



## Solid-phase extraction followed by liquid chromatography quadrupole time-of-flight tandem mass spectrometry for the selective determination of fungicides in wine samples

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### ABSTRACT

In this work, a reliable and selective procedure for the determination of thirteen fungicides in red and white wine samples is proposed. Solid-phase extraction (SPE) and liquid chromatography (LC) tandem mass spectrometry (MS/MS), based on a hybrid quadrupole time-of-flight (QTOF) system, were used as sample preparation and determination techniques, respectively. Extraction and purification of target analytes was carried out simultaneously by using a reversed-phase Oasis HLB (200 mg) SPE cartridge combined with acetonitrile as elution solvent. Fungicides were determined operating the electrospray source in the positive ionization mode, with MS/MS conditions adjusted to obtain at least two intense product ions per compound, or registering two transitions per species when a single product was noticed. High selective MS/MS chromatograms were extracted using a mass window of 20 ppm for each product ion. Considering external calibration as quantification technique, the overall recoveries (accuracy) of the procedure ranged between 81% and 114% for red and white wine samples (10–20 mL), spiked at different concentrations between 5 and 100 ng mL<sup>-1</sup>. Relative standard deviations of the above data stayed below 12% and the limits of quantification (LOQs) of the method, calculated for 10 mL of wine, varied between 0.1 ng mL<sup>-1</sup> for cyprodinil (CYP) and 0.7 ng mL<sup>-1</sup> for myclobutanil (MYC). The optimized method was applied to seventeen commercial wines produced in Spain and obtained from local supermarkets. Nine fungicides were determined, at levels above the LOQs of the method, in the above samples. The maximum concentrations and the highest occurrence frequencies corresponded to metalaxyl (MET) and iprovalicarb (IPR).

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### 1. Introduction

The incidence of diseases in vineyards, particularly fungal infections, is responsible for important economical losses in the viticulture sector. The most common fungal infections are grey-mold (*Botrytis cinerea*), powdery mildew (*Uncinula necator*) and downy mildew (*Plasmopara viticola*) [1]. Fungicides are extensively used to prevent and control fungal attacks [2], representing the main group of pesticides applied on vineyards. The use of these agrochemicals has a special relevance in regions with high humidity and warm temperatures. Several factors, such as type and concentrations of fungicides applied to vines, time period and climatic conditions from the last spraying until vintage, determine the

occurrence and the concentration of fungicide residues in grapes. Thereafter, the characteristics of each pesticide and the wine-making operations affect the transfer of fungicides from grapes to wine [3–7]. The European Union (EU) has established the maximum permissible concentrations of several fungicides in vinification grapes, expressed as the Maximum Residue Limits (MRLs) [8]. Nevertheless, with a few exceptions [9], the presence of fungicides in wine is not regulated being an issue of concern for consumers and producers. In this sense, the determination of fungicide residues in wine is necessary for food safety monitoring and future regulatory purposes. Moreover, it can be also useful to select those agrochemicals showing the lowest transfer factors from grapes to wine.

Sample preparation plays an important role in the determination of fungicides due to the complexity of wine matrix and the low expected concentration of target analytes (ng mL<sup>-1</sup> range). The most common approaches are liquid–liquid extrac-

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tion using organic solvents [10–12] solid-phase extraction (SPE) [13,14], solid-phase microextraction [15,16], hollow-fiber liquid-phase microextraction [17] and QuEChERS [18]. Among the above techniques, SPE offers a good compromise among robustness, rapidity, clean-up efficiency, scope for automation and solvent consumption.

As regards the determination step, most procedures are based on gas chromatography (GC) [10,19,20] or liquid chromatography (LC) [2,11,21] coupled with different detection systems, with a high percentage of studies dealing with mass spectrometry (MS) detection. Particularly, LC–tandem mass spectrometry (MS/MS) is being increasingly used in food applications related to fungicide compounds [21–24]. Triple quadrupole (QqQ) mass analyzers are the most resorted instruments, providing sensitive detection of target analytes in the multiple reaction monitoring (MRM) mode. Hybrid mass spectrometers, such as quadrupole time-of-flight (QTOF) systems, are emerging as an appealing alternative to QqQ instruments providing accurate mass measurements and the possibility to record full scan MS/MS spectra [25–27]. This feature avoids the loss of structural information of QqQ systems when operated in the MRM mode [28]. Also, TOF instruments offer valuable information of non-target or unknown analytes, which might be of interest to investigate the presence of new fungicides and/or their transformation by-products in complex food samples [29]. Moreover, last generation LC–QTOF instruments show an improved sensitivity as well as wider linear response ranges [30].

The objective of this work was the development and validation of a method for the identification and quantification of thirteen fungicides, belonging to different chemical classes, in wines using SPE followed by LC–QTOF–MS/MS. Sample preparation conditions were optimized in order to maximize the yield and the selectivity of the extraction process, avoiding changes in the efficiency of the electrospray ionization (ESI) between standard solutions and sample extracts. LC–QTOF–MS/MS parameters were adjusted to allow the sensitive and unequivocal determination of selected fungicides. The performance of the proposed methodology was evaluated in terms of limits of quantification (LOQs), absolute recoveries, precision and linear working range. Moreover, the procedure was applied to the determination of target fungicides in commercial wine samples from different regions of Spain.

## 2. Experimental

### 2.1. Standards, solvents and sorbents

HPLC-grade acetonitrile and methanol were acquired from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Formic acid, acetic acid and sodium hydroxide were also provided by Merck and ammonium acetate was from Riedel de Haën (Seelze, Germany). Standards of azoxystrobin (99.9%), benalaxyl (99.9%), cyprodinil (99.8%), diniconazole (99.1%), difenoconazole (97%), flusilazole (99.8%), iprovalicarb (98.5%), metalaxyl (99%), myclobutanil (99.4%), penconazole (99.1%), propiconazole (98.6%), tebuconazole (99.6%) and triadimenol (100 µg mL<sup>-1</sup> in acetonitrile) were purchased from Riedel de Haën and Sigma–Aldrich (Milwaukee, WI, USA). Table 1 summarizes the abbreviated names, molecular formulas, exact masses and other relevant properties of target analytes. Stock solutions of the above fungicides were prepared in acetonitrile. Further dilutions and mixtures of them were made in the same solvent. Standards in acetonitrile were stored in the dark, at 4 °C, for a maximum of two months. Calibration standards were dissolved in acetonitrile:water (1:1) and used for a maximum of one week after preparation.

OASIS HLB (200 mg) and OASIS MAX (150 mg) solid-phase extraction (SPE) cartridges were provided by Waters (Milford, MA, USA).

### 2.2. Samples and sample preparation

Wine samples were purchased in local supermarkets. Sample preparation conditions were optimized with pooled samples of red (*Tempranillo*, *Mencía* and *Cabernet Sauvignon*) and white (*Albariño* and *Palomino*) wines, spiked with target analytes at different concentrations in the range from 5 to 100 ng mL<sup>-1</sup>. Spiked samples were maintained at 4 °C and used for a maximum of 48 h. Synthetic wine, considered as procedural blank, was prepared by addition of tartaric acid (3.5 g L<sup>-1</sup>) to a 12% ethanol solution in ultrapure water, followed by pH adjustment to 3.6 with NaOH (1 M).

Wine samples were diluted with ultrapure water (1:2) and passed through the considered SPE cartridge (ca. 5 mL min<sup>-1</sup>), which was previously conditioned with the elution solvent followed by ultrapure water adjusted at pH 3.6 with acetic acid (5 mL of each). After the concentration step, cartridges were rinsed with 10 mL of ultrapure water, dried for 20 min using a gentle stream of nitrogen and eluted with a reversed-phase LC compatible organic solvent. This extract was diluted with ultrapure water (1:1) before injection in the chromatographic system. Breakthrough studies were performed by passing the spiked samples through two cartridges connected in series. The elution volume was established by collecting consecutive 1 mL fractions of solvent from the SPE cartridge.

Under final working conditions, wine samples (10–20 mL) were concentrated using Oasis HLB cartridges (200 mg). Analytes were further recovered with 2 mL of acetonitrile.

### 2.3. LC–MS/MS analysis

Target fungicides were determined using a LC–ESI–QTOF system acquired from Agilent (Wilmington, DE, USA). The LC instrument was an Agilent 1200 Series, consisting of vacuum degasser unit, autosampler, two isocratic high pressure mixing pumps and a chromatographic oven. The QTOF mass spectrometer was an Agilent 6520 model, furnished with a Dual-Spray ESI source.

Compounds were separated in a reversed-phase Zorbax Eclipse XDB-C18 column (100 mm × 2.1 mm, 3.5 µm) acquired from Agilent and connected to a C18 (4 mm × 2 mm) guard cartridge supplied by Phenomenex (Torrance, CA, USA). Ultrapure water (A) and acetonitrile (B), both containing ammonium acetate 1 mM, were used as mobile phases. Under final working conditions, compounds were separated using the following gradient: 0–2 min, 30% B; 2–7 min, 50% B; 7–15 min, 50% B; 15–20 min, 65% B; 20–23 min, 75% B; 23–24 min, 100% B; 24–27 min, 100% B; 27–28 min, 30% B; 28–34 min, 30% B. The mobile phase flow was 0.2 mL min<sup>-1</sup>, and the temperature of the column 35 °C. The injection volume for standards and sample extracts was 15 µL.

Nitrogen (99.999%), used as nebulising (40 PSI) and drying gas (300 °C, 9 L min<sup>-1</sup>) in the dual ESI source, was provided by a high purity generator (ErreDue srl, Livorno, Italy). The QTOF instrument was operated in the 2 GHz Extended Dynamic Range resolution mode and compounds were ionized in positive ESI, applying a capillary voltage of 4000 V. A reference calibration solution (Agilent calibration solution A) was continuously sprayed in the source of the QTOF system, employing the ions with *m/z* 121.0509 (purine) and 922.0098 (HP-921) for recalibrating the mass axis ensuring the accuracy of mass assignments throughout the chromatographic run. The Mass Hunter Workstation software was used to control all the acquisition parameters of the LC–ESI–QTOF system and also to process the obtained data.

**Table 1**  
Abbreviated names, CAS numbers, molecular formulas, exact masses and relevant properties of target species.

Analyte	Abbreviation	CAS number	Chemical class	Molecular formula	Exact mass	<sup>a</sup> pKa	<sup>a</sup> log K <sub>ow</sub>
Metalaxyl-M	MET	70630-17-0	Phenylamide	C <sub>15</sub> H <sub>21</sub> NO <sub>4</sub>	279.147059	1.41	1.76
Benalaxyl-M	BEN	71626-11-4	Phenylamide	C <sub>20</sub> H <sub>23</sub> NO <sub>3</sub>	325.167794	1.52	3.88
Penconazole	PEN	66246-88-6	Triazole	C <sub>13</sub> H <sub>15</sub> Cl <sub>2</sub> N <sub>3</sub>	283.064301	2.83	4.64
Diniconazole	DIN	83657-24-3	Triazole	C <sub>15</sub> H <sub>17</sub> Cl <sub>2</sub> N <sub>3</sub> O	325.074866	2.19	4.34
Propiconazole	PRO	60207-90-1	Triazole	C <sub>15</sub> H <sub>17</sub> Cl <sub>2</sub> N <sub>3</sub> O <sub>2</sub>	341.069781	2.94	3.65
Difenoconazole	DIF	119446-68-3	Triazole	C <sub>19</sub> H <sub>17</sub> Cl <sub>2</sub> N <sub>3</sub> O <sub>3</sub>	405.064696	3.06	4.9
Tebuconazole	TEB	107534-96-3	Triazole	C <sub>16</sub> H <sub>22</sub> ClN <sub>3</sub> O	307.145139	3.39, 13.7	3.58
Flusilazole	FLU	85509-19-9	Triazole	C <sub>16</sub> H <sub>15</sub> F <sub>2</sub> N <sub>3</sub> Si	315.397506	2.91	3.70
Triadimenol	TRI	55219-65-3	Triazole	C <sub>14</sub> H <sub>18</sub> ClN <sub>3</sub> O <sub>2</sub>	295.108754	2.16, 13.29	2.97
Myclobutanil	MYC	88671-89-0	Triazole	C <sub>15</sub> H <sub>17</sub> ClN <sub>4</sub>	288.114173	2.30	3.07
Azoxystrobin	AZO	131860-33-8	Strobilurin	C <sub>22</sub> H <sub>17</sub> N <sub>3</sub> O <sub>5</sub>	403.116822	−0.67	5.13
Cyprodinil	CYP	121552-61-2	Anilinoimidazole	C <sub>14</sub> H <sub>15</sub> N <sub>3</sub>	225.126597	3.1	4.0
Iprovalicarb	IPR	140923-17-7	Carbamate	C <sub>18</sub> H <sub>28</sub> N <sub>2</sub> O <sub>3</sub>	320.209993	−0.9, 11.4	3.56

<sup>a</sup> Values obtained from the SciFinder Scholar Database.

The precursor [M+H]<sup>+</sup> ions for all compounds were obtained using a common fragmentor (in source CID) voltage of 160 V. Thereafter, collision energy was optimized with the aim of obtaining a minimum of two product ions for each precursor. In addition to the MS/MS spectra of target analytes (recorded within a time window of 2–3 min), the system provided full scan MS spectra, in the range from 50 to 1250 *m/z* units, during the whole chromatographic run. Acquisition rates for MS and MS/MS modes were set at 1.4 spectra s<sup>−1</sup>, with each spectrum being the combination of 9600 transients. The most intense fragment in the MS/MS spectra of each analyte was used for quantification purposes, whereas the rest of product ions were used for confirmation.

#### 2.4. Matrix effects, SPE efficiencies, absolute recoveries and sample quantification

Potential matrix effects (ME, %) occurring during ESI were evaluated as ME = [(A<sub>se</sub> − A<sub>ne</sub>)/A<sub>s</sub>] × 100, being A<sub>se</sub> and A<sub>ne</sub> the responses (peak areas) measured for spiked (addition was done after the SPE extraction step) and non-spiked extracts of the same wine sample, and A<sub>s</sub> the response obtained for a standard with the same concentration. Thus, ME values around 100% indicate no changes in the efficiency of the ESI ionization for wine extracts versus standards prepared in acetonitrile:water (1:1). On the other hand, values below or above 100% point to suppression or enhancement effects in the ionization step. The extraction efficiency of the SPE step (EE, %) was calculated as EE = [(A<sub>ss</sub> − A<sub>ns</sub>)/(A<sub>se</sub> − A<sub>ne</sub>)] × 100, being A<sub>ss</sub> and A<sub>ns</sub> the responses obtained for spiked (addition was carried out over wine samples, before SPE) and non-spiked aliquots of the same sample. Finally, the absolute recoveries (R, %) of the proposed method were calculated as the difference between the concentrations measured for extracts from spiked (C<sub>s</sub>) and non-spiked aliquots (C<sub>b</sub>) of wine divided by the theoretical concentration (C<sub>t</sub>) added to the sample, and multiplied by 100, R = [(C<sub>s</sub> − C<sub>b</sub>)/C<sub>t</sub>] × 100. C<sub>s</sub> and C<sub>b</sub> were established against calibration curves obtained for standards in acetonitrile:water (1:1). Under final working conditions, the absolute recoveries (accuracy) of the global method stayed between 81 and 114%. Thus, the concentrations of target fungicides in commercial wine samples were accurately determined by external calibration, without applying any correction factor.

### 3. Results and discussion

#### 3.1. LC–QTOF method development

Optimization of ESI–QTOF parameters was carried out with individual and mixture standard solutions of target analytes at the 1000 ng mL<sup>−1</sup> level. Departure separation conditions were taken

from previous articles dealing with the LC determination of fungicide compounds [17,31,32]. As regards the ionization mode, most compounds were not detected, or produced very weak signals, in negative ESI; thus, positive ionization was selected. In the next step, the effect of the fragmentor voltage in the signal of the [M+H]<sup>+</sup> ions was investigated. For all compounds, the highest responses were obtained for 160 V.

LC conditions were optimized using binary mixtures of methanol:water and acetonitrile:water. In both cases, MET and TRI showed the shortest retention times, which is in agreement with their relatively low K<sub>ow</sub> values (Table 1). The elution order for the rest of species varied depending on the organic phase (acetonitrile or methanol), with the best overall separation efficiency corresponding to acetonitrile. Thereafter, the effect of ammonium acetate and formic acid in the efficiency of the ionization and the LC separation step was assessed. The above modifiers were added to water and acetonitrile phases at increasing concentrations: up to 0.1% in the case of formic acid, and up to 5 mM for ammonium acetate. Fig. 1 shows the average responses (peak areas, n = 2 replicates) obtained under different experimental conditions. The addition of formic acid to the mobile phase led to a moderate reduction in the responses obtained for MET, IPR, AZO and BEN, whereas it played a non-significant effect in the peak areas measured for the rest of species (Fig. 1A). As regards the LC separation process, the retention of CYP was strongly affected by the concentration of acid in the mobile phase, shifting to lower retention times with the increase in the percentage of formic acid. Likely, the above trend is a consequence of the protonation of the amino moiety existing in the structure of CYP. On the other hand, ammonium acetate produced a slight reduction in the responses measured for most compounds (Fig. 1B), and a negligible influence on their chromatographic retention. Despite the above reported sensitivity reduction, ammonium acetate (1 mM) was added as modifier to the mobile phase in order to control its ionic strength and pH.

Once chromatographic and ESI (ionization mode and fragmentor voltage) conditions were established, the collision energy was optimized in order to obtain at least two intense fragments, maintaining also a fraction (10–20%) of the precursor ion, in the MS/MS spectrum of each fungicide. However, TRI, TEB and DIN rendered a single intense signal in their MS/MS spectra corresponding to the protonated azole moiety with a theoretical *m/z* of 70.0400. Considering the existence of chlorine atoms in the structure of these analytes, a second transition using the [M+2+H]<sup>+</sup> precursor ion was registered for qualification purposes. For AZO, two intense fragment ions could be obtained only at the expense of losing the information corresponding to the precursor ion in the MS/MS spectrum. Table 2 compiles the experimental *m/z* ratios measured for precursor ([M+H]<sup>+</sup>) and fragment ions of target compounds, the collision energy as well as the retention times and the chro-

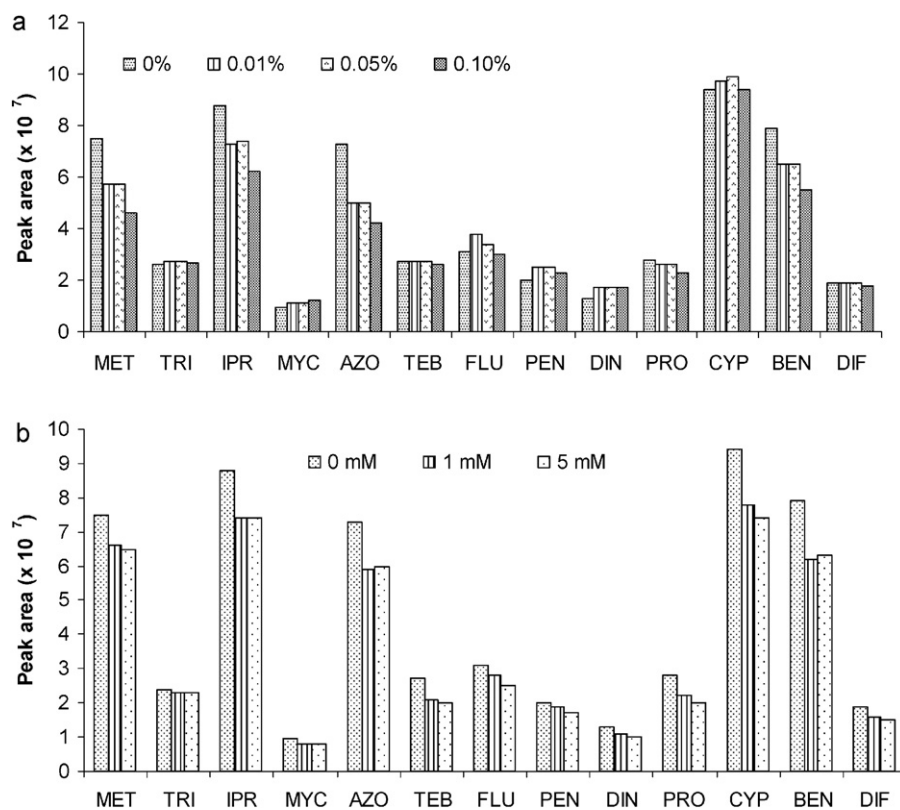


Fig. 1. Effects of type and amount of modifier in the responses (peak areas) of target analytes.

matographic acquisition window for each species. In general, the most intense fragment ions compiled in Table 2 have been also used for quantitative purposes in LC–MS/MS applications of triple quadrupole mass spectrometers, and their structures have been already described in the literature [21,31,33]. Fragmentation patterns of BEN and IPR are illustrated in Fig. 2. As observed, working under conditions summarized in Table 2, the relative errors in the assignment of  $m/z$  ratios of precursor and fragment ions remained below 4 ppm (absolute value) for a 1000 ng mL<sup>-1</sup> standard. The availability of accurate  $m/z$  data for fragment ions greatly simplifies the identification of their chemical structures and thus, the MS/MS fragmentation pattern of analytes.

The number of MS/MS spectra acquired through a chromatographic peak depended on (1) the number of precursor ions with overlapped chromatographic windows and (2) the acquisition rate of the TOF mass analyzer. Under the most un-favorable situation (5 precursors in the same window), 12 spectra were recorded per peak using an acquisition rate of 1.4 spectra s<sup>-1</sup>. Higher acquisition frequencies led to more spectra per peak, at the expense of reducing the number of transients per spectra. In order to assess the effect of this variable in the performance of the LC–QTOF instrument, some additional experiments were carried out using an acquisition rate of 6 spectra s<sup>-1</sup>, (ca. 2240 transients per spectra). Under these conditions, no differences were noticed in the accuracy of

**Table 2**  
LC-QTOF database including retention times (RT) and masses for precursor and fragment ions of target fungicides.

Analyte	RT (min)	Acquisition window (min)	<sup>a</sup> Precursor ion ( $m/z$ )	CE	<sup>a</sup> Quantification transition ( $m/z$ )	<sup>a</sup> Confirmation transition ( $m/z$ )	<sup>a</sup> Other fragments ( $m/z$ )
MET	10.31	3	280.1539	10	280.1539 > 220.1328	280.1539 > 192.1379	248.1277, 160.1114
TRI	13.16	3	296.1159, 298.1138	3	296.1159 > 70.0405	298.1138 > 70.0405	n.a.
IPR	14.97	2	321.2173	8	321.2173 > 119.0853	321.2173 > 203.1386	186.1127, 144.0651
MYC	15.08	2	289.1218	18	289.1218 > 70.0405	289.1218 > 125.0147	n.a.
AZO	15.53	2	404.1242	20	404.1242 > 372.0968	404.1242 > 344.1023	n.a.
TEB	16.52	3	308.1517, 310.1503	18	308.1517 > 70.0405	310.1503 > 70.0405	n.a.
FLU	17.22	2	316.1079	20	316.1079 > 247.0745	316.1079 > 165.0694	219.0428, 80.9971
PEN	17.42	2	284.0719	15	284.0719 > 70.0406	284.0719 > 158.9758	n.a.
DIN	18.78	3	326.0826, 328.0797	22	326.0826 > 70.0406	328.0797 > 70.0405	n.a.
PRO	19.04	3	342.0776	20	342.0776 > 69.0705	342.0776 > 158.9756	n.a.
CYP	19.72	3	226.1337	35	226.1337 > 93.0574	226.1337 > 108.0794	210.1022, 118.0551, 77.0390
BEN	21.91	3	326.1746	10	326.1746 > 148.1116	326.1746 > 208.1329	294.1485, 266.1535, 91.0544
DIF	22.59	3	406.0725	20	406.0725 > 251.0020	406.0725 > 337.0381	n.a.

CE, collision energy, eV.

n.a., not available.

<sup>a</sup> Experimental  $m/z$  values obtained for a 1000 ng mL<sup>-1</sup> standard.

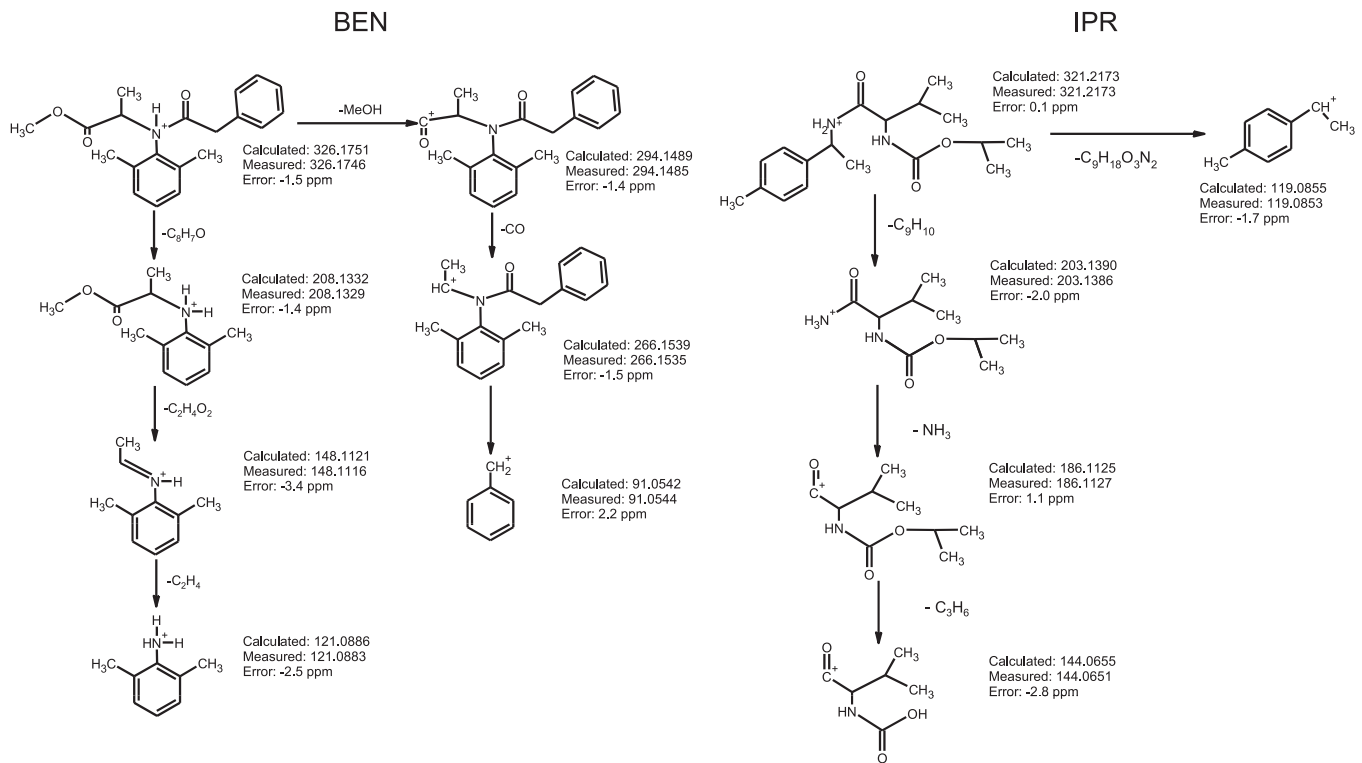


Fig. 2. MS/MS fragmentation patterns for BEN and IPR.

mass assignment to MS/MS fragments; however, the sensitivity of the LC-QTOF instrument underwent a four-fold reduction (data not shown). Obviously, in this study, the acquisition rate was maintained at 1.4 spectra s<sup>-1</sup>. However, the above finding arises as a relevant limitation of the LC-QTOF instrument, operated in the MS/MS mode, for the sensitive multi-residue quantification of more complex samples, containing many chromatographic overlapped analytes.

### 3.2. LC-QTOF-MS/MS instrumental performance

Table 3 summarizes the data related to the performance of the LC-QTOF system, without considering the sample preparation step. The mass window used to extract the MS/MS chromatograms, corresponding to quantification ions reported in Table 2, was 20 ppm. The plots of peak area versus concentration followed a linear trend, within the range of concentrations comprised between LOQs and 1000 ng mL<sup>-1</sup> for MET, IPR, AZO, FLU, PEN and BEN and up to 2000 ng mL<sup>-1</sup> for the rest of species. The determination coefficients (*R*<sup>2</sup>) of the obtained graphs ranged from 0.991 to 0.999. Fig. 3 depicts the MS/MS chromatogram corresponding to a standard solution (25 ng mL<sup>-1</sup>) of target species. LOQs calculated for a signal to noise ratio of 10 (*S/N* = 10) varied between 0.2 ng mL<sup>-1</sup> for CYP and 1.7 ng mL<sup>-1</sup> for MYC. Globally, the LOQs summarized in Table 2 are similar to those reported for these and other similar fungicides using LC-QQQ systems, operated in the MS/MS mode [31,33], and LC-TOF in the single MS mode [30]. The variability in the response of the system was investigated with standards at two concentration levels (10 and 100 ng mL<sup>-1</sup>) for intra-day precision and at 25 ng mL<sup>-1</sup> for inter-day precision studies. Relative standard deviations (RSDs, %) for consecutive injections (*n* = 5 replicates) made in the same day ranged between 0.6 and 9.5%, whereas values between 4.2 and 10.1% were obtained for injections (*n* = 12 replicates) carried out in three consecutive days, Table 3.

### 3.3. SPE optimization

SPE conditions were optimized with a pooled matrix of red wines, considered as the most complex sample, spiked at the 100 ng mL<sup>-1</sup> level. Initially, methanol and acetonitrile were selected as elution solvents to recover target analytes from Oasis MAX and HLB cartridges. The former solvent rendered reddish extracts, particularly when used in combination with the reversed-phase HLB sorbent, with a high level of co-extracted pigments. On the other hand, independently of the SPE sorbent, transparent and colorless extracts were achieved with acetonitrile, which was selected as elution solvent. In a further step, it was verified that target species were recovered just in the first two fractions (1 mL, each) of acetonitrile collected from both SPE cartridges. Breakthrough problems were not detected with any of both sorbents for 10 mL of red wine; however, a breakthrough percentage around 5% was noticed for MET using the mixed-mode MAX sorbent for 20 mL of wine. The above result may be related to (1) the lower mass of sorbent included in the MAX cartridge (150 mg versus 200 mg for the HLB one) and/or to (2) saturation of the mixed-mode sorbent due to retention of acidic compounds, contained in wine samples, through electrostatic interactions with the positively charged amino groups existing in this sorbent [31]. On the basis of its higher breakthrough volume, the 200 mg Oasis HLB cartridges were selected for further extractions.

The extraction efficiency and the selectivity of the above SPE protocol was evaluated for pooled samples of red and white wines (10 mL), both fortified at 100 ng mL<sup>-1</sup>. The achieved extraction efficiencies ranged from 77% to 95%, with standard deviations values below 7%, Fig. 4. Matrix effects (MEs), evaluated as described in Section 2, stayed between 94% and 104% with standard deviations lower than 7%. The only exception was the value obtained for CYP (117 ± 4%) in the red wine matrix, Fig. 5. Overall, the above ME values indicate negligible, or low (case of CYP), variations among the efficiency of the ionization process for sample (10 mL of red

**Table 3**  
Linearity, instrumental limits of quantification (LOQs), intra- and inter-day precision of the LC-QTOF system for standards of fungicides.

Analyte	Linear range (ng mL <sup>-1</sup> )	R <sup>2</sup>	LOQ (ng mL <sup>-1</sup> )	Precision (RSDs, %)		
				Intra-day <sup>a</sup>		Inter-day <sup>b</sup>
				10 ng mL <sup>-1</sup>	100 ng mL <sup>-1</sup>	25 ng mL <sup>-1</sup>
MET	LOQ-1000	0.9990	0.4	2.9	0.6	6.1
TRI	LOQ-2000	0.9998	1.2	5.8	8.8	6.4
IPR	LOQ-1000	0.9914	0.7	5.9	1.2	4.2
MYC	LOQ-2000	0.9945	1.7	7.4	4.0	9.7
AZO	LOQ-1000	0.9947	0.9	1.5	1.2	4.2
TEB	LOQ-2000	0.9990	0.8	4.2	4.9	8.5
FLU	LOQ-1000	0.9989	0.7	4.4	1.1	9.2
PEN	LOQ-1000	0.9994	1.0	7.3	3.3	6.4
DIN	LOQ-2000	0.9951	0.4	7.9	4.2	8.1
PRO	LOQ-2000	0.9989	0.9	9.5	4.5	10.1
CYP	LOQ-2000	0.9919	0.2	9.2	3.8	7.0
BEN	LOQ-1000	0.9975	0.5	4.0	0.9	6.2
DIF	LOQ-2000	0.9987	0.4	4.6	1.5	5.7

<sup>a</sup> *n* = 5 injections in the same day.

<sup>b</sup> *n* = 12 injections in 3 consecutive days.

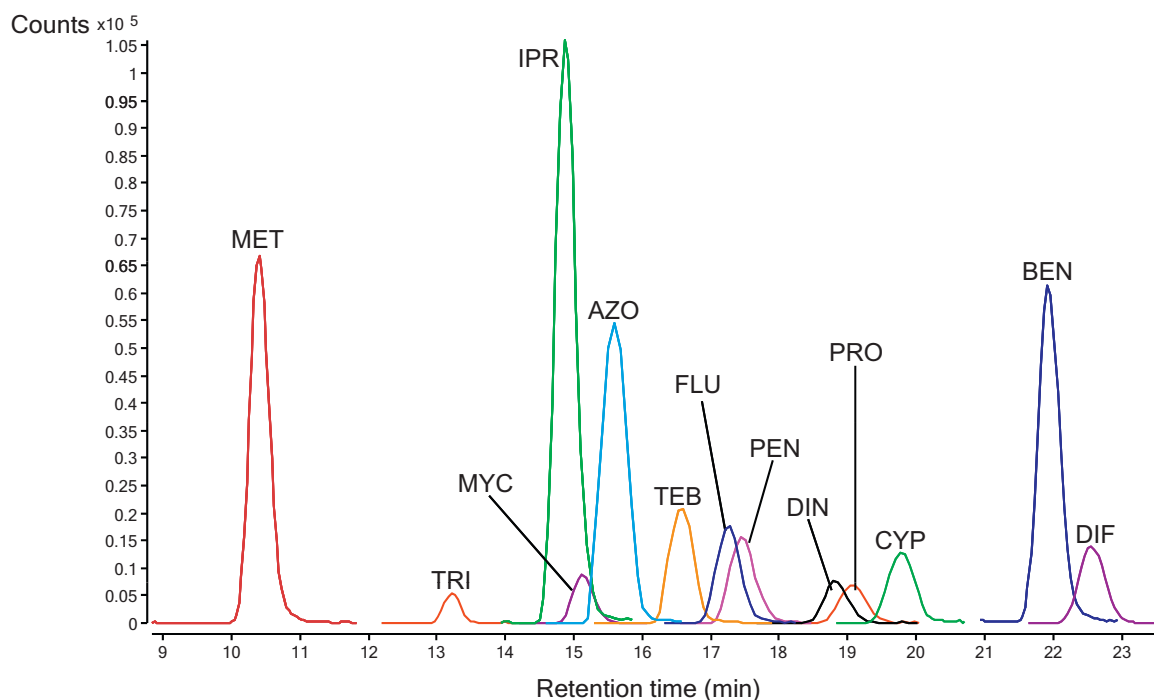
or white wine) extracts versus standards dissolved in acetonitrile:water (1:1). Thus, it appears feasible to use pure standards in acetonitrile:water, instead of matrix-matched standards, to quantify the levels of fungicides in wine samples.

#### 3.4. Performance of the analytical procedure

The analytical figures of merit of the optimized method are summarized in Table 4. Precision and accuracy were assessed using red and white wines spiked at four different concentration levels: 100, 25, 10 and 5 ng mL<sup>-1</sup>. Data reported for the lowest addition level (5 ng mL<sup>-1</sup>) correspond to 20 mL samples of individual red and white wines, selected on the basis of their low fungicide contents. The rest of recoveries were obtained for 10 mL aliquots of two pooled matrices of red and white wine. In all cases, spiked and non-spiked aliquots were processed in triplicate and the concentrations of fungicides in the corresponding extracts determined by

external calibration. The absolute recoveries (*R*, %) of the overall procedure, considered as an estimation of the accuracy, for 10 mL samples ranged between 84% and 114%, whereas values from 81% to 113% were achieved for 20 mL of wine. In both cases, the associated standard deviations varied between 1% and 12%. The inter-day accuracy and precision were assessed with 10 mL aliquots of the pooled matrices of red and wine samples, spiked at the 20 ng mL<sup>-1</sup> level and processed in triplicate during 3 consecutive days. In this case, the absolute recoveries of the method ranged from 86% to 106%, with standard deviations between 6% and 12%, data not given.

The analysis of procedural blanks, corresponding to synthetic wine samples, demonstrated the absence of contamination problems during sample preparation; thus, the LOQs of the method are controlled by the instrumental LOQs of the LC-ESI-QTOF system, sample and final extract volume. Considering a sample intake of 10 mL, LOQs varied between 0.1 ng mL<sup>-1</sup> for CYP and 0.7 ng mL<sup>-1</sup> for MYC (Table 4), with a linear response range up to 250 ng mL<sup>-1</sup> for



**Fig. 3.** LC-QTOF extracted chromatogram for a standard solution of target fungicides (25 ng mL<sup>-1</sup>).

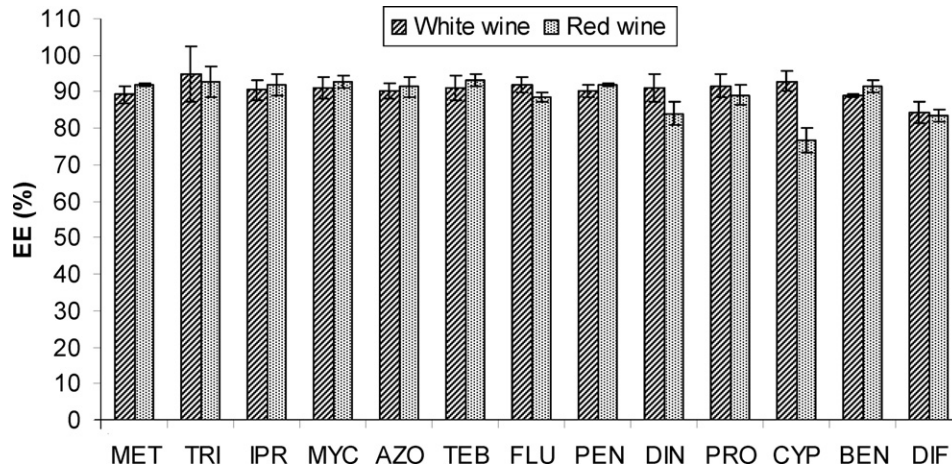


Fig. 4. Extraction efficiency (EE, %) of SPE for 10 mL wine samples spiked at 100 ng mL<sup>-1</sup>, n = 3 replicates.

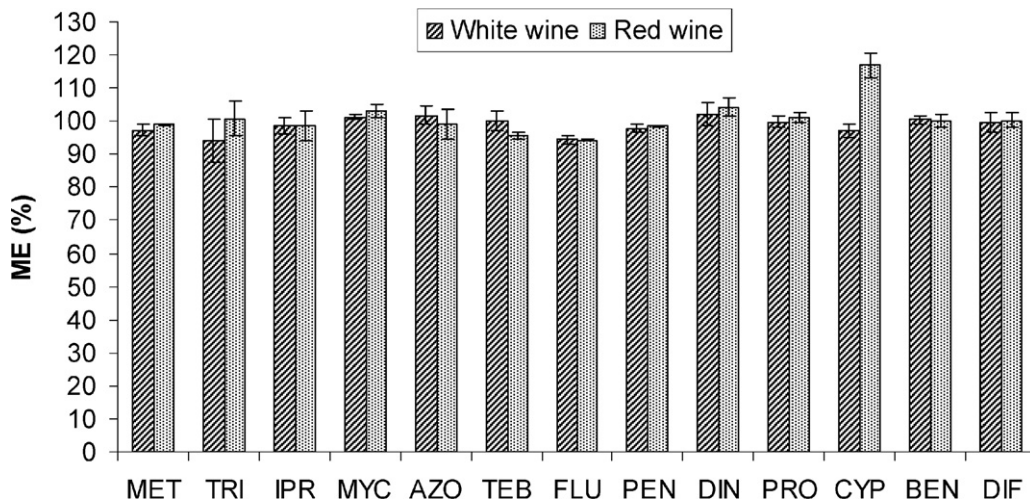


Fig. 5. Assessment of matrix effects (ME, %) during ESI for red and white wine extracts, n = 3 replicates.

MET, IPR, AZO, FLU, PEN and BEN, and up to 500 ng mL<sup>-1</sup> for the rest of analytes. LOQs compiled in Table 4 remain far below the MRLs established by the EU for these fungicides in vinification grapes (from 0.05 to 2 µg g<sup>-1</sup>) and those defined for FLU and TEB in wine

(0.2 and 2 µg g<sup>-1</sup>, respectively) [8,9]. They also fulfil the recommendation of the Organisation Internationale de la Vigne (OIV), which suggests taking for wine 1/10 of the MRLs established for grapes. Additionally, it is worth noting that the LOQs provided by the pro-

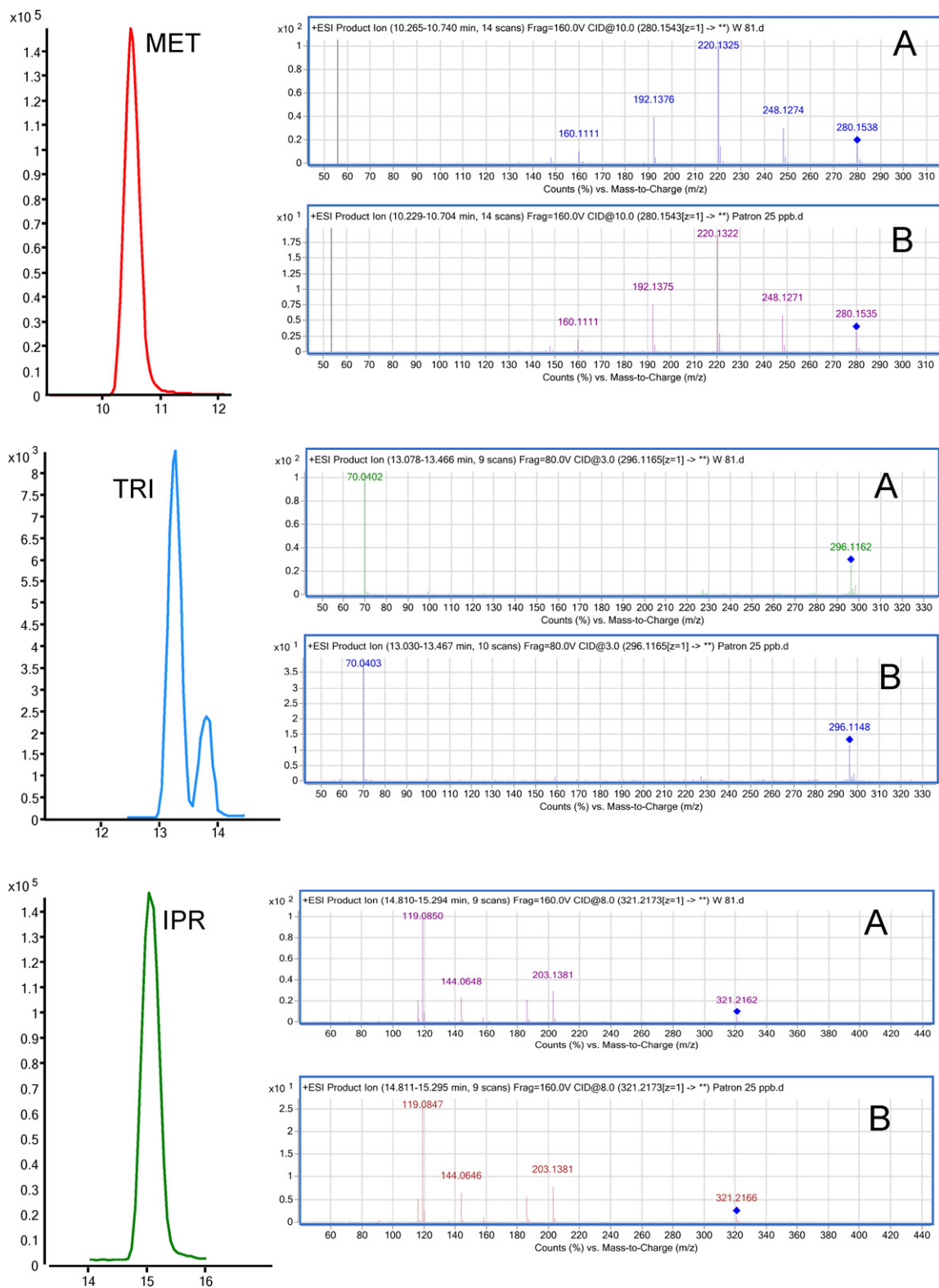
Table 4

Accuracy, expressed as absolute recoveries (%), and precision, given as standard deviation (SD), of the proposed method for red and white wine samples spiked at different concentration levels and LOQs (S/N = 10) calculated for 10 mL volume samples.

Analyte	Recovery (%) ± SD, n = 3 replicates								LOQs (ng mL <sup>-1</sup> )
	<sup>a</sup> 100 ng mL <sup>-1</sup>		<sup>a</sup> 25 ng mL <sup>-1</sup>		<sup>a</sup> 10 ng mL <sup>-1</sup>		<sup>b</sup> 5 ng mL <sup>-1</sup>		
	White wine	Red wine	White wine	Red wine	White wine	Red wine	White wine	Red wine	
MET	86.8 ± 2.7	90.9 ± 0.7	90.4 ± 0.4	95.1 ± 1.4	107.2 ± 2.7	100.3 ± 6.8	104.7 ± 5.6	107.0 ± 3.3	0.2
TRI	89.2 ± 11.8	93.6 ± 3.4	100.9 ± 0.8	107.9 ± 9.0	98.5 ± 8.0	80.8 ± 6.7	112.8 ± 10.3	112.4 ± 9.2	0.5
IPR	89.2 ± 2.6	90.6 ± 1.2	93.4 ± 0.2	97.2 ± 1.5	111.6 ± 4.8	103.8 ± 5.0	105.9 ± 5.3	108.1 ± 3.9	0.3
MYC	92.2 ± 5.2	95.6 ± 1.3	92.0 ± 4.0	105.5 ± 3.2	100.5 ± 7.6	98.1 ± 3.2	108.2 ± 10.3	106.2 ± 2.6	0.7
AZO	91.7 ± 1.6	90.5 ± 1.1	96.0 ± 4.7	100.9 ± 1.9	110.4 ± 1.3	107.6 ± 2.4	103.9 ± 5.7	106.1 ± 2.1	0.4
TEB	91.2 ± 3.4	89.1 ± 2.6	97.0 ± 7.5	103.0 ± 3.7	97.8 ± 7.1	113.5 ± 4.4	112.3 ± 2.4	103.2 ± 7.4	0.3
FLU	86.6 ± 3.1	83.3 ± 2.3	93.6 ± 1.4	99.7 ± 5.6	97.2 ± 7.4	89.7 ± 10.2	101.8 ± 5.0	95.5 ± 7.2	0.3
PEN	88.1 ± 2.5	90.6 ± 0.4	97.9 ± 4.3	103.1 ± 1.6	100.2 ± 2.1	105.7 ± 8.4	101.3 ± 1.9	101.3 ± 5.4	0.4
DIN	92.7 ± 4.1	87.6 ± 3.7	98.9 ± 6.7	102.5 ± 3.5	91.8 ± 6.9	88.0 ± 8.0	105.9 ± 1.2	103.5 ± 1.9	0.2
PRO	91.5 ± 4.6	90.0 ± 3.9	88.1 ± 5.9	97.0 ± 6.2	104.0 ± 8.9	100.5 ± 9.8	102.1 ± 3.3	89.9 ± 7.7	0.4
CYP	90.1 ± 3.3	89.5 ± 3.2	91.9 ± 1.4	83.6 ± 3.2	95.2 ± 6.6	86.1 ± 6.3	97.3 ± 8.1	81.0 ± 9.4	0.1
BEN	89.2 ± 3.8	91.4 ± 1.8	94.5 ± 3.4	98.5 ± 3.4	108.6 ± 2.1	105.5 ± 2.7	102.2 ± 2.4	101.5 ± 5.5	0.3
DIF	83.7 ± 2.9	83.6 ± 0.9	92.9 ± 1.2	89.6 ± 5.5	99.5 ± 2.3	92.4 ± 3.5	109.0 ± 3.6	108.5 ± 3.9	0.2

<sup>a</sup> Recoveries for 10 mL aliquots of two pooled samples of red and white wines.

<sup>b</sup> Recoveries for 20 mL aliquots of two individual samples of red (*Mencia*) and white (*Palomino*) wine.



**Fig. 6.** Extracted chromatograms for a white wine sample (code W7, Table 5) and MS/MS spectra for fungicide species in the wine extract (A) and in a 25 ng mL<sup>-1</sup> standard (B).



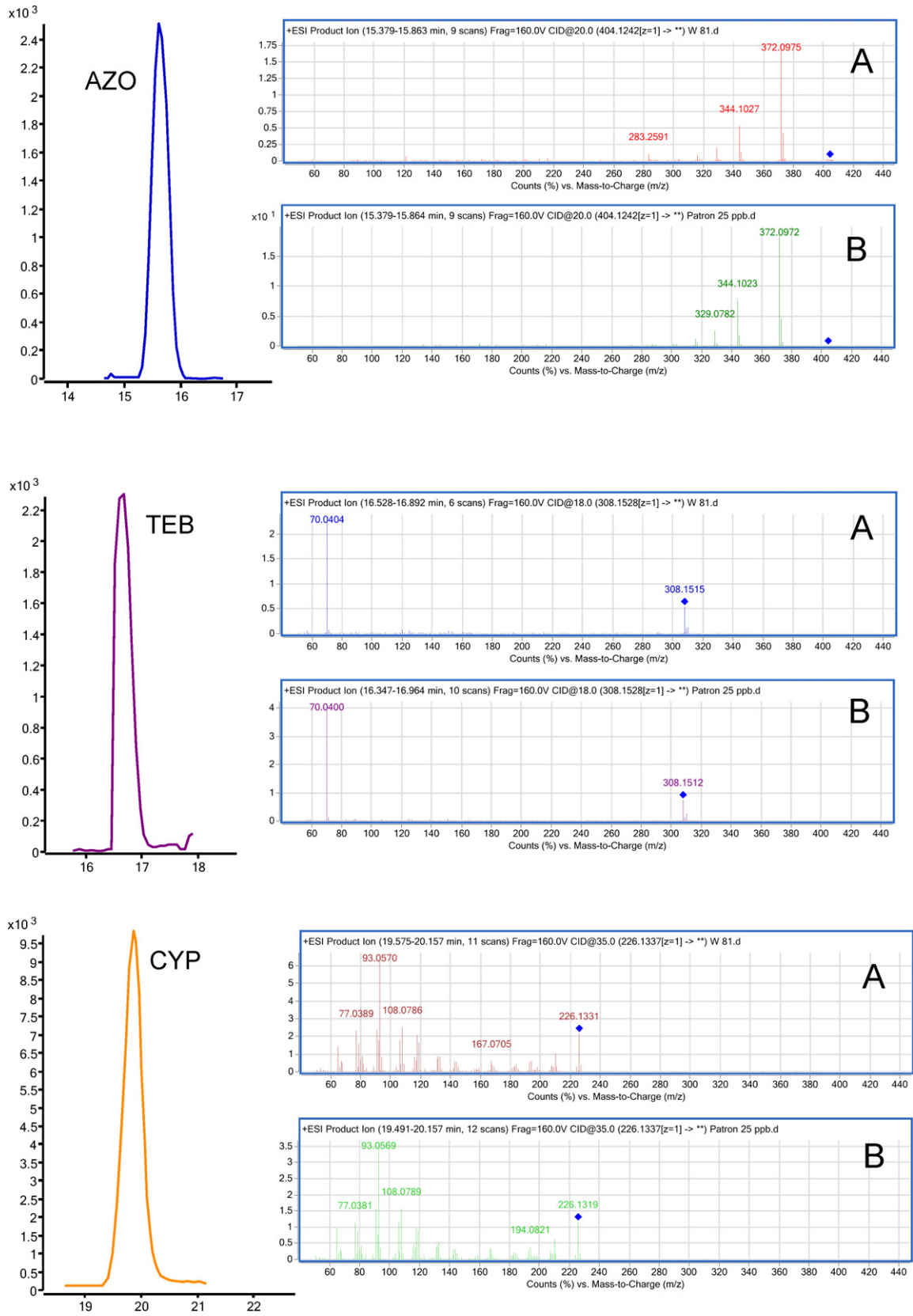


Fig. 6. (Continued)

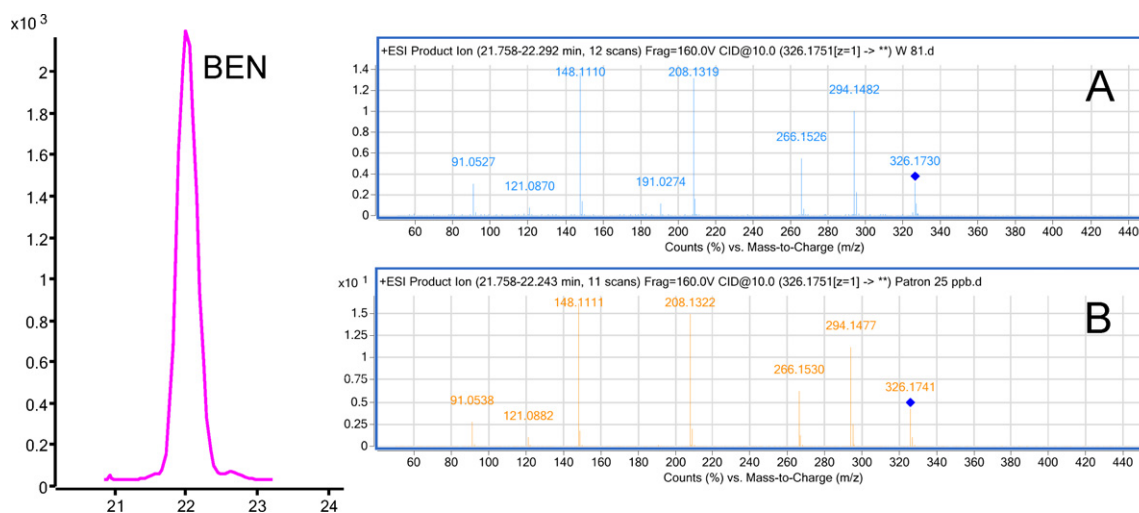


Fig. 6. (Continued).

posed methodology are similar to, or lower than, those reported in previous applications of LC–MS/MS based on triple quadrupole instruments [21,31,33]. They are also in the same range of values than those achieved for pesticide residues in fruit juices by LC–TOF after a 10-fold concentration step [34].

### 3.5. Real sample analysis

The proposed method was applied to a total of seventeen samples of white (9 specimens) and red (8 specimens) wines from different geographic regions in Spain. Four triazol type fungicides (FLU, PEN, DIN and PRO) remained below the LOQs of the method in all the processed samples. Concentrations measured for the rest of analytes are summarized in Table 5. Six fungicides were present in more than 20% of the analyzed samples, with maximum individual concentrations comprised between 23 ng mL<sup>-1</sup> for TEB and 125 ng mL<sup>-1</sup> for MET. It is also worth noting that all the processed samples contained measurable levels of at least one fungicide, nine of them showed total concentrations above

10 ng mL<sup>-1</sup> and three stayed above 100 ng mL<sup>-1</sup>. Globally, data compiled in Table 5 are similar to those found in the literature. As example, Trösken et al. [21] have reported a maximum concentration of 33 ng mL<sup>-1</sup> and an occurrence frequency of 55% for TEB in wines from different European countries. Also, CYP and MET have been detected at levels up to 30 ng mL<sup>-1</sup> in white wines produced in Galicia [19,31,35]. The presence of fungicide residues in wines is related to several variables, such as doses sprayed on vineyards, wine making technology, persistence of each particular compound during wine elaboration and their trend to remain associated with solid wastes (peels and seeds) or staying dissolved in wine [36]. In this sense, transfer factors from grapes to wine higher than 0.2 have been reported for IPR [4], MET [5], AZO and CYP [3], which are the compounds showing the highest levels in the analyzed samples, Table 5. Fig. 6 shows the extracted ion chromatograms and the MS/MS spectra for a non-spiked wine sample (code W7, Table 5), together with the MS/MS spectra for a standard (25 ng mL<sup>-1</sup>). The accurate measurement of the *m/z* ratios for precursor and fragment ions guaranteed the unambiguous identi-

**Table 5**  
Levels of target fungicides in non-spiked wine samples. Average concentrations (ng mL<sup>-1</sup>) with their standard deviations, *n* = 3 replicates.

Code	Geographic region	MET	TRI	IPR	MYC	AZO	TEB	CYP	BEN	DIF	Total conc. (ng mL <sup>-1</sup> )
W1	Galicia	30.6 ± 0.7	<LOQ	3.9 ± 0.2	<LOQ	27 ± 2	1.14 ± 0.05	<LOQ	<LOQ	<LOQ	63
W2	Galicia	3.9 ± 0.1	2.8 ± 0.2	3.6 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10
W3	Galicia	46 ± 2	12 ± 2	110 ± 7	<LOQ	3.3 ± 0.1	<LOQ	36.7 ± 0.4	<LOQ	n.d.	208
W4	Galicia	10.4 ± 0.7	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	n.d.	<LOQ	n.d.	10
W5	Galicia	48.7 ± 0.7	15.5 ± 0.7	50.9 ± 0.3	<LOQ	1.76 ± 0.02	4.8 ± 0.5	n.d.	12.9 ± 0.6	0.51 ± 0.02	135
W6	Galicia	10.7 ± 0.2	8.31 ± 0.08	22.9 ± 0.2	n.d.	<LOQ	6.7 ± 0.6	5.0 ± 0.3	<LOQ	n.d.	54
W7	Galicia	125 ± 2	57.5 ± 0.4	85.7 ± 0.6	n.d.	2.2 ± 0.1	6.4 ± 0.5	42 ± 2	2.2 ± 0.1	n.d.	321
W8	Castilla-La Mancha	0.98 ± 0.01	<LOQ	<LOQ	n.d.	n.d.	n.d.	0.7 ± 0.2	n.d.	n.d.	2
W9	Castilla-La Mancha	n.d.	n.d.	n.d.	n.d.	<LOQ	<LOQ	<LOQ	n.d.	1.6 ± 0.3	2
R1	Castilla-León	n.d.	n.d.	n.d.	<LOQ	<LOQ	<LOQ	2.3 ± 0.2	n.d.	n.d.	2
R2	Castilla-León	1.97 ± 0.08	3.9 ± 0.2	<LOQ	<LOQ	<LOQ	n.d.	n.d.	<LOQ	<LOQ	6
R3	Galicia	3.49 ± 0.03	n.d.	12.1 ± 0.5	n.d.	n.d.	n.d.	<LOQ	<LOQ	n.d.	16
R4	Galicia	0.55 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1
R5	Galicia	2.4 ± 0.4	n.d.	50 ± 9	4.4 ± 0.3	<LOQ	23 ± 3	0.38 ± 0.12	n.d.	n.d.	80
R6	Rioja	1.75 ± 0.03	2.4 ± 0.1	<LOQ	<LOQ	<LOQ	1.2 ± 0.3	n.d.	n.d.	n.d.	5
R7	Rioja	5.9 ± 0.3	3.66 ± 0.07	4.4 ± 0.1	<LOQ	n.d.	<LOQ	<LOQ	n.d.	n.d.	14
R8	Rioja	15.7 ± 0.4	<LOQ	<LOQ	<LOQ	<LOQ	n.d.	0.8 ± 0.3	<LOQ	n.d.	17
Occurrence frequency (%)		88%	47%	53%	6%	24%	35%	41%	12%	12%	
Maximum concentration (ng mL <sup>-1</sup> )		125	58	109	4	27	23	42	13	1.6	

n.d., not detected.

<LOQ, below the limit of quantification of the method.

Codes W and R correspond to white and red wines, respectively.

fication of target fungicides. As shown in Fig. 6, two peaks were obtained for TRI in the extracts from all processed wine samples. Likely, they correspond to diastereoisomers of this fungicide. TRI concentrations reported in Table 5 correspond to the first eluting isomer, showing the same retention time as the quantification standard.

#### 4. Conclusions

The SPE LC–QTOF developed methodology allows the selective determination of selected fungicides in red and white wine samples, showing LOQs far below the current MRLs reported for grapes and wine, suitable precision and linear response ranges. The sample preparation method provides quantitative recoveries, which remain unaffected by the type of sample; moreover, the ionization efficiency is similar for sample extracts and standard solutions, allowing the use of external calibration as quantification technique. The QTOF MS/MS spectra permit the unequivocal identification and quantification of target compounds based on the accurate mass determination of precursor and fragment ions. Altogether, the above features guarantee the ruggedness of the proposed methodology for the routine determination of target fungicides in commercial wine samples, with the aim of evaluating exposure of wine consumers to these compounds. Data obtained in this study, for a limited number of samples, confirmed the often occurrence of significant levels of several fungicides, particularly MET and IPR, in commercial wines.

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