



Optimisation of headspace solid-phase microextraction for the analysis of volatile phenols in wine

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Received 1 October 2002; received in revised form 12 March 2003; accepted 25 March 2003

Abstract

Headspace solid-phase microextraction has been applied to the analysis of volatile phenols in wine. Silica fibre coated with Carbowax–divinylbenzene was found to be more efficient at extracting these compounds than other fibres such as those coated with polydimethylsiloxane, polyacrylate, carboxen–polydimethylsiloxane, and polydimethylsiloxane–divinylbenzene. Different parameters such as extraction time, temperature of the sample during the extraction, ionic strength and sample volume were optimised using a two-level factorial design expanded further to a central composite design, in order to evaluate several possibly influential and/or interacting factors. The headspace (HS)-SPME procedure developed shows adequate detection and quantitation limits, and linear ranges for correctly analysing these compounds in wine. The recoveries obtained were close to 100%, with repeatability values lower than 16%. The method was applied to a variety of white and red wines. © 2003 Elsevier Science B.V. All rights reserved.

Keywords: Wine; Headspace solid-phase microextraction; Phenols

1. Introduction

Volatile phenols represent a large family of substances, some of which possess a strong odorous activity which can influence the aroma of numerous fermented beverages. These compounds are considered part of the aroma composition of wines. These are principally vinylphenols in white wines and ethylphenols in red wines [1]. Some of these compounds can produce unpleasant odours, affecting negatively the quality of the wine. Ethylphenols

(4-ethylphenol and 4-ethylguaiacol) are responsible for animal and smoky odours [2], while vinylphenols (4-vinylphenol and 4-vinylguaiacol) can be responsible for heavy pharmaceutical odours [3]. Vinylphenols are produced by yeasts of the genus *Brettanomyces/Dekkera*, through decarboxylation of *trans* ferulic and *trans p*-coumaric acids. Later, vinylphenols are transformed by reduction reactions into ethylphenols [4,5]. So, it is important for winemakers to control the concentrations of these compounds in their wines.

The analysis of volatile phenols is carried out by gas chromatography after a previous extraction and concentration stage. De Santis et al. [6] determined the volatile phenols of “Aleatico di Gradoli Riserva”

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red liqueur wines by SPE using 100 ml of wine. The extracts obtained were collected and diluted in 0.5 ml of 1:1 diethyl ether/*n*-hexane.

Chatonnet et al., [5] employed a liquid–liquid extraction by dichloromethane to measure this type of compound in wine. 4-ethylphenol and 4-ethylguaiaicol were quantified in wine employing gas chromatography–mass spectrometry after a liquid–liquid extraction with pentane–diethyl ether and using [²H₄]4-ethylphenol as internal standard [7].

As can be seen, there are several methods, mainly by liquid–liquid or solid-phase extraction, for determining volatile phenols in wine [3,5,7–9].

All these sample preparation methods present several disadvantages, such as excessive cost, volume of sample and time, the possible generation of artefacts, etc.

A preparation method, solid-phase microextraction (SPME) has recently been developed [10,11]. This rapid and inexpensive sample preparation method has been used routinely in combination with GC and GC–MS, and successfully applied to a wide variety of compounds, in particular for the extraction of volatile organic compounds from environmental, biological and food samples [12–14].

Two types of SPME techniques can be used: headspace (HS)-SPME and direct immersion (DI)-SPME. However, when direct SPME is applied to a variety of matrices, several problems may arise such as irreversible adsorption of major components leading to fibre deterioration [15,16].

The HS-SPME has already been used to determine ethylphenols in red wine, but it has not yet been optimised for vinylphenols in white and red wine [17].

The most important parameters affecting the SPME method are: type of fibre employed, extraction temperature and time, salt concentration and sample volume [18].

The purpose of the work reported here is to optimise the conditions of SPME for detection and quantification of volatile phenols in wine using a chemometric approach. After selecting the type of fibre, the effects of experimental parameters such as sample volume and temperature, sampling time and NaCl concentration on the SPME of these compounds were evaluated using a two-level factorial design expanded further to a central composite

design. This chemometric approach allows the simultaneous variation of all experimental factors studied, and the distinguishing of interactions among them [19].

After this, validation of the analytical method based on HS-SPME–GC for the analysis of these compounds was carried out.

2. Experimental

2.1. Samples

Individual stock standard solutions of each volatile phenol were prepared by weight in a model wine solution (Milli-Q water containing 15% (v/v) ethanol and 3 g/l tartaric acid).

Working solutions used in further studies were prepared by diluting different amounts of each stock standard solution in the model wine solution.

All these solutions were stored at 4 °C.

After validation, the method was applied to various white and red wine samples supplied by different producers.

2.2. Chemicals and reagents

All standards used in this study were supplied by Sigma–Aldrich (St Louis, MO, USA). 3,4-Dimethylphenol was employed as internal standard. NaCl was purchased from Scharlau (Barcelona, Spain).

2.3. SPME

2.3.1. Fibre screening

Before carrying out the optimization of the conditions of SPME for detection and quantification of volatile phenols, a fibre screening was carried out.

The silica fibres used in this study were purchased from Supelco (Bellefonte, PA, USA). Seven fibres were tested and compared: polydimethylsiloxane (PDMS, 100 μm), carboxen–polydimethylsiloxane (CAR–PDMS, 75 μm and 85 μm), StableFlex Carbowax–divinylbenzene (CW–DVB, 70 μm), polyacrylate (PA, 85 μm), polydimethylsiloxane–divinylbenzene (PDMS–DVB, 65 μm), and Stableflex

divinylbenzene–carboxen–polydimethylsiloxane (DVB–CAR–PDMS, 50/30 μm).

A 15-ml aliquot of a model wine solution spiked with the volatile phenols considered in this study were extracted, in triplicate, with these fibres using the HS-SPME mode. HS-SPME was carried out under magnetic stirring. The samples were saturated with NaCl, 6.14 g for this process of fibre screening. Before the extraction, the sample vials were equilibrated for 5 min at the extraction temperature, 50 °C. The sampling time was 30 min.

Each fibre was conditioned prior to use according to the supplier's instructions by inserting them into the GC injector.

2.3.2. Optimization. Experimental design

A two-level factorial design expanded further to a central composite design was used to determine the optimum experimental conditions for analysing volatile phenols in wine by SPME–GC using a CW–DVB fibre.

A sequential exploration of the response was chosen, carried out in two stages. In the first stage, we wished to establish the relative influence of the factors and their interactions on the chromatographic area obtained for each volatile phenol. Four factors were selected as potentially affecting the SPME efficiency: temperature and time of extraction, sample volume and ionic strength effect from adding different amounts of NaCl.

Consequently, a factorial design of 2^4 was selected. This design involves 16 experiments undertaken in random order to provide protection against the effects of hidden variables.

After this, the two-level factorial design was expanded to a star design. A central composite design (CCD, with $\alpha=1.287$) was obtained, since the centres of the two separate designs were coincidental. The values corresponding to the high (+),

low (–), centre, and axial points for each factor are shown in Table 1.

For data manipulation, the Statgraphics statistical computer package “Statgraphics Plus 5.0” for Windows 98 was used.

Each experiment was carried out in triplicate and with constant magnetic stirring because this has a positive effect on the analyte transference to the coated fibre [20].

2.3.3. Optimised headspace-SPME procedure

After optimisation, and for each SPME analysis, 12 ml of sample (natural or synthetic wine) was pipetted and placed into a 50-ml glass vial with 2.81 g of NaCl (so NaCl concentration used was 4 M). Each sample was spiked with 14 μl of a solution of 3,4-dimethylphenol (1.53 g/l in the model wine solution). A small magnetic stirring bar was also added. The vial was tightly capped with a PTFE-faced silicone septum and placed in a thermostatted block on a stirrer. After 5 min at 60 °C, the CW–DVB fibre was exposed to the headspace of the sample for 50 min. During this time, the sample was stirred at constant speed. After completion of sampling, the fibre was removed from the sample vial and inserted into the injection port of the GC.

2.4. Chromatography

The samples were analysed using a GC 8000 chromatograph with a FID detector (Fisons Instruments, Milan, Italy).

The injection was made in the splitless mode for 2 min. For the desorption of the analytes inside the GC injection port, the temperature employed was the conditioning one recommended by the supplier.

The GC was equipped with a DB–Wax capillary column (J&W Scientific, Folsom, CA, USA), 60 m \times 0.25 mm I.D., with a 0.25 μm coating. The

Table 1
Factor levels used for the optimisation of experimental conditions

Factor	Low (–)	High (+)	Centre	Axial ($-\alpha$)	Axial ($+\alpha$)
Sample volume (ml)	15	35	25	12.13	37.87
Sampling temperature (°C)	25	70	47.5	18.54	76.46
Sampling time (min)	15	60	37.5	8.54	66.46
NaCl (M)	3	6			

carrier gas was helium at a flow-rate of 1.1 ml/min. The detector temperature was 250 °C. The GC oven was programmed as follows: held at 35 °C for 10 min, then ramped at 5 °C/min to 100 °C. Then it was raised to 210 °C at 3 °C/min and held for 40 min.

The compounds were identified by mass spectrometric analysis. In these analyses, the same GC coupled to a MD 800 mass detector (Fisons Instruments, Milan, Italy) was used. The mass detector operated in EI+ mode at 70 eV in a range of 30–450 a.m.u. GC analytical conditions were the same as described above.

The signal was recorded and processed with Masslab software supplied with the Wiley 6.0 MS library. Peak identification was carried out by analogy of mass spectra and confirmed by retention indices of standards. Quantitative data from the identified compounds were obtained by measuring the relative peak area in relation to that of 3,4-dimethylphenol, the internal standard.

3. Results and discussion

3.1. Fibre screening

The results of the fibre screening showed that the highest responses for the volatile compounds studied were attained when using Stableflex CW–DVB and PA fibres (Table 2). For vinylphenols, the chromatographic responses obtained were better when a CW–DVB fibre was used, so this type of fibre was selected for the method optimisation.

3.2. SPME parameter optimisation

3.2.1. Screening by a 2⁴ factorial design

This first design was used to detect those variables presenting the greatest influence on the chromatographic area obtained for each volatile phenol.

The data obtained were evaluated by ANOVA at the 5% significance level. These results can be shown by bar charts (Fig. 1).

Extraction temperature was, in general, the most important parameter (at $P < 0.05$), with a positive effect, for all the volatile phenols studied. Monje et al. [17] found that, using a 85 µm polyacrylate fibre, the peak areas of 4-ethylguaiaicol and 4-ethylphenol increased with sample temperature. In the case of 4-ethylguaiaicol, the highest peak area was observed at 55 °C, decreasing at temperatures higher than 55 °C.

They explained this on the basis of the difference between the boiling points of the two compounds (99 °C for 4-ethylguaiaicol and 213 °C for 4-ethylphenol). 4-Vinylguaiaicol and 4-vinylphenol have higher boiling points than ethylphenols, so this would explain the fact that the extraction efficiency for these analytes, in our case, increases with the extraction temperature.

The next most influential factors were sampling time, with a positive effect, and sample volume, with a negative effect on the four volatile phenols. NaCl was only significant for 4-ethylphenol. For ethylphenols, this factor has a positive effect, whereas for vinylphenols its effect is negative, that is, as the NaCl concentration increases, the chromatographic responses obtained for these last compounds are lower.

Table 2
Peak areas (mean values) obtained for each volatile phenol

Fibre	Volatile phenol			
	4-Ethylguaiaicol	4-Ethylphenol	4-Vinylguaiaicol	4-Vinylphenol
PDMS	485 197	251 173	175 730	22 988
CW–DVB	2 156 034	2 148 226	1 208 702	194 212
PDMS–DVB	1 844 689	1 296 882	1 110 201	157 633
CAR–PDMS (75 µm)	666 752	1 053 688	353 260	138 082
CAR–PDMS (85 µm)	2 076 687	2 001 038	599 154	148 515
PA	2 685 822	2 214 656	1 152 416	165 941
DVD–CAR–PDMS	2 571 892	1 743 823	659 730	110 098

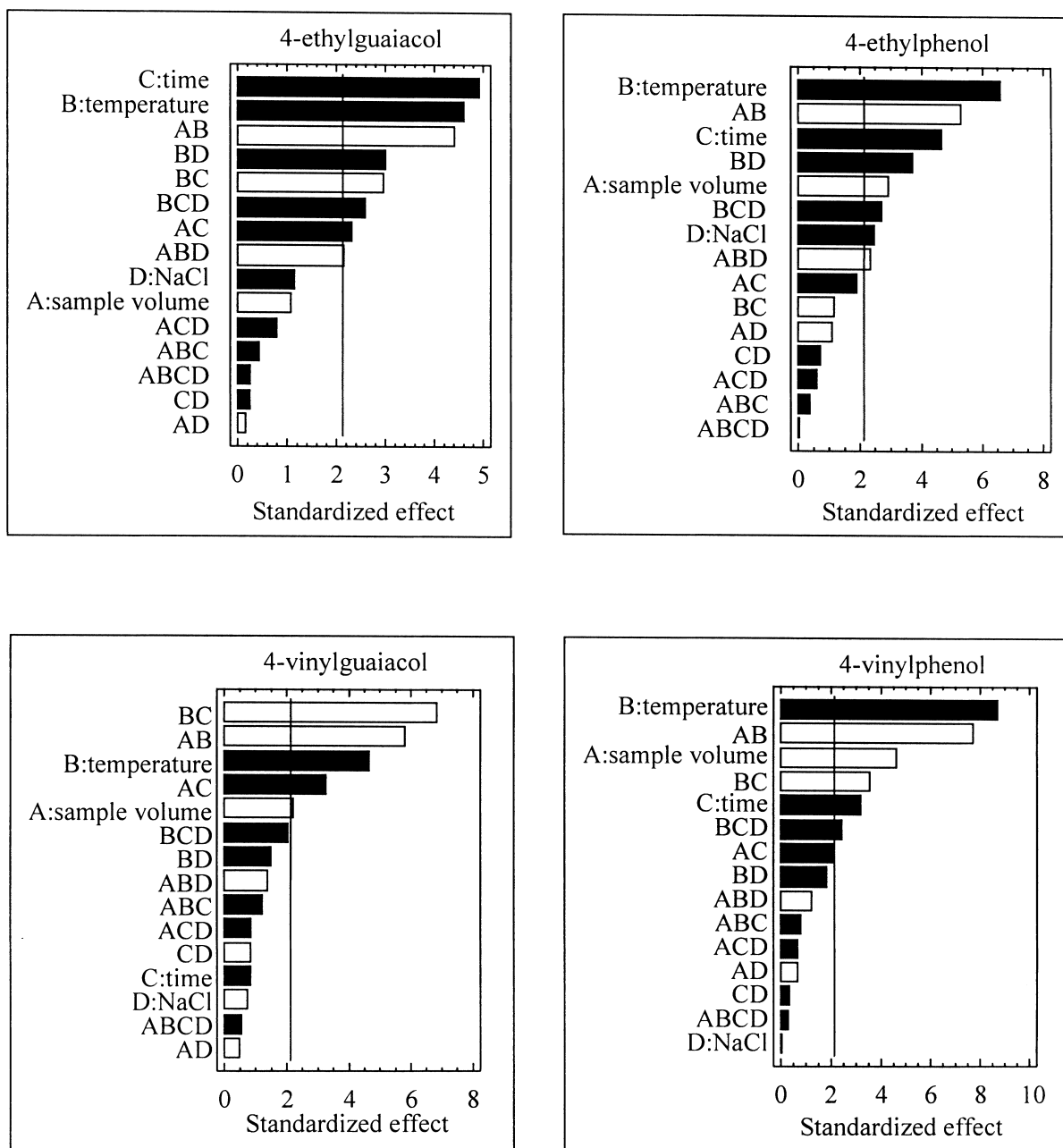


Fig. 1. Pareto chart of the main effects in the factorial 2^4 design for the volatile phenols studied: ■, positive effect; □, negative effect.

The SPME efficiency is also affected by the interrelated variables, as shown in Fig. 1. The interaction between the factors sample volume and extraction temperature appears statistically significant, with a negative effect for all the compounds

studied. For vinylphenols, the temperature–sampling time interaction has also a significant negative effect.

3.2.2. Optimisation by a central composite design

As can be seen, the SPME technique for analysing

volatile phenols is significantly affected by interrelated parameters. For an optimisation design, it is advisable to keep the number of parameters as small as possible and bearing in mind that the NaCl concentration was not shown to have a significant influence on the chromatographic responses obtained for the compounds studied with the exception of 4-ethylphenol, in order to estimate curvature in response surfaces, we decided not to keep this factor. For the central composite design (CCD), the three parameters used were: sampling temperature and time, and sample volume. The axial values for the considered parameters are located on a sphere surrounding the two-level factorial design (Table 1). The NaCl concentration was set at 4 M, taking into account the fact that the initial values selected for this parameter were 3 and 6 M and that its effect was positive for ethylphenols and negative for vinylphenols (Fig. 1).

After applying the CCD, the sample temperature and the sampling time appeared as statistically significant main effects for 4-ethylguaiaicol and 4-vinylphenol, having a strong positive influence (Table 3).

Sample volume only showed a significant negative influence on 4-vinylphenol. For all the compounds studied, the interaction between sample volume and extraction temperature was statistically significant. Fig. 2 shows the response surface plots for the volatile phenols considered in this study, obtained by plotting sample volume versus extraction temperature.

The influence of extraction temperature depends on the values of sample volume. At low sample volume (15 ml), better experimental responses were obtained as extraction temperature increased. At high sample volume (35 ml), poorer and more curved responses were obtained.

Extraction increases as the headspace volume decreases [21]. Zhang et al. [22] pointed out that an increase in sampling temperature increases the headspace concentration of aroma compounds, favouring their extraction, but that an excessively high temperature decreases the fibre/headspace partition coefficient, reducing it. In our case, for low sample volumes, only slight decreases in the chromatographic responses were obtained at high temperature.

In summary, after evaluation of the main factors and their interactions, the experimental results showed that the optimum conditions, within the range studied, to obtain the best extraction of the four volatile phenols considered were 60 °C for temperature, 50 min for sampling time, 4 M for NaCl concentration, and 12 ml for sample volume.

3.3. Performance characteristics

3.3.1. Calibration. Linearity

Five levels of concentration for each volatile phenol were tested in triplicate, covering the concentration ranges expected.

The [volatile phenol/internal standard] peak area ratio was used for each compound. The ranges of linearity studied are shown in Table 4. Excellent

Table 3
Main effects and interactions in the central composite design for ethylphenols and vinylphenols

Effect	4-Ethylguaiaicol		4-Ethylphenol		4-Vinylguaiaicol		4-Vinylphenol	
	F ratio	P-value	F ratio	P Value	F ratio	P-value	F ratio	P-value
A: sample volume	1.29	0.2696	0.22	0.6460	2.51	0.1284	6.80	0.0164*
B: temperature	9.72	0.0052*	0.02	0.8780	3.03	0.0964	11.85	0.0024*
C: time	12.14	0.0022*	6.91	0.0157*	2.64	0.1191	7.53	0.0121*
AA	0.12	0.7369	0.20	0.6611	0.01	0.9314	0.80	0.3824
AB	8.02	0.0100*	6.89	0.0158*	6.10	0.0221*	10.93	0.0034*
AC	1.79	0.1946	3.47	0.0764	2.00	0.1717	1.01	0.3254
BB	14.77	0.0009*	0.46	0.5054	17.45	0.0004*	11.29	0.0030*
BC	0.03	0.8711	1.61	0.2184	2.74	0.1127	0.17	0.6854
CC	0.69	0.4157	0.22	0.6429	0.98	0.3326	0.41	0.5270

*Values are significant at $P < 0.05$.

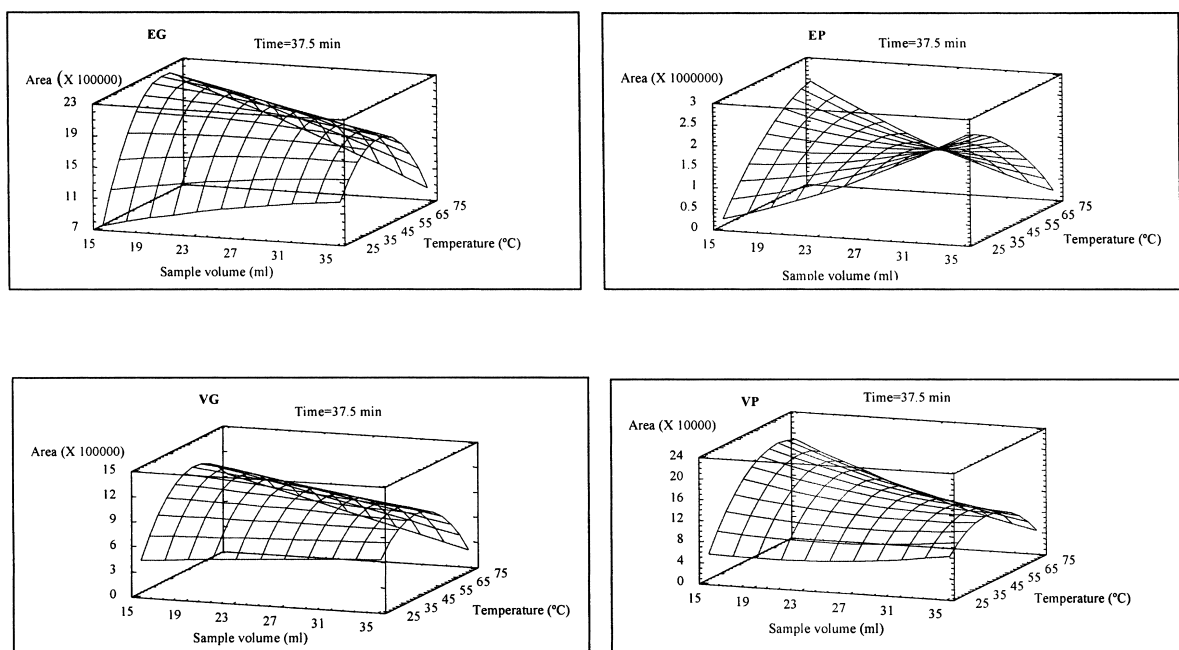


Fig. 2. Estimated response surfaces obtained using the central composite design by plotting temperature versus sample volume. EG, 4-ethylguaiaicol; EP, 4-ethylphenol; VG, 4-vinylguaiaicol; VP, 4-vinylphenol.

linearity was obtained for all volatile phenols ($r > 0.99$). It was also corroborated by the “on-line linearity (LOL)” [23], with values higher than 98% (Table 4). This parameter is determined by the following equation in which RSD(b) is the relative standard deviation of the slope (expressed as a percentage).

$$\text{LOL} (\%) = 100 - \text{RSD}(b)$$

3.3.2. Detection and quantitation limits and recovery

Detection and quantitation limits (Table 4) were calculated from the calibration curves constructed for

Table 4
Calibration curves and performance characteristics

	EG	EP	VG	VP
Linear range (mg/l)	0.015–3.011	0.017–3.041	0.050–3.144	0.048–3.853
r	0.9991	0.9989	0.9995	0.9990
Linearity (LOL; %)	98.95	98.97	99.21	99.83
Slope \pm SD	0.396 \pm 0.0041	0.837 \pm 0.0086	0.124 \pm 0.0020	0.018 \pm 0.0000
Intercept \pm SD	0.029 \pm 0.0055	0.202 \pm 0.0115	–0.023 \pm 0.0004	–0.000 \pm 0.0001
Detection limit (mg/l)	0.018	0.019	0.015	0.005
Quantitation limit (mg/l)	0.080	0.081	0.068	0.015
Recovery (%)				
White wine	112.6	82.1	115.8	114.1
Red wine	109.3	91.6	118.1	80.79

each volatile phenol, using the Alamin computer program [24].

The limits are estimated by extrapolating to zero concentration from the calibration curve, using the relative standard deviation of the analytical signal corresponding to a zero concentration value. In this way, these limits are calculated as three and 10 times, respectively, the relative standard deviation of the analytical blank values obtained from the calibration curve. In our case, the values obtained are low enough to permit the determination of these compounds in real wine samples (Table 4).

The technique of standard addition was used to check the accuracy of this analytical method. A representative sample of white wine and another of red wine were taken as matrices and known quantities of each standard solution were added at five levels and in triplicate. The slopes of the lines thus obtained for each of the volatile phenols were compared with the corresponding slopes obtained in the calibration with standards (*t* criterion). No significant differences were found between them at a significance level of 5%. Table 4 gives the data for the recovery of each compound, determined by the slope of the line plotting the concentration found against the concentration expected. Recoveries near 100% were obtained for all the volatile phenols. In wines, the ethanol content appears to interfere in the SPME technique but, for quantitative analysis, the compound area/internal standard area ratios may be used [25,26]. Whiton and Zoeklein [26] observed that the recoveries of 4-ethylphenol and 4-ethylguaiaicol were poor when they were extracted using a 65- μ m Carbowax–divinylbenzene coating for 30 min at room temperature. They suggested that components of the wine other than ethanol, possibly polyphenolic compounds, could alter the SPME extraction efficiency.

In relation to other extraction techniques, López et al. [27] developed a method for the quantitative determination of minor and trace volatile compounds in wine by SPE and GC with mass spectrometric detection. In it, the elution is carried out with dichloromethane. In this case, the recoveries for volatile phenols were, in general, higher than 90% (81% for 4-vinylphenol).

In our case, the results obtained demonstrate that the sample matrix does not interfere in the HS-SPME

for the analysis of volatile phenols in wine when a longer sampling time and a high temperature are used. Similar recoveries to other analytical techniques [27] are obtained in this case.

3.3.3. Repeatability and reproducibility

Fibre to fibre variation has been recognised as a problem in quantitative analysis using SPME. Yang et al. [28] found significant fibre to fibre variations when they employed a CW–DVB fibre to analyse volatile components in tobacco. Here, the repeatability and reproducibility have been evaluated by means of three sets of five extractions of a global standard solution using different fibres. The measurements ([analyte/internal standard] peak area ratio, $n=5$) were found to be repeatable with RSD values of 4–16%. The inter-fibre accuracy showed RSD values slightly higher than intra-fibre accuracy (5.7–17%). In comparison with other isolation analytical techniques, López et al. [27], using SPE, found reproducibility values below 10% for most of the volatile compounds considered, but poor RSD values were obtained for 4-vinylphenol and 4-vinylguaiaicol. Monje et al. [17] compared the efficiency of HS-SPME with a liquid–liquid extraction method for the quantitation of 4-ethylphenol and 4-ethylguaiaicol in red wine. At lowest concentrations, both compounds exhibited lower RSD values when they were isolated by SPME. In addition the HS-SPME technique demonstrated that it was a more efficient procedure to concentrate these compounds. However, other authors, using an extraction with pentane–diethyl ether obtained RSD values lower than 3% for 4-ethylphenol and 4-ethylguaiaicol [7].

As can be seen, in our case, the SPME method generates repeatable and reproducible results, similar to other analytical techniques.

3.4. Determination of volatile phenols in wines

The volatile phenol content of various white and red wines from different wineries and different vintages was quantified using the optimised SPME method. The analyses were carried out in duplicate.

The mean results obtained are shown in Table 5. These values agree with the results found in the literature [5,27,29,30] and, in most cases, the concentrations obtained lie within the calibrated inter-

Table 5

Volatile phenols (mean values, mg/l) found in white (samples 1–5) and red (samples 6–10) wines

Wine	Volatile phenol			
	4-Ethylguaiaicol	4-Ethylphenol	4-Vinylguaiaicol	4-Vinylphenol
Sample 1	0.071	0.002 ^a	0.324	2.802
Sample 2	0.125	0.090	0.200	1.341
Sample 3	nd	nd	0.281	0.628
Sample 4	0.003 ^a	0.228	0.267	1.483
Sample 5	0.238	0.005 ^a	0.222	2.005
Sample 6	0.255	0.682	0.282	1.430
Sample 7	0.072	0.134	nd	nd
Sample 8	0.088	0.097	0.880	2.174
Sample 9	0.149	0.782	nd	nd
Sample 10	0.081	0.278	nd	nd

nd, not detected.

^a Values lower than LOQ.

vals of the method. For vinylphenol, the peak of another unidentified compound interferes with its quantitation because the retention times are very similar. The analysis of these wines from different wineries and vintages showed a wide concentration range in volatile phenols (Table 5), and, as was to be expected, the red wines analysed showed a higher content of ethylphenols than in vinylphenols while the white ones were characterised by low levels of ethylphenols and high levels of vinylphenols.

4. Conclusions

The HS-SPME method described here is appropriate for the quantitative analysis of volatile phenols in wine. After optimising the experimental conditions to analyse this type of compound, the performance characteristics were determined. The detection and quantitation limits, the RSD values and the recoveries obtained for all the volatile phenols are adequate for their quantitation in wine. In addition, the HS-SPME procedure is a solvent-free method presenting major advantages: small sample volume, high sensitivity and simplicity.

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