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Determination of fungicides in wine by mixed-mode solid phase extraction and liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

A novel procedure for the determination of nine selected fungicides (metalaxyl-M, azoxystrobin, myclobutanil, flusilazole, penconazole, tebuconazole, propiconazole, diniconazole and difenoconazole) in wine samples is presented. Sample enrichment and purification is simultaneously performed using mixed-mode, anion exchange and reversed-phase, OASIS MAX solid-phase extraction (SPE) cartridges. Analytes were determined by liquid chromatography coupled to tandem mass spectrometry using atmospheric pressure electrospray ionization (LC–ESI-MS/MS). Parameters affecting the chromatographic determination and the extraction-purification processes were thoroughly investigated. Under optimized conditions, 10 mL of wine were firstly diluted 1:1 with ultrapure water and then passed through the mixed-mode SPE cartridge at a flow of ca. 5 mL min^{-1}. After a washing step with 5 mL of an aqueous NH_4OH solution (5%, w:v), analytes were recovered with just 1 mL of methanol and injected in the LC–MS/MS system without any additional purification. The selective extraction process avoided significant changes in the ionization efficiency for red and white wine extracts in comparison with pure standards in methanol. Performance of the method was good in terms of precision (RSDs < 11%) and accuracy (absolute recoveries > 72%, determined against pure standards in methanol) reporting method LOQs in the range of 0.01–0.79 ng mL^{-1} for target compounds, which are far below the EU maxima residue levels (MRLs) for fungicides in vinification grapes and wine. Several commercial wines from different geographic areas in Spain were analyzed. In most samples, metalaxyl-M and azoxystrobin were found at concentrations up to several ng mL^{-1}.

1. Introduction

Pesticides are used all over the world to protect vines against diseases caused by insects, fungi, molds and other agents. Fungicides belong to this group of high volume production agrochemicals, being mainly used for treating grey rot, downy and powdery mildew and oidium [1,2]. It is well-known that fungicide residues existing in vinification grapes can be removed in a high degree by different dissipation mechanisms (sorption, degradation, etc.) during the wine production process; however, depending on factors such as their physico-chemical properties and wine-making operations, some of these agrochemicals can be also found at low concentration levels in the final commercial product [2–6]. The maxima residue limits (MRLs) allowed in the European Community for different fungicides in vinification grapes are well established and legislated [7]; however, despite of being a direct exposure source to consumers, MRLs for wine are still scarcely regulated [8]. Thus, in order to establish future legal measures, it is mandatory to perform both monitoring and toxicological studies to assess the background levels of these chemicals in different wines and to evaluate the chronic exposure of wine consumers to these contaminants, respectively.

Analysis of wine samples is a challenging issue due to the high complexity of the matrix. Depending on the fungicides nature, either gas chromatography (GC) [9–11] or liquid chromatography (LC) [12–14] coupled to different detectors are frequently employed as determination techniques. In terms of selectivity, mass spectrometry (MS) represents, at present, one of the most powerful detection tools for both chromatographic techniques. Particularly, LC-tandem MS appears increasingly in food applications related to this type of compounds [15–20] and it has been proven to provide better performance than GC–MS [21].

Traditionally, sample preparation is performed either by liquid–liquid extraction (LLE) using organic solvents [10,13] or solid-phase extraction (SPE), using reversed-phase materials [11,14,20]. LLE and commonly used reversed-phase SPE sorbents, such as C18 or Oasis HLB, usually render extracts containing too many interferences, which require further clean-up before anal-

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ysis and an increase in time and cost of the sample preparation. Purification approaches can be based on additional SPE steps using either normal-phase sorbents [12,22] or cartridges containing a combination of materials such as aminopropyl-$MgSO_4$ [11], or graphitized carbon black/primary secondary amine (GCB/PSA) [6,23]. Recently, the application of dispersive liquid–liquid microextraction (DLLME) has also been successfully reported for the purification of wine extracts obtained by SPE [24]. Ion-exchange based sorbents have been used by Schermerhorn et al. during fungicides determination for purification of fruit and water extracts [25]; however, to the best of our knowledge, no analytical methodology based on ion-exchange SPE has been optimized to perform simultaneous wine samples enrichment and purification.

More recently, miniaturized enrichment techniques such as solid-phase microextraction (SPME) have also been applied to the determination of fungicides in wine using polyacrylate (PA) [26], polydimethylsiloxane (PDMS) [9] or PDMS–divinylbenzene (DVB) fibers [27]. Despite SPME normally provides a higher selectivity than SPE, factors such as the ethanol content and the complexity of wine matrices reduce its applicability in terms of extraction efficiency compared to water samples [28,29]. These limitations are also common to stir-bar sorptive extraction (SBSE) [30], which, in addition, is only valid for the concentration of low polar fungicides.

In this work, a SPE methodology based on the use of a mixed-mode sorbent, that combines ionic exchange and reversed-phase mechanisms, for the extraction of nine selected fungicides (belonging to azolic, phenylamide and strobilurine classes) from wine samples is presented. The combination of the above retention mechanisms during sample preparation improved the selectivity of the process, avoiding further tedious and labour intensive clean-up steps. Different parameters affecting the extraction process (sorbent/eluent type and amount) have been optimized. Analysis was performed by LC-tandem MS after optimization of chromatographic conditions and MS parameters. Under final conditions, good performance was obtained for both red and white wines. Levels of fungicides in some commercial wines are also reported.

2. Experimental

2.1. Standards, solvents and material

Standards of azoxystrobin (99.9%), diniconazole (99.1%), difenoconazole (97%), flusilazole (99.8%), metalaxyl-M (99%), myclobutanil (99.4%), penconazole (99.1%), propiconazole (98.6%) and tebuconazole (99.6%), as well as ammonium acetate were purchased from Riedel de Haën (Seelze, Germany). The chemical structures of these compounds and some properties of relevance to optimize extraction (SPE) and LC separation processes are compiled in Table 1. Acetic and formic acid, acetone, ammonium hydroxide and HPLC-grade methanol were acquired from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Stock solutions of each compound were prepared in methanol. Further dilutions and mixtures of them were made in the same solvent. Standards in methanol were stored in the dark, at 4 °C for a maximum of two months.

SPE cartridges containing either OASIS HLB (60 mg) or OASIS MAX sorbents (60 and 150 mg) were provided by Waters (Milford, MA, USA).

Cellulose acetate membrane filters (0.45 μm pore size) were purchased from Millipore (Bedford, MA, USA).

2.2. Samples and sample preparation

Red and white wine samples obtained from local supermarkets were used in this study. After opening the bottles, wine was filtered and processed within 48 h. Optimization of sample preparation conditions was performed using two spiked pooled samples of red (*Grenache and Cabernet Sauvignon*) and white (*Albariño and Palomino*) wines. Spiked samples were maintained at 4 °C and used for a maximum of 48 h. Unless otherwise stated, during optimization of extraction conditions samples were spiked at the 50 ng mL^{-1} level.

Spiked and non-spiked samples were diluted (1:1) with ultrapure water and passed through the SPE cartridges at a flow of approximately 5 mL min^{-1}. After a washing step, the sorbent was dried using a stream of nitrogen and analytes were eluted with methanol. Breakthrough studies were carried out passing the spiked samples through two serially connected cartridges. The minimum elution volume was established by collecting consecutive fractions of methanol (1 mL each) from SPE cartridges.

Under optimal conditions, 10 mL wine samples were diluted with the same volume of ultrapure water and concentrated using Oasis MAX cartridges (150 mg), previously conditioned with methanol, ultrapure water (pH 6) and ultrapure water (pH 4) (5 mL each). Afterwards, cartridges were washed with 5 mL of a 5% (w:v) ammonium hydroxide solution, dried during 15 min with a gentle nitrogen stream and eluted with just 1 mL of methanol.

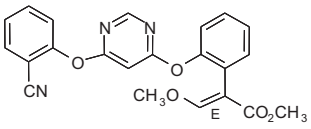
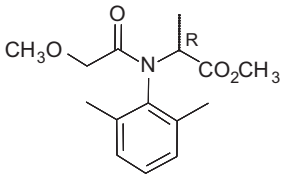
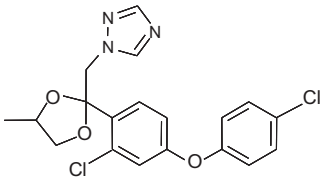
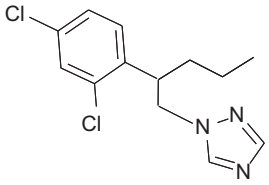
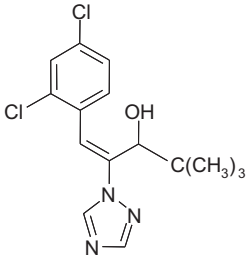
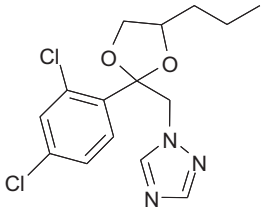
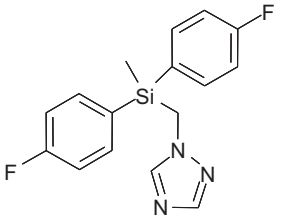
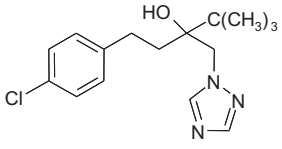
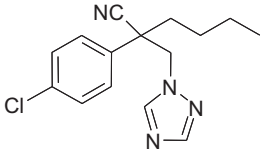
2.3. Equipment

Analytes were determined using a Varian (Walnut Creek, CA, USA) LC–MS/MS system. The LC instrument comprised two isocratic, high-pressure mixing pumps (Varian ProStar 210), an autosampler and a thermostated compartment for the column (Varian ProStar 410). The mass spectrometer was a triple quadrupole (Varian MS 1200L) furnished with an electrospray ionization (ESI) interface. A Varian ProStar 335 diode array detector (190–950 nm) connected in series after the column was occasionally used to test the complexity of the extracts obtained from red and white wines. The LC–MS/MS instrument was entirely controlled by the Varian MS Workstation Version 6.9 software. The same software was used to monitor the signal of the diode array detector.

Compounds were separated using a Kromasil C18 column (5 μm; 2.1 mm × 100 mm) acquired from Sugelabor (Madrid, SPAIN) and connected to a C18 (2 mm × 4 mm) guard cartridge supplied by Phenomenex (Torrance, CA, USA). Ultrapure water (A) and methanol (B), containing different amounts of formic acid or ammonium acetate as modifiers, were employed as mobile phases. Under final conditions, both phases were modified with formic acid (0.01%) and compounds were separated using the following gradient: 0–2 min, 15% B; 6 min, 40% B; 10 min, 70% B; 18 min, 75% B; 21–24 min, 100% B; 27–36 min, 15% B. The mobile phase flow was set at 0.25 mL min^{-1} and the temperature of the column fixed at 35 °C. The injection volume for standards and sample extracts was 15 μL.

Nitrogen (99.999%), used as nebulising (50 PSI) and drying gas (300 °C, 21 PSI) in the ESI source, was provided by a high purity generator (Domnick Hunter, Durham, UK). The ESI interface was operated in the positive mode and the voltage of the ESI needle fixed at 5000 V. The temperature of the ESI housing was set at 50 °C. Argon (99.999%) was employed as collision gas (2.2 mTorr) in the mass spectrometer. Fungicides were recorded in the multiple reaction monitoring (MRM) mode, using two transitions per compound and a dwell time of 0.18 s per transition. The most intense one was used to quantify the response of each species in standards and SPE extracts from real wine samples. Table 2 summarizes retention times, most intense MS/MS transitions, capillary voltages and collision energies for target species. Conversely to GC, the LC column was not able to separate the isomers of propiconazole and diniconazole [24].

Table 1
Names, CAS numbers, structures, pK_a and octanol–water partition coefficients of selected fungicides.

Name (abbreviation), CAS number and properties	Structure	Name (abbreviation), CAS number, pK_a and log Kow	Structure
Azoxystrobin (AZO) CAS: 131860-33-8; pK_a: −0.67; log Kow: 5.13		Metalaxyl-M (MET) CAS: 70630-17-0; pK_a: 1.41; log Kow: 2.15	
Difenoconazole (DIF) CAS: 119446-68-3; pK_a: 3.06; log Kow: 4.92		Penconazole (PEN) CAS: 66246-88-6; pK_a: 2.83; log Kow: 3.67	
Diniconazole (DIN) CAS: 70217-36-6; pK_a: 12.89, 2.19; log Kow: 4.23		Propiconazole (PRO) CAS: 60207-90-1; pK_a: 3.06; log Kow: 3.88	
Flusilazole (FLU) CAS: 85509-19-9; pK_a: 3.02; log Kow: 3.84		Tebuconazole (TEB) CAS: 107534-96-3; pK_a: 13.70, 3.39; log Kow: 3.58	
Myclobutanil (MYC) CAS: 88671-89-0; pK_a: 2.30; log Kow: 2.82			

2.4. Extraction efficiency, matrix effects and LOQ calculations

Absolute recoveries (as percentages) provided by the proposed method were calculated as: $\%R = [(C_s - C_b)/C_t] \times 100$, where C_s is the measured concentration in the extracts from spiked wine samples, C_b is the concentration measured in un-spiked aliquots of the same wine and C_t is the theoretical concentration, being C_s and C_b values established by external calibration. Matrix effects (%) during electrospray ionization were evaluated as recoveries using the difference of peak areas measured for spiked (A_{se}) and non-spiked extracts (A_{ne}) from 10 mL wine samples (addition was done after SPE) divided by the response obtained for

Table 2
Retention times and optimized ESI(+)-MS/MS conditions for selected fungicides. CV (capillary voltage, V); CE (collision energy, eV).

Compound	Ret. time (min)	MRM1 (quantification)	CV/CE	MRM2 (confirmation)	CV/CE	MRM1:MRM2 ratio
MET	12.27	280 > 220	72/8	280 > 192	72/11.5	64:36
AZO	12.93	404 > 372	39/10	404 > 329	39/25.5	82:18
MYC	14.54	289 > 70	70/12.5	289 > 125	70/24.5	72:28
FLU	16.42	316 > 165	63/18.5	316 > 247	63/13	51:49
PEN	17.22	284 > 159	71/21	284 > 70	71/11	55:45
TEB	17.62	308 > 70	65/12	308 > 125	65/27	83:17
PRO	17.92	342 > 159	56/20.5	342 > 69	56/13	94:6
DIN	19.79	326 > 70	60/14	326 > 159	60/23	80:20
DIF	20.19	406 > 251	39/18.5	406 > 337	39/12	82:18

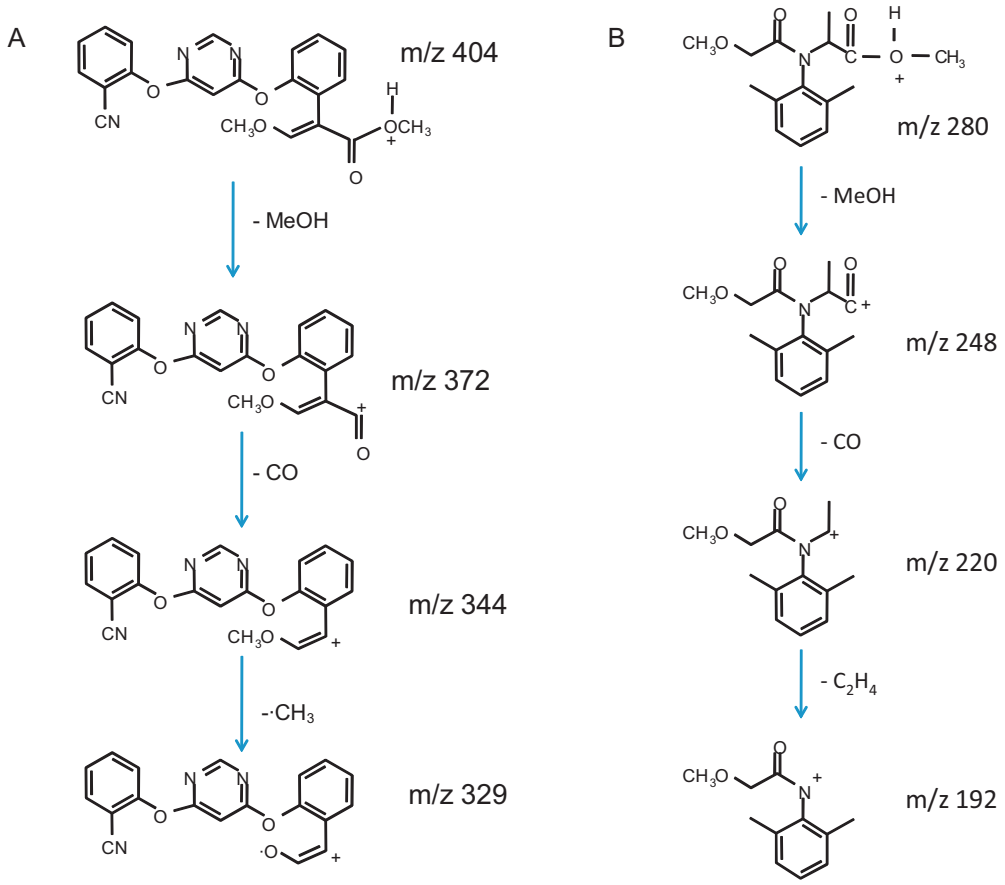


Fig. 1. LC–ESI-MS/MS fragmentation pattern for A, azoxystrobin and B, metalaxyl-M.

a standard with the same concentration prepared in methanol (A_{ss}): %ME = $((A_{se} - A_{ne})/A_{ss}) \times 100$. Therefore, the ion suppression percentage corresponds to 100 − %ME. LOQs of the whole methodology ($LOQ_{SPE-LC-MS/MS}$) were calculated from instrumental $LOQ_{LC-MS/MS}$, taking into account mean absolute recoveries: $LOQ_{SPE-LC-MS/MS} = LOQ_{LC-MS/MS}$ $(V_{ext}/V_s \cdot R)$, being V_{ext} and V_s the extract and sample volumes, respectively.

3. Results and discussion

3.1. Optimization of LC–MS/MS parameters

3.1.1. Ionization and fragmentation conditions

The optimization of ESI-MS/MS parameters was performed by direct infusion of 5 μg mL^{-1} individual standards in methanol using an isocratic flow of methanol–ultrapure water (1:1) (50 μL min^{-1}). Both, positive and negative ESI were investigated; however, compounds were only ionized in a significant extent in the positive mode, which agrees with their basic character. The capillary voltage was fixed at the maximum parent ion $[M+H]^+$ intensity, and afterwards, the collision energy for the most intense daughter ions was also optimized. Once main MS/MS transitions were identified for each compound, the fragmentation pattern was investigated. In the case of azoxystrobin, the main transition (404 → 372 *m/z*) corresponds to the methanol loss [17–21]. Additional losses of carbonyl and methyl groups produce 344 and 329 *m/z* ions, respectively (Fig. 1A). Although, the 404 → 344 *m/z* transition has been used as qualifier by most authors [17–21], the *m/z* 372 showed higher intensity and was chosen in our case.

Methanol loss is also observed during the fragmentation of metalaxyl-M. This yields the minority *m/z* 248 fragment and it is followed by a CO elimination giving the quantification ion *m/z* 220 [17,19–21] (Fig. 1B). Afterwards, the cleavage of the C–N bond takes place producing the *m/z* 192 species. Some authors have used the *m/z* 160 ion, corresponding to a further methanol loss from the *m/z* 192 fragment as primary product ion [21], but under our ionization conditions it presented lower intensity.

The cleavage of the C–N bond to release the triazolic ring is a common fragmentation pattern in the case of triazolic compounds [15]. However, there are some differences in fragmentation depending on fungicide structure. On one hand, PEN, DIN, MYC and TEB follow different rearrangements to produce the *m/z* 70 product ion [15] which is commonly used as quantification (MYC, TEB and DIN) or as confirmation (PEN) ion. The other positively charged fragment for the above compounds corresponded to a di- or monochlorinated tropylium ion (*m/z* 159 and 125, respectively) (Table 2). On the other hand, PRO, FLU and DIF undergo a C–N bond simple cleavage to release the much less intense *m/z* 69 product ion. The $[M-C_2H_3N_3]^+$ ions correspond to *m/z* 247 and *m/z* 337 for FLU and DIF, respectively. In case of PRO, a transposition of the methylene group, followed by a CO_2 loss, yields the *m/z* 159 ion [15,18], a similar mechanism yields the *m/z* 251 ion for DIF (see Table 2).

3.1.2. Selection of modifier

The ionic strength and the pH of the mobile phase are parameters that can affect notoriously the ionization efficiency and the chromatographic separation. Two commonly used modifiers in LC–MS, one with acidic nature (formic acid) and another with saline character (ammonium acetate), were separately evaluated for signal enhancement. With this purpose, a standard of 250 ng mL^{-1} was injected in the chromatographic system in consecutive runs increasing the amount of modifier in the mobile phase up to 0.5% of

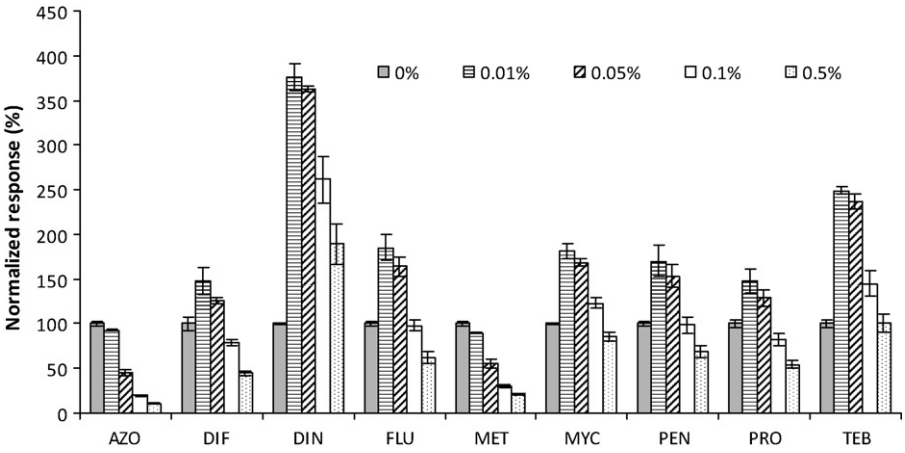


Fig. 2. Influence of formic acid percentage on the ionization efficiency.

formic acid, or up to 3 mM ammonium acetate. Fig. 2 shows the signal variation with the increase of formic acid content in the mobile phase. As it can be noticed, just a small percentage of acidic modifier (0.01%) is enough to provide a significant signal increase for the seven triazolic species, which show a higher basic character than AZO and MET. The ionization is favored because a slightly acid medium improves the formation of the $[M+H]^+$ precursor ions, and consequently, the intensity of MRM transitions. On the other hand, higher percentages lead to a progressive decrease in the monitorized responses, possibly due to ionic suppression phenomena.

The addition of ammonium acetate did not produce a significant improvement in the response, and slightly reduced the obtained peak areas at concentrations higher than 0.05 mM, data not shown. No changes in the LC separation efficiency were observed for the evaluated modifiers concentration range. Thus, based on the above comments, a percentage of 0.01% formic acid was selected as mobile phase modifier.

3.1.3. LC–MS/MS performance

Instrumental performance is summarized in Table 3. Calibration standard solutions at six different levels, in the range LOQ–1000 ng mL^{-1}, were injected in the system, providing a linear relationship between peak area and concentration with determination coefficients (R^2) higher than 0.997 for all analytes.

Limits of quantification determined by a signal to noise ratio of 10 (S/N = 10) were in the range 0.1 ng mL^{-1} obtained for AZO till 7 ng mL^{-1} for DIN. Fig. 3 shows a chromatogram obtained for a standard solution of target fungicides (20 ng mL^{-1}) monitoring the primary transitions. As it can be noticed, after optimization of the mobile phase gradient, fungicides were separated in a chromatographic run of about 20 min.

Instrumental response precision was investigated with standards at two concentration levels: 20 and 500 ng mL^{-1}. Relative standard deviations (RSD, %) for consecutive injections (n = 3 replicates) made in the same day ranged between 0.4 and 10.2%, and a maximum RSD of 10.6% was obtained for injections (n = 9 replicates) in three consecutive days.

3.2. Optimization of SPE conditions

3.2.1. Evaluation of sorbent selectivity

Two polymeric materials were evaluated as potential sorbents to concentrate wine samples. They were (1) the hydrophilic–lipophilic balance OASIS HLB sorbent, commonly found in environmental and food applications, and (2) the OASIS-MAX material which combines the same reversed-phase interactions as OASIS HLB (Van der Waals and π–π interactions) with an additional mechanism of anionic exchange, based on quaternary amine functionalized groups.

SPE experiments were performed with 20 mL aliquots of spiked, pooled red and white wine samples. Methanol was selected as elution solvent taking into account (1) its compatibility with reversed-phase LC separation process and (2) elution strength. In a first series of extractions, HLB and MAX cartridges (60 mg) were just washed with ultrapure water (5 mL). After being dried, analytes were recovered with 1 mL of methanol. The visual appearance of the corresponding extracts was related to the type of wine and the nature of the SPE sorbent. The most complex ones corresponded to the concentration of red wine using the HLB sorbent, which rendered intense red extracts. For the same matrix, light reddish extracts were noticed using the MAX cartridge, which kept a dark appearance after being eluted with methanol.

Table 3
Linearity, instrumental limits of quantification (LOQs), repeatability and reproducibility of the LC–MS/MS system.

Compound	Linearity, R^2 (LOQs–1000 ng mL^{-1})	LOQs (ng mL^{-1})	Repeatability (RSD, %)[a]		Reproducibility (RSD, %)[b]	
			20 ng mL^{-1}	500 ng mL^{-1}	20 ng mL^{-1}	500 ng mL^{-1}
MET	0.9987	1	7.1	1.1	8.8	5.2
AZO	0.9978	0.1	3.1	2	6.3	6.7
MYC	0.9996	2.5	1.4	0.5	6.9	4.6
FLU	0.9998	1	4.7	1.0	8.5	6.8
PEN	0.9992	2.2	3.5	1.4	8.9	4.3
TEB	0.9997	2	10.2	0.4	9.5	3.9
PRO	0.9998	2.5	7.8	0.5	8.3	5.4
DIN	0.9997	7	7.4	1.2	10.6	7.4
DIF	0.9999	1	9.0	1.9	7.3	5.4

[a] n = 3 injections in the same day.
[b] n = 9 injections in three consecutive days.

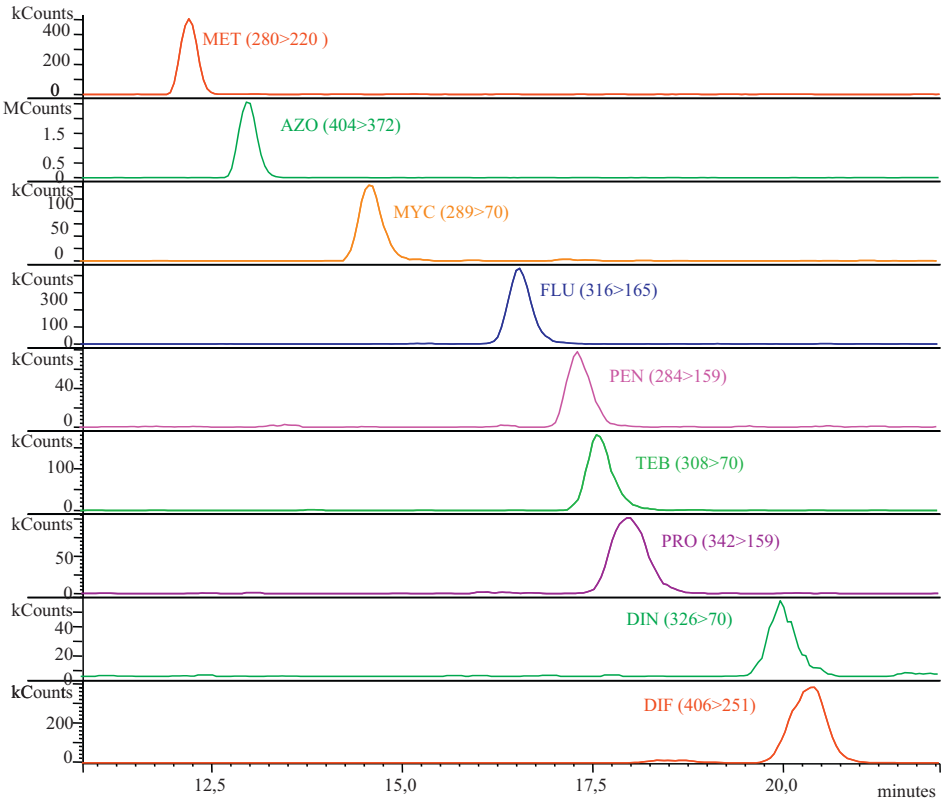


Fig. 3. MRM chromatogram for a standard solution of target fungicides (20 μg L^{-1}).

In a further series of extractions, the SPE cartridges were washed with 5 mL of an aqueous 5% (w:v) NH_4OH solution. Modification of the clean-up step did not change the color of the HLB extracts; however, it allowed obtaining colorless, completely transparent extracts from the MAX sorbent (Fig. 4). Both types of extracts were injected in the LC column using UV detection at 254 nm. As depicted in Fig. 4 for red wine, a significantly better baseline, showing less interferences between 6 and 15 min, was achieved with the mixed mode MAX cartridge.

Using any of both sorbents, phenols, polysaccharides and organic acids are retained together with target fungicides by the reversed-phase mechanism. After concentration of the sample, the basic washing step allows acids and phenols to become ionized and retained strongly by electrostatic interactions with the quaternary amine groups in MAX cartridges. When this sorbent is further eluted with 1 mL of MeOH, just neutral and weak bases, as our target analytes, are recovered. This selective extraction does not occur with the HLB sorbents and therefore is the responsible for cleaner extracts. Consequently, the MAX sorbent was selected to continue this research.

Breakthrough studies showed that the 60 mg MAX sorbent failed to quantitatively retain MET, the most polar of the selected compounds (Table 1), from 20 mL wine samples, with a significant percentage of the fungicide appearing in the second cartridge. Even when the sample intake was reduce to 10 mL, around 10% of the compound passed to the second cartridge. The 150 mg MAX cartridges allowed concentrating up to 10 mL of wine without noticeable breakthrough problems for any compounds. On the other hand, 1 mL of methanol suffices for the elution step.

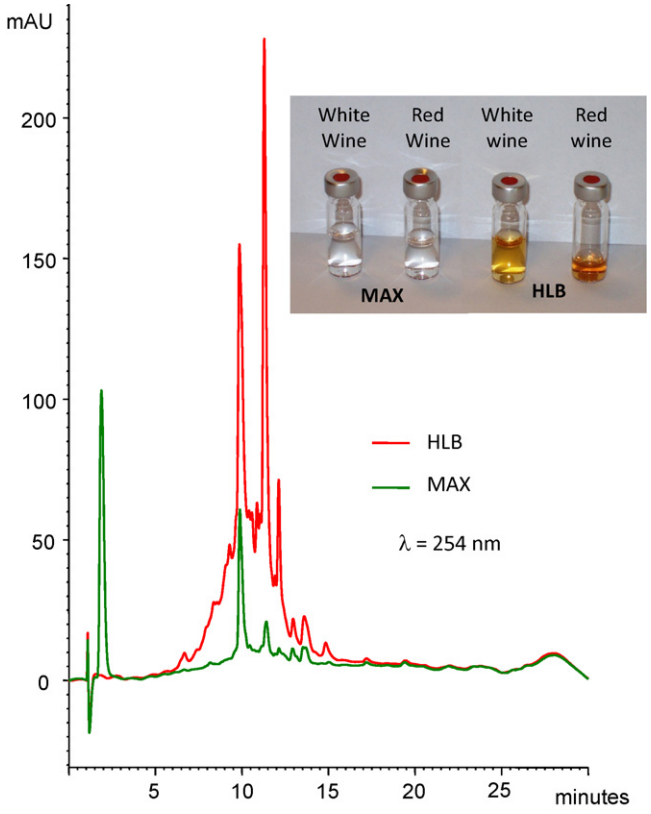


Fig. 4. LC-UV chromatograms (λ = 254 nm) corresponding to red wine extracts obtained after SPE with OASIS HLB and MAX cartridges. Inserted picture shows different extracts appearance.

3.3. Matrix effects

One of the main drawbacks of LC–MS atmospheric pressure ionization interfaces, particularly ESI, is the signal suppression caused

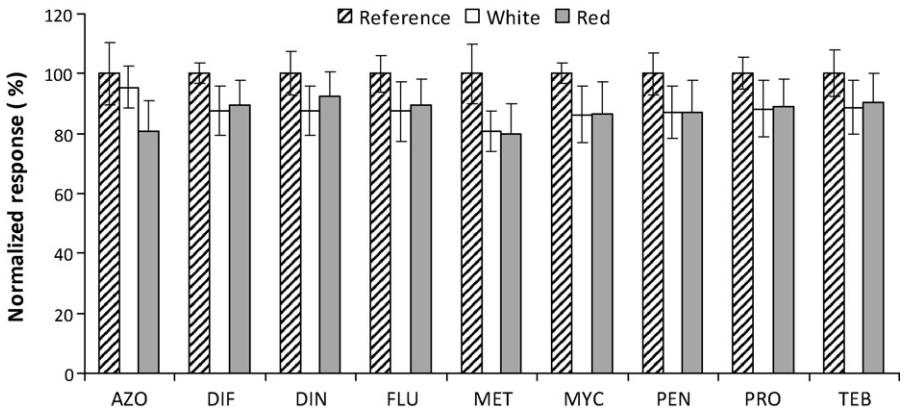


Fig. 5. Fungicide recoveries affected by matrix effects for both white and red wines as normalized areas.

by matrix co-eluting components competing with target analytes during the interface ionization process.

Fig. 5 shows the normalized responses obtained for 10 mL spiked wine samples versus a reference standard in methanol. For most compounds, relative responses around 90% were noticed in the two types of wine. Responses for MET, the earliest eluting species from the LC column, corresponded to 80% of those obtained for this compound in the reference standard. Globally, data in Fig. 5 proved the existence of just weak (between 10 and 20%) ionic suppression effects for red and white wine samples, pointing to the feasibility of considering external calibration as a valid, straightforward quantification approach.

3.4. Validation of the analytical methodology

Figures of merit of the proposed methodology are presented in Table 4. Precision and accuracy were assessed with the analysis of non-spiked red/white wine samples and also with spiked samples at two levels of concentration: 2 ng mL^{-1} and 20 ng mL^{-1}. The absolute recoveries of the whole procedure ranged from 72% to 97%. These values, combined with the previous evaluation of matrix effects confirmed that (1) compounds are quantitatively recovered (retained and then eluted) in the sample preparation process and (2) external calibration is a valid quantification strategy. Acceptable repeatability was also obtained for both wine types, with SD lower than 9 for all fungicides.

Method LOQs, calculated for free of fungicides wines, were mainly controlled by the instrumental LOQs of the LC–MS/MS system and the enrichment factor of the sample preparation process. The calculated method LOQs ranged from 0.01 μg L^{-1} for AZO to 0.8 μg L^{-1} for DIN; thus, they remain far below the EU MRLs considered for this type of fungicides in grapes (0.05–2 μg g^{-1}) and wine (0.2 and 2 μg g^{-1} for FLU and TEB, respectively) (Table 4). They would also fulfill the *Organisation Internationale de la Vigne* (OIV) recommendation which suggests taking for wine 1/10 of grapes MRLs [31]. Moreover, these LOQs are of the same range or better than those reported by other authors using SPE coupled to GC–MS (0.02–0.12 μg L^{-1}) [24], LC–MS (9–28 μg L^{-1}) [14] or LC–MS/MS (2–9 μg L^{-1}) [20] for wine analysis.

3.5. Application to real samples

The developed methodology was applied to the analysis of different commercial red and white wines mostly from northwestern Spain. Table 5 summarizes the type of grape and geographic denomination of the different processed wines together with found fungicide concentrations. Fig. 6 shows the MRM chromatograms obtained for compounds detected in the red wine sample code R2, a procedural blank and a 20 ng mL^{-1} standard solution.

In most samples, AZO and MET were detected in concentrations in the range of 0.2–2.9 ng mL^{-1} and 0.15–30.2 ng mL^{-1}, respectively. These findings are in agreement with the observation of Cabras et al. [1], who reported the incomplete removal of both agrochemicals during wine-making from grapes proceeding from treated vineyards. Detection of TEB also reveals its just partial elimination during the wine-making as postulated by González-Rodríguez et al. [6].

Although at present EU MRLs for MET and AZO have not yet been established in wine, an adequate analytical methodology and reliable occurrence data are powerful tools to develop future legislation. Thus, other European country, Switzerland, has already regulated these fungicides in wine at 0.6 and 0.5 μg g^{-1}, respectively [32].

Table 4
Accuracy, precision and quantification limits for the proposed methodology for red and white wine at two different concentration levels.

Compound	Absolute recoveries (%) (N = 3)				EU MRLs grape, wine (ng g^{-1})	$LOQ_{SPE-LC-MS/MS}$ (ng mL^{-1})
	White wine		Red Wine			
	2 ng mL^{-1}	20 ng mL^{-1}	2 ng mL^{-1}	20 ng mL^{-1}		
MET	83 ± 9	78 ± 5	77 ± 1	73 ± 7	1000	0.1
AZO	78 ± 8	88 ± 2	83 ± 3	77 ± 8	2000	0.01
MYC	80 ± 6	93 ± 1	89 ± 6	81 ± 7	1000	0.3
FLU	72 ± 4	76 ± 2	72 ± 2	74 ± 5	200, 200[a]	0.1
PEN	78 ± 5	96 ± 1	96 ± 4	84 ± 8	200	0.2
TEB	82 ± 4	97 ± 1	87 ± 6	84 ± 9	2000, 2000[a]	0.2
PRO	82 ± 6	97 ± 1	88 ± 8	84 ± 8	50	0.3
DIN	80 ± 6	97 ± 2	96 ± 5	81 ± 7	200	0.8
DIF	80 ± 3	94 ± 1	93 ± 4	81 ± 7	500	0.1

[a] These MRLs are for wine produced after 1 September 2008.

Table 5
Levels of different fungicides in non-spiked wine samples. Average concentrations (ng mL^{-1}) with their standard deviations, $n = 3$ replicates.

Wine code	Geographic denomination	Type of grape	AZO	MET	MYC	TEB
W1	–	*Albariño*	n.d.	n.d.	n.d.	n.d.
W2	–	*Palomino*	0.279 ± 0.002	0.96 ± 0.03	n.d.	n.d.
W3	Rías Baixas	*Albariño*	2.9 ± 0.2	28.6 ± 0.3	n.d.	n.d.
W4	Rías Baixas	*Albariño*	0.299 ± 0.006	21.7 ± 0.7	n.d.	n.d.
W5	Ribeiro	*Palomino*	0.3 ± 0.1	20.5 ± 1.3	n.d.	n.d.
W6	Ribeiro	*Dona Branca*	n.d.	30.2 ± 0.5	n.d.	n.d.
W7	Bierzo	*Dona Branca and Palomino*	0.52 ± 0.03	1.8 ± 0.1	n.d.	n.d.
R1	Ribeiro	*Garnacha*	2.6 ± 0.2	6.5 ± 0.4	n.d.	n.d.
R2	Ribeira Sacra	*Mencía*	0.56 ± 0.02	8.8 ± 0.7	1.12 ± 0.07	<LOQ
R3	Valdepeñas	*Tempranillo and Cabernet Sauvignon*	0.390 ± 0.002	1.79 ± 0.06	0.35 ± 0.05	n.d.
R4	Penedés	*Tempranillo and Cabernet Sauvignon*	0.2 ± 0.1	0.15 ± 0.05	0.35 ± 0.01	0.43 ± 0.04

n.d. not detected; Codes W and R correspond to white and red wines, respectively.

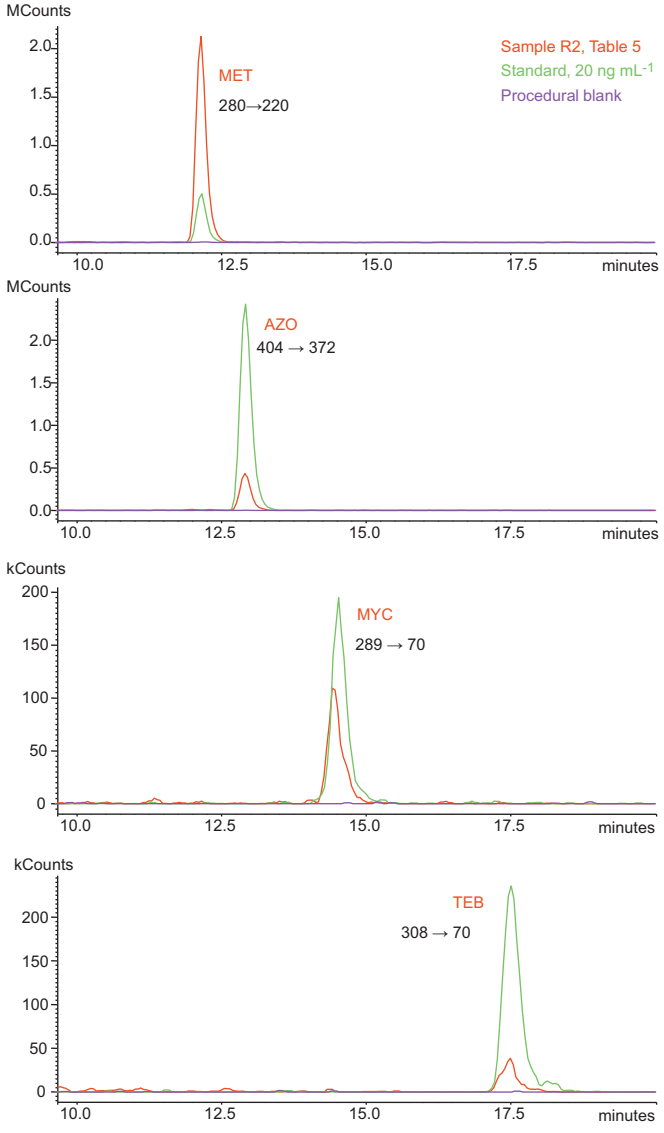


Fig. 6. MRM chromatograms showing selected transitions for MET, AZO, MYC and TEB in a red wine sample (code R2, Table 5), a standard solution (20 ng mL^{-1}) and a procedural blank.

It must be considered that most data in Table 5 corresponds to wines from a region with wet weather, where fungi are a common problem in vineyards, and a high number of fungicide treatments are applied before harvest. Anyhow, mean values reported for these samples are in agreement with those measured by other authors. For instance, Trösken et al. [15] found TEB levels between 0.25 and 33 ng mL^{-1} and MYC levels of 0.5–35 ng mL^{-1} in red and white wines from different origins. TEB measured concentration was far below the legal EU (2 μg g^{-1}) and Switzerland (0.3 μg g^{-1}) MRLs for wine.

4. Conclusions

The analytical methodology for the determination of 9 fungicides in wine samples has been successfully developed. For sample preparation, SPE was optimized using divinylbenzene-N-vinylpirrolidone funcionalized with quaternary amine groups as sorbent and analysis was performed by LC–ESI-tandem MS. The developed method presents good linearity, accuracy and precision with LOQs in the range of 0.01–0.8 ng mL^{-1} for target fungicides in wine, which are far below EU MRLs for vinification grapes and wine. The high selectivity of the sorbent material makes the SPE based on MAX cartridges a promising alternative to conventional extraction techniques for determination of fungicides in complex matrices such as wine. Its ionic exchange capacity allows the selective retention of matrix interferences and provides purified extracts that can be quantified by external calibration. AZO and particularly MET were found in most of the processed wine samples, with the latter compound being measured at concentrations as high as 30 ng mL^{-1}.

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References

[1] P. Cabras, E. Conte, Food Addit. Contam. 18 (2001) 880.
[2] R.M. González-Rodríguez, B. Cancho-Grande, J. Simal-Gándara, J. Sci. Food Agric. 89 (2009) 2625.
[3] P. Cabras, A. Angioni, J. Agric. Food Chem. 48 (2000) 967.
[4] P. Cabras, A. Angioni, V.L. Garau, F.M. Pirisi, J. Espinoza, A. Mendoza, F. Cabitza, M. Pala, V. Brandolini, J. Agric. Food Chem. 46 (1998) 3249.
[5] S. Navarro, J. Oliva, A. Barba, G. Navarro, M.A. García, M. Zamorano, J. Agric. Food Chem. 48 (2000) 3537.
[6] R.M. González-Rodríguez, B. Cancho-Grande, A. Torrado-Agrasar, J. Simal-Gándara, J. Mazaira-Pérez, Food Chem. 117 (2009) 529.
[7] Off. J. Eur. Union L70 (2005) 1.
[8] European Food Safety Authority Scientific Report 227 (2009) 1.
[9] M. Correia, C. Delerue-Matos, A. Alves, Fresenius J. Anal. Chem. 369 (2001) 647.
[10] J. Oliva, S. Navarro, A. Barba, G. Navarro, J. Chromatogr. A 833 (1999) 43.
[11] J.W. Wong, M.G. Webster, C.A. Halverson, M.J. Hengel, K.K. Ngim, S.E. Ebeler, J. Agric. Food Chem. 51 (2003) 1148.
[12] S. de Melo Abreu, M. Correia, P. Herbert, L. Santos, A. Alves, Food Addit. Contam. 22 (2005) 549.
[13] S. de Melo Abreu, P. Caboni, P. Cabras, V.L. Garau, A. Alves, Anal. Chim. Acta 573–574 (2006) 291.

[14] M.J. Nozal, J.L. Bernal, J.J. Jiménez, M.T. Martín, J. Bernal, J. Chromatogr. A 1076 (2005) 90.
[15] E.R. Trösken, N. Bittner, W. Völkel, J. Chromatogr. A 1083 (2005) 113.
[16] A. Sannino, J. AOAC Int. 87 (2004) 991.
[17] M.D. Hernando, C. Ferrer, M. Ulaszewska, J.F. García-Reyes, A. Molina-Díaz, A.R. Fernández-Alba, Anal. Bioanal. Chem. 389 (2007) 1815.
[18] K. Greulich, L. Alder, Anal. Bioanal. Chem. 391 (2008) 183.
[19] A. Sannino, L. Bolzoni, M. Bandini, J. Chromatogr. A 1036 (2004) 161.
[20] A. Economou, H. Botitsi, S. Antoniou, D. Tsipi, J. Chromatogr. A 1216 (2009) 5856.
[21] L. Alder, K. Greulich, G. Kempe, B. Vieth, Mass Spectrom. Rev. 25 (2006) 838.
[22] D.T. Likas, N.G. Tsiropoulos, Int. J. Environ. Anal. Chem. 89 (2009) 857.
[23] R.M. González-Rodríguez, B. Cancho-Grande, J. Simal-Gándara, J. Chromatogr. A 1216 (2009) 6033.
[24] R. Montes, I. Rodríguez, M. Ramil, E. Rubí, R. Cela, J. Chromatogr. A 1216 (2009) 5459.
[25] P.G. Schermerhorn, P.E. Golden, A.J. Krynitsky, W.M. Leimkuehler, J. AOAC Int. 88 (2005) 1491.
[26] C.G. Zambonin, A. Cilenti, F. Palmisano, J. Chromatogr. A 967 (2002) 255.
[27] S. Millán, M.C. Sanpedro, N. Unceta, M.A. Goicolea, E. Rodríguez, R.J. Barrio, J. Chromatogr. A 995 (2003) 135.
[28] D.A. Lambropoulou, I.K. Konstantinou, T.A. Albanis, J. Chromatogr. A 893 (2000) 143.
[29] R. Rial Otero, C. Yagüe Ruiz, B. Cancho Grande, J. Simal Gándara, J. Chromatogr. A 942 (2002) 41.
[30] P. Viñas, N. Aguinaga, N. Campillo, M. Hernández-Córdoba, J. Chromatogr. A 1194 (2008) 178.
[31] OIV, Résidus de Pesticides, Limites Autorisées: Classement par Pays et par Pesticide, Office International de la Vigne et du Vin, Paris, 1995.
[32] http://www.awri.com.au/industry_support/viticulture/agrochemicals/mrls.