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Determination of ethylphenol compounds in wine by headspace solid-phase microextraction in conjunction with gas chromatography and flame ionization detection

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Abstract

Headspace solid phase microextraction (HS-SPME) was investigated as a solvent-free alternative method for the extraction and determination of 4-ethylphenol (EP) and 4-ethylguaiacol (EG) in red wine by capillary gas chromatography with flame ionization detection (FID) and compared to liquid–liquid extraction.

For HS-SPME, better results were obtained with saturated sodium chloride samples, at 55 °C, using a 85 μm polyacrylate fiber. An absorption time of 40 min was needed to reach the absorption equilibrium for EG. This 40-min duration corresponds to the beginning of EP equilibrium and was selected for the experiments. In these conditions, the calibration graphs were linear in the range 5–5000 $\mu g\, l^{-1}$ and the sensitivity was nearly the same for the two compounds. The detection limits were in the low $\mu g\, l^{-1}$ range. In model wine solutions, result obtained with the liquid–liquid extraction method exhibit a linear calibration between 25 and 10,000 $\mu g\, l^{-1}$ with a detection limit of 1 $\mu g\, l^{-1}$, but, the relative standard deviations of the EP and EG result in the low concentration range ($<50\ \mu g\, l^{-1}$) are higher than those obtained by HS-SPME (15% compared to 2% for EP and 12% compared to 5% for EG). Taking into account the numerous volatile compounds in wine, HS-SPME is a rapid and valid alternative technique for use in the determination of ethylphenols at trace levels.

Keywords: Ethylphenols; Red wine; Headspace analysis; Solid phase microextaction; Gas chromatography

1. Introduction

Among the volatile phenols contributing to the aroma of wine, some may be produced by yeasts. For example, *Brettanomyces/Dekkera* produces 4-ethylphenol (EP) and 4-ethylguaiacol (EG) (Fig. 1) [1–4]. The aroma associated with EP in red wine has been described as "animal"-, "horsy"-, "barnyard"-, "medicinal"-like [5–7] while EG has been described as a "smoky"-, "clove"-like aroma [5,6,8–10]. These volatile phenols are almost ubiquitous in red wines but their concentrations vary considerably giving, at higher levels, heavy and undesirable odors. So, winemakers have to control yeast development and understand how winemaking and storage processes may affect the concentration of EP and EG. For this purpose, procedures have to be developed to facilitate rapid wine sample analysis both in the laboratory and on-site.

Analysis of volatile compounds such as aromas, pollutants and solvents is a continuous challenge in the food and beverage industries. Although various

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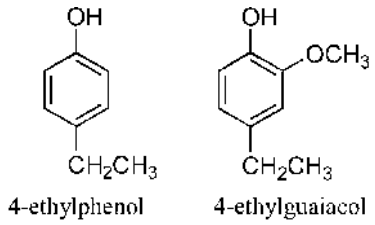

Fig. 1. Structures of 4-ethylphenol and 4-ethylguaiacol.

methods, using highly efficient instrumental techniques such as gas chromatography (GC), liquid chromatography (LC) and their combination with mass spectro-metry (MS), can be used for food analysis, most analytical instruments cannot handle the sample matrices directly. Consequently, several multistep sample preparations, including liquid–liquid extractions, have been developed but they are time-consuming, involve expensive and hazardous solvents, and produce low accuracy due to the manual handling. Some of them have been adapted for the determination of volatile phenols in wine. Chatonnet and Boidron in 1988 were the first to describe an extraction with dichloromethane coupled with a GC-flame ionization detection (FID) analysis [5]. Recently, Pollnitz et al. used a GC–MS procedure to analyze pentane:diethylether (2:1) extracts of wine containing EG and EP [11]. In both cases, these methods were useful to correlate ethylphenol concentrations with *Brettanomyces*/*Dekkera* contamination and determine the ethylphenol concentration range in a large variety of wine samples [1,2,11].

Recently, headspace solid phase microextraction (HS-SPME) coupled to GC has gained a sturdy reputation [12–14] as a valid alternative to other methods previously used for the analysis of volatile compounds in food. The HS-SPME–GC method has been used to determine aroma compounds in wine but has still not been optimized for ethylphenol determination [11,15,16].

The purpose of this work was to optimize the HS-SPME parameters for capillary GC–FID analysis of ethylphenols and to compare the efficiency of HS-SPME with a liquid–liquid extraction method through calibration graphs for model wine solutions and through quantitation of volatile phenols in red wine.

2. Experimental

2.1. Reagents

The gases for chromatographic analysis were purchased from AGA (Toulouse, France). The polyacrylate 85 μm fiber and the polydimethylsiloxane 100 μm fiber were from Supelco (Saint Quentin Fallavier, France). Ethanol was obtained from SDS (Peypin, France), and 4-ethylguaiacol from Lancaster (Strasbourg, France). All other reagents were purchased from Sigma–Aldrich Chemical Co. (Saint Quentin Fallavier, France).

2.2. Preparation of model wine solution and standard solutions

The model wine solution was prepared in distilled water containing 11% (v/v) ethanol, 6 g l^{-1} glycerine, 2.5 g l^{-1} tartaric acid, 3 g l^{-1} lactic acid and 1 g l^{-1} potassium phosphate. The pH of the resulting solution was 3.

A standard stock solution of each volatile phenol (EP, EG and 3,4-dimethylphenol) was prepared by mass at 1000 mg l^{-1} in the model wine solution. Secondary standard solutions of EP and EG were prepared by dilution of the stock solution in the model wine solution to obtain concentrations of 10 mg l^{-1}.

2.3. Procedure

2.3.1. Calibration

Calibration graphs for EP and EG in the range 5–10,000 μg l^{-1} were plotted in model wine solutions using primary and secondary standard solutions. 3,4-Dimethylphenol was used as internal standard (IS) for calibration and quantitation of the ethylphenol compounds. Its concentration in the sample was 5 mg l^{-1} for the HS-SPME procedure and 10 mg l^{-1} for the liquid–liquid extraction method.

2.3.2. Chromatographic analyses

Analyses were carried out on a DANI GC 1000 gas chromatograph equipped with a hydrogen flame ionization detector and an EC-WAX column (30 m × 0.32 mm × 0.25 μm) from Alltech (Templeuve, France). Nitrogen was used as carrier gas at a flow rate of 1 ml min^{-1}. A split/splitless injector was

used in the splitless mode (ratio 1:25) and maintained at 250 °C. The detector was kept at 250 °C. The column program was as follows: initial temperature 40 °C for 1 min, first ramp to 180 °C at 10 °C min^{-1}, kept at 180 °C for 0.5 min, second ramp to 205 °C at 3 °C min^{-1}, kept at 205 °C for 0.5 min, third ramp to 230 °C at 10 °C min^{-1}, kept at 230 °C for 5 min. A nitrogen purifier was fitted between the bottle of carrier gas and the GC apparatus to trap oxygen and water present in the nitrogen.

2.3.3. Solid-phase microextraction procedure

The sample (final volume: 2 ml) was placed in a 5 ml vial containing NaCl (1 g), 3,4-dimethylphenol (5 mg l^{-1}) and sealed with a septum-type cap. HS-SPME was carried out under magnetic stirring. The solutions were heated to 55 °C and extracted with a 85 μm polyacrylate fiber for 40 min by insertion into the vial headspace. The compounds were desorbed by inserting the fiber into the GC injector for 3 min.

2.3.4. Liquid–liquid extraction procedure

Volatile phenols were extracted from samples according to the method previously described by Chatonnet and Boidron [5]. First, the IS (3,4-dimethylphenol, 10,000 μg l^{-1}) was added to the sample (50 ml). Then 15 g of ammonium sulfate were added and well dissolved prior to extraction three times with 10, 5 and 2.5 ml of dichloromethane, successively. The combined organic extracts were centrifuged (10,000 × *g* for 5 min) to break any emulsion, and washed twice with 5% sodium bicarbonate, then extracted twice with 25 and 12.5 ml of 0.5% sodium hydroxyde solution. The combined alkaline extracts were acidified to pH 1 by adding 20% hydrochloric acid, then extracted with 7.5, 2.5 and 2.5 ml of diethyl ether, successively. The combined ether extracts were slowly concentrated to 0.5 ml at room temperature by evaporation under a nitrogen gas flow in vials capped with a septum. Gas chromatographic analysis was performed by injecting 1 μl of the concentrated extract.

2.4. Statistical analysis

Results are expressed as means ± S.E. of *n* experiments. Statistical comparisons were made using the unpaired Student's *t*-test. The *P*-value was considered to be statistically significant when $P < 0.05$.

3. Results and discussion

The study and optimization of HS-SPME conditions need about 10 experimental parameters to be taken into consideration. However, there have been numerous papers written on the extraction of semivolatile and volatile molecules from food and beverages, as well as from waters using SPME. These reports allowed us to rationalize the study on selected parameters [14–18].

In preliminary studies, two types of different polarity fibers were tested: a polydimethylsiloxane fiber and a more polar one, a polyacrylate fiber. For volatile phenol analysis, the polyacrylate fiber showed the best results (data not shown) which is in agreement with previous studies [12,13,17]. Moreover, two main types of SPME procedures were compared: the headspace (HS) and the direct immersion (DI) procedures. In model wine solutions, extraction efficiency with each technique depends on the analyte: the HS-SPME procedure is more sensitive for EG whereas DI-SPME seems to be better for EP and IS (data not shown). However, red wine has a very complex matrix and also contains non-volatile compounds. For these reasons, we selected the HS-SPME procedure to increase the life-time of the fiber and to lower the background compared to DI-SPME, as reported in the literature [12,13,15,17].

3.1. Optimization of HS-SPME parameters

3.1.1. Influence of ionic strength

The effect of ionic strength on the headspace ethyl phenol quantitation was determined by saturating a sample with NaCl (1 g) under magnetic stirring. Results obtained for EP and EG at 55 °C with or without NaCl are shown in Fig. 2. Whatever the compound studied, saturation of the samples with NaCl increased peak areas about four times indicating an increase of extraction efficiency for volatile phenols. Indeed, it is well known that supersaturation of the sample with salt is more effective for the extraction of analytes onto the fiber due to the salting-out effect [15]. This effect decreases the solubility of analytes and thus increases their absorption by the fiber [13]. So, samples saturated with 1 g of NaCl have been used for subsequent experiments.

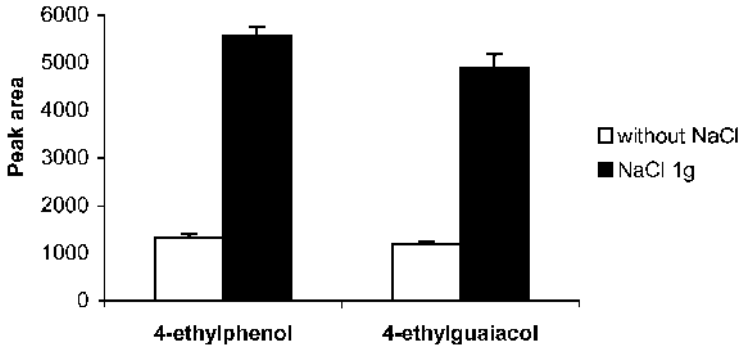

Fig. 2. Ionic strength effect on HS-SPME of volatile phenols ($5\,mg\,l^{-1}$) from a 2 ml model wine solution by a polyacrylate fiber at 55 °C (extraction time: 40 min). Each result represents the mean (±S.E.) of three independent experiments.

3.1.2. Effect of temperature

HS-SPME is controlled by the equilibria between the sample and the headspace and between the headspace and the fiber. These equilibria are affected by temperature.

To optimize HS-SPME, the temperature of the solution was varied from 25 to 75 °C using an absorption time of 40 min. Results concerning EP and EG are shown in Fig. 3. Peak areas of IS exhibited the same variations as EP (data not shown).

Below 55 °C, peak areas of volatile phenols increased with the sample temperature indicating an increase of the headspace concentration. The peak area of EG was a maximum at 55 °C and then decreased while EP and IS peak areas continued to increase with increasing temperature. These different temperature effects on volatile phenols could be explained in part by the boiling point of the compounds studied (99 °C for EG, 213 °C for EP and 227 °C for IS). Indeed, the fact that an increase in temperature reduced the response of the most volatile compound EG indicates a decrease in the fiber/headspace partition coefficient. At the same time, the responses for the higher boiling compounds, EP and IS, increase corresponding to the increased headspace/solution partition coefficients. These observations are in agreement with previous studies with different volatile compounds [16,19,20] and demonstrate that for a complex matrix, selection of an optimum sampling temperature depends strongly on which analytes are of greatest interest or require the greatest sensitivity. Moreover, as reported by Chatonnet et al. [2], the EG:EP ratio in red wine is 1:10.

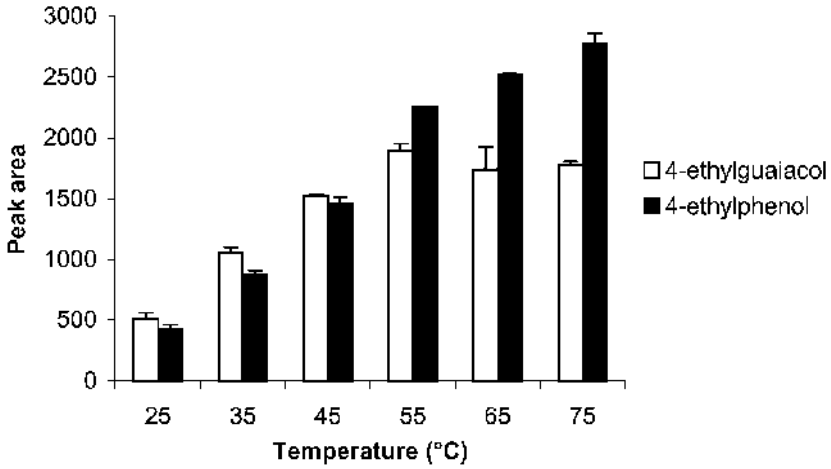

Fig. 3. Temperature effect on HS-SPME of volatile phenols ($2\,mg\,l^{-1}$) from a 2 ml model wine solution containing 1 g of NaCl by a polyacrylate fiber (extraction time: 40 min). Each result represents the mean (±S.E.) of three independent experiments.

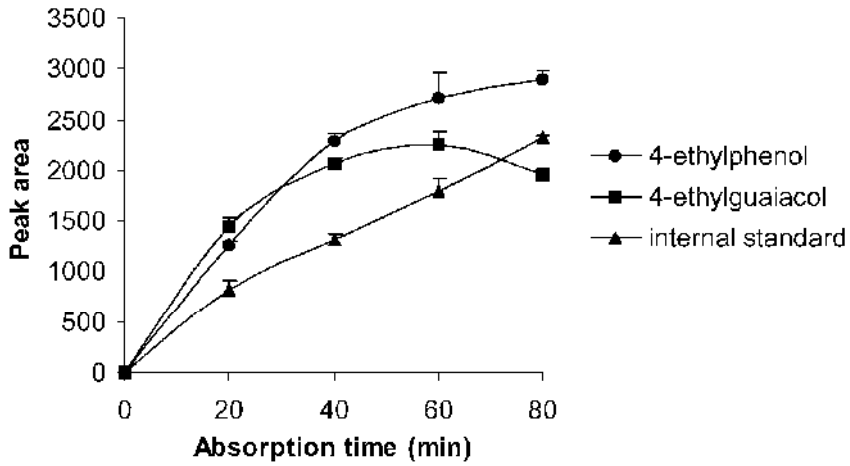

Fig. 4. Absorption time profiles of ethylphenol compounds on a polyacrylate fiber at 50 °C. Each test compound is dissolved at 2 mg l^{-1} in a 2 ml model wine solution containing 1 g of NaCl. Each point represents the mean (±S.E.) of three independent experiments.

Considering these data and experimental results, a temperature of 55 °C, giving the highest sensitivity for EG, was selected for subsequent experiments.

3.1.3. Absorption time on the polyacrylate fiber with stirring

The absorption-time profiles obtained at 55 °C for the ethylphenol mixtures are shown in Fig. 4. The responses of the compounds are affected by increasing the sampling time from 20 to 80 min corresponding to the amount of analyte absorbed by the fiber coating [13,15,16]. The equilibrium period required for EG was the shortest (40 min). For EP and IS, the less volatile components of the mixture, increasing the sampling time from 40 to 80 min increased the response, indicating slower equilibration. However, for EP the increase in response between 40 and 60 min was less than between 20 and 40 min (18% versus 82% increase) indicating the beginning of the equilibration. These results confirmed previous studies showing that in saturated salt solutions, the time required for phenols to reach equilibrium was >60 min [12,13]. Indeed, this is probably due to the slow diffusion of the compounds through a saturated salt solution. Although an absorption time of 40 min is not enough to reach equilibrium for all compounds, it was chosen for the subsequent experiments since it gave the highest sensitivity for EG. Moreover, this time was approximately equivalent to that required for a gas chromatographic run.

3.2. Comparison of HS-SPME and liquid–liquid extraction for the model wine solution

Validation of extraction methods coupled with GC analysis were performed for both EP and EG in the presence of IS with three independent calibration graphs. The calibration graphs obtained by HS-SPME–GC for EP and EG are linear over the range 5–5000 μg l^{-1} (10 data points, $n = 3$). These calibration graphs present the following regression coefficients and linear regression equations: $r^2 = 0.998$ and $y = 0.00025x$ for EP; $r^2 = 0.989$ and $y = 0.00024x$ for EG (where y is the peak area, in arbitrary units, and x is the concentration, in μg l^{-1}). The sensitivity represented by the slopes of the calibration graphs, was nearly the same for EP and EG. When the EP and EG concentrations were 50 μg l^{-1}, the R.S.D. was 6% for EP and 5% for EG ($n = 3$ independent determinations). When the EP and EG concentrations were 5000 μg l^{-1}, the R.S.D. was 3% for EP and 2% for EG ($n = 3$ independent determinations).

For liquid–liquid extraction, the calibration graphs were linear over a higher concentration range (25–10,000 μg l^{-1}, nine data points, $n = 3$) for EP and EG. The graphs present the following regression coefficients and linear regression equations: $r^2 = 0.999$ and $y = 0.00010x$ for EP; $r^2 = 0.997$ and $y = 0.00008x$ for EG. The sensitivity of the method (calibration slope) for EP is a little higher than for EG. When the EP and EG concentrations

were 50 $\mu g\,l^{-1}$, the R.S.D. was 15% for EP and 12% for EG ($n = 3$). When the EP and EG concentrations were 5000 $\mu g\,l^{-1}$, the R.S.D. was 2% for EP and 5% for EG ($n = 3$ independent determinations). Whatever the compounds and methods used, the detection limits (signal to noise ratio = 3) were in the low $\mu g\,l^{-1}$ range.

For EP and EG, the reproducibility of analysis at 50 and 5000 $\mu g\,l^{-1}$ were determined ($n = 3$) with different model wine solutions containing IS. At the lowest concentration, both compounds exhibited lower R.S.D. values within the SPME procedure than with liquid–liquid extraction (~2.5 times less) while the R.S.D. values were similar for the two methods at higher concentrations. It is important to note that all these optimization experiments were done with model wine solutions. The extraction efficiency of ethylphenol compounds by the two techniques was then checked in red wine.

3.3. Comparison of HS-SPME and liquid–liquid extraction for red wine

The efficiency of the HS-SPME and liquid–liquid extraction methods was compared for red wines through the chromatogram profiles and EG and EP quantitation. As shown in Figs. 5 and 6, the retention times of EG and EP were 18.3 min for EG and 20.6 min for EP. These values are close to those obtained in model wine solutions (data not shown). For a given wine (Wine-1), the EP and EG peaks were higher by HS-SPME than by liquid–liquid extraction indicating that HS-SPME is the more efficient procedure to concentrate volatile phenols. Then, the chromatogram obtained after liquid–liquid extraction presented fewer peaks than that obtained after HS-SPME.

The ability of both methods to quantify EP and EG was evaluated on two different wines, one rich in ethylphenols (Wine-1), and one poor in ethylphenols (Wine-2). The results are shown in Table 1. Whatever the wine tested and the method employed, the concentrations determined for EP were similar ($P = 0.43$ in Wine-1 and 0.2 in Wine-2, $n = 3$) indicating the good selectivity of both methods for EP. However, the variability of EP concentration in Wine-2 was higher for liquid–liquid extraction (28%) than HS-SPME (3%). For EG concentrations, whereas there was no difference between the two methods for Wine-1 ($P = 0.68$, $n = 3$) indicating the good selectivity of both methods for EG, the liquid–liquid extraction procedure showed lower values than HS-SPME ($P < 0.05$, $n = 3$) for Wine-2. This can be explained by the

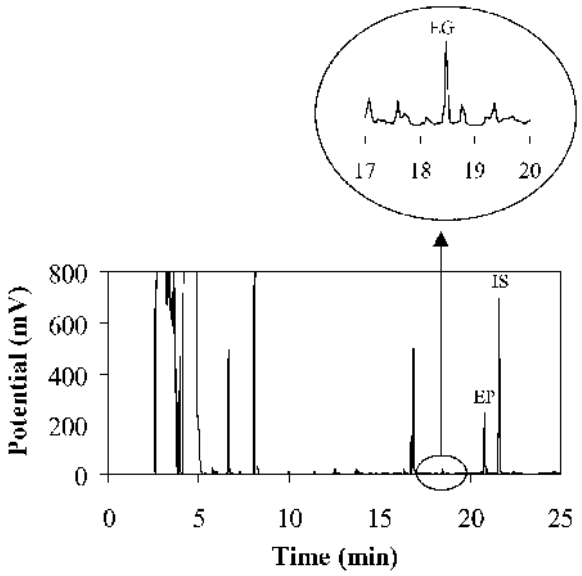

Fig. 5. Chromatogram obtained by capillary GC–FID after liquid–liquid extraction of ethylphenol compounds from red wine (Wine-1). EP: 4-ethylphenol; EG: 4-ethylguaiacol; IS: internal standard.

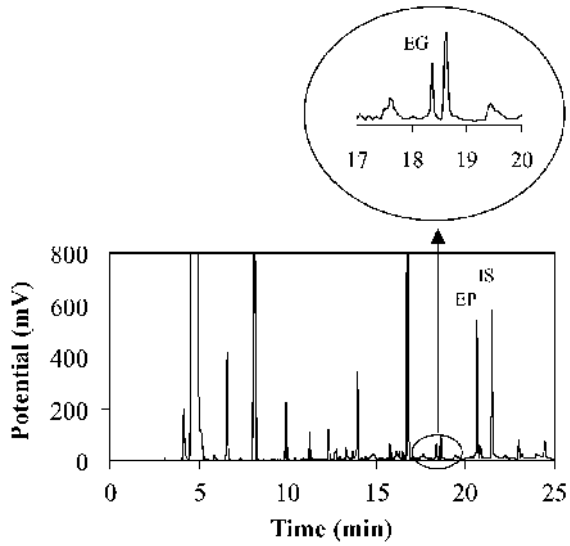

Fig. 6. Chromatogram obtained by capillary GC–FID after HS-SPME of ethylphenol compounds from red wine (Wine-1). EP: 4-ethylphenol; EG: 4-ethylguaiacol; IS: internal standard.

Table 1
Determination of EP and EG concentrations ($\mu g\,l^{-1}$) in two wines by HS-SPME and liquid–liquid extraction[a]

Extraction method	Wine-1		Wine-2	
	EP	EG	EP	EG
HS-SPME	3536 (±152)	280 (±8)	109 (±2)	56 (±2)
Liquid–liquid extraction	3350 (±84)	247 (±8)	90 (±15)	36 (±4)

[a] Concentrations of EP and EG are expressed as means (±S.E.) of three independent experiments.

fact that the EG concentration in Wine-2 is close to its lower quantitation limit ($25\,\mu g\,l^{-1}$) evaluated by liquid–liquid extraction. However, it is important to note that this quantitation limit was determined in model wine solutions, a less complex matrix than red wine. Moreover, the variability of EG concentration in Wine-2 was higher for liquid–liquid extraction (19%) than HS-SPME (6%).

The important variability of the results obtained by liquid–liquid extraction in Wine-2, poor in ethylphenol compounds, could be due to the uncontrolled temperature of extraction. In this procedure, the volatility of the compound studied and the liquid–liquid partition coefficients appeared to be dependent on temperature. In contrast, HS-SPME always allows the same temperature (55 °C) to be used, thus limiting this problem.

4. Conclusions

The HS-SPME procedure is a solvent-free method presenting major advantages: simplicity, rapidity, high sensitivity and small sample volume. The HS-SPME method is appropriate for the quantitative analysis of ethylphenols in red wine even if the gas chromatograms obtained by HS-SPME present more peaks than those resulting from liquid–liquid extraction. In contrast to liquid–liquid extraction, the solvent free HS-SPME method can be combined with an autosampler. We are now examining the possibility of developing a fast monitoring method for the routine analysis of volatile phenols in wine for quality management in winemaking.

Acknowledgements

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