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Analytica Chimica Acta 458 (2002) 95–102

ANALYTICA CHIMICA ACTA

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Determination of volatile phenols in fino sherry wines

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Received 20 June 2001; received in revised form 25 September 2001; accepted 27 November 2001

Abstract

An easy, fast and reliable analytical method is proposed to determine the concentration of volatile phenols (ethyl- and vinylphenols) in fino sherry wines. The technique employed is a single stage sample treatment by solid-phase extraction (SPE) following a simple, fast procedure that enables 12 samples to be extracted simultaneously and requires a small volume sample and little time. Subsequently, the extracts are analyzed by gas chromatography (GC) with flame ionization detection. The method proposed has been applied to the study of fino sherry wines affected by microbial contamination with yeasts of the *Brettanomyces* genus, and the relationship of these yeasts with the concentrations of volatile phenols present in this wine.
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Keywords: Wine; Ethylphenols; Vinylphenols; *Brettanomyces* yeast; Solid-phase extraction

1. Introduction

Generally speaking, volatile phenols are considered among the usual components of the aroma of wine. However, some of these compounds may negatively affect the quality of the wine, producing unpleasant odours. Specifically, the presence of ethylphenols (4-ethylphenol and 4-ethylguaiacol) in red wines has led professional tasters to describe "phenolic", animal and stableslit odours in these wines [1]. The vinylphenols (4-vinylphenol and 4-vinylguaiacol), however, are present in higher concentrations in white wines than in red ones, and are thought to be responsible for medicinal and spicy odours. The precursors of these compounds are the cinnamic acids, *trans*-ferulic and *trans-p*-coumaric, that give rise to vinylphenols through decarboxylation. These are then transformed by reduction into the corresponding ethylphenols [2,3]. Yeasts of the *Brettanomyces* genus, which have mainly been studied in red wines, seem to be involved in the aromatic deterioration of the wine caused by these compounds [4,5]. Recently, these yeasts have also been isolated in fino sherry wines [6], in which to date there exists only analytical evidence of their proliferation from the high volatility acidity detected in barrels of deteriorated wine; the possible presence of volatile phenols in such wines has not been studied before.

The analysis of wines for volatile phenols is carried out by gas chromatography (GC), preceded by an extraction stage. There are a variety of different sample treatments that can be applied to the wine for subsequently determining their content of volatile phenols. Liquid–liquid extraction, traditionally employed in the analysis for compounds related to the aroma of wine, seems to be the most common [7,8]. However, in recent years, other less tedious methods have been developed, such as, for example, liquid–liquid

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PII: S0003-2670(01)01581-1

micro-extraction [9,10], and the combination of this with a solid-phase extraction (SPE) [11].

The method proposed in this paper is simple, fast and reliable, for application to the determination of the volatile phenols in fino sherry wine by GC with a single sample treatment stage performed by SPE. The adsorbent employed is very selective and the SPE device allows twelve samples to be processed in the same session, using a small volume of sample. This extraction method was selected as the best of three tested. Two of these methods were based on liquid–liquid extraction, one was the method proposed by Chatonnet and Boidron [8] and the other a method developed by our research group for the extraction of phenolic compounds from wine [12]. The third method consisted of a SPE, the procedure for which was developed by our research group for the determination of diverse phenolic compounds in wine [13–15]. This method has been applied to the determination of the concentrations of volatile phenols in various different samples of fino sherry wine affected by microbiological contamination, specifically by yeasts of the *Brettanomyces* genus.

2. Experimental

2.1. Reagents and standards

Standards of 4-ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol and 4-vinylphenol were acquired from Sigma–Aldrich. The 3,4-dimethylphenol, employed as internal standard, also was supplied by Sigma–Aldrich. The dichloromethane, of analytical quality, was from Panreac Quimica, S.A. (Barcelona, Spain). The ethanol, of chromatographic quality, and the tartaric acid were from Merck (Darmstadt, Germany). The methanol, of HPLC quality, was supplied by Scharlau Chemie, S.A. (Barcelona). The water employed was previously purified in a Milli-Q system (Millipore, Bedford, MA).

2.2. Samples

For this study, four different soleras of fino sherry wine were selected. From each solera, two samples were taken, using sensorial analysis criteria carried out by qualified tasters of the wine-making producer, not by microbiological evaluation. One of both samples was from a cask of wine considered relatively lightly contaminated by the *Brettanomyces* yeast and the other sample was from a cask known to be severely contaminated by this yeast. All of them were provided by Bodegas Osborne and Cía. (El Puerto Sta. María, Cádiz, Spain).

2.3. Instrumentation and conditions

The analyses were performed using a HP 5890 gas chromatograph (Hewlett-Packard, Wilmington, DE) by injecting 1 μl of the extract in split mode (ratio 1:30, 230 °C) with an HP autosampler, 7673 model into a DB–WAX column (60 m × 0.25 mm i.d., 0.5 μm film thickness (J&W Scientific Inc., Folsom, CA). The carrier gas was helium at 0.6 ml min^{-1}. The temperature program was: 45 °C for 5 min, then raised to 230 °C at a rate of 3 °C min^{-1}, with a final isotherm of 30 min. The detection was performed with a flame ionization detector (FID) at 230 °C.

2.4. Preparation of the samples

The SPE stage was performed in a Visiprep SPE vacuum manifold, 12-port model from Supelco (Supelco Park, Bellefonte, PA) in which there are twelve positions available for conducting the SPE simultaneously. The cartridges used were LiChrolut EN (Merck), containing 200 mg of the polymeric adsorbent, polystyrene-divinylbenzene. These were conditioned first with 5 ml of methanol and then with 3 ml of water. A sample of 10 ml of wine, to which 100 μl of 1000 mg l^{-1} solution of 3,4-dimethylphenol as internal standard had been added, was loaded into the previously conditioned cartridge. The column was rinsed with 0.6 ml of water and dried with helium for 150 s. Finally, the compounds of interest were eluted with 2.5 ml of dichloromethane.

2.5. Calibration

Calibrations were carried out for each volatile phenol from a stock solution with the four volatile phenols in ethanol each at 100 mg l^{-1}, by dilution in a solution of synthetic wine (15% ethanol and 3 g l^{-1} tartaric acid) to different concentrations between 0.25 and 20 mg l^{-1}. These solutions of four volatile phenols

were then subjected to the previously described SPE process, with the prior addition of the internal standard as done with any sample. Their extracts were analysed by GC using the method already explained, and the peak area results obtained were used to construct calibration graphs, representing for each volatile phenol the area relative to the internal standard against the different concentrations.

3. Results and discussion

3.1. Selection of the extraction method

In the light of both the bibliography consulted and the various studies conducted by our research group in respect of the determination of phenolic compounds in wine, it was decided to test three methods for the extraction of volatile phenols from fino sherry wines. First, the extraction method for the analysis of volatile phenols in wine published by Chatonnet et al. [8] was tested. This method has been widely employed in other research studies related to these compounds, and it involves a liquid–liquid extraction of 100 ml of wine with dichloromethane using a magnetic agitator. In addition, the possibility was considered of applying two extraction methods that had previously been developed by our research group for the determination of polyphenols in sherry wines. One of these also consists of a liquid–liquid extraction in which the 100 ml of wine is brought into contact with a mixture of diethyl ether and *n*-pentane (2:1, v/v) in a specially designed device for performing a rotary and continuous extraction [12]. The other is a SPE method in which 10 ml of wine is loaded in a cartridge that selectively retains the polar compounds with high efficiency [13–15]. To compare the extractive capacity of each method for volatile phenols, samples of the same fino sherry wine spiked with volatile phenols were subjected to the three extraction methods and subsequently the extracts obtained were analysed by GC under the conditions described in Section 2 of this paper.

Fig. 1 shows the chromatograms of the extracts obtained for each extraction method. Considering first the general extractive capacity of the methods compared, it can be deduced that the rotary, continuous liquid–liquid extraction method [12] extracts the largest number of compounds, since the chromatogram of the extract obtained is the one presenting the most peaks (Fig. 1b). However, the particular interest of this study is centred on the analysis for the volatile phenols, therefore what is important is that the extraction method selected should be the most selective for these particular compounds, and that in addition it should extract the maximum quantity of these compounds. On these criteria, the rotary, continuous liquid–liquid extraction method has to be discounted, for although it extracts the volatile phenols very efficiently, it is similarly efficient for other compounds that make it difficult to quantify the volatile species of interest. In other words, it is not a selective extraction method. The other two methods tested meet to a similar degree this important condition of selectivity. However, the SPE method (Fig. 1c) is seen to be the method that extracts the greater quantity of volatile phenols, and thus meets the second of the criteria laid down for this study.

Other aspects of a more practical nature that influence the choice of extraction method are the sample volume required, the number of samples that can be extracted in a single extraction session, and the length of time needed for each session, defining this period as the time required from the preparation of the sample until the final extract is obtained, ready for injection into the gas chromatograph.

Table 1 presents the practical aspects offered by each of the extraction methods. It can be observed that with the method of Chatonnet and Boidron [8] it is possible to extract at least four samples simultaneously, with an approximate duration of 4 h per extraction session. However, these values can be improved upon, given a laboratory with a plentiful supply of glassware, together with several magnetic agitators that can be operated simultaneously. In comparison, the rotary, continuous liquid–liquid device [12] allows six samples to be extracted at the same time, requiring about 6 h for each extraction session. In contrast, the SPE can be performed in only 2.5 h, offering 12 positions for simultaneous extraction [13]. In respect of the volume of sample needed, the two liquid–liquid methods use 100 ml of wine, while the SPE method needs only 10 ml.

Since both the liquid–liquid extraction method proposed by Chatonnet and Boidron [8] and the SPE method developed by our research group [13–15] satisfy in equal measure the necessary conditions for

conducting our study, and since the SPE method is simpler, faster and more convenient, from a practical point of view we selected the SPE method for the purposes of our study.

Nevertheless, it was decided to investigate the maximum extractive capacity for volatile phenols provided by the method selected, by testing different solvents in the elution stage. For this, a sample of wine to which the four volatile phenols under study had been added was eluted from the SPE cartridge with the same volume (2.5 ml) of each of ten different solvents.

The results are given in Fig. 2, in which it can be observed that dichloromethane is the solvent that offers the best results among all the organic solvents tested. As was to be expected, *n*-hexane and *n*-pentane, both

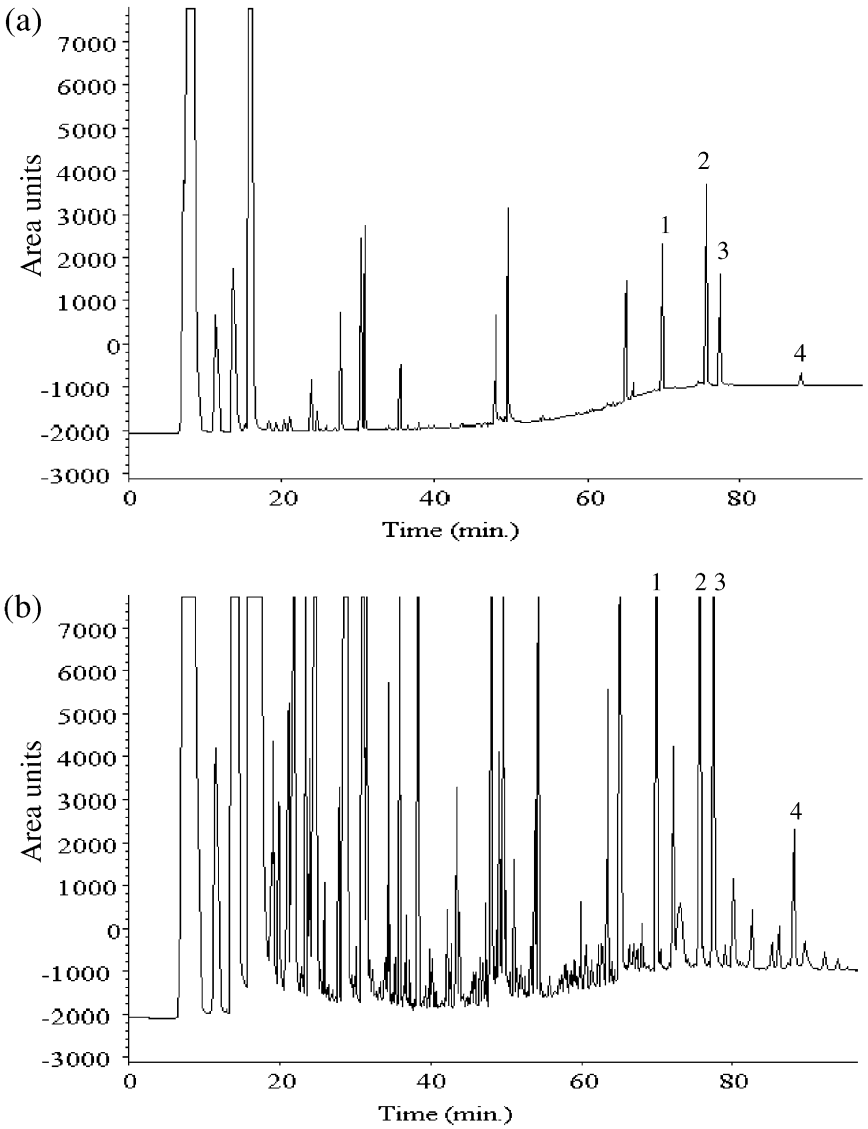

Fig. 1. Chromatograms of the extracts from the same wine, obtained by the three different methods of extraction tested: 1, 4-Ethylguaiacol, 2, 4-Ethylphenol, 3, 4-Vinylguaiacol, 4, 4-Vinylphenol. (a) by the extraction method of P. Chatonnet et al., [8]. (b) by the rotary and continuous liquid-liquid extraction method [12] and (c) by the solid phase extraction method [13–15].

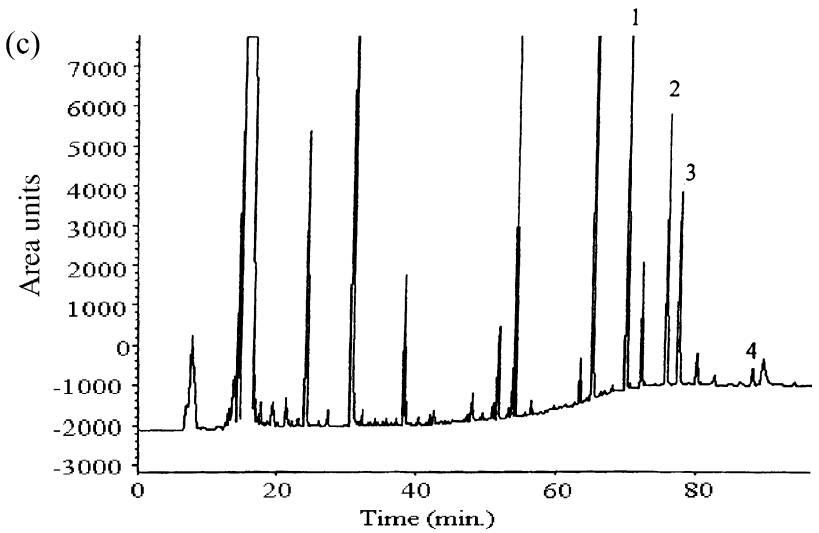

Fig. 1. (*Continued*).

Table 1
Practical aspects of each extraction method

	L–L[a] extraction [8]	Rotary, continuous L–L[a] extraction [12]	Solid-phase extraction [13–15]
Initial sample volume (ml)	100	100	10
Final extract volume (ml)	1	2	2.5
Number of samples/extraction session	4	6	12
Total time of extraction session (h)	4	6	2.5

[a] Liquid–liquid.

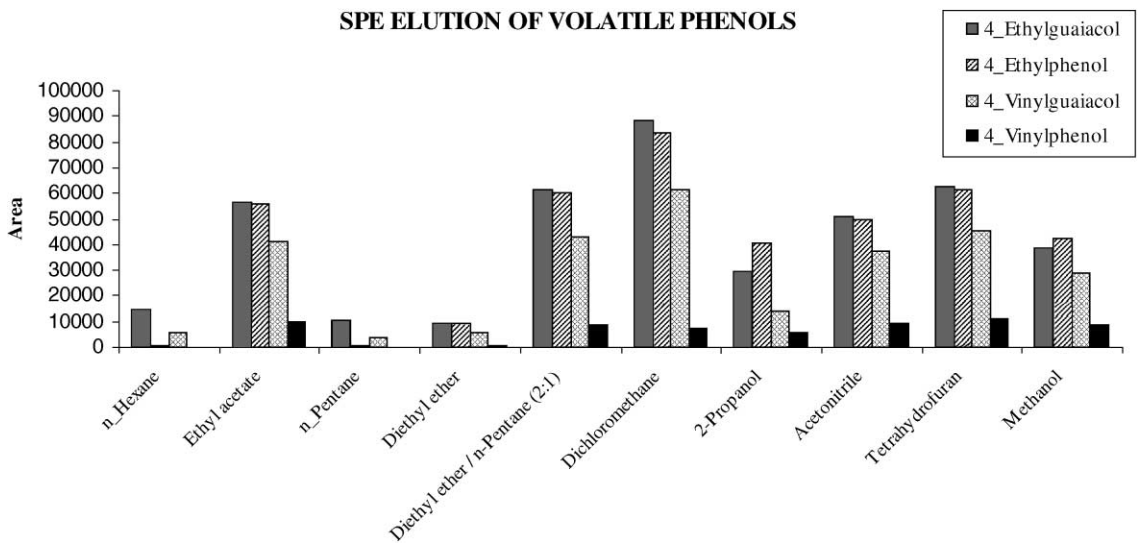

Fig. 2. Extractive capacity for volatile phenols, revealed in the solid-phase extraction tests conducted with different elution solvents.

Table 2
Analytical characteristics of the method

	4-Ethylguaiacol	4-Ethylphenol	4-Vinylguaiacol	4-Vinylphenol
Concentration range ($mg\,l^{-1}$)	0.275–22.00	0.287–22.96	0.383–30.64	0.668–26.72
r^2 ($n = 5$)	0.9979	0.9997	0.9958	0.9973
Linearity curve (%)	98.14	99.24	97.35	97.67
Detection limit ($mg\,l^{-1}$)[a]	1.13	0.48	2.24	1.59
Quantitation limit ($mg\,l^{-1}$)[b]	3.77	1.60	7.45	5.28
Analytical sensitivity	0.41	0.17	0.80	0.58

[a] 3σ.
[b] 10σ.

low polarity solvents, do not elute efficiently polar compounds, such as volatile phenols. The non-elution of 4-vinylphenol with both organic solvents, compared to the other three phenols, may be because of the chemical structure of this compound. It is very similar to the polymeric adsorbent contained in the SPE cartridge, so it may be strongly retained inside the cartridge and resists elution with *n*-hexane or *n*-pentane.

3.2. Validation of the analytical method

Having selected the extraction method and the elution solvent, the complete method for the analysis of volatile phenols was calibrated and validated. The calibration graphs were constructed from the results of the analyses of the extracts of several calibrated solutions of the four volatile phenols at different concentrations, the results of which are presented in Table 2. Taking into account the wide range of concentrations considered, the results of the calibration are fairly good, with the best being given by 4-ethylphenol.

The method was validated by standard addition, in triplicate, to identical samples of the same fino sherry wine of the four volatile phenols, at three different concentrations (2, 5 and 10 $mg\,l^{-1}$), some of them similar to real levels present in the wine analysed. At the same time, a repeatability study of the method was carried out, analyzing twelve repetitions of the same wine with the four volatile phenols added at a known concentration. In this way, an evaluation was also made of the repeatability provided by the twelve positions of the SPE vacuum manifold. The repeatability and recovery results of the method are presented in Table 3, from which it can be observed that the repeatability is fairly good for the four phenols, with a relative standard deviation of <10% for all of them, indicating a low dispersion of data.

Recoveries of >80% were achieved for 4-ethylguaiacol, 4-ethylphenol and 4-vinylphenol, but for 4-vinylguaiacol the recovery was only 66%. However, it must be borne in mind that these are very volatile compounds and therefore losses during the treatment of the sample are considerable.

3.3. Application to the study of contaminations by yeasts of the Brettanomyces genus in fino dry sherry wines

The eight samples drawn from four soleras of fino sherry wine selected for this study were analyzed according to the proposed method and the concentrations of volatile phenols found in them are shown in Fig. 3. For each compound of interest, a comparison is made between the concentrations found in the samples lightly contaminated and severely contaminated by yeasts of the *Brettanomyces* genus in each solera studied.

It can be observed that, at the higher level of contamination by the *Brettanomyces* yeast, a greater quantity of 4-ethylphenol is found in the sample; the

Table 3
Repeatability and recovery study of a fino sherry wine spiked with the four volatile phenols

Compound	Mean ($mg\,l^{-1}$)	S.D.	R.S.D. (%)	Recovery (%)
4-Ethylguaiacol	6.96	0.4325	6.21	80.8
4-Ethylphenol	5.64	0.1648	2.92	87.1
4-Vinylguaiacol	7.74	0.4982	6.43	66.4
4-Vinylphenol	9.73	0.7046	7.24	87.8

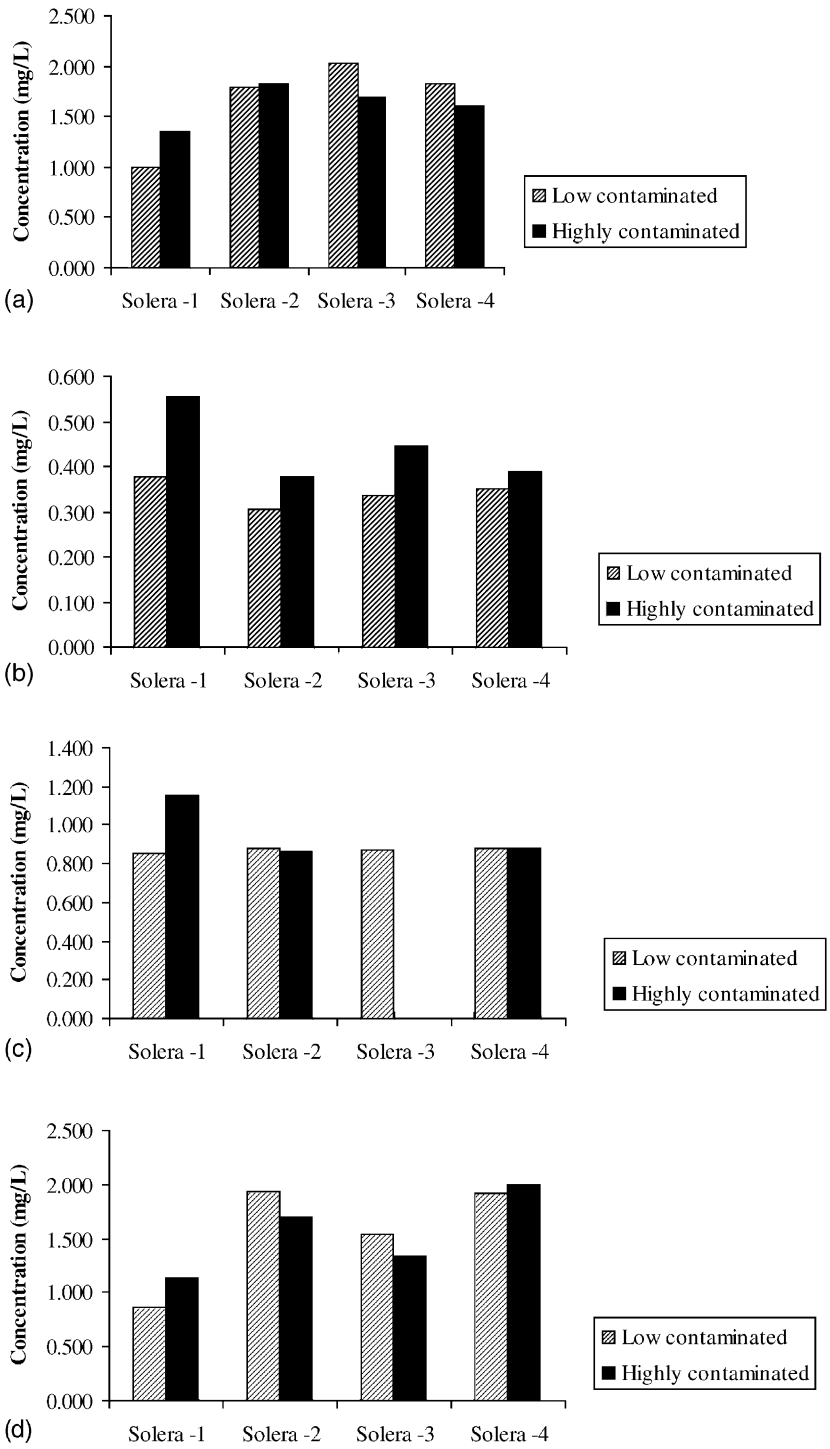

Fig. 3. Comparative concentrations of volatile phenols in lightly/severely contaminated wine by *Brettanomyces* yeast from four soleras of fino sherry wine studied: (a) 4-ethylguaiacol; (b) 4-ethylphenol; (c) 4-vinylguaiacol; (d) 4-vinylphenol.

concentration of this phenol is higher in all the samples known to be severely contaminated compared with the concentration in samples from the same solera but known to be only lightly contaminated. A similar result, however, is not found with the other three volatile phenols; in the comparison a lower concentration is found in some cases, and higher in others. Therefore, if it is proposed to monitor contamination by *Brettanomyces* in a solera of fino sherry wine, only 4-ethylphenol would serve as a reliable indicator of the degree of development of such contamination.

In spite of the fact that volatile phenols concentrations are not very different between lightly and severely contaminated wine samples, from an organoleptic point of view this difference can be sufficient for samples to be classified by sensorial analysis as different.

4. Conclusions

An easy, fast and reliable analytical method is proposed for the determination of the concentration of volatile phenols (ethyl- and vinylphenols) in fino sherry wines. A single sample treatment stage of SPE is employed, following a simple, fast procedure that allows twelve samples to be extracted simultaneously; very small sample volumes and very little time are required.

This method has been applied to the study of microbiological contamination of fino sherry wine by yeasts of the *Brettanomyces* genus. From this, it is observed that in all the samples of wine with a high degree of contamination by this yeast, the concentration of 4-ethylphenol is higher than that found in samples drawn from the same solera but with only a slight degree of contamination by this yeast.

Acknowledgements

This study was supported by FEDER of the European Union. (Project 1FD97-0875-C02-02). The authors are indebted to Bodegas Osborne & Cia. for supplying samples and for the facilities granted to use the installations and to Juan Díez Martín for his technnical support.

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