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Development of a solid-phase extraction method for the simultaneous determination of chloroanisoles and chlorophenols in red wine using gas chromatography–tandem mass spectrometry

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Abstract

A procedure for the determination of three chloroanisoles (2,4,6-trichloro, 2,3,4,6-tetrachloro and pentachloroanisol), as well as their precursor chlorophenols (2,4,6-trichloro, 2,3,4,6-tetrachloro and pentachlorophenol), involved in the presence of cork taint in red wine has been developed. Samples, up to 1 l, were concentrated using a 200 mg Oasis HLB solid-phase extraction (SPE) cartridge. Chlorophenols were quantitatively eluted from this sorbent with 3 ml of methanol. Chloroanisoles were mainly recovered in a second fraction of *n*-hexane (2 ml). Both fractions were combined and mixed with an aqueous solution of sodium bicarbonate and 50 μl of acetic anhydride. Chlorophenols were acetylated in the aqueous-methanolic phase and extracted to *n*-hexane. Chloroanisoles remained unaffected in the *n*-hexane layer. Both groups of compounds were determined by gas chromatography–tandem mass spectrometry in the same chromatographic analysis. Using a temperature programmable vaporization injector detection limits from 0.2 to 2.4 ng l^{-1}, below their sensorial threshold level in red wine, were obtained for all compounds. Average recoveries higher than 80% and acceptable precision were achieved using red wine samples spiked with the analytes at different concentration levels.

Keywords: Wine; Cork taint; Chloroanisoles; Chlorophenols; Solid-phase extraction; Gas chromatography–electron-capture detection; Gas chromatography–tandem mass spectrometry; CarboFrit™

1. Introduction

One of the most critical problems in the enological industry is associated to a serious defect in bottled wine perceived as a musty, mouldy and earthy off-flavour. The occurrence of so-called cork taint is normally 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 (2,4,6-trichloroanisole (TCA), 2,3,4,6-tetrachloroanisole (TeCA) and pentachloroanisole (PCA)), at the ng l^{-1} level [1–4]. Chloroanisoles present very low olfactory threshold levels in wine samples [2,5]. Particularly, TCA is perceivable at concentrations as low as 10 ng l^{-1} [4].

Anisoles are mainly formed due to the microbial induced methoxylation of phenols. The latter compounds can be directly or indirectly introduced in wine cellars through different sources, such as the use of wooden pallets, cartons and packing materials previously treated with polychlorophenolic biocides; the employment of chlorophenolic compounds during production of bark cork and the further elaboration of cork stoppers; the use of hypochlorite solutions in the cleaning of wooden made barrels [6,7]. Polluted bark cork, cork stoppers and different wooden materials, employed in the environment of wine cellars, may transfer the native

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pollutants, and thus the earthy–musty defect, to wine samples.

Analytical procedures for the determination of chloroanisoles and chlorophenols normally include an extraction and/or pre-concentration step followed by the chromatographic determination of the analytes using appropriate detection techniques, e.g. gas chromatography with mass spectrometry (GC–MS). Anisoles are volatile and thermally stable compounds easily separated by GC. In the case of phenols their previous derivatization is recommended in order to reduce peak tails and thus to improve the sensitivity of the method [8]. Liquid–liquid and solid–liquid extraction with organic solvents [2,9–12] and, most recently, pressurised liquid extraction [13], supercritical fluid extraction (SFE) [14], solid-phase microextraction (SPME) [15–23], stir bar sorptive extraction (SBSE) [24] and pervaporation [25,26] have been used as sample preparation techniques for the determination of chlorophenols and chloroanisoles in wine, cork stoppers and other materials related to wine elaboration.

SPE has been successfully applied to the concentration of natural components of wine [27,28]; however, from our knowledge, only two works have applied this technique to investigate the cork taint defect in wine samples [18,29]. In one case, the study was only focussed on the determination of chlorophenols in white wines and cork macerates [18], and in the other one, only TCA and TCP were considered as target compounds [29].

The final aim of this research was to develop an analytical method, based on SPE, for the simultaneous determination of the precursors, chlorophenols and the odorous compounds, the chloroanisoles, related to the presence of cork taint in red wine. After the concentration step chlorophenols were acetylated in the extract of the SPE cartridge. Both, anisoles and acetylated phenols were determined in the same injection using GC in combination with ECD or with MS–MS detection. The injection of medium sample volumes, using a programmable temperature vaporization injector, was also considered to decrease the detection limits of the method. Quantification limits of the developed method are compared to those obtained using previously reported approaches.

2. Experimental

2.1. Reagents, standards and samples

TCA, PCP and 2,4,6-tribromoanisole (TBrA) (internal standard) were supplied by Aldrich Chemie (Steinheim, Germany). TeCA was obtained from Ultra Scientific (North Kingstown, RI, USA); PCA, TeCP and TCP were purchased from Supelco (Bellefonte, PA, USA). The suppliers stated purities higher than 95% for all standards. Methanol, *n*-hexane, ethanol, acetic anhydride and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Individual standards of each compound and mixtures of them were prepared in methanol. These solutions were stored in the dark at 4 °C. Red wine without cork taint, according to sensory analysis, was spiked with different volumes of these methanolic standards to prepare the samples employed in this study.

2.2. Solid-phase extraction (SPE)

Samples were concentrated using Oasis HLB cartridges (60 and 200 mg) purchased from Waters (Milford, MA, USA). Cartridges were conditioned with methanol and Milli-Q water adjusted at pH 3.5. Volumes of 3 and 5 ml of each solvent were used for 60 and 200 mg cartridges, respectively. It was not necessary to acidify the sample because the evaluated red wine samples showed pH values below the pK_a of the three considered phenolic compounds.

Wine samples were passed through SPE cartridges at constant flow (40 ml min^{-1}). The sorbent was then dried for 30 min using a stream of nitrogen. Methanol and *n*-hexane were considered as elution solvents. In the case of 60 mg cartridges, retained analytes were desorbed with 3 ml of methanol. For the larger 200 mg ones, a further elution step using 2 ml of *n*-hexane was necessary to recover chloroanisoles.

2.3. Derivatization of chlorophenols

Acetylation was chosen as the derivatization method since it is one of the most efficient, simplest and fastest reactions for chlorophenolic species. The process was carried out at basic pH using acetic anhydride as the derivatization reagent. A previous procedure developed for water samples was adapted for the derivatization of chlorophenols in the organic extracts obtained from red wine [30,31]. In brief, 2 ml of a $KHCO_3$ solution (8.34 mg ml^{-1}), 2 ml of *n*-hexane and 50 μl of acetic anhydride were mixed with the 3 ml extract from 60 mg SPE cartridges. An aliquot of the internal standard solution was also added, and the mixture shaken manually for 2 min, allowing CO_2 to be released. Finally, the upper *n*-hexane phase containing the acetyl-chlorophenols, chloroanisoles and the internal standard was dried over anhydrous sodium sulphate and injected into the chromatographic system. For the 200 mg SPE cartridges, 3 ml of methanol followed by 2 ml of *n*-hexane were necessary to elute quantitatively the analytes from the sorbent. In this case, the $KHCO_3$ solution, the derivatization reagent and the internal standard were added directly to the combined SPE extract. After shaking the mixture, an aliquot of the *n*-hexane layer was injected in the chromatographic system.

2.4. Equipment and chromatographic conditions

Two GC systems equipped with ECD and MS–MS detectors were used in this work. In both instruments, injections were done using an autosampler device equipped with 10 μl

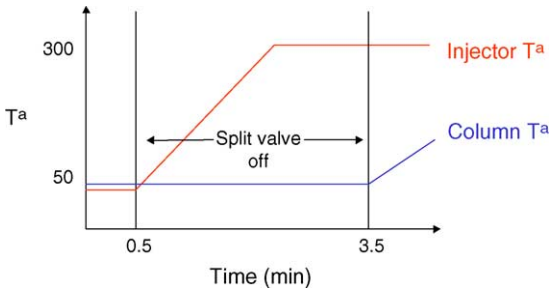

Fig. 1. Conditions for split valve, oven temperature and injector temperature for high volume injection in a CarboFrit™ packed liner.

syringes. The GC–ECD system was a HP 5890 Series II Plus gas chromatograph (Hewlett-Packard, Avondale, MA, USA) furnished with a split/splitless injection port and a ^{63}Ni electron-capture detector. Separations were carried out using a HP-5MS capillary column (30 m × 0.25 mm i.d. and 0.25 μm film thickness) acquired from J&W Scientific (Folsom, CA, USA). Helium was used as carrier gas at a constant flow of 1 ml min^{-1}. Injections (2 μl volume) were performed in the splitless mode (purge time 1 min) and the split flow was set to 30 ml min^{-1}. Oven temperature was programmed as follows: 50 °C for 1 min, heated at 15 °C min^{-1} to 115 °C, heated to 150 °C at 3 °C min^{-1} and kept for 8 min, and finally, raised to 250 °C at 15 °C min^{-1} and held for 6 min. Injector and detector temperatures were fixed to 250 and 300 °C, respectively.

GC–MS–MS analysis were carried out using a Varian 3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a programmable temperature vaporizing injector (Varian 1079) and connected to an ion-trap mass spectrometer (Varian Saturn 2200). Compounds were separated using a VF-5ms capillary column (30 m × 0.25 mm i.d. and 0.25 μm film thickness) from Varian. A split liner (3.4 mm i.d.) packed with 0.5 cm of CarboFrit™ (Restek, Bellefonte, PA, USA) was placed into the injection port. The injected volume was 9 μl. The injector temperature was programmed as follows: 52 °C for 0.5 min, heated at 100 °C min^{-1} to 300 °C and kept for 10 min. The split valve was opened until 0.5 min (split flow 50 ml min^{-1}), then closed for 3 min and finally opened again (split flow 100 ml min^{-1}) (Fig. 1). Helium at 1 ml min^{-1} was used as carrier gas. The oven temperature program was the same as in the GC–ECD system with the only difference that the initial temperature was set at 52 °C for 3.5 min. 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 window and subjected to further collision-induced dissociation (CID). Retention times and MS–MS detection parameters for acetyl-chlorophenols and chloroanisoles are shown in Table 1.

3. Results and discussion

3.1. Optimisation of solid-phase extraction

Wine samples were initially concentrated using 60 mg cartridges. Methanol was selected as elution solvent due to its compatibility with the subsequent acetylation reaction and capability to elute organic compounds from reversed-phase sorbents. Consecutive 1 ml fractions of methanol were collected from cartridges used for the concentration of spiked red wine samples (200 ml volume). Each fraction was derivatized, according to conditions described in Section 2 and injected in the GC–ECD system. Analytes were detected only in the first three fractions (data not shown). Therefore, the elution volume, for the 60 mg cartridges, was fixed to 3 ml of methanol.

The breakthrough volume of the sorbent (60 mg) was investigated using increasing volumes of spiked red wine samples (0.5, 1 and 2 l containing the same mass of analyte) passed through two cartridges connected in series. After finishing the concentration step, they were disconnected and eluted separately. Normalised responses corresponding to the fraction of each compound retained in both cartridges are given in Table 2. Up to 1 l of wine was concentrated without significant losses of the three chloroanisoles, TeCP and PCP; however, TCP showed a breakthrough volume below 500 ml. Conversely to these results, Morales and Cela have reported quantitative recoveries of TCP from 500 ml volume spiked water samples [32]. The difference between both findings could be related either to the ethanol content of red wine (up to 13%), either to the presence of organic compounds which compete with the analytes for the reversed-phase sor-

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	13.72	195	167	100	90
2,4,6-TCP	16.68	198	99	85	93
2,3,4,6-TeCA	19.39	246	203	110	95
2,4,6-TBrA[a]	22.91	346	303	110	90
2,3,4,6-TeCP	23.07	232	131	90	93
PCA	27.95	280	237	105	88
PCP	30.26	266	165	95	95

[a] Internal standard.

Table 2
Evaluation of breakthrough volumes for 60 mg Oasis HLB cartridges

Sample volume (ml)	Normalised peak areas (%)											
	TCA		TeCA		PCA		TCP		TeCP		PCP	
	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2
500	100	0	100	0	100	0	89.99	10.01	99.42	0.58	100	0
1000	99.05	0.05	99.16	0.84	100	0	80.84	19.16	96.21	3.79	99.34	0.66
2000	91.52	8.48	97.79	2.21	99.06	0.94	54.97	46.03	78.15	21.85	91.69	8.31

Normalised responses for each compound in the extracts from two cartridges sequentially connected; C1, first cartridge; C2, second cartridge.

bent. The first possibility was evaluated using ethanol–water solutions (13% of ethanol), spiked with the selected analytes, and adjusted at the same pH as wine samples. Even considering 1 l samples, all compounds were quantitatively retained in the first 60 mg SPE cartridge. Therefore, it was assumed that competitive processes were responsible for the poor retention of TCP in the sorbent.

In order to improve the enrichment factor achieved in the SPE step, a higher amount of sorbent was considered in further experiments. Using 200 mg cartridges, up to 1 l of wine could be concentrated without breakthrough problems for any compound (data not shown). Chlorophenols were recovered using 3 ml of methanol; however, up to 12 ml were required for the quantitative elution of chloroanisoles (Table 3). A re-optimisation of the elution step was therefore necessary. Three millilitres of methanol, followed by 2 ml of *n*-hexane, achieved the quantitative elution of chlorophenols and chloroanisoles. Both fractions were combined and submitted to the derivatization procedure. After that, the upper *n*-hexane layer containing anisoles and acetylated phenols was injected in the GC system. Using this approach, a 500-fold enrichment factor was achieved in the whole, extraction plus derivatization, sample preparation step.

3.2. SPE followed by GC/ECD

Detection limits (LOD), defined for a signal-to-noise ratio of 3, achieved after the concentration of 1 l wine samples using GC–ECD detection were 10 ng l^{-1} (TCA), 3 ng l^{-1} (TeCA), 8 ng l^{-1} (PCA and TCP), 20 ng l^{-1} (TeCP) and 11 ng l^{-1} (PCP). Values below odour thresholds were achieved for TeCA (odour threshold 25 ng l^{-1} in white wine [33]) and PCA (threshold of 4000 ng l^{-1} in water [34]). Also, rather low detection limits were obtained for chlorophenols. Globally, these values are in the same range than those achieved using SPME as the pre-concentration technique and GC–ECD detection: from 10 to 66 ng l^{-1} for chlorophenols [22] and between 4 and 8 ng l^{-1} for anisoles [23]. The main limitation of the proposed method is that for TCA, which plays the most critical role in cork taint, the achieved detection limit is just similar to its sensorial threshold [4].

Table 3
Normalised peak areas for chlorophenols and chloroanisoles in methanol fractions (3 ml each one) obtained from 200 mg OASIS cartridges

Compound	Normalised peak areas (%)			
	Fraction 1	Fraction 2	Fraction 3	Fraction 4
TCA	90.80	4.90	2.90	1.40
TeCA	74.00	15.50	5.50	5.00
PCA	41.20	36.40	14.30	8.10
TCP	100	–	–	–
TeCP	100	–	–	–
PCP	100	–	–	–

3.3. SPE with GC–MS–MS detection

Detection limits given in the above paragraph may be improved by injecting a larger volume of the extract from wine samples and/or by considering a more selective detection technique. Both possibilities were evaluated by using a GC–MS–MS system furnished with a programmable temperature vaporization (PTV) split/splitless injector. Optimal PTV operating parameters were investigated with standard solutions of chloroanisoles and acetyl-chlorophenols in *n*-hexane. Under working conditions described in Section 2, relative standard deviations for consecutive injections of a standard at the 25 ng ml^{-1} level remained between 2 and 5%. Moreover, a good linearity was observed in the concentration range from 0.5 to 2000 ng ml^{-1} (data not shown).

Experiments comparing the responses produced by pure standard solutions of the analytes and extracts from spiked wine samples showed a significant matrix effect. Both acetylation and extraction to the *n*-hexane phase appeared affected by the presence of co-extracted compounds in the extract of SPE cartridges. Thus, the standard addition technique was applied for quantification. Table 4 summarised the data corresponding to calibration graphs for the analytes in the whole procedure. Also, recoveries for samples spiked at two different concentration levels have been included in the last columns of Table 4. Globally, recoveries higher than 80% were obtained for all compounds. Fig. 2 depicts the GC–MS/MS chromatograms corresponding to a red wine sample spiked with the analytes at low levels, in some cases, closed to their sensory thresholds.

Detection limits of the whole method remained below 0.5 ng l^{-1} for all compounds except TCA (2.4 ng l^{-1}) (Table 5). These values are similar or even slightly lower than those previously reported using different sample concentration techniques, such as pervaporation, solid-phase microextraction and solid-phase extraction. In all cases, GC–MS or

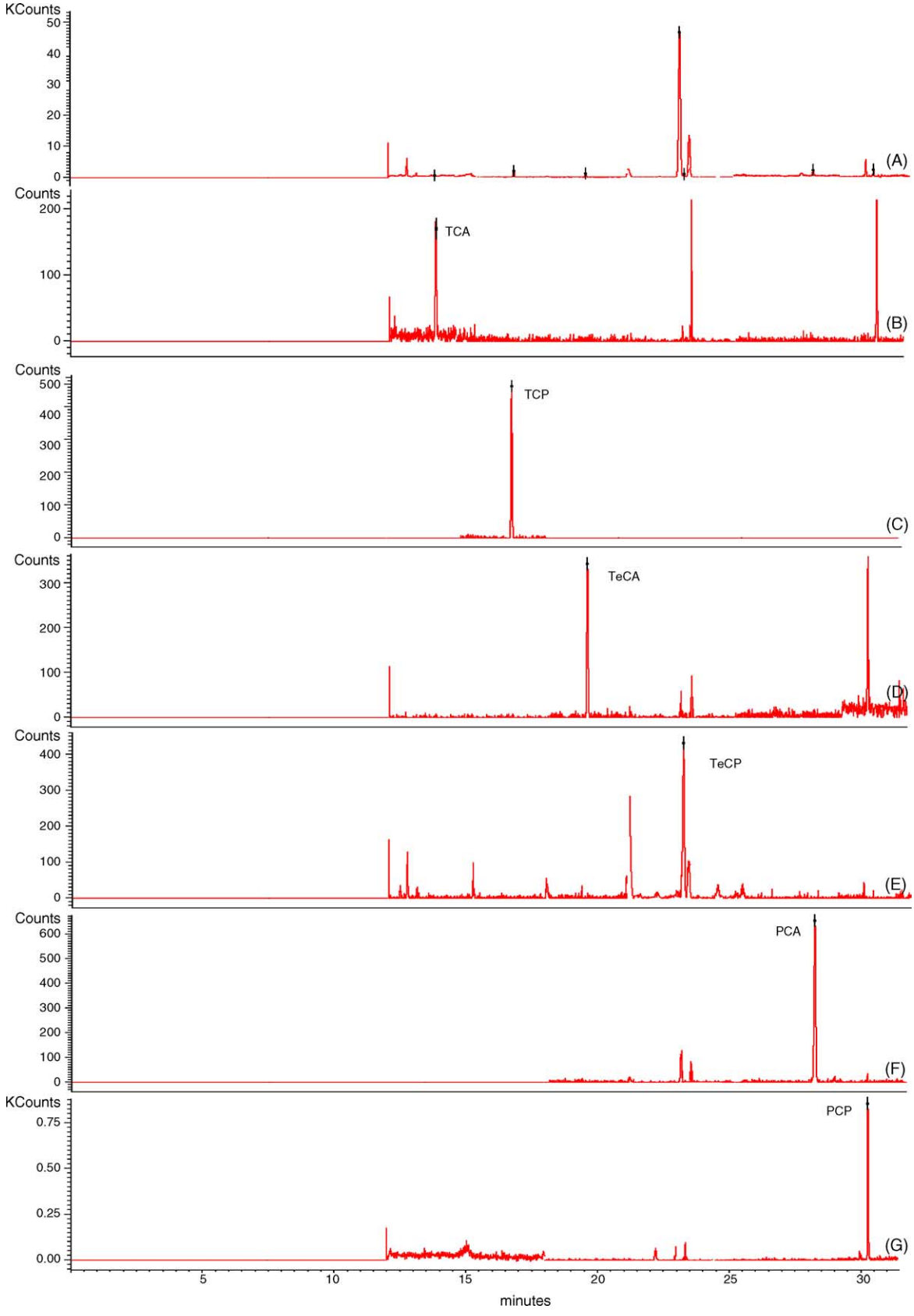

Fig. 2. GC–MS–MS chromatograms for a spiked red wine sample (4 ng l^{-1}): (A) total ions chromatogram (TIC), (B) chromatogram *m*/*z* 167, (C) chromatogram *m*/*z* 99, (D) chromatogram *m*/*z* 203, (E) chromatogram *m*/*z* 131, (F) chromatogram *m*/*z* 237 and (G) chromatogram *m*/*z* 165.

Table 4
Linearity and recoveries for the whole procedure (SPE followed by GC–MS–MS)

Compound	Linearity			Average recoveries ± R.S.D. (%)	
	r	Slope ± S_m	Intercept ± S_b	Low level[a]	High level[b]
TCA	0.995	0.184 ± 0.006	−0.045 ± 0.083	102.7 ± 9.5	96.3 ± 0.6
TeCA	0.996	0.504 ± 0.015	−0.155 ± 0.185	102.8 ± 10.7	94.1 ± 7.7
PCA	0.999	0.798 ± 0.010	−0.032 ± 0.117	85.4 ± 15.1	95.3 ± 12.5
TCP	0.996	0.155 ± 0.005	−0.021 ± 0.059	85.2 ± 7.6	95.1 ± 4.8
TeCP	0.999	1.452 ± 0.009	0.032 ± 0.113	68.4 ± 13.1	92.3 ± 7.1
PCP	0.996	0.458 ± 0.015	0.180 ± 0.178	97.4 ± 11.0	92.8 ± 4.9

[a] Spiked concentration, 40 ng l^{-1} for anisoles and 80 ng l^{-1} for phenols.
[b] Spiked concentration, 500 ng l^{-1} for anisoles and 1400 ng l^{-1} for phenols.

Table 5
Comparison of achieved detection limits (S/N 3) for chloroanisoles and chlorophenols in wine using different sample concentration techniques and GC–MS or GC–MS–MS detection

Compound	Detection limits (ng l^{-1})					
	This work	SPE (C18)[a] (Ref. [29])	Pervaporation (Ref. [27])	Pervaporation (Ref. [26])	SPME[a] (Ref. [19])	SBSE[a] (Ref. [25])
TCA	2.4	2	4.2	5	0.4	0.2
TeCA	0.3				0.3	0.02
PCA	0.4					0.02
TCP	0.5	4				0.1
TeCP	0.2					0.04
PCP	0.3					10000

Empty cells mean that analytes were not included in the study.
[a] Quantification limits.

GC–MS–MS detection was employed. In addition most of the published methods did not deal with the determination of the six compounds considered in this study, and potentially related to the cork taint problem in wine. Sorption of the analytes in polydimethylsiloxane (PDMS) coated stir bars followed by their thermal desorption is the only technique which, globally, improves the detection limits of this work (Table 5). The drawback of this approach is that it requires the use of a thermal desorption unit to transfer analytes from the stir bar to the chromatographic column. Moreover, because of the low affinity of PCP to PDMS, an extremely high detection limit was reported for this compound [24].

4. Conclusions

A procedure for the simultaneous determination of three chlorophenols and three chloroanisoles, considered as the main responsible for the *corkiness* problem, in red wine samples has been presented. The use of 200 mg Oasis HLB cartridges allowed to concentrate up to 1 l samples without losses of the analytes and achieving a 500-fold enrichment factor. This, added to the selectivity of tandem MS–MS detection and the use of a temperature programmable GC injector, allowed the simultaneous detection of chlorophenols and chloroanisoles at the ng and even sub-ng l^{-1} level. As expected, taking into account the complexity of wine samples, the yield of the acetylation reaction and the further extraction of the analytes in *n*-hexane was lower for wine extracts than for standards in methanol. Therefore, quantification was performed using the standard addition method. Further efforts should be focussed on improving the selectivity of the concentration step in order to allow the quantification of the analytes using external calibration.

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