optimization of the parameters affecting the extraction, separation of extracts from spiked samples was performed by CE coupled to a diode array detector. Using this technique, a good separation of the selected analytes was achieved in very few minutes. However, although the developed extraction technique was able to concentrate the sample in a few microliters, limits of detection were in general poor and far from the requirements of the restrictive regulation for drinking water. In order to solve this drawback, LC–MS was selected to evaluate the analytical performance of the extraction procedure. Thus, extracts from spiked orange juice samples were analyzed by LC–MS using the conditions described in the experimental section. Extracts from non-spiked orange juice samples were also analyzed. One peak was observed at the retention time corresponding to CBZ and with the same m/z (in positive mode, $m/z = 192$). Different kind of non-spiked orange juice samples were checked and such peak was present in all of them. Therefore, CBZ was excluded from LC–MS analysis and the evaluation was carried out with TBZ and IMZ ($m/z = 202$ and $m/z = 297$, respectively).

3.3.1. Linearity

Linearity was checked with fortified orange juice samples in the range of 0.1–10 μg L^{-1}, and extracts were analyzed by LC–MS. Table 2 shows the equations corresponding to the obtained calibration curves for both TBZ and IMZ. Good correlation was observed in the range of concentrations studied, with $r = 0.999$ for TBZ and $r = 0.998$ for IMZ.

3.3.2. Precision

The repeatability was studied in terms of the relative standard deviation of the recoveries obtained at different spiking levels. Although extractions were not carried out under conditions of

Table 2
Equation and analytical parameters obtained from the calibration curves of thiabendazole and imazalil. Analysis were carried out by LC–MS.

Analyte	Equation	RSD slope	RSD intercept	r^2	RSD		LOD ($\mu g L^{-1}$)	LOQ ($\mu g L^{-1}$)
					Spiking level[a]	Spiking level[b]		
TBZ	$A = 2 \times 10^6 c + 2{,}57{,}183$	2.1	9.3	0.999	7.9	10.6	0.05	0.17
IMZ	$A = 3 \times 10^6 c + 4{,}06{,}589$	1.9	12.5	0.998	3.4	6.3	0.10	0.33

RSD is the relative standard deviation of the recoveries obtained using the calculated calibration curves; LOD is the limit of detection of the method for each analyte.
[a] Spiking level 0.5 $\mu g L^{-1}$.
[b] Spiking level 5 $\mu g L^{-1}$.

equilibrium, the RSD ranged between 3.4 and 10.6%, as shown in Table 2. These data were comparable with data obtained for plasma, whole blood, urine or breast milk [21,47–49] and can be considered acceptable for a manual microextraction technique without any previous sample treatment, using a home-built equipment and working in non-equilibrium conditions.

3.3.3. Limits of detection

The limits of detection were calculated as three times the average signal of the background noise obtained in the analysis of three blank orange juice samples at the retention times of the corresponding analytes. As it can be seen in Table 2, the limits of detection achieved following the present method were suitable for the analysis of pesticides in drinking water (LOD $\leq 0.1\ \mu g L^{-1}$). Limits of detection were indeed confirmed with experimental work.

4. Conclusions

The present work has demonstrated that three-phase LPME may be successfully utilized for the extraction of the fungicides TBZ, CBZ and IMZ, from different orange juices without any sample pretreatment step such as filtration, centrifugation, etc. These procedures might involve losses of analytes linked to the solid material. Following the proposed method, the analytes were extracted despite the solid material present in the samples. The matrix affected the LPME from orange juices and when the extractions were made from real samples, higher recoveries were obtained for TBZ and especially for CBZ than those obtained from water samples. The presence of a natural carrier in the matrix has been proposed, but the mechanism is uncertain. The analytical performance of the optimized method was tested and good linearity and repeatability was observed. Despite the fact that recoveries obtained were far from being quantitative, the sensitivity of the method was suitable for the limits of detection required for drinking water, which is the most restrictive regulation applicable for these kinds of samples.

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