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Filter-vial dispersive solid-phase extraction as a simplified clean-up for determination of ethylphenols in red wines

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ABSTRACT

In-vial filtration with dispersive solid-phase extraction (d-SPE) clean-up of QuEChERS (quick, easy, cheap, effective, rugged and safe) extracts is proposed for the determination of ethylphenols (EPs) in red wines.

Analytes were extracted from 5 mL wine sample (previously alkalinized with 0.5% sodium hydroxide) using 5 mL acetonitrile. For phase separation, 1.5 g NaCl and 4 g anhydrous $MgSO_4$ were added. Then, a 0.5 mL aliquot of the partitioned supernatant was cleaned-up using d-SPE and in-vial filtration with a combination of anhydrous $CaCl_2$ (100 mg) and primary-secondary amine (PSA, 25 mg) as sorbents.

The proposed method provided limits of quantification (LOQs) ranging from 0.024 to 0.04 mg L^{-1}. Considering matrix-matched calibration as quantification technique, the recoveries (accuracy) ranged between 73% and 116%. The method was applied for the determination of EPs in 15 commercial wines of Argentina, where 4-EP was quantified at concentrations ranging from 0.25 to 3.01 mg L^{-1}.

1. Introduction

[*Brettanomyces*] sp. is a microorganism related to several wine faults, most notably those known as phenolic off-flavor or Brett character (Caboni, Sarais, Cabras, & Angioni, 2007). This flavor can be described as horsey, leathery, medicinal, smoky or savory being caused by the presence of ethylphenols (EPs) (Tempère et al., 2014). Although *Brettanomyces* sp. are considered spoilage organisms that cause an objectionable flavor in red wine when their related rot compounds are present in relatively high levels, minimum amounts of EPs may add complexity to a wine (Caboni et al., 2007; Rayne & Eggers, 2007; Tempère et al., 2014). The EPs, 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG), are formed from the transformation of p-coumaric and ferulic acids with 4-vinylphenol (4VP) and 4-vinylguaiacol (4VG) as intermediates. These transformations occur by a chain reaction of cinnamate decarboxylase generating vinylphenols, and then ethylphenols via vinylphenol reductase (Chatonnet, Viala, & Dubourdieu, 1997; Heresztyn, 1986). These reactions take place during wine elaboration and maturation, particularly when aging in wooden barrels (Carpinteiro, Abuín, Rodríguez, Ramil, & Cela, 2012); thus, organoleptic defects related to EPs are usually more significant in red wines than in white ones. Taking into account the relevant organoleptic impact of EPs on wine quality as off-flavor compounds, and therefore the convenience of monitoring their evolution during wine aging, the development of analytical methodologies for their determination in wine samples is a subject of interest.

Sample preparation has a critical role in the determination of EPs, particularly in complex samples such as wines. It has to reduce the complexity of extracts, concentrate target analytes and, in some cases, improve the determination performance by eliminating matrix interferences. The most common approaches reported for volatile phenols are liquid-liquid extraction, solid-phase extraction (SPE), solid-phase microextraction, stir bar sorptive extraction and dispersive liquid-liquid micro-extraction (Boutou & Chatonnet, 2007; Carpinteiro et al., 2012; Castro Mejías, Natera Marín, de Valme García Moreno, & Garcıa Barroso, 2003; Fariña, Boido,

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Carrau, & Dellacassa, 2007; Franc, David, & de Revel, 2009; Pizarro, Sáenz-González, Párez-del-Notario, & González-Sáiz, 2011; Rayne & Eggers, 2007; Rodríguez-Cabo, Rodríguez, Ramil, Silva, & Cela, 2016; Valente, Santos, Moreira, & Rodrigues, 2013; Zhou, Qian, & Qian, 2015). Recently, Han et al. introduced a new concept known as filter-vial dispersive-SPE (d-SPE), which simultaneously provides d-SPE clean-up and in-vial filtration of extracts (Han, Sapozhnikova, & Lehotay, 2014). Filter-vial d-SPE combines a common practice in LC analysis with clean-up, thus allowing further streamlining of numerous applications in sample preparation (Schneider, Lehotay, & Lightfield, 2015). The common practice of d-SPE by shaking the extract with adsorbents is followed by a centrifugation step. Then, an aliquot of the final extract is either filtered into auto-sampler vials using a syringe filter or a commercial in-vial filter device. A simple and effective option eliminates the centrifugation step, combining d-SPE and in-vial filtration. This approach allows the saving of several minutes of time and labor from each analysis, as well eliminating the requirement for a (mini-) centrifuge. These saving processes that can be achieved often have significant benefits when the measures can be implemented in routine operations (Zou et al., 2016). The filter vial d-SPE was recently applied for the determination of residues of pesticides, veterinary drugs and environmental contaminants in bovine muscles, fish, poultry and shrimp (Han, Sapozhnikova, & Lehotay, 2016; Han et al., 2014; Sapozhnikova & Lehotay, 2015; Sapozhnikova, Simons, & Lehotay, 2015; Schneider et al., 2015). However, its application for the sample preparation of EPs in wine samples has not been reported yet.

The objective of this work was the development and validation of a sample preparation method based on in-vial filtration with simultaneous d-SPE clean-up of QuEChERS extracts for the determination of EPs in red wines by LC-MWD. Sample preparation conditions were optimized in order to maximize the yield and selectivity of extraction process. The analytical performance of the proposed method was evaluated in terms of limits of quantification (LOQs), absolute recoveries, precision and linear range of work. Finally, the method was used to determine the levels of target analytes in commercial red wines.

2. Experimental

2.1. Standards, solvents and sorbents

Standards of 4-EP (99.8%), 4-EG (≥98%), 2-methoxy-4-vinylphenol (4-VG, ≥98%) and 4-VP (10% wt solution in propylene glycol) were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions of the above phenols were prepared in methanol (MeOH) at concentration levels of 1000 mg L^{-1}. Further dilutions were prepared monthly in methanol and stored in brown bottles at −20 °C to ensure stability.

HPLC-grade acetonitrile (MeCN), acetone and formic acid (FA) were purchased from Mallinckrodt Baker (Inc. Pillispsburg, NJ, USA). Ethyl acetate was from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

Analytical grade sorbents (50 μm particle size) for filter-vial d-SPE, including primary-secondary amine (PSA) and octadecylsilane (C_{18}) were both obtained from Waters (Milford, MA, USA). The 0.2 μm poly-tetrafluoroethylene (PTFE) filter vials used for filter-vial d-SPE were from Restek Corporation (Bellefonte, PA, USA). Reagent grade NaCl, sodium hydroxide (NaOH), $MgSO_4$, and $CaCl_2$ for QuEChERS development were purchased from Biopack (Buenos Aires, Argentina). Sodium phosphate monobasic (NaH_2PO_4) and o-phosphoric acid were obtained from Sigma-Aldrich. The NaH_2PO_4 buffer at 0.05 mol L^{-1} used for chromatographic phase was prepared by dissolving 5.99 g of salt in ultrapure water, adjusting pH to 3.15 with concentrated phosphoric acid and bringing to 1 L final volume.

2.2. Wine samples

The wine samples studied in this work were obtained from a wine company (Catena-Zapata, Agrelo, Mendoza, Argentina) and from local supermarkets. Samples included red wines of the varieties Malbec, Cabernet Sauvignon, Cabernet Franc, Nebbiolo, Anchelota and Merlot. It is important to mention that with the aim to test the method with commercially sourced samples naturally, some of them had the Brett character as organoleptic defect. This was assessed by personnel of the wine company with sommelier expertise that stated the prevalence of these aroma notes in wines of lots aged in certain oak barrels. The selected samples also included some young wines, without oak barrel aging, with the aim to test the proposed method with a wide range of matrices.

2.3. Sample preparation

The sample preparation method developed in this study involves two consecutive steps. First, wine samples were extracted through QuEChERS approach in order to extract and separate target analytes from other matrix components. Afterwards, an aliquot of the achieved extract was cleaned-up by in-vial d-SPE prior to drying and reconstitution in the initial mobile phase.

Under optimized conditions, 5 mL of wine and 50 μL NaOH (500 g L^{-1} solution) were placed into a 15 mL PTFE centrifuge tube. Then, 5 mL MeCN were added and the tube was vigorously hand-shaken for 30 s to ensure homogenization of sample and extraction solvent. Phase separation was achieved by adding 1.5 g of NaCl and 4 g of $MgSO_4$; followed by shaking for 1 min and centrifuging for 10 min at 8000 rpm (6450 rcf). Thereafter, 0.5 mL aliquot of the upper MeCN phase was pipetted into the shell portion of the in-filter vial containing 100 mg $CaCl_2$ and 25 mg PSA. The filter plunger was partially depressed into place to seal the bottom piece, vortexed for 30 s and the vial plunger was then depressed fully to achieve filtration. Finally, the cleaned and filtered extract was taken from the vial and evaporated to dryness under gentle N_2 stream. The residue was reconstituted with 400 μL of LC initial mobile phase (0.05 M NaH_2PO_4 in Milli-Q water and 20% of MeCN) and 20 μL were injected in the LC-MWD system.

2.4. Determination

Target EPs were determined using a LC-MWD system (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany). The LC instrument was a Dionex Ultimate 3000 consisting of vacuum degasser unit, autosampler, quaternary pump and chromatographic oven. The detector was a Dionex MWD-3000 (RS) model with an analytical flow cell operated with a data collection rate of 5 Hz, a band width of 4 nm and a response time of 1.000 s. The working wavelengths for the different analytes were 280 nm (4-EP and 4-EG) and 260 nm (4-VP and 4-VG). The Chromeleon 7.1 software was used to control all the acquisition parameters of the system and also to process the obtained data.

HPLC separations were carried out in reversed-phase Accucore C_{18} column (2.1 mm × 100 mm, 2.6 μm) Thermo Scientific (Bellefonte, PA, USA). The mobile phases were 0.05 M NaH_2PO_4 pH 3.15 (A) and MeCN (B). Analytes were separated using the following gradient: 0 min, 20% B; 0–6 min, 50% B; 6–6.5 min, 20% B; 6.5–9 min, 20% B. The mobile phase flow was 0.7 mL min^{-1}. The column temperature was 35 °C and the injection volume 20 μL. The identification and quantification of EPs in the wines were based on the comparison of the retention times (tR) and absorbance val-

ues of detected peaks in samples with those obtained by injection of pure standards of each analyte. Additionally, samples were spiked with known concentrations of compounds in order to verify the peak identification and the absence of interferences at the analytes tR. Levels of target species in wine samples were determined with matrix-matched standards, corresponding to aliquots of red wine (Malbec variety) spiked with increased concentrations of target species, from 0.05 to 5 mg L^{-1}, and submitted to the overall sample preparation method.

2.5. Interferences effect

Matrix effects (ME) are defined as positive or negative responses produced by compound/s (interferences) other than the analyte of interest that impact the measurement of its concentration or mass (European Commission, 2013). The interference is also referred to as "chemical noise" and ME are a subtle form of interference that could be minimized by a better detector selectivity. If the interference cannot be eliminated or compensated, its effects may be acceptable if the impact on accuracy is not significant (European Commission, 2013). Potential ME (%) for EPs produced by interferences occurring during HPLC-MWD analyses were evaluated by comparing the responses from solvent and matrix-matched standards; and were calculated as follows:

$$ME\% = \left(1 - \frac{Slope\ MM\ curve}{Slope\ Solvent\ curve}\right) \times 100$$

with MM being the matrix-matched calibration standards (Fontana & Bottini, 2014).

3. Results and discussion

3.1. Optimization of extraction conditions

Different extraction solvents were evaluated including MeCN, ethyl acetate and acetone. No differences in analytical responses were observed for 4-EP and 4-EG by using these solvents. However, for 4-VP and 4-VG higher responses were achieved using MeCN as solvent, and so MeCN was selected for further studies. In this sense, MeCN was the selected solvent for further studies. To determine the influence of extraction solvent volume, a series of separate sets of extractions were performed using 5 mL of red wine with different MeCN volumes (1, 2.5 and 5 mL). The best results for the studied EPs were achieved when 5 mL of MeCN were used. Thus, taking into account the achieved results, a sample to solvent ratio of 1:1 was selected to perform additional assays.

The EPs have pkas between 9.5 and 10. When the pH of the sample solution is lower than 8, more than 99% of the phenols will be present in the un-dissociated form, acting as weak hydrogen donors (Zhou et al., 2015). Sample pH determines the dissociation status of EPs in solution and directly affects their extraction in MeCN. As well, the pH determines the extractability of matrix components present in wine samples which are directly related to the presence of interferences during target analytes determination. The pH effect on the EPs extraction was evaluated by adding different concentration of NaOH (0.1, 0.5, 1 and 2.5% w/v) or FA (2.5% v/v) to samples and compared with sample without pH modification (raw wine). Fig. 1a shows the results for the four EPs under different pH conditions. As can be observed, a significant increase in the responses of 4-VP, 4-EP and 4-EG was achieved at pH 9.5 (0.5% NaOH). Considering the mentioned pkas of analytes, working at a basic pH shifts the phenol-phenolate equilibrium toward the less polar phenol form and EPs are mostly as neutral molecules facilitating the extraction with MeCN. At neutral or acid pH, EPs are mostly as ionic compounds, which difficult their extraction in the

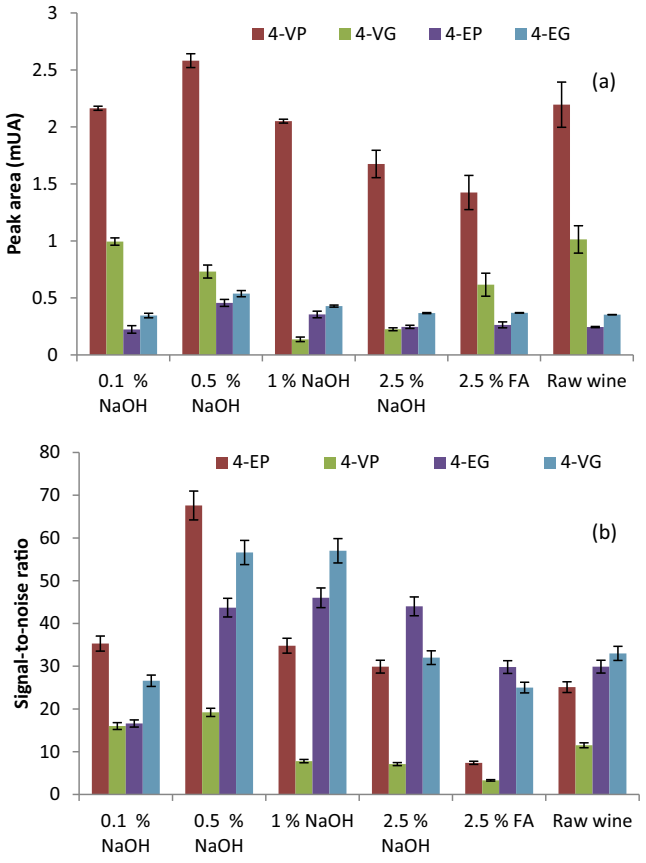

Fig. 1. (a) Effect of different pH conditions on the analytical response of EPs; (b) Effect of pH condition on the S/N ratio of EPs. Extraction conditions: 5 mL wine: 5 mL MeCN (NaOH and FA according to each assay); phase partitioning: 4 g anhydrous $MgSO_4$ + 1.5 g NaCl. Clean-up: 100 mg $CaCl_2$ + 25 mg PSA + 25 mg C_{18}. Filter-vial d-SPE as described in Section 2.3.

solvent. The 4-VG reported relatively higher analytical responses in the sample without pH modification (pH 3.7 for natural wine). Further pH increase to 11 (1% NaOH) or 12 (2.5% NaOH) showed a reduction in the responses for analytes. The other relevant aspect of studding the sample pH is those related to the co-extraction of interferences. The signal to noise ratio (S/N) achieved at each pH are summarized in Fig. 1b. The S/N achieved at pH 9.5 was much higher than the one obtained at the others studied pH, probably because at low pH more matrix co-extractives of wine are present as neutral analytes (i.e. phenolic compounds, free fatty acids and anthocyanins) remaining in MeCN phase after the salting-out step. It was evidenced by a dark appearance of extracts and higher background signals. On the contrary, at more alkaline pH's, the S/N were higher and more clear extracts were observed because the most of co-extractives from wine are present as ionic compounds, so reducing their mass transfer to the MeCN phase. To balance the sensitivity of all the EPs and eliminate interferences of the wine matrix, samples were adjusted at a pH value of approximately 9.5 by adding 0.5% NaOH.

3.2. Comparison of different sorbents in filter-vial d-SPE approach

The traditional QuEChERS approach often combines an additional efficient clean-up of sample extract using d-SPE after the salting-out extraction (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003). This is needed, particularly for complex MeCN extracts coming from wine samples, in order to reduce the content of sugars and remaining phenolic compounds that could negatively

affect the identification of EPs. For the clean-up optimization, the effects of sorbent type and composition of sorbent mixture on the purification efficiency and background of chromatograms were evaluated. As well, the most suitable sorbent amounts for maximizing clean-up effectiveness without affecting the realization of in-vial filtration approach were selected for this purpose. For the development of filter-vial d-SPE, PSA and C_{18} were evaluated alone and in different combinations. Aliquots (0.5 mL) of the extracts were placed into 0.2 μm PTFE filter vials for d-SPE cleanup as follows (each in triplicate): (1) 100 mg anhydrous $CaCl_2$ (2) 100 mg anhydrous $CaCl_2$ + 25 mg of PSA, (3) 100 mg anhydrous $CaCl_2$ + 25 - mg of C_{18}, (4) 100 mg anhydrous $CaCl_2$ + 25 mg of PSA + 25 mg of C_{18}, (5) 25 mg of PSA + 25 mg of C_{18}. Additionally, an extract without clean-up was processed similarly to evaluate the necessity of the clean-up. The results are summarized in Fig. 2. In terms of responses of analytes by using different sorbents and combinations, significant differences were observed when the d-SPE mixture contained PSA. This effect was particularly high for 4-VP (Fig. 2a). By observing the S/N (Fig. 3b) obtained for each d-SPE combination, the mixture of $CaCl_2$ and PSA showed better results for 4-VP and 4-VG. On the contrary, a little higher S/N for 4-EP and 4-EG was obtained by using only $CaCl_2$. The other combinations, particularly those not having $CaCl_2$ presented lower S/N and dirty chromatograms. The $CaCl_2$ has a favorable effect due to the removal of water that reduces the polarity of the final extracts thus producing the precipitation of certain polar co-extractives. As well, anhydrous $CaCl_2$ increases the ionic strength of the medium. In fact, the lower amounts of water and the high ionic strength favor the partition of neutral analytes (EPs are well above their pKa's) to the acetonitrile phase. Taking into account the achieved results, a compromise situation between sensitivity and clean-up was selected by using a combination of 100 mg $CaCl_2$ and 25 mg PSA.

3.3. Performance of the method

The analytical figures of merit of the optimized method are summarized in Table 1. The overall performance of the developed method was evaluated using spiked and non-spiked samples. The accuracy and precision of the method were assessed through recovery experiments of wine aliquots presenting low, medium and high EPs addition levels (0.1, 1 and 2.5 mg L^{-1}). In all cases, spiked and non-spiked aliquots were processed in triplicate and the concentrations of EPs in the corresponding extracts determined by the calibration curve obtained for matrix-matched standards. Relative recoveries between 73% and 116%, with associated standard deviations below 9, were achieved. The inter-day accuracy and precision were assessed with 5 mL aliquots of wine, spiked at the 0.5 mg L^{-1} level and processed in triplicate during 3 consecutive days. In this case, the absolute recoveries of the method ranged from 81% to 114%, with standard deviations between 7% and 13% (data not shown).

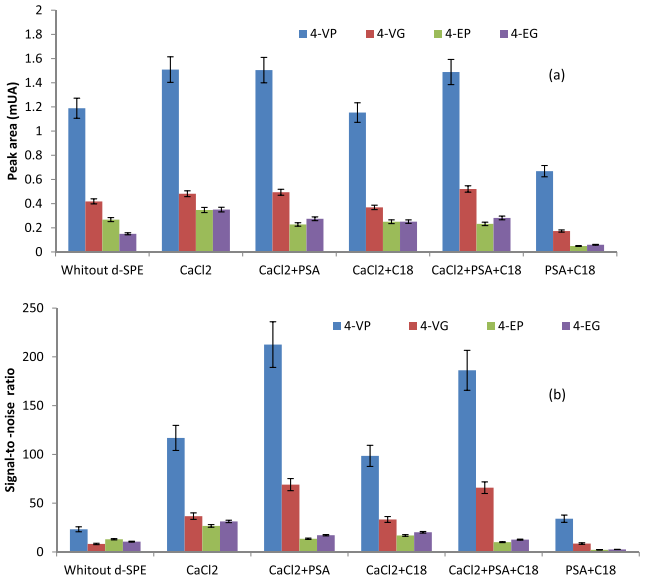

Fig. 2. (a) Effect of d-SPE sorbent combination on the analytical response of EPs; (b) Effect of d-SPE sorbent mixture on the S/N ratio of analytes. Extraction conditions as described in Fig. 1. Amounts of sorbents in each assay are explained in Section 3.2.

The slopes of the calibration graph obtained with matrix-matched standards were compared with those obtained with solvent-based standards, calculating the matrix to solvent slope ratios and then ME% as was described in Section 2.5 for each of the studied analytes. According to established parameters, ME values from −20 to +20% are considered suitable indicating minor ME (Ferrer, Lozano, Agüera, Girón, & Fernández-Alba, 2011; Fontana & Bottini, 2014). Usually, this ±20% range is used as a cutoff value to justify using solvent calibration in place of matrix-matched standards because it is considered to be a mild signal suppression or enhancement effect. The comparison of the slopes from solvent and matrix-matched calibration showed ME ranged between 52% for 4-VP and 73% for 4-VG (matrix signal enhancement for the four target EPs) caused by interferences occurring during LC-MWD analyses (see Table 1). These values of ME are considered to exert a strong effect during quantification of analytes. Thus, in order to compensate the errors associated with the observed interferences, matrix-matched standards were used as calibration technique to achieve accurate quantification of the target analytes. Linearity was assessed using a young wine without oak barrel aging (Malbec) sample presenting low concentrations of EPs species. Aliquots of this sample were spiked at seven concentration levels from 0.05 to 5 mg L^{-1}. Within the above interval, linearity was observed with determination coefficients higher than 0.9938 for all the studied analytes (See Table 1). Thus, method LOQs were estimated from the S/N values corresponding to chromatographic peaks in the lower level of the linearity study. Values obtained for S/N = 10 stayed between 0.024 mg L^{-1} for 4-VP and 0.04 mg L^{-1} for 4-EG (Table 1). These LOQs remain about a half of magnitude below those achieved by Valente et al. (2013) applying the QuEChERS technique with traditional d-SPE clean-up and without sample pH adjustment, followed by HPLC-UV-FLD determination. The LOQs reported were higher than to those attained by GC–MS/MS methods using different sample preparation and/or derivatization steps (Carpinteiro et al., 2012; Pizarro et al., 2011; Zhou et al., 2015). However, these methods require more laborious sample preparation as well as expensive equipment that not always are accessible to average routine laboratories, especially for small wineries.

The selectivity of the QuEChERS method with filter-vial d-SPE for the determination of EPs was evaluated by the comparison of tR and spectral behavior achieved by analyzing a standard solution of polyphenols and a QuEChERS extract of wine after applying the optimized method (Fontana, Antoniolli, & Bottini, 2016). As can be observed from Fig. 3, the tR obtained after analyzing a positive and a spiked wine sample, the tR obtained did not show significant differences with the obtained for the spiked as well as any interference was detected at the EPs tR.

3.4. Wine samples analysis

The developed and validated QuEChERS-LC-MWD method with filter-vial d-SPE was applied for the determination of EPs to a total of 15 samples of red wines from different grape varieties cultivated

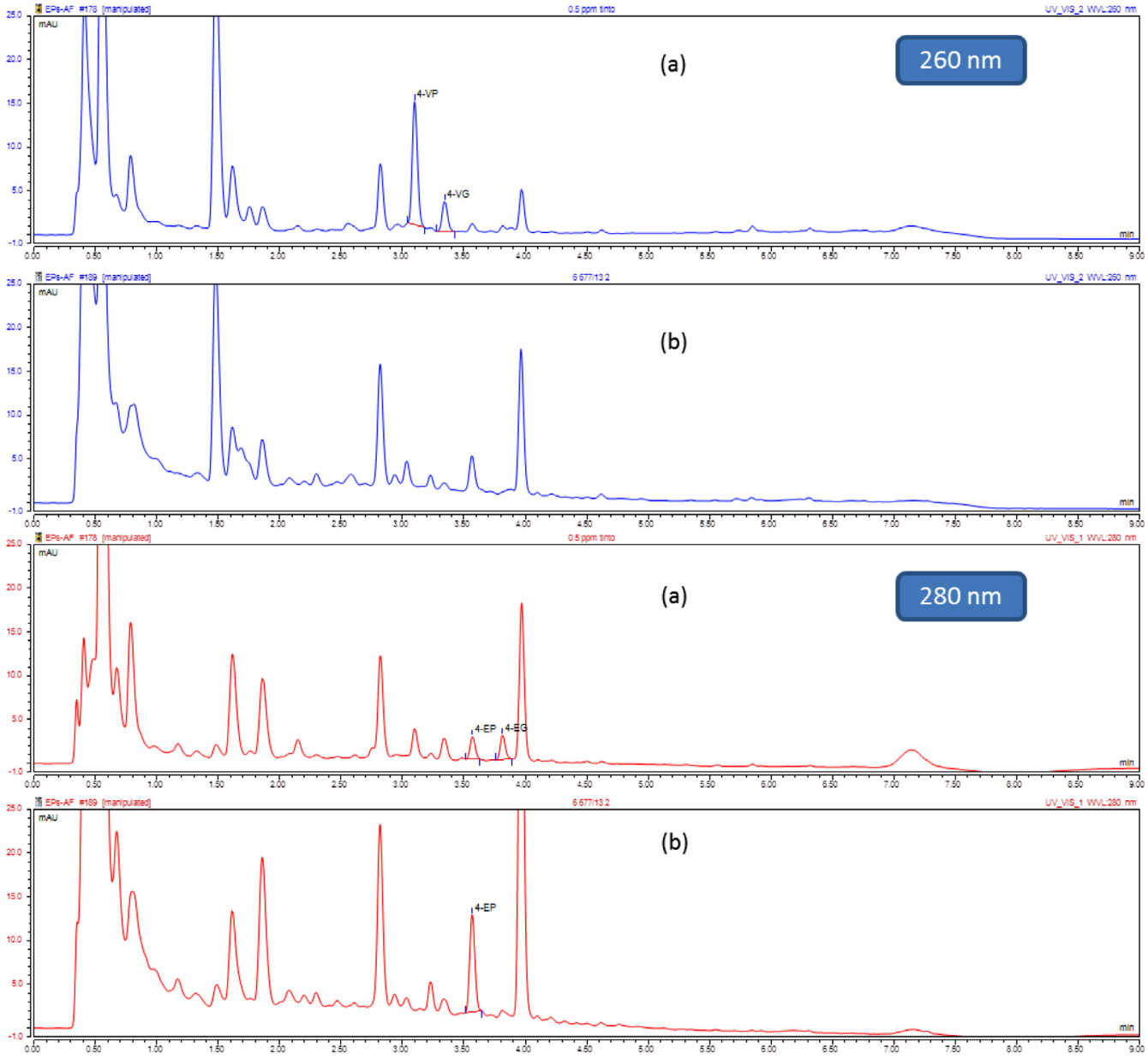

Fig. 3. Chromatograms at each detection wavelength, (a) Red wine sample spiked with 0.5 mg L^{-1} of EP standards; (b) Red wine sample without addition of standards.

Table 1
Analytical performance, absolute recoveries (%, as an estimation of accuracy) and precision of the proposed method for red wines spiked at different concentration levels.

	Linear range (mg L^{-1})	r^2	LOD (mg L^{-1})	LOQ (mg L^{-1})	ME[a] (%)	Recovery (%) ± RSD, n = 3 replicates		
						0.1 mg L^{-1}	1 mg L^{-1}	2.5 mg L^{-1}
4-VP	LOQ-5	0.9994	0.007	0.024	52	73 ± 5	85 ± 5	108 ± 4
4-VG	LOQ-5	0.9938	0.010	0.033	73	99 ± 9	84 ± 2	115 ± 8
4-EP	LOQ-5	0.9976	0.009	0.031	59	109 ± 6	100 ± 7	111 ± 7
4-EG	LOQ-5	0.9981	0.012	0.040	53	74 ± 3	104 ± 1	116 ± 4

[a] Matrix effect calculated as $ME\,\% = \left(1 - \frac{Slope\ MM\ curve}{Slope\ Solvent\ curve}\right) \times 100$.

in Argentina. Among the samples, 10 with an organoleptic defective Brett character were obtained from a local winery after a sensory analysis in order to validate the methodology with commercially sourced samples. The prevalence of these aroma notes in wines of lots aged in certain oak barrels was mentioned by the personnel of the winery and corresponded to 9 of these samples. As well, one sample was not oak aged but also presented the defect. The rest of samples from commercial origin corresponded to 1 oak aged and 4 young wines. The 4-EP was quantified in 10 of the 15 processed samples, whereas the rest of species were below the LODs of the method in all of them. The 4-EP concentrations varied between 0.25 and 3.01 mg L^{-1}. When the total concentration of EPs is greater than 0.62 mg L^{-1}, the "Brett" or phenolic character becomes too pronounced for the wine to be acceptable

(Caboni et al., 2007). If the levels are lower than 0.4 mg L^{-1}, EPs contributes favorably to the complexity of the wine aroma by imparting aromatic notes of spices, smoke, and leather (Caboni et al., 2007). The presented approach has high sample throughput and usefulness in screening studies for wines Brett character analysis.

4. Conclusions

A simplified, fast and robust sample preparation method for the quantification of EPs in red wines based on a combination of in-vial filtration with d-SPE as a convenient clean-up of QuEChERS extracts was developed and validated. The proposed method allows the selective determination of studied EPs in wine samples, showing sensitivity good enough to guarantee reliable determination at levels of organoleptic interest in wines, suitable precision and linear response ranges. The results obtained in the validation procedure at three spike levels of EPs showed adequate accuracy and precision. The applicability of the proposed approach was confirmed by the suitable quantification of analytes in commercially sourced samples. The proposed methodology has potentiality for the routine determination of the target EPs with the aim of evaluating its content in wines and helping in making commercial decisions in commercial wineries.

Conflict of interest

Ariel R. Fontana declares that he has no conflict of interest. Rubén R. Bottini declares that he has no conflict of interest.

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