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# Multiple solid-phase microextraction in a non-equilibrium situation Application in quantitative analysis of chlorophenols and chloroanisoles related to cork taint in wine

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**Abstract**

Multiple HS-solid-phase microextraction (MHS-SPME) is a modification of SPME developed for quantitative analysis that avoids possible matrix effects based on an exhaustive analyte extraction from the sample. In this paper, the theory of this process associated with a non-equilibrium situation has been presented. The application of an optimised HS-SPME-based method in the analysis of chloroanisoles and chlorophenols, previously acetylated, associated with the occurrence of cork taint in different red, white and rosé wine samples, has revealed the existence of matrix effects. This fact determines the choice of standard addition as the adequate technique for the quantification of these compounds in real samples. MHS-SPME is proposed as a good alternative technique with respect to HS-SPME because it avoids matrix effects, simplifies the quantification of these compounds in real samples and reduces analysis time, providing sensitivity below chloroanisole sensory threshold with acceptable precision.


*Keywords:* Wine; Cork taint; Chloroanisoles; Chlorophenols; Multiple solid-phase microextraction; GC/MS/MS

## 1. Introduction

Multiple HS-solid-phase microextraction (MHS-SPME) is a technique based on carrying out several consecutive extractions from the same vial until all analytes are removed from the sample. After these extractions, analyte concentration will decrease exponentially and the total peak area for this compound after a complete extraction will be calculated as the sum of the areas of each individual extraction. Besides the advantages associated with SPME, such as simplicity, short preparation time and the non-use of solvents, the use of MHS-SPME enables the complete recovery of the target compounds and therefore the matrix effect, which commonly appears in SPME-based analysis, is avoided. Multiple headspace extraction (MHE) was developed by Kolb [1–5]. MHS-SPME has been described elsewhere [6,7] and applied in the determination of volatiles and BTEX in several matrices [7,8], pharmaceuticals in wine [9], odour-causing volatile organic compounds in cork stoppers [10] and 2-cyclopentyl-cyclopentanone in polyamide 6.6. [11].

Apart from the theoretical description of this technique for a specific situation, this paper presents an application of MHS-SPME in the analysis of the presence of a sensorial alteration (referred to as “cork taint”) in wine. The occurrence of this organoleptic defect in wine causes very serious financial losses for this industry [12,13]. The main compounds responsible for this alteration are various chloroanisoles, 2,4,6-tricholoanisole (TCA), 2,3,4,6-tetrachloroanisole (TECA) and pentachloroanisole (PCA), which are synthesised by fungal methylation of their corresponding chlorophenols, 2,4,6-trichlorophenol (TCP), 2,3,4,6-tetrachlorophenol (TECP) and pentachlorophenol (PCP) [12,14–16]. The presence of these contaminants in wine, which becomes tainted, stems from multiple sources related to biocide treatments of different packaging materials

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based on polychlorophenols, certain washing products used in wood barrels and several treatments included in cork stopper processing [17,18].

Chloroanisoles can damage organoleptic profile of wine when they are present at the ng/l level [14,16,19]. Therefore, most analytical methods developed for determining these compounds include a preconcentration step prior to its chromatographic analysis, generally by gas chromatography (GC) coupled to an adequate detection technique (electron-capture detection (ECD) or mass spectrometry (MS)). Since chlorophenols cannot be directly determined by GC due to their high polarity, a prior derivatisation step is recommended [20].

Liquid–liquid and solid–liquid extraction with organic solvents have been the most commonly employed methods for determining chloroanisoles and chlorophenols in wine, cork stoppers and other materials related to the wine industry [14–19,21–25]. But currently, there is a tendency to focus on the development of alternative methods such as pressurised liquid extraction [26], supercritical fluid extraction (SFE) [27], pervaporation [28,29] and perevaporation [30], solid-phase extraction (SPE) [31,32], stir bar sorptive extraction (SBSE) [33,34] and solid-phase microextraction (SPME) [31,32,35–41].

This research had two main objectives: the development and presentation of the theory related to MHS-SPME for non-equilibrium situations, which are very common when SPME is used as extraction technique, and its application for chloroanisoles and acetyl-chlorophenols quantification in wine samples in order to avoid the matrix effect shown when a HS-SPME-based method, previously optimised, was employed for their analysis in different red, white and rosé wines.

## 2. MHS-SPME theory

Two previous studies have described the theory of MHS-SPME for an equilibrium situation between the three phases of the system [7] and the theory for multiple SPME working at immersion mode for a non-equilibrium situation [6]. Similarly, it is possible to develop the theory associated with MHS-SPME in a situation where the equilibrium of analytes has not yet been achieved [6,42]. In this case, it is imperative that not only the distribution constants of the target compounds between the fibre and the sample ($K_{fs}$) and between the headspace and the sample ($K_{hss}$), and the volume of the three phases, sample ($V_s$), headspace ($V_{hs}$) and coating ($V_f$), are constant but also the rest of parameters which influence SPME extraction (agitation sample, exposition time, etc.) must be constant in each individual extraction.

The analyte mass extracted for a determined period of time ($m_{f,t}$) can be defined as:

$$m_{f,t} = m_{f,e}(1 - e^{-at}) \quad (1)$$

where $m_{f,e}$ is the mass extracted by the fibre when equilibrium has been reached and $a$ is a measure of how fast the partition equilibrium can be reached. Parameter $a$ is dependent on the mass transfer coefficient, the evaporation rate constant, the partition constant and the physical dimension of the HS-SPME system. It can be expressed as:

$$a = 2Am\frac{kK_{fs}V_f + kK_{hss}V_{hs} + kV_s}{2mK_{fhs}V_fV_s + kK_{hss}V_fV_{hs} + kV_fV_s} \quad (2)$$

where $A$ is the surface area of the SPME polymer film, $m$ the mass transfer coefficient of the analyte in the SPME polymer phase, $k$ the evaporation rate constant and $K_{fhs}$ the equilibrium partition constant of each analyte between the SPME polymer phase and the headspace gas phase. The larger the parameter $a$, the faster the partition equilibrium can be reached.

Parameter $m_{f,e}$ can be defined as follows:

$$m_{f,e} = \frac{K_{fs}V_f}{K_{fs}V_f + K_{hss}V_{hs} + V_s}m_0 \quad (3)$$

By substituting Eq. (3) into Eq. (1), the latter becomes:

$$m_{f,t} = \frac{K_{fs}V_f}{K_{fs}V_f + K_{hss}V_{hs} + V_s}m_0(1 - e^{-at}) \quad (4)$$

Eq. (4) is the base for quantitative analysis before equilibrium will be reached. If equilibrium partition constants, three phases volume, extraction time and the other SPME extraction parameters remain constant, $m_{f,t}$ can be expressed as:

$$m_{f,t} = \alpha m_0 \quad (5)$$

where $\alpha$ ($0 \leq \alpha \leq 1$) is defined as:

$$\alpha = \frac{K_{fs}V_f}{K_{fs}V_f + K_{hss}V_{hs} + V_s}(1 - e^{-at}) \quad (6)$$

Eq. (5) correlates the amount of extracted analyte with its initial concentration in the sample. There is a directly proportional relationship between them so SPME quantification is feasible before reaching a partition equilibrium once the SPME conditions and sampling time are held constant. Taking into account the expression described in Eq. (5), the mathematical development for a non-equilibrium situation is the same as described for a system in equilibrium [7].

In summary, the total area ($A_T$) corresponding to a cumulative extraction yield after multiple extraction at a specific time can be determined as the sum of the areas obtained for each individual extraction when the extraction is exhaustive or can be also expressed as follows:

$$A_T = \sum_{i=1}^{N} A_i = \frac{A_1}{1 - \beta} \quad (7)$$

where $A_i$ is the peak area obtained in the $i$th extraction, $A_1$ the peak area obtained after the first extraction and $\beta$ a constant calculated from the linear regression represented in Eq. (8):

$$\ln A_i = (i - 1)\ln \beta + \ln A_1 \quad (8)$$

## 3. Experimental

### 3.1. Reagents, standards

2,4,6-Trichloroanisole (TCA) and pentachlorophenol (PCP) were supplied by Aldrich Chemie (Steinheim, Germany), 2,3,4,6-tetrachloroanisole (TeCA) by Ultra Scientific (North Kingstown, RI, USA) and pentachloroanisole (PCA), 2,3,4,6-tetrachlorophenol (TeCP) and 2,4,6-trichlorophenol (TCP) by Supelco (Bellefonte, PA, USA). The suppliers stated purity of all standards was above 95%. Methanol, ethanol and L(+)-tartaric acid were purchased from Merck (Darmstadt, Germany). Potassium hydrogen carbonate, sodium hydroxide and acetic acid anhydride were purchased from Aldrich Chemie (Steinheim, Germany) and ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Individual stock standard solutions of each compound were prepared in methanol. They were stored in darkness at 4 °C. Diluted solutions and mixtures of the compounds were also prepared in methanol.

### 3.2. Wine samples and synthetic wine solutions

For the matrix effect study, the HS-SPME-based method was applied to different commercial wines produced in Rioja: four red wines, two white wines and two rosé wines. The absence of taint defect in these samples was checked by sensory analysis.

According to the specific characteristics of the different types of wine, two synthetic wine solutions were prepared at the laboratory by dissolving 5 g/l for red wines and 6 g/l of L(+)-tartaric acid, for white and rosé wines, in a hydroalcoholic solution (13%, v/v, ethanol). Finally, the pH of these solutions was adjusted at the mean pH of the considered samples (pH 3.6 for red wine and pH 3.1 for white and rosé wine) with NaOH.

Both real and synthetic samples were spiked with different amounts of work solutions containing the target analytes.

### 3.3. Derivatisation-HS-SPME procedure

The conditions associated with the derivatisation-HS-SPME method applied to the analysis of red and synthetic samples have been optimised in previous researches in which it was evaluated the influence on these two procedures of several relevant parameters [40,41].

Before the analysis by HS-SPME, 4 ml of samples were placed in a 20 ml headspace vial and derivatised by adding 25 mg/ml of potassium hydrogen carbonate and 34 μl/ml of acetic acid anhydride. The mixture was shaken manually for 3 min. Then, the samples were incubated at 70 °C for 15 min before 60 min extraction in the headspace of the vial with a DVB/CAR/PDMS 50/30 μm. During extraction, the coating was automatically agitated at 250 rpm. Finally, the SPME device was removed from the vial and immediately inserted into the injection port of a GC/MS system for thermal desorption during 5 min.

For calibration in the MHS-SPME study, 50 μl of aqueous chloroanisoles and acetyl-chlorophenols solutions were used. The HS-SPME procedure used to analyse calibration standards was the same as described above, the only difference being the extraction time; in this case, 30 min was selected as exposure time.

### 3.4. Equipment and chromatographic conditions

The HS-SPME-GC/MS/MS and MHS-SPME-GC/MS/MS analyses of acetyl-chlorophenols and chloroanisoles were performed with a Varian 3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a Combipal Autosampler (CTC Analytics) and connected to an ion-trap mass spectrometer (Varian Saturn 2200). Compounds were separated using a VF-5 ms capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) from Varian (Walnut Creek, CA, USA). Helium, at a flow of 1 ml/min, was used as carrier gas. Oven temperature was programmed as follows: 50 °C for 2 min, heated at 15 °C/min to 115 °C, heated to 150 °C at 3 °C/min and kept for 8 min; and finally raised to 250 °C at 15 °C/min and held for 1 min. Injection was performed in splitless mode for 2 min and then split flow was set at 30 ml/min. An inlet of 0.75 mm I.D. was used and injector temperature was fixed at 270 °C. The manifold, GC–MS interface and ion trap temperatures were set at 60, 280 and 200 °C, respectively. Mass spectra were obtained using electron impact ionization (70 eV). Precursor ions were isolated using a 3 amu isolation window and subjected to collision-induced dissociation (CID). For operating in MS/MS mode,

Table 1
Retention times and MS/MS detection parameters for acetyl-chlorophenols and chloroanisoles

| Compound | Retention time (min) | Precursor ion (*m*/*z*) | Quantification ion (*m*/*z*) | CID parameters | |
|---|---|---|---|---|---|
| | | | | Storage level (*m*/*z*) | Amplitude (V) |
| 2,4,6-TCA | 12.350 | 195 | 167 | 100 | 90 |
| 2,4,6-TCP | 15.315 | 198 | 99 | 85 | 93 |
| 2,3,4,6-TeCA | 18.016 | 246 | 201 | 110 | 95 |
| 2,3,4,6-TeCP | 21.693 | 232 | 131 | 90 | 93 |
| PCA | 26.567 | 280 | 237 | 105 | 88 |
| PCP | 28.893 | 266 | 165 | 95 | 95 |

Table 2
Slopes and their corresponding standard deviations of the linear calibration functions obtained for red wine samples and synthetic wine solution

| Compound | Red wine A $b_A \pm s_{bA}$ | Red wine B $b_B \pm s_{bB}$ | Red wine C $b_C \pm s_{bC}$ | Red wine D $b_D \pm s_{bD}$ | Synthetic wine $b \pm s_b$ |
|---|---|---|---|---|---|
| 2,4,6-TCA | 2681 ± 39 | 2758 ± 172 | 2954 ± 26 | 2955 ± 29 | 3253 ± 51 |
| 2,3,4,6-TeCA | 4127 ± 72 | 5295 ± 291 | 5193 ± 85 | 5362 ± 144 | 5368 ± 65 |
| PCA | 3162 ± 57 | 4606 ± 323 | 4206 ± 137 | 4471 ± 254 | 4644 ± 112 |
| 2,4,6-TCP | 761 ± 7 | 867 ± 23 | 948 ± 14 | 1000 ± 36 | 1027 ± 36 |
| 2,3,4,6-TeCP | 889 ± 16 | 931 ± 52 | 1359 ± 41 | 1522 ± 84 | 1153 ± 81 |
| PCP | 441 ± 10 | 366 ± 34 | 751 ± 32 | 917 ± 61 | 530 ± 34 |

the emission current was fixed at 80 μA and scan time at 0.53 s/scan. The rest of MS/MS parameters are summarised in Table 1.

## 4. Results and discussion

### 4.1. Matrix effect study

After validating an analytical procedure, a quantification approach must be chosen. The optimum method will depend on the sample matrix. In order to study the relevance of a matrix effect, the slopes of the linear calibration functions obtained for the different spiked red, white and rosé wines and for the two specific synthetic wine solutions were compared using a t-student test. Linear calibration functions were generated from the addition of the target analytes at two concentration levels (25–100 ng/l for chlororoanisoles and 80–800 ng/l for chlorophenols) within the linear range studied previously (data not shown). Each level was analysed in triplicate. Before evaluating the matrix effect for the determination of chloroanisoles and acetyl-chlorophenols in wine samples, it was necessary to compare, using a *F*-test, the estimated variance for the residuals of both lines in order to establish whether there were any significant differences between them. If this test demonstrates that both are comparable, it is possible to calculate an estimation of a pooled variance as defined in Eq. (9) and *t* value as defined in Eq. (10).

$$s^2_{b_{1,2}} = \frac{(n_1 - 2)s^2_{b_1} + (n_2 - 2)s^2_{b_2}}{n_1 + n_2 - 4} \tag{9}$$

$$t_{cal} = \frac{b_1 - b_2}{s^2_{b_{1,2}}\left(1/\sum (x_{i1} - \bar{x}_1)^2 + 1/\sum (x_{i2} - \bar{x}_2)^2\right)^{1/2}} \tag{10}$$

If, on the contrary, there are significant differences between them, that the abovementioned *t* value can be calculated using Eq. (11) for the *t*-student test. In this case, $t_{cal}$ is compared to a critical *t* value defined in Eq. (12).

$$t_{cal} = \frac{b_1 - b_2}{(s^2_{b_1} + s^2_{b_2})^{1/2}} \tag{11}$$

$$t = \frac{t_1 s^2_{b_1} + t_2 s^2_{b_2}}{s^2_{b_1} + s^2_{b_2}} \tag{12}$$

The results summarised obtained for chloroanisoles and chlorophenols in red wine samples are shown in Table 2. All the results of the statistic tests are not included in this paper because they are too long. According to statistical data analysis, certain conclusions may be drawn. The characteristic behaviour for each group of compounds was quite similar. In the determination of chloroanisoles, the matrix effect appeared when the slope for red wine A was compared with the synthetic solution slope. The same occurred for TCA when this compound was analysed in every red wine. The special behaviour with respect to red wine A is coherent with the results obtained in the comparison of the different red wine sample slopes. Statistically significant difference were only observed between the slope of red wine A and other red wine slopes. In the case of chlorophenols, significant differences were not only observed in the comparison between associated slopes and the different red wine samples with synthetic solution but also in the comparison between different red wines.

Data obtained for chloroanisoles and chlorophenols determination in white and rosé wines are shown in Table 3. The conclusions that could be drawn in respect of the white and rosé wines were similar to those for the red wine. From the comparison between each individual wine slope with the synthetic solution slope and the comparison between slopes associated with the different samples, the matrix effect was

Table 3
Slopes and their corresponding standard deviations of the linear calibration functions obtained for white and rosé wine samples and synthetic wine solution

| Compound | White wine A $b_A \pm s_{bA}$ | White wine B $b_B \pm s_{bB}$ | Rosé wine C $b_C \pm s_{bC}$ | Rosé wine D $b_D \pm s_{bD}$ | Synthetic wine $b \pm s_b$ |
|---|---|---|---|---|---|
| 2,4,6-TCA | 3101 ± 33 | 3010 ± 32 | 3059 ± 56 | 2081 ± 12 | 3161 ± 48 |
| 2,3,4,6-TeCA | 5311 ± 46 | 5276 ± 51 | 4844 ± 28 | 3378 ± 89 | 4581 ± 33 |
| PCA | 4375 ± 122 | 4285 ± 73 | 3712 ± 98 | 2362 ± 94 | 3532 ± 44 |
| 2,4,6-TCP | 1022 ± 12 | 1018 ± 8 | 824 ± 6 | 872 ± 27 | 1020 ± 10 |
| 2,3,4,6-TeCP | 1491 ± 44 | 1473 ± 17 | 1116 ± 26 | 1174 ± 39 | 1136 ± 15 |
| PCP | 784 ± 31 | 749 ± 20 | 576 ± 26 | 544 ± 18 | 516 ± 11 |

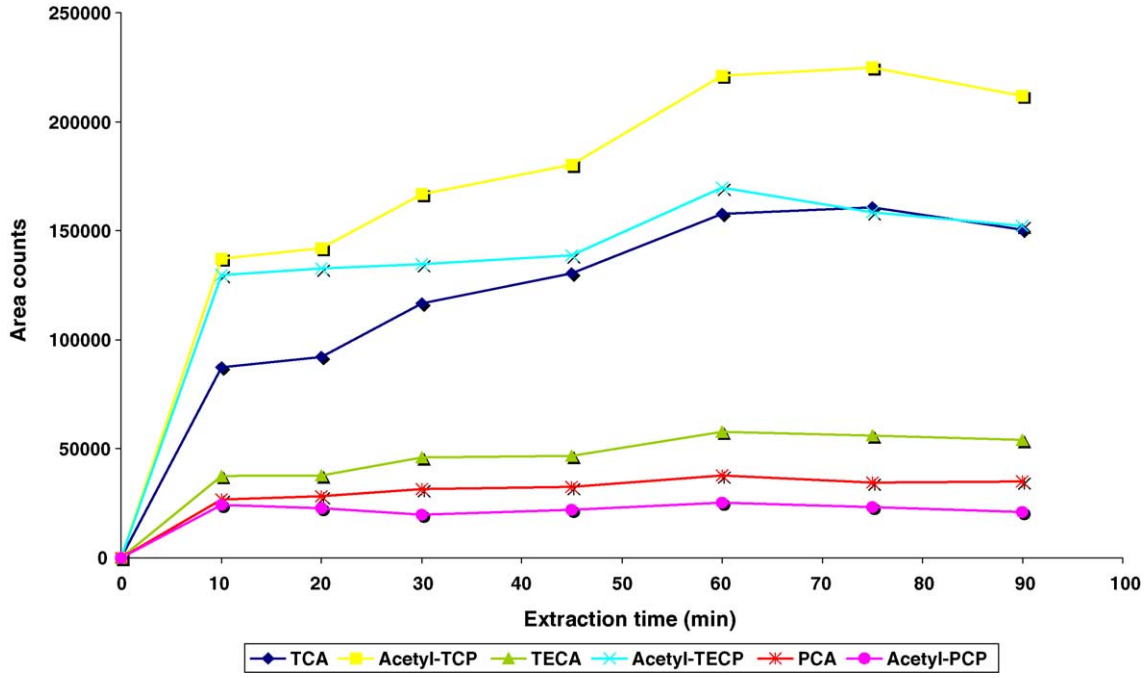


Fig. 1. Extraction time profile obtained for a standard solution analysed by HS-SPME at 70 °C using a 50/30 μm DVB/CAR/PDMS fibre.

demonstrated in most of the cases, with certain exceptions which did not correspond to a specific group of compounds.

In conclusion, the standard addition technique is proposed as the most suitable quantification method for the simultaneous determination of chloroanisoles and chlorophenols in red, white and rosé wines by HS-SPME.

### 4.2. MHS-SPME

As described in the previous study, matrix effect appeared in the determination of acetyl-chlorophenols and chloroanisoles in different wine samples by HS-SPME under the conditions described in the experimental section. MHS-SPME is proposed as a suitable alternative in order to avoid this.

MHS-SPME is a modification of the usual HS-SPME. The principle of the MHS-SPME procedure is based on a stepwise extraction at equal time intervals. If carried out until exhaustive extraction, the various peaks areas for a certain compound must be summed up in order to achieve a total area which corresponds to the total amount of that compound in the sample vial and which is therefore independent from its distribution between the three phases. Apparently, the influence of the sample matrix on the phase equilibrium is completely eliminated [2].

#### 4.2.1. Selection of extraction time for the analysis of calibration solutions

In order to study the influence of exposition time in the extraction of calibration solutions and select the optimum value, 50 μl of aqueous solutions containing between 1 and 2 ng of the target compounds were placed in a headspace vial. In order to obtain time extraction profile, these standard solutions were extracted for progressively longer periods of time (0–90 min) at 70 °C with a DVB/CAR/PDMS 50/30 μm under coating agitation. These conditions were the optimised conditions for chloroanisoles and chlorophenols in wine samples. The results were shown in graphs that included a comparison of the peak area against extraction time (Fig. 1). Thirty minutes was considered an adequate value because,

Table 4
Linearity, correlation coefficients, LOD and LOQ and recovery study of the MHS-SPME-GC/MS/MS proposed method

| Compound | Linear range (ng) | Correlation coefficient ($r$) | Slope ± $S_m$ | Intercept ± $S_b$ | LOQ S/N = 10 (ng) | LOD S/N = 3 (ng) | Average recoveries (%RSD) spiked amount 0.5 ng |
|---|---|---|---|---|---|---|---|
| 2,4,6-TCA | 0.060–18.363 | 0.9946 | 73828 ± 1245 | −22965 ± 12073 | 0.035 | 0.011 | 102.35 (4.56) |
| 2,3,4,6-TeCA | 0.056–16.921 | 0.9921 | 32179 ± 659 | −9087 ± 5884 | 0.020 | 0.006 | 97.42 (7.35) |
| PCA | 0.055–16.752 | 0.9941 | 23817 ± 422 | −4183 ± 3733 | 0.055 | 0.016 | 98.38 (5.27) |
| 2,4,6-TCP | 0.112–18.095 | 0.9954 | 40657 ± 633 | −13219 ± 5991 | 0.015 | 0.004 | 91.27 (8.15) |
| 2,3,4,6-TeCP | 0.116–18.817 | 0.9939 | 33161 ± 650 | −8151 ± 4776 | 0.046 | 0.014 | 102.53 (10.14) |
| PCP | 0.260–17.325 | 0.9920 | 5645 ± 127 | −1842 ± 857 | 0.258 | 0.077 | 95.35 (9.33) |

after this time, some analytes had already reached equilibrium and the sensitivity obtained at that point for the rest of other analytes was acceptable.

*4.2.2. Method performance*

The result of the MHS-SPME procedure is an area value for each compound in the sample corresponding to the total amount of that compound in the sample vial. As is normal with the GC procedure, it must be calibrated in order to derive the relationship between this area value and the corresponding amount of that compound. In order to determine the mass range of acetyl-chlorophenols and chloroanisoles in which there was a linear relationship between the total area obtained from the MHS-SPME process and its initial amount in the sample, standard solutions at seven concentration levels were prepared and analysed in triplicate. In this case, the target compounds were exhaustively extracted from the sample after two to five extractions, depending of its concentration; hence, the total area value was calculated as the sum of the area obtained from each individual extraction. Linear ranges, regression equations and correlation coefficients are shown in Table 4. The correlation coefficients obtained ranged from 0.9920 to 0.9954, and were therefore considered acceptable in all cases.

Quantification (LOQ) and detection (LOD) limits were calculated for a signal-to-noise ratio (S/N) of 10 and 3, respectively, from the first extraction of the most diluted standard solution, close to these limits (Table 4). Detection limits associated with chloroanisoles were lower than the olfactory thresholds provided by expert tasters [43–45].

Recoveries for samples spiked with 0.5 ng of the target analytes, analysed in triplicate, have been included in Table 4. Recoveries higher than 90% were obtained for all compounds.

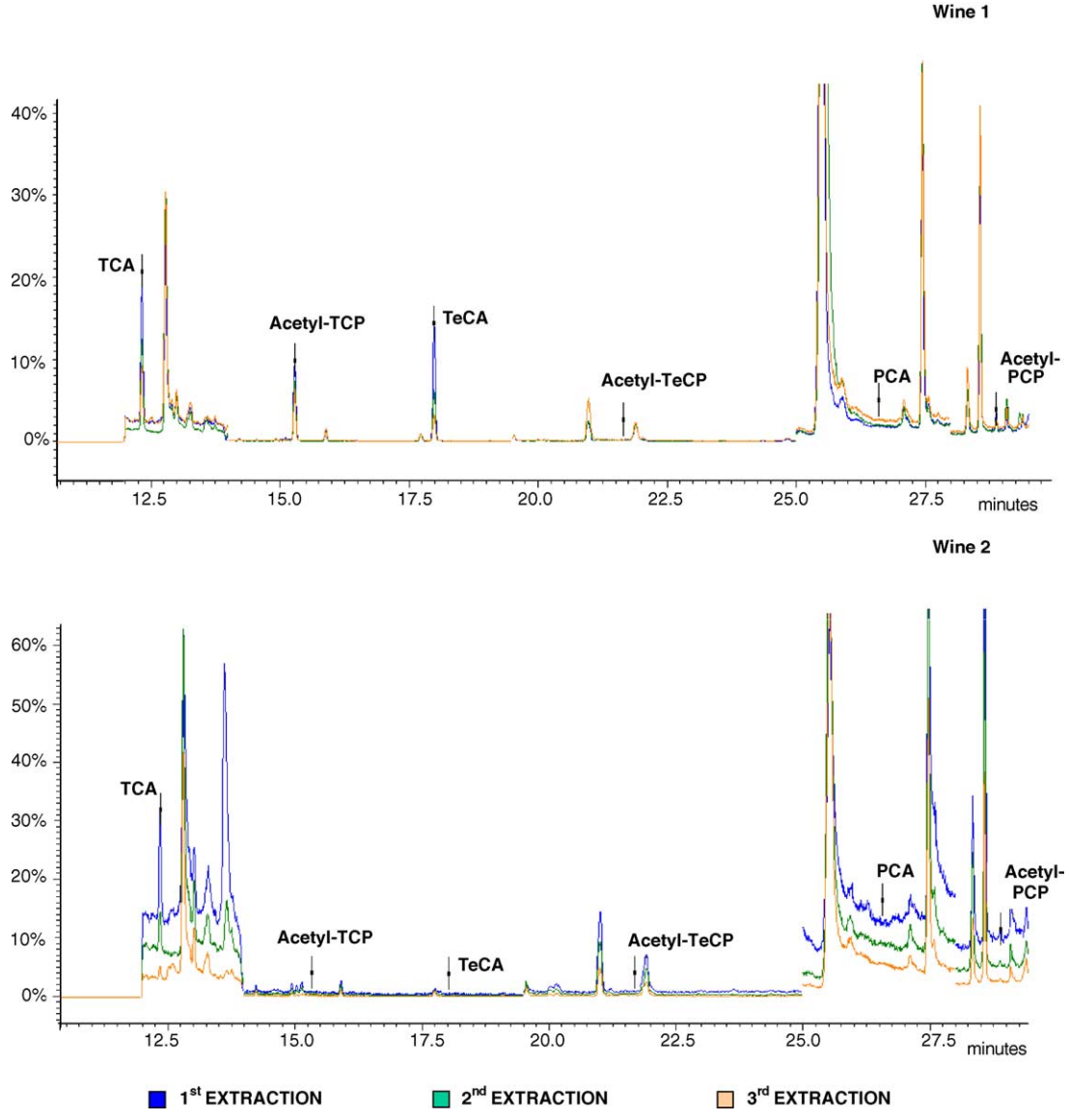


Fig. 2. Total ions chromatograms (TIC) normalised obtained after 3 consecutive extractions by HS-SPME of two-tainted red wine samples.

Table 5
Summary of repeatability study for the simultaneous determination of acetylchlorophenols and chloroanisoles in wine

| Compound | Repeatability %RSD ($n = 5$) | | |
|---|---|---|---|
| | 14 ng | 4 ng | 0.2 ng |
| 2,4,6-TCA | 2.62 | 4.07 | 4.25 |
| 2,3,4,6-TeCA | 0.80 | 6.92 | 9.37 |
| PCA | 1.20 | 7.91 | 8.96 |
| 2,4,6-TCP | 0.11 | 3.16 | 12.54 |
| 2,3,4,6-TeCP | 2.40 | 6.27 | 13.19 |
| PCP | 3.00 | 3.65 | 7.89 |

Table 6
Results of the analysis of two tainted red wine samples by MHS-SPME-GC/MS/MS proposed method ($n = 3$).

| Compound | Concentration ± SD (ng/l) | |
|---|---|---|
| | Sample 1 | Sample 2 |
| 2,4,6-TCA | 137 ± 7 | 38 ± 5 |
| 2,3,4,6-TeCA | 157 ± 16 | – |
| PCA | – | – |
| 2,4,6-TCP | 142 ± 6 | – |
| 2,3,4,6-TeCP | – | – |
| PCP | 180 ± 2 | 122 ± 7 |

The repeatability of the MHS-SPME procedure was evaluated after five consecutive analyses of standard solutions prepared at three different concentration levels. The results are shown in Table 5, expressed as relative standard deviations (RSD), and ranged from 0.11 to 13.19%. Results dispersion was higher when chloroanisoles and chlorophenols concentration was near to LOQ. However, the repeatability obtained can be considered acceptable in all cases.

#### 4.2.3. Analysis of real wine sample

Two different red wine samples, in which taint defect had been detected by sensory analysis, were analysed by the proposed MHS-SPME method, previously optimised and validated. Each sample was analysed in triplicate. In this case, three consecutive extractions were performed and the chromatograms obtained are shown in Fig. 2. The total area was calculated using Eq. (7). The chloroanisole concentrations obtained in each sample were coherent with the sensory trial (Table 6). According to this sensory analysis, sample 1 displayed a strong alteration and TCA and TeCA concentrations measured in this sample were substantially higher than its organoleptic threshold. The lower concentration of TCA measured in red wine 2 can be justified by the fact that tasters had detected the presence of the defect but only slightly.

## 5. Conclusions

According to the MHS-SPME theory presented in this paper, this technique can be successfully applied to quantitative analysis even if the partition equilibrium of the extracted analyte has not been reached. This situation appears frequently when SPME is used as extraction method.

On the other hand, to our knowledge, this research reports the first application of MHS-SPME in the analysis of chloroanisoles and chlorophenols in wine samples. Although HS-SPME has been successfully applied, the matrix effect appeared after the analysis of these compounds in different red, white and rosé wines from La Rioja, hence standard addition is required as a quantitative approach for these samples.

MHS-SPME coupled to GC/MS/MS has been evaluated and presented as an alternative to HS-SPME. The results achieved here demonstrate its suitability for removing the matrix effect, simplifying compound quantification and its potential application in the analysis of real samples in non-equilibrium situation with acceptable sensitivity and precision.

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