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Original Research Article

Determination of pesticide residues in wine by solid-phase extraction on-line combined with liquid chromatography tandem mass spectrometry

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ABSTRACT

Liquid chromatography tandem mass spectrometry (LC—MS/MS) has been combined with different sample preparation strategies to determine potential pesticide residues existing in wine. Herein, we propose the on-line coupling between solid-phase extraction (SPE), based on a reversed-phase polystyrene-divinyl benzene (PS-DVB) sorbent, and LC—MS/MS for the automated monitoring of a selection of 48 pesticides (37 fungicides and 11 insecticides) in this beverage. Parameters affecting the sensitivity, selectivity, accuracy and robustness of the method are evaluated as function of sample volume, clean-up conditions, and number of extraction-desorption cycles performed with the same SPE cartridge. Considering a sample volume of 3 μL, the on-line SPE LC—MS/MS method was free of MEs, attaining LOQs below 2.5 μg L^{-1}, and providing recoveries in the range from 70 to 120 % for 90 % of investigated compounds. Increasing the volume of sample loaded in the on-line SPE cartridge to 25 μL permitted a further reduction of LOQs, with a linear response range (0.1–25 μg L^{-1}), covering the analysis of low residue samples. Twelve out of 48 investigated compounds were noticed at individual concentrations above 10 μg L^{-1} in at least one out of 30 processed wines. In white wines, the sum of concentrations for iprovalicarb, fenhexamide and metalaxyl represented more than 50 % of total residues. As regards red wines, tebuconazole, cyprodinil and metalaxyl were the predominant species.

1. Introduction

Vineyards, managed under conventional production practices, receive considerable amounts of different plant protection products (PPPs). Among them, fungicides and insecticides are directly sprayed on plant leaves and canes. Depending on their application rates and dates, a fraction of these compounds might remain in grapes at harvest time and reach the elaborated wines, in percentages controlled by their processing factors (PFs) during vinification operations (Pazzirota et al., 2013; Santana-Mayor et al., 2020; Schusterova et al., 2021).

Despite the maximum residue limits (MRLs) of pesticides in wine are not regulated yet, it is generally accepted that they should not exceed 10 % of MRLs established for vinification grapes. PFs provide a direct estimation of MRLs applied to wine from values set for vinification grapes; unfortunately, consensus values have been obtained for a limited number of pesticides, to date (Spanish Agency of Food Safety, 2021). In case of non-authorized, or phased-out PPPs (i.e. certain neonicotinoids and chlorpyrifos insecticides; and an increasing number of fungicides, such as thiophanate methyl and iprodione), a generic limit of 10 μg Kg^{-1} is set (European pesticide database, 2021). Sensitive analytical methods, enabling the determination of pesticide residues well below this generic MRL, are also required for the analysis of ecological wines in order to verify their compliance with the production and elaboration standards of this agriculture practice (Castro et al., 2018). In addition to legal requirements, there are increasing evidences that some pesticides modulate the microbiota of yeasts involved in the vinification process (Agarbati et al., 2019), which might have a negative impact in the sensorial (Sieiro-Sampedro et al., 2020) and the polyphenolic profiles (Briz-Cid et al., 2018) of wines. In this regard, in-vitro studies pointed out to a negative correlation between the bioavailability of the phenolic antioxidants existing in wine and the content of fungicide residues (Camara et al., 2019). To sum up, a number of issues justify the development of multiresidue, sensitive, reliable and cost-affordable analytical methodologies, to monitor pesticide residues in commercial wines. These analytical tools are also required to assess the performance of treatments implemented at caves, aiming to reduce pesticide residues in wine (Doulia et al., 2016; Nicolini et al., 2016).

Currently, LC–MS/MS is accepted as one of the two gold standards

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for the determination of PPPs residues in wine (Wang and Cheung, 2016). Although the wine matrix is compatible with reversed-phase conditions employed in most LC–MS/MS methods, a sample preparation step is still recommended to remove those components of wine which might reduce the performance and/or the useful life of chromatographic columns. Sample preparation is also expected to limit matrix effects during electrospray ionization (ESI), improving method accuracy. Solid-phase extraction (SPE) (Castro et al., 2018; Pelajić et al., 2016) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), under conventional (He et al., 2019) or miniaturized (Bernardi et al., 2020) modalities, have replaced liquid-liquid methodologies for the extraction of pesticides from wine. SPE and QuEChERS improve the performance attained by direct injection approaches, which normally require sample dilution (Berset et al., 2017; Dias et al., 2019), and they are better established than microextraction approaches in official food analysis laboratories (Kalogiouri and Samanidou, 2019). Despite their excellent performance, off-line SPE and QuEChERS extraction still present some limitations, such as a moderate consumption of organic solvents and automation difficulties, particularly in case of the later technique. In addition, single use cartridges and bulk disposable sorbents increase their operation costs and the generation of solid wastes.

SPE on-line coupled to LC–MS/MS has been proposed for the determination of trace levels of pesticides in liquid matrices, as it is the case of environmental water samples (Barbieri et al., 2020; Mann and Pock, 2016). Same combination has been applied to the analysis of natural toxins in wine (Campone et al., 2018); however, as far as we could trace, applications to pesticide analysis in this matrix have not been found. Herein, we evaluate the analytical features of the on-line combination of SPE, using a commercially available polymeric sorbent, with LC–MS/MS for the automated and sensitive quantification of fungicide and insecticide residues in commercial wines. The major expected advantages versus off-line SPE, followed by LC–MS/MS, are an increased sample workflow, the reduction of solvent consumption, and an improved precision. Effects of operational parameters, such as sample volume and clean-up conditions, in the performance of the method are described. Attention was mainly focused on (1) achieving limits of quantification at least below 10 μg L^{-1}, in order to obtain a suitable method for the analysis of ecologic production wines and/or non-authorized pesticides; (2) assessing the accuracy of the method for wine samples with different characteristics; (3) evaluating the reusability of sorbents, and (4) studying potential cross-contamination problems between samples.

2. Material and methods

2.1. Solvents, standards and sorbents

Methanol and acetonitrile (LC–MS purity grade), ethanol (trace analysis quality) and formic acid were supplied by Merck (Darmstadt, Germany). Ultra-pure deionized water (18.2 MΩ cm^{-1}) was obtained from a Genie U system (Rephile, Shangai, China).

Individual standards for a suite of 48 pesticides (37 fungicides and 11 insecticides, minimum purity 98 %) were acquired form Sigma-Aldrich (Milwaukee, WI, USA). Isotopically labelled analogues of 15 compounds, used as surrogate standards (SSs) added to wine samples before SPE, were either provided by Sigma-Aldrich, or by Toronto Research Chemicals (Ontario, Canada). The list of species involved in the current study is given as supplementary information, Table S1. Individual stock solutions of above compounds, at concentrations comprised between 1000 and 3000 mg L^{-1}, were prepared in methanol. Mixtures of native and labelled pesticides were made in the same solvent. A first set of solvent-based calibration solutions, employed during optimization of chromatographic separation and ionization conditions, was prepared in a mixture of acetonitrile: methanol (8:2, v:v). Another set of solvent-based calibration standards was made in ethanol: water (12: 88, v:v). Unless otherwise stated, the concentration of isotopically labelled compounds in the above calibration solutions was maintained at 50 μg L^{-1}.

Off-line SPE was carried out using OASIS HLB (copolymer of divinylbenzene and N-vinyl pyrrolidone, 30 μm particle size, 80 Å pore size) 200 mg cartridges acquired from Waters (Milford, MA, USA). Polystyrene-divinyl benzene (PS-DVB) cartridges (12.5 mm x 2.1 mm, 15−20 μm particle size, 100 Å pore size) for on-line SPE (maximum operational pressure 250 bar) were purchased from Agilent (Wilmington, DE, USA).

2.2. Samples and sample preparation

Wines employed in this study were acquired in local markets. Most of them were elaborated with grapes produced during last two years (2019 and 2020). Table S2 summarizes some data corresponding to processed samples. Out of 30 different wines, 5 samples were marketed with the ecological label stamp, meaning that no synthetic PPPs were applied to vineyards at least in the previous 3 years.

Off-line SPE was carried out using a previously optimized and validated method (Castro el al., 2018). In brief, 2 mL of filtered wine were diluted with the same volume of ultrapure water, spiked with SSs (50 μg L^{-1}), and passed through the OASIS HLB sorbent previously conditioned with an 8:2 (v:v) mixture of acetonitrile: methanol, followed by a 12:88 (v:v) ethanol: water solution, 2 mL each. After sample loading, cartridges were rinsed again with 2 mL of the above ethanolic solution, dried using a stream of nitrogen and eluted with 2 mL of acetonitrile: methanol (8:2, v:v). This extract was filtered, using a 0.22 μm PTFE (polytetrafluoroethylene) syringe filter, and injected in the LC-QqQ-MS system.

For on-line SPE concentration, wine was also spiked with SSs (50 μg L^{-1}), diluted with ultrapure water (1:1) and filtered. The volume of this solution withdrawn by the LC autosampler and loaded in the PS-DVB packed sorbent, varied from 3 to 80 μL. Compounds elution was carried out using the same mobile phase employed in the LC separation process.

2.3. Equipment and determination conditions

The LC system was an Agilent 1290 Infinity II connected to a triple quadrupole (QqQ) mass analyzer (MS), Agilent 6495, through a jet stream electrospray ionization source (ESI). The system includes an autosampler, furnished with a 100 μL needle loop, and a temperature controlled column compartment. In addition to the binary analytical pump, it incorporates a quaternary pump to deliver samples, conditioning or clean-up solvents through the on-line connected SPE cartridges. An additional module in the LC–MS/MS instrument includes two 10-port (2-position) valves. The first valve directs the flow from the analytical pump either to the LC column (direct injection mode), or to the second valve, which is connected with two identical PS-DVB cartridges. Thus, during on-line SPE, the mobile phase passes through one of the cartridges, desorbing previously retained compounds; whilst the auxiliary quaternary pump pushes sample, conditioning or clean-up solvents through the 2nd SPE cartridge to waste, Fig. S1.

Compounds were separated using a Zorbax Eclipse XDB-C18 column (100 mm x 2.1 mm, 3.5 μm). The mobile phase employed in the LC chromatographic process consisted on ultrapure water (A) and acetonitrile (B) both 0.1 % in formic acid. Its composition was programmed as follows: 0–3.5 min (10 % B), 10 min (30 % B), 15 min (70 % B), 20−25 min (100 % B), 25.1−30 min (10 % B). Mobile phase flowrate and column temperature were 0.3 mL min^{-1} and 35 °C, respectively. Nitrogen was employed as nebulizing (25 psi), drying (130 °C, 11 L min^{-1}) and sheath gas (400 °C, 12 L min^{-1}) in the ESI source. Voltages of the ESI source were 3500 and 3000 V for positive and negative ionization modes, respectively. In the off-line LC–MS/MS mode, the injection volume was 1.5 μL (Castro el al., 2018). Retention times, quantifier and qualifier ions are included in Table S1.

Solutions delivered by the quaternary pump during on-line SPE were

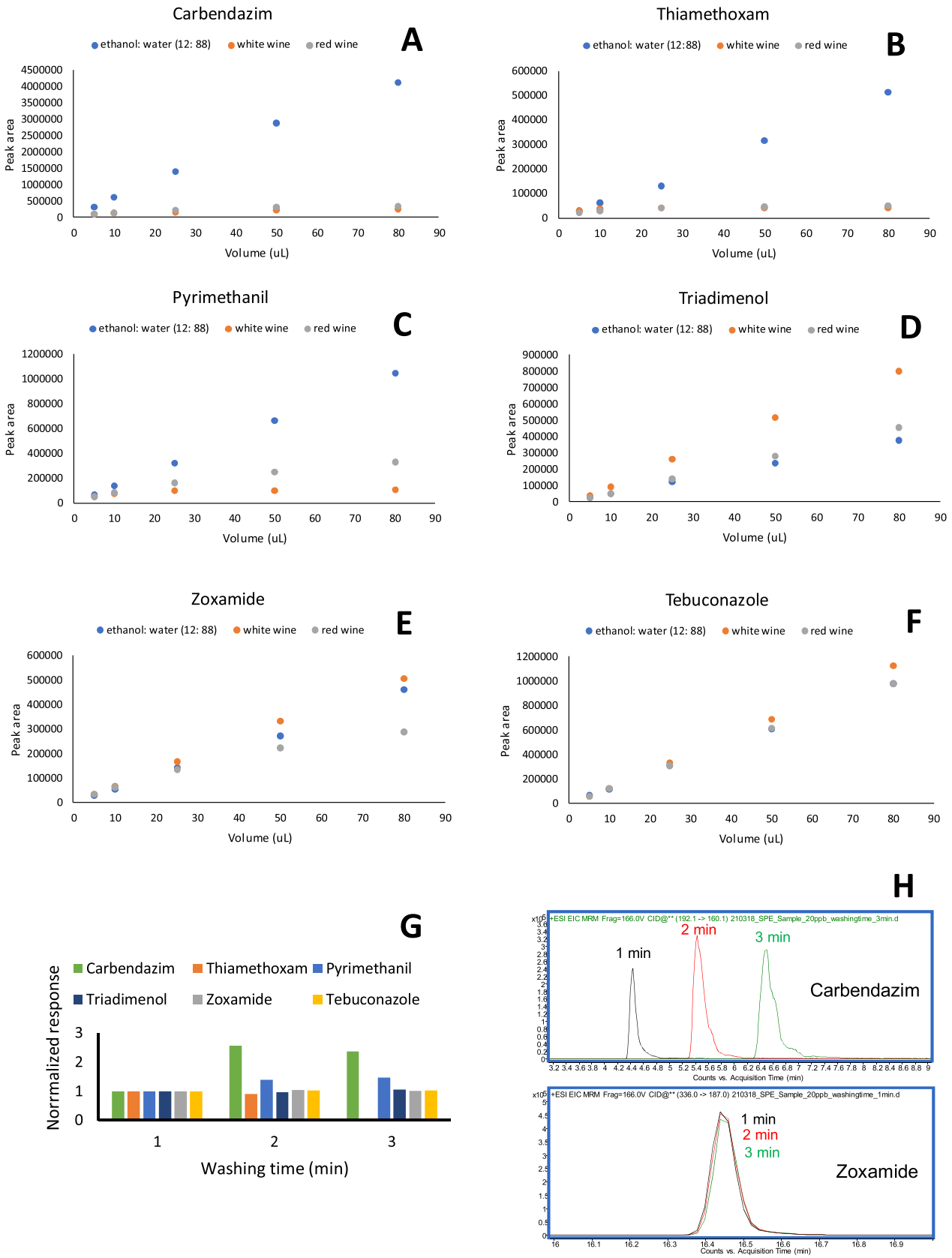

Fig. 1. A to F, effect of loaded sample volume in the responses measured for selected compounds in different matrices, spiked at 5 μg L^{-1}, by SPE on-line connected to LC–MS/MS. G, normalized responses for increasing washing times of the on-line SPE cartridge (1 mL min^{-1} of ethanol: water, 12:88, v:v) after loading 25 μL of spiked red wine. H, effect of washing time on peak shape and retention times of carbendazim and zoxamide.

ultrapure water, (C); ethanol: water (12:88, v:v), (D); and methanol (E). First, solvent C was pumped through the injection loop to transfer the withdrawn sample volume (up to 80 μL) in the on-line PS-DVB SPE cartridge. Thereafter, interferences were rinsed pumping solution D through the cartridge. After turning the 10-port valve, connected to both SPE cartridges, the chromatographic mobile phase elutes the retained compounds, whilst the auxiliary pump prepares the 2nd cartridge for next loading cycle, using solvent E followed by C. A scheme of the valve connecting on-line SPE cartridges with LC and auxiliary pumps, and the detailed SPE program, is provided as supplementary information, Fig S1.

2.4. Matrix effects and accuracy assessment

Matrix effects (MEs) were evaluated as the ratio between the slopes of calibration curves corresponding to spiked samples, or sample extracts (n = 6 levels, 1−50 μg L^{-1}), for on-line and off-line SPE methods, respectively; and those obtained for solvent-based standards in the same range of concentrations. Whatever the SPE mode, ratios around 1 point out to similar ionization efficiencies for samples and solvent-based standards. On the contrary, values below and above 1 mean suppression and enhancement of compounds ionization, respectively.

Accuracy was investigated using samples spiked at three different levels: 1 μg L^{-1} (using a volume of sample of 25 μL), and 10 and 50 μg L^{-1} (for a volume of sample of 3 μL). The difference of concentrations for spiked (n = 4 replicates) and non-spiked aliquots (n = 2 replicates) of the same matrix were divided by the nominal values added to wine before filtration and on-line SPE. Depending on the loaded sample volume, two different approaches were followed to calculate concentrations in wine samples. Using 3 μL of sample, concentrations were established against solvent-based standards, prepared in ethanol: water (12:88, v:v), in the range of concentration from 1 to 200 μg L^{-1}. When increasing the volume of sample used for on-line SPE to 25 μL, two series of matrix-matched standards were prepared using spiked aliquots of red and white wines, respectively.

2.5. Samples quantification

Identification of residues in commercial wines was made considering retention times match (maximum difference below 0.1 min), a minimum signal to noise (S/N) value of 10 for the quantifier product ion, and response ratios between quantification and qualification transitions similar to those observed for solvent-based standards (± 30 %). Procedural blanks, corresponding to ethanol: water (12:88, v:v) solutions, were processed every 5 samples. On-line SPE using a loading volume of 3 μL was used as the standard sample preparation strategy; moreover, ecological labelled wines and those where some pesticides stay in the limit of quantification of the above approach were re-processed increasing the volume of sample loaded in the on-line SPE cartridge to 25 μL.

3. Results and discussion

3.1. Optimization/characterization of on-line SPE conditions

Performance of SPE on-line connected to LC-ESI-MS/MS depends on the retention efficiency of the SPE sorbent, the selectivity of the extraction, and the compatibility between extraction and chromatographic conditions. Fig. 1A to F show the plots of responses (peak areas) versus the volume of sample loaded in the PS-DVB sorbent, in three matrices with different complexity: ethanol: water (12: 88, v:v), white wine and red wine. All of them were spiked at 5 μg L^{-1}. Depicted data correspond to a selection of 6 compounds, belonging to different chemical families and showing different functional groups and polarities, which reproduce the different trends observed for the set of 48 pesticides included in this research. After loading the corresponding volume of sample in the PS-DVB cartridge (loading flow 0.75 mL min^{-1}), cartridges were rinsed for 1 min, at 0.75 mL min^{-1}, with an ethanolic solution in ultrapure water (12:88, v:v), Fig. S1. For the spiked ethanol: water solution, responses of all compounds increased linearly with the sample volume. On the other hand, trends observed for the two wine matrices were compound dependent. For relatively polar pesticides, such as carbendazim, thiamethoxam and even pyrimethanil (Fig. 1A to C), little improvement was observed as function of the concentrated volume for both wines. On the contrary, for tebuconazole (Fig. 1F) similar responses were noticed for real wine samples and the ethanolic solution. In case of triadimenol and zoxamide (Fig. 1D and E), responses (peak areas) increased with the volume of sample loaded in the SPE cartridge; however, different slopes were noticed depending on the type of sample. The above data suggests that, the wine matrix might modify (in most cases attenuate) the efficiency of ionization of some compounds in the ESI source, reinforcing the need to optimize clean-up conditions.

Thus, in further experiments, the flowrate of the washing solution (ethanol: water, 12: 88 v:v) was increased from 0.75 to 1.0 mL min^{-1} and different washing times (1, 2 and 3 min) were evaluated. Fig. 1G shows the effect of this factor in the normalized responses for above compounds. Data correspond to a loading volume of 25 μL of a spiked red wine (duplicate extractions). Again different trends were noticed depending on the compound. In some cases, responses remained unaffected by the washing time. For carbendazim and pyrimethanil, normalized responses increased with this parameter, and for thiamethoxam the maximum peak area was attained at 1 min. Analysis of the washing fraction, eluting from the on-line PS-DVB cartridge, demonstrated the absence of breakthrough problems for all the investigated washing times. As illustrated in Fig. 1H, raising the washing time led to an increase in the retention times, and in the peak widths, of those compounds showing short chromatographic times, as it is the case of carbendazim. These trends (band broadening and retention time variations) can be understood considering that washing (ethanol: water, 12: 88) and elution (mobile phase) solutions flow through PS-DVB cartridges in opposite directions, Fig. S1. So, long washing periods cause diffusion of the distribution band of most polar compounds in the SPE sorbent. Consequently, in the further elution step, they are slowly transferred into the LC column resulting in wide chromatographic peaks. Obviously, those compounds displaying strong interactions with both sorbents (PS-DVB and C_{18} in SPE cartridges and the LC column, respectively) are not affected by band broadening processes. This behaviour is illustrated with chromatograms obtained for carbendazim and zoxamide (as representatives of poorly and strongly retained compounds, respectively) as function of the washing time, Fig. 1H. In further experiments, whatever the loading volume, the washing step was limited to 2 min, using a 1.0 mL min^{-1} flowrate of ethanol: water (12:88, v:v).

Two key parameters to demonstrate the performance of the on-line connection between SPE and LC–MS/MS are the identification of potential cross-contamination problems between consecutive samples, and the repeatability of the SPE process depending on the number of loading-elution cycles per cartridge. The 1st parameter was assessed using samples of red and white wines spiked at a relatively high concentration level (50 μg L^{-1}) considering a loading volume of 25 μL. Two aliquots of above matrices were concentrated using both PS-DVB cartridges on-line connected to the LC system (Fig. S1), followed by two procedural blanks. Carry-over effects accounted for less than 0.1 % for all compounds. Likely, the continuous flow of the LC mobile phase (acetonitrile: water: FA) through the elution cartridge, during the entire chromatographic separation program, followed by a further conditioning of same cartridge with methanol before the next loading step (Fig. S1), guaranteed the absence of cross-contamination problems between samples.

The cost of PS-DVB cartridges used in this research is around 10-times that of the off-line SPE ones (containing 200 mg of OASIS HLB), employed in a previous publication from our group (Castro et al., 2018). So, a relevant question to evaluate the applicability of the on-line

Table 1
Normalized ratios (RRs) between slopes of calibration curves corresponding to spiked wine samples (on-line SPE, considering a sample volume of 3 μL), or spiked SPE extracts (off-line SPE); versus solvent-based standards prepared in ethanol: water (12:88, on-line SPE), or acetonitrile: methanol (8:2, off-line SPE).

Name	White wine		Red wine	
	On-line SPE	Off-line SPE	On-line SPE	Off-line SPE
[a]Acetamiprid	107 %	117 %	95 %	117 %
[b]Ametoctradin	109 %	109 %	104 %	103 %
[b]Azoxystrobin	111 %	112 %	112 %	97 %
[b]Benalaxyl	112 %	97 %	118 %	92 %
[b]Bitertanol	106 %	116 %	120 %	118 %
[b]Boscalid	108 %	102 %	109 %	95 %
[b]Carbendazim	96 %	81 %	101 %	83 %
[a]Chlorantraniliprole	159 %	181 %	135 %	124 %
[a]Chlorpyrifos-methyl	114 %	82 %	110 %	70 %
[a]Chlorpyriphos	110 %	96 %	124 %	88 %
[a]Clofentezine	119 %	87 %	105 %	81 %
[a]Clothianidin	61 %	21 %	75 %	68 %
[b]Cyflufenamid	117 %	88 %	110 %	80 %
[b]Cymoxanil	111 %	94 %	105 %	96 %
[b]Cyprodinil	111 %	98 %	102 %	94 %
[b]Difenoconazole	111 %	109 %	120 %	106 %
[b]Dimethomorph	121 %	107 %	122 %	97 %
[b]Diniconazole	111 %	115 %	114 %	110 %
[b]Fenamidone	109 %	116 %	118 %	99 %
[b]Fenhexamid	113 %	101 %	112 %	94 %
[b]Fenpropidin	108 %	96 %	90 %	90 %
[b]Fludioxonil	105 %	109 %	80 %	94 %
[b]Fluopicolid	110 %	96 %	114 %	90 %
[b]Flusilazol	110 %	112 %	112 %	110 %
[b]Imazalil	92 %	102 %	85 %	96 %
[a]Imidacloprid	125 %	104 %	109 %	116 %
[b]Iprovalicarb	114 %	102 %	113 %	97 %
[b]Mandipropamid	110 %	108 %	115 %	100 %
[b]Metalaxyl	112 %	100 %	100 %	96 %
[a]Methiocarb	108 %	99 %	111 %	93 %
[b]Metrafenone	118 %	91 %	128 %	86 %
[b]Myclobutanil	111 %	113 %	113 %	103 %
[b]Penconazole	109 %	109 %	115 %	103 %
[b]Prochloraz	118 %	121 %	115 %	114 %
[b]Propiconazole	111 %	117 %	113 %	114 %
[b]Pyraclostrobin	113 %	101 %	114 %	95 %
[b]Pyrimethanil	92 %	92 %	83 %	77 %
[b]Quinoxyfen	112 %	106 %	132 %	97 %
[b]Tebuconazole	110 %	118 %	112 %	116 %
[a]Tebufenozide	106 %	90 %	120 %	85 %
[b]Thiabendazole	105 %	74 %	102 %	66 %
[a]Thiacloprid	111 %	127 %	101 %	111 %
[a]Thiamethoxam	124 %	122 %	101 %	114 %
[b]Thiophanate-methyl	119 %	160 %	102 %	159 %
[b]Triadimefon	111 %	111 %	116 %	104 %
[b]Triadimenol	126 %	128 %	125 %	116 %
[b]Trifloxystrobin	118 %	98 %	118 %	92 %
[b]Zoxamide	113 %	95 %	123 %	90 %
RRs range	**Number of compounds**			
< 70 %	1	1	0	3
> 130 %	1	2	2	1
70 %-130 %	46	45	46	44

[a] Insecticides.
[b] Fungicides.

methodology is to assess for how long cartridges can be used. Fig. S2 shows the repeatability of the on-line SPE process, considering two different loading volumes. Data (as relative standard deviations, RSDs, without any correction with responses for isotopically labelled compounds) correspond to wine samples spiked at 1 μg L^{-1} (n = 6 replicates), processed with two PS-DVB cartridges used for 150 loading-desorption cycles, each. RSDs of responses measured for above 90 % of the considered compounds remained below 10 %, whilst the maximum RSD value was 13 %. Thus, under experimental conditions reported in the Material and Methods section, the performance of

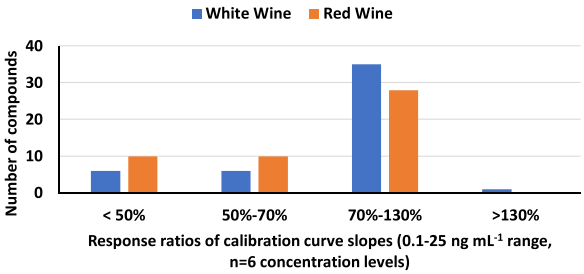

Fig. 2. Distribution of matrix effects (MEs), as normalized (0-100) ratios of calibration curves (0.1 -25 μg L^{-1}, n = 6 levels) for spiked wine samples and ethanol: water (12:88, v:v) solutions. Data corresponding to on-line SPE of 25 μL volume samples.

on-line SPE cartridge is maintained at least during 150 extraction-desorption cycles.

Table 1 compiles MEs data corresponding to off-line and on-line SPE, using the disposable HLB cartridges (Castro et al., 2018) and the reusable PS-DVB ones, respectively. In both cases, sorbents were employed just for wine extraction, without considering compounds concentration or dilution. Thus, sample and extract volumes were 2 mL for off-line SPE. Then, 1.5 μL of extract were injected in the LC–MS/MS system, following conditions reported during validation of the off-line method (Castro et al., 2018). For on-line SPE, 3 μL of 1:1 diluted wine were loaded in the PS-DVB cartridge. Whatever the SPE mode, most compounds displayed normalized response ratios in the range from 70 to 130 %, pointing out to a low effect of the sample matrix in the efficiency of ESI ionization (± 30 %).

Fig. 2 summarizes the distribution of normalized (0–100) ratios between slopes of calibration curves (0.1–25 μg L^{-1} range, referred to the undiluted wine) obtained for spiked samples of red and white wine and ethanol: water solutions, when the volume of sample loaded in PS-DVB cartridges was increased to 25 μL. In this case, the percentage of compounds showing response ratios between 70 and 130 % decreased significantly; moreover, severe signal attenuation effects were found in some cases, with response ratio values below 50 %. Additionally, for several compounds, their response ratios differed significantly between red and white wines, Table S3.

3.2. Performance of on-line SPE

SPE followed by on-line LC-ESI-MS/MS determination was characterized in terms of linearity, accuracy and limits of quantification (LOQs), loading 3 and 25 μL of sample (1:1 diluted wine) in the PS-DVB cartridges. In the first case, quantification was carried out with responses obtained for spiked ethanol: water (12:88) solutions. In the second one, two series of matrix-matched calibration solutions, using spiked aliquots of red and white wines with low pesticide residues, were proposed to assess the accuracy of the method with spiked samples. For all compounds, the plots of their corrected responses versus concentration fitted a linear model in the range of concentrations from 1 to 200 μg L^{-1} (n = 6 levels, loaded volume 3 μL), and from 0.1–25 μg L^{-1} (n = 6 levels, loaded volume 25 μL), with determination coefficients (R^2) above 0.990, Table S4.

Accuracy was estimated with recoveries obtained for samples spiked at different concentration levels. Whatever the added concentration, and the volume of sample loaded in the on-line SPE cartridge, a minimum of 39 species, out of 48 considered compounds, showed recoveries in the range between 70 and 120 %, Table 2. On average, for the 3 different considered addition levels and the two wine matrices, 90 % of the compounds presented recoveries comprised between 70 and 120 %. Thus, the selection of SSs employed in the study allowed the effective correction of changes (in most cases attenuation) in the efficiency of the ionization noticed for some compounds, particularly when loading

Table 2
Accuracy assessment of SPE on-line combined with LC-ESI-MS/MS for wine samples spiked at different concentration levels. Mean recoveries (%) with associated standard deviations (SD).

Compound	Sample volume 3 µL				Sample volume 25 µL		LOQs (µg L^{-1})		[c]MRL vinification grapes
	Red wine		White wine		Red wine	White wine			
	[a]50 µg L^{-1} Mean (SD, %)	[a]10 µg L^{-1} Mean (SD, %)	[a]50 µg L^{-1} Mean (SD, %)	[a]10 µg L^{-1} Mean (SD, %)	[a]1 µg L^{-1} Mean (SD, %)	[a]1 µg L^{-1} Mean (SD, %)	Sample volume 3 µL	Sample volume 25 µL	(µg Kg^{-1})
Acetamiprid	57 % (11)	83 % (19)	76 % (3)	132 % (9)	119 % (5)	119 % (1)	0.1	0.05	500
Ametoctradin	72 % (3)	98 % (10)	86 % (0)	120 % (2)	87 % (1)	100 % (2)	0.1	0.05	6000
Azoxystrobin	118 % (1)	115 % (8)	125 % (9)	118 % (2)	150 % (1)	102 % (1)	0.2	0.03	3000
Benalaxyl	95 % (1)	102 % (3)	98 % (1)	101 % (2)	100 % (1)	100 % (2)	0.1	0.01	300
Bitertanol	91 % (3))	100 % (6)	90 % (5)	107 % (1)	82 % (4)	78 % (2)	0.7	0.1	10
Boscalid	98 % (2)	107 % (4)	105 % (1)	116 % (11)	116 % (1)	104 % (1)	0.5	0.1	5000
Carbendazim	89 % (1)	108 % (6)	90 % (2)	89 % (8)	113 % (1)	115 % (12)	0.9	0.1	500
Chlorantraniliprole	102 % (10)	99 % (1)	103 % (3)	96 % (1)	100 % (2)	111 % (1)	0.5	0.05	1000
Chlorpyrifos-methyl	101 % (4)	84 % (3)	112 % (9)	71 % (19)	68 % (4)	81 % (6)	2.5	0.5	10
Chlorpyriphos	96 % (2)	72 % (3)	106 % (6)	61 % (1)	76 % (2)	81 % (1)	0.5	0.05	10
Clofentezine	85 % (2)	71 % (4)	100 % (5)	80 % (2)	87 % (3)	81 % (2)	0.3	0.05	1000
Clothianidin	90 % (1)	115 % (1)	92 % (1)	109 % (6)	117 % (8)	110 % (1)	0.6	0.5	700
Cyflufenamid	86 % (6)	116 % (4)	89 % (2)	115 % (1)	85 % (8)	96 % (9)	0.3	0.04	200
Cymoxanil	93 % (4)	99 % (2)	97 % (1)	94 % (1)	104 % (3)	112 % (1)	1	0.2	300
Cyprodinil	93 % (1)	121 % (4)	91 % (0)	123 % (5)	115 % (9)	83 % (10)	0.5	0.08	3000
Difenoconazole	70 % (3)	82 % (8)	85 % (0)	97 % (3)	101 % (2)	85 % (1)	0.1	0.03	3000
Dimethomorph	86 % (3)	107 % (3)	76 % (12)	96 % (9)	112 % (0)	80 % (0)	0.2	0.03	3000
Diniconazole	92 % (0)	100 % (4)	92 % (2)	100 % (2)	82 % (1)	87 % (1)	0.4	0.08	10
Fenamidone	116 % (2)	136 % (8)	112 % (4)	103 % (23)	117 % (0)	110 % (1)	0.2	0.03	600
Fenhexamid	86 % (1)	117 % (4)	84 % (4)	101 % (26)	110 % (5)	103 % (1)	1	0.05	15,000
Fenpropidin	80 % (3)	95 % (17)	89 % (1)	118 % (11)	98 % (4)	116 % (8)	0.2	0.03	10
Fludioxonil	105 % (4)	108 % (3)	107 % (2)	105 % (4)	120 % (5)	112 % (1)	0.7	0.1	4000
Fluopicolid	87 % (1)	104 % (5)	88 % (3)	101 % (0)	88 % (1)	96 % (1)	0.5	0.05	2000
Flusilazol	77 % (1)	99 % (6)	82 % (1)	105 % (0)	82 % (3)	90 % (1)	0.2	0.05	10
Imazalil	94 % (1)	123 % (3)	93 % (1)	122 % (5)	113 % (8)	110 % (2)	0.3	0.06	10
Imidacloprid	89 % (1)	114 % (6)	94 % (5)	122 % (11)	117 % (2)	103 % (4)	1	0.1	1000
Iprovalicarb	95 % (2)	116 % (6)	94 % (2)	105 % (6)	102 % (1)	96 % (2)	0.1	0.02	2000
Mandipropamid	95 % (2)	108 % (5)	103 % (1)	112 % (2)	111 % (0)	76 % (2)	0.1	0.02	2000
Metalaxyl	90 % (3)	114 % (5)	89 % (1)	113 % (1)	123 % (0)	107 % (4)	0.2	0.1	1000
Methiocarb	95 % (2)	103 % (4)	97 % (2)	104 % (3)	94 % (0)	109 % (2)	0.4	0.04	300
Metrafenone	84 % (3)	91 % (8)	93 % (2)	94 % (3)	93 % (2)	91 % (4)	0.2	0.02	7000
Myclobutanil	96 % (1)	114 % (4)	96 % (0)	115 % (4)	108 % (2)	108 % (1)	0.2	0.03	1500
Penconazole	79 % (1)	97 % (5)	83 % (1)	101 % (2)	87 % (2)	92 % (1)	0.1	0.05	500
Prochloraz	64 % (8)	70 % (17)	85 % (5)	123 % (12)	85 % (1)	81 % (4)	0.3	0.05	30
Propiconazole	89 % (1)	96 % (6)	95 % (1)	101 % (1)	82 % (2)	85 % (0)	0.3	0.05	300
Pyraclostrobin	75 % (3)	83 % (7)	88 % (2)	93 % (1)	86 % (1)	85 % (1)	0.1	0.03	2000
Pyrimethanil	128 % (4)	135 % (3)	113 % (6)	129 % (4)	114 % (7)	71 % (8)	0.2	0.04	5000
Quinoxyfen	85 % (4)	73 % (6)	101 % (3)	80 % (1)	75 % (2)	70 % (2)	0.2	0.03	1000
Tebuconazole	89 % (2)	108 % (3)	89 % (0)	106 % (3)	[b]n.e.	105 % (2)	0.5	0.05	1000
Tebufenozide	80 % (9)	123 % (4)	97 % (3)	116 % (10)	88 % (8)	128 % (9)	1	0.1	4000
Thiabendazole	88 % (5)	96 % (8)	102 % (2)	102 % (27)	110 % (1)	115 % (0)	0.6	0.1	10
Thiacloprid	62 % (16)	70 % (21)	83 % (5)	108 % (9)	125 % (2)	96 % (2)	0.1	0.02	10
Thiamethoxam	88 % (1)	112 % (3)	89 % (1)	110 % (2)	108 % (1)	103 % (1)	0.7	0.1	400
Thiophanate-methyl	99 % (4)	98 % (1)	98 % (2)	100 % (1)	103 % (3)	126 % (1)	0.4	0.05	3000 (10 from oct-20)
Triadimefon	90 % (1)	104 % (2)	91 % (1)	105 % (1)	93 % (1)	77 % (1)	0.4	0.05	10
Triadimenol	82 % (2)	114 % (4)	87 % (13)	127 % (15)	95 % (5)	89 % (2)	0.6	0.08	300
Trifloxystrobin	78 % (4)	92 % (8)	86 % (4)	87 % (2)	78 % (3)	87 % (5)	0.1	0.02	3000
Zoxamide	84 % (0)	85 % (5)	90 % (4)	80 % (1)	80 % (2)	91 % (0)	0.2	0.03	5000
Recoveries range	**Number of compounds**								
< 70 %	3	–		1	1	–			
70–120%	44	43	47	40	44	46			
> 120 %	1	5	1	7	3	2			

[a] Added concentration.
[b] n.e., not evaluated since added concentration represented less than 10 % of native concentration.
[c] European Pesticide Database for wine grapes (available 20/04/2021). https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/products.

25 µL of sample in the PS-DVB cartridge, Fig. S2. The LOQs of the proposed method, considering extraction volumes of 3 and 25 µL, are included in the last two columns of Table 2. LOQs were estimated from the S/N values observed for the lowest concentration standard, either prepared in ethanol: water (1 µg L^{-1}), or spiked in pesticide-free wines (0.1 µg L^{-1}) for sample volumes of 3 and 25 µL, respectively. In the latter case, the reported LOQs correspond to the wine matrix (red or white wine, depending on the compound) rendering the lowest (poorest) S/N ratio. Globally, the obtained LOQs are three orders of magnitude below MRLs established in the EU for vinification grapes (assuming that wine density stays very close to 1 g mL^{-1}, LOQs reported in µg L^{-1} are equivalent to same value given in µg Kg^{-1}). For those pesticides whose use has been phased-out, a common MRL of 10 µg Kg^{-1} is fixed by the EU for vinification grapes. Even in this situation, both on-line SPE

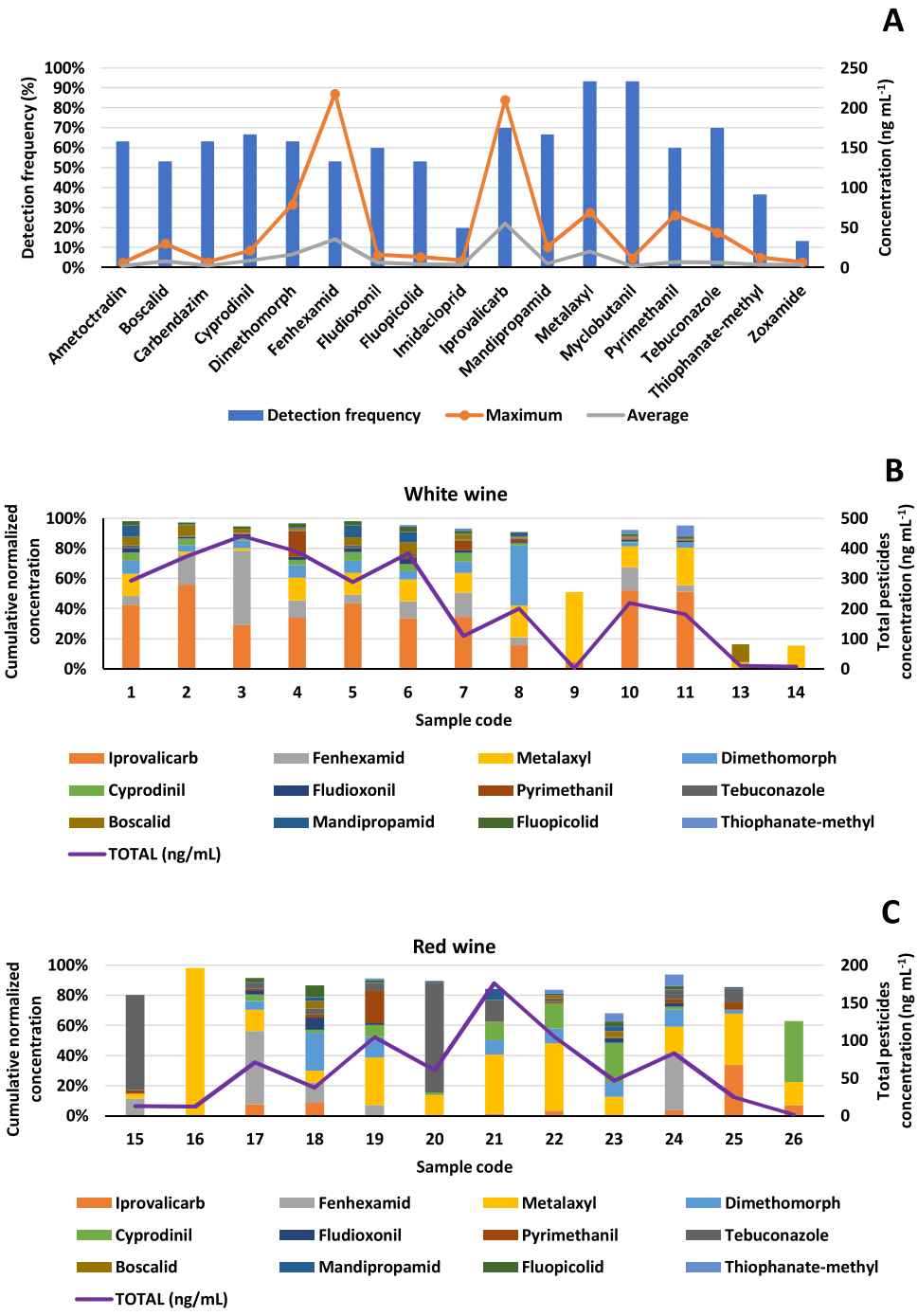

Fig. 3. Distribution of pesticide residues in conventional wine samples. A, detection frequency, maximum and average concentrations of compounds found at concentrations above 5 μg L^{-1} in at least one sample. Total concentration and normalized contribution of compounds with concentrations above 10 μg L^{-1} in at least one sample of conventional white (B) or red (C) wine.

approaches provide suitable LOQs to investigate the presence of these species in commercial wines. Thus, with the exception of some particular cases, commented in the next section, limiting the extracted sample volume to 3 μL suffices to quantify potential pesticide residues in commercial wines, with the advantages of (1) using the same set of solvent-based standards to quantify red and white wines, and (2) making possible to prepare authentic procedural blanks.

A summary of sample and organic solvent volumes involved in previous LC–MS/MS methods dealing with determination of pesticide residues in wine sample is given as supplementary information, Table S5. The procedure reported in this study combines most advantages of direct injection approaches, improving the so-far reported LOQs, and extending the life of the analytical column, as well as reducing maintenance at the ESI source in the LC–MS instrument. To the best of our knowledge, it constitutes the first application of SPE on-line combined with LC–MS/MS for the determination of fungicide and insecticide residues in wine samples. In comparison with previous on-line combinations of SPE and LC–MS/MS dealing with analysis of pesticides in other matrices, such as environmental water samples (Barbieri et al., 2020; Mann and Pock, 2016), an obvious advantage of the reported methodology is the reusability of SPE cartridges, which reduces sample preparation costs. Moreover, the use of two identical SPE cartridges permits increasing the

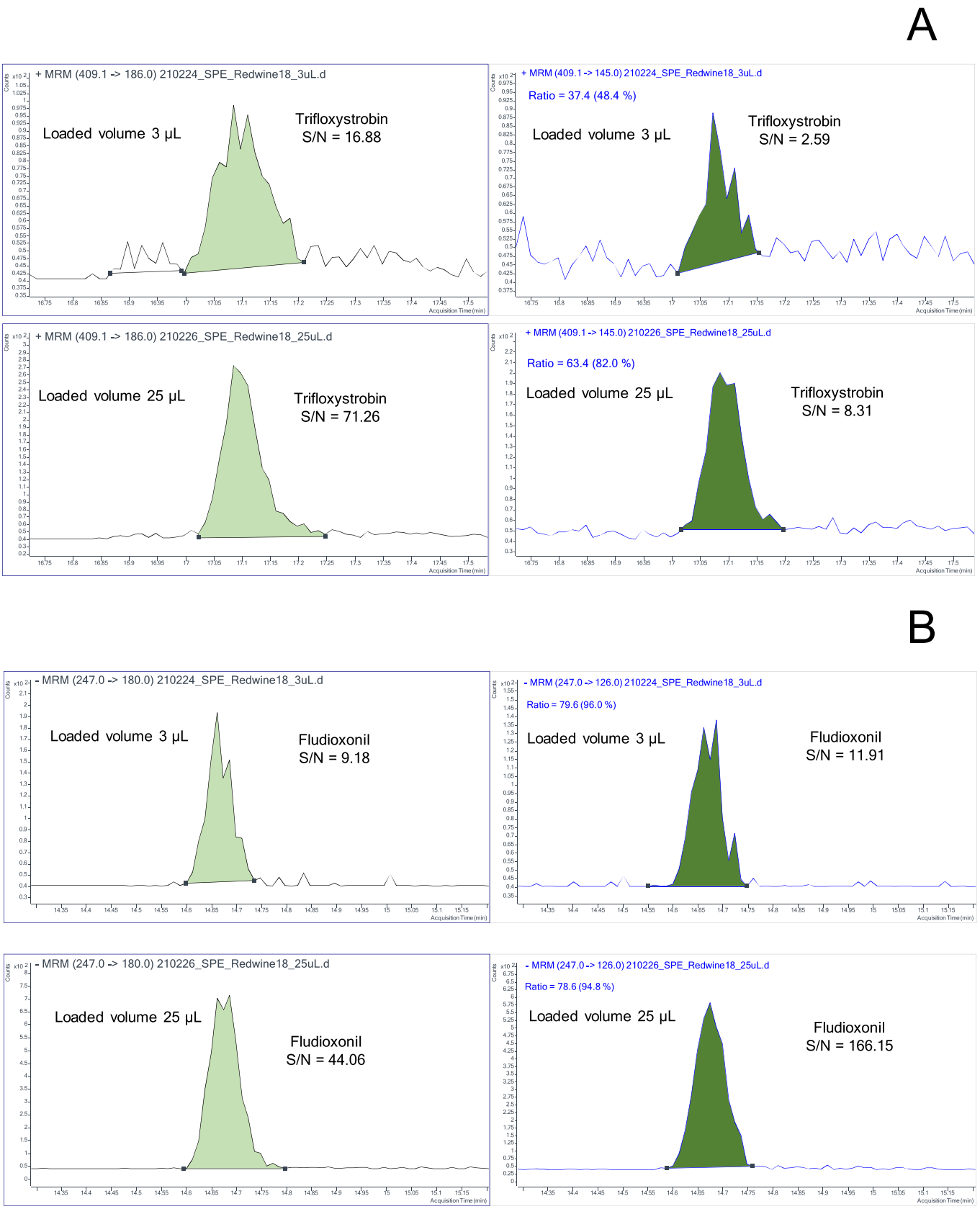

Fig. 4. Effect of loaded sample volume (3 vs 25 µL) in the signal to noise (S/N) ratio of quantification (left) and qualification (right) transitions of trifloxystrobin (A) and fludioxonil (B) in a non-spiked red wine processed by on-line SPE. Estimated concentrations: 0.08 and 0.4 µg L^{-1} for trifloxystrobin and fludioxonil, respectively.

sample workflow, since one of the sorbents is conditioned whilst the other is being desorbed. Operational conditions employed in the current method also avoided the need to dilute the extract from the SPE cartridge, as reported during determination of toxins in wine by online SPE (Campone et al., 2018) to re-focussed eluted species in the analytical column, and thus to improve the shape of the chromatographic peaks.

3.3. Application to wine samples

SPE on-line connected with LC-ESI-MS/MS was applied to investigate the existence of residues of selected compounds in a set of 30 wines, 5 of them displaying the ecological production stamp. Since one of the aims of the study was to assess the evolution of pesticide residues compared to our previous study published in 2018 (Castro et al., 2018), most of the selected samples were elaborated with grapes harvested in 2019 and 2020. Whenever possible, same commercial brands as in the previous study were acquired. Further details of the analyzed samples are included in Table S2. Wines were processed in duplicate, considering loading volumes of 3 and 25 μL for conventional and ecological wines, respectively. A procedural blank was processed, in duplicate, every 5 samples. Concentrations above method LOQs and showing differences lower than 10 % between duplicate extractions are gathered in Table S6. Residues of target compounds stayed below MRLs set in Europe for vinification grapes in all samples; moreover, fourteen compounds were never detected, and the maximum residues of 17 additional pesticides stayed below 5 μg L^{-1} in all the wines.

Fig. 3A summarizes detection frequencies, maximum and average residue levels for the other seventeen pesticides included in this research. Values displayed in this figure remain similar to those obtained for wines elaborated some years ago and processed by off-line SPE LC–MS/MS (Castro et al., 2018) except in case of pyrimethanil and boscalid. The maximum residues of these two compound underwent a slight reduction. Detection rates shown in Fig. 3A are significantly higher than those reported for wines produced in other areas of Spain (Santana-Mayor et al., 2020); however, it must be considered that the percentage of positive samples depends also on the LOQs achieved by the analytical methodologies employed in each study. Even the maximum concentrations displayed in Fig. 3A remain below 10 % of the MRL for vinification grapes; thus, they are within limits recommended by the OIV for commercial wines. Fig. 3B and 3C compile total pesticide concentrations and the normalized contributions of a selection of 12 compounds (all of them fungicides) with levels above 10 μg L^{-1} in at least one of the conventional wines. Overall residues in white wines (Fig. 3B) were higher than those found in the red wine samples (Fig. 3C); with iprovalicarb, fenhexamide and metalaxyl representing 50 % of total concentrations found in eleven out of thirteen white wines, Fig. 3B. In the case of red wines, iprovalicarb presented a lower contribution to the total fungicide residues; however, those of cyprodinil and tebuconzole increased significantly, Fig. 3C.

Out of 5 processed wines displaying the ecological production stamp, four samples contained detectable levels of several fungicides with total residues in the range from 0.2–26 μg L^{-1}. Regarding their individual concentrations, measured values stayed below 0.6 μg L^{-1} in three samples, whilst wine code 29 contained concentrations close to 6 μg L^{-1} of boscalid and metalaxyl. Fig. 4 illustrates sensitivity improvement derived from loading a higher volume of sample in the SPE cartridge for two compounds remaining below method LOQs when concentrating 3 μL of wine. Thus, increasing the volume of sample loaded in the on-line SPE cartridge is particularly suitable when extremely low concentrations need to be determined.

4. Conclusions

The on-line combination of SPE and LC-ESI-MS/MS, permits the sensitive determination of pesticide residues in wines, limiting sample handling to filtration, reducing sample preparation costs and the generation of solid wastes compared to off-line SPE extraction with disposable sorbents. Considering a sample volume as low as 3 microliters, the proposed method provides LOQs between 0.1 and 1 μg L^{-1} (except chlorpyrifos methyl, LOQ 2.5 μg L^{-1}), and a satisfactory accuracy (average recoveries comprised between 70 and 120 % for 90 % of investigated compounds) using solvent-based calibration standards to quantify pesticide residues in red and white wines. If required (i.e. for analysis of ecological labelled wines), a further reduction of above LOQs can be attained increasing the volume of sample loaded in the polymeric SPE cartridge to 25 μL. Within the set of processed samples, residues remained well-below MRLs defined by the EU for vinification grapes; however, twelve fungicides out of the 48 pesticides considered in the current study showed concentrations above 10 μg L^{-1} in at least one sample. Some of them could be quantified even in ecological production wines at concentrations in the range from 0.1–6 μg L^{-1}.

Authors contributions

L. Pérez-Mayán: Investigation; Methodology; Original Draft.
M. Ramil: Data curation; Formal analysis; review & editing.
R. Cela: Project administration; Funding acquisition; review & editing
I. Rodríguez: Conceptualization; Supervision; Funding acquisition; Original Draft

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2021.104184.

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