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Evaluation of solid-phase extraction and stir-bar sorptive extraction for the determination of fungicide residues at low-μg kg^{-1} levels in grapes by liquid chromatography–mass spectrometry☆

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Abstract

A liquid chromatography–mass spectrometry method has been developed for determining bitertanol, carboxin, flutriafol, pyrimethanil, tebuconazole and triadimefon. The evaluation of both atmospheric pressure interfaces (API), atmospheric pressure chemical ionization (APCI) and electrospray (ESI) using positive and negative ionization modes, clearly shows that the studied pesticides are more sensitive using APCI in positive mode. Two procedures based on solid-phase extraction (SPE) and stir-bar sorptive extraction (SBSE) have been assessed for extracting these compounds in grape. The recoveries obtained by SPE in samples spiked at the limit of quantification (LOQ) level ranged from 60 to 100% with relative standard deviation (R.S.D.s) from 7 to 17%. With the SBSE the recoveries obtained from samples spiked at LOQ level were between 15 and 100% and the R.S.D.s between 10 and 19%. The LOQs of most compounds are better by SPE (0.003–0.01 mg kg^{-1}) than by SBSE (0.01 mg kg^{-1} for all fungicides). Although SPE provided higher recoveries, lower R.S.D.s, best LOQs and is more rapid to carry out compared with SBSE, this last one has some advantages such as lower organic solvent consumption, and cleaner extracts. Results obtained applying both techniques to real samples are analogous.

Keywords: Food analysis; Solid-phase extraction; Stir-bar sorptive extraction; Fungicides; Pesticides

1. Introduction

Fungicides as bitertanol, flutriafol, triadimefon and tebuconazole (triazoles), carboxin (anilide) and pyrimethanil (pyridine) are intensively applied to grapes at various stages of cultivation and during post-harvest storage to provide protection against rotting [1,2]. Triazines, anilines and pyridines are important classes of fungicides with a wide range of useful activities. Many are systemic and they are highly active with as little as 60 g ha^{-1} being required (compared to the 250 g ha^{-1} for other fungicides as dithiocarbamates). They act by interfering with the synthesis of sterols, which are essential for the construction of normal cell membrane [3–5].

Although all these compounds have low mammalian toxicity, fungicide residue levels in foodstuffs are generally legislated to minimise the exposure of consumers to the harmful or unnecessary intake of pesticides; to control their correct use in terms of the authorisations or registrations granted (application rates and pre-harvest intervals); and to permit the free circulation of products treated with them as long as they comply with the maximum residue limits (MRLs) fixed [4–6]. MRLs are not toxicological limits but are toxicologically acceptables. Exceeded MRLs are strong indicators of violations of good agricultural practices. If MRLs are exceeded, comparison of the exposure with admissible daily intake (ADIs) will indicate whether or not there are possible chronic or

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acute health risks, respectively. Because of the reasons indicated above, monitoring residues of these fungicides in fruits and vegetables is still required [6].

The analysis of fungicides has been widely described in the recent literature and usually utilises the established multiresidue methods (MRM) of analysis [7,8]. These methods involve solvent extraction and partitioning followed by solid-phase or gel permeation cleanup to achieve removal of co-extractives present in the sample extract. Most analytical methods developed in the literature are modification and variations that can improve these extraction and cleanup methods through changes in technologies to reduce the analysis time because sample preparation is still the bottleneck in the analytical laboratory, occupying more than 60% of the analyst's time [8].

Advances could make by simplifying clean-up [9–12], improving extraction and miniaturization [9,12], increasing the use of liquid chromatography (LC) [11,13–18], intensifying automation [9], and introducing mass spectrometry (MS) detection [14–22]. A valid alternative is the enrichment on solid-phases cartridges, glass columns or disks packed with C_{18} [9,13,14], mixed cation exchange [10,11], hydrophilic/lipophilic balance phases [10] or polymeric resins [22]. Detection limits attained ranged from 0.1 to 180 $\mu g\,kg^{-1}$ depending on the compound and the determination technique used.

Another very elegant enrichment technique for aqueous extraction is the recently developed stir-bar sorptive extraction (SBSE). In SBSE, analytes are adsorbed into a magnetic rod coated with polydimethylsiloxane (PDMS) by stirring for a given time. After that, the stir-bar is either thermally desorbed on-line with capillary GC–MS or by organic solvents. SBSE has been already verified for analysing dicarboximide fungicides in wine [23], organophosphorus and carbamates in oranges [24], and organophosphorus pesticides in honey [25].

In the study, SPE and SBSE were evaluated to analyze bitertanol, carboxin, flutriafol, pyrimethanil, tebuconazole and triadimefon in grapes in combination with liquid chromatography–mass spectrometry (LC–MS). Several parameters controlling the recovery efficiency of the analytes from the samples are optimized. Both procedures were compared to establish the most suitable technique for quantifying these pesticides. The methods were applied to measure the levels of fungicides in grape samples taken from the market.

2. Experimental

2.1. Chemicals

Fungicides carboxin, flutriafol, tebuconazole and bitertanol were purchased from Riedel-de Haën (Seelze, Germany), pyrimethanil and triadimefon from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions of 1 $mg\,ml^{-1}$ were prepared by weighting and dissolving each pesticide in methanol and stored in glass-stopper bottles at 4 °C. The stock solutions were stable in the stored conditions for more than 3 months. Standard working solutions at various concentrations were daily prepared by appropriate dilution of aliquots of the stock solutions in methanol.

HPLC-grade methanol and organic trace analysis grade dichloromethane were purchased from Merck (Darmstadt, Germany). Deionized water (<8 cm MΩ resistivity) was obtained from the 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_{18} solid phase sorbent (particle diameter in the range of 45–55 μm and pore diameter 60 Å) was acquired from Análisis Vínicos (Tomelloso, Spain). The solid-phase (500 mg amount, 900 μl volume) was placed into a 100 mm × 9 mm i.d. glass column fitted with a coarse frit (No. 3). The column was preconditioned by passing through it 10 ml of methanol and 10 ml of deionized water.

The stir bars (Twister) were from Gerstel (Mülheim, Germany) with a length of 10 mm and coated with a 1 mm PDMS layer, that correspond to an amount of 55 μl of PDMS. Prior to use, stir bars were conditioned into a vial containing 15 ml of methanol, and treated for 5 min by sonication, then the solvent was rejected and the procedure was repeated three times.

2.2. Extraction and clean-up procedures

2.2.1. Extraction of grapes

A representative portion of the sample (ca. 200 g of grapes) was chopped and homogenized in a food chopper. Then, a 5 g portion was placed in 250 ml glass beaker and homogenized with 25 ml of water by sonication over 15 min. The resulting suspension was filtered through Albet 40 μm folded filters (Barcelona, Spain).

Linearity, percentage of recovery and the repeatability (within-day precision) were determined by adding 50 μl of the appropriate working mixture to the 5 g portion placed in a jar. The spiked sample was allowed to stand for 1 h before extraction to attain the pesticide distribution in grapes.

2.2.2. Solid-phase extraction (SPE)

The solution was passed under vacuum through a column containing 0.5 g of solid-phase C_{18}. The filtrate was discarded and the pesticides retained in the solid phase were eluted with 10 ml of dichloromethane–methanol (50:50, v/v). The eluent was collected in a graduated conical tube (20 ml) and concentrated at 50 °C, under a stream of nitrogen, to dryness. After that it was redissolved with 0.5 ml of methanol.

2.2.3. Stir-bar sorptive extraction (SBSE)

The filtrate was place into a 50 ml glass beaker and stirred with the stir bar, coated with PDMS, for 2 h at 900 rpm.

After the extraction, the stir bar was removed from the aqueous sample with a magnetic stirring bar and tweezers.

Table 1
Time scheduled SIM conditions for monitoring pesticides

	Group	Time (min)	SIM ion	Gain	Fragmentor (V)	Dwell time (ms)
Carboxin	1	0–8	236	1	100	132
			143			132
Flutriafol	2		302	1	100	132
Pyrimethanil	3	8–14	200	1	100	132
Triadimefon	4		294	1	100	132
			197			132
Tebuconazole	5	14–30	308	1	60	98
Bitertanol	6		269	1	60	98
			338			98

Then, the analytes were desorbed into 2 ml vial filled with 1 ml of methanol. Desorption of the pesticides was performed by sonication for 15 min.

2.3. 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 5 μl, and a mass-selective detector (MSD) with atmospheric pressure chemical ionization (APCI) coupled with an HPChem work station was used. Operating conditions of the APCI interface in positive ion mode were vaporizer temperature 325 °C; nebulizer gas (nitrogen) pressure of 60 psi (1 psi = 6894.76 Pa); drying gas (nitrogen) flow rate 4 l min^{-1}; drying gas temperature 250 °C; capillary voltage 4000 V; and corona current 10 μA.

Chromatographic separation was performed on a C_{18} column (15 cm × 0.4 cm i.d., 5 μm) from Phenomenex (Madrid, Spain) with a methanol–water gradient that started with 65% of methanol increasing linearly during 15 min until 80% of methanol. The flow rate was 0.8 ml min^{-1}.

Full-scan LC–MS chromatograms were obtained by scanning from *m*/*z* 80–340; with a scan time of 0.68 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.

3. Results and discussion

3.1. Mass spectrometry remarks

Although there are a lot of methods described now in the literature, established procedures for choosing the most sensitive interface or the best ionization mode do not exist.

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 six studied fungicides using APCI and electrospray (ESI) interfaces in positive ionization (PI) and negative ionization (NI) modes. The studied compounds gave response in positive and negative mode by both interfaces, except that bitertanol did not give a signal in ESI.

The ESI interface in PI mode provided mainly the protonated molecules and strong signal for sodium adducts. Only carboxin presents a fragment ion corresponding to the neutral loss of aniline. The sodium adduct is the main ion for flutriafol, triadimefon and tebuconazole whereas pyrimethanil did not form sodium adducts, confirming the theory that the sodium adduct formation requires a group that can donate a lone pair of electrons. Carboxin, flutriafol, triadimefon and tebuconazole contain carboxyl and hydroxyl groups that are absent in the pyrimethanil molecule.

The ESI interface in NI mode provided the deprotonated molecules as main ion for triadimefon and pyrimethanil. The main ion in the spectrum of flutriafol is the fragment obtained by the neutral loss of fluorobenzene, in the spectrum of carboxin the fragment obtained by the loss of ethene, and the only ion in the spectrum of tebuconazole is the fragment resulting from the loss of methyltriazole moiety.

The studied fungicides have similar mass spectra in APCI to those provided in ESI. However, in PI mode, there were no sodium adducts and some fragment ions can be obtained. Carboxin provides the fragment by neutral loss of aniline in higher proportion. Flutriafol, triadimefon and bitertanol commonly suffered the neutral loss of 69 u of the molecule that corresponds to the triazole ring. APCI in NI mode provided similar mass spectra for carboxin, flutriafol and tebuconazole to those obtained by ESI. Triadimefon provided a characteristic fragment ion at *m*/*z* 127 corresponding to the loss of *p*-chlorophenol, and bitertanol gave a fragment at *m*/*z* 169 that correspond to the *p*-phenylphenol. Fig. 1 illustrates some examples of the mass fragmentation observed.

A summary of the results in terms of limits of detection (LODs) obtained using full scan mode is shown in Table 3. The response varied from 250 pg for the six compounds in the APCI in PI mode, which are the most sensitive interface and mode, to no response in ESI for bitertanol. ESI was between 25 and 100 times less sensitive for the studied compounds than APCI. Taking into account these data, APCI in PI mode was chosen for further experiments. The system sensitivity was fully optimized using SIM. The time-schedule of SIM was performed following the procedure reported in Table 1.

Table 2
Molecular and fragment ions and their relative abundance both API interface in NI and PI modes at voltage fragmentor 100 V

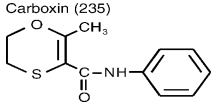
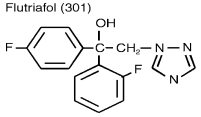
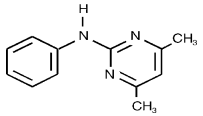
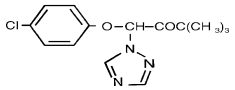
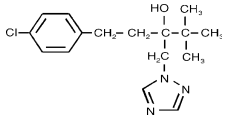
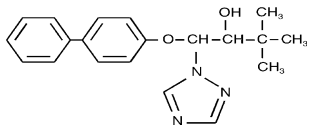
Compound (M_w)	ES				APCI			
	Positive (PI)		Negative (NI)		Positive (PI)		Negative (NI)	
	m/z tentative ion	*R*(%)	*m/z tentative ion*	*R*(%)	*m/z tentative ion*	*R*(%)	*m/z tentative ion*	*R*(%)
Carboxin (235) 	143 $[M + H - C_6H_6NH_2]^+$ 236 $[M + H]^+$ 258 $[M + Na]^+$	25 100 25	206 $[M - H - CH_2CH_2]^-$ 234 $[M - H]^-$	100 50	236 $[M + H]^+$ 143 $[M + H - C_6H_6NH_2]^+$	100 80	234 $[M - H]^-$ 206 $[M - H - CH_2CH_2]^-$	100 20
Flutriafol (301) 	302 $[M + H]^+$ 324 $[M + Na]^+$	20 100	300 $[M - H]^-$ 204 $[M - H - FC_6H_5]^-$	10 100	302 $[M + H]^+$ 233 $[M + H - C_2\ HN_3]^+$	100 15	300 $[M - H]^-$ 204 $[M - H - FC_6H_5]^-$	10 100
Pyrimethanil (199) 	200 $[M + H]^+$	100	198 $[M - H]^-$	100	200 $[M + H]^+$	100	198 $[M - H]^-$	100
Triadimefon (293) 	294 $[M + H]^+$ 316 $[M + Na]^+$	10 100	292 $[M - H]^-$	100	294 $[M + H]^+$ 225 $[M + H - C_2HN_3]^+$ 197 $[M + H - C_2HN_3C_2H_4]^+$	100 50 50	292 $[M - H]^-$ 127 $[M - H - ClC_6H_4O]^-$	100 10
Tebuconazote (307) 	308 $[M + H]^+$ 330 $[M + Na]^+$	25 100	223 $[M - H - C_3N_3H_5]^-$	100	308 $[M + H]^+$	100	306 $[M - H]^-$ 223 $[M - H - C_3N_3H_5]^-$	25 100
Bitertanel (337) 	–		–		338 $[M + H]^+$ 269 $[M + H - C_2HN_3]^+$ 99 $[CH_2COHC(CH_3)_3]^+$	20 100 100	169 $[C_{12}H_9O^-]^-$	100

Table 3
Detection limits (ng injected) obtained using full-scan mode with both interfaces APCI and ESI in PI and NI modes at fragmentor voltage of 100 V

	APCI		ESI	
	PI	NI	PI	NI
Carboxin	0.25	1	10	25
Flutriafol	0.25	0.25	10	25
Pyrimethanil	0.25	0.25	5	25
Triadimefon	0.25	0.25	10	125
Tebuconazole	0.25	0.25	5	25
Bitertanol	0.25	125	–	–

Table 4
Influence of water volume on the extraction efficiency

Pesticide	SPE volume (ml)				SBSE volume (ml)			
	10	25	50	100	10	25	50	100
Carboxin	88	105	95	52	55	58	20	28
Flutriafol	87	100	95	102	65	63	30	11
Pyrimethanil	95	102	97	101	57	50	25	17
Triadimefon	96	99	93	114	97	97	50	29
Tebuconazole	60	111	102	111	60	53	26	11
Bitertanol	103	112	110	105	44	44	26	18

3.2. Optimization of the extraction procedures

SPE and SBSE are significantly influenced by the aqueous volume selected, the amount of sample processed and the ionic strength of the medium. A set of experiments to determine the effect of these parameters in the recoveries of the studied compounds was designed. The elution step has already been widely studied in the literature for both techniques, and it is state that the best eluent for SPE is a mixture of dichloromethane–methanol since it provides highest recoveries and cleanest extracts, and that the best desorption of the compounds from the stir bar is accomplished sonicating 15 min with 0.5 ml of either methanol or acetonitrile [9,13,14,23–25].

Extraction efficiencies for a wide variety of compounds (depending on the polarity) can be improved increasing ionic strength since high ionic strength reduces their water solubility. As SBSE provided recoveries below 90% for most of the studied fungicides, this effect was tested adding 10, 20 and 30% (w/w) of sodium chloride (NaCl). The recovery of all fungicides was increased in proportion to the amount of sodium chloride, and double recovery percentages are achieved using the highest amount of 30%, which was added to the samples processed by SBSE. The recoveries obtained

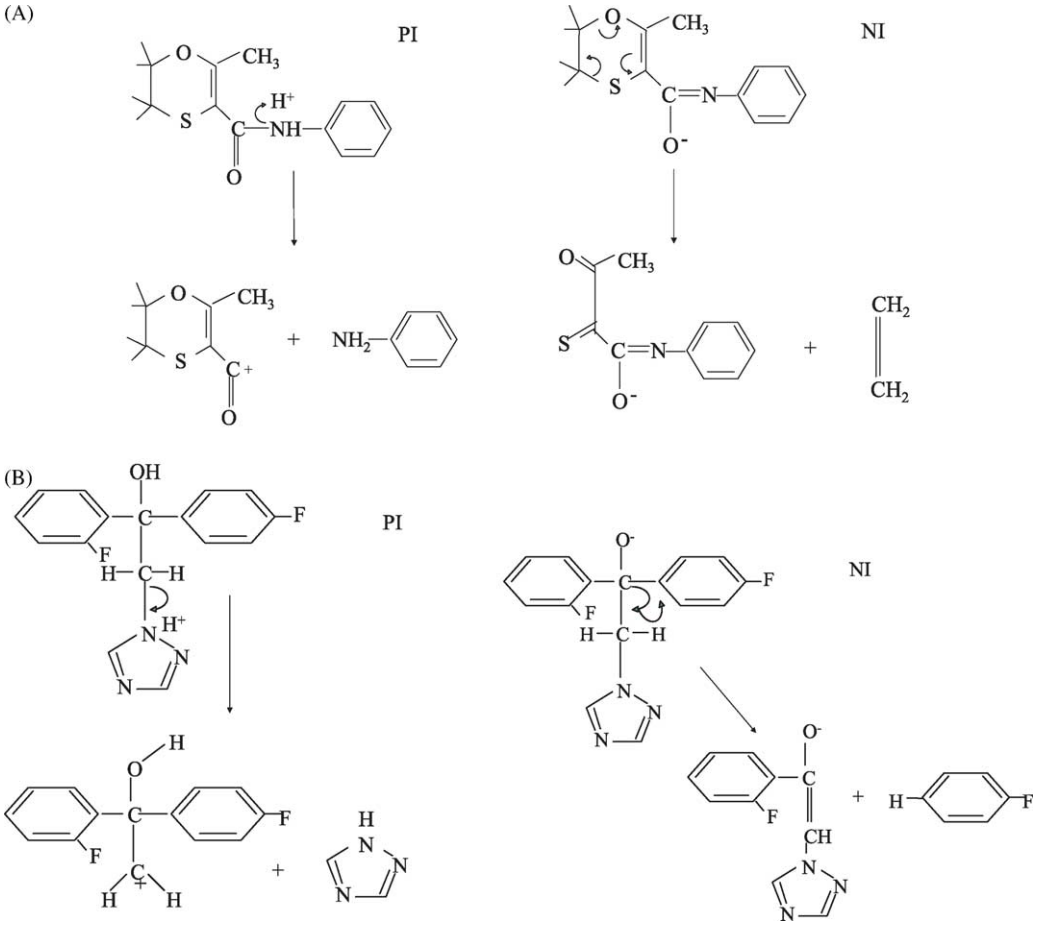


Fig. 1. Mass fragmentation patterns observed in PI and NI modes for (A) carboxin and (B) flutriafol.

Table 5
Influence of grape amount on the extraction efficiency

Pesticide	SPE amount of matrix (g)				SBSE amount of matrix (g)			
	2	5	7	10	2	5	7	10
Carboxin	13	33	10	35	3	15	14	13
Flutriafol	95	100	33	93	80	45	9	14
Pyrimethanil	14	60	28	57	69	65	13	12
Triadimefon	79	72	65	72	100	105	26	9
Tebuconazole	68	98	99	95	92	51	31	13
Bitertanol	96	97	100	79	27	21	13	10

by SPE (see the data presented below) do not require the salt addition.

Different water volumes (10–100 ml) were tested as it is shown in Table 4. The recoveries obtained using SPE for these range of volumes are around 100% and almost independent of the aqueous volume passed through it, except for carboxin, the recovery of which is reduced to the half for volumes up to 50 ml, and for tebuconazole that is recovered on a 40% less when the sample volume is less than 25 ml. For SBSE, the results are quite different. The lower the sample volume is, the higher the recovery obtained. SBSE recoveries ranged from 42 to 98% using 10 ml of water, and are maintained for 25 ml of water, suffering an important decrease for higher volumes that lead to recoveries from 20 to 50% for 50 ml and from 5 to 25% for 100 ml. A water volume of 25 ml was selected for further experiments as a compromise to obtain appropriate sensitivity with a water volume that achieved the dissolution of an appropriate quantity of grape.

The influence of grape matrix on the extraction efficiency of SPE and SBSE was checked diluting different amounts of grape in 25 ml of water. Table 5 illustrates the results in terms of recovery for SPE and SBSE. The matrix reduces the recovery obtained by SPE for carboxin, pyrimethanil and triadimefon whereas that obtained for flutriafol, tebuconazole and bitertanol are scarcely affected. Although the amount of grape tested (between 2 and 10 g) shows negligible effect on recovery, the variability of the results is greater with amounts up to 5 g. The effect of the grape matrix in the recoveries attained by SBSE presented a strong relation with the grape amount. Grape amounts of 2 g only reduced the recovery of carboxin and bitertanol respect to those obtained in pure water, grape amounts of 5 g also decreased the recovery

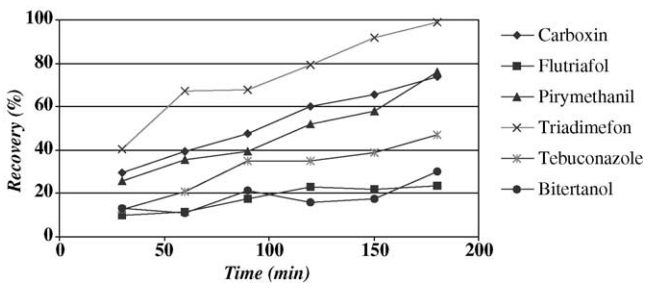


Fig. 2. Effect of stirring time with the PDMS stir-bar on the recovery of the studied pesticides. Amount of each pesticide in solution: 50 ng.

of flutriafol, and higher grape amounts (from 7 to 10 g) were negative for all fungicides. The amount of 5 g of grape was used for the following experiments, since it provided acceptable recoveries and good sensitivity for all studied fungicides by both extraction techniques.

The pH of the spiked samples oscillated from 4.1 to 4.3. The pH of the unspiked grape matrix and that of the spiked grape matrix were controlled to ensure that are equivalent. Optimization of sample pH was not carried out because all the analytes are protonated at low pH because they contain basic nitrogens, which enhanced their water solubility. These analytes are stables in aqueous solutions at slightly acid pH. The sample pH lower 4 can negatively affected the solid phase stability. Because of this, the sample pH was considered appropriate since it provides acceptable recoveries, which are comparable for flutriafol, tebuconazole and bitertanol to those obtained in the experiments performed with distilled water.

In addition, SBSE is an adsorption equilibrium and it is very influenced by the extraction time and temperature. Different extraction times were studied to obtain the sorption time profiles, which are presented in Fig. 2. A 120 min extraction time was selected for SBSE to avoid unreasonable analysis time. Equilibrium was not reached for any of the studied pesticides. However, quantitative analysis can be carried out because the samples are extracted exactly the same time and analytical sensitivity is rather satisfactory.

In quantitative analysis one of the major problems is the suppression/enhancement of the analyte signal in presence of matrix components, which has been reported by many authors [19–21]. Response suppression caused by sample matrix components using the ESI interface has been widely discussed in the literature [20–21]. However, the information about the effects of this class of interferences on APCI interface is more conflicting.

This interference can be established comparing the signal intensity obtained in a standard solution (methanol) with those obtained in matrix matched standards. This was carried out for both procedures. Using SPE a slight enhancement of the response (ranging from 0 to 15%) depending on the compound was noted whereas using SBSE the response of the standard prepared in methanol and the standard prepared in matrix extract was the same. The absence of matrix effect using SBSE is an interesting characteristic of this technique that has already been reported in the literature [24].

The use of matrix-matched calibration standards was not necessary to compensate for signal enhancement of target analytes in matrix solution compared to their response in pure solvent since the enhancement is really low.

3.3. Validation

Table 6 shows the mean recovery and precision obtained by SPE from samples spiked at the limit of quantification (LOQ) levels and at around 10 times the LOQ levels. LOQs were calculated according to the European Union Guidelines as the lower concentration that provides repeatabilities lower

Table 6
Recovery and R.S.D.s of the studied fungicides in grape samples spiked at LOQ and at 0.1 mg kg^{-1} (ca. LOQ × 10) obtained by SPE

Compound	Concentration (mg kg^{-1})	Recovery (%) ± R.S.D., n = 5	Concentration (mg kg^{-1})	Recovery (%) ± R.S.D., n = 5
Carboxin	0.003	67 ± 17	0.1	91 ± 12
Flutriafol	0.005	100 ± 8	0.1	99 ± 6
Pyrimethanil	0.008	60 ± 10	0.1	107 ± 9
Triadimefon	0.01	71 ± 9	0.1	102 ± 5
Tebuconazole	0.005	98 ± 8	0.1	98 ± 5
Bitertanol	0.003	96 ± 7	0.1	107 ± 4

than 20%. Fungicide recoveries were between 60 and 100% at the lowest concentration, and between 91 and 107 at the highest one. The relative standard deviations (R.S.D.s) were from 7% (bitertanol) to 17% (carboxin) and from 4% (bitertanol) to 12% (carboxin) for the lowest and highest concentration. The LOQ obtained, considering it as the lowest concentration for which the recovery and repeatability were acceptable, ranged from 3 to 10 μg kg^{-1}. Recovery only depends on concentration for three compounds carboxin, pyrimethanil and tebuconazole. The difference in recovery was only applied to the determination of the analyte concentration in the real samples when its concentration is close to the LOQ. Chromatograms of the SBSE–LC–MS analysis of an unspiked grape sample and grape sample spiked at 0.01 mg kg^{-1} of each compound are illustrated in Fig. 3A and B.

Table 7 reports the same data but corresponding to the SBSE. The recoveries ranged from 15% for carboxin to 100% for triadimefon and the R.S.D.s from 10% for triadimefon to 19% for carboxin and bitertanol at the lowest concentration whereas at the higher one, the recoveries were between 17% for carboxin to 101% for triadimefon with R.S.D.s < 17%. The LOQs were 10 μg kg^{-1} for all studied fungicides. Characteristic examples of LC–MS chromatograms of grapes spiked at LOQ level and non-spiked grapes samples are shown in Fig. 4. It is remarkable the lack of interfering peaks and the low background noise compared with the chromatogram obtained by SPE. The comparison of both chromatograms also pointed out the higher sensitivity of SPE.

Table 8 compares the parameters indicative of the analytical performance of the two methodologies described. SPE provided LOQs slightly lower than those obtained by SBSE (three times as much), recoveries higher and R.S.D.s lower than those obtained by SBSE. The low recoveries, higher R.S.D.s and worse LOQs obtained by SBSE compared to those from the SPE can be explained because the SBSE is based on reaching adsorption equilibrium whereas SPE is a

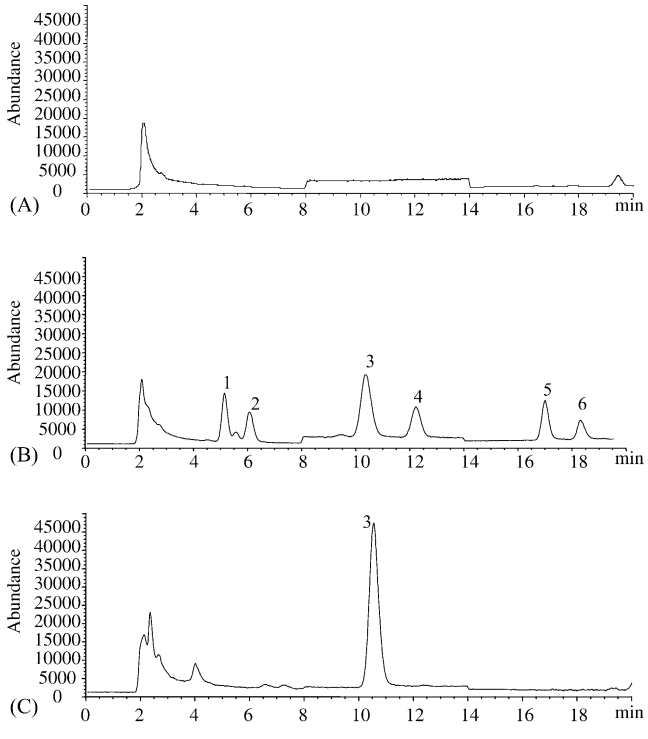


Fig. 3. LC–MS chromatograms in SIM mode obtained after SPE of (A) untreated grape sample, (B) untreated grape sample spiked at 0.01 mg kg^{-1} of each compound and (C) grape sample that contains 0.05 mg kg^{-1} of pyrimethanil. Peak identification: (1) carboxin, (2) flutriafol, (3) pyrimethanil, (4) triadimefon, (5) tebuconazole and (6) bitertanol.

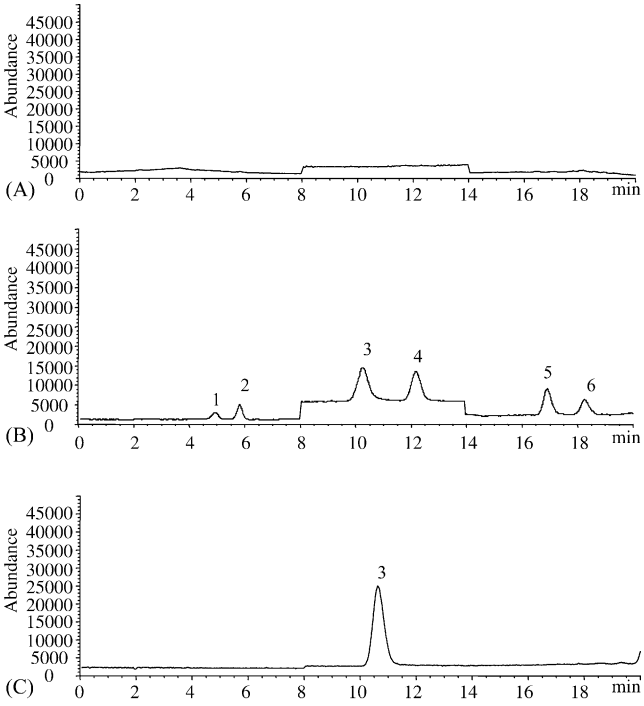


Fig. 4. LC–MS chromatograms in SIM mode obtained after SBSE of (A) untreated grape sample, (B) untreated grape sample spiked at 0.01 mg kg^{-1} of each compound and (C) grape sample that contains 0.05 mg kg^{-1} of pyrimethanil. Peak identification as in Fig. 3.

Table 7
Recovery and R.S.D.s of the studied fungicides in grape samples spiked at LOQ and 10 times LOQ obtained by SBSE

Compound	Concentration (mg kg^{-1})	Recovery (%) ± R.S.D., n = 5	Concentration (mg kg^{-1})	Recovery (%) ± R.S.D., n = 5
Carboxin	0.01	15 ± 19	0.1	17 ± 17
Flutriafol	0.01	45 ± 17	0.1	59 ± 16
Pyrimethanil	0.01	65 ± 16	0.1	73 ± 14
Triadimefon	0.01	100 ± 10	0.1	101 ± 8
Tebuconazole	0.01	55 ± 12	0.1	57 ± 15
Bitertanol	0.01	20 ± 19	0.1	25 ± 16

non-equilibrium process, based on partitioning between the aqueous extract of the sample and the solid-phase.

The linearity was evaluated at five concentrations, from the LOQ to 100 times the LOQ, showing correlation coefficients higher than 0.995 for SPE and 0.994 for SBSE. These coefficients (0.99) are relatively poor compared to conventional calibration techniques (0.999) because the extraction is included as it has been previously reported [25].

Other advantage of SPE is that is more rapid to perform that SBSE since it is not dependent on the sample equilibrium time. However, SBSE presents some advantages with respect to SPE as it reduces the organic solvent required, provides cleanest chromatogram and less matrix interference effect (in spite that this effect can be considered negligible in both techniques).

LOQs obtained by both procedures were always lower than MRLs established by the EU [6], Codex Alimentarius Commission of FAO/WHO [26], Food and Drug Administration (FDA) from the USA [27] and Spanish legislation [28], which are in the interval of 0.05–2 mg kg^{-1} for bitertanol, 0.2 mg kg^{-1} for carboxin, 0.01 mg kg^{-1} for flutriafol, 5 mg kg^{-1} for pyrimethanil, 2 mg kg^{-1} for tebuconazole and 2 mg kg^{-1} for triadimefon.

3.4. Application

SPE and SBSE procedures were applied for determining these fungicides in 15 commercial grape samples from different markets (these samples include red and white grapes of different varieties). Only pyrimethanil was detected in one Muscat grape. This sample was extracted by triplicate and each replicate was injected twice. The mean concentration value and the standard deviation were 0.05 ± 0.002 mg kg^{-1} by SPE and 0.05 ± 0.003 mg kg^{-1} by SBSE. Fig. 3C shows the chromatogram of the sample extracted by SPE and Fig. 4C displays the chromatogram of the sample obtained by SBSE. Excellent conformity is obtained by both procedures.

Table 8
Comparison of both methods features

	SPE	SBSE
Spiking concentration (mg kg^{-1})	0.01–1	0.01–1
Accuracy (% recovery)	67–106	15–100
Repeatibility (R.S.D., %)	4–17	8–19
Linearity (r^2)	>0.995	>0.994
Sensitivity (LOQ)	0.003–0.01	0.01
Organic solvent used to desorb analytes (ml)	10	0.5
Time required to process a sample (min)	90	150

4. Conclusion

The studied compounds have been well characterized by all the atmospheric pressure interfaces (API) sources and in PI and NI mode. The analysis of the six studied fungicides by SPE and SBSE has demonstrated to be an interesting alternative to more conventional methods that are usually more time consuming. SPE is more effective than SBSE to extract triazole, anilides and pyridines from grapes because it provides higher recoveries, lower R.S.D.s and best detection limits. In addition, the SPE procedure described is relatively simple and rapid. However, both procedures can be applied to determine bitertanol, carboxin, flutriafol, pyrimethanil, tebucanozole and triadimefon in real grape samples with comparable results.

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