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Optimisation of the derivatisation reaction and subsequent headspace solid-phase microextraction method for the direct determination of chlorophenols in red wine

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Abstract

An acetylation reaction for the derivatisation of the three chlorophenols involved in cork taint was optimised using a Doehlert design for direct application in wine samples. In this first step, the optimum reaction pH, by adding different amounts of $KHCO_3$, and the required quantity of derivatisation reagent were fixed. Then a series of parameters relevant for the headspace solid-phase microextraction process, such as desorption conditions, salt addition and agitation sample were evaluated. A simultaneous study of the type of fibre and extraction temperature was performed at five levels and based on the results obtained the rest of factors (sample volume and exposition time) that could potentially affect the extraction yields were optimised by a central composite design. According to the validation of the method, we propose here, to our knowledge, the first application of solid-phase microextraction for the direct analysis of chlorophenols in red wine samples.

Keywords: Wine; Cork taint; Chlorophenols; Acetylation; Solid-phase microextraction; Experimental design

1. Introduction

Chlorophenols are used widely in industry as intermediates in the production of dyes, plastics and pharmaceuticals, and are commonly found in pulp and paper mill wastewater and as groundwater pollutants [1–3]. In the 1950s and 1960s they were used as general biocides and wood preservatives. These pesticides belong to the most important contaminants in the environment, aqueous system and soils [4], and they can represent serious health hazards due to their moderate bioaccumulation and high toxicity, which increase with the increment of chlorination [3,5]. In fact, that group of compounds is included in the US Environmental Protection Agency (EPA) list of priority pollutants and the European Union has fixed maximum concentrations for these analytes in drinking water [2,5–7]. Moreover, they cause earthy-musty off-flavour problems in water [5,8–9] and wine at trace levels.

One of the most critical problems in the enological industry is associated with a serious defect in bottled wine perceived as a musty, mouldy and earthy off-flavour [10]. The occurrence of so-called cork taint is generally related to the presence in wine of certain chlorophenols [2,4,6-trichlorophenol (TCP), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP)] and chloroanisoles [above all 2,4,6-trichloroanisole (TCA) but also 2,3,4,6-tetrachloroanisole (TeCA) and pentachloroanisole (PCA)]. The latter compounds mainly damage the organoleptic profile of the wine at trace levels and these come from the previous ones [11–13]. The presence of chlorophenols in soil and in the environment of wine cellars is associated with the use of polychlorophenolic biocides in wooden pallets, cartons and packaging material. The main constituent in these commercial preparations is PCP, together with TeCP and minor quantities of TCP. Chlorophenols may originate from the use of these biocides in cork-oak forest or bleaching in cork processing [14–15]. Moreover, hypochlorite washing of barrels can contaminate wine contained in these barrels. A chemical

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synthesis from chlorophenols and a biologically methylation of them by certain mould species as a means of detoxification [13] are the two mechanisms that account for the presence of compounds responsible for that organoleptic disorder [11–12,16].

Taking into account the widespread use of chlorophenols in industry as described above, the analysis of these compounds in several matrices is justified. As regards the presence of chlorophenols in the environment, air samples [1,17], water (river and seawater [2,8,18,19], natural [20,21], waste [3,6,7,19,22–27] and groundwater [6–8]) and soil samples [4,28–30] have been processed. Because of their potential toxicity, numerous studies have focused on the analysis of chlorophenols in drinking water [5,9,31–33] and the study of their bioaccumulation by the processing of urine samples of sawmill workers [34]. Finally, in connection with their capacity to cause earthy-musty off-flavour in food, certain authors have studied chlorophenol contamination in wine, corks and different wooden materials obtained from wine cellars [10–16,36] or used for food transport [37,38].

Gas chromatography (GC) in combination with electron-capture detection (ECD), flame ionisation detection (FID), microwave induced plasma atomic emission detection (MIP-AED) and mass spectrometry (MS) [1,3–9,12–17, 20–30,32–40] and high-performance liquid chromatography (HPLC) associated with ultraviolet (UV) or electrochemical (ED) detection [18,19,31] have been used as analytical techniques for the determination of phenols in solid and liquid matrices. The main difference between GC and HPLC is the fact that chlorophenols cannot be directly analysed when GC is used. Because of their high polarity, these compounds can hydrogen bond with GC column stationary phase, which results in a tendency to give broad and tailed peaks, making it necessary to perform a prior derivatisation step [41]. The final aim of this reaction consists in a transformation of chlorophenols into less polar compounds thus improving their chromatographic performance. As result of derivatisation reactions, chlorophenols may be transformed into the corresponding esters using pentafluorobenzoyl chloride [5] or acetic anhydride [1,6–8,17,20–23,25–29].

Chromatographic analysis is always preceded by a preconcentrate step in order to obtain the required sensitivity for an analytical method applied in the determination of chlorophenols. Liquid–liquid or solid–liquid extraction with organic solvents have been the most common methods selected for determining chlorophenols in water samples [20], packaging material [37,38] and for analysing chlorophenols related to cork taint in wine and cork samples [10,12–16,39]. Supercritical fluid extraction (SFE) has also been used as an extraction method [28]. Recently, there is a tendency to replace the traditional method with techniques such as solid-phase extraction (SPE) [1,2,8,17,21,25,32,33,35], solid-phase microextraction (SPME) [3–7,9,18,19,22–24,26,29–31,34], stir bar sorptive extraction (SBSE) [36] and pervaporation [40] because they are simpler, reduce preparation time and some of them do not require the use of solvents. Although SPME methods have also been applied to analyse various components, off-flavours and contaminants in wine samples (i.e. volatile components, sulphur aroma, diacetyl, methylisothiocyanate, pesticides, chloroanisoles) [42–46], to our knowledge, no SPME-based method has been applied in the analysis of chlorophenols in wine samples.

Hence, the ultimate aim of this research was to optimise and validate a headspace (HS) SPME method for the determination of the chlorophenols involved in corky taste in red wine samples. To this aim, the influence of different parameters such as desorption conditions, agitation, salting-out effect, absorption time, type of coating and extraction temperature was studied. The latter two factors were evaluated more extensively than in previous studies focused on water samples as this study also included the evaluation of the mostly recent developed coatings and a wider range of temperatures. Moreover, acetylation conditions for chlorophenol derivatisation were also optimised. This prior step was completely necessary because although previous studies had reported that optimal conditions had already been fixed at this stage, these had focused mainly on water samples. Thus, selection of the optimum amount of acetic anhydride and $KHCO_3$ was required because wine samples are much more complex and on account of the fact that acetylation is a pH-dependent reaction and the initial pH of the reaction was different.

2. Experimental

2.1. Chemicals

Pentachlorophenol (PCP) was supplied by Aldrich Chemie (Steinheim, Germany) and 2,3,4,6-tetrachlorophenol (TeCP) and 2,4,6-trichlorophenol (TCP) by Supelco (Bellefonte, PA, USA). The purity of all standards was above 95%. Sodium chloride, potassium hydrogen carbonate and acetic acid anhydride were purchased from Aldrich Chemie, methanol, ethanol and L(+)-tartaric acid from Merck (Darmstadt, Germany) and sodium sulphate anhydrous from Prolabo (Fontenay sous Bois, France).

2.2. Standard solutions and samples

Individual stock standard solutions of each chlorophenol were prepared in methanol. Work solutions used in further studies were prepared by diluting different amounts of each stock standard solution. Standard and work solutions were stored in darkness at 4 °C.

The synthetic wine used for the optimisation of chlorophenols and matrix effect study was obtained by dissolving 5 g/l of L(+)-tartaric acid in a hydroalcoholic solution (13%, v/v, ethanol) with the same pH as the wine sample.

For the HS-SPME-based method, a red wine without cork taint was spiked with different amounts of working solutions in order to prepare the samples for the different studies.

2.3. Derivatisation of chlorophenols

Aqueous acetylation was chosen as a derivatisation method because it is one of the most efficient, simplest and fastest reactions. This reaction makes the compounds less polar by replacing the hydroxyl group with an acetate group, avoiding the problems associated with chromatography and consisting in the addition of the derivatisation reagent to the aqueous sample in the presence of a base because alkaline conditions are required for transformation to take place. Optimum reaction conditions were studied by adding different amounts of $KHCO_3$ and acetic anhydride to 5 ml of a hydroalcoholic solution containing the chlorophenols in the concentrate range 39–42 μg/l. The acetic anhydride was added to 2 ml of hexane used for the extraction of acetylchlorophenols obtained. The mixture was shaken manually for 90 s allowing CO_2 to be released. Finally, the organic phase was separated, dried over anhydrous sodium sulphate and analysed in the GC–ECD system.

2.4. SPME procedure

The evaluated fibres were purchased from Supelco and coated with different stationary phases: polydimethylsiloxane (PDMS, 100 μm), polydimethylsiloxane–divinylbenzene (PDMS–DVB, 65 μm), polyacrylate (PA, 85 μm), Carboxen–polydimethylsiloxane (CAR–PDMS, 75 μm), divinylbenzene–Carboxen–polydimethylsiloxane (DVB–CAR–PDMS, 50/30 μm). Before use, they were conditioned in accordance with the manufacturer's specifications.

Preliminary studies were performed exposing the PDMS coating—the most widespread fibre—in the headspace of the vial at 60 °C. Taking into account the matrix complexity, headspace mode was selected over direct extraction in order to minimise matrix effects. Initial experiments were centre on the selection of desorption conditions and the study of the influence of agitation and salt addition. Samples were prepared by adding 5 ml of red wine, spiked with the chlorophenols considered in this study, into a 20 ml headspace vial sealed with a PTFE septum. The final concentrations in the wine samples were between 3.9 and 4.2 μg/l. Depending on the experiment, 0–2 g of NaCl was added in order to alter the ionic strength of the matrix. In some cases, the sample was stirred with a 8 mm × 3 mm stir bar at 1000 rpm. Before extraction, the vial was equilibrated for 10 min. Sampling time was fixed at 30 min.

Then, the type of fibre and extraction temperature, which are the main parameters that affect the SPME process, were studied using a screening design. To this aim, extraction was performed for 30 min and the sealed vial was immersed in a water bath at (25–100 °C). Every day, before using the fibres, a blank desorption was performed to ensure that the fibre and needles were free of contaminants.

From these previous experiments, the most suitable coating, i.e., PDMS 100 μm, and an adequate range of temperatures (55–95 °C) were chosen for the most efficient extraction of acetylated chlorophenols from red wine samples. With these results, a central composite design (CCD with spherical experimental domain) was used to optimise the temperature associated with a compromise situation for the extraction of the three compounds and the Vs (sample volume)/Vt (total volume) ratio that provided the highest response for all of them. This design had a full factorial 2^k (k = the number of factors) design to which a star or axial design was added. The length of the arms of the star was $\alpha = \sqrt{2}$. The CCD was completed with a central point used to estimate the experimental variance and the test-points. These last additional experiments are used to verify the predictive capabilities of the model by comparing the experimental results obtained for these points with the predictions of the model. The extraction time for the design experiments was set at 30 min.

Finally, the kinetics of the microextraction performed with the selected fibre and under optimum experimental conditions were studied to determine the ideal sampling time. Time extraction profiles were obtained by preparing a set of spiked wine samples and then extracting them for progressively longer periods of time from 10 to 120 min in static mode.

2.5. Equipment and chromatographic conditions

The SPME procedure was carried out using an IKAMAG RET control visc (Staufen, Germany) with heating and stirring function. After extraction, the SPME device was removed from the vial and inserted immediately into the injection port for thermal desorption during 5 min.

Chromatographic analysis was performed with a Hewlett-Packard 5890 Series II gas chromatograph equipped with a split/splitless injector, electronic pressure control in the injector and an electron capture detector. A capillary column HP-5MS (30 m × 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific (Folsom, CA, USA) was used. 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 the 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 differed according to the coating type: 280 °C for PA and CAR–PDMS and 270 °C for PDMS, PDMS–DVB and DVB–CAR–PDMS. ECD temperature was held at 260 °C.

3. Results and discussion

3.1. Doehlert design for the optimisation of chlorophenol acetylation in wine samples

The choice of the optimum amounts of acetic anhydride and $KHCO_3$ is the most critical point because for successful

Table 1
Doehlert's experimental matrix, the corresponding experimental conditions and response values for the two variables optimised

No. exp.	Coded experiments matrix		Experimental plan		Results (area counts)		
	$KHCO_3$	Acetic anhydride	$KHCO_3$ (mg/ml)	Acetic anhydride (μl/ml)	Acetyl-TCP	Acetyl-TeCP	Acetyl-PCP
1	+1	0	60	22	294440	358780	241803
2	−1	0	4	22	429791	869941	1060976
3	0.5	0.866	46	39.8	462712	970657	1229290
4	−0.5	−0.866	18	4.2	118454	75569	20723
5	0.5	−0.866	46	4.2	32381	15652	0
6	−0.5	0.866	18	39.8	462926	944803	1221946
7	0	0	32	22	481659	1023052	1276366
8	0	0	32	22	429631	841217	952168
9	0	0	32	22	444317	856896	958237

derivatisation to occur, the acetylation rate of the phenolate ion must be fast with respect to the hydrolysis rate of acetic anhydride and it depends on that selection. If the pH is too high, acetic anhydride will be destroyed before the acetylation process is complete; if pH is not high enough, reactive phenolate ion concentration will be lower, resulting in worse yields [1,25]. Some authors have proposed the use of excess base to neutralise the acid formed because of anhydride hydrolysis [21,23]. Taking into account these premises, it may be concluded that despite the fact that acetylation has been successfully applied for the analysis of chlorophenols in water samples [21,26], it has to be optimised for wine samples because the complexity of the matrix is much greater and, above all, the starting pH is lower; therefore, the optimum quantities of acetic anhydride and base are different from those required for acetylation in water.

A Doehlert design was applied in order to determine the amounts of acetic anhydride and $KHCO_3$ required for the reaction to take place with desirable, often optimal, characteristics with a minimum of experiments. Doehlert's experimental matrix, the corresponding experimental conditions and the experimental responses studied are shown in Table 1. The experiments were carried out randomly in order to minimise the effect of systematic errors. The intervals for the two factors were chosen according to the bibliography [21,26] and preliminary experiments. For that research, the design matrix consisted in nine experiments that were scattered uniformly in the space of the coded variables and located in the corners of a hexagonal polyhedron [47].

The experimental responses studied were expressed as area counts associated with the derivatised chlorophenols. Each response can be described by a second order model suitable for predicting the responses in all experimental regions; the coefficients of that postulated model were calculated on the basis of the experimental responses by least squares regression using NEMROD-W software [48]. The validity of the model chosen is justified by the correlation coefficients: 0.992 for acetyl-TCP, 0.954 for acetyl-TeCP and 0.922 for acetyl-PCP. Examination of the isoresponse curves revealed that the tendency was the same for all compounds. Fig. 1 shows the variation of area counts for derivatised PCP versus the two factors evaluated. The other response surface plots were very similar. The effect of the derivatisation reagent was more significant than the effect of $KHCO_3$. The increase in the amount of acetic anhydride produced a greater response for acetylated derivatives. However, big quantities of base decreased derivatisation efficiency. The interactions between them were not statistically significant in any case.

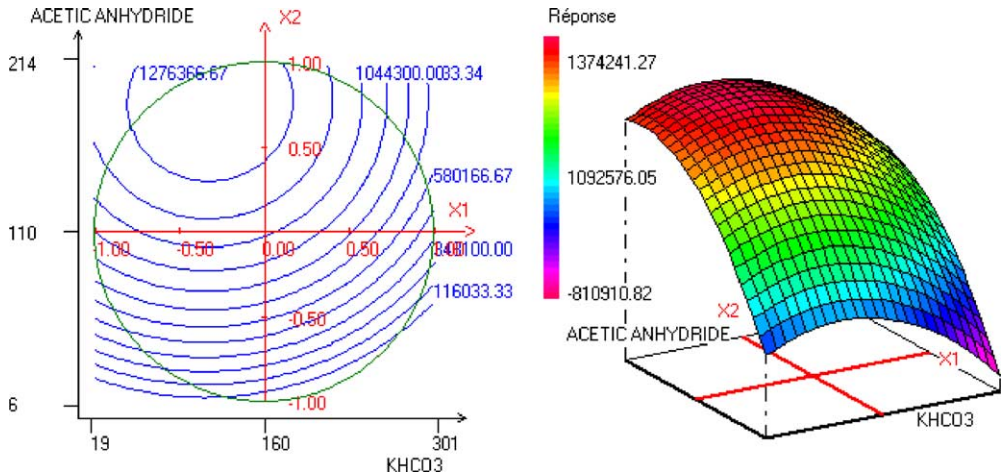

Fig. 1. Variation of area counts for acetyl-PCP vs. amount of base and derivatisation reagent.

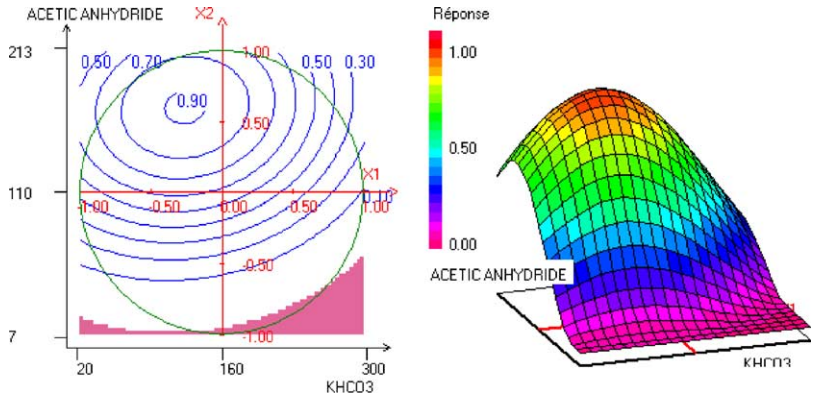

Fig. 2. Variation of the function of desirability as a function of quantities of $KHCO_3$ and acetic anhydride.

Following the analysis of design results, in order to optimise all responses under the same conditions desirability functions were built as linear partial functions for the different responses using NEMROD-W software. Global desirability represents the global quality of the compromise; this was calculated by combining single desirability functions. The optimal point indicated by the model is shown in Fig. 2 and corresponded to 25 mg/ml of base and 34 μl of acetic anhydride.

For the optimisation of derivatisation conditions, the experiments were carried out using a "synthetic and hypothetical wine solution" for mimic wine matrix. In order to guarantee that the reaction took place in the same way in wine samples with a comparable percentage of ethanol and pH, the derivatisation process for real wine samples was evaluated. Six samples of spiked wine were processed under the same conditions, previously optimised, and a repeatability study was performed. Horwitz's criterion was selected for the acceptation of the accuracy method [49]. The aforementioned theory establishes the accepted RSD for an analytical method based on the percentage of analyte in the sample. The Horwitz test was passed for every case studied. It may therefore be concluded that the proposed derivatisation method is valid for the acetylation of chlorophenols in wine samples.

3.2. Development of the HS-SPME procedure

3.2.1. Desorption conditions

Temperature and time desorption are important factors to take into account during SPME method development in order to avoid the carryover effect. After absorption, analytes were desorpted in the centre of the hot injector zone at the centre of the insert for 5 min (but only 2 min of splitless). Temperature desorption was fixed according to the fibre used and coincided with the highest allowable coating temperature. It was ensured that these parameters guaranteed the complete desorption of the analytes.

3.2.2. Agitation sample

It is well known that sample agitation is a factor that influences microextraction because it can foster the transfer of analytes from the sample matrix to the fibre coating, but this does not always occur. Magnetic stirring was selected for sample agitation and each experiment was performed in triplicate. Fig. 3 shows the differences between the efficiency of extraction obtained with and without agitation. From the one-factor analysis of variance (ANOVA) performed in order to evaluate stirring influence (data not shown) it can be concluded that agitation does not have a significant effect on the acetyl-chlorophenol responses of interest. Therefore, in subsequent experiments, wine samples were not stirred.

3.2.3. Salting-out effect

Salt addition is another parameter that must be taken into account because the enhancement of ionic strength of the matrix brings a decrease of the analyte solubility increasing, therefore, the sensitivity of the analytical method. The influence of this factor was evaluated at three different levels: no NaCl addition, undersaturation (1 g/5 ml spiked wine) and supersaturation (2 g/5 ml spiked wine). After performing Levene's test in order to evaluate the homogeneity of variance across the samples, an analysis of variance for salting-out effect (one-factor ANOVA) was performed. Moreover, pairwise comparisons of the means of these three groups based on the *least significant difference* (LSD) were also performed. The results are shown in Table 2. For every analyte, there were significant differences among the three evaluated levels. Salt ad-

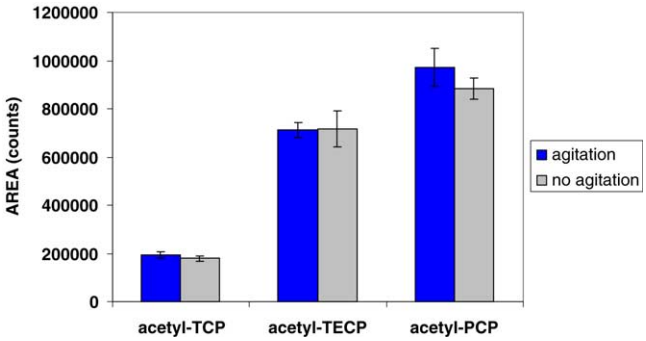

Fig. 3. Effect of agitation on the HS-SPME of 2,4,6-trichlorophenol (TCP), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) using a 100 μm PDMS fibre (n = 2).

Table 2
Influence of the addition of NaCl (expressed as area counts) on the HS-SPME of 2,4,6-trichlorophenol (TCP), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) acetylated in spiked samples of wine using a 100 μm PDMS fibre at 60 °C (n = 3)

(I)g NaCl	(J)g NaCl	Acetyl-TCP			Acetyl-TeCP			Acetyl-PCP		
		Difference between means ($I - J$)	Error	Significance	Difference between means ($I - J$)	Error	Significance	Difference between means ($I - J$)	Error	Significance
0	1	−26681[a]	7317	0.011	107925[a]	11651	0.000	243349[a]	27741	0.000
	2	−43467[a]	7317	0.001	153079[a]	11651	0.000	304241[a]	27741	0.000
1	0	26681[a]	7317	0.011	−107925[a]	11651	0.000	−243349[a]	27741	0.000
	2	−16786	7317	0.062	45154[a]	11651	0.008	60892	27741	0.071
2	0	43467[a]	7317	0.001	−153079[a]	11651	0.000	−304241[a]	27741	0.000
	1	16786	7317	0.062	−45154[a]	11651	0.008	−60892	27741	0.071

[a] α = 0.05.

dition improved significantly the sensitivity of the method for acetyl-TCP extraction and under or over-supersaturation conditions could be used because there were not significant differences between them. However for acetyl-TeCP and PCP, the addition of different amounts of NaCl above and under saturation decreased the extraction efficiency associated with these compounds. It was decided to carry out the extraction without the addition of salt.

3.2.4. Screening design for the study of the influence of type of fibre and extraction temperature

Two of the most critical parameters that affect extraction efficiency are the type of coating and extraction temperature. Previous experiments related to SPME had revealed that an interaction between the type of coating and temperature may occur, and therefore the evaluation of extraction efficiency performed with every fibre at different work temperatures was justified. A 5^2 experimental design specific to qualitative variables was chosen for this screening study of five fibres and five temperatures. The particular model associated with the design takes one of the levels as reference and the variation of the response is described with respect to that level [47]. The estimates of the coefficients are related to the selected reference state. In this work, the reference was established for the conditions type of fibre (PDMS), and temperature (60 °C). These conditions had already been proposed in one of the cited papers [26], which included the analysis of the same chlorophenols in water samples by SPME after acetylation. In this case, the screening involved two factors at five levels each one; factor A was "type of fibre" and was studied at five levels (1: PDMS–DVB, 2: PA, 3: CAR–PDMS, 4: DVB–CAR–PDMS, 5: PDMS) and factor B was "temperature" and was studied at five levels too (1: 25 °C, 2: 40 °C, 3: 80 °C, 4: 100 °C, 5: 60 °C). The proposed model is described in the Eq. (1).

$$Y = \beta_0 + \beta_{A1}(X_{A1}) + \beta_{A2}(X_{A2}) + \beta_{A3}(X_{A3}) + \beta_{A4}(X_{A4}) + \beta_{A5}(X_{A5}) + \beta_{B1}(X_{B1}) + \beta_{B2}(X_{B2}) + \beta_{B3}(X_{B3}) + \beta_{B4}(X_{B4}) + \beta_{B5}(X_{B5}) \quad (1)$$

After selecting the reference states, i.e., PDMS as coating and 60 °C as extraction temperature, the variables associated with the reference states were eliminated from the equation and the coefficients were replaced with new ones denoted by β'. That new model was called "reference state model" and is described in Eq. (2).

$$Y = \beta'_0 + \beta'_{A1}(X_{A1}) + \beta'_{A2}(X_{A2}) + \beta'_{A3}(X_{A3}) + \beta'_{A4}(X_{A4}) + \beta'_{B1}(X_{B1}) + \beta'_{B2}(X_{B2}) + \beta'_{B3}(X_{B3}) + \beta'_{B4}(X_{B4}) \quad (2)$$

The data obtained after the 25 experiments were evaluated by ANOVA with the NEMROD-W program [48]. The results are shown in Figs. 4 and 5. These bar charts compare the effect on the response caused by the change of level for the studied factor. The coefficient associated with the reference state is set at 0 and the relative effects to that level are represented in the charts. The bars length is proportional to the amplitude of the relative effect. If the bars tend to the right, the relative effect is positive; if the bars tend to the left, the relative effect is negative. Moreover, there is a broken line that shows the results of the statistic significance study for each estimate (5% significance level).

As it can be seen, when the CAR–PDMS fibre was used, the efficiency for the extraction of acetylated chlorophenols was significantly worse than that obtained when PDMS and the rest of coatings were used. This characteristic was common to the extraction of the three chlorophenols considered. However, the use of DVB–CAR–PDMS provided very different and opposite results depending on the analyte. This coating showed the best yield extraction for acetyl-TCP and the difference was significant in terms of response when PDMS was used. On the other hand, this fibre presented an affinity worse than the others for acetylated TeCP and PCP. There were not significant differences between PDMS and PDMS–DVB or PA. Note that for an efficient extraction of chlorophenols it is necessary to perform microextraction at a temperature higher than 40 °C and lower than 100 °C. The bigger the number of chlorines, the higher the temperature associated with the highest response. Extreme temper-

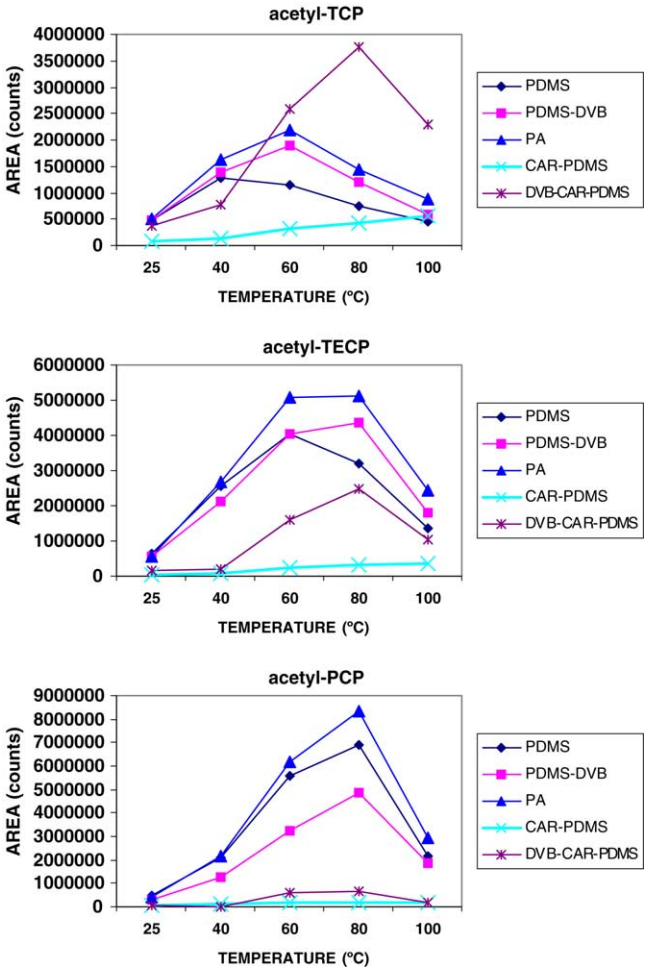

Fig. 4. Influence of type of fibre and extraction temperature on the HS-SPME process.

atures are not recommended for the efficient extraction of these compounds. SPME is an exothermic process governed by kinetics and thermodynamics mechanisms. Although extraction yield increases with the increase of temperature due to the enhancement of mass transfer, extreme temperatures decrease the efficiency of extraction due to thermodynamic reasons (decrease of distribution constant).

To conclude, the use of PDMS, PDMS–DVB or PA as coating for the extraction of acetylated chlorophenols from wine samples might be a good alternative to obtain good sensitivity results. But PDMS was selected because it had already provided good yields for the extraction of acetylated chlorophenols from water samples [26], it was a rugged liquid polymeric coating so its diffusion coefficients were higher than PA and it was not associated with any displacement effect like PDMS–DVB [50]. Based on these preliminary studies, the experimental domain was successfully reduced for a subsequent optimisation step of the sample volume and extraction temperature aimed at obtaining a compromise situation for all compounds and the exposition time.

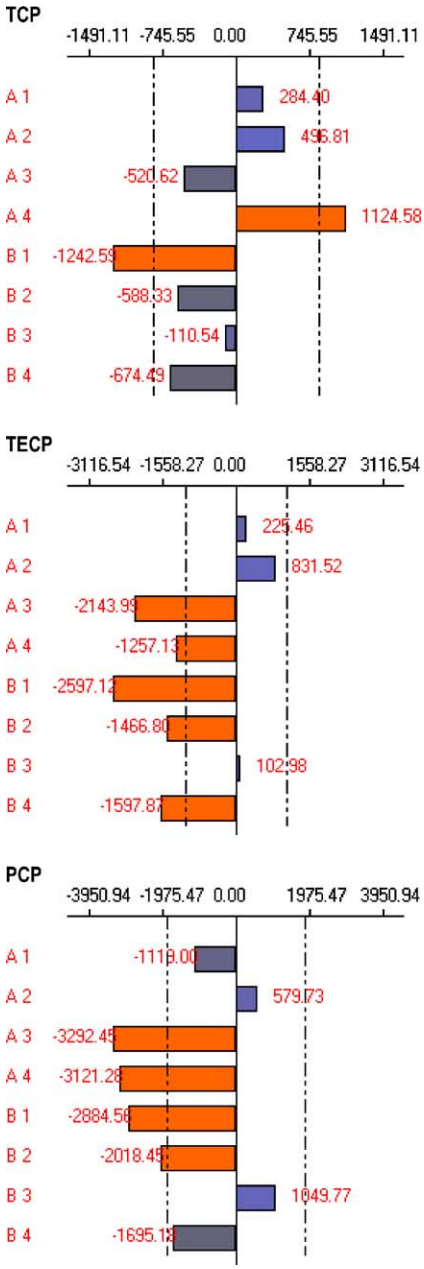

Fig. 5. Bar charts showing the comparison of the effect on the response associated with the change of level for each factor. The *x*-axis shows the relative effect to the reference state (PDMS, 60 °C) and *y*-axis represents the estimates of the model coefficients (α = 0.05): A1 (PDMS–DVB), A2 (PA), A3 (CAR–PDMS), A4 (DVB–CAR–PMS); B1 (25 °C), B2 (40 °C), B3 (80 °C), B4 (100 °C).

3.2.5. CCD (central composite design) for the optimisation of extraction temperature and Vs/Vt

Each factor was evaluated at five levels: 55–95 °C for extraction temperature and 4/20–12/20 for Vs/Vt. Thus, eight experiments, five replicates of the centre of the domain plus three test-points, were needed. These experiments were randomly carried out. The experimental matrix, experimental conditions and experimental responses studied are shown in Table 3.

Table 3
CCD experimental matrix, plan and experimental responses for the three acetylated chlorophenols

No. exp.	Coded experiments matrix		Experimental plan		Results (area counts)		
	T^a (x_1)	Vs/Vt (x_2)	T^a (°C)	Vs/Vt	Acetyl-TCP	Acetyl-TeCP	Acetyl-PCP
1	−1.000	−1.000	61	5/20	813357	2883270	3304850
2	1.000	−1.000	89	5/20	459888	1575730	2762270
3	−1.000	1.000	61	11/20	742004	2916770	3926960
4	1.000	1.000	89	11/20	426586	1527930	2831160
5	−1.429	0.000	55	8/20	822587	2216090	1390810
6	1.429	0.000	95	8/20	337286	1145840	2033220
7	0.000	−1.333	75	4/20	608306	2445970	4188330
8	0.000	1.333	75	12/20	474839	2149050	4550200
9	0.000	0.000	75	8/20	602483	2576530	3768430
10	0.000	0.000	75	8/20	547174	2410850	4558050
11	0.000	0.000	75	8/20	651223	2809530	4951380
12	0.000	0.000	75	8/20	721088	2707800	3919890
13	0.000	0.000	75	8/20	667727	2733080	4812390
14	−0.643	−0.333	66	7/20	694362	2445210	2516390
15	0.643	−0.333	84	7/20	529473	2090290	3956920
16	0.000	0.667	75	10/20	626696	2668420	4823280

Table 4
Response variables and estimates of the model coefficients for the optimisation of the HS-SPME method

Coefficient	y_1, acetyl-TCP (area counts)	y_2, acetyl-TeCP (area counts)	y_3, acetyl-PCP (area counts)
b_0	**632.4439**	**2610.0668**	**4288.7271**
b_1	**−163.2806**	**−500.9711**	28.3682
b_2	−32.0557	−28.6456	232.5783
b_{11}	−15.8280	**−412.7862**	**−1274.5064**
b_{22}	−33.0390	−95.0975	104.8488
b_{12}	5.9641	−35.0396	−217.4553

Significant effects (5%) are printed in bold type.

The estimates of coefficients for the second-order models were calculated by least squares linear regression and these models were analysed and validated by ANOVA; the test points were included in the experimental matrix using the NEMROD-W software [48]. These points were accepted because the regressions associated with each one were significant and the analysis of variance did not reveal significant lack of fit in any case. Model coefficients for each response are shown in Table 4. The influence of the evaluated factors on the three responses was similar. The Vs/Vt ratio had no impact on the yield extraction for any compound, and thus it could be fixed at any level. 8/20 was selected for subsequent experiments. The negative values for all coefficients corresponding to factor X_1, i.e., extraction temperature, suggested that high temperatures decrease significantly the recovery rate of acetyl-chlorophenols. The optimum extraction temperature for each analyte could be determined by plotting the evolution of the three responses throughout the experimental domain (Fig. 6). Optimum extraction temperatures were 55, 65, and 75 °C for TCP, TeCP and PCP, respectively. As the considered variables showed important differences in terms of magnitude, an autoscaling of the data was proposed in order to equal the starting weight and reach a compromise situation for all the responses. After this pretreatment, the autoscaled results were plotted (graphic not shown) to establish a common favourable level. According to that, 65 °C was proposed as optimum extraction temperature for the simultaneous analysis of the three chlorophenols from red wine samples.

Table 5
Quality parameters of the proposed analytical procedure

Compound	Precision (n = 5) RSD (%)			Quantification limits (S/N = 10, μg/l)	Detection limits (S/N = 3, μg/l)
	0.025 μg/l	0.9 μg/l	3.4 μg/l		
2,4,6-TCP	2.02	6.77	5.05	0.066	0.020
2,3,4,6-TeCP	4.24	5.68	3.47	0.054	0.016
PCP	8.73	4.03	3.26	0.010	0.003

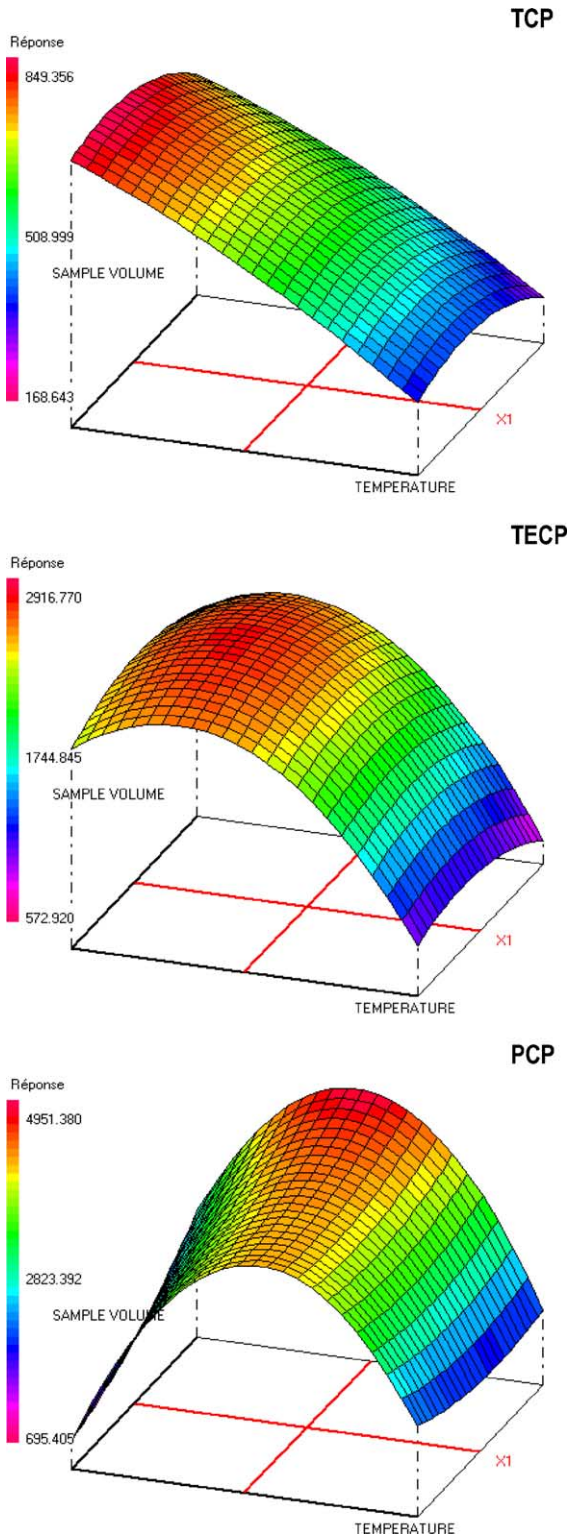

Fig. 6. Response surface for extraction temperature vs. Vs/Vt for the three acetylated chlorophenols.

3.2.6. *Extraction time profile*

For determining the effect of sampling time the results are represented in graphs that include a comparison of the peak area against the extraction time. This study was performed under the optimum conditions shown in this paper: 8 ml of wine were prepared in a 20 ml headspace vial sealed with a PTFE septum and immersed in a water bath at 65 °C after derivatisation reaction. Extraction was performed with a 100 μm PDMS for 10–120 min. Then, the fibre was immediately inserted into the injector for thermal desorption at 270 °C for 5 min. The splitless mode was used for the first 2 min. Extraction time profiles are shown in Fig. 7. Acetylated TCP and TeCP reached equilibrium after 20 and 30 min, respectively. However, the kinetics of SPME for PCP was slower and it did not reach equilibrium after 2 h. Forty-five minutes was selected as sampling time because TCP and TeCP had reached equilibrium at that point and the response associated with PCP was good enough in terms of sensitivity.

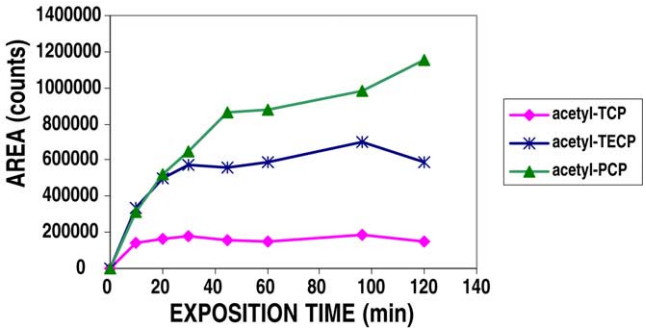

Fig. 7. Extraction time profile obtained by HS-SPME at 65 °C using a 100 μm DVB–CAR–PDMS fibre.

3.3. *Performance evaluation and validation of the proposed acetylation-HS-SPME method*

The linearity of the proposed method was evaluated by preparing spiked red wine with the three chlorophenols at five levels from 0.025 to 4.8 μg/l. These samples were analysed in triplicate. The correlation coefficients (r^2) obtained were 0.996 for acetyl-TCP and TeCP and 0.986 for PCP acetylated. Those results were acceptable taking into account that this method included a previous step of derivatisation. Quantification and detection limits shown in Table 5 were calculated for a ratio S/N of 10 and 3, respectively. The precision of the experimental procedure was evaluated at three levels. A series of five consecutive experiments provided relative standard deviations from 2.02 to 8.73% (Table 5). In order to study the importance of the matrix effect, the slopes of the standard addition curves for spiked red wine and synthetic wine were compared by a Student's *t*-test (data not shown). For all compounds no matrix effects were observed.

4. Conclusions

In this paper, the optimum concentrations of acetic anhydride and base were established using a Doehlert design for an adequate aqueous acetylation of chlorophenols in wine samples. The behaviour of the three chlorophenols in rela-

tion to this reaction was similar and the optimum amounts were the same for all of them. The amount of base required for derivatisation was virtually the same as the one proposed for chlorophenol acetylation in water samples. However, approximately eight times more derivatisation reagent was required for acetylation to take place successfully. In order to develop a method based on HS-SPME for the determination of acetylated chlorophenols in wine samples, the impact of the different parameters on extraction efficiency was studied. In general, altering the ionic strength of the matrix by adding NaCl or stirring the sample did not increase significantly the analyte responses. According to the evaluation of coating affinities for the analytes when extraction was performed at different temperatures, 100 μm PDMS was selected as the most suitable one. In these preliminary studies, the range of temperatures was successfully reduced for its further optimisation by CCD design together with the Vs/Vt ratio. The modification of the sample volume in a 20 ml headspace vial had no impact on the evaluated responses. On the other hand, extraction temperature, which had a relevant influence on the process, was fixed at 65 °C as a compromise for the different optimum temperatures associated with acetylated chlorophenols. Finally, a kinetic study under optimum experimental conditions was performed in order to set sampling time.

This research work proposes SPME as an adequate and potential alternative to the traditional technique based on liquid–liquid extraction for the direct determination of chlorophenols in wine samples. This method is free of organic solvents, simpler and faster than that used so far.

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