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Short communication

Determination of 4-ethylguaiacol and 4-ethylphenol in red wines using headspace-solid-phase microextraction-gas chromatography

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Abstract

A method for analysing 4-ethylguaiacol and 4-ethylphenol in the aroma of red wines using headspace-solid-phase microextraction is presented. The fibres used were coated with 100 μm of polydimethylsiloxane. Parameters like ionic strength, agitation of the sample, sample volume, temperature of the sample and adsorption/desorption times were studied and optimised to obtain the best extraction results. The linearity of the response was studied in the usual concentration ranges in wines (4-ethylguaiacol, 40–400 μg/l; 4-ethylphenol, 200–1800 μg/l). Repeatability of the method was determined, and the relative standard deviation was about 10%. Limits of detection and limits of quantification were also determined, and the values found were 1 and 5 μg/l for 4-ethylguaiacol and 2 and 5 μg/l for 4-ethylphenol, respectively. All these values were under the sensory thresholds established for these volatile phenols. The presence of interferences due to the matrix composition implies the use of the standard addition technique for both compounds quantification.

Keywords: Wine; Solid-phase microextraction; Headspace analysis; 4-Ethylguaiacol; 4-Ethylphenol

1. Introduction

Volatile phenols like phenol, guaiacol, cresols, ethylphenols, vinylphenols, eugenol and vainilline, are usually present in wine aroma [1]. The main characteristic of this kind of compounds is that their sensory thresholds are often lower than the habitual concentration ranges in wines, so their contribution to wine aroma is usually significant. Depending on the concentration levels and the aromatic properties, some of them contribute positively to wine aroma, but others are responsible for wine off-flavours. Among these off-flavours, it has to be taken into account, mainly, ethylphenols and vinylphenols [2].

In red wines the amount of ethylphenols is always higher than the amount of vinylphenols, and sometimes their concentrations are high enough to become an organoleptic defect that make consumers to refuse them [3]. The origin of ethylphenols in wine aroma is due to different sources, but the most usual ways of formation are enzymatic processes of wine yeast and ageing. Some parameters like hygienic conditions of the cellar and SO_2 treatments of the barrels can avoid the formation of these volatile phenols [2]. From an enological point of view, the most important ones are 4-ethylguaiacol and 4-ethylphenol

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due to their unpleasant organoleptic properties. In fact, 4-ethylphenol, gives a horsy character to wine aroma, and 4-ethylguaiacol has smoky or spice characteristics [1,2].

Due to the aforementioned unacceptable organoleptic properties of red wines which contain certain levels of ethylphenols, some cellars and wine producers show special interest in the determination of these two compounds, because their presence in wine aroma is quite regular, and sometimes their concentration levels should be controlled in order to export their products to some foreign countries. All these issues involve the need for a method that allows the determination of both of them with accuracy. The bibliography reviewed shows generic methods of wine aroma analysis, that use mainly gas chromatography (GC) [4–6]. This separation technique is always preceded by an extraction step, which is often a liquid–liquid extraction [7], a distillation [8], a headspace technique (either static or dynamic) [9] or, recently, a solid-phase microextraction [10–13]. However, there is a lack of methodologies for the exclusive analysis of 4-ethylphenol and 4-ethylguaiacol.

Considering all these general issues, the aim of this paper is to establish a methodology to evaluate the concentration levels of 4-ethylguaiacol and 4-ethylphenol in red wine aroma. The method proposed consists of the application of solid-phase microextraction (SPME) to sample headspace (HS), subsequently performing a separation using gas chromatography with flame ionisation detection (FID). The fibres used in this method were coated with polydimethylsiloxane (PDMS) because, after a previous fibre-coating screening, the results showed that this kind of coating was the most sensitive for the extraction of phenols.

2. Experimental

2.1. Chemicals and reagents

4-Ethylguaiacol (4-ethyl-2-methoxyphenol) [2785-89-9] (98%) and *p*-cresol (internal standard, I.S.) [106-44-5] (99%) standards were supplied by Lancaster (Morecambe, UK). The 4-ethylphenol [123-07-9] (99%) standard was supplied by Sigma–Aldrich (Madrid, Spain).

The auxiliary reagents used were: ethanol HPLC grade, L-(+)tartaric acid, NaOH and NaCl, all supplied by Scharlab (Barcelona, Spain).

2.2. Preparation of standard and working solutions

Individual standard solutions of 1000 mg/l of 4-ethylphenol, 4-ethylguaiacol and *p*-cresol were prepared by direct dilution in ethanol and stored at 5 °C. Working solutions were prepared by dilution of the standard solutions, using either synthetic wine solution or commercial wines.

The synthetic wine solution was prepared by dissolving 3.5 g of L-(+) tartaric acid and 120 ml of ethanol, in a suitable amount of Milli-Q quality water to give a 1-l solution. The pH was adjusted to 3.5 with NaOH 1 M.

2.3. Equipment

2.3.1. SPME fibres

The fibres used in this study were coated with polydimethylsiloxane PDMS, (100 μm), and were supplied by Supelco (Bellefonte, USA). The holder used was for manual injection and was also supplied by Supelco.

2.3.2. Chromatography

Chromatographic experiments were performed using a Hewlett-Packard 6890 gas chromatograph with a flame ionization detector (FID). The injection port was a split-splitless one, working at 250 °C in splitless mode for 1 min, and the injector liner used was of 0.75 mm I.D. Separation was performed using a CP-WAX 57-CB (50 m×0.25 mm×0.2 μm), and the carrier gas was helium with a flow-rate of 1 ml/min.

The oven temperature program was as follows: 60 °C (1 min), 10 °C/min to 150 °C, 3 °C/min to 210 °C (30 min), while the detector temperature was set at 250 °C. The detector signals were sent to a HP

Chemstation, where they were collected, integrated and recorded.

2.4. Optimised headspace-SPME procedure

For sample preparation, 25 ml of sample (either commercial wine or synthetic wine solution) were placed into a 50-ml vial, with a magnetic stirrer and a suitable amount of NaCl to get a 6 *M* solution. Finally, the vial was hermetically sealed with a PTFE-faced silicone septum.

Before the extraction step, samples were equilibrated for 15 min at 25 °C and magnetically stirred at 300 rpm. Then, the PDMS fibre was inserted through the vial septum and exposed to the sample headspace during 60 min to perform the extraction, under the same conditions of temperature and agitation. Finally, the fibre was removed from the sample headspace and inserted into the injection port of the gas chromatograph, for thermal desorption at 250 °C during 1 min.

3. Results and discussion

3.1. SPME parameters optimisation

The SPME technique involves the optimisation of some experimental parameters that can affect the extraction procedure. Ionic strength, sample volume, stirring conditions, sample temperature, extraction time and desorption time are very important to get a high sensitivity SPME extraction, so all them were studied and optimised [13].

For all the optimisation steps, identical solutions of synthetic wine spiked with 4-ethylphenol (200 μg/l) and 4-ethylguaiacol (1000 μg/l) were analysed.

In order to obtain the best extraction results, firstly we made successive trial and error experiments using different values of desorption time, sample volume, ionic strength and speed of sample agitation [17]. The results showed that the most effective extraction conditions were obtained with 25 ml of sample in a 50-ml vial with NaCl (6 *M*) and stirred at medium agitation conditions (300 rpm). Furthermore, the experiments also showed that 1 min of fibre desorption on the GC-port (250 °C) is enough to transfer the analytes to the chromatographic column.

Finally, the extraction temperature and the extraction time were jointly optimised using an experimental design, because it is well known that when working under optimum conditions of temperature, the extraction time reduces significantly [13–15]. To study the analytes behaviour with these variables, we defined a suitable experimental domain which ranged between 30 and 120 min and 10 and 50 °C. To determine the best values of time and temperature we used a 2^2 factorial design with an additional experiment in the center of the domain [16,17]. In that way we performed five experiments with identical spiked synthetic wine solutions corresponding to the four extreme points of the experimental domain and an experiment in the center of it. To take into account the variability between different fibers coated with the same stationary phase, we studied the behaviour of two PDMS fibers, analysing different spiked wines with both fibers, and doing several replicates. The results showed values of relative standard deviation (RSD) about 10% for both phenolic compounds. To consider this variability in the method, each experiment was performed with two PDMS fibers, and solutions were analysed twice with each one, so we made four analyses for each experiment [18]. The results showed that higher temperatures and longer times gave better extraction conditions. However, when we worked with commercial wines under these conditions, some interfering compounds were extracted and their chromatographic peaks overlapped those corresponding to the volatile phenols studied. To avoid this fact, the extraction conditions were performed at 25 °C and 60 min, because these values allowed extraction of the phenolic compounds and minimized the presence of interferences due to other compounds (Fig. 1).

The ethanol content in wine also affects the extraction of the analytes, because higher concentrations of this compound give less extraction of volatile phenols [18–20]. After a previous experimental study that confirmed the theoretical results, the alcoholic grade in commercial samples was adjusted at 13% (v/v) with water or ethanol, depending on the percent (v/v) of the wine analysed, in

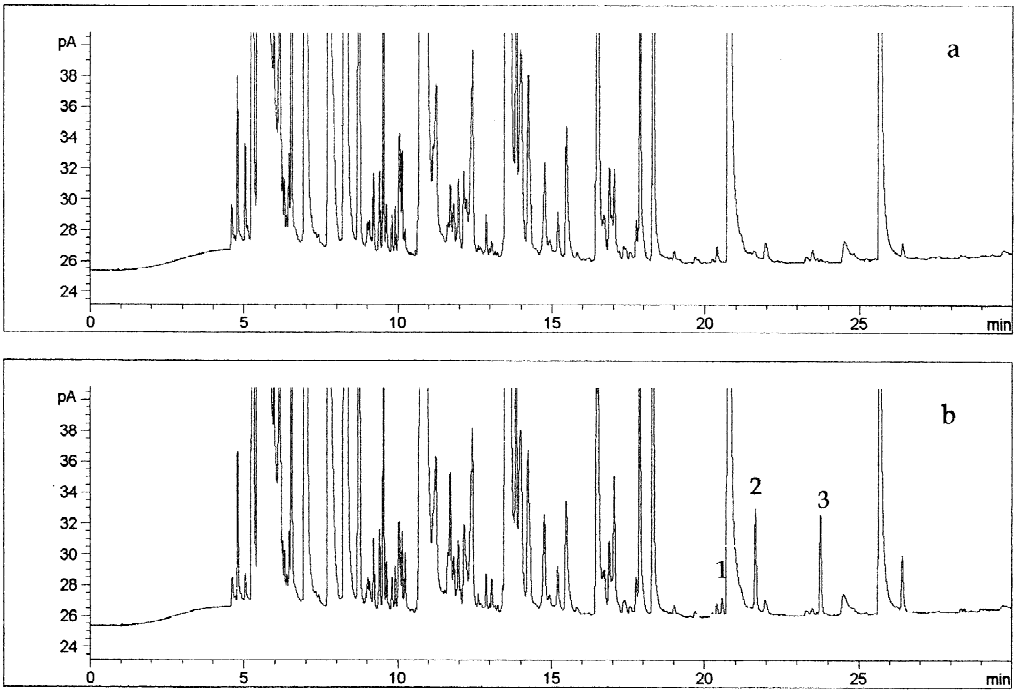

Fig. 1. (a) Chromatogram of a red wine sample analysed under the optimum conditions established (25 °C–60 min). (b) Chromatogram of the same sample, spiked with 120 μg/l of 4-ethylguaiacol (1), 2000 mg/l of *p*-cresol (I.S.) (2) and 750 μg/l of 4-ethylphenol (3), and analysed under optimum conditions.

order to reproduce the ethanol effect in every analysis.

3.2. Method validation

After the SPME optimisation, the precision and the linearity of the method were examined using the internal standard technique. For the linearity study, calibration graphs were established with synthetic wine spiked with five different concentrations of both analytes and the internal standard. Each level of concentration was analysed twice with two different PDMS fibres, so there were a total of four replicates. The concentration ranges studied were the regular ones for these compounds in red wines (4-ethylguaiacol, 40–400 μg/l; 4-ethylphenol, 200–1800 μg/l). The linearity of the responses was checked using the graphical analysis of residuals (the residuals are defined as the differences between the observed values and values predicted by the model) [21], and the slope (*a*), the intercept (*b*) and the determination coefficient (r^2) were calculated by linear least-squares regression. In both cases, a good determination coefficient was obtained ($r^2 \geq 0.980$) (Table 1).

In very complex samples, like wines, the matrix composition usually affects the extraction procedure, and the results become different when working with synthetic wine or commercial wines [22,23]. In order to determine the matrix effect, we evaluated the differences between the calibration graphs obtained with each kind of matrix. With that purpose, we analysed six commercial wines spiked with five different concentration levels in the usual ranges, using two different PDMS-fibres, and analysing samples twice with each fibre. Then, we established calibration graphs for each single wine and the slopes of each commercial wine were compared with the slope of the synthetic wine (Table 1). That comparison was performed using the ULC programme [24], that compares two different slopes using a Student *t*-test, after a previous comparison of

Table 1
Calibration graphs parameters, obtained for synthetic wine and for each commercial wine samples

	4-Ethylguaiacol						4-Ethylphenol					
	$10^3 \times a$	$10^3 \times b$	r^2	$10^4 \times S_a$ (μg/l)	$10^2 \times S_b$ (μg/l)	$t_{cal} > t_{tab}$	$10^3 \times a$	$10^2 \times b$	r^2	$10^5 \times S_a$ (μg/l)	$10^2 \times S_b$ (μg/l)	$t_{cal} > t_{tab}$
Synth. wine	2.526	0.826	0.997	1.128	1.244	–	1.453	−1.911	0.998	4.829	3.328	–
Sample 1	1.832	−4.739	0.986	1.254	1.351	Yes	1.198	−2.277	0.989	7.023	4.727	Yes
Sample 2	2.065	−11.301	0.980	1.729	1.826	No	1.164	1.710	0.993	5.269	3.547	Yes
Sample 3	1.486	−1.285	0.989	0.993	0.998	Yes	1.185	3.799	0.991	6.632	4.464	Yes
Sample 4	1.597	−2.546	0.989	0.964	1.039	Yes	1.181	−0.301	0.991	6.342	4.269	Yes
Sample 5	1.689	6.460	0.984	1.252	1.349	Yes	1.256	−0.188	0.998	3.628	2.442	Yes
Sample 6	1.685	0.012	0.990	1.116	1.202	Yes	1.202	0.210	0.997	4.160	2.800	Yes

$t_{cal} > t_{tab}$ means that there are significant differences between the slopes of commercial wine and synthetic wine ($\alpha = 0.05$).

the standard deviations of these slopes using an *F*-test.

The comparison showed significant differences between the slopes, so the presence of the matrix effect involved the use of the standard addition technique for the quantification of the analytes (Fig. 2).

Due to the matrix effect, the repeatability of the method was studied working with different matrices,

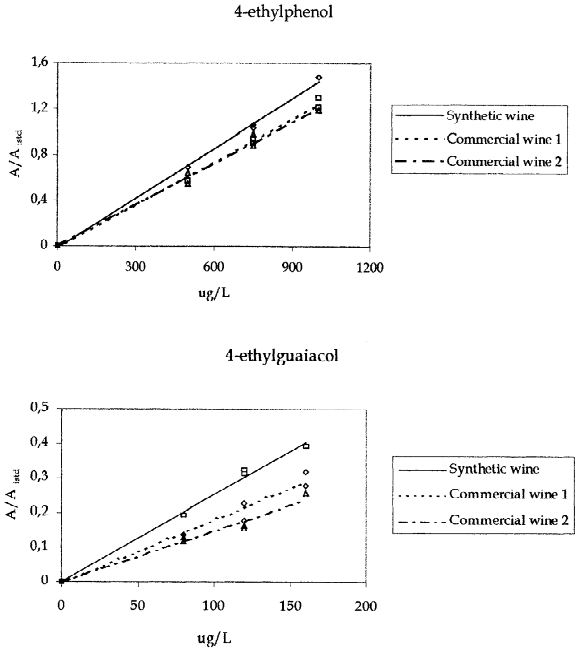

Fig. 2. Calibration graphs obtained after analysing a spiked synthetic wine and two spiked commercial wines. The figure shows the differences between the slopes of commercial wines and the slope of synthetic wine.

so we analysed six different commercial wines spiked with 120 μg/l of 4-ethylguaiacol and 750 μg/l of 4-ethylphenol. Five replicates of each wine were analysed under the optimised conditions, by the same analyst. The RSDs were calculated for each wine and the results obtained were about 10% for both analytes.

To determine the limits of detection (LOD), we analysed six different commercial wines spiked with decreasing concentration levels of the phenols studied, under optimised conditions. From the results obtained after these experiments, the LODs, defined as the amount of analyte which gives a signal three times higher than noise signal ($S/N = 3$), were established at 1 μg/l for 4-ethylguaiacol and at 2 μg/l for 4-ethylphenol. On the other hand, limits of quantification (LOQ), defined as the concentration level that gives a signal 10 times higher than noise signal ($S/N = 10$), were established about 5 μg/l for both 4-ethylguaiacol and 4-ethylphenol.

The method developed was successfully applied to different red wine samples, and the results showed the absence of both volatile phenolic compounds. These results were the expected ones because all the samples analysed were high quality products, free of aromatic defects.

4. Conclusions

The analysis of 4-ethylguaiacol and 4-ethylphenol in red wines using headspace-solid-phase microextraction technique is a very simple and fast methodology, that shows good linearity, repeatability and

detection limits. Matrix interferences were observed, so the standard addition technique should be used to quantify the analytes in order to avoid the matrix effect.

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