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Journal of Chromatography A, 977 (2002) 1–8

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Determination of 2,4,6-trichloroanisole in wines by headspace solid-phase microextraction and gas chromatography–electron-capture detection

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Received 4 February 2002; received in revised form 7 August 2002; accepted 14 August 2002

Abstract

One of the most important problems in the wine world, today, is cork taint, which often has been chemically identified as 2,4,6-trichloroanisole (TCA). The perception limit of this compound is very low (close to 10 and 40 ng/l for white and red wines, respectively), so, even at such low concentrations, its presence becomes a problem in wine quality. A method for the analysis of TCA in white and red wines has been developed in our laboratory, using headspace solid-phase microextraction and gas chromatography with electron-capture detection. The method, which has been optimized using an experimental design, involves the use of fibres coated with polydimethylsiloxane (PDMS) and allows the analysis of TCA at very low concentrations (under 500 ng/l) with good accuracy (RSD≤10%). The limits of quantification of the method are 5 and 8 ng/l for white and red wines, respectively, while the limit of detection is 1 ng/l for both types of wine.

Keywords: Wine; Solid-phase microextraction; Headspace analysis; 2,4,6-Trichloroanisole

1. Introduction

There is a growing concern in the wine industry about the quality of the products it manufactures, partly motivated by the increasing consumer awareness on quality issues. Nowadays, consumers can detect different organoleptic defects. One of those is cork taint [1].

Among other compounds such as geosmine, guaiacol, 1-octen-3-one, 1-octen-3-ol and 2-methyl-isoborneol, it has been shown that chloroanisoles can give a musty taint to a variety of foods and beverages, including wine [2–4]. The most odorant of the chloroanisoles is 2,4,6-trichloroanisole (TCA) [5].

In 1996, a study set up by the European Cork Confederation, the QUERCUS project, investigated the causes and origins of taints in corks. It found that TCA was responsible for the musty/mouldy taint in, at least, 80% of cases when it was detected in bottled wines. Taking into account the estimated incidence of cork taint in wine bottles which ranges from 0.5 to 7%, this implies large economic losses [6,7]. However, the true cork taint in wines is rare. This denotation gives the idea that the origin of this defect is only the cork; however there is evidence that the

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01276-1

compounds causing cork taint may also appear from other sources [4,8,9].

Several microorganisms such as moulds, yeasts or bacteria are involved in the formation of TCA [1–4,10,11]. There are also many other causes that explain the presence of TCA in wine: use of fungicides, biocides, herbicides and wood preservatives containing pentachlorophenol (PCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP) and 2,4,6-trichlorophenol (TCP) [4,8,12], contamination coming from the cardboard used in the transport of the corks [13], use of hypochlorite as cork bleaching agent [4,8,12], and so on.

The sensory threshold of TCA is close to 10 ng/l in white wine and 40 ng/l in red wine [1,4,5,7,8]. Taking into account that the usual concentrations of TCA in wines range in ng/l levels, it is necessary to apply a preconcentration technique before analytical determination, because even gas chromatography with electron-capture detection (GC–ECD) is not sensitive enough. The classical methods as distillation [12], solvent extraction [3,5,13], simultaneous distillation–extraction (SDE) [14,15] or Soxhlet [16] are time consuming, require large volumes of samples and solvents and are prone to losses of analytes. So, solid-phase microextraction (SPME) appears as an alternative to these techniques since it is a solvent-free technique, which saves preparation time, solvent use and cost [17,18]. The results found in previous work, which presented some applications for analysing TCA in wine using SPME–GC, coupled either with mass spectrometry [7,19,20] or electron-capture detection [21], encouraged us to validate a method to determine TCA concentration in white and red wines by using headspace (HS) SPME and GC–ECD.

2. Methods and materials

2.1. Instrumental analysis

Chromatographic analyses were carried out on a Hewlett-Packard (HP) 5890 series II gas chromatograph equipped with a HP ECD system. The injection was made in the splitless mode for 1 min at 250 °C using an inlet of 0.75 mm I.D. that improved the GC resolution. Separation was made using a Chrompack CP-Wax 57 CB (50 m×0.25 mm, 0.20 μm) fused-silica column. Carrier gas was high-purity helium flowing through the column at 0.8 ml/min (head pressure, 175 kPa). The injector was held at 250 °C and the detector at 300 °C. The oven temperature was 60 °C for 2 min, then it was raised at 8 °C/min to 200 °C. Finally, the temperature was raised at 30 °C/min to 220 °C (10 min).

2.2. Chemicals and reagents

TCA ([87-40-1], 99%) was supplied by Aldrich. The internal standard (I.S.) used was 2,3,6-trichlorotoluene (TCT [2077-46-5], 97%), supplied by Riedel-de Haën. An individual stock solution of 1000 mg/l for each compound was prepared in ethanol and stored at 4 °C. From these stock solutions, we prepared standard solutions of 100 and 1 μg/l, by diluting with ethanol, which were also stored at 4 °C. Working solutions used in further studies were prepared by diluting different amounts of the standard solutions in either synthetic wine or real wine (white and red wine). The synthetic wine was obtained dissolving 3.5 g/l of L-(+)-tartaric acid and 120 ml/l of ethanol in a suitable amount of deionised water to 1 l. Finally the pH was adjusted to 3.5 with 1 *M* NaOH.

2.3. HS-SPME

The manual SPME device used was purchased from Supelco. We tested fibres coated with different kinds of stationary phases, all of them also supplied by Supelco. The fibres were conditioned before use according to the instructions of the supplier.

2.3.1. SPME procedure

Headspace sampling was done using 50-ml vials, each containing 20 ml of liquid sample (either commercial or synthetic wine), with addition of a suitable amount of NaCl (99.8%) to get 5 *M*. The vials were tightly capped with a PTFE-faced silicone septum and placed in a thermostatic bath. SPME was carried out under constant magnetic stirring (300 rpm). The sample vials were preequilibrated for 30 min at 25 °C. Afterwards, the stainless steel needle, in which the fibre is housed, was pushed through the vial septum, allowing the coating to be

exposed to the headspace over the sample for 30 min. Then, the fibre was pulled into the needle sheath and the SPME device was removed from the vial and inserted into the injection port for thermal desorption at 250 °C during 3 min.

2.4. Statistical package

The statistical package used for the optimization of the method was Multisimplex 2.0 [22]. The ULC (univariate linear calibration) computer programme [23] was used to calculate, by linear least-squares regression, the slope and intercept with the determination coefficient (r^2), the standard errors of the coefficients and to compare the slopes of the calibration lines.

3. Results and discussion

3.1. Nature of fibre

We checked different fibres commercially available coated with: polydimethylsiloxane (PDMS) 100 μm, PDMS–divinylbenzene (DVB) 65 μm, polyacrylate (PA) 85 μm, Carboxen (CAR)–PDMS 75 μm, Carbowax (CW)–DVB 65 μm and StableFlex DVB–CAR–PDMS 50/30 μm. To determine which of these coatings gave the best results we compared the results of the experiments that were carried out using two fibres (to verify the repeatability of the response using different fibres) of each kind and making duplicates with every one. The samples analysed were identical and obtained from 20 ml of synthetic wine spiked with 400 ng/l of TCA and TCT, in a 50-ml vial. The HS-SPME conditions used in this study were taken from the bibliography (30 min of extraction at 25 °C, with magnetic stirring and sample saturation with NaCl) [7,19–21]. The two most suitable coatings for TCA extraction are the PDMS and the DVB–CAR–PDMS. The DVB–CAR–PDMS coating presents better sensitivity (100% better than PDMS), but the repeatability becomes better with PDMS coating (4 versus 12%, expressed in RSD terms), so we decided to use the PDMS stationary phase.

3.2. Optimization of the method

First of all we checked the experimental parameters which could affect the extraction process. These parameters are temperature and time of extraction, sample volume and ionic strength. It is well known that sample magnetic stirring is another parameter that influences the extraction because it helps the transfer of analytes from sample matrix to the fibre coating [19–21,24], so all the experiments were made under constant magnetic stirring (at the faster speed allowed).

A full two-level factorial design (using duplicated experiments) was applied to check the influence of the four above mentioned factors and to estimate the magnitude of their effects in the suitable experimental domain specified in Table 1. The number of experiments required was 16 (2^4) [25,26]. Every experiment was carried out twice using two different fibres and analysing identical samples of synthetic wine spiked with 400 ng/l of TCA.

The results obtained can be graphically seen in the Pareto chart (Fig. 1). This chart shows that the ionic strength, the sample volume and the extraction time play a positive influence on the process when their values increase, but the temperature of extraction has a negative effect, because the higher the temperature the lower the peak area. Furthermore it can be also observed that the effect of each factor has an absolute value larger than the confidence interval (in the chart this interval is pointed out with a dotted line) which was calculated from the variance of each effect, 16 degrees of freedom and $\alpha = 0.05$ [25]. This fact illustrates that the effect of each of the four experimental parameters checked are significant so the four experimental parameters must be really taken into account for the extraction process. On the

Table 1
Factors and levels studied to check the influence of the experimental parameters on the TCA extraction

Factor		Level	
		Low (−)	High (+)
Extraction temperature (°C)	(A)	10	40
Extraction time (min)	(B)	10	60
Ionic strength (*M*)	(C)	0	6
Sample volume (ml)	(D)	10	25

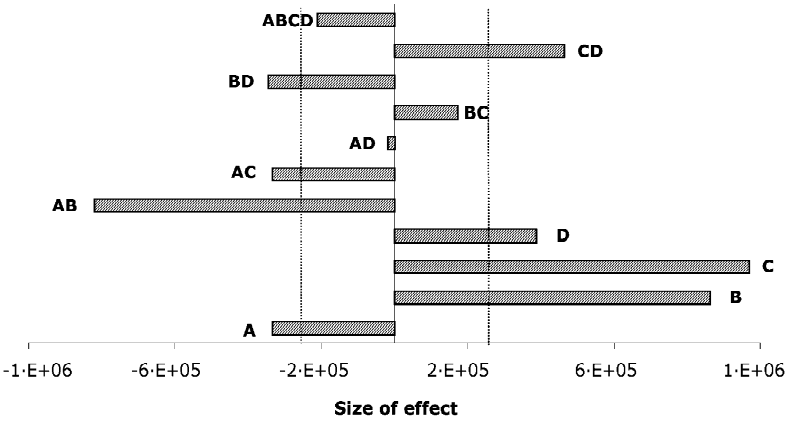

Fig. 1. Pareto chart showing the magnitude of the effects on the extraction of TCA by HS-SPME. The *x*-axis shows the absolute magnitude of the effect of each factor determined by the statistical analysis and the *y*-axis shows the different factors: (A) extraction temperature (10–40 °C); (B) extraction time (10–60 min); (C) ionic strength (0–6 *M*); (D) sample volume (10–25 ml). The confidence limits of the effects are represented graphically by a dotted line ($\alpha = 0.05$ and 16 degrees of freedom).

other hand, in this Pareto chart it can also be observed that there is an interaction between the different factors (mainly between A and B factors) so, all of them should be studied together.

Once we knew which parameters had to be taken into account, we applied a mathematical model based on a Simplex method (Multisimplex 2.0) to optimize the extraction conditions. The experimental matrix used by this mathematical model was defined according to the literature [7,19–21] and some previous experiments. The experimental parameters and their working intervals, given in parentheses, to develop the Simplex method are: temperature of extraction (10 to 40 °C, step of 10 °C), extraction time (15 to 60 min, step of 15 min), ionic strength (0 to 6 *M*, step of 2 *M*) and sample volume (5 to 25 ml, step of 5 ml). The results showed that the optimal conditions were obtained when 20 ml of sample with addition of 5 *M* NaCl was poured into a 50-ml glass vial and the TCA was extracted by means of SPME fibre from its headspace for 30 min at 25 °C (conditions described in Section 2.3).

The distribution constants between the liquid phase and the coating are strongly dependent on the matrix [17]. In wine analysis we have to take into account the ethanol, because since it is a majority component of wine, it can interfere with the extraction of TCA by the fibre. In order to determine its contribution we analysed synthetic wine, with different ethanol contents between 10 and 15% (v/v) (four replicates for every level using two fibres), and we found that when the ethanol content increased, the extraction extent of TCA decreased (Fig. 2). Taking into account that 12% is one of the most usual contents of ethanol in wines we fixed this value in order to minimize the sample handling, so we worked with a constant ethanol content of 12% (v/v).

Some authors state that matrix effects are different for red and white wine, because the presence in red wine of some characteristic substances as polyphenolic compounds can induce changes on the distribution constants [24,27,28], so, it seemed important to check if any difference between synthetic, white and red wines appeared. Therefore we evaluated the chromatographic response of TCA in spiked synthetic, white and red wines at concentrations under 400 ng/l by comparing the slopes (and their confidence intervals) of the regression lines synthetic wine vs. white wine, synthetic wine vs. red wine and white wine vs. red wine, two on two. The slopes of the first comparison (synthetic vs. white) were statistically comparable for a significance level of 0.05. The other two were not statistically comparable for the same significance level. So, we have to work with different methods to build the calibration lines

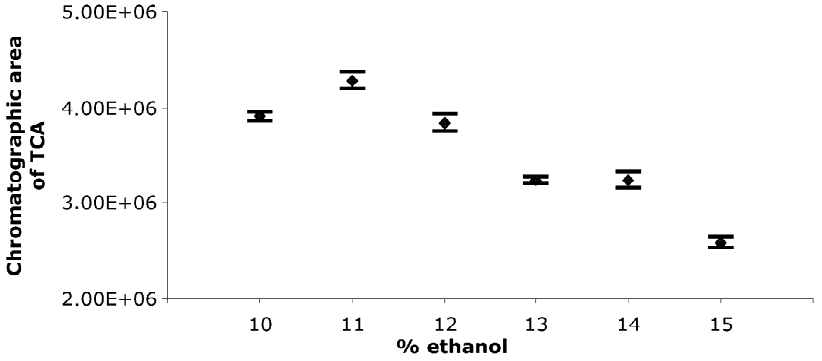

Fig. 2. Influence of ethanol concentration on the extraction of TCA in a synthetic wine.

for every kind of wine. With white wine we could use spiked synthetic wine, but for red wine we have to use the standard addition technique. To avoid this laborious technique for each different kind of red wine it is also possible to build the calibration lines by using a spiked mixture of different red wines from different origins and ethanol contents, as it has been done in previous studies [27]. In this way, a matrix of red wine as representative as possible was obtained.

Figs. 3 and 4 show, respectively, examples of

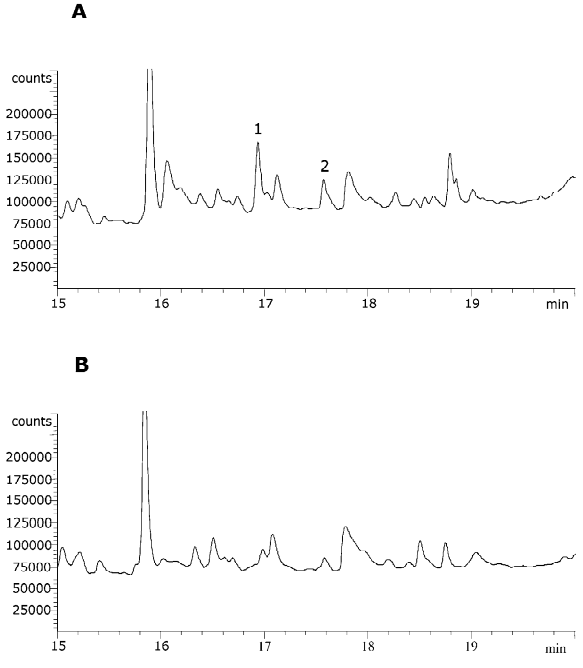

Fig. 3. Chromatograms obtained under the optimized conditions of the method. (A) White wine with an addition of 75 ng/l TCT (1) and 10 ng/l TCA (2). (B) Blank chromatogram of the white wine.

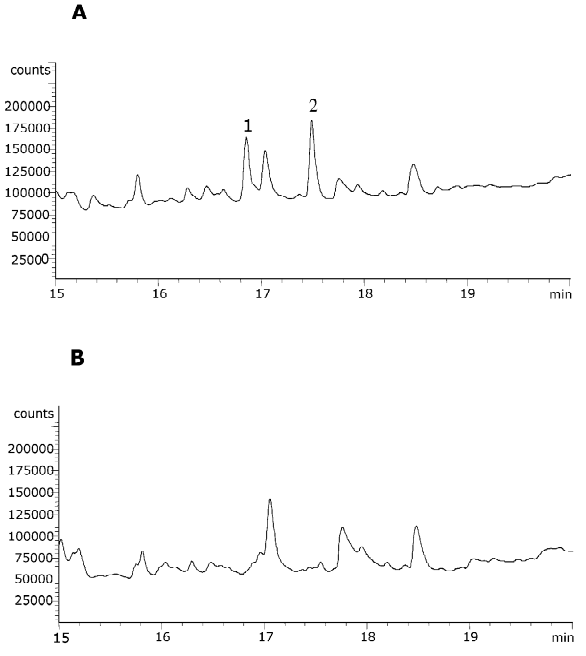

Fig. 4. Chromatograms obtained with the optimized conditions of the method. (A) Red wine with an addition of 50 ng/l TCT (1) and 40 ppt TCA (2). (B) Blank chromatogram of the red wine.

chromatograms (white and red wine with/without addition of TCA) obtained under the optimized conditions of the method described.

4. Parameters of quality

4.1. Limits of detection and quantification

According to the IUPAC recommendations, limits of detection and quantification were determined considering the standard deviations of the lines at concentration 0 and considering α and β errors [29,30], so calibration lines at low concentrations should be built.

For white wine, the calibration line was calculated with synthetic wine spiked with six different concentrations of TCA, in the range of 0–15 ng/l, with an I.S. concentration of 25 ng/l. For red wine, the calibration line was calculated by using the mixed red wine described in Section 3.2. The mixture was spiked with five different concentrations of TCA, in the range of 0–15 ng/l, with an I.S. concentration of 50 ng/l. In both cases four replicates using two fibres were done. The determination coefficients were good (r^2=0.976 for white wine and r^2=0.964 for red wine), taking into account the low concentration range of these lines. The calculated values of limits of detection for both kind of wines are 1 ng/l. As for calculated values of limits of quantification, these are 5 ng/l in white wines and 8 ng/l in red wines. All these values were experimentally checked.

4.2. Calibration lines

In all cases, the calibration lines were constructed by plotting the peak-area ratios (TCA area/I.S. area) against the concentration ratios (TCA concentration/I.S. concentration). The parameters of the calibration lines are shown in Table 2. For white wine, the calibration line was built with synthetic wine spiked with seven different concentrations of TCA in the range of 5–500 ng/l and with an I.S. amount of 75 ng/l. Each point of the calibration line was obtained from four replicates analysed using two fibres. The determination coefficient of the calibration line obtained is good (r^2=0.990).

Table 2
Parameters of the calibration lines ($y=a+bx$) built for quantification of TCA (white wine and red wine)

Wine	a	S_a	b	S_b	r^2	$S_{y/x}$
White wine	0.12	0.05	2.49	0.15	0.990	0.57
Red wine (low concentrations)	−0.12	0.05	2.32	0.05	0.994	0.14
Red wine (high concentrations)	0.28	0.04	1.98	0.03	0.995	0.17

For red wine, we had to built two calibration lines (two linear ranges observed): one at high levels of TCA and another at low levels, but both were constructed using the mixed red wine described in Section 3.2. For high concentrations of TCA calibration line, we analysed four replicates of samples spiked with six different concentration levels in the range of 30–500 ng/l, all of them with an I.S. content of 150 ng/l and working with two fibres. For low concentrations of TCA calibration line we also used four replicates of a mixed wine spiked with six different concentration levels. The concentration range of the samples was 8–100 ng/l, with an I.S. content of 50 ng/l. The determination coefficients obtained in this study were also good (0.995 and 0.994, respectively).

4.3. Recoveries

The recovery of the method was determined by addition of different quantities of TCA to four red and four white commercial wines, all of them adjusted to 12% (v/v). The TCA was added to each wine at three different concentrations: at lower, middle and higher levels of the calibration range. Four samples of each level were extracted using two fibres. The recoveries were calculated as the percentage ratio between the total concentration of TCA found, using the calibration lines, and the real concentration added to wine (matched matrix standard). Table 3 shows the recoveries obtained. As can be seen, the values are good (up to 90%) with good standard deviation (RSD≤10%).

4.4. Repeatability and reproducibility (intermediate precision)

To evaluate the repeatability we analysed, con-

Table 3
Recoveries of TCA in commercial wines (white and red wine) of different origins

	TCA spiked (ng/l)	TCA found (ng/l)	Recovery (%)	RSD (%)
White wine	10	10	100	7.8
	200	186	93	4.2
	450	409	91	0.5
Red wine	40	37	92	4.6
(high concentrations)	200	192	96	5.2
	450	491	109	10.0
Red wine	10	9	90	6.1
(low concentrations)	40	40	100	6.3
	75	74	99	4.6

secutively, six different samples (synthetic and mixed red wine) spiked with 40 ng/l of TCA and with I.S. concentrations of 75 ng/l (synthetic wine) and 50 ng/l (red wine). The intermediate precision was evaluated analysing a pair of identical samples prepared as it has been described before, on 3 different days, at different hours [25,31]. For white wine we found an RSD of 2% for both parameters. For red wine, RSDs of 5 and 7% were found for repeatability and intermediate precision, respectively.

The method proposed was successfully applied to wines produced in the experimental cellar (Mas dels Frares) of the Faculty of Enology of Tarragona. Wines analysed presented low quantities of TCA, all of them below the perception limit of TCA in wine. The main causes of the absence of this organoleptic defect in these wines were the control of every production step and the good handling and storage of these wines in the cellar.

5. Conclusions

This method presents a good alternative to the classical methods for analysing TCA in red and white wines at subthreshold levels, with good repeatability and intermediate precision (2–7%), in the range of concentrations between 5 and 500 ng/l (white wine) or 8–500 ng/l (red wine). The detection limit is 1 ng/l for both white and red wine and the quantification limits are 5 and 8 ng/l, respectively. The recoveries found are good (up to 90%) with acceptable RSDs ($\leq$10%). Because of the simplicity and speed of the technique used without the use of organic solvents, this method seems to be adequate for routine analysis.

Acknowledgements

We wish to thank the Comisión Interministerial de Ciencia y Tecnología/CICYT (project ALI97-0765) and Ministerio de Ciencia y Tecnología/INIA (project VIN00-045) for the financial support given.

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