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Optimisation of a dispersive liquid–liquid microextraction method for the simultaneous determination of halophenols and haloanisoles in wines

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ABSTRACT

A dispersive liquid–liquid microextraction (DLLME) method has been optimised for simultaneously extracting 2,4,6-trichloranisole (TCA), 2,3,4,6-tetrachloroanisole (TeCA), 2,4,6-tribromoanisole (TBA), pentachloroanisole (PCA), 2,4,6-trichlorophenol (TCP), 2,3,4,6-tetrachlorophenol (TeCP), 2,4,6-tribromophenol (TBP) and pentachlorophenol (PCP) from wine. The haloanisoles and halophenols were automatically determined using a gas chromatography-electron-capture detection (GC-ECD) system. Derivatisation of halophenols was performed at the same time as DLLME. Firstly, disperser and extraction solvents, salt addition and temperature conditions were selected. Then, the volume of disperser solvent, extraction solvent and derivatisation agent, and the percentage of base were optimised by means of a central composite design combined with desirability functions. The optimal extraction–derivatisation conditions found were 1.3 mL of acetone, 150 μL of carbon tetrachloride, 75 μL of acetic anhydride and a percentage of base of 0.7%; with no salt addition and at room temperature. Under these conditions, the proposed method showed satisfactory linearity (with correlation coefficients over 0.994), repeatability (below 9.7%) and reproducibility (below 9.9%). Moreover, detection limits were lower than the olfactory threshold of the compounds. The developed method was successfully applied to the analysis of red wine samples. To our knowledge, this is the first time that DLLME has been applied to determine cork taint responsible compounds in wine.

1. Introduction

The enological industry needs to produce high quality wines capable of standing out in the highly competitive current market. The quality of wines is related to its sensory properties and is conditioned by the appearance of negative attributes during the several processes that take place during wine elaboration. Among the off-flavours that might appear in wine, the so-called cork taint is one of the most important problems in wine making, characterized by mouldy–musty off-flavour [1].

Haloanisoles (2,4,6-trichloroanisole (TCA), 2,3,4,6-tetrachloroanisole (TeCA), pentachloroanisole (PCA) and 2,4,6-tribromoanisole (TBA)), are the main compounds responsible for cork taint [2–6]. They originate from a defensive reaction of some microorganisms through the biomethylation of their corresponding halophenols, present in wine due to the use of polluted materials in cellars. Chlorophenols derive from reactions between lignin breakdown products and chlorinated compounds, such as chlorinated solutions used to bleach cork and washing barrels, chlorinated biocides used in oak forests, and wood preservatives [4,7,8]. Tribromophenol (TBP) is widely used as a flame retardant and fungicide, and may be formed chemically in wastewater treated with chlorine in the presence of bromide ions and traces of organic phenols [6,9,10].

The presence of haloanisoles is a great enological problem because of their extraordinary low sensory threshold and their determination along with their halophenols precursors is of great interest to the wine industry [2,4,11].

Analytical procedures for the determination of haloanisoles and halophenols usually include an extraction and/or preconcentration step, followed by the chromatographic determination of the analytes using appropriate detection techniques. Halophenols need to be derivatised prior to their chromatographic analysis due to their high polarity, which can cause broad and tailed peaks. Usually, the derivatisation procedure is aqueous acetylation because it is one of the most efficient, simplest and fastest derivatisation reactions [12–14]. As a result of derivatisation reaction, halophenols may be transformed into their corresponding esters using acetic anhydride in alkaline conditions.

Regarding the extraction step, several approaches to the quantitative determination of haloanisoles and halophenols have been

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reported. Traditionally, liquid–liquid extraction (LLE) methods have been employed for determining haloanisoles and halophenols in wines and other enological matrices [15–17]. However, LLE is time-consuming and requires large amounts of organic solvent. To solve these problems alternative methods have been applied, such as supercritical fluid extraction (SFE) [18], pressurised liquid extraction (PLE) [19], pervaporation [20], solid-phase extraction (SPE) [21–23], solid-phase microextraction (SPME) [24–27], stir bar sorptive extraction (SBSE) [28–30] and, more recently, single drop microextraction (SDME) [31].

In recent years, dispersive liquid–liquid microextraction (DLLME) has been introduced as a novel liquid–liquid microextraction method [32]. In this method, a mixture of extraction and disperser solvents is injected into the aqueous sample forming a cloudy solution, in such a way that the large contact surface between the sample and the extractant allows to perform the extraction in little time. Then, the organic extract is separated from the aqueous phase by centrifugation. This method has been successfully applied to the determination of several organic and inorganic compounds in different matrices [33–37]. Moreover, chlorophenols have been determined in water using DLLME [38,39]. In wines, DLLME has been previously used to analyse volatile phenols [40] and fungicides [41].

The aim of this study was the optimisation of a simultaneous dispersive liquid–liquid extraction–derivatisation method for determining haloanisoles and halophenols in wine. For this purpose, the different parameters affecting the whole process such as type of extraction and disperser solvents, salt addition and temperature effect were studied. The volumes of extraction solvent, disperser solvent, derivatisation agent and base were evaluated by experimental design methodology combined with desirability functions. The haloanisoles and halophenols were determined using a gas chromatography-electron-capture detection (GC-ECD) system. The selection of an adequate extraction solvent volume enabled the automatic injection of the sample, decreasing the experimental effort and saving time. Once it had been optimised, the quality parameters of the DLLME-GC/ECD method were established and it was used for the simultaneous determination of haloanisoles and halophenols in real samples. To our knowledge, this is the first time that DLLME has been used to determine haloanisoles and halophenols in wine.

2. Experimental

2.1. Chemicals

2,3,4,6-Tetrachloroanisole (TeCA) was supplied by Ultra Scientific (North Kingstown, RI, USA). 2,4,6-Trichloroanisole (TCA), 2,4,6-tribromoanisole (TBA), 2,4,6-tribromophenol (TBP), pentachlorophenol (PCP) and 4-iodoanisole (IA) (internal standard) were supplied by Aldrich Chemie (Steinheim, Germany). Pentachloroanisole (PCA), 2,4,6-trichlorophenol (TCP) and 2,3,4,6-tetrachlorophenol (TeCP) were supplied by Supelco (Belfonte, PA, USA). The purity of all standards was above 95%.

Dichloromethane, chloroform and tetrachloroethylene were supplied by Scharlau (Barcelona, Spain). Carbon tetrachloride was purchased from Aldrich Chemie and chlorobenzene and carbon disulfide from Acros Organics (Geel, Belgium). Ethanol, acetone, acetonitrile and tartaric acid were purchased from Merck (Darmstad, Germany) and acetic anhydride, potassium carbonate and sodium chloride from Aldrich Chemie.

Ultrapure water was obtained from a Mili-Q system (Milipore, Bedford, MA, USA).

2.2. Standard solutions and samples

Individual stock standard solutions of each compound were prepared in methanol at concentration level of 400 mg/L. Work solutions used for further studies were prepared by diluting different amounts of each stock standard solution. Standard and work solutions were stored in darkness at 4 °C.

Red wine without cork taint was selected for the different studies. The absence of cork taint defect in these samples was checked by sensory analysis. The synthetic wine solutions were prepared by dissolving 5 g/L of L(+)-tartaric acid in a hydroalcoholic solution (13%, v/v ethanol). The pH of these resulting solutions was adjusted to 3.5 with NaOH. Both real and synthetic samples were spiked at 200 ng/L with different amounts of work solutions containing the target analytes.

2.3. Extraction–derivatisation procedure

The extraction and derivatisation procedures were carried out simultaneously. For each DLLME analysis, an aliquot of 5 mL of spiked wine was placed in a 10 mL glass test tube with a conic bottom. Under optimised conditions, 0.5 mL of K_2CO_3 solution (final concentration 0.7%) was added to the wine sample to create the alkaline conditions required for acetylation reaction. Then, a mixture containing 1.3 mL of acetone as disperser solvent, 150 μL of CCl_4 as extraction solvent and 75 μL of acetic anhydride as derivatisation agent, was prepared immediately before injection. The extraction mixture was rapidly injected into the sample solution by using a micropipette, thus forming a cloudy solution that was stable for a long time [32]. As a result, halophenols reacted with acetic anhydride and were extracted together with haloanisoles into the dispersed fine droplets of carbon tetrachloride. Then the mixture was centrifuged for 2 min at 5000 rpm in a Rotina 38 (Hettich, Tuttlingen, Germany). The centrifugation allowed the separation of organic phase in the bottom of the conical test tube, which was then totally removed with a microsyringe and poured into a 0.15 mL glass insert that was placed into an autosampler vial to be analysed into GC-ECD system. All experiments were performed in triplicate.

2.4. Chromatographic conditions

Chromatographic analysis was performed with a Hewelett-Packard 5890 Series II gas chromatograph equipped with a splitless injector, electronic pressure control in the injector, 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 1 min, heated at 15 °C/min to 115 °C, heated to 150 °C at 3 °C/min and kept for 10 min; and finally raised to 250 °C at 15 °C/min and maintained for 4 min. For each analysis, injection of 0.5 μL of organic extract was performed in splitless mode for 1 min using an autosampler. Injector temperature was set to 250 °C. ECD temperature was kept at 300 °C.

2.5. Software

The construction and analyses of the experimental design, the response surface and the desirability functions for reaching the optimum conditions were carried out using the Nemrod-W statistical package [42].

3. Results and discussion

The optimisation of the simultaneous DLLME-derivatisation procedure involves several factors. The selection of the suitable

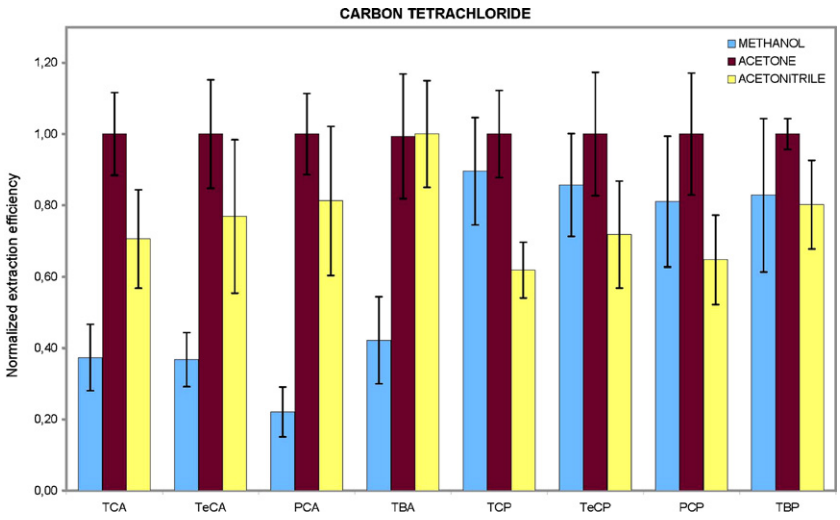

Fig. 1. Normalized extraction efficiency for the combination of carbon tetrachloride with the disperser solvents: methanol, acetone and acetonitrile ($n = 3$).

extraction and disperser solvents and their volumes is a critical step. Optimum amounts of derivatisation agent and base are needed for successful derivatisation reaction. Moreover, the addition of salt and temperature play an important role because of their effects in the extraction yield. Taking into account the high number of factors to be studied, the optimisation was performed in different steps. Firstly, the selection of disperser and extraction solvents was made. Then the study of the salting out and temperature effects was performed. Once these parameters were fixed, an experimental design was constructed to simultaneously examine the effect of the volume of disperser solvent, extraction solvent, acetic anhydride and K_2CO_3 on extraction efficiency.

Since it has been previously demonstrated that the large surface contact formed in the cloudy solution allows both extraction and derivatisation procedures to reach equilibrium in a few seconds, extraction time was not considered in this work [32].

3.1. Solvent selection

The first step in the development of a DLLME procedure is to select the appropriate extraction mixture. The extraction mixture consists of (a) an extraction solvent, responsible for extracting the analytes from the aqueous sample and (b) a disperser solvent that disperses the extraction solvent in the aqueous phase in very fine droplets, thus forming a cloudy solution and increasing the contact surface between the aqueous sample and the organic solvent.

The extraction solvent must satisfy certain requirements: it must be suitable for the extraction of the analytes of interest and have good chromatographic behaviour; besides, the extraction solvent must have higher density than water and low water solubility. Dichloromethane, chloroform, carbon tetrachloride, tetrachloroethylene, chlorobenzene and carbon disulfide are commonly used organic solvents that meet these requirements [33,34].

The main characteristic that a suitable dispersant should have is good solubility in both the aqueous phase and the organic phase. Taking this into account, methanol, acetone and acetonitrile were selected as disperser solvents.

In order to select the appropriate extraction mixture, an exhaustive study was carried out by examining all combinations of disperser and extraction solvents considered. For this purpose, 5 mL of synthetic wine, together with 0.5 mL of K_2CO_3 solution (final concentration 0.5%), and extraction mixtures consisting of 1 mL of disperser solvent and 60 μL of extraction solvent and 50 μL of derivatisation agent were used.

Phase separation was not observed for any combination of dichloromethane, probably due to its higher water solubility. When tetrachloroethylene was used as extraction solvent, a broad and tailed solvent front appeared on the chromatograms. Carbon disulfide produced a progressive increase in the background signal of the ECD system. Chloroform and chlorobenzene generated chromatograms with much interference that could not be separated from peaks corresponding to the compounds of interest. However, carbon tetrachloride is more selective and the results obtained were more reproducible. On the basis of these results carbon tetrachloride was selected as the extraction solvent. Once the extraction solvent had been selected, it was proceeded with the selection of the most suitable disperser solvent. Fig. 1 shows the normalized extraction efficiency obtained for combinations of carbon tetrachloride with methanol, acetone and acetonitrile. Regarding the halophenols, acetonitrile produced the lower extraction efficiencies. However, for haloanisoles extraction efficiency was significantly lower when using methanol as disperser solvent. For most compounds, the best results were obtained using acetone as disperser solvent so, for further experiments, the mixture of carbon tetrachloride and acetone was selected as the extraction mixture.

3.2. Salt addition

Addition of salt is an important parameter in extraction processes. Addition of salt may produce a decrease in the solubility of the extraction solvent in water thus increasing the volume of sedimented phase. On the other hand, addition of salt may also affect the solubility of analytes in the aqueous phase [43]. To determine the effect of salt addition in DLLME process, extraction efficiency was evaluated at three different sodium chloride concentrations (no addition, 5% and 10%). The volume of sedimented phase remained practically constant when increasing the amount of salt added but, as it can be observed in Fig. 2, there were not significant differences in extraction efficiency of halophenols. Nevertheless, extraction efficiency of haloanisoles strongly decreased with the addition of salt. Taking these results into account, no salt addition was carried out in further experiments.

3.3. Temperature effect

Temperature also plays an important role in extraction processes. It can affect the solubility of extraction solvent in the aqueous phase and the distribution coefficients of the analytes between the two phases [44]. To prove the effect of temperature in

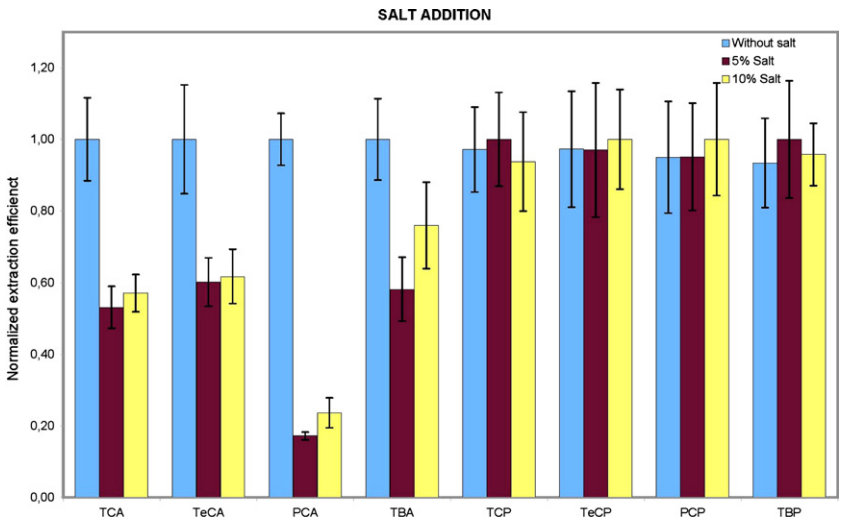

Fig. 2. Effect of salt addition on the efficiency of DLLME-derivatisation procedure for haloanisoles and halophenols ($n = 3$).

the DLLME-derivatisation process, extractions were carried out in at 0 °C, at room temperature and at 50 °C.

The sedimented phase volume slightly decreased due to the increase in the solubility between phases by raising the temperature from 0 to 50 °C. Even so, no significant differences in extraction efficiency were observed at the three temperatures (Fig. 3) except for TeCA and TeCP that clearly showed the best results at room temperature. According to these results, room temperature was considered suitable for working.

3.4. Experimental design and response surface modeling

Once the types of solvents, the salt addition and the temperature conditions have been fixed, a chemometrical approach, based on experimental design methodology, was applied to simultaneously optimise the rest of the parameters affecting DLLME. The variables considered in the DLLME-derivatisation process were: extraction solvent volume, disperser solvent volume, derivatisation agent volume and percentage of K_2CO_3). Sample volume (5 mL) was constant in all experiments.

The experimental domain was defined taking into account preliminary experiments and instrumental and operative limits. Firstly, it is necessary to bear in mind that the lower the extraction solvent volume, the lower the sedimented phase volume. In order to be able to obtain the minimum volume of organic extract needed to allow the automation of the injection, the minimum extraction solvent volume was set at 60 μL. On the other hand, if the extraction solvent volume is too high the enrichment factor strongly decreased. Thus, the maximum extraction solvent volume was established at 200 μL. In case of disperser solvent volume, it must be high enough to form the dispersion properly but an increase in its volume produced a decrease in extraction efficiency (due to an increase in the miscibility of the extraction solvent in the aqueous sample). As a result, the disperser solvent volume studied ranged from 0.5 to 2 mL. To produce the derivatisation of halophenols it was proved that they were necessary at least 30 μL of derivatisation agent. The upper value of acetic anhydride volume was set at 100 μL. Finally, base addition is necessary for successful derivatisation to occur, but it produced the formation of a precipitate. Increasing percentages of base produced an increase of this precipitate until it was not feasible to separate the sedimented phase. Consequently, percentage of base was studied from 0.25 to 1.50%.

In order to perform the optimisation, a central composite design type 2^4 plus star, involving 24 runs, 4 central points and 5 test-points, was used to determine the effect of the four experimental factors on the simultaneous DLLME-derivatisation process efficiency. This model was used to obtain the surface response fitting the data to a polynomial model, the evaluation of the effects of each factor and also the interaction effects between factors [45]. All experiments were performed randomly to minimise the effects of uncontrolled factors that may introduce bias into the measurements. Eq. (1) shows the most general function for central composite design:

$$Y = b_0 + \sum_{I=1}^{n} b_i X_i + \sum_{i=1}^{n}\sum_{j=1}^{n} b_{ij} X_i X_j \quad (1)$$

where X_i were the studied factors (X_1: disperser solvent volume; X_2: extraction solvent volume; X_3: acetic anhydride volume; X_4: percentage of K_2CO_3) and the response Y was the extraction efficiency calculated as the mathematical product of the area peak and the volume of the sedimented phase. The experimental matrix, experimental conditions and results obtained, are presented in Table 1.

The estimates of the coefficients for the second-order models of each response were calculated by least squares linear regression and these models were analysed and validated by analysis of variance (ANOVA) and the test points using Nemrod-W software [42]. For all compounds, the proposed mathematical models were significant and correctly explained the behaviour of the compounds in the experimental domain. Therefore, the models were accepted. Model coefficients for each response are shown in Table 2.

Since several interaction coefficients were significant, the effects of the factors cannot be studied separately and response surfaces must be analysed. Regarding the disperser solvent, an increase in its volume produced the increase in the extraction efficiency. However, when the volume of disperser solvent was too high, the raise on the solubility of the analytes in the aqueous phase produced a decrease in the extraction efficiency. The raise on the extraction solvent volume had a positive effect on the extraction efficiency, but at large extraction solvent volumes the extraction efficiency decreased along with the enrichment factor. The effect of acetic anhydride addition was similar for both families of compounds. The addition of acetic anhydride encouraged the derivatisation of halophenols and produced an increase in the DLLME efficiency for haloanisoles and halophenols, probably be due to the contribution of acetic anhydride on the volume of sedimented phase. When acetic anhydride volume is too high, this effect, together with the increase in the acidity of the solution caused by the

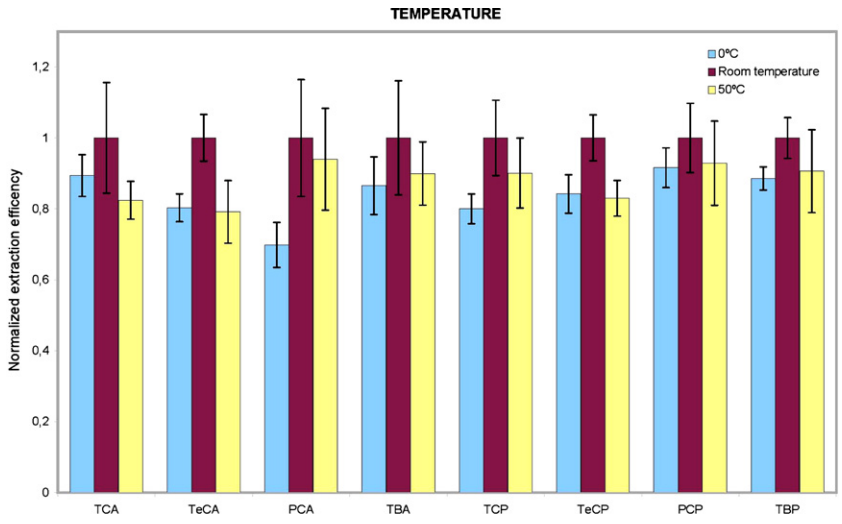

Fig. 3. Effect of temperature on the efficiency of DLLME-derivatisation procedure for haloanisoles and halophenols ($n = 3$).

hydrolysis of acetic anhydride, produced a decrease in the DLLME-derivatisation efficiency. Finally, the addition of K_2CO_3 showed opposite effects depending on the type of compounds. Extraction of haloanisoles presented better yields at lower percentages of base added, maybe due to the decrease of the sedimented phase volume, while halophenols need higher values to increase their extraction–derivatisation efficiency.

When several responses have to be optimised, it is difficult to find a common optimum for all of them. In these cases, it is necessary to find experimental conditions of compromise so that each one of the responses is within an acceptable range. For this purpose, desirability functions were included in the experimental design methodology. Desirability functions methodology consists in transforming the measured property of each response to a dimensionless partial desirability function, d_i, which varies from zero (undesirable response) to one (optimal response). The overall objective function D (Eq. (2)), representing the global desirability function is defined as the weighted geometric average of n individual desirability functions [45]. This allows optimisation to take into account the relative importance of each response, while selecting the most appropriate

Table 1
Experimental design matrix and extraction efficiency for studied compounds.

No. exp	Disperser solvent volume (mL)	Extraction solvent volume (μL)	Acetic anhydride volume (μL)	% K_2CO_3	Extraction efficiency							
					TCA	TeCA	TBA	PCA	TCP	TeCP	PCP	TBP
1	0.9	95	48	0.56	5649	8862	10,220	13,388	1473	3751	7068	3384
2	1.7	95	48	0.56	5763	9387	11,133	15,851	1208	3055	4481	2745
3	0.9	165	48	0.56	6263	10,659	11,755	17,467	1938	3735	7618	3445
4	1.7	165	48	0.56	6285	10,601	12,147	18,058	1873	4161	6933	3795
5	0.9	95	82	0.56	5616	8764	11,037	15,964	2550	5779	9557	5104
6	1.7	95	82	0.56	5683	8916	11,169	16,579	2672	5965	9720	5192
7	0.9	165	82	0.56	6830	11,866	13,145	19,424	2486	5257	8817	4715
8	1.7	165	82	0.56	6090	10,169	12,838	18,996	3270	6625	12,851	6031
9	0.9	95	48	1.19	4651	6414	8523	10,770	2966	6960	10,807	5783
10	1.7	95	48	1.19	5534	8664	10,663	14,102	3752	7406	14,535	6956
11	0.9	165	48	1.19	5570	7372	10,132	14,895	3004	6973	11,619	5427
12	1.7	165	48	1.19	6077	8971	11,344	17,062	4270	8012	17,425	7714
13	0.9	95	82	1.19	5104	7169	9410	13,696	3016	7138	11,029	5906
14	1.7	95	82	1.19	5402	8748	10,779	15,918	3912	8579	17,377	8082
15	0.9	165	82	1.19	5752	7625	11,239	17,274	2481	6880	9894	6094
16	1.7	165	82	1.19	5638	8502	11,735	17,697	4050	8854	19,056	8043
17	0.5	130	65	0.88	5396	8046	10,727	13,229	1984	6156	8481	3735
18	2.0	130	65	0.88	5739	9336	12,163	16,687	3539	7766	14,719	6603
19	1.3	60	65	0.88	4313	6686	8546	9121	2835	6140	12,479	5422
20	1.3	200	65	0.88	5869	8056	11,703	16,086	3296	7004	15,482	6313
21	1.3	130	30	0.88	5336	8544	10,482	13,121	2712	5327	13,226	4787
22	1.3	130	10	0.88	5551	8521	11,940	17,627	3758	7926	17,155	6945
23	1.3	130	65	0.25	7036	11,785	13,560	20,062	695	2123	5355	2065
24	1.3	130	65	1.50	5978	7675	9925	16,732	3668	7331	14,140	6392
Central	1.3	130	65	0.88	6068	10,090	12,386	17,773	3707	7299	15,671	7019
Central	1.3	130	65	0.88	5805	9917	12,296	17,705	3781	7626	15,806	7022
Central	1.3	130	65	0.88	6092	9967	12,464	17,582	3784	7373	16,505	6911
Central	1.3	130	65	0.88	5990	10,045	12,351	17,138	3688	7733	15,593	6809
Test	1	114	59	0.8	5917	9926	11,487	15,957	3133	6729	13,281	5674
Test	1.5	114	59	0.8	5957	10,333	12,357	16,907	3476	6953	14,717	6138
Test	0.9	164	59	0.8	6240	10,030	12,619	17,403	3514	7029	15,092	6540
Test	0.9	130	82	0.8	6103	10,059	12,735	18,411	3710	7785	16,763	7481
Test	0.9	130	65	1.19	5928	9516	11,458	16,458	4233	8380	17,676	7671

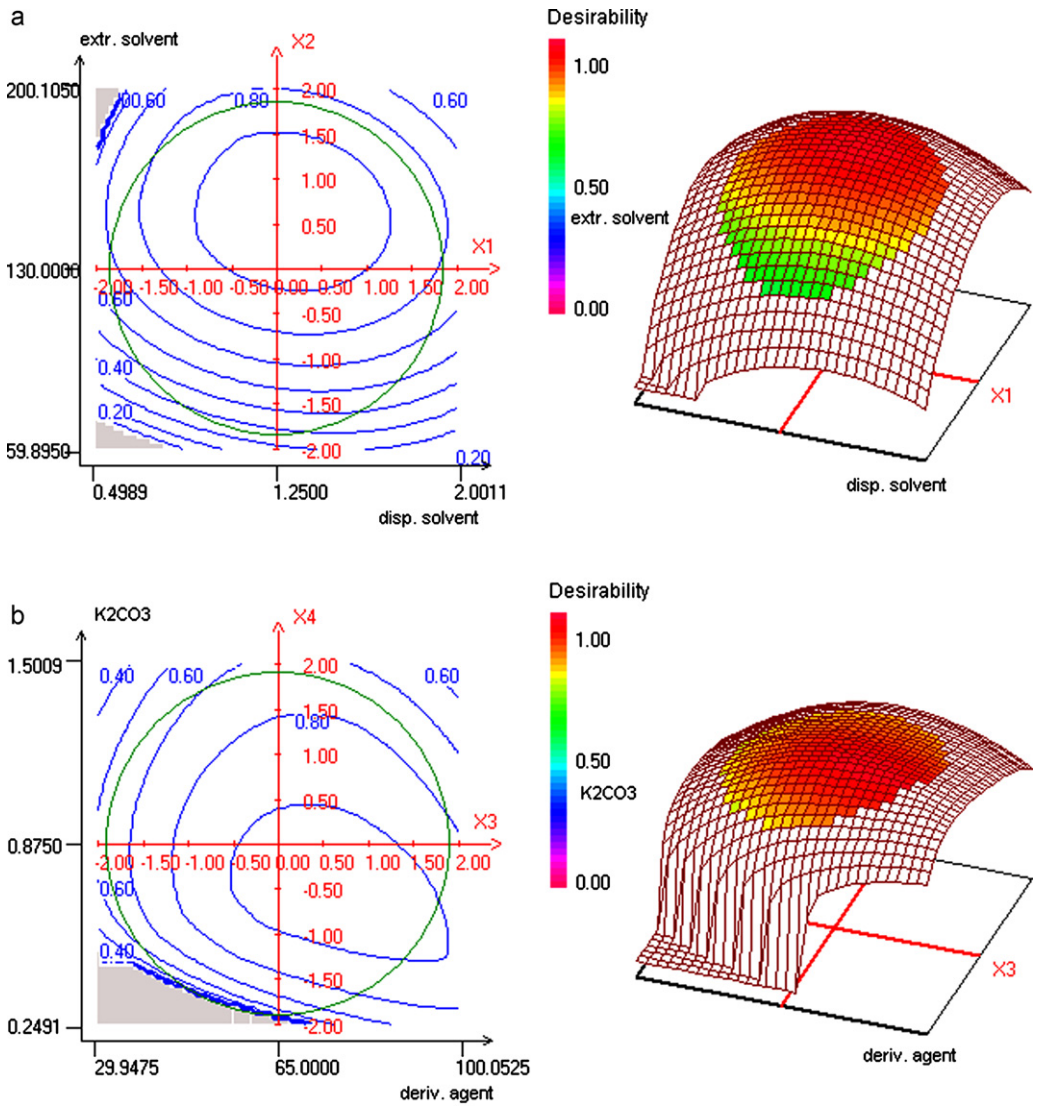

Fig. 4. Response surfaces of global desirability (a) as a function of extraction and disperser solvent with fixed volume of acetic anhydride (75 μL) and percentage of base (0.7%); (b) as a function of acetic anhydride volume and base amount with fixed volumes of extraction solvent (150 μL) and disperser solvent (1.3 mL).

form of the partial desirability functions.

$$D = \left[\prod_{i=1}^{n} d_i^{p_i}\right]^{1/n} \quad (2)$$

where p_i is the weighting of the ith, normalized so that $\sum_{i=1}^{n} p_i = 1$.

Due to their lower olfactory threshold, the most important compounds responsible for cork taint are TCA and TBA. Therefore, the weight of the partial desirability functions for TCA and TBA was

Table 2
Estimates of the model coefficients.

Coefficients	TCA	TeCA	PCA	TBA	TCP	TeCP	PCP	TBP
b_0	**6048.411**	**10151.087**	**17499.704**	**12415.997**	**3797.801**	**7613.302**	**16260.111**	**7013.812**
b_1	**66.940**	**319.118**	**744.868**	**388.563**	**343.048**	**390.928**	**1614.372**	**598.072**
b_2	**338.703**	**458.916**	**1573.566**	**735.133**	**111.861**	**149.283**	**641.421**	**171.225**
b_3	31.211	26.103	**961.350**	**349.140**	**245.893**	**672.105**	**1080.157**	**608.649**
b_4	**−275.590**	**−986.774**	**−896.119**	**−709.544**	**669.054**	**1370.881**	**2608.768**	**1179.685**
b_{11}	**−102.467**	**−299.508**	**−498.997**	**−248.901**	**−257.549**	**−164.958**	**−1404.148**	**−426.906**
b_{22}	**−224.018**	**−645.561**	**−1109.644**	**−581.145**	**−183.239**	**−262.551**	**−816.143**	**−246.127**
b_{33}	**−134.749**	**−351.980**	**−402.748**	**−307.512**	**−144.217**	**−251.435**	**−502.456**	**−238.408**
b_{44}	**129.619**	−40.191	**334.233**	**−179.684**	**−400.802**	**−724.235**	**−1857.584**	**−653.221**
b_{12}	**−101.987**	**−232.465**	**−355.163**	**−175.635**	**124.985**	**215.014**	**658.072**	**196.440**
b_{13}	**−123.479**	**−209.797**	**−349.050**	**−187.628**	**102.424**	**235.090**	**834.350**	**149.000**
b_{23}	−4.256	29.540	−117.962	109.135	**−95.549**	−96.884	−230.448	−61.503
b_{14}	**133.773**	**463.732**	**313.018**	**253.810**	**245.850**	**226.385**	**1502.746**	**405.547**
b_{24}	−24.441	**−359.244**	29.621	−77.071	**−93.130**	−36.899	−67.822	−66.519
b_{34}	−12.079	28.602	95.661	−27.563	**−311.679**	**−425.118**	**−746.211**	**−344.946**

Bold numbers indicate significant effects (5%).

Table 3
Correlation coefficients, repeatability, reproducibility, detection and quantification limits of the proposed method.

Compound	Correlation coefficient R^2	Repeatability RSD%			Reproducibility RSD%			LOD S/N = 3 (ng/L)	LOQ S/N = 10 (ng/L)
		25 ng/L	150 ng/L	400 ng/L	25 ng/L	150 ng/L	400 ng/L		
TCA	0.998	7.5	9.2	8.8	9.5	9.3	9.0	2.3	7.7
TeCA	0.994	9.0	9.1	9.2	9.8	9.3	9.1	2.2	7.3
PCA	0.995	9.3	9.7	8.3	9.4	9.8	9.0	2.7	9.0
TBA	0.995	8.7	8.9	9.2	9.0	9.7	9.9	2.6	8.7
TCP	0.996	9.6	9.3	9.3	9.6	9.3	9.4	3.9	13.0
TeCP	0.997	9.2	9.6	7.9	9.7	9.9	9.5	4.2	14.0
PCP	0.994	9.4	9.6	8.1	9.6	9.8	9.6	5.3	17.7
TBP	0.996	8.1	9.1	9.0	9.2	9.4	9.9	5.2	17.3

fixed at 5, while it was fixed at 1 for the rest of the studied compounds. The plots of global desirability obtained are shown in Fig. 4. Isoresponse curves showed that maximum desirability was at the centre of the experimental domain for disperser solvent volume and derivatisation agent volume; at medium–high volumes of extraction solvent and at medium–low percentages of K_2CO_3. In this zone desirability was close to 1. The optimum compromise situation was found at 1.3 mL of disperser solvent, 150 μL of extraction solvent, 75 μL of derivatisation agent and a percentage of base of 0.7%.

3.5. Validation of the method

This is the first time that DLLME has been applied to the simultaneous determination of haloanisoles and halophenols in wine samples. In order to confirm that the proposed method was suitable, it had to be evaluated. Table 3 shows the method performance obtained under optimal conditions. Linearity was evaluated at nine levels from 10 to 500 ng/L using internal standard. Over the linear range the correlation coefficients varied between 0.994 and 0.998. The precision of the DLLME method was evaluated studying reproducibility and repeatability for all the compounds at three different concentration levels. For repeatability, five extractions were performed on the same day under optimum conditions. Reproducibility was assessed by performing extractions on five different days. The RSDs for both of them are below 9.9%. Recoveries for red and white wines spiked with the target analytes, analysed in triplicate, are shown in Table 4. Recoveries higher than 84% were obtained for all compounds. Quantification and detection limits (Table 3) were calculated at the lowest concentration level for ratio S/N of 10 and 3, respectively. For haloanisoles, detection limits, ranging from 2.2 to 2.7 ng/L, were lower than their olfactory thresholds [24–26]. For all compounds detection limits were similar or even lower than those obtained with other analytical methods [26,27,31,38]. These results showed the suitability of the proposed derivatisation-DLLME procedure as a simple and fast method. The proposed method allows reducing the use of toxic organic solvents and avoiding the use of expensive devices with a limited lifetime as in SPE, SPME and SBSE methods [32].

Table 4
Recoveries of haloanisoles and halophenols in red and white wines using DLLME ($n = 3$).

Compound	Recoveries ± RSD (%)					
	Red wine			White wine		
	25 ng/L	150 ng/L	400 ng/L	25 ng/L	150 ng/L	400 ng/L
TCA	95 ± 6	93 ± 3	89 ± 5	90 ± 4	93 ± 7	91 ± 3
TeCA	89 ± 3	95 ± 6	96 ± 2	87 ± 6	89 ± 6	92 ± 2
PCA	93 ± 4	93 ± 2	90 ± 5	95 ± 4	90 ± 4	88 ± 6
TBA	96 ± 4	89 ± 3	91 ± 3	97 ± 7	95 ± 5	93 ± 5
TCP	89 ± 5	90 ± 4	91 ± 6	88 ± 8	91 ± 3	94 ± 2
TeCP	84 ± 7	95 ± 5	89 ± 3	90 ± 5	96 ± 8	94 ± 4
PCP	87 ± 6	89 ± 3	92 ± 4	91 ± 3	93 ± 4	97 ± 5
TBP	94 ± 9	93 ± 4	90 ± 7	91 ± 5	89 ± 3	87 ± 4

Table 5
Results of an analysis of commercial wine samples by the DLLME proposed method ($n = 3$).

Compound	Concentration ± SD (ng/L)		
	Sample 1	Sample 2	Sample 3
TCA	63 ± 3	58 ± 6	34 ± 4
TeCA	88 ± 6	–	110 ± 10
PCA	–	–	–
TBA	47 ± 9	–	–
TCP	–	169 ± 7	134 ± 9
TeCP	–	–	–
PCP	157 ± 5	–	184 ± 7
TBP	–	–	–

3.6. Application of the method to real samples

Once the proposed DLLME method had been optimised and evaluated, it was used to determine the content of cork taint responsible compounds in wines. Three different red wines from different origins in which taint defect had been detected were extracted under the optimised DLLME conditions and the extracts were analysed in the GC-ECD system. Each determination was made in triplicate. Fig. 5 shows the chromatogram obtained for sample 3. The results obtained with their relative standard deviation are shown in Table 5. Considering the halophenols, only TCP and PCP were

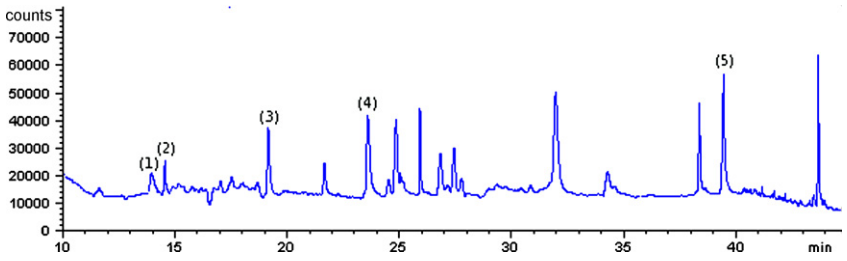

Fig. 5. GC-ECD chromatogram of sample 3. (1) 4-Iodoanisole, (2) 2,4,6-trichloroanisole, (3) 2,4,6-trichlorophenol, (4) 2,3,4,6-tetrachloroanisole, (5) pentachlorophenol.

found in these wines. All the wines presented contamination with TCA at levels above its odour threshold in wine. Sample 1, which tasters had defined as the most defective, also presented high concentrations of TBA and TeCA. Among the studied wines, sample 3 had the lowest concentration of TCA. However, tasters detected that sample 3 was strongly cork tainted, similar to sample 2, may be due to the presence of levels of TeCA higher than its organoleptic threshold.

4. Conclusions

In this study, simultaneous DLLME-derivatisation method has been optimised for the determination of haloanisoles and halophenols in wine. In order to optimise the procedure, the influence of different parameters was evaluated. Acetone and carbon tetrachloride were selected as disperser and extraction solvents, respectively. After the optimisation step, optimum conditions for the DLLME-derivatisation procedure were found at 1.3 mL of disperser solvent, 150 μL of extraction solvent, 75 μL of acetic anhydride and a percentage of base of 0.7%, at room temperature and without salt addition, with a fixed volume of wine of 5 mL. The proposed method showed satisfactory linearity, precision and detection limits. Regarding the haloanisoles, detection limits were below their olfactory thresholds. The applicability of the proposed method was demonstrated by analysing red wines contaminated with the target compounds. These results confirm the suitability of the proposed DLLME-derivatisation method for the determination of cork taint responsible compounds in wine. This technique is a simple, fast and inexpensive method that reduces the organic solvent consumption and extraction time. Moreover, the possibility of automating the injection of extracts in the chromatographic system allows the reduction of the experimental effort. To our knowledge, this is the first time that DLLME has been used to determine cork taint responsible compounds in wine and could be a suitable alternative to previously reported methods.

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