Journal of Chromatography A, 1217 (2010) 7208–7214

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Sorptive extraction with in-sample acetylation for gas chromatography–mass spectrometry determination of ethylphenol species in wine samples

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ARTICLE INFO

Article history:
Received 12 July 2010
Received in revised form 9 September 2010
Accepted 13 September 2010
Available online 18 September 2010

Keywords:
Ethylphenolic off-flavours
Wine
Acetylation
Sorptive extraction
Gas chromatography
Mass spectrometry

ABSTRACT

An inexpensive and effective sample preparation procedure for the determination of three ethylphenolic off-flavours (4-ethylphenol, 4-ethylguaiacol and 4-ethylcathecol) in wine samples is presented. Analytes were in situ acetylated and concentrated using a disposable silicone sorbent (DSS) exposed to the diluted sample. After that, the analytes were recovered with ethyl acetate and determined by gas chromatography with mass spectrometry. The influence of different parameters (volume of acetic anhydride, basic catalyst, ionic strength, sorbent format, sampling mode and extraction time) on the efficiency of derivatization and extraction steps is discussed. Under optimized conditions, 2 mL of wine were diluted with 15 mL of an aqueous solution of potassium bicarbonate (5%, m/v) in a 22 mL vessel, containing 2 g of sodium chloride. The volume of acetic anhydride and the extraction time were set at 90 μL and 2 h, and the extraction was carried out at room temperature (20 ± 2 °C). Analytes were concentrated using a silicone disc (5 mm diameter × 0.5 mm thickness) and further desorbed with 0.2 mL of ethyl acetate. The achieved limits of quantification (LOQs), defined as the concentration of each compound providing a signal 10 times higher than the baseline noise, stayed between 5 and 15 ng mL^{-1}. The method provided a linear response range of up to 5000 ng mL^{-1} and relative recoveries from 91% to 116%. The 4-ethylphenol off-flavour was detected in most red wine samples at concentrations of up to 2700 ng mL^{-1}.

1. Introduction

Organoleptic parameters greatly affect the quality of wine and the acceptance of this product by consumers. Ethylphenol species, e.g. 4-ethylphenol (EP) and 4-ethylguaiacol (EG), contribute to the complexity of wine aroma; however, when their concentrations exceed a given threshold, which depends on the type of wine and the perception skills of the individual consumers, they provide undesirable off-flavours to wine, described as horse sweat or medicinal, and normally referred as *Brett* character [1,2]. The above species and 4-ethylcathecol (EC) are generated from the enzymatic decarboxylation and the further reduction of hydroxycinnamic acids contained in grapes must. These reactions are enhanced by yeasts of the *Brettanomyces* genus and occur during the aging of wine in wood barrels [3,4] and, to a lesser extent, in bottles [5]. Thus, monitoring the levels of the above ethylphenols is recommended to understand and to control those variables affecting their formation during winemaking and maturation steps.

Recently, many efforts have been focussed on the optimization of improved and/or simplified sample preparation methodologies for the determination of volatile phenols in wine. Thus, solid-phase microextraction (SPME) [6–10] and stir bar sorptive extraction (SBSE), using polydimethylsiloxane (PDMS) coated magnetic stirrers [11–13], have been proposed as appealing alternatives to conventional liquid–liquid extraction (LLE) [14] and solid-phase extraction (SPE) [15]. In most applications, SPME and SBSE are combined with gas chromatography–mass spectrometry (GC–MS) for the direct determination of EP and EG, using medium-polarity capillary columns. The higher polarity of EC limits its extraction yield and also impairs the determination of this species by GC-based methods. To overcome this drawback, in-sample acetylation has been combined with SPME for the simultaneous determination of EP, EG and EC in wine by GC–MS [10]. SPME and SBSE avoid completely the use of organic solvents and provide limits of quantification (LOQs) below the commonly accepted thresholds for ethylphenol species (from 33 ng mL^{-1} for EG to 440 ng mL^{-1} for EP) [10,13]. On the other hand, they also show some inherent limitations, such as the fragility of SPME coatings and fibres, the risk of cross-contamination problems between samples and the relatively high cost of fibres and PDMS coated stir bars.

Sorptive extraction, using disposable pieces of silicone polymers produced on an industrial scale, offers interesting features

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doi:10.1016/j.chroma.2010.09.036

to overcome the above reported limitations. Popp and co-workers [16,17] first proposed the use of silicone rods for the extraction of environmental pollutants from water samples, demonstrating that partition of the analytes between the matrix and the bulk sorbent was governed by the same equations as those used to predict the yield of SPME and SBSE processes. Following these early studies, different applications have been reported for direct and headspace extraction of organic species from aqueous matrices, liquid foodstuffs and air [18–23]. Bulk silicone sorbents are available in different formats (cord, hollow tube and sheets), they are suitable for thermal and organic solvents desorption, their dimensions (length and thickness) can be adapted for each particular application and they are inexpensive [24]. An immediate advantage of the latter feature is that they are considered as single-use sorbents (similar to SPE cartridges), which avoids carry-over and cross-contamination problems. In addition, some analytes show an excellent stability once they have been taken up into the silicone sorbent [25], thus samples can be processed and concentrated in situ. Thereafter, only the pieces of sorbent are transported to the laboratory for further desorption and analysis.

The aim of this study was to evaluate the suitability of sorptive extraction, using disposable silicone sorbents (DSS), for the determination of EP, EG and EC in wine samples, at concentrations below their sensorial thresholds. In-sample acetylation was considered in order to increase the affinity of the analytes for the sorbent and to improve the performance of their further GC–MS determination. Parameters affecting the efficiency of the sample preparation process are discussed and the performance of the method is compared with that reported for other microextraction approaches followed by GC–MS determination.

2. Experimental

2.1. Solvents, standards and sorbents

HPLC-grade methanol, trace analysis ethanol, acetone and ethyl acetate, sodium chloride, sodium hydroxide, potassium carbonate, potassium bicarbonate, tartaric acid and acetic anhydride were supplied by Merck (Darmstadt, Germany). Standards of EP, EG, EC and 3,4-dimethylphenol (DMP), used as internal standard (I.S.) [10,11] in the extraction process, were purchased from Aldrich (Milwaukee, WI, USA) and TCI Europe (Zwijndrecht, Belgium). Individual solutions of each compound (ca. 1000 $\mu g\,mL^{-1}$) were prepared in methanol, further dilutions and mixtures of the three ethylphenolic off-flavours were made in the same solvent. Diluted solutions of DMP were also prepared in methanol. Aqueous solutions of potassium carbonate, potassium bicarbonate and sodium hydroxide were made in ultrapure (Milli-Q) water.

Silicone sorbents were acquired from Goodfellow (Bad Nauheim, Germany) in two different formats: cord with a diameter of 1 mm and sheets with a 0.5 mm thickness. Rods (12 mm length × 1 mm diameter) were cut using a sharp blade; discs (5 mm diameter × 0.5 mm thickness) were obtained by pressing the silicone sheet with a sharp hollow punch (internal diameter 5 mm). The obtained pieces of silicone were weighed and those differing by more than 2% in weight were discarded. Those fulfilling the above requirement were soaked twice with a methanol:acetone (1:1) solution, for 15 min, and then conditioned overnight at 250 °C, before use.

Red and white wine samples were acquired from local markets. Synthetic wine was obtained by addition of tartaric acid (3.5 $g\,L^{-1}$) to 12% ethanol solutions in ultrapure water, followed by pH adjustment to 3.5 with NaOH (1 M).

2.2. Sample preparation

Extraction experiments were carried out in 22 mL volume, cylindrical glass vessels furnished with PTFE-lined septa and aluminium caps. An aliquot of wine was poured in each vessel, together with an aqueous solution of potassium carbonate or bicarbonate, a PTFE-covered magnetic stirrer (6 mm × 3 mm) and a certain volume of acetic anhydride. The silicone sorbent (rod or disc) was fixed to the septum of the vessel with a stainless steel pin (i.d. 0.4 mm) and immersed into the liquid sample or suspended in the headspace (HS) of the vessel, depending on the selected conditions. Normally, extractions were performed at room temperature (20 ± 2 °C); however, some assays were carried out at higher values, using a water bath to control the temperature of sample vessels. After finishing the extraction step, the pieces of silicone were rinsed with ultrapure water, dried with a lint free tissue and analytes were recovered with a small volume of ethyl acetate. Discs were desorbed in a GC autosampler vial (2 mL). Rods were first introduced into a glass insert (0.3 mL) and then desorbed with the same solvent.

Under optimized conditions, 2 mL of wine were transferred to extraction vessels containing 2 g of sodium chloride, 15 mL of potassium bicarbonate (5%, m/v) and a magnetic stirrer. After homogenization of the mixture, 90 μL of acetic anhydride was added. Analytes were in situ acetylated and extracted with a silicone disc exposed directly to the stirred sample, for 2 h at room temperature. Desorption was also performed at room temperature, for 15 min with 0.2 mL of ethyl acetate. After this period, the silicone disc, still attached to a piece of stainless steel, was removed with tweezers, and the ethyl acetate extract injected directly in the GC–MS system. Further details describing the handling of silicone sorbents have been reported in a previous work [18].

2.3. GC–MS determination

Analytes were determined by GC–MS, using a Varian (Walnut Creek, CA, USA) 450 GC instrument connected to an ion-trap Varian 240 mass spectrometer (MS), furnished with an electron impact (EI) ionization source in the external configuration mode. Separations were carried out in an Agilent (Wilmington, DE, USA) HP-5 ms 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 pulsed splitless mode (25 psi, 1.1 min), with the solenoid valve switched to the split position after 1 min. Transfer line, ion source and trap temperatures were set at 290, 200 and 150 °C, respectively. The helium damping gas flow was set at 2.5 $mL\,min^{-1}$.

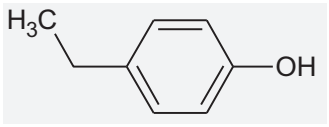
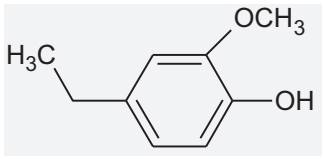
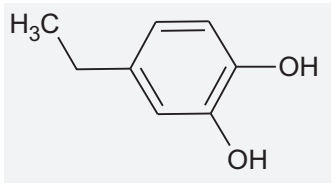
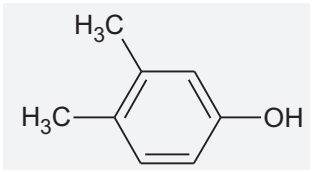
The mass spectrometer was operated in the electron impact ionization mode (70 eV) with a filament emission current of 50 μA. MS spectra were acquired in the *m/z* range between 80 and 400 a.m.u. The electromultiplier voltage was set at 1700 V. The sum of responses for the two most intense ions in the 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.

3. Results and discussion

3.1. Preliminary extractions

The analytes involved in this research (EP, EG and EC) were of high to medium polarity ($\log K_{ow}$ values from 1.87 to 2.47 units) and thus had a limited affinity for silicone. In addition, the ethanol content of wine increases their solubility reducing even more their

Table 1
Structures, CAS numbers and relevant properties of selected compounds.

Abbreviation	Structure	CAS	Log K_{ow}	pK_a
EP		123-07-9	2.47	10.2
EG		2785-89-9	2.18	10.3
EC		1124-39-6	1.87	9.7
DMP (I.S.)		95-65-8	2.40	10.4

partitioning between the non-polar sorbent and the sample. Moreover, phenols are difficult to determine using GC columns coated with non-polar stationary phases. This problem is particularly relevant for EC, which contains two hydroxyl moieties in its structure, Table 1. Therefore, in situ acetylation was used in order to improve the performance of extraction and determination steps. In practise, this means that the parameters affecting the yield of both steps have to be considered simultaneously.

Preliminary extractions were carried out in order to obtain an initial overview of the effects of the sampling mode (direct versus HS) and the base used as catalyst of the acetylation reaction (potassium carbonate versus bicarbonate, both 5%, m/v) on the efficiency of the sample preparation process. Assays were accomplished with 1 mL aliquots of a red wine sample previously spiked with target compounds (500 ng mL^{-1}) and diluted with 8 mL of the selected base aqueous solution (5%, m/v). The volume of acetic anhydride and the sampling time were 100 μL and 1 h, respectively. A silicone disc was used as extraction device and it was subsequently desorbed with ethyl acetate. EC was detected in the sorbent extracts only when potassium bicarbonate was used as catalyst of the acetylation reaction, but not in the presence of potassium carbonate. On the other hand, responses obtained in the HS mode, at room temperature and 60 °C, remained far below of those measured by dipping the silicone disc into the diluted wine sample. Consequently, direct exposure was adopted as the sampling mode; moreover, extractions were carried out at room temperature (1) to simplify the setup of the extraction process, and (2) to prevent changes in the composition of wine during extraction. In further experiments, in order to reduce the HS volume in the extraction vessel, wine and potassium bicarbonate volumes were up-scaled to 2 and 15 mL, respectively.

The effects of the volume of ethyl acetate used for desorption of the analytes and the duration of this step were further evaluated. The first variable was investigated between 15 and 60 min. Within this range, the desorption time did not affect the response of target analytes and, therefore, it was set to 15 min. Silicone pieces were desorbed twice with 0.2 mL or 0.1 mL of ethyl acetate. For the larger volume of solvent, the relative responses in the second desorption represented less than 3% of those measured in the first one; whereas, values around 10% were obtained with 0.1 mL of ethyl acetate. Thus, 0.2 mL of ethyl acetate was adopted as working value for the elution solvent volume. Under the above conditions desorption was not quantitative; however, as silicone discs were considered as single-use sorbents, there was not risk of cross-contamination problems.

Fig. 1 compares the responses (peak areas, without internal standard correction) obtained using silicone sorbents in two different formats (discs and rods), exposed to spiked aliquots of red wine for 2 h. Both formats have similar volumes and surfaces (ca. 10 μL and 39 mm^2, respectively); however, under the conditions used, discs rendered higher responses than rods. This suggests that not only the total volume and surface, but also the format of the sorbent affects the performance of the extraction process. Obviously, discs were therefore used as extraction devices.

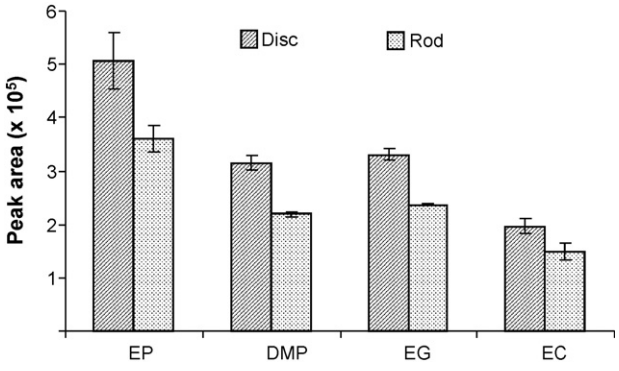


Fig. 1. Responses (peak areas) provided by silicone rods and discs for 2 mL aliquots of spiked (500 ng mL^{-1}) red wine. Direct extraction at room temperature for 2 h, $n = 3$ replicates. EP, 4-ethylphenol; DMP, 3,4-dimethylphenol; EG, 4-ethylguaiacol; EC, 4-ethylcathecol.

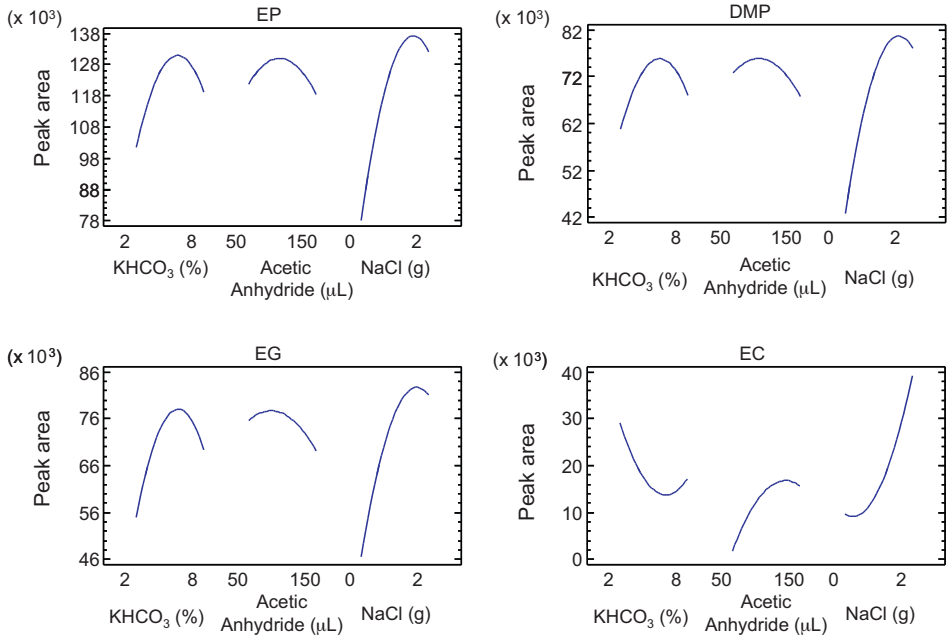


Fig. 2. Main effect graphs corresponding to the Box-Behnken experimental factorial design.

3.2. Ionic strength, acetic anhydride volume and potassium bicarbonate concentration

The influence of these parameters on the efficiency of the sample preparation process was simultaneously investigated using a Box-Behnken type, experimental factorial design with each variable considered at three levels (0, 1 and 2 g for the amount of NaCl; 50, 100 and 150 μL as acetic anhydride volume; 2%, 5% and 8% as the concentration of $KHCO_3$). The above design involved a total of 15 experiments, including a triplicate of the central point, and it allows estimating the main effects associated with each experimental factor, their quadratic terms, and the two-factor interactions. The experimental domain of the design was selected on the basis of the previous results reported by Carrillo and Tena [10], using SPME as the extraction technique. Graphs showing the standardized main effects for target compounds, and the internal standard, are shown in Fig. 2. The length of depicted lines is proportional to the variation in the response obtained for each compound when the corresponding factor varied within the domain of the design. As observed, increasing the ionic strength (mass of NaCl in the extraction vessel) favoured the efficiency of the extraction for all species. Analysis of variance (ANOVA) of responses obtained in the experiments involved in the Box-Behnken design demonstrated that the amount of NaCl was the only variable with a statistical significant effect (95% confidence level) on the extraction efficiency; whereas, the concentration of $KHCO_3$ and the volume of acetic anhydride did not achieve the threshold of statistical significance, data not given. As shown in Fig. 2, most compounds were better extracted when the above variables were set at the medium level of the design. The exception was EC, which preferred low $KHCO_3$ concentrations and high acetic anhydride volumes. Two-factor interactions remained below the level of statistical significance at the 95% confidence level. The best compromise conditions, which maximized the efficiency of the extraction for all compounds, were calculated with a global desirability (D) function. This function is defined as the geometric mean of the normalized (between 0 for the minimum and 1 for the maximum) predicted responses (d_i) for each compound, by the Box-Behnken design. The maximum value of D (0.70) was obtained for 2 g of NaCl, 90 μL of acetic anhydride and 5% (m/v) of $KHCO_3$, Fig. 3.

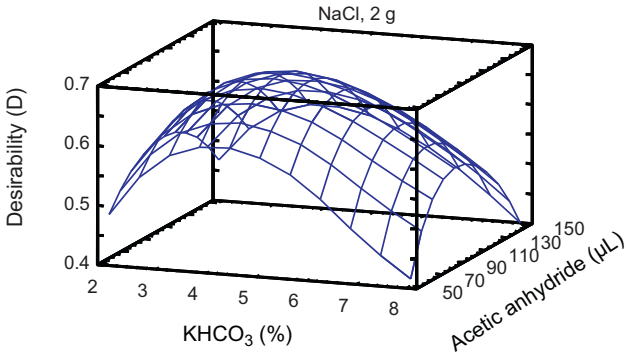


Fig. 3. Plots of the global desirability function.

3.3. Stirring and extraction kinetics

Stirring favours migration of the analytes from the sample in the extraction vessel to the interface with the silicone disc; thus, it is expected to improve the efficiency of the extraction under non-equilibrium conditions. Considering a sampling time of 2 h, 10-fold higher responses were measured for stirred samples versus non-stirred ones; however, no differences were noticed working at three different stirring speeds: 300, 900 and 1500 rpm. The same behaviour has been reported for PDMS coated stir bars [13]. A working value of 900 rpm was adopted for this variable.

The time-course of the sorptive extraction at room temperature was investigated with spiked aliquots (2 mL) of red wine, considering sampling times between 10 min and 8 h. As normally occurs with sorptive techniques, the extraction process showed a slow kinetics [20]. In the case of EC, 2 h of sampling was enough to obtain equilibrium conditions, whereas the other two analytes and the I.S. required 4 h, Fig. 4. It seems that, even after in situ acetylation, EC remains as the most polar species and therefore migrates more rapidly from the sample to the silicone disc. In this study, the sampling time was limited to 2 h in order to increase the sample throughput, assuming the corresponding loss of efficiency for EP and EG.

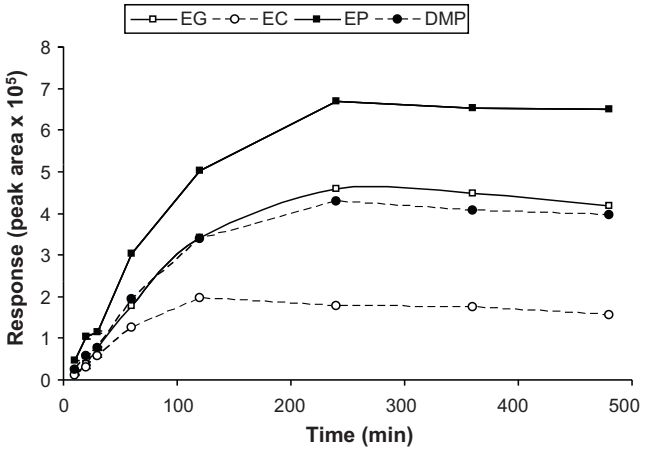


Fig. 4. Kinetics of sorptive extraction. Direct sampling, at room temperature.

3.4. Performance of the method

Table 2 summarizes some data related to the linearity and the precision of the proposed method. The linearity was evaluated with a young red wine (*Mencia* variety), which did not contain detectable levels of target analytes. Aliquots of this matrix were spiked at eight different levels, prepared in duplicate, between LOQs and 5000 ng mL^{-1}. This interval covers the range of concentrations reported for target analytes in aged samples of red wines [10]. The concentration of the I.S. was maintained at 200 ng mL^{-1}. The plots of the ratios between the peak areas measured for each compound and the I.S. versus their concentrations in the wine sample fitted a linear model, with determination coefficients (R^2) higher than 0.997, Table 2. Precision was investigated with spiked samples of red and white wines processed on the same day (repeatability) and on different days (reproducibility). The relative standard deviations (RSDs) of the corrected responses (peak area divided by the I.S. peak area) ranged from 2% to 9%, Table 2. On the other hand, RSDs up to 16% were obtained before I.S. correction, data not shown.

Procedural blanks, corresponding to synthetic wine solution, demonstrated the absence of contamination problems; thus, the limits of quantification of the method, defined for a S/N ratio of 10, were controlled by the efficiency of the sample preparation process and the sensitivity of the GC–MS instrument. Achieved LOQs, estimated for S/N ratios of chromatographic peaks in the lower levels of the linearity study, ranged from 5 to 15 ng mL^{-1}, depending on the particular analyte, Table 2. These values are similar to or lower than those obtained for EP and EG with SBSE followed by thermal desorption and GC–MS determination [11–13], HS-SPME with in situ derivatization [10] and dispersive liquid–liquid microextraction (DLLME) [26], Table 3. Thermal desorption of PDMS coated stir bars allows transferring the whole entire extracted amount of each compound to the GC column; thus, the achieved LOQs are expected to be much lower than those attained in this work

Table 2
Linearity, precision and LOQs of the proposed method.

Analyte	Linearity (LOQs–5000 ng mL^{-1})			Precision (RSDs, %)						LOQs (ng mL^{-1})
	Slope (SD)	Intercept (SD)	R^2	Intra-day (n = 4 replicates)				Inter-day (n = 9 replicates, 3 days)		
				Red wine		White wine		Red wine	White wine	
				100 ng mL^{-1} [a]	500 ng mL^{-1} [a]	100 ng mL^{-1} [a]	500 ng mL^{-1} [a]	200 ng mL^{-1} [a]	200 ng mL^{-1} [a]	
EP	0.0046 (4E-5)	0.01 (0.04)	0.998	3.8	6.0	8.9	4.5	2.4	1.9	5
EG	0.0032 (4E-5)	−0.01 (0.05)	0.998	4.2	5.3	6.0	6.9	4.5	8.6	5
EC	0.0019 (9E-6)	0.026 (0.009)	0.997	6.6	7.5	6.8	5.0	6.2	6.5	15

[a] Added concentration.
SD, standard deviation.

Table 3
Summary of sample preparation conditions and LOQs reported for ethylphenolic off-flavours in wine samples using different microextraction techniques followed by GC–MS determination.

Technique	Wine vol. (mL)	Extraction time and temperature	In situ acetylation	LOQs (ng mL^{-1})			Ref.
				EP	EG	EC	
SBSE	4	60 min, room temperature	No	21	529	n.a.	[11]
SPME	4	70 min, 70 °C	Yes	30	3	6	[10]
SBSE	10	60 min, room temperature	No	1.1	0.9	n.a.	[13]
SBSE	25	90 min, room temperature	No	21	0.1	n.a.	[12]
DLLME	5	5 min, room temperature	No	147	95	n.a.	[26]
Sorptive extraction	2	120 min, room temperature	Yes	5	5	15	This work

n.a.: not available

Table 4
Relative recoveries obtained for spiked wine samples, n = 4 replicates.

Compound	Recovery (%) ± SD			
	Added concentration			
	150 ng mL^{-1} [a]	100 ng mL^{-1} [b]	300 ng mL^{-1} [b]	1000 ng mL^{-1} [b]
EP	105 ± 2	103 ± 4	105 ± 9	108 ± 5
EG	101 ± 10	92 ± 9	116 ± 12	100 ± 4
EC	91 ± 4	103 ± 5	99 ± 9	112 ± 9

[a] White wine.
[b] Red wine.

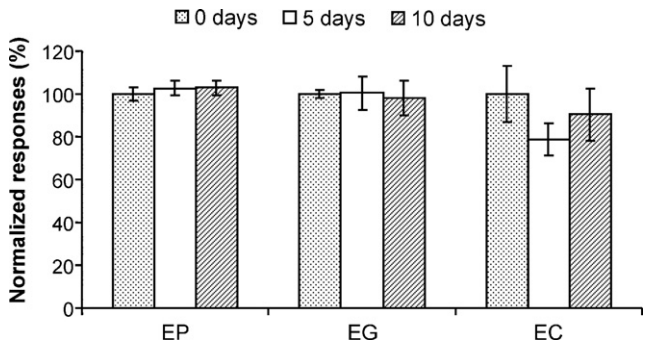


Fig. 5. Stability of acetylated species in the silicone discs stored at 4 °C. Normalized values to those measured for silicone discs desorbed immediately after the extraction step, $n = 4$ replicates.

using solvent desorption followed by injection of a fraction of the extract (1%, considering injection and desorption volumes of 2 and 200 μL, respectively) in the GC–MS system. However, on the other hand, the affinity of the polar ethylphenol species for the sorbent increases significantly after in situ acetylation, which contributes significantly to reduce the LOQs of the proposed method. Most authors have reported sensorial thresholds for ethylphenol species of between 33 ng mL^{-1} for EG and 440 ng mL^{-1} for EP [10,13]. These values are higher than the LOQs of the methodology optimized in this study.

The effect of the type of sample on the efficiency of the extraction was first evaluated by comparing the corrected responses (peak areas after subtraction of the signals obtained for non-spiked aliquots of each matrix) measured for spiked aliquots of red and white wine samples, fortified at two different concentrations. Despite considerable dilution of samples (2 mL of wine plus 15 mL of a 5% potassium bicarbonate solution), the yield of the extraction for target analytes decreased around 35% for red wines versus white ones. This may be explained by the higher complexity of the former matrix, and the significant increase in the viscosity of red wines when adjusted at basic pHs in comparison with white wines. The signal measured for DMP was reduced to a similar extent as those corresponding to the rest of species. Thus, after I.S. normalization, the use of matrix-matched standards, instead of the standard addition methodology, appears as a feasible quantification approach. In order to confirm the accuracy of the latter methodology, samples of white (*Palomino*) and red (*Grenache*) wines were spiked at different levels and quantified against spiked aliquots of another red wine (*Mencia*). The obtained relative recoveries varied between 91% and 116% (Table 4), confirming the usefulness of DMP to compensate for changes in the efficiency of the sorptive extraction, as well as the possibility of using matrix-matched standards for quantification purposes.

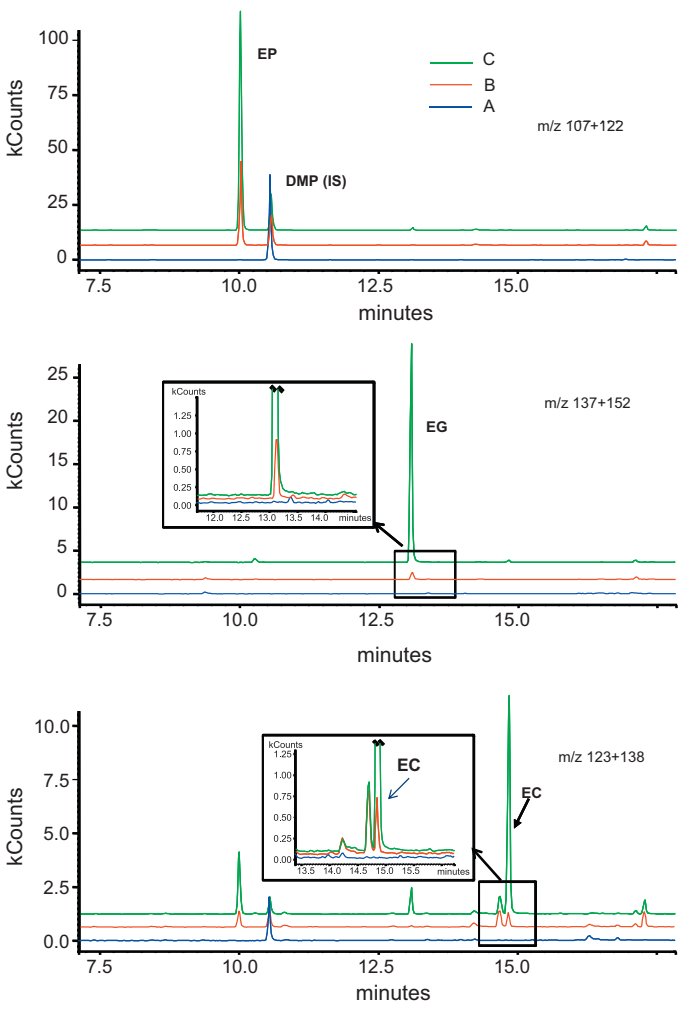


Fig. 6. Selected ion GC–MS chromatograms for a procedural blank of synthetic wine (A), sample code 3 in Table 5 (B), and same sample spiked at 300 ng mL^{-1} per compound (C). The I.S. was added to all samples at 200 ng mL^{-1}.

The combination of inexpensive extraction devices with simplified sample preparation conditions opens the possibility for in situ sample preparation, avoiding potential changes in the concentration of ethylphenol species during transport and/or storage of wine samples in the laboratory. In practise, the feasibility of this alter-

Table 5
Summary of concentrations measured in red wine samples, $n = 3$ replicates.

Code	Wood-aged	Year	Concentration (ng mL^{-1}) ± SD		
			EP	EG	EC
1	n.a.	2008	2414 ± 53	114 ± 11	292 ± 25
2	Yes	2008	1892 ± 139	171 ± 22	162 ± 18
3	Yes	2007	359 ± 13	34 ± 2	62 ± 6
4	Yes	2002	2708 ± 115	204 ± 6	344 ± 39
5	Yes	2002	212 ± 18	n.d.	n.d.
6	No	2009	n.d.	n.d.	n.d.
7	No	2008	34 ± 4	n.d.	n.d.
8	No	2009	132 ± 10	n.d.	n.d.
9	No	2009	311 ± 24	9 ± 1	n.d.
10	No	2009	191 ± 7	6 ± 1	n.d.
Sensorial threshold (ng mL^{-1})			440	33	n.a.

n.a., not available information.
n.d., below detection limits.

native is determined by the stability of the acetylated species in the extraction discs. Fig. 5 shows the normalized responses corresponding to the extracts from a spiked (300 ng mL^{-1}) wine sample. Three series of extractions ($n = 4$ replicates, each) were carried out by desorbing the silicone discs immediately after finishing the sample preparation step, after 5 and 10 days of storage in 2 mL amber vessels, at 4 °C. As observed in Fig. 5, no differences were noticed among the obtained responses.

3.5. Application to commercial wines

The proposed method was applied to samples of red and white wines, acquired in local supermarkets, from different geographic areas in Spain. None of the compounds was detected in white wines of the Albariño, Ribeiro, Rueda and Valdepeñas geographic regions. The levels measured in red wines are summarized in Table 5. The maximum concentration in all the processed samples corresponded to EP, with a level between 10 and 20 times higher than that of EG. The latter species was found at similar concentrations to EC. Overall, the above ratios matched with those reported for Rioja and other red wines form different countries aged in wood containers [10,13]. Fig. 6 shows the GC–MS chromatograms corresponding to a procedural blank, a red wine sample (code 3, Table 5) and same sample fortified with target species at 300 ng mL^{-1}.

It is worth noting that EP was not only detected in wood-aged wines (codes 2–5, Table 5), but also in most of the young wine samples (codes 6–10, Table 5) at levels up to 310 ng mL^{-1}. On the other hand, EG and EC were only found at relevant concentrations in the first type of wines.

4. Conclusions

In-sample acetylation followed by sorptive extraction with inexpensive silicone discs was found to be an effective, low cost method for extraction of ethyl phenolic off-flavours from wine samples. When combined with GC–MS determination, the developed method provides LOQs below the sensorial threshold of target phenols, it presents a limited consumption of organic solvents, it is free of cross-contamination problems and sample preparation can be in situ performed. Moreover, the use of DMP as internal standard allows compensating for changes in the extraction efficiency among different wine samples; thus, calibration with matrix-matched standards, instead of the standard addition methodology, can be used for samples quantification. The 4-ethylphenol compound was found in most of the analyzed red wine samples, even when they had not been aged in wood barrels. On the other hand, none of the ethylphenol off-flavours was detected in white wines. Finally, EG and EC were mainly present in wood-aged wines.

Acknowledgements

This study has been supported by the Spanish Government and E.U. FEDER funds (projects CTQ2009-08377 and DE2009-0020). I.C. and M.R. thank their FPU and Isidro Parga Pondal contracts to the Spanish Ministry of Science and Innovation and the Xunta de Galicia Government, respectively.

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