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Comparison of microextraction procedures to determine pesticides in oranges by liquid chromatography–mass spectrometry

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Abstract

A liquid chromatographic–mass spectrometric method has been developed for the determination of bitertanol, carbendazim, fenthion, flusilazole, hexythiazox, imidacloprid, methidathion, methiocarb, pyriproxyfen and trichlorfon. Two procedures, based on stir bar sorptive extraction (SBSE) and matrix solid-phase dispersion (MSPD), have been evaluated for the extraction of these compounds in oranges. Their respective advantages and disadvantages are also discussed. The recoveries obtained by MSPD ranged from 47 to 96% and the relative standard deviations (RSDs) ranged from 1 to 15%, whereas with the SBSE method the recoveries were between 8 and 84% and the RSDs between 4 and 16%. Although, the limits of quantitation of most compounds are much better (0.001–0.05 mg kg^{-1}) by SBSE, it is not suitable to determine some polar pesticides as carbendazim, imidacloprid and trichlorfon. Results obtained by both methods were compared, in terms of sensitivity and selectivity, with a classical ethyl acetate extraction method, and the three methods were applied to analyze real samples. As MSPD is easier to perform, faster than the organic solvent extraction, and shows equal accuracy and resolution, its application for analyzing pesticides in oranges is recommended.

Keywords: Food analysis; Matrix solid-phase dispersion; Stir bar sorptive extraction; Pesticides

1. Introduction

The risk of pesticide residues depends on their ability to cause adverse health effects and the potential human exposure to their residues in the diet [1]. There is a strict legislative framework controlling the use of such substances with the aim of minimizing the risk to human health associated with the consumption of their residues. The European Union (EU) and the Spanish government have set tolerance levels for these compounds as maximum residue limits (MRLs), which are in the range of part-per-billion [2,3].

Although methods for determining pesticides in fruits, vegetables and other complex food matrices number in the thousands (based on i.e., gas chromatography and liquid chromatography), the pesticide residues analysis still represent an analytical challenge [4–6]. An adequate method for residue analysis should be sensitive, selective, accurate, precise, automated, cheap, applicable to a wide range of pesticides and matrices and capable of providing unambiguous structural information. However, such perfect methods are not encountered in practice [7].

Among the analytical approaches used in residue control, liquid chromatography (LC) is effective in separating non-volatile and thermally labile com-

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pounds as well as those gas chromatography (GC) compatible pesticides [8]. Mass spectrometry (MS) detectors have provided the most powerful confirmatory tool for screening applications. LC–MS has become a dominant analytical technique for the identification and quantitation of pesticides. However, its potential for multiresidue analysis and its compatibility with the fruits sample preparation procedures has not yet been totally checked [9–11].

The key step is the pretreatment of the sample to isolate interesting compounds from the matrix using a correct and efficient method. Over the years, several procedures have been developed with this aim such as liquid–liquid extraction, supercritical fluid extraction and extraction assisted by microwaves. Liquid–liquid extraction (LLE) methods to determine pesticide residues have been largely employed with satisfactory results. However, they are rather laborious and time consuming and large volumes of toxic extraction solvents are used [4–6].

With the currents trends toward miniaturization of sample preparation, several new methods have been introduced, e.g., matrix solid-phase dispersion (MSPD), solid-phase microextraction (SPME), and stir bar sorptive extraction (SBSE). These techniques offer environmentally safe extraction, essentially obviate the use of hazardous solvents, generate little waste and reduce time, space and glassware required for extraction [12,13].

MSPD conducts simultaneous disruption and extraction of solid and semi-solid samples [14]. The method involves the dispersal of the sample over a solid support, followed by washing and eluting with a small amount of sorbent. The method has been applied to isolate carbamates [15], benzoylureas [16] and fungicides [17] from fruits and vegetables followed by LC–MS.

SBSE is a new sampling technique, developed to extract organic analytes from liquid samples, which is based on the sorption of analytes onto a thick film of polydimethylsiloxane (PDMS) coated on an iron stir-bar. The stir bar is inserted into an aqueous sample and extraction takes place during stirring. Combined with thermodesorption–GC–MS, it enables low detection limits [18–20]. The capability of SBSE to determine organochlorine pesticides and chlorobenzenes in fruit and vegetables has been successfully tested [21,22]. As an alternative, the stir bar can be desorbed by liquid extraction, and the extract injected in the LC system [23].

The scope of this study is to evaluate SBSE and MSPD for the extraction of bitertanol, carbendazim, fenthion, flusilazole, hexythiazox, imidacloprid, methidathion, methiocarb, pyriproxyfen and trichlorfon in oranges followed by LC–MS determination. Several parameters governing the recovery of the analytes from the samples are optimized. Both procedures and a traditional organic solvent extraction method were compared to establish the most suitable technique for quantifying these pesticides. The methods were applied to measure the levels of pesticides in orange samples taken from the market.

2. Experimental

2.1. Chemicals

Bitertanol, carbendazim, fenthion, flusilazole, hexythiazox, imidacloprid, methidathion, methiocarb, pyriproxyfen and trichlorfon were obtained from Supelco (Madrid, Spain). The individual stock solutions were prepared by dissolving 100 mg of each compound in 100 ml of methanol, except for carbendazim, which was prepared in 10 *M* HCl instead of methanol. They were stored in glass-stopper bottles at 4 °C. Standard working solutions at various concentrations were prepared daily by appropriate dilution of aliquots of the stock solution in methanol.

Methanol and acetonitrile (gradient grade for liquid chromatography), and ethyl acetate, hexane, dichloromethane (organic trace analysis) were purchased from Merck (Darmstadt, Germany). Deionized water ($<$18 MΩ cm resistivity) was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All the solvents were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use.

MFE C_8 and MFE C_{18} solid phases (particle diameters in the range of 45–55 μm and pore diameter 60 Å) were acquired from Análisis Vínicos (Tomelloso, Spain).

The stir bars (Twister) were from Gerstel (Mülheim, Germany) with a length of 10 mm and coated with a 1 mm PDMS layer. Prior to use, stir bars were conditioned into a vial containing 15 ml of

acetonitrile, and treated for 5 min by sonication, then the solvent was rejected and the procedure repeated three times.

2.2. Liquid chromatography–mass spectrometry

A Hewlett-Packard (Palo Alto, CA, USA) HP-1100 Series LC–MS system equipped with a binary solvent pump, an autosampler, with the volume injection set to 20 μl, and a mass-selective detector with atmospheric pressure chemical ionization (APCI) coupled with an HPChem work station was used. Operating conditions of the APCI interface in the positive ion mode were vaporizer temperature, 350 °C; nebulizer gas (nitrogen) pressure of 60 p.s.i. (1 p.s.i.=6894.76 Pa); drying gas (nitrogen) flow-rate, 4 l min^{-1}; drying gas temperature, 350 °C; capillary voltage, 4000 V; and corona current, 4 μA.

Separation was performed on a Luna C_{18} column (150×4.6 mm I.D., particle size 5 μm) protected by a Securityguard cartridge C_{18} (4×2 mm I.D.), both from Phenomenex (Madrid, Spain). The mobile phase was methanol–water (40:60, v/v) and a gradient was used (from t=0 to 5 min, MeOH was set at 40%, from t=5 to 8 min MeOH was increased to 80%, from t=8 to 18 min MeOH was set at 80%, from t=18 to 20 min MeOH was increased to 90%, and then from t=20 to 25 min MeOH was set at 90%). The flow-rate was 0.8 ml min^{-1}.

Full-scan LC–MS chromatograms were obtained by scanning from m/z 50 to 400; with a scan time of 0.75 s. Time-scheduled selected-ion monitoring (SIM) of the most abundant ions of each compound was performed as is reported in Table 1 using the high resolution setting.

2.3. Sample preparation

A portion of sample (200 g of orange) was chopped and homogenized for 3 min at high speed using a Bapitaurus food chopper (Taurus, Berlin, Germany).

2.3.1. Stir-bar sorptive extraction

A 5-g portion was weighed and placed into a 25-ml Erlenmeyer flask and homogenized with 5 ml of methanol and 5 ml of water by sonication over 15 min. The resulting suspension was filtered through Whatman 40 μm filter and the filter cake was washed with 5 ml of water. The filtrate (15 ml) of the orange extract was placed in a 25-ml glass beaker and then, extracted using the stir bar for 2 h (stirring speed 900 rpm). After extraction the stir bar was removed from the aqueous sample with a tweezers and dipped for 1 min in clean water to remove the excess of sample matrix. Then the stir bar was placed into 2-ml vial that was filled with 500 μl of acetonitrile. Desorption of the pesticides was per-

Table 1
Time scheduled SIM conditions for monitoring pesticides

Pesticide	Group	Time (min)	SIM ion	Gain	Fragmentor (V)	Dwell time (ms)
Imidacloprid	1	0.0–6.5	256.0 212.0	1	60	199 199
Trichlorfon	2	6.5–8.7	111.0 257.0	1	60	199 199
Carbendazim	3	8.7–11.0	134.1 192.1	1	60	199 199
Methiocarb Methidathion	4	11.0–13.2	169.1 145.1	1	60	199 199
Flusilazole	5	13.2–14.5	316.0	1	100	400
Fenthion Bitertanol	6	14.5–18.0	279.0 269.0	1	80	199 199
Pyriproxyfen Hexythiazox	7	18.0–25.0	322.1 353.1	1	80	199 199

formed with an ultrasonic device for 10 min. After desorption, the stir bar was removed by a magnetic road.

2.3.2. Matrix solid-phase dispersion

A sample of 0.5 g placed into a glass mortar (50 ml capacity) was gently blended with 0.5 g of C_8 for 5 min, using a pestle, to obtain a homogeneous mixture. This mixture was introduced into a 100×9 mm I.D. glass column and conditioned with 0.2 ml of distilled water; then, 10 ml of dichloromethane was added to the column and the sample was allowed to elute dropwise by applying a slight vacuum. The eluent, which do not contain water, was collected in a graduated conical tube (15 ml). A 1-ml volume of methanol was added to the eluent to avoid evaporation to dryness and then, it was concentrated, under stream of nitrogen at ≤50 °C, to 0.5 ml.

2.3.3. Ethyl acetate extraction

A 50-g amount of chopped sample was placed in a 250-ml glass beaker and mixed thoroughly with 100 ml of ethyl acetate and 50 g of anhydrous sodium sulfate, using a Bapitaurus food chopper during 2 min. The homogenate was allowed to settle and the supernatant was passed through a filter paper into a 500-ml rotary-evaporation flask. The solid residue was again homogenized with 100 ml ethyl acetate, filtered through the anhydrous sodium sulfate and collected with the first extraction fraction. Twice 25 ml ethyl acetate was used to rinse the glass beaker and the rinsings were passed through the filter and collected. A rotary evaporator set at 40 °C and 250 mbar was used to evaporated the extract to less than 5 ml, and then, the extract was passed to a graduated conical tube (15 ml) and evaporated to dryness under a stream of nitrogen at ≤50 °C. The sample was reconstituted in 10 ml of methanol.

2.3.4. Recovery, precision and linearity studies

These studies were performed using a different working mixtures of the compounds.

A standard solution at concentrations of 10 μg ml^{-1} of carbendazim, flusilazole and pyriproxyfen, 25 μg ml^{-1} of imidacloprid, 50 μg ml^{-1} of methiocarb and trichlorfon, 75 μg ml^{-1} of hexythiazox, 100 μg ml^{-1} of bitertanol and methidathion, and 150 μg ml^{-1} of fenthion was used to calculate recovery and precision.

The percentage of recovery and the precision of the SBSE were determined at four spiked levels, by spiking with 12.5, 50, 500 and 1000 μl of the working mixture to 5 g of chopped untreated orange samples. Recovery and precision studies for MSPD were also carried out at four concentration levels by adding 2.5, 10, 25 and 50 μl of the working mixture to 0.5 g of orange. For the ethyl acetate the four concentration levels were obtained by adding 50, 200, 500 and 1000 μl. The spiked samples were allowed to stand for 1 h before extraction. Five replicated samples of each spiked level were extracted and analyzed.

Moreover, the recovery and the precision were calculated also at the limit of quantitation (LOQ) levels according to the EU guidelines. For this, different working mixtures were prepared at adequate concentration to spike the sample with 50 μl for each checked method.

LC–MS analysis may be complicated by suppression or enhancement of the response by matrix components, to avoid this, quantification was performed using a matrix matched standards and a five-point calibration curve.

The linearity was determined in the same range of concentrations than those indicated above for each method. The calibration was carried out using standard solutions and matrix matched standards.

3. Results and discussion

3.1. Optimization of MS fragmentation for pesticides

Table 2 summarizes the chemical structures, molecular weights, base peaks and the most abundant ions (with their relative abundance) of the mass spectra of the ten studied pesticides. Base peaks were most often the protonated molecules, except for trichlorfon, the mass spectra of which gave always the fragment m/z 110 corresponding to the characteristic fragment of organophosphorus pesticide molecules $[(CH_3O)_2POH]^+$. The highest responses were obtained at fragmentor voltages between 60 and 100 V depending on the compounds.

Table 2
Molecular and fragment ions and their relative abundance obtained by LC-APCI-MS at different fragmentor voltages

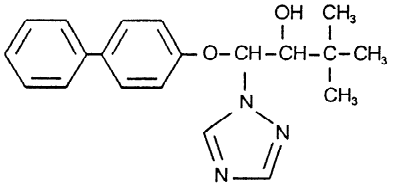
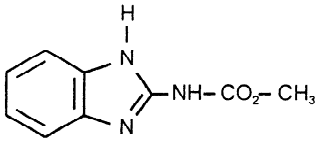
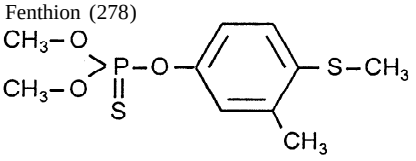
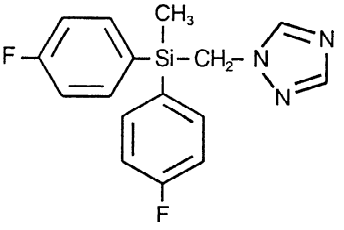
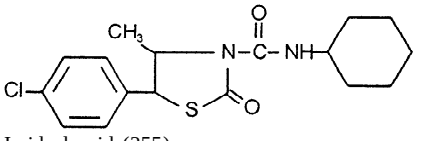
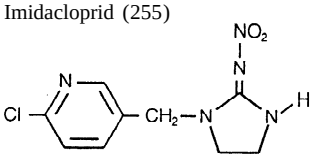
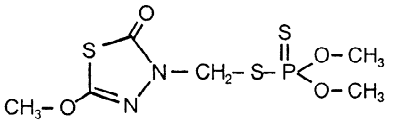
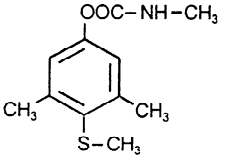
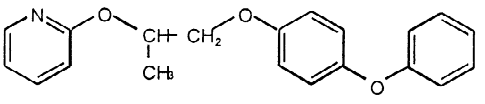
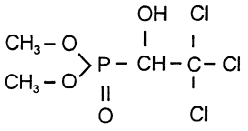
Pesticide (Mw)	*m/z* and tentative ion	Abundance (%)			
		20V	40V	60V	80V
Bitertanol (337) 	338 $[M+H]^+$	100	100	100	25
	269 $[M+H\text{-}C_2H_3N_3]^+$	–	25	80	100
Carbendazim (191) 	192 $[M+H]^+$	100	100	100	100
	160 $[M+H\text{-}CH_3OH]^+$	–	5	15	30
	134 $[M+H\text{-}CH_2{=}O\text{-}CO]^+$	70	72	70	60
Fenthion (278) 	279 $[M+H]^+$	100	100	100	100
	247 $[M+H\text{-}CH_3OH]^+$	–	–	–	15
Flusilazole (315) 	316 $[M+H]^+$	100	100	100	100
	247 $[M\text{-}C_2H_3N_3]^+$	–	–	–	10
Hexythiazox (352) 	353 $[M+H]^+$	100	100	100	100
	271 $[M+H\text{-}C_6H_{10}]^+$	–	–	12	25
	228 $[M+H\text{-}C_6H_{11}N{=}C{=}O]^+$	40	55	60	100
	168 $[M+H\text{-}C_6H_{11}N{=}C{=}O\text{ -SCO}]^+$	–	–	12	28
Imidacloprid (255) 	256 $[M+H]^+$	100	100	100	100
	212 $[M+H\text{-}N_2O]^+$	5	10	20	40
	175 $[M+H\text{-}NO_2\text{-}Cl]^+$	–	–	10	40
Methidathion (302) 	303 $[M+H]^+$	100	75	15	–
	145 $[M+H\text{-}HS_2P(OCH_3)_2]^+$	–	100	100	100

Table 2. Continued

Pesticide (Mw)	m/z and tentative ion	Abundance (%)			
		20V	40V	60V	80V
Methiocarb (225) 	226 $[M+H]^+$	100	100	50	–
	169 $[M+H\text{-}CH_3N{=}C{=}O]^+$	–	10	100	100
	149 $[M+H\text{-}H_2C{=}NH\text{-}CH_3SH]^+$	–	–	10	20
	121 $[M+H\text{-}CH_3N{=}C{=}O\text{-}CH_3SH]^+$	–	–	–	20
Pyriproxyfen (321) 	322 $[M+H]^+$	100	100	100	100
	227 $[M+H\text{-}C_5H_4NOH]^+$	–	–	5	20
	185 $[M+H\text{-}C_5H_4NO\text{-}CH(CH_3)_2]^+$	–	–	–	5
Trichlorfon (257) 	257 $[M+H]^+$	80	75	60	60
	110 $[(CH_3O)_2POH]^+$	100	100	100	100

As it can be seen in the SIM schedule for each group of ions (Table 1), the optimum fragmentor voltage can be selected. Carbendazim, imidacloprid and trichlorfon showed some molecular fragmentation, and the quantification can be achieved choosing the protonated molecule and one fragment ion. For fenthion, flusilazole, hexythiazox and pyriproxyfen, the protonated molecule was the only ion selected because their poor fragmentation, and for bitertanol, methiocarb and methidathion were selected the fragment ions m/z 269 $[M+H-C_2H_3N_3]^+$, m/z 169 $[M+H-CH_3N{=}C{=}O]^+$ and m/z 145 $[M+H-HS_2PO_2C_2H_6]^+$, respectively, which gave the highest sensitivity.

3.2. Optimization of the analytical procedures

3.2.1. SBSE

Preliminary experiments using SBSE show that imidacloprid, trichlorfon and carbendazim, which are the most polar of the studied compounds, are not recovered by this method and were not included in further experiments with SBSE. Parameters as the ion strength in the aqueous sample, pH value, desorption time of the fiber, and desorption solvent were evaluated. Experiments were performed by spiking 15 ml of a methanol–water (5 plus 10 ml) sample with 50 μl of the working mixture.

Ultrasonic treatment was used to accelerate desorption of the compounds from the stirrers, and a sonication time of 10 min was found sufficient for complete desorption. The best desorption profile and the smallest memory-effects were obtained desorbing the pesticides of the stir bar with 500 μl of acetonitrile. Under these conditions no carry-over effect was observed.

The addition of salt to the aqueous sample shows different effects on the extraction efficiency, as is demonstrated in Fig. 1. The recovery of the more polar compounds (methiocarb and methidathion) was

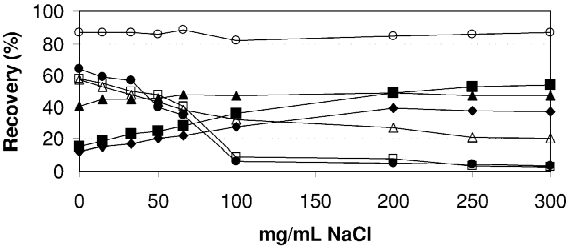


Fig. 1. Effect of salt concentration on the extraction yield of the pesticides studied. Compound identification: ○ bitertanol, △ fenthion, ▲ flusilazole, □ hexythiazox, ■ methidathion, ♦ methiocarb, and ● pyriproxyfen.

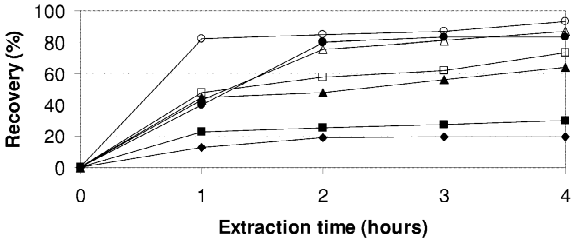


Fig. 2. SBSE exposure time profiles of the pesticides studied extracted from spiked water samples. Compound identification as in Fig. 1.

increased in proportion to the amount of sodium chloride because increasing ionic strength decreases their solubility in water. However, the recoveries of more apolar compounds (fenthion, hexythiazox and pyriproxyfen) decreased with the addition of salt. The recovery of flusilazole and bitertanol are not affected by this parameter. The best compromise was achieved adding 50 mg ml^{-1} of sodium chloride.

The influence of the pH value on the extraction yield was investigated choosing an exposure time of 60 min and a concentration of 50 mg ml^{-1} of NaCl in the sample. The extraction efficiency was independent of the pH of the sample. Thus, pH adjustment is not required.

Fig. 2 shows the exposure times profile of the individual pesticides. The extraction time was varied within 1–4 h. The extraction time was set at 2 h because the equilibrium was almost reached.

To demonstrate the influence of the orange matrix on the extraction efficiency of the SBSE, both an orange extract and a methanol–water sample were spiked with the analytes at the same concentration level (50 μl of the working mixture). After an interaction time of 2 h, the samples were analyzed using LC–MS. As is shown in Fig. 3, the influence of the matrix was negative for all pesticides. The lower extraction yield in presence of matrix compared with those obtained from the standards in the methanol–water mixture was apparently because prior the SBSE, the pesticides are not totally removed from the orange matrix with the methanol–water mixture.

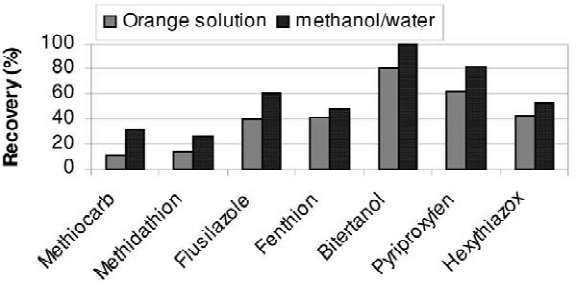


Fig. 3. Influence of the orange matrix on the extraction efficiency of SBSE.

The developed method was evaluated with respect to accuracy, precision and quantitation limits (Table 3). Analyte recoveries through the whole method (methanol–water extraction and SBSE) between 8 and 84% were calculated. The relative standard deviations (RSDs) are between 4% (hexythiazox) and 16% (fenthion). Although according to EU guidelines [24], the mean recoveries at each fortification level should be in the range 70–110%, SBSE is a non-conventional extraction technique and recoveries outside of the established range can be accepted. The LOQs obtained for the studied compounds were in the μg kg^{-1} level (see Table 3). The LOQ was defined as the lowest level for which acceptable recoveries and repeatabilities (<20%) are obtained [24]. The linearity was characterized by a coefficient of correlation better than 0.997, at least, three orders of magnitude in concentration. Carryover was controlled by a blank control desorption showing any remaining for pesticides.

SBSE combined with solvent desorption LC–MS detection enables the sensitive determination of methiocarb, methidathion, flusilazole, fenthion, bitertanol, pyriproxyfen and hexythiazox in oranges. Fig. 4 shows two SBSE–LC–MS chromatograms. Fig. 4a indicates a typical orange blank. Fig. 4b illustrates a spiked orange sample.

Main advantages of SBSE are the simplicity and the low LOQs obtained, but the technique present important disadvantages as the null or low recovery of some polar pesticides. Moreover, it is extremely hard to obtain commercially the twisters; however, sometime in the future this difficulty will be overcome, and even new types materials to cover the stir-bar will be developed, widening the range of pesticides that can be determined by this technique.

3.2.2. MSPD

The most suitable extraction conditions (type of

Table 3
Concentration range, recovery and relative standard deviations (RSDs) of the studied compounds in oranges obtained by SBSE

Compound	Concentration range[a] (mg kg^{-1})	Recovery[b] (%)	RSD[c] (%)
Methiocarb	0.02–2	8–13	6–9
Methidathion	0.05–4	10–15	7–10
Flusilazole	0.001–0.4	38–43	8–13
Fenthion	0.02–6	39–43	10–16
Bitertanol	0.01–4	77–84	6–10
Pyriproxyfen	0.002–0.4	60–64	9–13
Hexythiazox	0.008–3	37–43	4–8

[a] Five levels of concentration were tested, the lowest was the LOQ of the compound.
[b] Minimum and maximum values of the mean obtained from quintuplicate measurements for each spiked level.
[c] Minimum and maximum value of RSDs, which do not show dependence with concentration (n=5) for the different spiked levels tested.

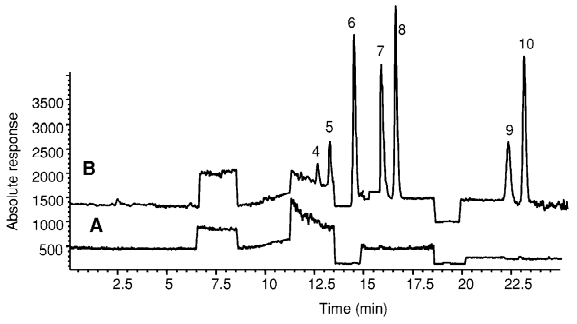


Fig. 4. Chromatograms of the SBSE–LC–MS analysis of (a) blank orange and (b) spiked oranges. Peak identification and concentration: (4) methiocarb (0.025 mg kg^{-1}), (5) methidathion (0.05 mg kg^{-1}), (6) flusilazole (0.005 mg kg^{-1}), (7) fenthion (0.075 mg kg^{-1}), (8) bitertanol (0.05 mg kg^{-1}), (9) pyriproxyfen (0.05 mg kg^{-1}), and (10) hexythiazox (0.037 mg kg^{-1}). For other conditions, see the Experimental section.

solid phase and eluent) were assessed by spiking orange samples at concentrations between 0.2 and 3 mg kg^{-1} depending on the pesticide. The results reported in Fig. 5 show that the recoveries were similar using either C_{18} or C_8 as dispersant agent. C_8 was preferred because it provided the cleanest chromatogram.

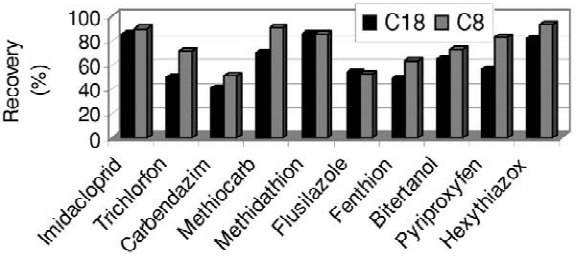


Fig. 5. Effect on the pesticide recoveries of C_8 and C_{18} using dichloromethane as elution solvent.

Dichloromethane, ethyl acetate, hexane and methanol were tested as elution solvent (Fig. 6). Hexane is only useful to elute the most nonpolar pesticides providing the worst recoveries and methanol achieves only the partial elution of hexythiazox and pyriproxyfen. Dichloromethane and ethyl acetate gave similar recoveries. Dichloromethane was considered optimal for the extraction because it gave the cleanest extracts.

Table 4 shows the recovery, precision and quantitation limits of MSPD. The recoveries ranged from 47% for carbendazim to 96% for the hexythiazox and the RSDs ranged from 1% for bitertanol to 15% for hexythiazox. The LOQs were between 0.008 and 0.3 mg kg^{-1}, always below the MRLs. Characteristic examples of LC–MS chromatograms of spiked and non-spiked orange samples are shown in Fig. 7.

3.3. Method comparison

Table 5 summarizes several parameters indicative of the analytical performance of the three methodologies described. As it has been already commented, the SBSE technique do not recover the most polar pesticides—imidacloprid, trichlorfon and carbendazim—but it allows the determination of non-polar and semi-polar organic compounds in aqueous matrices by liquid chromatography providing quantification limits similar to those obtained by LLE and MSPD.

MSPD provides better accuracy (recoveries were

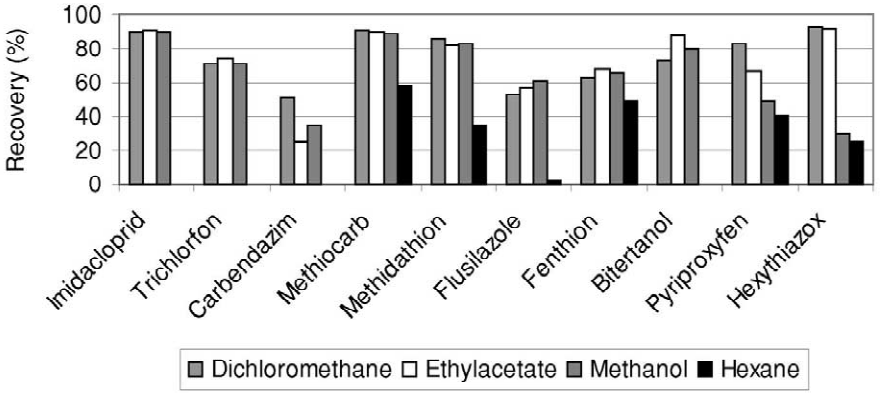


Fig. 6. Effect on the pesticide recoveries of different elution solvents using C_8 as dispersing material.

between 47 and 96%), repeatability (RSDs<15%) and extracts all the studied pesticides. When both microextraction methods were compared with traditional ethyl acetate extraction the main advantages are the avoidance of long concentration procedures and the significant reduction of the required volume of organic solvents.

The linearity of the calibration curves constructed from the analysis of spiked samples was good in all three procedures, with correlation coefficients always greater than 0.99. Matrix interference studies were carried out for the three procedures comparing these calibration curves with those obtained for standard solution (MSPD and ethyl acetate extraction) or spiked water samples (SBSE). Using ethyl acetate extraction, an important enhancement in the response owing to the matrix effect is observed for the majority of the compounds, more evident at high concentrations, whereas using MSPD also a slight enhancement of the response (ranging from 0 to 15%) dependent on the compound is noted. The amount of matrix per ml in the final extract varies between 10 g/ml for SBSE to 1 g/ml for MSPD. Taken this into account, the absence of matrix effect when using SBSE is an interesting feature of this technique.

Table 4
Concentration range, recovery and relative standard deviations (RSDs) of the studied compounds in oranges obtained by MSPD and maximum residue limits (MRLs) established by the EU [2] and the Spanish legislation [3]

Compound	Concentration range[a] (mg kg^{-1})	Recovery[b] (%)	RSD[c] (%)	MRL (mg kg^{-1})
Imidacloprid	0.02–2.5	87–93	6–12	1
Trichlorfon	0.12–5	68–74	3–6	0.5[d]
Carbendazim	0.008–1	47–53	3–5	5[d]
Methiocarb	0.06–5	88–94	2–3	0.05
Methidathion	0.1–10	82–88	3–6	2[d]
Flusilazole	0.01–1	49–55	2–3	0.01
Fenthion	0.3–15	60–65	2–5	0.5
Bitertanol	0.1–10	72–73	1–2	0.05
Pyriproxyfen	0.008–1	81–85	1–3	0.5
Hexythiazox	0.05–7.5	85–96	7–15	1

[a] Five levels of concentration were tested, the lowest was the LOQ of the compound.
[b] Minimum and maximum values of the mean obtained from quintuplicate measurements for each spiked level.
[c] Minimum and maximum value of RSDs, which do not show dependence with concentration ($n=5$) for the different spiked levels tested.
[d] Existence of EU legislation.

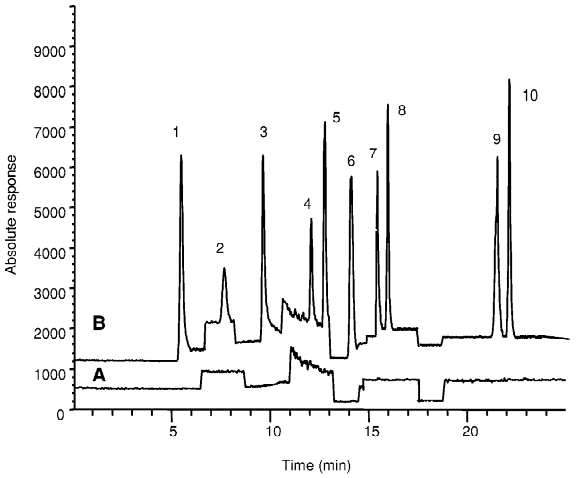


Fig. 7. Chromatograms of the MSPD–LC–MS analysis of (a) blank orange and (b) spiked orange. Peak identification and concentration: (1) imidacloprid (0.12 mg kg^{-1}), (2) trichlorfon (0.25 mg kg^{-1}), (3) carbendazim (0.05 mg kg^{-1}), (4) methiocarb (0.25 mg kg^{-1}), (5) methidathion (0.5 mg kg^{-1}), (6) flusilazole (0.05 mg kg^{-1}), (7) fenthion (0.75 mg kg^{-1}), (8) bitertanol (0.5 mg kg^{-1}), (9) pyriproxyfen (0.05 mg kg^{-1}), and (10) hexythiazox (0.4 mg kg^{-1}). For other conditions, see the Experimental section.

The sensitivity is one of the most important parameters in pesticide residues determination. The corresponding LOQs are better using SBSE (concentration factor 10) and ethyl acetate extraction (concentration factor 5) than using MSPD (concentration factor 1). Comparison between LOQs and MRLs showed that sensitivity of the three methods were good enough to ensure a reliable determination.

Of the three methods studied for isolating pesticides, MSPD was preferred for determining the compounds in orange samples. The proposed method offers simplicity and less consumption of solvents as advantages when it is compared with a classical LLE method.

Table 6
Pesticide concentrations in oranges obtained from an agricultural cooperative

Sample	Pesticide	Content[a], mg kg^{-1} (RSD, %)		
		SBSE	MSPD	Ethyl acetate
1	Carbendazim	-	0.02 (5)	0.03 (15)
	Hexythiazox	0.1 (12)	0.1 (10)	0.1 (8)
3	Carbendazim	–	0.02 (7)	0.02 (16)
	Hexythiazox	0.06 (11)	0.07 (10)	0.05 (12)
	Pyriproxifen	0.02 (14)	0.02 (12)	0.02 (14)
4	Carbendazim	–	1 (3)	1 (6)
	Hexythiazox	0.08 (10)	0.06 (8)	0.06 (12)
5	Carbendazim	–	0.06 (9)	0.05 (8)
6	Carbendazim	–	0.03 (16)	0.04 (11)
	Trichlorfon	–	0.1 (12)	0.1 (9)
8	Methidathion	0.5 (8)	0.6 (4)	0.5 (8)
9	Carbendazim	–	0.02 (10)	0.02 (13)
	Hexythiazox	0.07 (14)	0.06 (13)	0.09 (10)
	Methidathion	0.1 (7)	0.1 (5)	0.2 (8)
	Pyriproxifen	0.1 (8)	0.08 (12)	0.06 (17)
10	Trichlorfon	–	0.4 (6)	0.5 (5)

[a] Triplicate measurements.

3.4. Application to real samples

The three procedures were verified by analyzing 10 orange samples taken from an agricultural cooperative located near Valencia city. The results of samples containing pesticides are given in Table 6, and reveal the presence of several of the studied pesticides in oranges for human consumption, at concentrations usually in the μg kg^{-1} range. It is interesting to note the good agreement between the results obtained by the three procedures, except for imidacloprid, carbendazim and trichlorfon. This study also demonstrated that carbendazim and hexythiazox are the most ubiquitous of the selected

Table 5
Method performance comparison

	SBSE	MSPD	Ethyl acetate
Spiking concentrations (mg kg^{-1})	0.001–4	0.008–10	0.005–6
Accuracy (% recovery)	8–84	47–96	32–98
Repeatability (RSD, %)	<16	<15	<18
Linearity (r^2)	>0.995	>0.998	>0.997
Sensitivity (LOQ)	0.001–0.05	0.008–0.3	0.002–0.2
Applicability	Non polar pesticide	Wide	Wide

compounds. The pesticide concentrations found in oranges were always lower than the limits established by the EU [2] or the Spanish legislation [3], which demonstrated the good quality of the Spanish oranges for human consumption.

Fig. 8A displays the chromatogram of the sample number 9 extracted with the ethyl acetate method, while Fig. 8B and C show the chromatogram of the same sample extracted by SBSE and MSPD, respectively. Differences in sensitivity between the three extraction methods can be clearly observed in this figure as well as the absence of carbendazim signal using SBSE.

4. Conclusions

LC–MS determination provided sensitive and selective identification and quantitation of bitertanol, carbendazim, fenthion, flusilazole, hexythiazox, imidacloprid, methidathion, methiocarb, pyriproxyfen and trichlorfon. It can be successfully combined with the state-of-art extraction procedures to be applied for monitoring control of oranges.

SBSE was introduced as an alternative method for the extraction of pesticides from oranges. Advantages are the simple handling (only a methanol–water homogenization and filtration of the samples is necessary), the small solvent and sample amounts needed and its high selectivity. A disadvantage is the limited enrichment capability of polar pesticides as imidacloprid, carbendazim and trichlorfon. At any case, it is a promising technique, that in the future, can be a rapid and sensitive option to commonly applied extraction methods.

However, the results presented in this report indicate that MSPD is an excellent extraction technique for preconcentrating a wide variety of pesticides. Of the three methods studied for isolating pesticides, it was preferred to determine the selected compounds in orange samples.

The main advantage of the described microextraction methods compared with a traditional method is the significant reduction of the required volume of organic solvent.

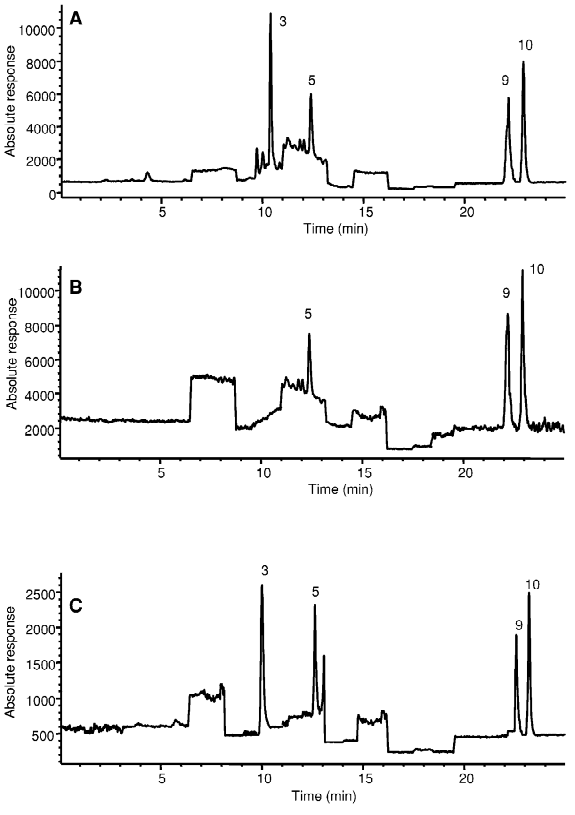


Fig. 8. LC–MS chromatograms of the orange sample number 9 after (A) ethyl acetate extraction, (B) SBSE and (C) MSPD. Peak identification as in Fig. 7. For concentrations see Table 6.

Acknowledgements

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