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Liquid–liquid microextraction methods based on ultrasound-assisted emulsification and single-drop coupled to gas chromatography–mass spectrometry for determining strobilurin and oxazole fungicides in juices and fruits

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ABSTRACT

Two procedures are proposed based on ultrasound-assisted emulsification and single-drop liquid–liquid microextraction for the sensitive determination of seven strobilurin and six oxazole fungicides in fruits and juice samples. Both miniaturized techniques are coupled to gas chromatography with mass spectrometry in the selected ion monitoring mode, GC–MS(SIM). The procedures use low density organic solvents, and several factors influencing the emulsification, extraction and collection efficiency are optimized. The detection limits obtained at a signal-to-noise ratio of 3 are below the MRLs set by the European Commission. Enrichment factors are between 140–1140 for the first technique used and 80–1600 for the latter. The recoveries obtained for spiked samples are satisfactory for all compounds. The methods are validated according to the Commission Decision 2002/657/EC. Different fruit and juices are analyzed by the proposed method and none of the samples contained fungicide residues above the detection limits.

1. Introduction

Fungicide residues are frequently found in food destined for human consumption, some of which are dangerous to health and can induce drug resistance. Hymexazol, drazoxolon, vinclozolin, chlozolinate, oxadixyl and famoxadone are included in the group of oxazoles. Strobilurins are a new class of fungicides of the Quinone outside Inhibitors (QoI) group, which includes synthetic compounds such as azoxystrobin, metominostrobin, kresoxim-methyl, trifloxystrobin and, more recently, picoxystrobin, dimoxystrobin and pyraclostrobin, which act in a similar way to the natural strobilurin A, produced by the *strobilurus tenacellus* fungus [1]. However, they can leave residues, which must be controlled for food safety reasons. The European Commission (EC) has specified [2] a maximum residue limit (MRL) of 10 $\mu g\,kg^{-1}$ for pesticide residues in processed foods. The MRLs of both the strobilurin and oxazole fungicides in products of plant origin, including fruits and vegetables, have been established [3,4]. Consequently, efficient, reliable and very sensitive analytical methods are required and, in this respect, the European Union has established the minimal performance acceptable for analytical methods and the interpretation of results for the control of residues [5].

The most useful ways for determining pesticide residues in food samples are gas (GC) or liquid (HPLC) chromatographic procedures with different sample preparation methods [6–8]. Classical techniques for sample preparation, such as liquid–liquid extraction (LLE), have their inconveniences since they are tedious and time-consuming and require great volumes of sample and organic solvents. Emerging methods for food matrices tend towards efficient and miniaturized techniques that share the priorities of green chemistry with respect to the environment through the use of chemical processes that do not produce residues and which use low amounts of safe solvents for dissolving or extracting analytes [9].

Liquid-phase microextraction (LPME) includes several miniaturized techniques based on the extraction of analytes in a liquid phase using very low amounts of organic solvents [10]. Jeannot and Cantwell [11] developed single-drop microextraction (SDME) in which extraction was achieved into a small drop of a water-immiscible organic solvent. A wide range of solvents are available and the solvent can be collected easily, although extraction is time-consuming. To overcome this disadvantage, ultrasound-assisted emulsification extraction (USAEE) [12] and ultrasound-assisted emulsification microextraction (USAEME) [13] were developed

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using a heterogeneous system of two immiscible liquid phases, in which the main effects of ultrasounds are the fragmentation of one of the phases to form an emulsion of submicron droplet size that extends the contact surface between both liquids. The main advantage of both techniques is the high extraction efficiency achievable in a short period of time.

Several gas chromatographic procedures have been proposed for oxazole fungicides, such as famoxadone [14–17], vinclozolin [18–23], chlozolinate and oxadixyl [24–31], or mixtures [32] using different approaches for sample treatment. No methods have been developed for the GC determination of drazoxolon and hymexazol. With respect to strobilurins, azoxystrobin was the first to be used on a commercial scale and is still the most frequently used. Several GC methods have been developed with pesticides from other groups using different sample treatment procedures [27,29,33–40]. Mixtures containing several strobilurins have also been determined by GC–MS [14,25,28,41–61]. Very few procedures have been proposed for the application of LPME to these kinds of fungicides. Thus, vinclozolin has been determined using hollow-fiber liquid microextraction (HFLPME) [23,62,63] and chlozolinate and oxadixyl by pressurized liquid–liquid extraction (PLLE) [29]. Strobilurin mixtures have been determined by LPME and LC–MS/MS [64], PLLE [65] and HFLPME [66]. The SDME technique has also been applied to the determination of vinclozolin, kresoxim-methyl and azoxystrobin in fruits by headspace [67] and trifloxystrobin [68].

This study deals with the comparison of two procedures based on SDME and USAEME coupled to GC–MS for determining six oxazoles and seven strobilurins in juices and fruits. The main significance of our work is that this is the first time that all these fungicides have been determined using green chemistry principles avoiding the use of high amounts of solvents and the generation of residues. The developed methods were validated according to the criteria described in Commission Decision 2002/657/EC.

2. Experimental

2.1. Reagents

The analytical-reagent grade solvents methanol, tetrachloromethane, dichloromethane, chloroform, n-hexane and ethylacetate were purchased from Lab-Scan (Dublin, Ireland). Petroleum ether, isoctane, cyclohexane, octanone, undecanone, undecanol and decanol were obtained from Aldrich. Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Seven commercially available strobilurin fungicides (all from Riedel-de-Haën, Steinheim, Germany, >99%) were included in the optimization of the procedure: picoxystrobin [methyl (E)-α-methoxymethylene-2-(3-trifluoromethyl-2-pyridyloxymethyl) phenylacetate], metominostrobin [(Z)-2-methoxyimino-N-methyl-2-(2-phenoxyphenyl)acetate], kresoxim-methyl [methyl (E)-α-(methoxyimino)-2-(2-methylphenoxymethyl)phenylacetate], trifloxystrobin [methyl (E)-α-methoxyimino-2-[(E)-1-(3-trifluoromethylphenyl)ethylidenaminooxymethyl]phenylacetate], dimoxystrobin [(E)-2-(2,5-dimethylphenoxymethyl)-α-methoxyimino-N-methylphenylacetamide], pyraclostrobin [methyl{2-[1-(4-chlorophenyl)-1H-pyrazol-3-yloxymethyl] phenyl} methoxycarbamate] and azoxystrobin [methyl (*E*)-2-[2-[6-(2-cyanophenoxy)pyrimidin-4-yl]oxyphenyl]-3-methoxy-prop-2-enoate]. Six commercially available oxazole fungicides were also studied: Hymexazol (5-methylisoxazol-3-ol or 5-methyl-1,2-oxazol-3-ol) from Sigma (Steinheim, Germany, 90%), drazoxolon ((*EZ*)-4-(2-chlorophenylhydrazono)-3-methyl-1,2-oxazol-5(4*H*)-one or (*EZ*)-4-(2-chlorophenylhydrazono)-3-methylisoxazol-5(4*H*)-one), vinclozolin ((*RS*)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione), chlozolinate (ethyl(*RS*)-3-(3,5-dichlorophenyl)-5-methyl-2,4-dioxo-1,3-oxazolidine-5-carboxylate), oxadixyl (2-methoxy-*N*-(2-oxo-1,3-oxazolidin-3-yl)acet-2′,6′-xylidide) and famoxadone ((*RS*)-3-anilino-5-methyl-5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione) all from Riedel-de-Haën (Steinheim, Germany, >99%). Stock solutions (100 μg mL^{-1}) were prepared by dissolving the commercial products, without previous purification, in methanol, except famoxadone which was supplied as a 100 μg mL^{-1} solution in acetonitrile. They were kept at 4 °C in dark bottles sealed with PTFE/silicone caps. A working standard mixed solution was prepared daily by diluting with methanol.

Table 1
Retention time and target and qualifier ions for the fungicides.

Fungicide	t_R (min)	T	Q_1 (Q_1/T %)	Q_2 (Q_2/T %)	Q_3 (Q_3/T %)
Drazoxolon	7.90	127	129 (30)	130 (14)	131 (10)
Hymexazol	8.08	99	100 (10)	101 (10)	127 (10)
Vinclozolin	8.68	212	198 (96)	285 (70)	178 (50)
Chlozolinate	8.99	188	259 (90)	331 (60)	295 (30)
Picoxystrobin	9.53	145	335 (50)	303 (30)	295 (20)
Metominostrobin	9.73	191	196 (70)	238 (40)	295 (15)
Kresoxim-methyl	9.84	116	131 (60)	206 (44)	191 (35)
Oxadixyl	10.10	105	163 (85)	132 (55)	205 (30)
Trifloxystrobin	10.47	116	131 (74)	222 (20)	186 (15)
Dimoxystrobin	11.19	116	202 (65)	259 (35)	295 (10)
Famoxadone	12.60	196	224 (90)	330 (37)	388 (15)
Pyraclostrobin	13.80	132	164 (40)	325 (20)	372 (10)
Azoxystrobin	15.20	344	388 (40)	372 (10)	325 (13)

2.2. Instrumentation

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source and provided with a split-splitless injection port and a 78.5 mm × 6.5 mm O.D. × 4 mm I.D. liner. The mass spectrometer was operated using electron-impact (EI) mode (70 eV). The carrier gas helium was maintained at a constant flow of 0.5 mL min^{-1}. An HP-5MS UI (5% diphenyl–95% dimethylpolysiloxane, Agilent) capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) was used. Injection was carried out in the splitless mode at 250 °C and using a 40 psi pulse. The GC temperature was programmed as follows: start temperature of 70 °C (3 min hold), increase to 250 °C at 50 °C min^{-1}, increase to 320 °C at 10 °C min^{-1} and hold for 2 min. The temperatures of the ion source and the transfer line were 230 and 325 °C, respectively. The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using one target and two or three qualifier ions. Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound (Table 1). Solutions were stirred with a magnetic stirrer (IKA RH KT/C, Supelco) using PTFE-coated magnetic stir bars (10 mm × 6 mm O.D.). To prevent analyte evaporation, vials sealed with hole-caps and PTFE/silicone septa were used. To control the extraction temperature, a home-made heating system consisting of a drilled block provided with an electronic temperature control system was used.

A 50 Hz and 110 W ultrasonic water bath was applied to emulsify the organic solvent. For extraction and collection procedures, 4 or 15 mL glass vials were used. A 10 μL Hamilton plunger protected syringe was used for the collection of floated organic solvent.

An ultra-turrax T-25 (Jane and Kunkel, Ika-Labortechnick) was used for the grinding and homogenization of samples.

2.3. Samples

The samples of different types of juices, musts, canned fruits and fresh fruits (peach, peach and grape, apple, pear, orange, pineapple and carrots) were obtained commercially. The solid samples were crushed using an ultra-turrax to obtain a homogeneous purée. Recovery experiments were carried out using samples spiked with a standard mixture of fungicides. The samples were allowed to equilibrate at 4 °C for at least half an hour before starting the extraction procedure.

2.4. Analytical procedure for SDME GC–MS

All analyses were performed with 4 mL glass vials containing 1 g of the homogenized sample, 0.4 g (10% m/v) sodium chloride, 1 mL of 0.1 M phosphate buffer (pH 5) and water up to 4 mL. A 10 μL volume of the 1:1 (v/v) octanone–undecanone mixture and a 10 mm magnetic stir bar was introduced in the vial. Then, it was placed in the home-made heating module previously programmed at 50 °C and was maintained under magnetic stirring (1500 rpm) for 30 min. After this extraction step, the supernatant organic solvent was collected, while stirring, with a micropipette and transferred to a 100 μL eppendorf tube. An aliquot of 3 μL was injected in the injection port of the GC in the splitless mode at 250 °C and using a 40 psi pulse. Each sampling was performed in duplicate. To avoid contamination between samples, magnetic bars were washed with methanol after each extraction.

2.5. Analytical procedure for USAEME GC–MS

All analyses were performed with 15 mL conical-bottom glass centrifuge tubes vials containing 1 g of the homogenized sample, 0.4 g (10% m/v) sodium chloride, 1 mL of 0.1 M phosphate buffer (pH 5) and water up to 4 mL. A 20 μL volume of undecanone was injected and the mixture was shaken manually for a few seconds. The vial was immersed in an ultrasonic water bath in such a way that the level of both liquids (bath and sample) was the same and extraction was performed at 50 Hz of ultrasound frequency and 110 W of power for 4 min at 25 °C. Thus, oil-in-water emulsions were formed. Emulsions were then disrupted by centrifugation at 3000 rpm for 3 min and the organic phase floating at the top of the vial was collected with a micropipette and transferred to a 100 μL eppendorf tube. An aliquot of 3 μL was injected through the injection port of the GC in the splitless mode at 250 °C and using a 40 psi pulse. Each sampling was performed in duplicate.

2.6. Validation

The EU Commission Decision 2002/657/EC was used as a guideline for the validation of the proposed method and the following parameters were determined: linear dynamic range, selectivity, limits of quantitation, accuracy and precision. For the calculation of the decision limits (CCα) and the detection capabilities (CCβ), the fungicides were included in two groups. In the case of substances for which no permitted limit has been established, CCα was calculated by fortifying twenty blank materials at the quantitation limit and the corresponding concentration at the *y*-intercept plus 1.64 times the standard deviation of the within-laboratory reproducibility of the intercept was taken as the decision limit. CCβ was calculated by fortifying twenty blank materials at the quantitation limit and the corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit was taken as the detection capability. In the case of substances with an established permitted limit, CCα was established by fortifying twenty blank materials around the permitted limit and the corresponding concentration at the permitted limit plus 1.64 times the standard deviation of the within-laboratory reproducibility was taken as the decision limit. CCβ was established by fortifying twenty blank materials around the permitted limit and the corresponding concentration at the value of the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility was taken as the decision capability.

3. Results and discussion

3.1. GC–MS separation

A comparison of the optimal chromatographic conditions for fungicides was performed using two capillary columns coated with non-polar stationary phases, HP-1MS (100% dimethylpolysiloxane) and HP-5MS UI (5% diphenyl–95% dimethylpolysiloxane). Best separation was achieved with the HP-5MS UI column. Several temperature programmes were tested in order to obtain the best separation of the fungicides in the lowest possible time using undecanone as the solvent. The programme selected is summarized in Section 2. The flow of helium gas was varied in the 0.5–4 mL min^{-1} range and an optimal value of 0.5 mL min^{-1} was selected. Table 1 shows the retention times as well as the target and the qualifier ions selected for the fungicides studied under the chromatographic conditions finally used in the SIM mode. The correct sequence of the ions selected as a function of the eluting time for the best chromatographic conditions was: drazoxolon (127, 7.90 min), hymexazol (99, 8.08 min), vinclozolin (212, 8.68 min), chlozolinate (188, 8.99 min), picoxystrobin (145, 9.53 min), metominostrobin (191, 9.73 min), kresoxim-methyl (116, 9.84 min), oxadixyl (105, 10.10 min), trifloxystrobin (116, 10.47 min), dimoxystrobin (116, 11.19 min), famoxadone (196, 12.60 min), pyraclostrobin (132, 13.80 min) and azoxystrobin (344, 15.20 min).

3.2. Optimization of SDME and USAEME procedures

The optimization of the extraction techniques was done using both aqueous standards and a fruit juice sample. Organic solvents of different chemical characteristics, poorly soluble in water to prevent dissolution into the aqueous phase, of low volatility to avoid solvent evaporation during extraction, and differing in polarity were tested to achieve the best extraction of all the fungicides. The optimal solvent for SDME was selected from among several low density organic solvents, including decanol, undecanol, octanone and undecanone, or different solvent mixtures. A constant volume of 30 μL was used and the sample was stirred at 1500 rpm while the drop was collected. The results showed that best efficiencies were obtained with ketones, the most volatile fungicides (drazoxolon and hymexazol) providing the best extraction when using undecanone. For less volatile analytes, extraction was higher with octanone (Fig. 1). Consequently, as a compromise solution, a 1:1 (v/v) mixture of octanone-undecanone was selected. For USAEME, various solvents of higher or lower density than water, were assayed. First experiments were carried out using carbon tetrachloride, chloroform, tetrachloroethane and dichloromethane as denser than water solvents, and petroleum ether, cyclohexane, n-hexane, isooctane, ethyl acetate, decanol, undecanol, octanone and undecanone as lighter than water solvents. The organic solvent volume was 100 μL. Lower extraction efficiencies for most analytes were obtained when using the denser solvents, while better results were reached using the low density solvents, which presented no difficulty as regards collection of the organic solvent floating on the surface of the aqueous samples. The best extraction for most fungicides was achieved with undecanone, which was selected.

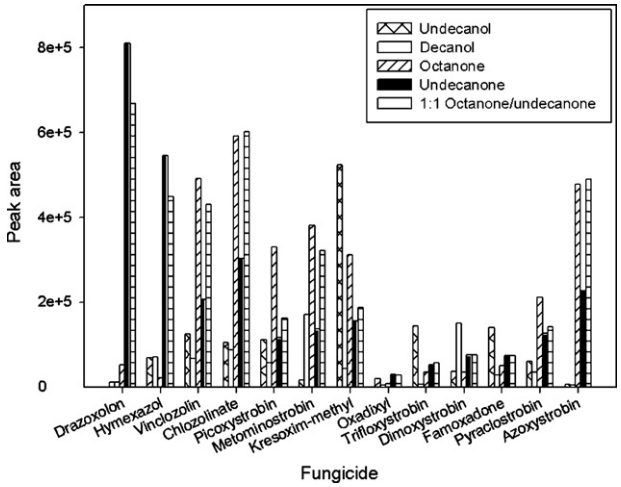

Fig. 1. Selection of extraction solvent for SDME. Organic solvent volume, 30 μL; extraction at 30 °C for 20 min. Analyte concentration: drazoxolon, hymexazol, pyraclostrobin, azoxystrobin, vinclozolin and chlozolinate 200 ng mL^{-1}, picoxystrobin, oxadixyl and famoxadone 100 ng mL^{-1}, kresoxim-methyl, dimoxystrobin, trifloxystrobin and metominostrobin 20 ng mL^{-1}.

The factors affecting the emulsification and extraction processes were then studied. The volumes of the donor and acceptor phases were optimized as the sensitivity of the method can be increased by decreasing the volume ratio of the acceptor/donor phase. When using SDME, the volume of the organic solvent was varied in the 10–70 μL range and extraction continuously decreased for higher volumes (Fig. 2). Volumes lower than 10 μL were tried but the single drop cannot be collected. For USAEME, the volume of the extraction solvent was varied between 20 and 100 μL. When using volumes smaller than 20 μL, the floating solvent is not discernible. Fig. 2 shows that extraction again decreased for all the analytes when higher volumes were used due to the dilution effect. Volumes of 10 and 20 μL were selected for SDME and USAEME, respectively.

The mass transfer between the aqueous and the organic phases is strongly affected by the temperature and the length of the extraction step. USAEME is a multiple microextraction technique because the organic solvent is dispersed into the aqueous phase, producing the emulsification and almost instantaneous mixture of the components. Thus, the equilibrium is rapidly reached. The diffusion coefficients of the analytes normally increased with temperature, although very high temperatures might decrease extraction due to the solvent evaporation. The effect of the temperature was studied between 25 and 80 °C and results indicated that solution temperature has no significant effect on extraction efficiency. When the extraction time was varied in the 1–10 min range (Fig. 3A), maximum extraction was achieved at 4 min for most compounds, because the contact surface was very large and the equilibrium was achieved in a few minutes. Thus, emulsification-extractions were performed at 25 °C and using a 4 min extraction time. Both parameters were also optimized for SDME. With this technique, the equilibrium of the compounds between the organic and the aqueous phases took longer and compromise time had to be selected to ensure high sample throughput. When the temperature was varied between 25 and 80 °C, for a time of 20 min (Fig. 3B), the extraction efficiency increased up to 50 °C for most fungicides and then decreased. When varying the extraction time between 5 and 60 min (Fig. 3C) maximum signals were obtained at 30–40 min for most compounds. Thus, a time of 30 min at 50 °C was selected in SDME to decrease the total analysis time. A comparison of the extraction time for both proposed miniaturized methods proves a clear advantage of USAEME over SDME.

The samples were shaken or stirred to accelerate the extraction kinetics. For USAEME, stirring was not necessary, as equilibrium was reached rapidly. For SDME, stirring rates in the 0–2000 rpm range were studied. Fig. 3D shows that extraction efficiency significantly increased for most compounds up to 1500 rpm, which was selected.

The effect of ionic strength on the extraction efficiency has been extensively studied in LPME. The pH of the donor aqueous solution can also be adjusted to decrease the solubility of target fungicides in the sample solution and to provide efficient transfer into the organic phase when they are mainly present in their neutral form. Consequently, the salting-out effect was studied by adding 0–36% (m/v) sodium chloride. Extraction improved for most compounds up to 10–12% m/v and then decreased using both enrichment methods, as the amount of collected solvent decreased. A 10% m/v concentration was selected. The pH effect was studied in the 2–7 range, using 0.01 M phosphate buffer solutions. Maximal extraction for most fungicides, except famoxadone, was obtained at pH 5, which was selected.

As indicated, when USAEME was used, the solvent was dispersed into the aqueous phase, producing emulsification of the components. To collect the extraction solvent it was necessary to break down the emulsion by including a centrifugation step, which was optimized. Optimal results for most of the analytes were obtained (Fig. 4) when centrifuging at 3000 rpm for 3 min.

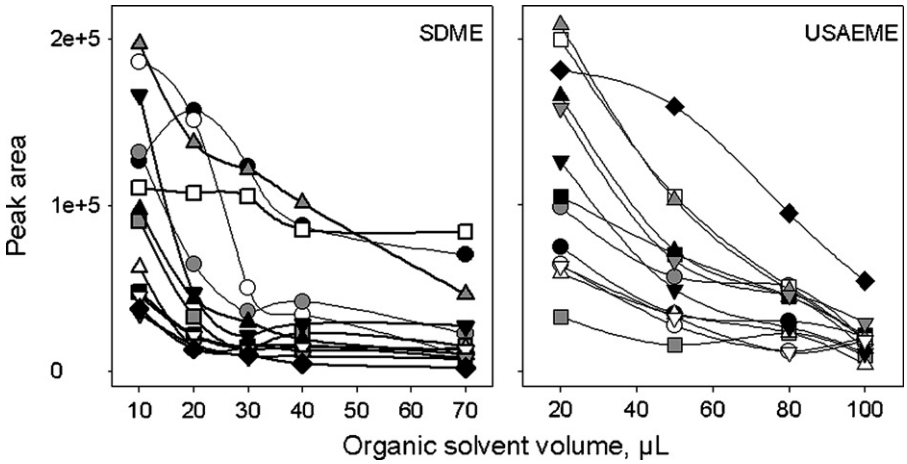

Fig. 2. Influence of the extraction solvent volume. SDME GC–MS, sample volume 4 mL, extraction at 30 °C for 20 min. USAEME GC–MS, sample volume 4 mL, extraction at 30 °C for 4 min. Symbols correspond to: Drazoxolon (●), hymexazol (○), vinclozolin (◉), chlozolinate (■), picoxystrobin (□), metominostrobin (▣), kresoxim-methyl (▲), oxadixyl (△), trifloxystrobin (◭), dimoxystrobin (▼), famoxadone (▽), pyraclostrobin (◬) and azoxystrobin (♦).

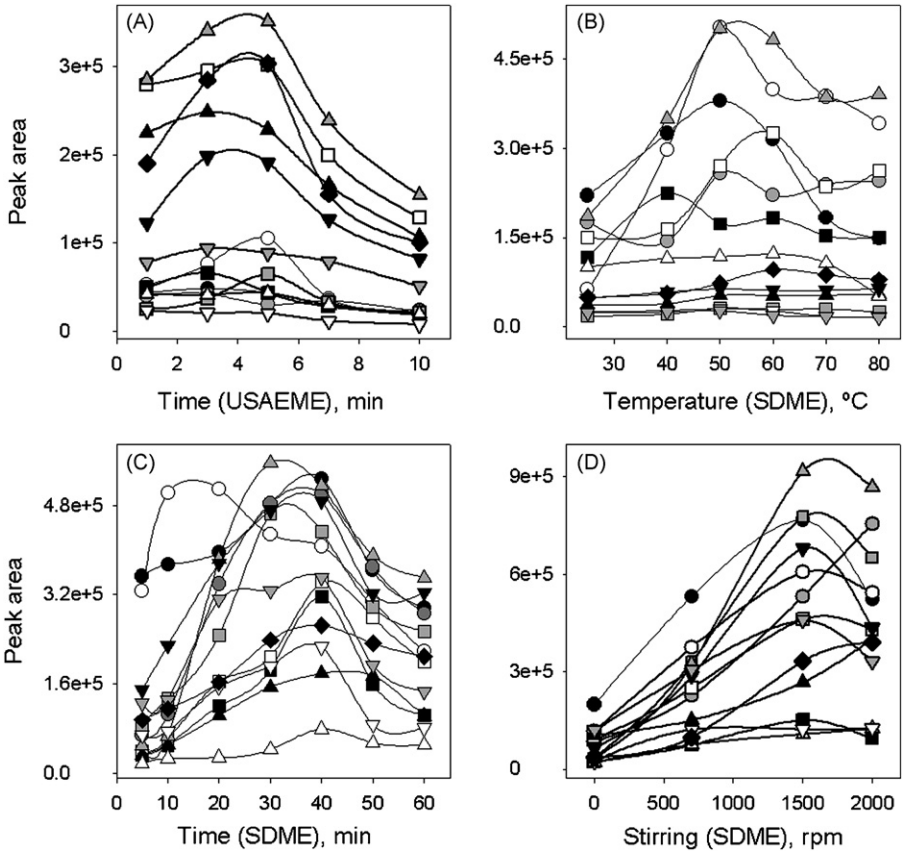

Fig. 3. Influence of the extraction time using USAEME (A) and the influence of the extraction temperature (B), extraction time (C) and stirring rate (D) using SDME. Symbols correspond to: Drazoxolon (●), hymexazol (○), vinclozolin (◍), chlozolinate (■), picoxystrobin (□), metominostrobin (◧), kresoxim-methyl (▲), oxadixyl (△), trifloxystrobin (◭), dimoxystrobin (▼), famoxadone (▽), pyraclostrobin (◬) and azoxystrobin (♦).

The volume to be injected in the GC was varied in the 1–4 μL range in order to increase sensitivity. When injecting volumes larger than 3 μL in splitless mode, column performance rapidly decreases and pulsed injection is recommended. A pressure was applied to the injector and the sample entered the column more rapidly than when there was no pulse. The best results were obtained with 40 psi and 3 μL, values which were selected for both extraction methods. The injection temperature was also varied between 180 and 300 °C and maximal signal for most compounds was achieved at 250 °C.

3.3. Study of the matrix effect

The response of the detector system to certain fungicides may be affected by the presence of co-extractives from the sample. This matrix effect of the different samples was evaluated by comparing the slopes of aqueous standards and standard additions calibration graphs for different fruit and juice samples, obtained by plotting concentration (at five levels) against peak area and following linear regression analysis. Table 2 shows the results obtained. Slopes for the samples were very similar to

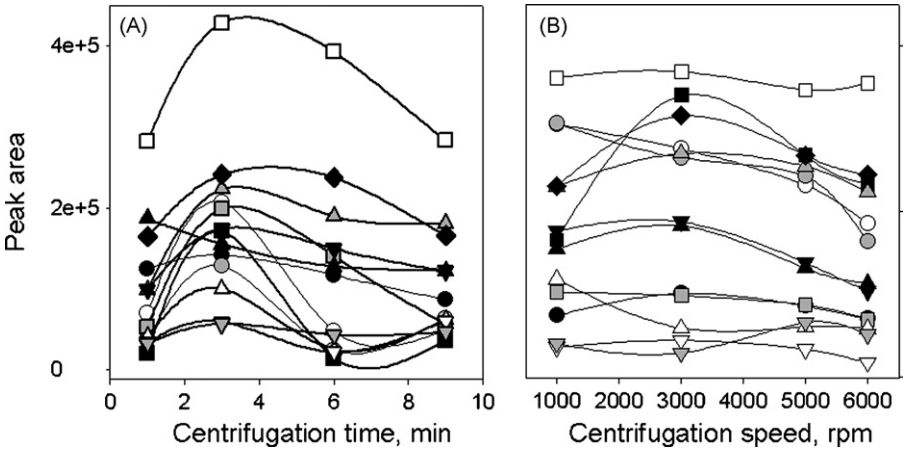

Fig. 4. Influence of the centrifugation time (A) and centrifugation speed (B) using USAEME. Symbols correspond to: Drazoxolon (●), hymexazol (○), vinclozolin (◍), chlozolinate (■), picoxystrobin (□), metominostrobin (◧), kresoxim-methyl (▲), oxadixyl (△), trifloxystrobin (◭), dimoxystrobin (▼), famoxadone (▽), pyraclostrobin (◬) and azoxystrobin (♦).

Table 2
Slopes of standard additions calibration graphs ($\times 10^{-3}$), mL ng^{-1}.

Fungicide	USAEME						SDME					
	Aqueous	Grape	Peach/grape	Apple	Canned fruit	Orange	Aqueous	Grape	Peach/grape	Apple	Canned fruit	Orange
Drazoxolon	75	74	65	66	71	61	20	19	18	15	19	18
Hymexazol	91	85	84	85	88	75	17	16	13	15	14	15
Vinclozolin	74	74	69	67	70	70	21	20	14	17	16	15
Chlozolinate	75	70	64	60	68	66	22	20	17	18	17	19
Picoxystrobin	322	316	314	299	299	262	160	155	158	155	154	146
Metominostrobin	249	248	221	194	175	183	154	137	119	133	124	129
Kresoxim-methyl	184	181	169	158	117	120	144	144	137	133	133	142
Oxadixyl	73	73	64	66	66	67	38	37	36	35	37	34
Trifloxystrobin	344	336	300	267	257	203	397	382	358	360	344	350
Dimoxystrobin	130	114	119	106	121	90	182	181	116	152	179	166
Famoxadone	47	47	42	44	43	42	29	28	25	26	28	29
Pyraclostrobin	41	37	40	37	36	31	29	27	26	24	24	26
Azoxystrobin	85	83	80	81	80	65	96	92	92	88	83	91

Table 3
Analytical characteristics of the methods.

Fungicide	Linearity (ng mL^{-1})		Detection limit (ng mL^{-1})		Enrichment factor (EF)	
	USAEME	SDME	USAEME	SDME	USAEME	SDME
Drazoxolon	0.50–100	1–100	0.075	0.21	341	91
Hymexazol	0.50–100	1–100	0.042	0.19	433	81
Vinclozolin	0.50–100	1–100	0.059	0.31	352	100
Chlozolinate	0.50–100	1–100	0.039	0.11	750	105
Picoxystrobin	0.06–100	0.1–100	0.010	0.029	520	258
Metominostrobin	0.06–60	0.1–100	0.017	0.050	452	466
Kresoxim-methyl	0.1–60	0.1–100	0.022	0.028	256	200
Oxadixyl	0.3–60	1–60	0.030	0.16	192	100
Trifloxystrobin	0.06–60	0.06–60	0.006	0.010	839	968
Dimoxystrobin	0.1–60	0.1–80	0.01	0.029	1141	1602
Famoxadone	0.5–100	1–100	0.051	0.15	142	108
Pyraclostrobin	0.5–200	1–300	0.059	0.13	1025	725
Azoxystrobin	0.5–100	0.5–300	0.071	0.04	405	480

Table 4
Validation of the procedure USAEME-GC–MS according to the criteria of Commission Decision 2002/657/EC.

Compound	Added (ng g^{-1})	Found ± SD (ng g^{-1})	Error α (1.64 × SD)	CCα (ng g^{-1})	Added (ng g^{-1})	Found ± SD (ng g^{-1})	Error β (1.64 × SD)	CCβ (ng g^{-1})
Drazoxolon	0.50[a]	0.66 ± 0.15	0.25	0.91	1	1.5 ± 0.56	0.92	2.4
Hymexazol	0.50[a]	0.43 ± 0.14	0.23	0.66	1	1.2 ± 0.30	0.49	1.7
Vinclozolin	50[b]	45 ± 4.8	7.9	53	53	59 ± 4.4	7.3	67
Chlozolinate	50[b]	49 ± 5.1	8.3	57	57	61 ± 2.7	4.5	66
Picoxystrobin	50[b]	56 ± 4.1	6.8	62	62	66 ± 8.8	14	81
Metominostrobin	0.06[a]	0.08 ± 0.016	0.026	0.11	0.1	0.30 ± 0.17	0.28	0.58
Kresoxim-methyl	50[b]	56 ± 6.4	10	67	67	69 ± 4.3	7.1	76
Oxadixyl	10[b]	11 ± 0.24	0.39	12	12	12 ± 1.2	1.9	14
Trifloxystrobin	20[b]	23 ± 0.94	1.5	25	25	25 ± 1.9	3.0	28
Dimoxystrobin	10[b]	10 ± 0.58	0.95	11	11	12 ± 1.2	2.0	14
Famoxadone	20[b]	19 ± 1.6	2.6	22	22	25 ± 1.9	3.1	28
Pyraclostrobin	20[b]	22 ± 2.1	3.5	26	26	30 ± 1.4	2.3	32
Azoxystrobin	50[b]	50 ± 5.0	8.2	59	59	64 ± 6.2	10	74

[a] LOQ for banned fungicides.
[b] MRL for fungicides with established permitted limits.

Table 5
Validation of the procedure SDME-GC–MS according to the criteria of Commission Decision 2002/657/EC.

Compound	Added (ng g^{-1})	Found ± SD (ng g^{-1})	Error α (1.64 × SD)	CCα (ng g^{-1})	Added (ng g^{-1})	Found ± SD (ng g^{-1})	Error β (1.64 × SD)	CCβ (ng g^{-1})
Drazoxolon	1.0[a]	1.6 ± 0.29	0.48	2.1	2	2.8 ± 0.17	0.28	3.0
Hymexazol	1.0[a]	0.93 ± 0.03	0.049	0.98	1	1.7 ± 0.25	0.41	2.1
Vinclozolin	50[b]	47 ± 2.1	3.3	51	51	47 ± 1.1	1.8	49
Chlozolinate	50[b]	53 ± 2.0	3.3	57	57	59 ± 1.6	2.7	62
Picoxystrobin	50[b]	54 ± 4.4	7.3	62	62	64 ± 3.3	5.4	70
Metominostrobin	0.1[a]	0.17 ± 0.033	0.054	0.22	0.20	0.34 ± 0.02	0.033	0.37
Kresoxim-methyl	50[b]	56 ± 2.7	4.5	60	60	62 ± 3.0	4.9	67
Oxadixyl	10[b]	8.8 ± 0.94	1.5	10	10	10 ± 1.0	1.6	12
Trifloxystrobin	20[b]	19 ± 1.8	3.0	22	22	21 ± 2.5	4.1	25
Dimoxystrobin	10[b]	10 ± 0.98	1.6	12	12	13 ± 0.76	1.2	14
Famoxadone	20[b]	18 ± 1.0	1.7	20	20	19 ± 1.1	1.8	21
Pyraclostrobin	20[b]	19 ± 1.7	2.9	22	22	20 ± 2.8	1.7	22
Azoxystrobin	50[b]	47 ± 3.0	4.8	52	52	50 ± 4.0	6.5	56

[a] LOQ for banned fungicides.
[b] MRL for fungicides with established permitted limits.

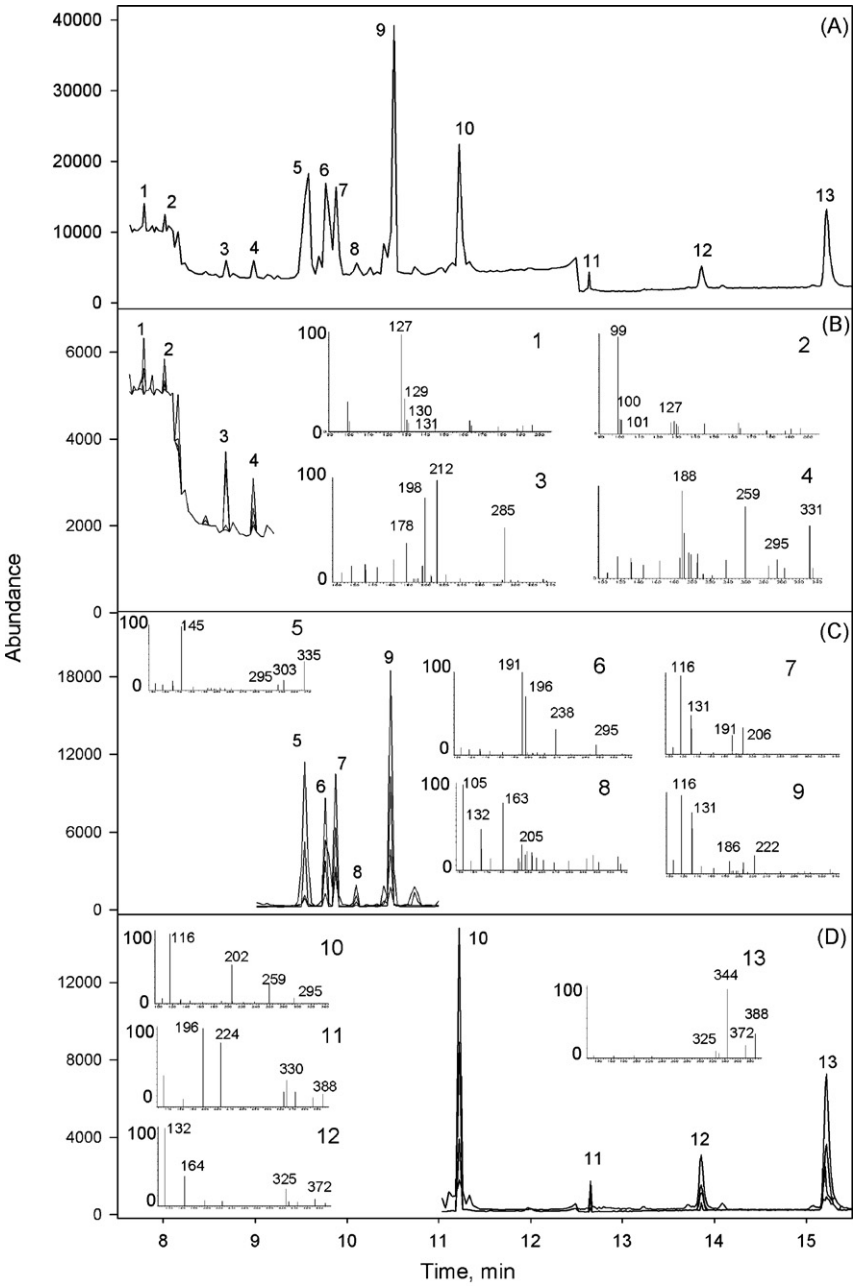

Fig. 5. (A) Elution profile obtained for a spiked canned pineapple sample by USAEME GC–MS. Sample volume, 4 mL; extraction time, 4 min; extraction temperature, 25 °C; injection temperature, 250 °C. Peaks correspond to: 1, drazoxolon; 2, hymexazol; 3, vinclozolin; 4, chlozolinate; 8, oxadixyl; 11, famoxadone; 13, azoxystrobin (10 ng mL^{-1}); 5, picoxystrobin; 6, metominostrobin; 7, kresoxim-methyl; 9, trifloxystrobin; 10, dimoxystrobin; 12, pyraclostrobin (5 ng mL^{-1}). (B–D) Extracted ion chromatograms showing the spectra of compounds.

those of aqueous standards for some fungicides when using both USAEME and SDME methods. However, the slopes were different for others, and a study by means of ANOVA test proved that there were statistically significant differences. Values can vary between 47–42 for famoxadone and 344–203 for trifloxystrobin using USAEME and in the ranges 38–34 for oxadixyl and 397–344 for trifloxystrobin using SDME. This matrix effect was not produced by the chromatographic procedure, while it was due to the pretreatment of the sample. Thus, the extraction efficiency for the analytes was different when applying aqueous standards or complex matrix samples. Once the pretreatment USAEME or SDME was performed, no analytes coeluting with the standards appeared and there was no matrix effect due to GC–MS. Consequently, to obtain a generally reliable procedure, the standard additions method is recommended for quantification purposes.

3.4. Method performance and validation according to the criteria of Commission Decision 2002/657/EC

The methods were validated for linearity, detection and quantification limits, selectivity, accuracy and precision. Calibration curves using USAEME GC–MS and SDME GC–MS were obtained by least-squares linear regression analysis of the peak area versus analyte concentration using five concentration levels in duplicate. The values of r^2 were good ($r^2 > 0.99$) and excellent linearity was obtained in the range studied for all fungicides (Table 3). The limits of detection (LOD, calculated as three times the signal-to-noise

Table 6
Recovery percentages of fungicides from fruit and juices samples.

Fungicide	Level (ng g^{-1})	USAEME				SDME			
		Grape	Peach/grape	Apple	Canned fruit	Grape	Peach/grape	Apple	Canned fruit
Drazoxolon	4	107 (4.6)	89.3 (3.2)	102 (6.6)	98.4 (9.7)	80.0 (3.8)	98.7 (9.8)	88.5 (8.1)	94.9 (6.5)
	40	90.4 (7.0)	89.6 (9.9)	98.5 (8.0)	105 (6.3)	97.2 (6.7)	101 (9.6)	86.1 (9.4)	104 (8.0)
Hymexazol	4	110 (4.0)	98.2 (5.7)	89.2 (9.8)	95.8 (6.5)	103 (6.5)	105 (8.4)	95.1 (7.8)	114 (9.9)
	40	89.0 (10)	108 (9.7)	101 (5.3)	101 (7.8)	89.4 (5.7)	115 (9.8)	103 (9.9)	115 (9.6)
Vinclozolin	4	90.5 (5.2)	99.6 (5.4)	117 (7.5)	106 (9.3)	94.1 (4.4)	91.2 (7.6)	80.5 (7.6)	104 (9.9)
	40	107 (5.3)	115 (6.3)	115 (6.3)	90.5 (9.9)	100 (3.3)	106 (3.6)	99.9 (9.7)	96.9 (4.9)
Chlozolinate	4	115 (3.5)	118 (4.8)	116 (8.9)	115 (9.5)	93.6 (5.3)	104 (7.5)	87.7 (8.1)	85.3 (7.1)
	40	84.3 (8.5)	100 (4.7)	115 (6.3)	89.0 (9.3)	94.6 (4.4)	107 (7.6)	96.4 (7.3)	109 (7.6)
Picoxystrobin	0.6	96.5 (7.1)	98.6 (3.7)	107 (5.4)	89.5 (9.0)	94.5 (5.2)	101 (5.5)	91.1 (9.3)	114 (9.8)
	6	88.0 (8.2)	105 (6.9)	91.1 (5.1)	80.0 (9.2)	104 (6.7)	96.9 (5.9)	104 (8.9)	104 (5.5)
Metominostrobin	0.6	109 (10)	100 (2.8)	92.8 (6.3)	111 (9.7)	102 (7.9)	109 (8.7)	107 (5.2)	113 (9.7)
	6	104 (10)	112 (5.0)	106 (2.9)	80.9 (4.8)	84.4 (6.8)	100 (9.1)	95.9 (8.4)	112 (9.9)
Kresoxim-methyl	4	98.9 (6.3)	94.4 (4.4)	110 (6.5)	97.5 (9.8)	92.3 (2.5)	83.7 (5.7)	103 (6.7)	90.6 (9.8)
	40	103 (9.3)	104 (9.9)	104 (6.1)	81.6 (6.9)	79.0 (3.7)	98.6 (9.9)	88.5 (4.1)	94.9 (6.5)
Oxadixyl	4	91.1 (7.1)	117 (3.7)	107 (9.3)	85.0 (7.5)	97.2 (6.7)	101 (9.7)	86.1 (5.4)	104 (8.1)
	40	103 (9.2)	106 (9.0)	107 (9.7)	87.4 (6.3)	103 (6.5)	115 (8.4)	95.1 (7.8)	95.9 (9.7)
Trifloxystrobin	0.6	112 (4.7)	107 (4.4)	101 (5.5)	109 (9.9)	89.4 (5.6)	117 (9.8)	108 (7.9)	115 (9.6)
	6	85.3 (6.5)	105 (5.3)	111 (9.6)	89.4 (9.8)	94.1 (4.4)	91.1 (7.6)	80.5 (9.8)	104 (9.9)
Dimoxystrobin	4	88.0 (6.8)	98.3 (5.7)	118 (6.3)	113 (8.9)	100 (3.2)	106 (3.6)	99.9 (9.7)	86.9 (5.0)
	40	109 (9.9)	116 (5.5)	96.7 (8.6)	88.5 (5.4)	93.6 (5.3)	103 (7.5)	87.7 (8.1)	88.3 (5.2)
Famoxadone	4	80.0 (2.9)	107 (6.7)	102 (4.3)	113 (9.6)	94.6 (4.4)	107 (6.6)	86.2 (6.2)	105 (3.6)
	40	86.7 (9.8)	114 (9.9)	119 (9.9)	93.0 (9.4)	94.5 (5.1)	101 (3.5)	98.1 (9.3)	114 (9.8)
Pyraclostrobin	4	102 (4.4)	106 (5.8)	111 (8.2)	106 (9.6)	104 (6.7)	96.9 (5.9)	111 (8.8)	91.5 (5.6)
	40	109 (6.8)	94.5 (6.2)	100 (7.3)	113 (8.9)	102 (7.9)	117 (8.7)	117 (9.1)	113 (9.7)
Azoxystrobin	4	97.1 (5.3)	104 (2.1)	106 (4.4)	115 (8.1)	84.4 (6.8)	100 (9.1)	95.9 (8.4)	92.8 (8.5)
	40	89.6 (7.7)	105 (6.6)	83.6 (8.6)	85.2 (9.7)	92.3 (2.5)	83.7 (5.7)	103 (6.7)	110 (9.8)

Values into brackets correspond to RSD for 10 measurements.

ratio) are included in Table 3 for aqueous standards and were dependent on the sample matrix. It can be seen from the data that the sensitivity and detection limits for all fungicides using the LLME sample pretreatment methods were very low and satisfied the MRLs set by the EC. The enrichment factor (EF) was calculated as the ratio between the analyte concentration in the floating organic phase after extraction and the initial concentration of analyte in the aqueous solution; values between 140–1140 for USAEME and 80–1600 for SDME were obtained. The EF obtained for famoxadone was lower, probably due to the higher solubility of this fungicide in the aqueous solution. Values of EF were generally higher for strobilurin than for oxazole fungicides, although the differences were not great.

The selectivity of the methods was judged from the absence of interfering peaks at the elution times of the analytes for blank chromatograms of different unspiked samples. No matrix compounds existed that might give a false positive signal in the blank samples.

The methods were also validated according to the criteria of the Commission Decision (2002/657/EC), which states that the spiking experiments for MRL substances should be around the MRL level, while validation of banned substances should focus around the quantification limit. Statistical analysis for CCα and CCβ was performed at the 95% confidence level using twenty replicate analyses. Tables 4 and 5 summarize the CCα and CCβ obtained for fruit samples using USAEME and SDME, respectively, at the LOQ levels of the method for drazoxolon, hymexazol and metominostrobin and at the MRL for the rest of the fungicides.

3.5. Recovery

In order to check the accuracy of the proposed methods, a recovery study was carried out by fortifying four fruit and juice samples (grape, apple, peach and grape juice and canned fruit) at two concentration levels corresponding to approximately 5 and 50 times the detection limit (Table 6). For USAEME, the recoveries of the fungicides from spiked samples were in the 80–119% range, with an average recovery ± SD (for $n = 104$) of 101 ± 10 and for SDME, the recoveries were in the 79–117% range, with an average recovery ± SD (for $n = 104$) of 98 ± 9. The similarity in recoveries obtained for each fungicide in the four samples indicates that the matrix effect was corrected by using standard additions calibration.

The repeatability of the method was also calculated from these experiments by obtaining the RSD of 10 replicate analyses of the above fortified fruit and juice samples, with RSD < 10% in all cases (Table 6). These values indicate that the precision of the method was satisfactory for residue control analysis.

3.6. Analysis of fruit and juice samples

Fig. 5A shows typical chromatograms obtained by USAEME GC–MS under SIM mode for a fortified canned pineapple sample under the selected conditions. Similar chromatograms were obtained for the other samples. The profiles demonstrated the absence of interfering peaks at the retention times for the analytes. The fungicides in the samples were identified by comparing the retention time, identifying the target (T) and qualifier ions (Q) and comparing the qualifier-to-target ratios (Q/T %) of the peaks in both the sample and the fungicide standard solution. The average values for the retention times of fungicides pointed to very good agreement between the retention data obtained in the different samples. The T and Q abundances were determined by injecting individual standards under the same chromatographic conditions, except in full scan mode. The Q/T percentage was determined by dividing the abundance of the selected qualifier ion by the target ion (see Table 1). Fig. 5B–D shows the ion corresponding to each peak, as well as the mass spectra, which confirmed the identity. The chromatograms corresponding to the aqueous standards and the different samples using the SDME GC–MS technique were similar and, again, the profiles demonstrated the absence of interfering peaks.

After identification of the peaks, different types of juices, musts, canned fruits and fresh fruits (peach, peach and grape, apple, pear, orange, pineapple and carrots) were analyzed using both USAEME

GC–MS and SDME GC–MS procedures. All samples were analyzed in triplicate. No fungicides were detected above their detection limits.

3.7. Comparison of SDME with USAEME

The use of low density organic solvents appeared appropriate for both miniaturized procedures. Comparison of the equilibrium time and extraction temperature indicated that USAEME is advantageous for extraction of fungicides as equilibrium was reached in a few seconds at room temperature meaning it is a more precise and robust method. Enrichment factors were between 140–1140 for the target fungicides using USAEME and in the 80–1600 range when using SDME. The extraction recoveries were similar for both methods, ranging from 80 to 119%. Detection limits were between 0.006–0.075 and 0.010–0.31 ng mL^{-1} for USAEME and SDME, respectively.

4. Conclusion

This study describes two miniaturized analytical procedures in which non-harmful environmentally pre-concentration techniques which avoid the use of high amounts of organic solvents, USAEME and SDME, were successfully applied to the GC–MS determination of six oxazole and seven strobilurin fungicide residues in fruit and juice samples. The procedures were based on the dispersion of micro volumes of low density organic solvents and collection of the floating organic solvent on the surface of aqueous samples and were validated according to 2002/657/EC Commission Decision. The results demonstrate that the methods are fast, effective, cheap and safe. They provide high selectivity, enrichment and reproducibility, and are suitable for analysing residues of fungicides in fruit samples for routine controls. USAEME GC–MS was the faster of the two procedures.

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