RESIDUES AND TRACE ELEMENTS

Optimization and Validation of a Simple and Fast Method for the Determination of Fungicides in Must and Wine Samples by SPE and GC/MS

LAURA LAGUNAS-ALLUÉ, JESÚS SANZ-ASENSIO, and MARIA-TERESA MARTÍNEZ-SORIA[1]
University of La Rioja, Department of Chemistry, Madre de Dios, 51, La Rioja, 26006, Spain

A rapid, simple, and low-cost method based on SPE was optimized and validated for simultaneous determination of eight fungicides belonging to different chemical classes in must and wine. The method involves extraction of 10 mL of must or wine samples with a C_{18} cartridge using 5 mL of dichloromethane as the elution solvent. Separation and final determination of the fungicides (vinclozolin, dichlofluanid, penconazol, captan, quinoxyfen, fluquinconazol, boscalid, and pyraclostrobin) was performed by GC coupled to single quadrupole MS. Recoveries at 10, 50, and 100 µg/L were between 71 and 106% in both matrixes for the fungicides evaluated. The calculated LOQ ranged from 1.5 to 3.4 µg/L in must and 1.1 to 3.8 µg/L in wine. Matrix effects observed for wine and must samples were overcome by using matrix-matched calibration. The developed method was linear at concentrations within the tested interval, with coefficients of determination higher than 0.999. The expanded uncertainties at 10 µg/L were <20% for all analytes. Intralaboratory precision in terms of the Horwitz ratio of the fungicides evaluated was below 0.5, suggesting the ruggedness of the method. The proposed method was applied to determine fungicide residues in must samples obtained from red grapes treated with two new commercial formulations, as well as in their corresponding final wines.

The misuse of pesticides may leave harmful residues in grapes after harvest that may pass to the must and eventually to the wine during fermentation, which involves a possible health risk. Therefore, maximum residue levels (MRLs) for pesticide residues in a variety of agricultural foods were established by the European Union (EU) to protect consumers' health. Nevertheless, for most of the studied fungicides, MRLs have not been established in wine. For wines elaborated in the EU from September, 2008 (1), MRLs were established for boscalid (5000 µg/L), captan (20 µg/L), and pyraclostrobin (1000 µg/L). Other countries, such as Italy or Switzerland, have also established MRLs (2–4) for some of the fungicides (vinclozolin, dichlofluanid, and boscalid in Switzerland at 1000 µg/L in all cases, and quinoxyfen, boscalid, and pyraclostrobin in Italy at 10, 1000, and 50 µg/L, respectively).

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[1] Corresponding author's e-mail: maria-teresa.martinez@unirioja.es
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Analytical methods for determining pesticide residues in wine production involve several extraction and purification steps to remove the potentially interfering compounds that are generally present at higher concentrations than the pesticide residues. SPE has been proposed for the extraction of pesticides from must and wine samples (5, 6) as alternative to liquid–liquid extraction (7, 8). In most applications, a volume of sample in the range from 10 to 50 mL is passed through an RP SPE sorbent, then analytes are recovered using an organic solvent. Other current extraction techniques, such as single-drop microextraction the Quick, Easy, Cheap, Effective, Rugged, and Safe method; hollow fiber liquid phase microextraction; solid-phase microextraction (SPME); and stir bar sorptive extraction (SBSE) have also been applied to determination of fungicides in wine (9–13). Among all of these techniques mentioned previously, SPME is the most widely used for analysis of pesticides in wine. Although this technique normally provides higher selectivity than SPE, the ethanol content of wine significantly reduces its extraction efficiency when compared to water samples (14, 15); moreover, the kinetics of the extraction is relatively slow, and there are differences in the yield of the process depending on the wine matrix. These disadvantages are also common to SBSE, the applicability of which is restricted to low-polarity fungicides showing a high affinity for the polydimethylsiloxane sorbent (16).

Chromatographic separation and identification were achieved by GC with a nitrogen-phosphorous detector, electron capture detector, or MS detection (10, 17) or by HPLC for compounds not volatile or thermally unstable. In this case, UV, diode array, and MS detector are the most-used detectors and give good results (18, 19).

Different kinds of fungicides against diseases of grapes—i.e., vinclozolin (dicarboximide), dichlofluanid (sulfamide), penconazol (triazole), captan (phthalimide), quinoxyfen (quinoline), and fluquinconazol (triazole), and two new generation fungicides, boscalid (carboxamide) and pyraclostrobin (strobilurin)—widely used in the Qualified Designation of Origin Rioja, were selected for the study.

In spite of the great number of SPE publications, well-described and validated SPE methods for the extraction of the tested fungicides in must and wine are scarce. A few articles have been reported using SPE for analysis of dichlofluanid (6, 19), penconazol (6, 20), and vinclozolin (19, 21–23), only one regarding SPE extraction of captan and fluquinconazol (24) and boscalid and pyraclostrobin in grapes and wines (25), and none for quinoxyfen.

To our knowledge, an instrumental method for the simultaneous determination of these eight multiclass fungicides, included in integrated pest management strategies in Spanish viticulture (except dichlofluanid), in must and wines has not been reported.

Table 1. Retention times, target ion, and qualifier ions for the target pesticides by GC/MS

Compounds	t_R window, min[a]	Target ion I_1, *m/z*	Qualifier ion I_2, *m/z*	Qualifier ion I_3, *m/z*	$[I_2]/[I_1]$ (RSD, %)[b]	Tolerances $[I_2]/[I_1]$ (RSD, %)[c]	$[I_3]/[I_1]$ (RSD, %)[b]	Tolerances $[I_3]/[I_1]$ (RSD, %)[c]
Vinclozolin	7.51–7.60	212	285	198	0.74 (7)	10	0.87 (6)	10
Dichlofluanid	8.66–8.73	123	224	167	0.34 (11)	15	0.43 (14)	15
Penconazol	10.00–10.07	248	159	—	0.86 (7)	10	—	
Captan	10.19–10.24	79	149	—	0.22 (13)	20	—	
Quinoxyfen	13.19–13.25	237	307	272	0.29 (7)	15	0.43 (11)	15
Tetradifon	14.92–15.00	159	356	111	0.50 (9)	15	0.90 (6)	10
Fluquinconazol	16.52–16.57	340	108	—	0.26 (14)	20	—	
Boscalid	17.28–17.33	140	342	112	0.45 (8)	15	0.33 (9)	15
Pyraclostrobin	18.21–18–27	132	164	325	0.36 (12)	15	0.14 (15)	20

[a] Retention time.

[b] Intensity ratio of the two ions, target and qualifier ion at 100 µg/L in must and wine matrix-matched standards.

[c] Default recommended maximum permitted tolerances for relative ion intensities (% of base peak) using GC/electron impact MS according to SANCO guidelines.

Efficient analytical methods for the determination of boscalid and pyraclostrobin are thus demanded because both fungicides have been recently introduced in viticulture. The method based on SPE and determination by GC/MS was optimized to obtain lower cost and more accuracy for determining residues of the eight fungicides in must and wine.

Experimental

Chemicals and Reagents

Pesticide analytical standards of vinclozolin, dichlofluanid, penconazol, captan, quinoxyfen, fluquinconazol, boscalid, and pyraclostrobin with purity higher than 99.0% were purchased from Riedel-de-Haën (Seelze, Germany). Tetradifon from Riedel-de-Haën with a purity of 99.5% was used as an internal standard (IS). All were stored at –20°C.

HPLC grade methanol, ethyl acetate, acetone, acetonitrile, and dichloromethane were obtained from Scharlab (Barcelona, Spain). Ultrapure water was obtained using a Milli-RO plus system together with a Milli-Q system from Millipore (Billerica, MA).

For SPE, 500 mg C_{18} (Bond Elut® LRC-C_{18} INT) cartridges were supplied by Varian (Middelburg, The Netherlands).

Two new commercial formulations, Cantus® (50% boscalid) and Cabrio Top® (5% pyraclostrobin), were supplied by BASF Española (Tarragona, Spain).

Standard Preparation

Fungicide stock solutions (500 mg/L) and intermediary solutions (10 and 1 mg/L) were prepared in methanol. Stock and intermediary standard solutions of the IS, tetradifon, were prepared in the same way in ethyl acetate. All standard solutions were stored at –20°C. They were stable over a period of at least 3 months (tested against newly prepared solutions by comparing the detector responses). Intermediary solutions were used to spike wine and must matrixes.

SPE Procedure

The sample preparation procedure and, in particular, the sorbent of the cartridge was chosen according to our experience in wine and must analysis (11). A Visiprep® SPE vacuum manifold from Supelco (Bellefonte, PA) was used to simultaneously process 12 tubes. A wine or must volume of 10 mL was percolated through a C_{18} cartridge, previously conditioned with 5 mL methanol and 3 mL water. Then, the cartridge was rinsed with 10 mL water–methanol (9 + 1, v/v) to clean up the cartridge and was dried under an applied vacuum for 20 min to remove excess water. Finally, the retained fungicides were eluted with 5 mL dichloromethane, evaporated to dryness under a gentle nitrogen stream, redissolved with 10 mL ethyl acetate, and 100 µg/L tetradifon was added. Tetradifon was used as an IS to compensate for any sample and injection volume changes and to correct the variability in GC injection and MS detection response.

Instrumentation and Chromatographic Conditions

The analysis of the target fungicides was carried out on an Agilent Technologies (Santa Clara, CA) GC 7890A chromatograph coupled to a 5975C MS quadrupole mass selective detector. Chromatographic separations were done by using an HP-5MS capillary column (30 m × 0.25 mm id × 0.25 µm film thickness). The initial oven temperature was set at 100°C, increased to 185°C at 40°C/min, kept for 5 min, increased at a rate of 10°C/min to 300°C, and held for 3 min. The volume of sample was 2 µL, injected in the splitless mode. The injector temperature was set at 250°C. Helium (99.9999% purity) was used as the carrier gas at a constant flow rate of 1.5 mL/min. The mass spectrometer was operated with electron ionization (70 eV) using a 6 min solvent delay. The interface temperature was 310°C, and ion source temperature was 230°C.

GC/MS Analysis

Initially, full-scan MS was performed by scanning *m/z* of 50–550 to confirm the retention times of the analytes and to

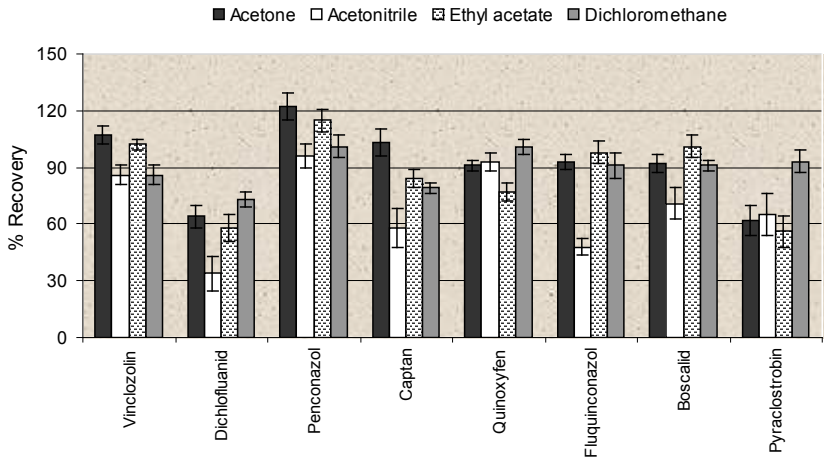

Figure 1. SPE recoveries using various extraction solvents for the elution step.

select the most abundant ion (base peak) and qualifier ions for each target compound. Once ions were selected for all analytes, selected ion monitoring was performed for determination of the fungicide residues.

Optimization of the SPE Method

Different parameters were studied in order to develop the SPE method to determine these fungicides in real must and wine samples. These included the following: eluent solvent parameters and volume, composition and volume of the solid-phase wash, and breakthrough volume.

(**a**) *Elution solvent.*—The following different solvents were initially considered for SPE elution because of the wide range of polarity and solubility exhibited by the compounds investigated: ethyl acetate, acetonitrile, acetone, and dichloromethane. Acetone, ethyl acetate, and acetonitrile were selected as solvents because of their effectiveness for extraction of polar and nonpolar pesticides from a diverse range of matrixes, and dichloromethane was also considered to be one of the investigated solvents because it has an ability to lower the extraction of polar coextractants.

The must and wine samples (10 mL) fortified at a level of 50 μg/L for each fungicide were extracted in triplicate by SPE with C_{18} cartridges, previously conditioned and eluted with 10 mL (5 mL + 5 mL) of the solvents mentioned above.

(**b**) *Composition and volume of the solid-phase wash.*—Once the elution solvent was chosen, a study of the wash step was carried out. Thus, 10 mL wine and must samples were both spiked at the same concentration of pesticides and percolated through the SPE cartridge. Later, these cartridges were washed with different proportions of water–methanol (10+0, 9+1, 7+3, and 5+5, v/v) before elution with organic solvent to check the influence of this step. In addition, different volumes (2, 5, and 10 mL) for the selected water–methanol mixture were checked in order to obtain the cleanest chromatograms without loss of compounds.

(**c**) *Breakthrough volume.*—An assay to determine breakthrough volume was performed according to the procedure described by Hennion (26) and Dopico-García et al. (27). It consisted of preconcentrating samples of increasing volumes, each containing the same amount of analytes. Volumes of samples of 5, 25, 50, 100, and 250 mL, spiked with a mixture of pesticide standards, were used to determine the breakthrough volume.

Method Validation

Validation was performed according to SANCO/10684/2009 guidelines (28) concerning the performance of methods for pesticide residue monitoring. Performance characteristics studied were selectivity, linearity, LOD, LOQ, recovery, precision, and matrix effects. Global uncertainty was determined for all the pesticides according to the EURACHEM/CITAC Guide (29).

According to the SANCO guidelines, a quantitative analytical method should be demonstrated at initial and extended validation as being capable of providing mean recovery values at each spiking level within the range 70–120%, and repeatability and reproducibility RSD ≤20%, for all compounds to be determined using the method.

(**a**) *Selectivity.*—The selectivity of the method was tested by injecting extracts of nonspiked must and wine samples (30).

(**b**) *Linearity.*—Different approaches to quantification of pesticide residues in fresh fruits and vegetables can be considered in order to reduce the quantitative errors from the matrix effects: use of the standard addition method; standards in residue-free matrix spiked with standards (matrix-matched standards); deuterated internal and/or surrogate standards; and analyte protectants (28). In this study, matrix-matched calibration was used. Must and wine samples obtained from untreated grapes were subjected to the SPE method described above. These blank extracts were spiked with variable amounts of fungicides. The calibration curves for all the compounds in must and wine matrixes were obtained by plotting the fungicide to the IS peak area ratio against the concentration for each compound of the corresponding calibration standards at six calibration levels ranging between close to each LOQ to 100 μg/L. Linearity was checked by calculating the determination coefficient, r^2, of the linear regression equations in matrix-matched standard solutions in the concentration ranges studied.

(**c**) *LOD and LOQ.*—LOD and LOQ of the overall method were calculated as the concentration giving S/N=3 and S/N=10, respectively. These limits were estimated using the SPE extract of must and wine samples spiked at 10 μg/L.

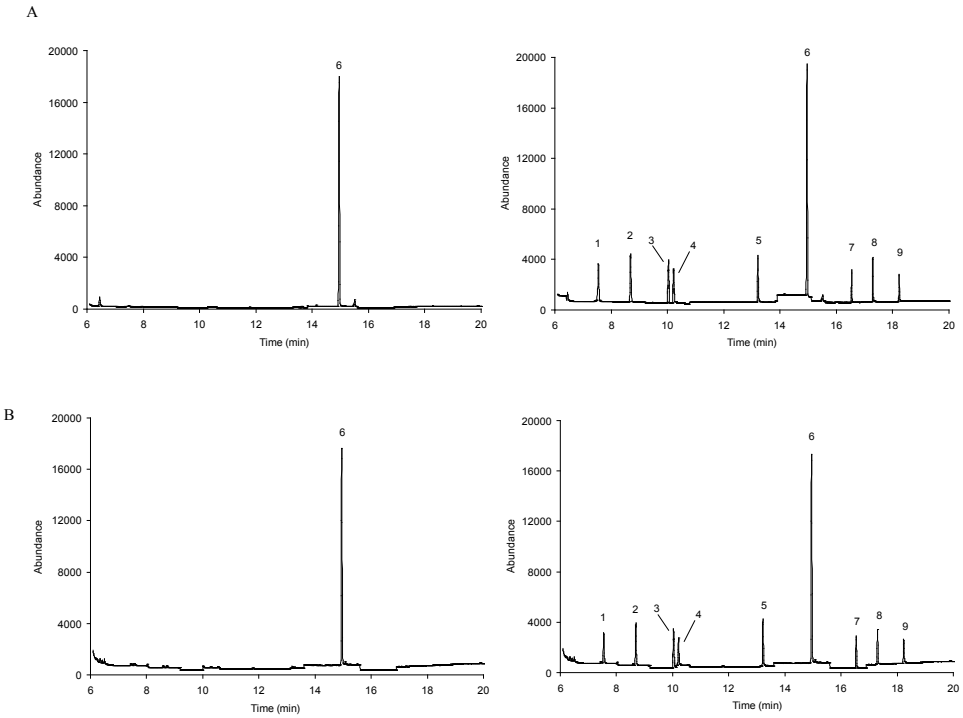

Figure 2. GC/MS chromatograms obtained after SPE of: (A) extract from blank and spiked must (10 µg/L); and (B) extract from blank and spiked wine (10 µg/L). Peak identification: 1, vinclozolin; 2, dichlofluanid; 3, penconazol; 4, captan; 5, quinoxyfen; 6, tetradifon (IS; 100 µg/L); 7, fluquinconazol; 8, boscalid; and 9, pyraclostrobin.

(**d**) *Precision.*—Precision was evaluated by means of repeatability and intermediate precision measurements. Repeatability was evaluated by way of five consecutive replicates of the analysis on the "blank" must and wine samples spiked with the analytes at three concentrations levels (10, 50, and 100 µg/L), on a single day.

Intermediate precision was determined separately at a fortification level of 10 µg/L for all the analytes by calculating the RSD of five analyses of the same must and wine samples performed over 5 days within 1 month. Horwitz ratio (HorRat) pertaining to intralaboratory precision, which indicates the acceptability of a method with respect to precision (31), was calculated for all the fungicides in the following way:

$$\text{HorRat} = \text{RSD}/\text{Prsd}$$

where Prsd is the predicted RSD calculated by the equation:

$$\text{Prsd} = 2C^{-0.15}$$

where C is the concentration expressed as mass fraction (e.g., 10 ng/g = 10×10^{-9}).

(**e**) *Recovery.*—To evaluate the accuracy of the present method, a standard mixture solution of the eight target fungicides was added to must and wine samples at three fortification levels (10, 50, and 100 µg/L). Quantification in the recovery samples was performed by internal calibration using matrix-matched standards.

Matrix Effects

(**a**) *Matrix effect in GC analysis.*—The main consequence of matrix effects is an increasing (ion enhancement) or decreasing analyte signal (ion suppression) in the presence of the matrix (real sample) with respect to the same analyte in solvent (standard solution; 32, 33). Therefore, matrix effects were evaluated by comparison of the slope of a calibration curve based on the matrix-matched standards of must or wine with the slope of the pure solvent-based calibration curve. A higher slope of the matrix calibration curve indicates matrix-induced signal enhancement, whereas a lower slope represents signal suppressions. Tetradifon (IS) was added to both calibration solutions.

(**b**) *Matrix effects between samples in the SPE method.*—In view of the change in composition of the samples during alcoholic fermentation (in the transformation of grape must into wine), the possible matrix effects in the sample treatment process must be studied. Therefore, to check the matrix effects, several samples of different matrixes (red, white, and rose wines and red and white must) were spiked with the target compounds at four different concentration levels within the linear range studied previously (analyses were performed in duplicate), and the slopes of the linear calibration functions obtained for the different spiked wines were compared by the application of statistical tests.

Uncertainty Evaluation

Global uncertainty was determined for all the fungicides at the level of 10 µg/L according to the statistical procedure of the EURACHEM/CITAC Guide CG 4 (29). Five individual sources of uncertainty were taken into account:

Table 2. Analytical features of the GC/MS method designed for analysis of fungicides in must and wine samples

	Must					Wine					Wine	
Fungicide	Linear range, µg/L[a]	$r^{2\,b}$	LOD, µg/L	LOQ, µg/L	Slope ratio, extract/solvent	Linear range, µg/L[a]	$r^{2\,b}$	LOD, µg/L	LOQ, µg/L	Slope ratio, extract/ solvent	t_{cal}[c]	MRL Italy, µg/L	MRL Switzerland, µg/L
Vinclozolin	3–110	0.9996	0.8	2.8	1.3	3–110	0.9991	1.1	3.2	1.4	6.49		1000
Dichlofluanid	3–105	0.9991	1.0	2.7	1.4	3–105	0.9990	0.6	2.4	1.4	12.29	—	1000
Penconazol	2–103	0.9990	0.7	2.1	1.2	2–103	0.9992	0.8	1.8	1.3	8.81	—	—
Captan	4–108	0.999	1.3	3.4	1.3	4–108	0.9993	1.5	3.8	1.4	14.60	—	—
Quinoxyfen	2–120	0.9991	0.7	1.5	1.1	1–120	0.9991	0.4	1.1	1.2	5.25	10	—
Fluquinconazol	3–105	0.9992	1.2	2.8	1.5	3–105	0.9992	1.2	2.9	1.4	8.71	—	—
Boscalid	3–112	0.9990	1.2	2.4	1.3	2–112	0.9991	0.9	2.1	1.2	7.53	1000	1000
Pyraclostrobin	4–133	0.9998	1.1	3.3	1.0	3–133	0.9991	1.3	3.1	0.9	0.10	50	—

[a] Concentration range for calibration curve.

[b] Coefficient of determination.

[c] $t_{cal} = \dfrac{(b_a - b_b)}{\sqrt{(s_a^2 + s_b^2)}}$

uncertainty associated with the calibration graph (u_1), day-wise uncertainty associated with precision (u_2), analyst-wise uncertainty associated with precision (u_3), day-wise uncertainty associated with accuracy/bias (u_4), and analyst-wise uncertainty associated with accuracy/bias (u_5). The uncertainties were calculated as follows:

$$u_1 = \frac{s}{b_1}\sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(c_o - \bar{c})^2}{\sum_{i=1}^{N}(c_i - \bar{c})^2}}$$

where s is the SD of the residuals of the calibration curve, b_1 is the slope of the calibration curve, p is the number of measurements of the unknown, n is the number of points used to form the calibration curve, c_0 is the calculated concentration of the analyte from the calibration curve, $\bar{c}$ is the average of all of the standards used to make the calibration curve, and c_i ($i = 1, 2\ldots, n$) is the concentration of each calibration standard used to create the calibration curve. $U_2 = s_1/n^{1/2}$ where s_1 is the SD of the results obtained from a single analyst on different days and n is the number of assays. $U_3 = s_2/n^{1/2}$ where s_2 is the SD of the results obtained from different analysts on a particular day, and n is the number of assays. $U_4 = s_1(\eta)/n^{1/2}$ where $s_1(\eta)$ is the SD of the percentage recoveries obtained from a single analyst on different days, and n is the number of assays. $U_5 = s_2(\eta)/n^{1/2}$ where $s_2(\eta)$ is the SD of the percentage recoveries obtained from different analysts on a particular day and n is the number of assays.

The global uncertainty (U) was calculated as:

$$U = \sqrt{u_1^2 + u_2^2 + u_3^2 + u_4^2 + u_5^2}$$

Evaluation of total uncertainty was done assuming that all the contributions were independent of each other. A coverage factor of 2 was considered at the confidence level of 95% to evaluate the expanded uncertainty at a 10 µg/L fortification level.

Application to Real Must and Wine Samples

(**a**) *Samples from local markets.*—In order to assess the performance of the method, 16 wine and must samples of Spanish origin were collected from the local markets and analyzed by SPE and GC/MS.

(**b**) *Vinification process samples.*—Another study to evaluate the applicability of the proposed method was carried out by determining residues of two new fungicides (boscalid and pyraclostrobin) in must and wine obtained from red grape samples (cv. Tempranillo).

Red grapes were harvested in September 2008 from cv. Tempranillo grapevines at a vineyard in Aldeanueva de Ebro, La Rioja, Spain. Two new commercial formulations against grey mold, downy mildew, and powdery mildew were applied on red grapes Cantus (50% boscalid) and Cabrio Top (5% pyraclostrobin) at the recommended doses (1 and 2 kg/ha, respectively). These applications were performed in recommended periods corresponding to different phenological stages; using the harvested grapes, microvinifications (50 kg) were performed with each treated grape as common

Table 3. Recovery (*n* = 5), repeatability (*n* = 5), and HorRat (*n* = 25) of analysis of must and wine by the GC/MS method

	Must; recovery, RSD, % ± SD				Wine; recovery, RSD, % ± SD			
Fungicide	10 µg/L[a]	50 µg/L[a]	100 µg/L[a]	HorRat, 10 µg/L[b]	10 µg/L[a]	50 µg/L[a]	100 µg/L[a]	HorRat, 10 µg/L[b]
Vinclozolin	92 ± 3	86 ± 6	90 ± 2	0.24	86 ± 6	84 ± 4	86 ± 6	0.26
Dichlofluanid	77 ± 6	74 ± 5	74 ± 8	0.27	75 ± 6	74 ± 6	71 ± 7	0.31
Penconazol	100 ± 5	103 ± 2	101 ± 4	0.18	98 ± 1	105 ± 4	106 ± 3	0.23
Captan	73 ± 3	76 ± 4	76 ± 6	0.31	71 ± 5	74 ± 9	77 ± 7	0.33
Quinoxyfen	95 ± 4	98 ± 4	90 ± 5	0.18	103 ± 4	97 ± 6	93 ± 5	0.26
Fluquinconazol	101 ± 6	91 ± 7	96 ± 3	0.22	101 ± 3	96 ± 3	98 ± 5	0.19
Boscalid	102 ± 4	98 ± 3	93 ± 7	0.22	97 ± 6	93 ± 4	92 ± 5	0.25
Pyraclostrobin	94 ± 4	96 ± 6	91 ± 8	0.27	98 ± 7	99 ± 8	98 ± 4	0.28

[a] *n* = 5.

[b] *n* = 25.

processing in Qualified Designation of Origin Rioja. Residue levels of pyraclostrobin and boscalid were analyzed by SPE and GC/MS during all steps of the vinification process.

Results and Discussion

GC/MS Analysis

The dwell time for ion monitoring was 100 ms/ion. The fungicides determined by GC/MS were eluted between 6 and 20 min. Selected ions (*m/z*) used for confirmation and quantification, target and qualifiers ion, and the intensity ratios are shown in Table 1. The intention was to select the most abundant ions of higher *m/z*, which provided more sensitivity and selectivity.

SPE

Elution solvent.—Figure 1 shows the average recoveries of the target compounds in wine matrix using different extraction solvents. The behavior of the target compounds in both must and wine matrixes was similar (therefore, results for must sample are not shown). Dichloromethane exhibited recoveries >70% for all of the investigated fungicides. Even in the case of the lowest recovery (73% for dichlofluanid), the overall repeatability of the method was good enough to ensure a reliable determination of the target compounds. On the other hand, acetonitrile provided the lowest recoveries for most of the fungicides (between 34 and 96%). In the case of acetone and ethyl acetate, recoveries were higher than 80% for almost all the fungicides, except for dichlofluanid and pyraclostrobin with values lower than 60%. In addition, the extracts obtained with the acetone, acetonitrile, and ethyl acetate were heavily pigmented, containing large amounts of matrix coextractants and providing high noise and low sensitivity together with poorer precision. Therefore, elution was performed with a total volume of 5 mL of dichloromethane.

Composition and volume of the solid-phase wash.—Once all the cartridges were washed with the different water–methanol proportions, elution was performed as explained above, and recoveries were calculated. By increasing the proportion of methanol in the wash solvent, a reduction in the extraction recoveries of the fungicides was observed. Water–methanol (5 + 5, v/v) and (7 + 3, v/v) led to low recoveries of 23–58% and 43–88%, respectively. The loss of compounds in these water–methanol mixtures was due to their greater solubility in the organic solvent.

Table 4. Slopes ± SD from standard curves obtained for the different matrixes[a]

Compound	White wine ($b_A \pm s_{bA}$)10^{-3}	Rose wine ($b_B \pm s_{bB}$)10^{-3}	Red wine ($b_C \pm s_{bC}$)10^{-3}	White must ($b_D \pm s_{bD}$)10^{-3}	Red must ($b_E \pm s_{bE}$)10^{-3}	Levene's test *P*-values	ANOVA *P*-values
Vinclozolin	8.45 ± 0.16	8.52 ± 0.10	8.48 ± 0.13	8.36 ± 0.12	8.38 ± 0.11	0.966	0.764
Dichlofluanid	12.57 ± 0.25	12.43 ± 0.15	12.70 ± 0.22	12.54 ± 0.19	12.82 ± 0.14	0.880	0.157
Penconazol	10.81 ± 0.14	10.52 ± 0.12	10.72 ± 0.19	10.82 ± 0.08	10.44 ± 0.11	0.797	0.09
Captan	7.43 ± 0.11	7.29 ± 0.08	7.32 ± 0.09	7.45 ± 0.14	7.47 ± 0.10	0.856	0.278
Quinoxyfen	13.98 ± 0.29	14.21 ± 0.23	14.34 ± 0.44	13.47 ± 0.43	13.81 ± 0.48	0.864	0.123
Fluquinconazol	9.53 ± 0.10	9.76 ± 0.13	9.74 ± 0.12	9.59 ± 0.15	9.45 ± 0.17	0.955	0.078
Boscalid	10.49 ± 0.11	10.38 ± 0.18	10.51 ± 0.16	10.29 ± 0.13	10.24 ± 0.09	0.924	0.082
Pyraclostrobin	7.74 ± 0.09	7.87 ± 0.09	7.86 ± 0.11	7.71 ± 0.12	7.70 ± 0.14	0.952	0.198

[a] b_A = Slope of white wine, b_B = slope of rose wine, b_C = slope of red wine, b_D = slope of white must, b_E = slope of red must. $S_{bA} = S_D$ of slope b_A, Sb_B = SD of slope b_B, Sb_C= SD of slope b_C, Sb_D = SD of slope b_D, and Sb_E= SD of slope b_E.

Table 5. Individual and global uncertainties for each pesticide expressed as relative measures, calculated at 10 µg/L

	Must							Wine						
	Calibration curve	Precision		Bias		Global uncertainty	Expanded uncertainty	Calibration curve	Precision		Bias		Global uncertainty	Expanded uncertainty
Compounds	u_1	u_2	u_3	u_4	u_5	U	2U	u_1	u_2	u_3	u_4	u_5	U	2U
Vinclozolin	0.090	0.018	0.024	0.018	0.031	0.102	0.204	0.099	0.016	0.026	0.015	0.026	0.108	0.216
Dichlofluanid	0.092	0.013	0.016	0.022	0.022	0.099	0.198	0.094	0.017	0.013	0.024	0.019	0.101	0.202
Penconazol	0.085	0.008	0.026	0.009	0.022	0.093	0.186	0.061	0.008	0.018	0.009	0.019	0.068	0.136
Captan	0.079	0.014	0.014	0.017	0.022	0.086	0.172	0.082	0.018	0.016	0.023	0.022	0.091	0.182
Quinoxyfen	0.061	0.012	0.020	0.011	0.025	0.070	0.140	0.072	0.015	0.015	0.017	0.019	0.079	0.158
Fluquinconazol	0.057	0.009	0.019	0.010	0.022	0.065	0.130	0.060	0.009	0.017	0.012	0.021	0.068	0.136
Boscalid	0.072	0.009	0.023	0.008	0.026	0.081	0.162	0.079	0.014	0.021	0.012	0.023	0.087	0.174
Pyraclostrobin	0.063	0.015	0.023	0.016	0.025	0.075	0.150	0.076	0.010	0.020	0.010	0.020	0.083	0.166

Conversely, water–methanol (9 + 1, v/v) showed recoveries from 78 to 98%, similar to pure water addition. Both mixtures provided similar recoveries, but the addition of a small volume of methanol led to cleanest chromatograms due to the elimination of most methanol-soluble interferences without loss of the target compounds.

Therefore, water–methanol (9 + 1, v/v) was selected for the C_{18} cartridge wash step. Different studied volumes of this mixture (2, 5, and 10 mL) showed no significant differences in the final recoveries. However, with volume increases, there were fewer matrix interferences. Therefore, 10 mL of water–methanol (9 + 1, v/v) was chosen as the mixture for solid-phase wash step.

Breakthrough volume.—An analysis of variance (ANOVA) performed with the raw data revealed the absence of significant differences (P >0.05) between the assayed volume of sample except for dichlofluanid (when the volume was 100 mL, the recoveries started to diminish in must samples). Thus, this method can be utilized for analyzing samples up to 250 mL of must and wine samples contaminated with these compounds, except for dichlofluanid in must, for which the breakthrough volume was 100 mL.

Method Validation

The proposed conditions generated narrow and reproducible chromatographic peaks; no interfering peaks were observed in blank sample chromatograms (Figure 2) of wine and must extracts fortified with the fungicides, proving sufficient selectivity for the analysis of the target fungicides.

The linear ranges and r^2 values, LODs, LOQs, and MRLs in the EU, Switzerland, and Italy are listed in Table 2. Chromatographic response was checked up to approximately 100 µg/L (according to the concentration of each fungicide in the stock solution) with $r^2 > 0.999$ showing, in all the cases, good linearity for the tested fungicides.

LODs ranged from 0.7 to 1.3 µg/L and 0.4 to 1.5 µg/L for must and wine, respectively. LOQs were between 1.5 and 3.4 µg/L in must and 1.1 to 3.8 µg/L in wine. From these data, it can be shown that, for the majority of the compounds, both limits were similar for must and wine samples. Moreover, LODs and LOQs tested for wines were lower than MRLs established by the EU, Switzerland, or Italy.

Recovery, repeatability expressed as SD, and intermediate precision expressed as HorRat values are summarized in Table 3 for three fortification levels. In all instances, satisfactory results were found, with recovery values between 71 and 106%, not related to the spiking level and complying with the requirements of SANCO/10684/2009 (28). As can be seen in Table 3, RSD values were within the acceptable range of <20%, according to SANCO guidelines. The HorRat values obtained were lower than 0.5 for all the compounds at 10 µg/L. Thus, the method provided a satisfactory level of intralaboratory precision.

Table 6. Pesticide concentrations (µg/L) for positive results of the analyzed real samples

	Must samples			Wine samples	
Compound	Must A	Must B	Must C	Wine A	Wine B
Captan	ND[a]	N.D.	ND	<LOQ[b]	<LOQ[b]
Fluquinconazol	ND	3.8 ± 0.2	ND	ND	<LOQ[c]
Penconazol	ND	5.1 ± 0.4	<LOQ[d]	3.7 ± 0.2	ND
Vinclozolin	4.0 ± 0.3	ND	ND	<LOQ[e]	ND

[a] ND = Not detected.
[b] LOQ = 3.8 µg/L.
[c] LOQ = 2.9 µg/L.
[d] LOQ = 2.1 µg/L.
[e] LOQ = 3.2 µg/L.

Matrix Effects

Matrix effects in GC analysis.—Table 2 summarizes the ratio values for slopes in sample extracts and solvent. Differences in response were observed for almost all fungicides. Most of them, except pyraclostrobin in wine, displayed enhancement of the signal. Vinclozolin, dichlofluanid, captan, and fluquinconazol showed the highest signal enhancement (ratio values of 1.4) in wine samples, while in must samples, only dichlofluanid showed this value. A Student's t-test (34) was done to compare the slope of regression lines in must, wine, and ethyl acetate obtained for each compound studied. The results for this test showed that there were significant differences at the 95% confidence level between the slopes obtained in ethyl acetate and must for all target

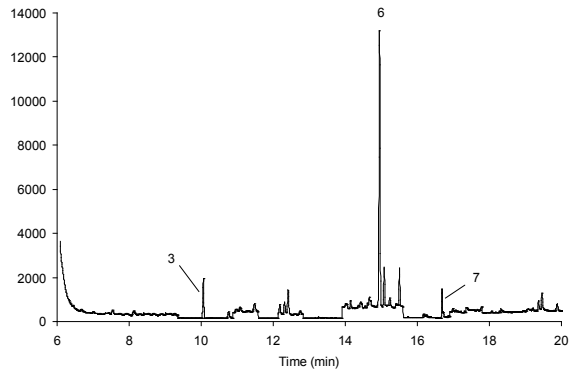

Figure 3. SIM chromatogram of a real must sample obtained by SPE containing penconazol at a concentration of 5.1 µg/L and fluquinconazol at 3.8 µg/L. For compound identification, *see* Figure 2.

analytes except captan, quinoxyfen, and pyraclostrobin, and only for pyraclostrobin and boscalid in wine samples.

Matrix effects between samples in the SPE method.—Table 4 shows the summarized results obtained for each compound in the different matrixes. Certain conclusions may be drawn from a statistical data analysis. First, Levene's test was applied in order to check variance homogeneity; *P*-values higher than 0.05 were obtained in all cases, indicating no statistically significant differences among the variances. According to these results, one-way ANOVA was carried out in order to compare the slopes between must and wine samples. *P*-values higher than 0.05 showed there were no matrix effects between different samples; therefore, the method could be used regardless of the matrix.

Uncertainty Evaluation

Global uncertainty of the fungicides evaluated varied up to 11%. The expanded uncertainties ranged from 13.0 to 20.4% and from 13.6 to 21.6% in must and wine samples, respectively. The uncertainty values for all the fungicides were similar in must and wine matrixes, except for penconazol. As seen in Table 5, higher uncertainties were observed for dichlofluanid and vinclozolin.

Uncertainties in precision and bias were low (0.8–2.6 and 0.8–3.1%, respectively); however, the uncertainty associated with the calibration curve (in each case, within 5.7–9.9%) contributed considerably toward the global uncertainty. Because the uncertainty level was equal to or below 11% for all the compounds in must and wine samples, the method performance could be considered satisfactory for the whole range of these fungicides.

Application to Real Must and Wine Samples

Samples from local markets.—Results of the positive analyzed must and wine samples are summarized in Table 6. A chromatogram of a positive real must sample (must B) found to contain penconazol (5.1 ± 0.4 µg/L) and fluquinconazol (3.8±0.2 µg/L) is shown in Figure 3. Confirmation criteria were that the retention times of the compounds in the sample be within ±0.5% of the respective retention times in matrix-matched calibration standards and the intensity ratios $[I_2]/[I_1]$ and $[I_3]/[I_1]$ of the target and qualifier ions in the sample be within 20% of the respective ratios in matrix-matched calibration standards (Table 1; 33). The concentrations of the fungicides in musts and wines analyzed were found to be lower than 10 µg/L, and only three fungicides—fluquinconazol, penconazol, and vinclozolin—were determined at concentrations slightly higher than the LOQs (Table 2).

Vinification process samples.—The proposed method was applied to determine residues of two new fungicides (boscalid and pyraclostrobin) in musts and wines obtained from red grape samples (cv. Tempranillo). The vinification process with red grapes was performed by following the winemaking process described before. Residue levels of pyraclostrobin and boscalid are presented in Table 7. To determine the dissipation of fungicide residues during the entire process, the total residual concentration present in the sample was calculated. The pesticide concentration present in pressed musts was considered as 100% in each case and the starting point to study the disappearance of the fungicides.

Boscalid and pyraclostrobin concentrations found in must pressed samples were very low. Once the must was pressed, alcoholic fermentation started. This vinification step had a considerable effect on the decrease in these fungicide residues; no residual levels were detected for pyraclostrobin, while the proportion of boscalid remaining in the racked wine was 65%.

Once alcoholic fermentation finished, malolactic fermentation took place. At the end of this step, the reduction of boscalid was 25%. The dissipation in this step was lower than in alcoholic fermentation.

The clarification and filtration processes, the last two winemaking stages, did not play an important role in the reduction of boscalid residues, showing a decrease of 10% for clarification and 7% for filtration. Boscalid concentration remaining in final wine was 8.8 µg/L, much lower than MRLs set in Switzerland and Italy (6–8), as summarized in Table 2.

Conclusions

The SPE and GC/MS method described in this paper allows the rapid determination of the fungicides vinclozolin, dichlofluanid, penconazol, captan, quinoxyfen, fluquinconazol, boscalid, and pyraclostrobin in must and wine samples. The method has been validated according to SANCO/10684/2009 guidelines and global uncertainties have been calculated. The method offers good recoveries, linearity, precision, and accuracy, and is highly sensitive. Matrix effects were overcome by using matrix-matched calibration. The uncertainty associated with the analytical method was lower than 10% for all compounds tested. The different composition of the matrixes, must and wine, does not affect the sensitivity of the method, giving similar LOD and LOQ values for both.

Captan, fluquinconazol, penconazol, and vinclozolin residues were found in five of the 16 analyzed samples. Boscalid and pyraclostrobin concentrations were determined in musts and wines obtained from red grapes previously treated with these substances. A total dissipation of pyraclostrobin was observed, while boscalid residues showed a decrease of 75% during all steps of the winemaking process, including clarification and filtration.

The proposed analytical procedure is low cost, rapid, and easy to perform, and could be utilized for regular monitoring of these pesticide residues to ensure food safety.

Table 7. Concentration of fungicide residues and percentage remaining (n = 3) in each stage

	Pyraclostrobin		Boscalid	
Samples	Concn, µg/L	Remaining, %	Concn, µg/L	Remaining, %
Pressed must	29.1 ± 1.2	100 ± 3.7	34.5 ± 1.6	100 ± 3.7
Racked wine	<LOD[a]	<LOD[a]	22.4 ± 1.1	65 ± 4.2
Final malolactic fermentation	<LOD[a]	<LOD[a]	14.6 ± 0.9	42 ± 2.1
Clarified wine	<LOD[a]	<LOD[a]	11.0 ± 0.6	32 ± 2.7
Filtered wine	<LOD[a]	<LOD[a]	8.8 ± 0.8	25 ± 1.3

[a] LOD_{wine} = 1.3 mg/L.

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