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Multiresidue method for fourteen fungicides in white grapes by liquid–liquid and solid-phase extraction followed by liquid chromatography–diode array detection

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Abstract

A quantitative, selective and sensitive HPLC method for the analysis of 14 fungicides in white grapes for vinification is described. The proposed method is based on liquid–liquid extraction (LLE) and solid-phase extraction (SPE) followed by liquid chromatography and diode array detection (HPLC–DAD). Dichloromethane–acetone (75:25, v/v) was the most appropriate solvent mix for extracting fungicides in white grapes. Silica cartridges resulted the most appropriate for extract purification purposes. Quality parameters of the proposed multiresidue method presented good recovery (ca. 85% for almost all target compounds) and precision (between 1.5 and 16%), and detection limits lower than maxima residual limits set by the 76/895/ECC and 90/642/ECC Directive. Five different white grapes for vinification produced in Rías Baixas area in Galicia (NW Spain) were analyzed in order to assess the performance of the method with real samples and to determine whether the concentration of the pesticides used exceed their maxima residue levels (MRLs). Results showed that grape concentrations for those identified fungicides were lower than those established by European legislation.

Keywords: Grapes; Fruits; Food analysis; Pesticides

1. Introduction

Grey mold (*Botrytis cinerea*), powdery mildew (*Uncicula necator*) and downy mildew (*Plasmopara viticola*) are the most common fungi encountered in vineyards control [1]. Several different fungicides are widely used in the treatment of diseases of grapes for vinification such as azoxystrobin, carbendazim, cymoxanil, cyprodinil, dichlofluanid, fenhexamid, folpet, fludioxonil, metalaxyl, thiophanate methyl, penconazol, pyrimethanil, procymidone and vinclozolin, studied in the present paper [2–10].

Although the correct use of fungicides does not cause problems of public concern in health and environmental areas, if inappropriate abusive treatments are applied without respecting safety recommendations, undesirable residues can remain on grapes after harvest. The presence of pesticides has also been associated with stuck and sluggish fermentations [11–13] and with problems in malolactic fermentation [14]. The activity of yeasts can be affected by the presence of pesticides residues. In

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this way, fermentation delays have been observed in the presence of pesticides such as folpet, dichlofluanid and thiophanate-methyl [14–16]. On the other hand, in some cases the presence of pesticides can also stimulate the yeasts, in particular *Kloeckera apiculata*, to produce more alcohol [17]. Nevertheless others authors pointed that yeasts can decrease the amount of pesticides by degradation and adsorption processes [18–24]. These fungicides residues can pass from grape to must, and later, to wine [5,6,8,24–29] with the resulting risk to the consumer's health and producing a decrease in the quality of wine because of the production of off-flavors [13,30]. In any case, the presence of fungicides in grapes and wines is a matter of public health concern.

The widespread consumer concern about pesticide use and consequent residues in food has led to increasingly strict regulations of pesticide use on grapes. The 76/895/ECC and 90/642/ECC European Directives [31,32] and their subsequent modifications have established maxima residue limits (MRLs) for practically all fungicides selected in this work for viniferous grapes except for cymoxanil, cyprodinil, fenhexamid, fludioxonil and penconazol; for the last five fungicides we have considered the MRLs established by the spanish legislation RD 280/1994 [33] and their later modifications.

Studies on determination of some pesticides in grapes, must and wine at residue levels have been published [34–40]. Analytical methods for determining pesticide residues in grapes for wine production involve several extraction and purification steps to remove the huge amount of potentially interfering compounds which are generally present at higher concentrations than the pesticide residues themselves. The method commonly used is a liquid–liquid extraction, as a prior step of isolation, with solvents such as acetonitrile [34], hexane [4,9], acetone [3,7], acetone–dichloromethane [2], acetone–petroleum ether [5], acetone–hexane [6], ethyl acetate–cyclohexane [10,40], although other solvents such as benzene, ethyl ether or isooctane have been used by other authors [41,42]. Solid-phase extraction (SPE) has been employed by other authors as an effective tool for purification procedures [2,3,26,27,35]. However, liquid–liquid extraction cannot be replaced by SPE.

Chromatographic techniques are the most suitable to identify and quantify pesticides residues in grapes, in particular gas chromatography (GC) equipped with selective and sensitive detectors [2,5,6,10,14,24,25,27,28,34,35,43,44]. Nevertheless, some pesticides can not be determined directly by GC due to their lack of thermal stability and/or their insufficient volatility without further derivatization (e.g. benomyl, carbendazim or thiophanate-methyl) [43,45]. In this cases the use of high-performance liquid chromatography (HPLC) is particularly useful for determining these residues in grapes [1,3,4,7,36,45].

The main aim of this work was to develop a multiresidue method for the determination of the 14 fungicides cited above, what makes the method very valuable for screening purposes. Some of them (fenhexamid, cyprodinil, fludioxonil and pyrimethanil) are only used since 2–3 years ago and therefore there are no methods available in the scientific literature for such a screening. The proposed method is based on organic solvent extraction (LLE), purification by SPE and liquid chromatography followed by diode array detection (HPLC–DAD). Different organic solvents and SPE sorbent compositions were tested, resulting the dichloromethane–acetone mix and silica cartridges the most appropriate in terms of simplicity of sample treatment and quantitative recoveries. Quality parameters of the method such as precision, linearity and detection limits were evaluated by spiking uncontaminated white grapes for wine production. Finally, white grape samples produced in Rías Baixas area (Galicia, NW Spain) were analyzed in order to correct for sample matrix effects and to screen for the presence of these fungicides.

2. Experimental

2.1. Chemicals, solvents and disposables

Pestanal grade standards of azoxystrobin [CAS No. 131860-33-8], carbendazim [10605-21-7], cymoxanil [57966-95-7], cyprodinil [121552-61], dichlofluanid [1085-98-9], fenhexamid [126833-17-8], folpet [133-07-3], fludioxonil [131341-86-1], metalaxyl [057837-19-1], thiophanate methyl [23564-05-8], penconazol [66246-88-6], pyrimethanil [53122-28-0], procymidone [32809-16-

8] and vinclozolin [050471-44-8] (purity >99%, for all of them) were purchased from Riedel–de-Haën (Seelze, Germany). Carbofuran (99.5%), used as an internal standard, was purchased from Dr Ehrenstorfer (Augsburg, Germany).

Dichloromethane and acetone for gas chromatography; *n*-hexane, methanol and water for liquid chromatography were purchased from Merck (Darmstadt, Germany); acetonitrile for instrumental analysis from Panreac (Barcelona, Spain); isooctane for analysis from Scharlau (Barcelona, Spain). Another reagent used was anhydrous sodium sulphate ACS–ISO for analysis from Panreac (Spain).

Waters Sep-Pak cartridges (Milford, CT, USA) packed, separately, with silica (690 mg), alumina (910 mg; acid, basic and neutral), diol (360 mg) and cyano propyl (360 mg) as sorbents were used as solid-phase extraction (SPE) minicolumns for purification and concentration. A visiprep solid-phase extraction vacuum manifold (Supelco, San Diego, CA, USA) was used to simultaneously process up to 24 SPE tubes. The visidry drying attachment (Supelco) was used to dry up to 24 SPE tubes at one time, and can be used with any inert gas supply. It is also useful for evaporating and concentrating recovered samples. Nitrogen C-50 of analytical quality was supplied by Carburos Metálicos (Vigo, Spain).

A blender from Moulinex (Barcelona, Spain) was used to crumble and homogenize white grape samples. For LLE extraction, white grape samples were placed in 250-ml polypropylene carbonate containers from Nalgene (Rochester, NY, USA). Polypropylene tubes were centrifuged in a Beckman J2-HS centrifuge (San Jose, CA, USA). Organic extracts were placed into round-bottom flasks from Schott Duran (Germany) prior to be evaporated in a Heidolph WB 2000 vacuum rotary evaporator (Germany). Homogenization of final extract was achieved with Heidolph Reax Top vortex agitation (Germany). Final organic extracts were filtered through a 25-mm nylon filter membrane (0.22 μm) from Tracer (Barcelona, Spain) and placed in 350-μl inserts in 2-ml vials (Supelco) prior to the chromatographic analysis.

2.2. Stock standard solutions

A stock standard solution (ca. 1000 mg/l) of each fungicide was prepared in methanol by weighing approximately 0.025 g of the analyte into a 25-ml volumetric flask and diluting to volume. An intermediary mixed standard solution was prepared by dilution in acetonitrile of the stock standard solutions to give a concentration of ca. 100 mg/l for each fungicide. Stock and intermediary standard solutions of the internal standard, carbofuran, were prepared in the same way. All standard solutions were stored in the dark at 4 °C.

2.3. White grape sampling

Uncontaminated white grape samples used for developing and validating the proposed method were purchased at local markets in Ourense, Spain. Samples were analyzed unwashed, in raw state.

In order to assess the performance of the method with real samples and to screen the presence of these fungicides, white grape samples produced in five different vineyards in different districts of Rías Baixas area (Galicia, NW Spain) were collected. In these vineyards, fungicides were applied by using air blast sprayers and following the recommendations of manufacturers. Grapes were collected in 2001 and were stored in small portions at −18 °C until usage.

2.4. Extraction and purification

White grape samples were placed in a Moulinex Universal food cutter and chopped for 3 min. A portion (60 g) of the homogenized chopped grapes was weighed inside a 250-ml polypropylene carbonate container. The sample was spiked with carbofuran (30 μl of a 1000 mg/l methanolic solution). Carbofuran was chosen as internal standard due to its use is forbidden in Spanish grape crops. Then, a volume (100 ml) of dichloromethane–acetone (75:25, v/v) and anhydrous sodium sulphate (45 g) were added. The mixture was vigorously shaken for 5 min and then centrifuged for further 15 min at 10 000 rpm.

An aliquot of the organic extract (20 ml) was transferred to a 50-ml round-bottomed flask and evaporated at 40 °C to dryness on the rotary evaporator. The residue obtained was dissolved in isooctane (5 ml).

Extract purification was performed on Sep-Pak silica cartridges. Before use they were conditioned with isooctane (5 ml) without allowing the cartridges to dry out. The cartridges were loaded with the

isooctane extract (5 ml), dried by blowing N_2 for 20 min, eluted with a volume (5 ml) of dichloromethane–acetone (50:50, v/v) into a 25-ml round-bottom flask and evaporated at 40 °C to dryness on the rotary evaporator. The residue was finally re-dissolved in acetonitrile (1 ml), homogenized with vortex agitation. and filtered through a 22-μm nylon filter prior to the chromatographic analysis.

2.5. HPLC–DAD system and operating conditions

High-performance liquid chromatography (HPLC) analyses were carried out on a Thermo HPLC system equipped with a SCM1000 vacuum membrane degasser, a P2000 binary pump, an AS 1000 autosampler, a column heater from Jones chromatography (Model 7981) and a UV6000LP detector linked to a PC computer running the ChromQuest-version 2.51 software programme (TermoQuest, Italy).

The analytical column (250×4.6 mm I.D.) used was an Ultracarb 5 μm ODS 30% C (Phenomenex, CA, USA). The guard column (50×4.6 mm I.D.) was packed with dry 40 μm Pelliguard LC-18 (Supelco, Switzerland). For HPLC analysis an aliquot (50 μl) was injected into the column and eluted at 40 °C, with a constant flow-rate of 1.5 ml/min at the following gradient conditions for the mobile phase—acetonitrile (A)–ultrapure water (B)—t=0 min, A:B (25:75, v/v); t=15 min, A:B (25:75, v/v); t=20 min, A:B (30:70, v/v); t=40 min, A:B (30:70, v/v); t=60 min, A:B (50:50, v/v); t=78 min, A:B (50:50, v/v); t=79 min, A:B (90:10, v/v); t=89 min, A:B (90:10, v/v); t=90 min, A:B (25:75, v/v); t=110 min, A:B (25:75, v/v). Detection was carried out at wavelengths between 200 and 380 nm, and quantification was done at 200 nm for practically all target compounds; at 204 nm for azoxystrobin, fenhexamid and fludioxonil; at 224 nm for folpet; at 240 nm for cymoxanil; and at 270 nm for cyprodinil and pyrimethanil.

3. Results and discussion

3.1. HPLC –DAD performance

The mobile phase acetonitrile–HPLC water was initially considered. Acetonitrile is the most transparent organic solvent at 200 nm, the maximum absorption wavelength for practically all target compounds. On the other hand, ultrapure water obtained from Milli-Ro water purification system (USA) originated an unacceptable drifting baseline which was solved with the use of HPLC water. The separation of all target compounds with different gradient combinations of such a mobile phase was not possible with an Ultracarb 5 μm ODS 30% C analytical column shorter (150×4.6 mm I.D.) than the used in this work (250×4.6 mm I.D.). The gradient conditions described above and the column thermostated (at 40 °C) allowed to resolve correctly all target compounds (Fig. 1); column heater programmed at temperatures higher than 45 °C produced the thermal degradation of some fungicides.

3.2. Sample extraction performance

Uncontaminated grapes, once chopped and homogenized, were spiked at 17 mg/kg level with the following fungicides: carbendazim, cymoxanil, dichlofluanid, fenhexamid, folpet, metalaxyl, penconazol, procymidone, thiophanate-methyl and vinclozolin. After equilibration for 5 min prior to extraction, they were initially processed according to the following procedure: a portion of spiked grape sample (30 g) was weighed inside a 250-ml polypropylene container. A volume of the organic mix (100 ml) and anhydrous sodium sulphate (45 g) were added. This amount of sodium sulphate resulted to be the minimum necessary to facilitate the separation between the organic and aqueous phases. The mixture was vigorously shaken for 5 min and then centrifuged for further 15 min at 10 000 rpm. An aliquot of the organic extract (5 ml) was transferred to a 25-ml round-bottomed flask and evaporated at 40 °C to dryness on the rotary evaporator. The residue obtained was dissolved in acetonitrile solvent (1 ml). Duplicate analyses were performed for each organic solvent portion.

Different equilibration times (5, 30 min and 24 h) prior to extraction were evaluated when uncontaminated grapes were spiked with the fungicides. After applying the analytical procedure described above, no significant differences in recovery values were observed. To minimize the analysis time, further

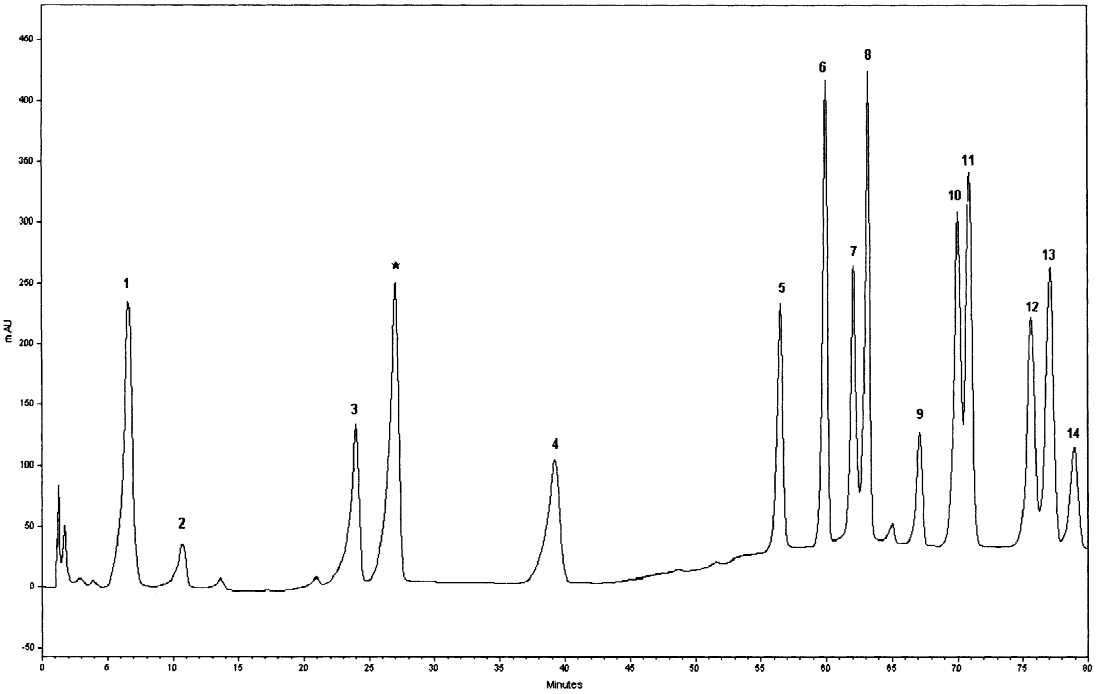

Fig. 1. HPLC–DAD chromatogram registered at 200 nm for a fungicide mix standard solution (6 mg/l, in acetonitrile). Peaks: *: internal standard, carbofuran (t_R=26.8 min), **1**: carbendazim (t_R=6.5 min), **2**: cymoxanil (t_R=10.7 min), **3**: thiophanate-methyl (t_R=23.7 min), **4**: metalaxyl (t_R=39.1 min), **5**: pyrimethanil (t_R=56.4 min), **6**: fludioxonil (t_R=59.9 min), **7**: fenhexamid (t_R=61.9 min), **8**: azoxystrobin (t_R=63.1 min), **9**: folpet (t_R=67.0 min), **10**: procymidone (t_R=69.9 min), **11**: penconazol (t_R=70.8 min), **12**: cyprodinil (t_R=75.6 min), **13**: vinclozolin (t_R=77.0 min) and **14**: dichlofluanid (t_R=78.9 min) (Chromatographic conditions as described).

experiments were performed considering an equilibration time prior to extraction of 5 min.

Mixed organic solvents tested for quantitative extraction purposes by partition were acetone–dichloromethane (at 75:25, 50:50 and 25:75, v/v) and hexane–dichloromethane (at 75:25, 50:50 and 25:75, v/v). Acetone is a commonly used extractant due to its capability for extracting non-polar and polar pesticides [45] and its miscibility with grape material [46]. However, acetone allows to extract many interfering compounds from the sample matrix due to its polarity. To reduce these interferences, the effect of dichloromethane mixed with acetone at different portions was evaluated. Hexane is an organic solvent considered by other authors [6] and the effect of dichloromethane when they were mixed was also considered.

For hexane–dichloromethane, recoveries ranged from 24% (carbendazim) to 90% (dichlofluanid) at 75:25 (v/v); from 65% (carbendazim) to 100% (dichlofluanid) at 50:50 (v/v); from 54% (carbendazim) to 70% (dichlofluanid) at 25:75 (v/v). Higher recoveries were obtained for acetone–dichloromethane ranging between 32% (carbendazim) to 58% (procymidone) at 75:25 (v/v); between 67% (carbendazim) to 91% (penconazol) at 50:50 (v/v); and between 84% (carbendazim) to 100% (dichlofluanid) at 25:75 (v/v).

Further quantitative white grape sample extractions were performed using acetone–dichloromethane (25:75, v/v), while other authors used 50:50 (v/v) proportions [39].

3.3. Extract purification performance

Organic solvent extracts from white grape samples (60 g) have many interfering compounds from the sample matrix. To remove matrix interferences, the purification efficiency of amino, cyano propyl and diol sorbents which can work in reversed or normal-

phase mode; alumina (acidic, basic and neutral) and silica sorbents which act in normal-phase were tested on organic extracts from uncontaminated grapes samples.

Acetone–dichloromethane (25:75, v/v) extracts (20 ml) were spiked with a standard mix solution of all fungicides at level of 0.5 mg/l. The experimental procedure after passage of the extract through the sorbent cartridges and elution with isooctane is described above. Duplicate analyses were performed for each sorbent cartridge.

Most of the matrix pigments were removed with SPE procedure and isooctane eluted pale yellow. Recoveries obtained are summarized in Table 1. Purification by alumina (acidic, basic and neutral) was not efficient for carbendazim, cymoxanil, thiophanate-methyl, fenhexamid, folpet, vinclozolin and dichlofluanid due to their low retention. Amino sorbent was also not efficient for fenhexamid, folpet, vinclozolin and dichlofluanid; however thiophanate-methyl was recovered at 100%. Insufficient sorption for some fungicides was observed with cyano propyl and diol sorbents. Alumina (acidic, basic or neutral) sorbents gave recoveries higher than 150%; it could be explained with the presence of not-removed interferences. Silica was effective in removing interfering compounds and quantitative in recovering all studied fungicides, with values close to 100%, except for thiophanate methyl which has not been recovered, as can be seen in Table 1. On the other hand, HPLC–DAD chromatograms obtained with the use of silica cartridges were much cleaner compared with those obtained with the use of other sorbents.

Fig. 2(top) shows the chromatogram obtained at 200 nm (a wavelength suitable for detection of many target pesticides) from an uncontaminated grape sample, and Fig. 2(bottom) shows the chromatogram obtained from a spiked grape sample at 0.5 mg/kg level, both treated following the experimental procedure described. Identification of peaks is described in Table 1. The chromatogram shows the purification efficiency of silica cartridges. Lower detection limits can be reached due to the final acetonitrile extract contains very few interfering peaks. Fungicide identification was confirmed by recording their absorption spectra between 200 and 380 nm and by comparing these with spectra previously obtained from standard samples.

3.4. Method performance

Method performance was assessed by evaluating quality parameters such as recovery values, repeatability, reproducibility, linearity and limits of detection and quantitation. All values obtained are summarized in Table 2. For this purpose, uncontami-

Table 1
Recovery efficiencies of different normal-phases to determine fungicides in white grape samples

No.	Fungicides	Sorbent composition of SPE cartridges						
		Amino	Cyano	Diol	Acidic alumina	Neutral alumina	Basic alumina	Silica
1	Carbendazim	83	77	93	27	–	–	70
2	Cymoxanil	97	92	94	–	–	–	92
3	Thiophanate-methyl	114	120	–	–	–	–	–
4	Metalaxyl	104	103	97	102	>150	142	102
5	Pyrimethanil	88	103	100	111	>150	>150	101
6	Fludioxonil	90	89	105	111	>150	–	98
7	Fenhexamid	–	65	102	–	–	–	84
8	Azoxystrobin	129	110	83	112	>150	>150	99
9	Folpet	–	38	31	–	–	–	112
10	Procymidone	118	>150	>150	116	>150	>150	85
11	Penconazol	102	108	106	77	>150	–	103
12	Cyprodinil	85	94	103	113	>150	>150	103
13	Vinclozolin	–	28	35	72	–	49	117
14	Dichlofluanid	–	39	30	15	–	–	101

(n=2) determinations. –: not recovered.

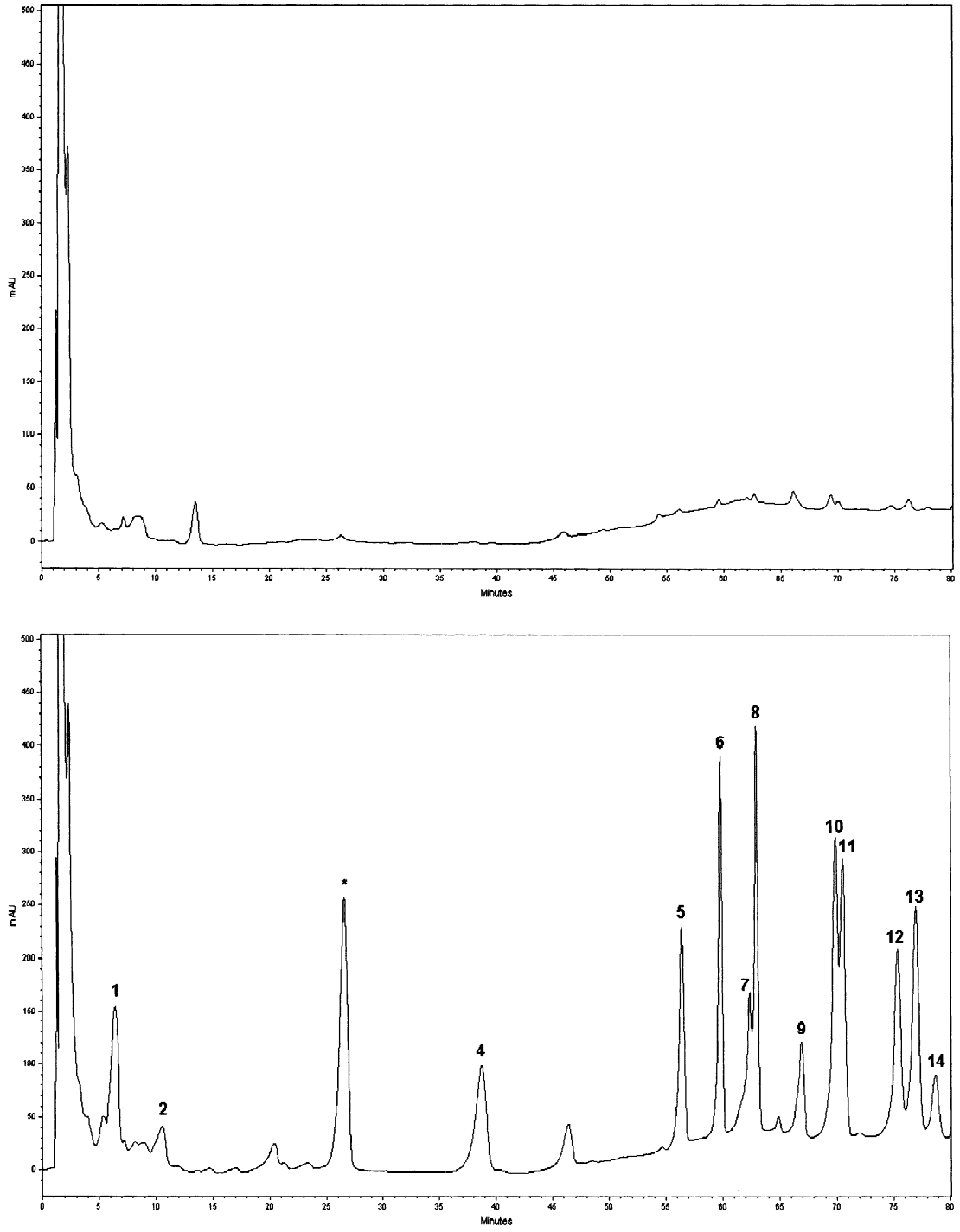

Fig. 2. LLE–SPE/HPLC–DAD chromatograms registered at 200 nm for an uncontaminated white grape sample (top) and a spiked white grape sample at a 0.5 mg/kg level (bottom), both processed following the experimental procedure described. Peaks: *: internal standard, carbofuran (t_R = 26.83 min); fungicides identification correspond to Table 1 (Chromatographic conditions as described).

Table 2
Recoveries, repeatabilities, reproducibilities, linear dynamic ranges, determination coefficients (r^2), limits of detection (LODs) and limits of quantitation (LOQs) of the optimized method based on LLE–SPE and HPLC–DAD

Fungicide	Recovery± Repeatability (%) ±RSD (%) (n=7)	Reproducibility ±RSD (%) (n=6)	Linear range (mg/kg) (n=10)	Determination coefficient (r^2)	LOD (mg/kg) (n=7)	LOQ (mg/kg) (n=7)
Carbendazim	68±7	11	0.05–9.6	0.998	0.07	0.15
Cymoxanil	84±6	7	0.05–9.6	0.995	0.02	0.05
Thiophanate-methyl	n.r	n.r	1.01–10.1	0.976	0.31	0.64
Metalaxyl	86±6	7	0.05–9.9	0.998	0.04	0.09
Pyrimethanil	88±3	5	0.05–9.6	0.996	0.01	0.02
Fludioxonil	84±2	6	0.05–1.9	0.996	0.02	0.03
Fenhexamid	41±8	15	0.05–9.9	0.991	0.05	0.11
Azoxystrobin	84±5	25	0.05–7.9	0.999	0.01	0.02
Folpet	58±15	16	0.05–3.8	0.993	0.01	0.03
Procymidone	85±10	28	0.05–5.6	0.997	0.05	0.13
Penconazol	65±6	7	0.05–3.9	0.99	0.04	0.07
Cyprodinil	88±3	8	0.05–10.1	0.997	0.02	0.03
Vinclozolin	86±4	11	0.05–5.3	0.998	0.06	0.15
Dichlofluanid	63±16	15	0.05–9.6	0.998	0.25	0.58

n.r.=not recovered.

nated white grape samples were previously fortified with fungicides listed in Table 2 and treated following the experimental conditions described.

The repeatability and reproducibility of the method were assessed by analyzing seven spiked uncontaminated grape samples in the same day and a total of nine spiked uncontaminated grape samples along 3 days in two different weeks, respectively. All samples were spiked at a levels of ca. 0.5 mg/kg of each fungicide. The relative standard deviation (RSD %) for repeatability was lower than 10%, except for folpet and dichlofluanid; and for reproducibility was lower than 16%, except for azoxystrobin and procymidone, as can be seen in Table 2. These values show the good precision of the multiresidue method proposed. On the other hand, recoveries are higher than 85% for practically all target compounds. The lower recoveries observed for some fungicides

Table 3
Measured concentrations and standard deviations of fungicides in uncontaminated white grapes spiked at ca. 500 μg/kg of each fungicide and determined by LLE–SPE/HPLC–DAD in order to assess matrix effects

Fungicide	White grape concentration (mg/kg)±SD		
	A Grape sample	B Grape sample	C Grape sample
Carbendazim	0.51±0.13	0.48±0.01	0.32±0.02
Cymoxanil	0.37±<0.01	0.28±<0.01	0.41±0.01
Metalaxyl	0.42±0.01	0.44±0.07	1.43±0.02
Pyrimethanil	0.32±0.03	0.35±<0.01	0.35±<0.01
Fludioxonil	0.47±0.01	0.45±0.01	0.30±0.02
Fenhexamid	0.95±<0.01	0.40±0.04	0.87±0.01
Azoxystrobin	0.55±0.03	0.34±0.02	0.53±<0.01
Folpet	0.40±0.01	0.26±0.03	3.10±0.03
Procymidone	0.50±<0.01	0.42±0.05	0.47±0.01
Penconazol	0.49±0.02	0.51±0.02	0.32±0.04
Cyprodinil	0.43±0.04	0.38±0.01	0.12±0.04
Vinclozolin	0.59±0.01	0.55±0.03	0.58±0.03
Dichlofluanid	0.47±0.01	0.64±0.04	0.50±0.01

(n=2) determinations.

can be explained due to partial adsorption to the sample matrix or degradation during the extraction process; they are recovered at 100% from silica cartridges as can be seen in Table 1.

Calibration curves for the fungicides were prepared by plotting area relative to that of the internal standard (carbofuran) vs. the analyte concentration using a total of ten spiked uncontaminated grape samples (0.05–10 mg/kg). Analysis of a unspiked grape sample did not give any response at the retention time of the studied fungicides. Linear ranges and determination coefficients (r^2) corresponding to each fungicide are shown in Table 2.

Limits of detection and quantitation were evaluated following the recommendations of the American Chemical Society [47]. As tested experimentally detection and quantitation limits were ten-hundred times lower than LMRs established by European legislation.

3.5. Matrix effects assessment

Various types of white grapes (A, used during the method performance; B and C) purchased at different local markets were examined to study the matrix effect. It was initially confirmed that all grape samples were not contaminated with the studied fungicides. Grape samples, once chopped and homogenized, were spiked at a level of 0.5 mg/kg and treated following the experimental procedure described. Triplicate analysis were performed for each white grape sample. Quantitation was performed using the calibration line for each fungicide with carbofuran as internal standard. Results are given in Table 3.

Significant differences have been found between concentrations determined in B (e.g. cymoxanil, fenhexamid, azoxystrobin, folpet, procymidone and dichlofluanid) and C (e.g. carbendazim, metalaxyl, fludioxonil, folpet, penconazol and cyprodinil) grape samples respect to concentrations determined in A grape sample. Matrix effects were also detected by other authors [27,39,46]. These detected matrix effects could be explained as a result of the different grape sample origin. Then, the standard addition method is necessary for quantifying fungicide residues to avoid matrix effects and was applied as follows: grape samples were directly analyzed twice and subsequently two standard additions of fungicide with a mix standard were performed at levels of 1 and 2 mg/kg for further analysis. The four-point calibration equation was calculated in order to estimate the fungicide concentrations in grape samples according to Miller and Miller [48].

3.6. Analysis of white grape samples from Rías Baixas

Galician white wines is VQPRD (Vino di Qualitá Prodotto in Regione Determinata) or ''denominación de origen'' certifications Rías Baixas. Five different growing districts, named and numbered in Fig. 3 as

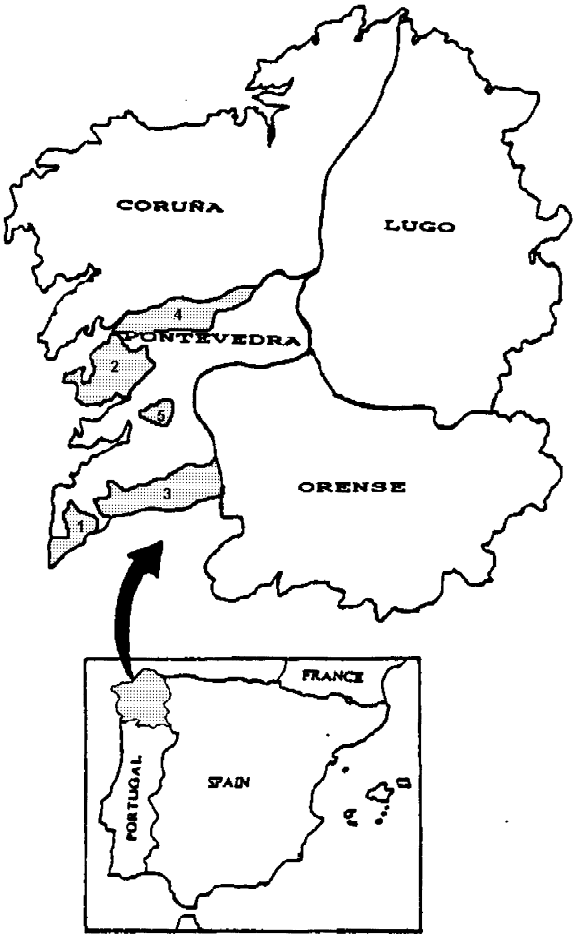

Fig. 3. Location of the five growing districts which compose the VQPRD of Rías Baixas: (1) O Rosal, (2) Val do Salnés, (3) Condado do Tea, (4) Ribeira do Ulla and (5) Soutomaior.

O Rosal (1), Val do Salnés (2), Condado do Tea (3), Ribeira do Ulla (4) and Soutomaior (5), compose the total producing Rías Baixas area. White grape samples (A–E) produced in different vineyard districts (A and B in 1, C in 2, D and E in 3) of Rías Baixas area were analyzed. Standard addition method was applied for quantitation.

As shown in Table 4, all Rías Baixas grapes presented some of the target fungicides used in the treatment of downy mildew and grey mold. Folpet and metalaxyl are used against downy mildew in the first treatment of grape crops; their presence indicate their chemical persistence. Cyprodinil, fludioxonil, fenhexamid, pyrimethanil and procymidone are used against grey mold and their presence is due to their use in the last treatment of grape crops before harvest. Concentrations determined in all grape samples are lower respect to MRLs (mg/kg) established by European and Spanish legislations [31–33]. This could be explained by the correct timing of the safety intervals (defined as the time elapsed between last application and harvest).

Results obtained are in the same order than those determined by other authors [8,9,49]. Cabras and co-workers [8,9] determined the following concentrations and their decay rates in 21 days after treatment for cyprodinil (from 5.54 to 1.08 mg/kg), fludioxonil (from 1.86 to 1.20 mg/kg), penconazol (from 0.08 to 0.02 mg/kg), pyrimethanil (from 1.62 to 1.19 mg/kg) [8]; and fenhexamid (from 2.05 to 0.8 mg/kg) [9]. García-Cazorla and Xirau determined procymidone in grapes at levels of 0.61 mg/kg [49].

4. Conclusions

The multiresidue method proposed is suitable to determine 14 fungicides in white grape samples. The use of acetone–dichloromethane (25:75, v/v) as extraction mix and silica cartridges as a purification step allows to determine them quantitatively without interferences. The method has good linearity, precision and accuracy, and is highly sensitive. Quantitation process required the use of standard addition in order to avoid matrix effects. Application to white

Table 4
LLE–SPE/HPLC–DAD analysis of five Rías Baixas white grape samples in the search for the studied fungicides

Fungicides	MRLs (mg/kg)	Rías Baixas white grape samples (mg/kg)				
		A albariño grape	B loureira grape	C albariño grape	D albariño grape	E treixadura grape
Carbendazim (1)	2	–	<LOQ	–	–	–
Cymoxanil	0.2*	–	–	–	–	–
Thiophanate-methyl (1)	2	–	–	–	–	–
Metalaxyl	1	–	–	–	0.21	0.14
Pyrimethanil	5	–	2.33	–	–	–
Fludioxonil	1*	–	0.61	0.17	0.25	0.43
Fenhexamid	2*	0.52	–	–	–	–
Azoxystrobin	2	–	–	–	–	–
Folpet (2)	10	–	–	0.10	0.40	0.09
Procymidone	5	–	–	2.41	–	–
Penconazol	0.2*	–	–	–	–	–
Cyprodinil	2*	–	1.45	0.14	0.38	0.44
Vinclozolin	5	–	–	–	–	–
Dichlofluanid	10	–	–	–	–	–

MRLs (mg/kg) established by 76/895/EEC and 90/642/EEC Directives and their subsequent modifications.

* MRLs (mg/kg) established by RD 280/1994, the Spanish legislation, and their subsequent modifications due to the non-existence of MRLs for all in the European Community.

(1): sum of carbendazim, benomyl and thiophanate-methyl.

(2): sum of captan and folpet.

–: not detected.

grapes of Rías Baixas area (Galicia, NW Spain) allows to confirm that levels determined in all grape samples analyzed are lower than MRLs established by European and Spanish legislations.

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