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Journal of Chromatography A, 1052 (2004) 145–149
JOURNAL OF CHROMATOGRAPHY A
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Determination of chloroanisole compounds in red wine by headspace solid-phase microextraction and gas chromatography–mass spectrometry☆

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Received 8 March 2004; received in revised form 29 June 2004; accepted 16 August 2004

Abstract

The objectives of this study, were the development and validation of an analytical method for the determination of 2,4,6-trichloroanisole (TCA), 2,3,4,6-tetrachloroanisole (TeCA) and pentachloroanisole (PCA) in red wine by headspace solid-phase microextraction and GC–MS as well as the application of the optimized and validated method for the quatification of chloroanisoles in different red wines from Navarra. To carry out this study, the extraction variables have been optimized. The fiber and the experimental design selected permit the determination of low analyte concentrations (ng/L) with good accuracy (<5%). Moreover, an analytical method for the determination of TCA and TeCA in wine by GC–MS has been validated. The results obtained in the validation step, recovery values, detection and quantitative limits, and precision were acceptable for all the analytes in the ranges of concentration studied (<5% and <10% for TCA and TeCA, respectively). This method has been used as an analytical method for the quantification of TCA and TeCA in red wine samples that were selected for this study, yielding good results.
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Keywords: Cork taint; Wine; Trichloroanisole; Tetrachloroanisole; 2,4,6-trichloroanisole (TCA); Head space microextraction; Solid-phase microextraction

1. Introduction

The presence of a musty taint in wine prevents its immediate comsuption. Some cellars have had to discard bottles due to the organoleptic alterations caused by the presence of the anisole family chlorine derivatives. Recent studies have concluded that the compounds, which are mainly responsible for this flavor are 2,4,6-trichloroanisole (TCA) and 2,3,4,6-tetrachloroanisole (TeCA) [1–6]. It has been found that pentachloroanisole may also be partially responsible. These compounds contribute to a corky off-flavor in wine. This is an organoleptic problem that affects 2–5% of bottled wine and which produces great economic losses in the viticulture and related industries.

The origin of the chloroanisoles can be found in methylation of the corresponding chlorophenols by certain fungi, such as the aspergilius family [7–11]. Said chlorophenols, in many cases, are used in the treatment of the barrels and also used in certain pesticides.

There are several possible sources of contamination of wine cork stoppers by TCA and other chloroanisoles. These microbial metabolites could have formed from chlorophenol compounds that arose during the manufacturing process of the corks, such as the washing processes, the desinfection with chloro, and in the use of hypochlorite as a cork-beaching agent. Nevertheless, the totality of the musty-off flavor found in the wine is not due to the cork. A good number of alterations of this type has been detected just before the wine is bottled and plugged. However, the investigations carried out in a study set up by the European Cork Confederation, the

☆ Presented at the 3rd Meeting of the Spanish Association of Chromatography and related Techniques and the European Workshop: 3rd Waste water Cluster, Aguadulce (Almeria), 19–21 November 2003.

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0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.08.046

Quercus Project [12], have revealed that while the cork stopper is not to be solely blamed, it is nonetheless, a direct source of contamination.

The odor threshold of these substances is in the ng/L range and so far can only be analyzed after extraction and concentration of the tainted samples [13–17].

2. Materials and methods

2.1. Reagents and chemicals

2,4,6-trichloroanisole ([23,539-3], 99%) was supplied by Sigma-Aldrich, 2,3,4,6-tetrachloroanisole ([RPC-05], 95) by Ultra scientific, and 2,3,4,5,6-pentachloroanisole ([PS1109], 98.2) by Chem service. Absolute ethanol grade UV-IR-HPLC for instrumental analylsis (Panreac ref: 361086) was used as the solvent.

2.2. Wine samples

Samples of a Navarrian commercial wine (wine of the current year) were used to optimize the extraction conditions and to validate the method.

2.3. Sample preparation

The weighing procedures have been carried out in liquid form in order to improve the precision of these compounds because they are more volatile in a solid state.

Stock solutions of 100 and 1 μg/L for each compound were prepared in absolute ethanol and stored in darkness at 3 °C until use. A standard solution containing 10 ng/L of each analyte was prepared by diluting the standard solutions in red wine. Finally, five calibration solutions, in the range 0.5–10 ng/L, and another five, in the range 10–60 ng/L, were prepared by suitable dilution of stock solution of 1 μg/L.

2.4. Equipment and chromatographic conditions

Chromatographic analyses were carried out on a Agilent 6890 gas chromatograph coupled to a 5973 quadrupole mass spectrometer from Agilent. The gas chromatograph was equipped with an HP-5MS capillary column (30 m × 0.25 mm i.d.) coated with a 0.25-μm film of stationary phase (5% phenyl–methylsiloxane). The carrier gas was high purity helium and was flowing through the column at 1.1 mL/min. The injector was held at 250 °C and the transfer line to detector, at 300 °C. The GC oven temperature was programmed at 45 °C (held for 2 min), raised at a rate of 40 °C/min until reaching 100 °C (held for 0.30 min), and then raised at 5 °C/min until reaching 200 °C. The temperature was finally raised at a rate of 60 °C/min, to 260 °C. The mass spectrometer was operated in electron impact mode (EI, 70 eV). A solvent delay of 4 min was used, in order to avoid overloading the mass spectrometer with EtOH. The selected-ion monitoring (SIM) conditions for the TCA were, ions (*m/z*) 195, 197, 199, 210 and 212, at a start time of 10.10 min for a retention time of 10.38 min and for the TeCA were, ions (*m/z*) 229, 231, 244 and 246 for a start time of 12.00 min and a retention time of 14.64 min.

2.5. HS–SPME

The manual SPME device used was purchased from Supelco. We tested fibers coated with different kinds of stationary phases, all of them supplied by Supelco. The following fibers were used in order to find the ones most suitable for analysis: polydimethylsiloxane (PDMS 100 μm), polydimethylsiloxane (PDMS 7 μm), polydimethylsiloxane–divinylbenzene (PDMS–DVB) 65 μm, polyacrylate (PA) 85 μm, and Carboxen–poly-dimethylsiloxane (CAR–PDMS) 75 μm. The fibers were conditioned before use according to the instructions from the supplier. They were immediately used in order to prevent contamination. The PDMS 100 μm was conditioned 30 min at 250 °C.

2.5.1. SPME procedure

The adequate technique for the determination of chloroanisole compounds in wine is the headspace solid-phase microextraction, because it allows both the extraction and the concentration of the analyzed compounds simultaneously, without using organic solvents [18,19]. Moreover, it could also be coupled to a gas chromatograph.

Solid-phase microextraction (SPME) involves absorption of the analytes on a fiber coating which is introduced into the gas phase above the sample [20,21]. At equilibrium, the amount of any volatile component initially present in the sample will be distributed in the three phases: fiber, headspace and sample ($C_0V_s = C_fV_f + C_hV_h + C_sV_s$, where C_0 is the initial concentration of the analyte in the sample solution, V_s the volume of the sample, C_f, C_h and C_s are the equilibrium concentrations of the analyte in the fiber, headspace and sample, respectively, V_f, V_h and V_s are the volumes of the fiber, headspace and sample). The ratio of the concentration of a component in each of the three phases can be described by the equilibrium constants: $K_{fh} = C_f/C_h$, $K_{hs} = C_h/C_s$, $K_{fs} = C_f/C_s$ (between the fiber and the headspace, the headspace and the sample, and the fiber and the sample, respectively).

The amount of analyte absorbed by the fiber ($n = C_fV_f$) at equilibrium is directly related to the concentration of the analyte in the sample [22,23]:

$$n = \frac{K_{fs}V_fC_0V_s}{K_{fs}V_f + K_{hs}V_h + V_s}$$

where n is the mass of analyte absorbed by the fiber. The equation also shows that if V_s very large ($V_s \gg K_{fs}V_f$ and $K_{hs}V_h$), the amount of analyte extracted by the fiber is not related to sample volume (it is independent of V_s and proportional to K_{fs} and V_f). This relationship is described as $n = K_{fs}V_fC_0$.

Several factors influence SPME efficiency are evaluated during method development. Solid-phase microextraction is optimized by adjusting parameters that impact analyte absorption and desorption. The primary parameters influencing analyte absorption into the stationary phase are fiber type, extraction time, ionic strength, pH, temperature, sample volume and stirring. For SPME–GC, analyte desorption is a function of time vs. temperature [22–24]. Different parameters were studied, including the effect of temperature, time, salt addition and fiber type.

Headspace sampling was carried out, using 50-mL vials, each one containing 25 mL of liquid sample (red wine). The vials were tightly capped with a Black Viton septum (Supelco) and placed in a thermostatic bath adjusted to the different temperatures tested. SPME was carried out under constant magnetic stirring (350 rpm). The sample vials were pre-equilibrated for 25 min at 35 °C.

Next the fiber was exposed to the headspace over the sample for 30 min and was inserted into the injection port for thermal desorption at 250 °C during 2 min. All analyses were made in triplicate.

2.6. Different wine samples

After the method was validated, several wine samples, originating from different grape varieties (Tempranillo, Cabernet-Sauvignon and Merlot), from different areas of Navarra with different climate (mountain, medium zone, and south zone) were analyzed. Different aged wines were also analyzed; they were classified as "raising" (1 year in barrel and at least another year in bottle), "reserve" (1 year in barrel and at least 2 years in bottle) and "great reserve" (3 years in barrel and at least 3 years in bottle).

3. Results and Discussion

3.1. Fiber optimization

Five different types of commercially available fibers with different types of coatings were investigated: PDMS, 100 and 7 μm; PDMS–DVB 65 μm; PA 85 μm and CAR–PDMS 75 μm. The equilibrium time of the system in terms of the polymeric coating used was found to be a fundamental parameter, determined in order to optimize recovery and reproducibility. Different coatings require different equilibration times in terms of the diffusion of the analytes throughout the system and the number of molecules extracted.

Fig. 1 shows the curves area/time at different temperatures for PDMS 100 μm fiber, thereby obtaining the times at which equilibration is reached at each temperature. The same procedure is used for the rest of the fibers that were examined and by this method, an equilibration temperature was selected for each fiber.

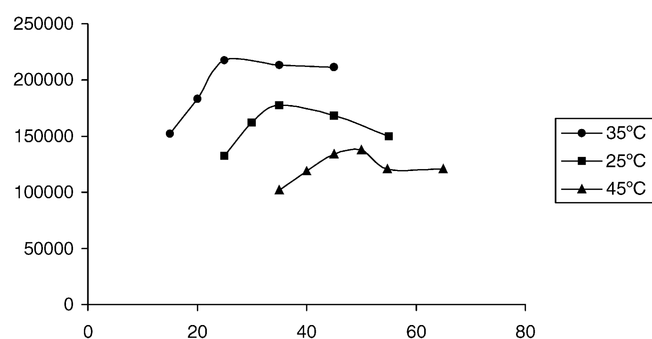

Fig. 1. Influence of time, at different temperatures, on the extraction of 2,4,6-trichloroanisole by PDMS 100 μm fiber.

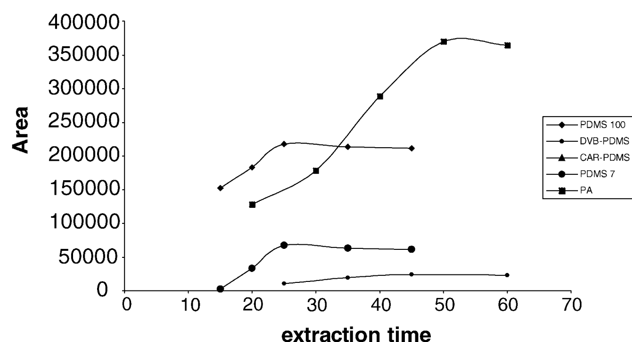

Fig. 2. Influence of time, at optimized equilibrium temperatures for each fiber, on the extraction of 2,4,6-trichloroanisole by PDMS 100 μm fiber, PDMS 7 μm fiber, PDMS–DVB 65 μm, PA 85 μm.

Fig. 2 shows the curves area/time for PDMS 100 μm fiber, PDMS 7 μm fiber, PDMS–DVB 65 μm and PA 85 μm fiber at each optimized equilibrium temperature.

For these optimized equilibrium conditions, Table 1 lists the extraction reproducibility of the five fibers examined.

It can be observed that the most appropriate fibers are the PDMS (100 μm) and the PA (85 μm). The PA (85 μm) fiber gives a greater peak area but its drawback is the fact that it requires a longer analysis time and offers less precision. Therefore, the PDMS 100 μm fiber was chosen.

Fig. 3 shows chromatograms of red wine obtained under the optimized conditions of the method described.

Table 1
Reproducibility of five SPME fibers used for head-space analysis of chloroanisole compounds

Fiber	Temperature (°C)	Time (min)	Reproducibility (%)
PDMS (100 μm)	35	30	3.8
PDMS (7 μm)	35	30	14.63
PDMS–DVB (65 μm)	45	45	28.3
PA (85 μm)	25	50	9.15
CAR–PDMS (75 μm)		No selectivity	

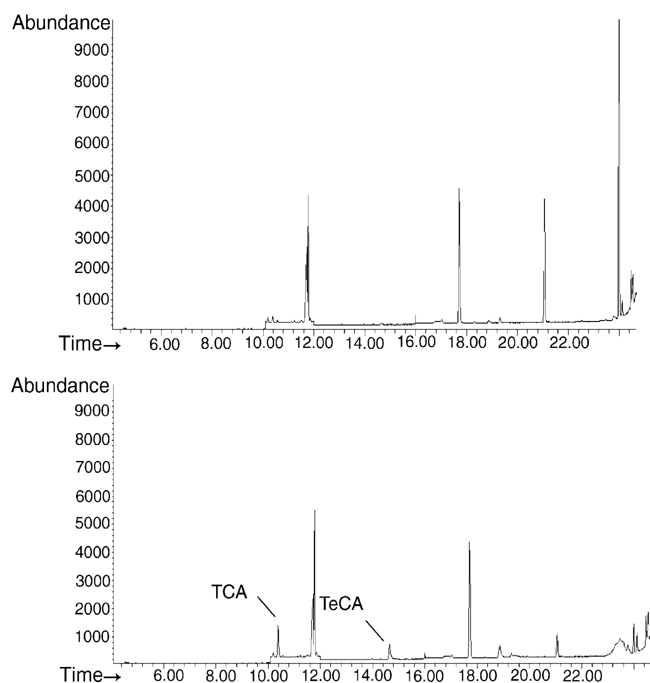

Fig. 3. Chromatograms obtained with the optimized conditions of the extraction method. Blank chromatogram of the red wine (top). Red wine with an addition of 10 ng/L TCA (bottom). Time scales in min.

Table 2
Parameters of the calibration lines (n = 3) for quantification of TCA and TeCA

ng/L	r^2	a	S_a	b	S_b
TCA					
0.5–10	0.9911	−134.516	1444.13	2246.00	260.6
10–60	0.9885	6310.89	7602.04	1599.52	222.36
TeCA					
0.5–10	0.9936	344.61	1234.83	2283.64	222.83
10–60	0.9886	9569.61	7508.11	1583.89	219.61

Table 3
Limits of detection and quantification of TCA and TeCA in red wine

	Detection limit (ng/L)	Quantification limit (ng/L)
TCA	0.18	0.4
TeCA	0.06	0.3

3.2. Parameters of quality

Having studied the extraction parameters, calibration curves were built and the linearity range and quantification of the method were estimated.

3.2.1. Calibration lines

It was necessary to build two calibration lines because two linear ranges were observed at two different levels. Six standard solutions in the range 0.5–10 ng/L and another five solutions in the range 10–60 ng/L of each analyte were analyzed. Each point of the calibration lines was obtained from analyzing three replicates. Table 2 shows the chromatographic retention times and the parameters of the calibration lines for each analyte. The determination coefficients obtained from the calibration lines are good (r^2 = 0.9885, calibration line at high concentrations and r^2 = 0.9911, at low concentration).

3.2.2. Limits of detection and quantification

A red wine sample spiked with low levels of TCA was used to establish the limits of detection and quantification of the overall method. Three replicates were carried out. Table 3 lists the lowest concentration detectable for TCA and TeCA at signal-to-noise ratios of 3 and 10 (limit of detection and quantification, respectively) [25].

3.2.3. Recoveries

The recovery of the method was investigated with a spiked red wine sample with different quantities of TCA and TeCA. Three samples were prepared in addition to three different concentrations. The recoveries were evaluated as the quotient between the amount of analyte extracted, determined from the calibration lines, and the real amount of the analyte added to the sample. Table 4 shows the results of the recoveries of each analyte. As can be observed, the analytes showed good recoveries (up to 95%) with good standard deviation (R.S.D. $<$ 5% for TCA and R.S.D. $<$ 10% for TeCA).

3.2.4. Repeatability and reproducibility

To evaluate the repeatability, three different red wine samples spiked with 10 ng/L were analyzed. The reproducibility was evaluated analyzing three identical red wine samples prepared on three different days [25]. For TCA, R.S.D. of 2.8 and 3.8% were found for repeatability and reproducibility, respectively; for TeCA, the R.S.D. values were 3.4 and 8.4%.

3.3. Different wine samples

The method proposed was successfully applied to commercial wines produced in wine cellars of Navarra. The wines analyzed presented low quantities of TCA and TeCA, all of them being below the perception limit of TCA in wine. The main causes of the absence of this organoleptic defect in these

Table 4
Mean recoveries and relative standard deviation (R.S.D.) in red wine samples spiked with different quantities of TCA and TeCA

TCA spiked (ng/L)	TCA found (ng/L)	Recovery (%)	R.S.D. (%)	TeCA spiked (ng/L)	TeCA found (ng/L)	Recovery (%)	R.S.D. (%)
4	3.85	96.25	1.03	4	4.32	108	7.1
50	48.27	96.54	3.2	50	53.32	106	4.5

wines were the control of every production step and the good handling and storage of these wines in the cellars.

We found significant differences among the different-aged wines, but these differences had no relevance with regard to the organoleptic properties.

4. Conclusions

The method described enables the chloroanisole compound studied to be determined in red wine at the ng/L levels. These levels are useful because they are below their olfactory threshold (for the TCA, it is about 20–40 ng/L) with good recoveries (up to 95%), with acceptable R.S.D. (<5% for TCA and <10% for TeCA). The detection and the quantification limits are 0.1 ng/L and 0.4 ng/L, respectively, for the TCA. The repeatability and reproducibility found are good for all of the analytes (<5% and <10% for TCA and TeCA, respectively) in the range of concentrations studied. Due to its simplicity and rapidity, the method seems to be adequate for routine analysis of wine of "Navarra denomination".

Acknowledgement

The authors thank the Navarra Government and the University of Navarra for financing this study.

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