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Mixed-mode solid-phase extraction followed by dispersive liquid–liquid microextraction for the sensitive determination of ethylphenols in red wines☆

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ABSTRACT

Selectivity of mixed-mode solid-phase extraction (SPE) was combined with the concentration power of dispersive liquid–liquid microextraction (DLLME) to obtain a sensitive, low solvent consumption method for gas chromatography-mass spectrometry determination of ethylphenol off-flavours (4-ethylphenol, EP; 4-ethylguaicol, EG and 4-ethylcathecol, EC) in complex red wine samples. Under optimized conditions, limits of quantification (LOQs) between 0.3 and 0.8 ng mL^{-1} were obtained using just 5 mL of wine and 0.06 mL of 1,1,1-trichloroethane (TCE) as extractant in the DLLME step. Analytes were acetylated after SPE and previously to DLLME concentration to enhance the performance of their GC–MS determination. The overall extraction efficiency of the method was unaffected by the particular characteristics of each wine; thus, accurate results (relative recoveries from 89 to 109% for samples spiked at concentrations from 20 to 1000 ng mL^{-1}) were obtained using matrix-matched standards, without requiring the use of the time consuming standard addition quantification methodology. The applicability of the method was demonstrated with the analysis of different red wines. Analytes concentrations varied from 6 to 2265 ng mL^{-1} (EP), 0.8 to 251 ng mL^{-1} (EG) and non-detected to 158 ng mL^{-1} (EC).

1. Introduction

The organoleptic characteristics of wine are the result of a complex balance of volatile and semi-volatile compounds, displaying different concentration ranges and contributions to wine aroma [1]. Among them, ethylphenol species are considered as responsible for organoleptic defects when present at levels above a given threshold, which varies from a few ng mL^{-1} for 4-ethylguaiacol (EG) up to several hundreds of ng mL^{-1} for 4-ethylphenol (EP) [2,3]. In addition to EP and EG, the presence of significant amounts of 4-ethylcatechol (EC) has also been correlated with negative aromatic notes (spicy or medicinal) in wine [4]. On the other hand, at lower concentrations, ethylphenols positively contribute to the aroma of wine [2,5]. Ethylphenol species are the result of hydroxycinnamic acids (coumaric, cafeic and ferulic) decarboxylation, followed by reduction of the intermediate vinylphenols [6]. The above reactions are enhanced by certain yeasts and take place during wine elaboration and maduration, particularly when ageing in wood barrels [7]; thus, organoleptic deffects related to ethylphenols are usually more significant in red wines than in white ones. Understanding the exact mechanisms controlling the formation of these off-flavours, and monitoring their evolution during wine maduration, requires sensitive analytical methods, able to provide reliable data at concentrations further below their sensorial thresholds.

Sample preparation plays several relevant roles in the determination of ethylphenol off-flavours, aiming (1) to reduce the complexity of wine matrices, (2) to concentrate target species and, in some cases, (3) to improve the performance of their further determination. Solid-phase extraction (SPE) [8–10] and several solid-phase type microextraction techniques [11–14] have been proposed to achieve the first two goals; whereas, analytes derivatization, usually with acetic anhydride, is advisable when considering gas chromatography–mass spectrometry (GC–MS) determination [12]. Overall, microextraction techniques, such a solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE), render similar limits of quantification (LOQs) to, or even lower than, those achieved by SPE, with the advantage of requiring lower wine volumes. On the other hand, SPME and SBSE show a low sample throughput due to (1) the limited kinetics of mass transference processes from the sample to the surface of the sorbent and

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(2) the use of standard addition as quantification technique, which is justified since the yield of the microextraction might change depending on the characteristics of each wine sample and/or the state of the polymeric coating.

The dispersive liquid–liquid microextraction technique (DLLME), first reported by Rezaee and co-workers [15], overcomes the problem associated with the slow kinetics of SPME and SBSE. However, on the other hand, the complexity of wine samples, particularly red wines, makes difficult obtaining a neat interface between the sample and the small drop of organic extractant. The first application of DLLME to the determination of ethylphenols in wine was proposed by Fariña et al. [16]. Using GC–MS, they achieved a LOQ for EG (95 ng mL^{-1}), which remains above the sensorial threshold of 33 ng mL^{-1}, reported for this compound in the bibliography [12]. Thereafter, EP and EG were included in a DLLME method dealing with GC tandem mass spectrometry (MS/MS) for the determination of several off-flavours in wine [5]. This method was further implemented replacing the dispersant solvent by ultrasound energy to achieve an emulsion of extractant droplets in the sample [17]. Unfortunately, the above methods are not applicable to EC, since they do not consider a derivatization step, required to improve the GC detectability of this dihydroxylated species. Also, as further explained, some doubts arise about their performance when transferred from hydro-alcoholic solutions, considered during optimization of DLLME, to red wines displaying a much higher complexity.

The aim of this research was to develop a sensitive and selective sample preparation method for the determination of three ethylphenol species in red wine samples, covering the range of concentrations from the sub ng mL^{-1} level (below their sensorial thresholds) up to the μg mL^{-1} range. The selectivity of SPE was combined with the concentration capability of DLLME to attain the above aims, minimizing sample and organic solvent consumption, as well as time requirements to obtain quantitative data. Moreover, acetylation of target species was also considered to improve the performance of their GC–MS determination. Parameters affecting the efficiency of extraction, derivatization and concentration steps were thoroughly investigated and their effects in the overall performance of the method discussed. Finally, the method was used to determine the levels of target species in commercial red wines.

2. Experimental

2.1. Standards, solvents and sorbents

Standards of EP, EG, EC and 3,4-dimethylphenol (DMP), used as internal surrogate (I.S.) in the sample preparation process, were purchased from Aldrich (Milwaukee, WI, USA) and TCI Europe (Zwijndrecht, Belgium). Individual solutions of each compound (ca. 1000 μg mL^{-1}) were prepared in ethanol, further dilutions and mixtures of the three ethylphenol off-flavours were made in the same solvent. Diluted solutions of DMP were also prepared in ethanol.

Ethanol, acetonitrile, methanol and acetone (chromatographic analysis grade), as well as isooctane, chlorobenzene (ClBz), carbon tetrachloride (CCl_4) and 1,1,1-trichloroethane (TCE), all trace analysis grade, were supplied by Merck (Darmstadt, Germany). Potassium hydrogen carbonate ($KHCO_3$), potassium carbonate (K_2CO_3) and di-potassium hydrogen phosphate (K_2HPO_4) were also purchased from Merck. The above salts were dissolved in ultrapure water to prepare 5% (p/v) solutions.

SPE cartridges, containing 60 mg of the mixed-mode (reversed-phase and anionic exchanger) OASIS MAX sorbent, were acquired from Waters (Milford, MA, USA).

Samples of red wine, elaborated at different geographic areas in Spain, were acquired from local markets. Bottles were protected from light and stored at room temperature until analysis.

2.2. Sample preparation

The sample preparation method developed in this study involves three consecutive steps. First, wine samples were passed through SPE cartridges in order to extract and separate target analytes from other matrix components. The extract from the cartridge was diluted with an aqueous alkaline solution and analytes were derivatized using acetic anhydride. Finally, acetylated species were concentrated by DLLME.

Under optimized conditions, 5 mL of wine were diluted with the same volume of ultrapure water and passed through the SPE cartridge, previously conditioned with methanol and water adjusted at pH 3.5 (2 mL each). After drying the sorbent with a gentle stream of nitrogen, analytes were recovered with 1 mL of acetone, which was collected in a conical bottom glass tube (12 mL volume). This extract was diluted with 8 mL of a 5% (p/v) K_2HPO_4 aqueous solution, 0.05 mL of acetic anhydride were added and tubes were shaken for 1 min. DLLME was carried out adding a binary mixture consisting of 0.06 mL of TCE in 1.5 mL of acetone. After centrifugation (3 min at 3000 rpm), the settled drop of TCE was transferred to an insert and 1–2 μL injected in the GC–MS system.

2.3. Determination

Analytes were determined by GC–MS, using a Varian (Walnut Creek, CA, USA) 3800 GC instrument connected to an ion-trap Varian 2000 mass spectrometer (MS), furnished with an electron impact (EI) ionization source. Separations were carried out in an Agilent (Wilmington, DE, USA) HP-5ms type capillary column (30 m × 0.25 mm i.d., d_f: 0.25 μm) operated at a constant helium flow of 1.2 mL min^{-1}. The GC oven was programmed as follows: 60 °C (held for 1 min), first rate at 8 °C min^{-1} to 245 °C, second rate at 25 °C min^{-1} to 285 °C (held for 15 min). The temperature of the injector was maintained at 270 °C. Injections (2 μL) were done in the splitless mode, with the solenoid valve switched to the split position after 1 min (split flow 50 mL min^{-1}). Transfer line, manifold and trap temperatures were set at 290, 50 and 220 °C, respectively. MS spectra were acquired in the *m/z* range between 80 and 400 a.m.u. The sum of responses for the two most intense ions in the spectra of acetylated compounds (*m/z* 107 + 122, EP and DMP; *m/z* 137 + 152, EG; and *m/z* 123 + 138, EC) was used for quantification purposes.

Levels of target species in wine samples were determined with matrix-matched standards, corresponding to aliquots of red wine (*Mencía* variety) spiked with increased concentrations of target species, from 1 to 5000 ng mL^{-1} (100 ng mL^{-1} for the I.S.), and submitted to the global sample preparation method. The ratios between the responses (peak areas) measured for each compound and the I.S. were plotted against the added concentration and fitted to a linear model.

3. Results and discussion

3.1. Sample preparation strategy

Previous studies from our research group have shown that chlorinated solvents used as extractants in DLLME render viscous extracts for undiluted wine samples, particularly for red wines, unsuitable for direct injection in the GC column [18,19]. Increasing the pH of wine, which is required for analytes acetylation, worsened the aforementioned problem. Replacing chlorinated extractants, typically employed in DLLME, by room temperature melting point, low density solvents (such as 1-undecanol and hexadecane) provides cleaner extracts [19]; however, these solvents might interfere with chromatographic peaks corresponding to volatile compounds,

as it is the case of ethylphenol off-flavours. Thus, SPE and DLLME were combined to improve the selectivity of the overall method by removing, during the SPE step, those matrix compounds which difficult phases separation in DLLME [18,20].

3.2. Solid-phase extraction

Optimization of the SPE step was performed with spiked (20 ng mL^{-1}) aliquots of red wine, diluted with the same volume of ultrapure water, passed through SPE cartridges previously conditioned with methanol and ultrapure water adjusted at pH 3.5. The mixed-mode OASIS MAX sorbent was preferred to reversed-phase polymers since the former has demonstrated a very high affinity for red wine matrix components, e.g. tannins, which remain retained in the sorbent after analytes elution [21]. As a result, colourless, transparent extracts are obtained using polar, water soluble solvents compatible with further steps of the analytical method. After passing a dry stream of nitrogen through the sorbent bed (ca. 10 min), analytes were recovered with methanol, acetone or acetonitrile. This extract was diluted with 8 mL of a 5% K_2HPO_4 aqueous solution and 50 μL of acetic anhydride were added, followed by manual shaking (5 min) and extraction of acetylated species with 2 mL of isooctane [22]. Recoveries of the SPE step were determined comparing the difference between responses measured for spiked and non-spiked fractions of the same wine versus standards, prepared in the same solvent used to elute MAX cartridges and derivatized under above reported conditions.

Breakthrough studies, performed passing the spiked samples through two cartridges connected in series, showed losses around 10% for EP considering 10 mL of wine, whereas, the rest of species were not detected in the extract from the second cartridge. Using 5 mL of wine, all compounds were quantitatively retained in the first SPE cartridge. As regards the elution solvent, acetone and acetonitrile rendered completely transparent extracts versus reddish ones obtained with methanol, which was discarded for further experiments. Table 1 summarizes the recoveries of the SPE step as function of the elution solvent (1 mL). Acetone provided absolute recoveries between 103% and 108%, with standard deviations below 12%. In the case of acetonitrile, recoveries ranged from 59% for EC to 102% for DMP, with standard deviations below 12% (Table 1). Moreover, EC was still detected in the 2nd fractions (1 mL) of acetonitrile eluted from SPE cartridges. Thus, 1 mL of acetone was selected as extraction solvent.

3.3. Acetylation and DLLME

Departure derivatization and liquid–liquid microextraction conditions were adopted from a previous work reporting the determination of chlorophenols in water samples after SPE and DLLME [23]. In brief, the acetone extract from the SPE cartridge was collected in a conical bottom glass tube, mixed with 0.1 mL of a chlorinated extractant and a few microliters (20–100 μL) of acetic anhydride. Then, 8 mL of an alkaline aqueous solution ($KHCO_3$ or K_2HPO_4, both 5%, p/v) were rapidly injected in the tube to obtain an emulsion of dispersed droplets of extractant. Under these conditions, it was expected that analytes were simultaneously acetylated and concentrated in the chlorinated extractant, which settled at the bottom of the conical tube after centrifugation [23]. Experimental findings showed that, whatever the volume of acetic anhydride, extractant type and alkaline catalyzer, extracts contained mixtures of derivatized and underivatized forms of EP, DMP and EG. Taking into account these unsatisfactory results, it was decided to perform first the acetylation reaction, in the aqueous phase, and then to concentrate the acetylated derivatives by DLLME.

Table 1
Absolute recoveries of the SPE step as function of the elution solvent (1 mL), $n = 4$ replicates. Data corresponding to 20 ng mL^{-1} spiked red wine samples.

Compound	Recovery (%) ± SD	
	Acetone	Acetonitrile
EP	108 ± 6	98 ± 7
EG	103 ± 6	99 ± 7
EC	107 ± 2	59 ± 12
DMP (I.S.)	103 ± 12	102 ± 10

3.3.1. Optimization of acetylation conditions

Derivatization conditions were optimized using a pool of SPE extracts obtained from unspiked aliquots (5 mL) of a red wine sample (*Tempranillo* variety) containing detectable amounts of target compounds. Fractions (1 mL volume) of this extract were diluted with 8 mL of an alkaline 5% aqueous solution, a given volume of acetic anhydride was added and then the mixture was manually shaken before DLLME extraction using, in all experiments, a mixture consisting of 0.9 mL of acetone and 0.1 mL of TCE. The effects of acetic anhydride volume (50–200 μL), type of alkaline catalizer ($KHCO_3$ versus K_2HPO_4) and derivatization time (1–10 min) in the responses (peak areas) obtained for each analyte and the I.S. were simultaneously investigated using a 2^3 type experimental factorial design, with four replicates of the central point. The Statgraphics statistical package was used to estimate the main effects associated with each variable and two-factor interactions. Fig. 1 shows the Pareto charts with standardized values of above-mentioned parameters. The length of depicted bars is proportional to the effect of the considered factor (or two-factor interaction) in the response of acetylated species. A positive sign points out to an improvement in the efficiency of the reaction when the factor changes from the low to the high level, within the domain of the design and, a negative one indicates the opposite behaviour. The vertical dotted line in the graphs represents the statistical significance bound, established for a 95% confidence level.

As appreciated in Fig. 1, the derivatization time was the less important variable, thus it was fixed at 1 min. The type of catalyzer played a positive influence on the acetylation process. Although the associated main effect did not reach the significance threshold for any compound, it is evident that the most favourable situation corresponded to the use of K_2HPO_4 (5%, p/v). This salt provides a higher pH (9.2 units) than $KHCO_3$ (pH 8.3) and thus, it shifts the acid-base equilibrium of ethylphenols towards phenolate forms, which are supposed to be more reactive than neutral species. Finally, the volume of acetic anhydride was the most relevant factor, with a significant negative influence on the performance of the derivatization step (Fig. 1). Likely, an excess of acetic anhydride reduces the pH of the aqueous solutions, which negatively affects the yield of acetylation. Thus, 50 μL was adopted as the working value for this variable. Some additional experiments were carried out using K_2CO_3 instead of K_2HPO_4; however, in this case EC could not be acetylated, thus K_2HPO_4 was retained as catalyzer in the derivatization reaction.

Fig. 2 compares the extracted GC–MS chromatograms obtained using above optimized acetylation conditions, followed by DLLME with 1 mL of a binary mixture of acetone-TCE (9:1), and considering simultaneous DLLME and acetylation, as proposed by Fattahi and co-workers for chlorophenols [23]. As observed, the two-step method, optimized in this work, provided higher peak areas for EP, DMP and EG without traces of underivatized species for any of the considered phenols.

3.3.2. DLLME parameters

3.3.2.1. Dispersant and extractant selection. Performance of DLLME methods is mainly affected by the composition of the binary extraction solution. The effects of both solvents (extractant and

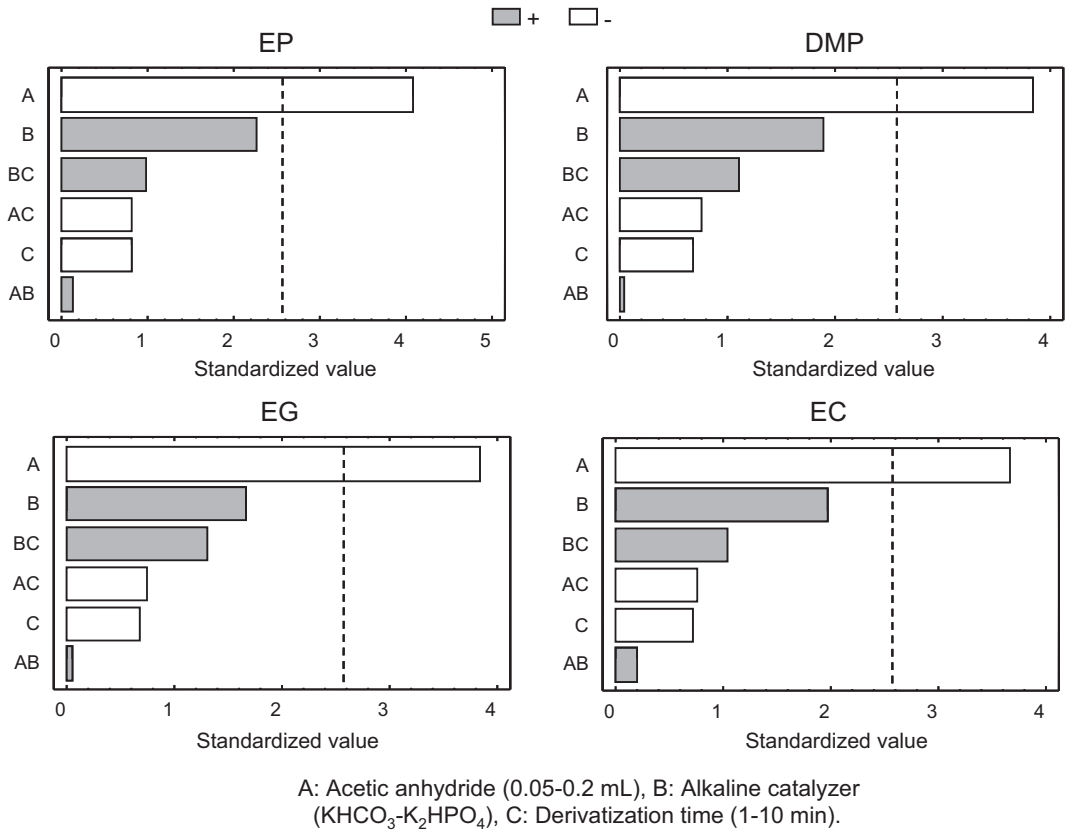

Fig. 1. Standardized Pareto charts corresponding to optimization of acetylation conditions.

dispersant) in the responses of target compounds were again investigated using the combined acetone extracts obtained after SPE of red wine. Comparison of responses obtained using methanol, acetone and acetonitrile (1 mL each) as dispersants, reflected non-significant differences between acetone and methanol, whereas around 30% lower peak areas were noticed for acetonitrile, figure not shown. Considering that acetone was also used for elution of SPE cartridges, it was selected as dispersant in the DLLME step.

Fig. 3 shows the peak areas measured using three different extractants (0.1 mL). Responses for ClBz and CCl_4 stayed below those obtained with TCE, which was maintained as extractant.

3.3.2.2. Dispersant and extractant volume. In DLLME, the volume of dispersant must be high enough to provide an emulsion of extractant droplets in the aqueous phase containing the acetylated ethylphenols. Larger volumes result in lower extraction yields due to an increase in the solubility of the analytes.

Fig. 4 shows the peak areas obtained for four different volumes of acetone (0.5, 1.0, 1.5 and 2.0 mL) combined with either 0.06 mL or 0.1 mL of TCE. The highest responses for all compounds were attained for the combination of 1.5 mL of acetone with 0.06 mL of TCE. Under these conditions, around 45 μL of TCE were recovered from the bottom of conical shaped derivatization-extraction tubes. This volume is large enough to be handled by the autosampler of the GC–MS system.

3.3.2.3. Extraction and centrifugation time. The extraction time is defined as the period comprised between addition of the extraction mixture to the aqueous sample, containing the acetylated compounds, and centrifugation. During this step DLLME tubes were manually shaken to maintain the emulsion of dispersed TCE droplets. Experimental data did not reveal any difference considering extraction times of 1, 5 and 10 min, which is in agreement with the extremely fast kinetics of the DLLME technique [20]. As regards the centrifugation time, values of 3, 10 and 15 min were tested. Again, this factor exerted a negligible effect in the responses of target compounds. Thus, extraction and centrifugation times were set at 1 and 3 min, respectively.

3.4. Performance of the method

The extraction efficiencies (EEs, %) of the optimized DLLME step were assessed comparing the differences between responses obtained for spiked (100 ng mL^{-1}, added to the extract of the SPE cartridge) and non-spiked extracts from the same red wine sample, submitted to acetylation and DLLME under optimized conditions, with those corresponding to pure standards prepared in acetone, acetylated and further extracted by conventional liquid–liquid extraction (LLE) using 3 mL of TCE. Concentrations found in DLLME extracts were divided by the theoretic expected one (2222 ng mL^{-1}, assuming a drop volume of 45 μL) and multiplied by 100 in order to obtain the EEs reported in Table 2. Considering the whole analytical procedure (SPE–acetylation–DLLME), enrichment factors (EFs) obtained for 5 mL wine samples varied between 57 and 78 times.

The overall performance of the developed method was evaluated using spiked and non-spiked samples. In all cases, DMP (I.S.)

Table 2
Extraction efficiencies (EEs, %) of the DLLME step and enrichment factors (EFs) of the whole procedure for 5 mL wine samples, $n = 3$ replicates.

Compound	EEs (%)	RSD (%)	EFs	SD
EP	70	11	78	8
EG	60	10	67	6
EC	60	11	67	7
DMP (I.S.)	51	5	57	5

Table 3
Precision of the proposed method for non-spiked red wine samples with different ethylphenol contents, linearity (1–5000 ng mL^{-1}) and limits of quantification (LOQs) defined for a signal to noise (S/N) of 10.

Compound	Intra-day precision (RSDs %, n = 4 replicates)		Inter-day precision (RSDs, %, n = 12 replicates)	Linearity (R^2, 1–5000 ng mL^{-1})	LOQs (ng mL^{-1})
	Low level sample	High level sample	Medium level sample		
EP	2.2	4.1	2.9	0.997	0.4
EG	7.8	3.7	9.6	0.998	0.3
EC	2.0	6.8	8.0	0.998	0.8

was added to wine at a constant concentration of 100 ng mL^{-1}. Precision was investigated with three red wines, corresponding to different geographic denominations and presenting low, medium and high ethylphenol contents. The intra-day precision (n = 4 replicates) varied between 2 and 8%, whereas under reproducibility conditions (n = 12 replicates, 3 days) relative standard deviations (RSDs) ranged from 3 to 10% (Table 3).

Linearity was assessed using a red wine (*Mencía*) sample, displaying low concentrations of ethylphenol species. Aliquots of this sample were spiked at nine concentration levels from 1 ng mL^{-1} to 5000 ng mL^{-1}. Fig. 5 shows the chromatograms for the non-spiked wine and a fraction of the same matrix fortified with 5 ng mL^{-1} per compound (the I.S. was maintained at 100 ng mL^{-1}). Within the above interval, a good linearity was observed with determination coefficients (R^2) above 0.997 (Table 3). Procedural blanks (corresponding to the extraction of 5 mL aliquots of synthetic wine) did not show signals at retention times of ethylphenol species. Thus, method LOQs were estimated from the signal to noise (S/N) values corresponding to chromatographic peaks in the lower levels of the linearity study. Values obtained for S/N = 10 stayed between 0.3 ng mL^{-1} for EG and 0.8 ng mL^{-1} for EC (Table 3). These LOQs remain two orders of magnitude below those achieved by Fariña et al. [16] applying the DLLME technique directly to wine samples followed by GC–MS determination and are similar to those attained by GC–MS/MS [5,17]. It is worth noting that, none of the above methods was tested for EC. LOQs reported in Table 3 are also significantly lower than those published using SPME (LOQs, 3–30 ng mL^{-1}) and sorptive microextraction with polydimethylsiloxane type sorbents (LOQs, 5–15 ng mL^{-1}) after analytes acetylation [12,24]. On the other hand, SPE based methods require the concentration of wine volumes between 50 and 100 mL to achieve LOQs in the sub ng mL^{-1} range [8,9].

As shown in Table 2, the EEs provided by the DLLME step are considerably high for a microextraction technique; however, the liquid–liquid microextraction process cannot be considered as exhaustive. Thus, quantification of ethylphenol levels in wine samples must rely either on the time-consuming standard addition method, or on matrix-matched standards. The accuracy of the later approach depends on whether the yield of the sample preparation process varies among wine samples, or if it remains basically unaltered. In order to answer this question, samples of young red wines, from three different geographic denominations, were spiked with increased concentrations of target compounds. After I.S. normalization, the maximum differences among the slopes of calibration curves accounted for a 15% for EP and EC, whereas, they remained below 10% for EG, see Supplementary information. On the basis of these moderate sensitivity differences, the matrix-matched quantification technique was considered to assess the accuracy of

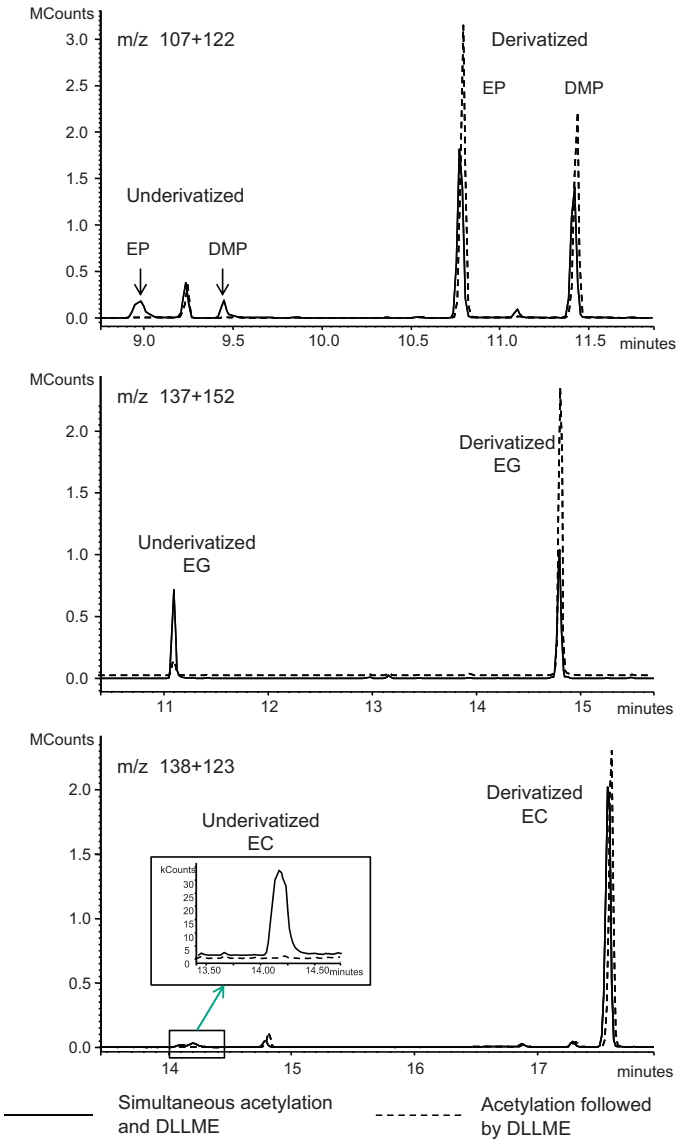

Fig. 2. GC–MS chromatograms corresponding to a spiked red wine (addition level 200 ng mL^{-1}) under optimized acetylation and DLLME conditions (dotted line) and performing simultaneously acetylation and DLLME (solid line).

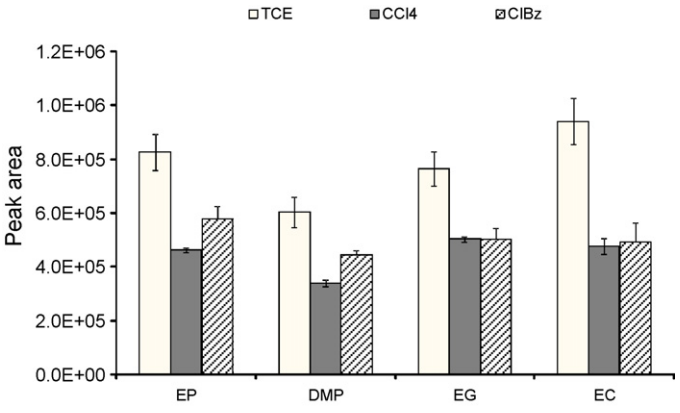

Fig. 3. Comparison of responses as function of the extractant used in the DLLME step, n = 4 replicates.

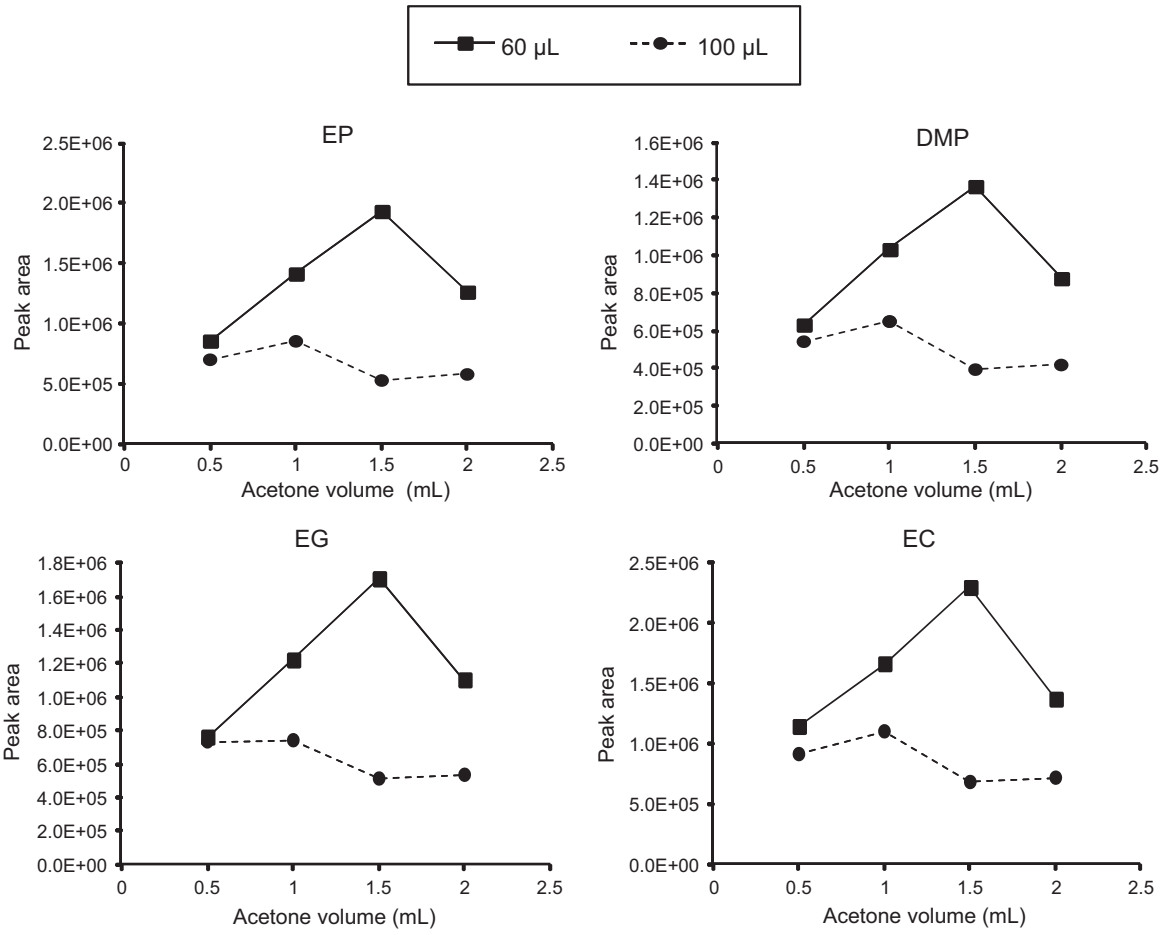

Fig. 4. Effect of dispersant (acetone) and extractant (TCE) volumes in the responses of target compounds. Average values for duplicate experiments.

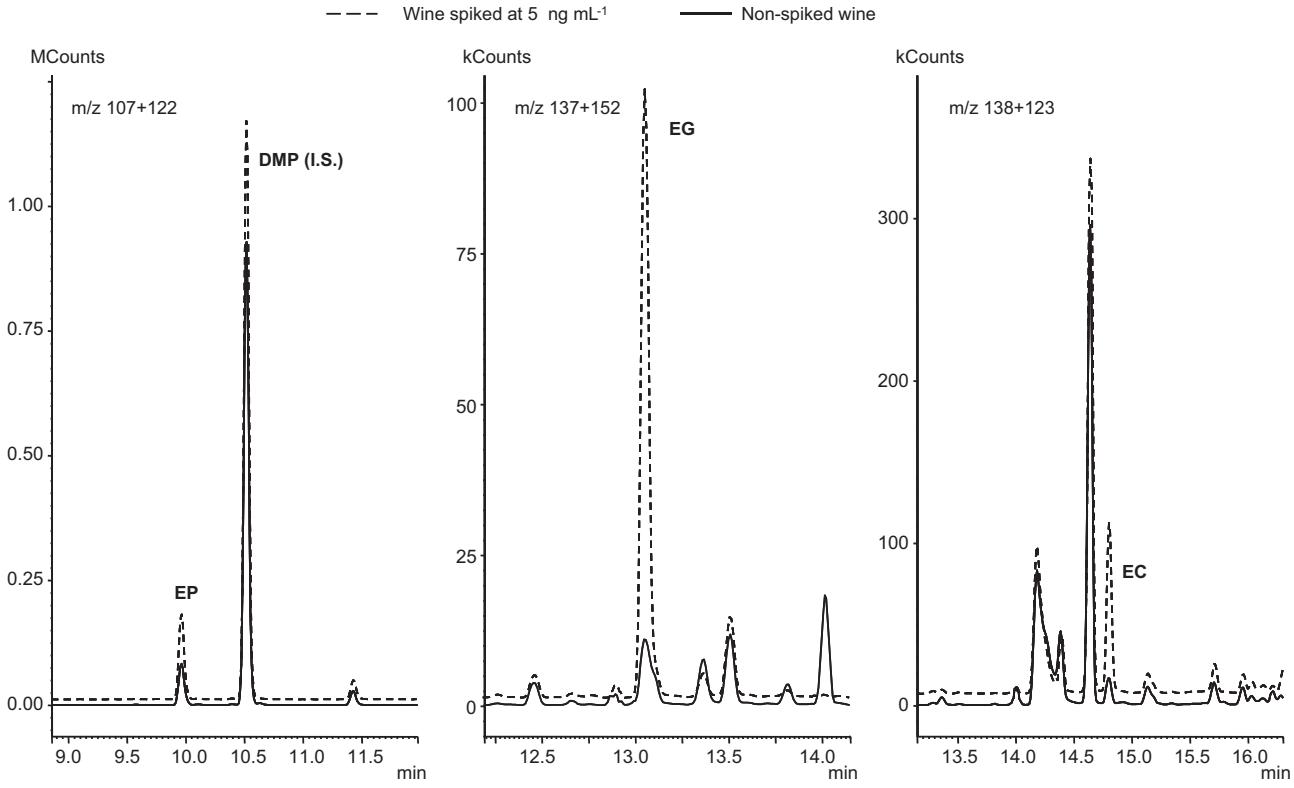

Fig. 5. Extracted ion chromatograms corresponding to a wine sample before and after addition of ethylphenols (5 ng mL^{-1}).

Table 4
Relative recoveries of the method (accuracy) for spiked aliquots of red wine.

Compound	Relative recovery (%) ± SD		
	20 ng mL^{-1} [a]	100 ng mL^{-1} [a]	1000 ng mL^{-1} [a]
EP	108 ± 5	97 ± 2	92 ± 3
EG	106 ± 10	95 ± 2	89 ± 4
EC	89 ± 3	98 ± 2	89 ± 4

[a] Added concentration.

Table 5
Concentrations (average values in ng mL^{-1} with their standard deviations) measured in red wine samples, $n = 3$ replicates.

Code	Wooden aged	Mean ± SD		
		EP	EG	EC
1	No	5.9 ± 0.1	0.81 ± 0.08	n.d.
2	No	151 ± 6	16.5 ± 0.7	19 ± 1
3	No	7.2 ± 0.7	1.2 ± 0.2	n.d.
4	No	150 ± 9	22 ± 2	22.9 ± 0.5
5	No	38 ± 2	6.1 ± 0.4	5.0 ± 0.6
6	No	65 ± 1	8.6 ± 0.5	7.0 ± 0.4
7	No	11.42 ± 0.02	1.1 ± 0.1	1.85 ± 0.04
8	No	81 ± 2	5.8 ± 0.1	9.6 ± 0.2
9	Yes	1177 ± 50	73 ± 2	43 ± 3
10	Yes	2265 ± 44	251 ± 9	158 ± 11

the method. Table 4 shows the relative recoveries for a red wine (*Grenache* variety) spiked at three different levels (20, 100 and 1000 ng mL^{-1}), quantified using a calibration curve corresponding to spiked aliquots of a different wine (*Mencía* variety). Relative recoveries between 89% and 108%, with associated standard deviations below 10, were achieved.

3.5. Application to real samples

The proposed method was applied to a total of 10 red wine samples. Six samples were young wines (codes 1–5, 8), two were table wine (codes 6 and 7), without geographic denomination and distributed in Tetra Pack type packages; finally, codes 9 and 10 correspond to red wines aged in wood barrels. EC was quantified in 8 of the 10 processed samples, whilst the rest of species overpass the LOQs of the method in all of them (Table 5). Globally, EP levels stayed one order of magnitude above those of EG and EC, which were measured at similar concentrations in most samples. As expected, the highest concentrations corresponded to wood aged wines (codes 9 and 10); however, the presence of target analytes in young and table wines, which were never in contact with wood, points out to ethylphenols as intrinsic components of the aromatic profile of red wines.

4. Conclusions

The combination of mixed-mode SPE and DLLME provides an un-matched performance for the sensitive and accurate determination of ethylphenol compounds in red wines, overcoming the limited selectivity and capability of DLLME to deal with complex samples, as well as the reduced enrichment factors of SPE when considered individually. Despite acetylation and DLLME have to be performed sequentially, the method shows an acceptable sample throughput (both steps require about 5 min and many samples can be simultaneously submitted to SPE); moreover, it provides accurate recoveries without using the time-consuming standard addition quantification methodology. Also, it permits the determination of target compounds in red wines at levels further below their sensorial thresholds, being suitable to investigate their evolution during wine maduration. Advantages of the proposed sample preparation approach (SPE followed by DLLME) are expected to be also useful for the determination of other volatile species, related to the organoleptic quality of wine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.01.044.

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