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Matrix effect during the application of a rapid method using HS-SPME followed by GC–ECD for the analysis of 2,4,6-TCA in wine and cork soaks

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Abstract

An off-flavor in wine known as 'cork taint' is of concern in the wine and cork industry. Cork taint imparts a musty flavor to the wine and is primarily due to the presence of 2,4,6-trichloroanisole [2,4,6-TCA] in cork stoppers. During this study, an instrumental method for 2,4,6-TCA analysis was developed and evaluated using headspace solid-phase microextraction (HS-SPME) and gas chromatography coupled with an electron capture detector (GC–ECD). 2,3,6-Trichlorotoluene [2,3,6-TCT] was assayed as the internal standard. The method was developed in synthetic wine and was applied in commercial wine samples, as well as in cork soaks obtained by the extraction of TCA from cork stoppers and cork barks using synthetic wine. The method performance was evaluated through the estimation of its linearity ($R^2 > 0.99$), repeatability (RSD value = 5.72%) and sensitivity (recovery > 86%, LOD = 0.177–0.368 ng/L) in different types of samples. Due to the complexity of the samples used, the study has been especially focused on the matrix effects that were identified causing significant bias to the quantitative analysis of 2,4,6-TCA in cork soaks, where there is a lack of previous studies.

Keywords: 2,4,6-Trichloroanisole; Cork taint; SPME; Matrix effect; Wine; Cork stoppers; Cork barks

1. Introduction

The most important off-flavour in wine, mainly caused by the aromatic compound 2,4,6-trichloroanisole [2,4,6-TCA], is called *cork taint*. The 2,4,6-TCA is the single most prevalent wine defect, associated with natural cork. In addition, 2,4,6-TCA can be generated by a variety of means-most commonly by fungal metabolism of chlorophenols (Alvarez-Rodriguez et al., 2002).

The occurrence of taint in bottled wine, causing unpleasant alterations in wine flavor or aroma, is responsible for economical losses in the wine and cork industry. It is reported that it affects 0.5–6% of total bottled wine (Hall, 1997), 0.5–2% of European bottled wines and 1–5.5% of Australian wines (e.g. Heyes, 1995; Lee & Simpon, 1993; Leske, Bruer, & Sefton, 1995). It has also been estimated that 5% of the wine produced in the United States becomes tainted with 2,4,6-TCA from natural cork, costing the wine industry approximately $100 million annually (Fuller, 1995). For the cork stoppers it is estimated that 2–5% of all produced natural corks are tainted (Fuller, 1995).

Current industry practices include a variety of evaluation techniques to screen corks for the 2,4,6-TCA presence, which are based on sensorial analysis. The human identification threshold for 2,4,6-TCA in cork soaks varies from 4 to 10 ng/L (e.g. Tanner, Zanier, Buser, & Schweiz, 1981; Amon, Vandepeer, & Simpson, 1989). On the other hand, the sensorial threshold of 2,4,6-TCA in wines is close to 4 ng/L according to Tanner et al. (1981), 2–5 ng/L in both

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white and red wines according to Liacopoulos et al. (1999) and 6 ng/L according to Sefton and Simpson (2005). The subjective value of 2,4,6-TCA that results from the sensorial evaluation is the most important disadvantage, leading to efforts for the development of reliable analytical techniques.

Gas chromatography coupled with electron capture detector (GC–ECD) and mass-spectroscopy (GC–MS) is usually used for the trace analysis of 2,4,6-TCA and other chloroanisoles (e.g. Alzaga, Ortiz, Sanchez-Baeza, Pilar Marco, & Bayona, 2003; Evans, Butzke, & Ebeler, 1997; Jonsson, Uusitalo, van Bavel, Gustafsson, & Lindstrom, 2006; Juanola, Subira, Salvado, Garcia Regueiro, & Antico, 2002; Riu, Mestres, Busto, & Guasch, 2002, 2005). In most of the cases, ECD is generally preferred, because of its lower cost and higher sensitivity. However, in order to analyze 2,4,6-TCA in wine or cork soak samples a previous extraction step is necessary. All the classical methods such as Soxhlet extraction (Bayonove & Leroy, 1994), distillation (Cantagrel & Vidal, 1990), solvent extraction (Amon et al., 1989) and simultaneous distillation–extraction (SDE) (Hill, Hocking, & Whitfield, 1995) use hazardous solvents, are time consuming and are prone to losses of analytes (Riu et al., 2002).

On the other hand, solid-phase microextraction (SPME) (Zhang, Yang, & Pawliszyn, 1994) is a relatively new analytical technique that can overcome these difficulties and can be applied to the detection of flavor volatiles in wine and cork. The first application of SPME was in the evaluation of pollutants in water (Belardi & Pawliszyn, 1989). Since then, SPME has been used in a wide range of fields including studies of flavors and taint, especially for quick screening of the volatile composition of a wide range of products. It has been applied to fruits (e.g. Penton, 1996; Yang & Peppard, 1994), vegetables oils (Yang & Peppard, 1994), coffee (Yang & Peppard, 1994), wine (Fischer & Fischer, 1997), cork (Butzke, Evans, & Ebeler, 1999; Fischer & Fischer, 1997), beer (Jelen, Wlazly, & Kaminski, 1998), meat (Ruiz, Cava, Ventanas, & Jensen, 1998), milk (Marsili, 1999) and biological fluids (Cardinali, Ashley, Wooten, McCraw, & Lemire, 2000). SPME is a solvent-free method of extracting analytes from a variety of matrices by partitioning them from a liquid or gaseous sample into an immobilized stationary phase. It uses a very simple setup and requires no additional instrumentation other than a conventional gas chromatograph (GC). SPME eliminates preconcentration steps by directly extracting the analytes into a poly-(dimethylsiloxane)-coated fiber, which is the most suitable fiber for the analysis of 2,4,6-TCA in wines (Riu et al., 2002).

During analysis of real samples, rich in organic compounds, using the SPME technique, matrix effects have again been reported. Fromberg et al. (1996) cited matrix effects during the analysis of 20 chloro- and nitro-anilines and benzenes in soil samples in their published work. Valor, Molto, Apraiz, and Font (1997) studied the matrix effects on SPME of organophosphorous from water. Llompart, Li, and Fingas (1998) observed matrix effects during the determination of polychlorinated biphenyls in water samples. Furthermore, matrix effects were observed by Alzaga et al. (2003) when analyzing Spanish wines.

During this work we first developed the conditions of the SPME in order to determine the concentration of the 2,4,6-TCA in different samples. The method was assessed in terms of recovery, repeatability and sensitivity. Secondly, the method was applied for the determination of the 2,4,6-TCA content not only in synthetic wine, but also in commercial red and white wines, as well as in soaks obtained from cork stoppers and cork barks. Finally, we focused on the matrix effects that cause significant bias to the quantitative analysis of 2,4,6-TCA in cork soaks obtained by the liquid–solid extraction of cork samples (stoppers and barks), which to our knowledge has not been studied so far.

2. Experimental

2.1. Reagents and samples

Analytical standards of 2,4,6-TCA [87-40-1] and 2,3,6-TCT [2077-46-5], which was used as an internal standard, were obtained from Aldrich and Riedel-de Haen, respectively. Both standards were of high purity (more than 97%). The choice of the internal standard in the current study was realized according to Riu et al. (2002). Other options referred in Alzaga et al. (2003), related with 2,4,6-tribromoanisole were not suitable, as this compound is known as only a potential cork taint compound (Chatonnet, Bonnet, Boutou, & Labadie, 2003; Jonsson et al., 2006). 2,3,6-TCT was used as an internal standard for the identification of 2,4,6-TCA in samples (wines and cork soaks) and the quantification was carried out via external calibration and standard addition.

All organic solvents were of analytical or HPLC grade from Merck. Water was prepared in a Milli-Q purification system (Millipore).

A stock solution of 100 mg/L of each compound was prepared in methanol. Work solutions with concentration of 100 μg/L were prepared from stock solution diluted with ethanol. The preparation of standard solutions for calibration was made by diluting different amounts of the work solutions in synthetic wine. The synthetic wine contained: 5.0 g/L tartaric acid in 12% ethanol–water solution acidified to pH 3.5.

Commercial red and white wines were purchased from local stores. Cork stoppers and cork barks were provided from Portuguese cork firms.

2.2. Sample preparation

Wine samples were measured without any preparation. In the case of cork stoppers and cork barks, the term of *releasable TCA* previously introduced by Hervé, Price, Burns, and Weber (2000) is used in this work. Releasable

TCA is defined as the equilibrium value of 2,4,6-TCA measured in the wine, following the application of liquid–solid extraction (Hervé et al., 2000). The releasable TCA of a cork stopper remains practically constant, even after multiple soaks (Hervé et al., 2000). Releasable TCA is a function of the 2,4,6-TCA content in the cork stopper, the localization of 2,4,6-TCA in the cork stopper and the specific wine characteristics (e.g. EtOH concentration) (Hervé et al., 2000). In this study, cork stoppers and cork barks, had been subjected to a liquid–solid extraction in synthetic wine, at ambient temperature with 24 h shaking (120 rpm). Cork soaks performed during the current study were based on the same ratio of synthetic wine (soak solution) to each cork (40 mL per cork stopper and 10 mL per 1 g of cork barks).

2.3. The SPME procedure

For SPME, 20 mL headspace crimp-top glass vials tapped with 20 mm crimp-top magnetic capsule with silicone/PTFE (opening diameter 8 mm) were used. A volume of 10 ml of the sample was transferred to each vial, 3 g NaCl were added and an appropriate amount of the internal standard (2,3,6-TCT) was spiked just before starting the extraction (50 μL from an ethanol solution of 10 μg/L of 2,3,6-TCT, in order to achieve a final concentration of 50 ng/L). After closing the vial, the fiber was adjusted in the headspace of the vial just above the liquid phase. The extraction was carried out at 25 °C (in a water bath), at 200 rpm (using a Teflon coating magnetic stirring bar). After 30 min, the fiber was removed and immediately injected to the GC–ECD. The fiber was held in the injector for 3 min.

2.4. Instrumentation

The solid-phase microextraction (SPME) was performed using PDMS fibers (100 μm) obtained from Supelco. Chromatographic analyses were performed in a Varian 3600 Gas Chromatograph equipped with a ^{63}Ni electron capture detector (Varian). Injection at 250 °C was accomplished in the splitless mode (2 min). Separation was achieved by using a CP-Sil5 CB 50 m × 32 mm × 0.20 μm, Chrompack capillary column, obtained from Varian. Carrier gas was high-purity helium at a flow of 2 mL/min. The oven temperature was held at 70 °C for 1 min, raised to 150 °C with 25 °C/min for 2 min, then to 200 °C at 5 °C/min for 2 min, and finally, to 250 °C at 5 °C/min for 5 min. The temperature of the ECD was set at 300 °C. Under these analytical conditions, 2,3,6-TCT appeared at 8.35 min while 2,4,6-TCA at 8.60 min.

2.5. Statistical analysis

All statistical analysis was performed using the software Mathematica 4.1.0.0 (The Mathematica Archive, Wolfram Research Inc. USA). The distribution of 2,4,6-TCA concentration in the two studied bales of naturally contaminated cork stoppers (i.e. stoppers that had been rejected by the Portuguese company due to their high 2,4,6-TCA released concentrations), was simulated using the Gamma distribution.

The general formula for the probability density function (PDF) of the Gamma distribution is

$$f(x) = \frac{\left(\frac{x-\mu}{\beta}\right)^{\gamma-1} \exp\left(-\frac{x-\mu}{\beta}\right)}{\beta\Gamma(\gamma)}, \quad x \geqslant \mu, \beta, \gamma \succ 0, \tag{1}$$

where γ is the shape parameter, μ is the location parameter, β is the scale parameter, and Γ is the Gamma function

$$\Gamma(a) = \int_0^\infty t^{a-1} e^{-t}\, dt. \tag{2}$$

The mean value of this distribution is equal to $\gamma\beta$ and the variation coefficient is equal to $\gamma\beta^2$.

The f cumulative density function (CDF) of the Gamma distribution is

$$F(x) = \frac{\Gamma_x(\gamma)}{\Gamma(\gamma)}, \quad x \geqslant 0, \ \ \gamma \succ 0, \tag{3}$$

where Γ is the gamma function defined above and Γ_x is the incomplete Gamma function. The incomplete Gamma function is given by

$$\Gamma_x(a) = \int_0^x t^{a-1} e^{-t}\, dt. \tag{4}$$

The cumulative density function describes the probability that a variate x takes on a value less than or equal to a number a.

$$P(x \leqslant a) = F(a). \tag{5}$$

The moments of the Gamma distribution are

$$\gamma = \left(\frac{\bar{x}}{s}\right)^2, \tag{6}$$

$$\beta = \frac{s^2}{\bar{x}}, \tag{7}$$

where $\bar{x}$ and s are the sample mean and standard deviation, respectively (Engineering Statistics).

3. Results and discussion

3.1. Optimized conditions of SPME

The extraction method was based on the protocol described by Riu et al. (2002) and was modified in order to achieve the best performance. Before initial analysis, the PDMS fiber was conditioned at 250 °C for 30 min. After the conditioning process, a fiber blank was run to confirm fiber cleaning. In order to determine the most appropriate conditions for the microextraction, several preliminary experiments were run. Desorption times were evaluated at 1 and 2 min, microextraction times were evaluated at 30 and 60 min and NaCl addition was evaluated at

3 and 5 g. According to the obtained results, the thermal desorption of 2,4,6-TCA (at concentrations of 20 and 100 ng/L) did not depend on the time duration of splitless (1 or 2 min). Also the measured concentration of 2,4,6-TCA for 30 and 60 min extraction times was the same. Finally, following the SPME procedure, addition of 3 g of NaCl, was sufficient to obtain repeatable results regarding the absorbed 2,4,6-TCA on the fiber. So the SPME procedure followed was as follows: 2 min desorption, in order to avoid possible injector contamination, 30 min extraction and salting addition: 3 g of NaCl.

3.2. Method performance

The performance of the method was evaluated through estimation of its linearity, repeatability and sensitivity, according to Standard methods (1995).

For quantification, two 5-point calibration curves were constructed for high and low concentrations, using least-square linear regression, from the GC–ECD analysis of standard solutions of 2,4,6-TCA in synthetic wine at concentrations ranging from 1 to 10 ng/L and 10 to 80 ng/L. The calibration curves were linear with correlation coefficients (R^2) higher than 0.99 for the target compound (see Table 1).

Different types of wine-samples were used in order to estimate the overall method recovery in three different concentration levels. The recovery in commercial wines was calculated as the percentage ratio of the measured concentration after the analysis of the sample (subtracting the background concentration of 2,4,6-TCA), over the real concentration that had been added. In the case of the synthetic wine, as it was free of 2,4,6-TCA, the total measured concentration was the added one. Table 2 shows the results of this study. As can be seen, the recovery in all samples is quite high (more than 86%). The recoveries calculated in the commercial red and white wine samples were slightly higher than the ones in synthetic wine. The recoveries calculated during this study in the three different wine types are in agreement with those reported by Riu et al. (2002).

The overall method repeatability, calculated as the relative standard deviation (RSD) of the replicate ($n = 6$) analysis of synthetic wine, spiked with a standard amount of 2,4,6-TCA in order to obtain a final concentration of 25 ng/L, was satisfactory, with an RSD value of 5.72 (Table 2).

The limits of detection (LOD) and quantification (LOQ) of the method were experimentally estimated from the analysis of synthetic wine samples and commercial red and white wines at the minimum concentration of the analyte, giving a signal to noise ratio of 3–5. As shown in Table 2, the LODs obtained for 2,4,6-TCA was 0.177 ng/L for both the synthetic wine and the red wine (free of background 2,4,6-TCA) and 0.368 ng/L, i.e. of the same order of magnitude but somewhat higher, for white wine. The LOQs, which were approximately 2.7 times higher than the corresponding LODs, were determined as 0.478 and 0.994 ng/L for the synthetic wine/red wine and white wine, respectively. The LODs determined in this study were of the same order of magnitude, compared to the ones reported by Riu et al. (2002) (1 ng/L for both wines types).

3.3. Matrix effect

During TCA analysis in soaks obtained from the extraction of 50 cork stoppers and cork barks (10 g), a strong matrix effect was observed. The analysis of 1, 10 and 50 cork stoppers of the same grade, showed that the obtained matrix (liquid–solid extraction soaks) from the extraction of 50 cork stoppers affected the measured peak area of the internal standard 2,3,6-TCT. In fact, the peak size of the internal standard 2,3,6-TCT decreased when the number of tested cork samples increased (Fig. 1).

The effects caused by the matrix in the extraction with SPME have not been adequately well studied. Partitioning of volatile substances, such as 2,4,6-TCA between the liquid and gas phases is mainly governed by the aroma compound volatility and solubility. However, these physicochemical properties may be influenced by other

Table 1
Calibration curves for the quantification of 2,4,6-TCA according to the internal standardization and for quantification of 2,4,6-TCA using the standard addition calibration technique in cork soaks obtained by the extraction of either 3 or 50 cork stoppers in synthetic wine

	a^*	b^*	R^2
Synthetic wine (low concentrations)	0.0588	0.1852	0.9919
Synthetic wine (high concentrations)	0.0538	0.2833	0.9986
3 corks soak	25,538	96,995	0.9950
50 corks soak	9791.5	1,04,167	0.9525

a, slope and b, intercept.

Table 2
Recoveries, repeatability and detection limits of 2,4,6-TCA in wine samples

	TCA spiked (ng/L)	TCA found (ng/L)	Recovery (%)	RSD (%)	Repeatability (RSD%)	Limit of detection (ng/L)[a]	Limit of quantification (ng/L)[a]
Synthetic wine	54	46.45	86.01	7.36	5.723	0.177	0.478
Synthetic wine	10.8	10.02	92.79	0.62			
Commercial white wine	54	58.99	109.24	6.70		0.368	0.994
Commercial red wine	54	49.78	92.18	1.52		0.177	0.478

[a] Selected wines with background free of TCA.

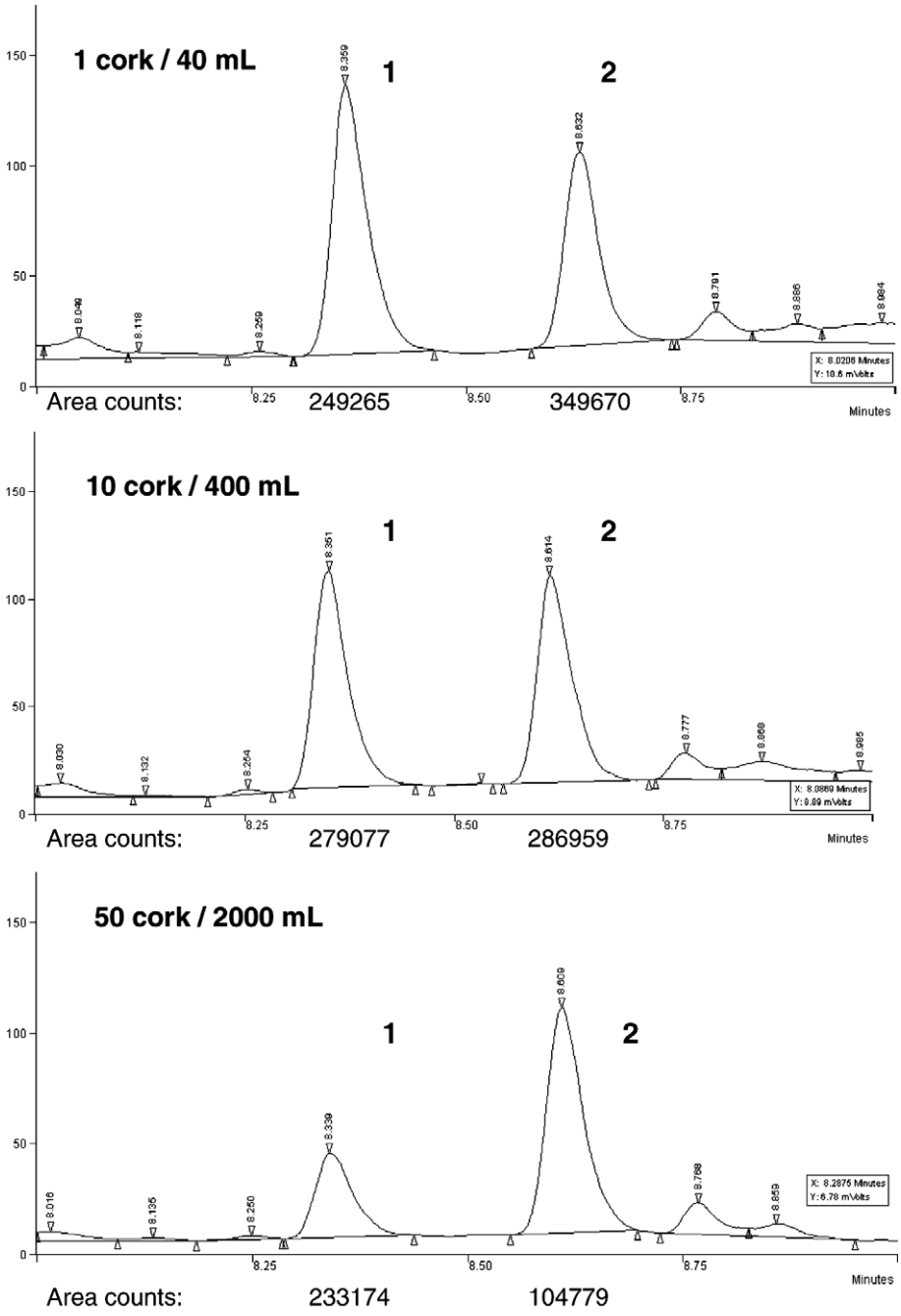

Fig. 1. Chromatograms of 2,4,6-TCA and 2,3,6-TCT during analysis of soaks obtained by the extraction of different number of cork stoppers in synthetic wine.

constituents present in the medium, such as polysaccharides, proteins and polyphenols. So, quantification of 2,4,6-TCA by SPME turned out to be highly dependent on the matrix composition. In order to overcome this difficulty in relative complex matrices (such as the one obtained from the extraction of 50 cork stoppers), the quantification of 2,4,6-TCA was carried out according to the method of standard addition. The performance of the method was again evaluated through estimation of linearity, repeatability and sensitivity, according to Standard methods (1995).

For quantification, one 5-point calibration curve was constructed using least-square linear regression of standard solutions of 2,4,6-TCA measured with GC–ECD. These solutions were prepared by spiking the matrix obtained from the extraction of 3 or 50 cork stoppers with 2,4,6-TCA at concentrations ranging from 2 to 50 ng/L. The

calibration curve after triplicate analysis of the standards was linear with correlation coefficients (R^2) higher than 0.95 for the target compound (see Table 1). The estimated recovery in the obtained matrix was high, more than 93% (Table 3).

The overall method repeatability, after analysis of the obtained matrix spiked with a standard amount of 2,4,6-TCA to a final concentration of 25 ng/L (six samples), was satisfactory, with an RSD value of 3.240 (Table 3). As shown in Table 3, the LOD obtained for 2,4,6-TCA was 0.366 ng/L and the LOQ was 2.7 time higher and equal with 1.258 ng/L.

Consequently, the qualitative analysis of 2,4,6-TCA in wine, cork stoppers and cork barks as well as the quantification of 2,4,6-TCA in wine and low numbers of cork stoppers (<3) were carried out according to the internal standardization. The quantification of 2,4,6-TCA in high numbers of cork stoppers (>3) and cork barks was carried out following the standard addition calibration technique.

3.4. Determination of 2,4,6-TCA in wine and cork samples

3.4.1. Wines and their cork stoppers

In order to determine 2,4,6-TCA in commercial wines with a cork taint off-flavor, eight wines (four red and four white) made of different grape varieties produced in Greece were selected. The target of this study was to examine the correlation between the 2,4,6-TCA in bottled wines and 2,4,6-TCA in the soaks of their cork stoppers. During these experiments, various quality parameters (pH, acidity, vol%) of the wines were also determined, according to the Standard methods (1995). The precise obtained experimental data are tabulated in Table 4. For example, pH values found between 3.18 and 3.72. Acidity was measured from 3081.4% to 4606.9% and vol% (which means the percentage of ethanol content in wine) was varied between 11 and 15. All the wines were purchased by local stores, where they were stored at shelves in vertical position. The placement prehistory of the wines during their storage in wine industry cellars and during their transportation is not well known. It is easily assumed that during their maturity period in wine cellars they were levelly mounted, which is a widely known and applicable practise in wine industry. Taking into account that wines are remaining at local shelves for three months at the most, the crucial period for 2,4,6-TCA migration from cork to wine or reversely is the time located before their arrival to the market store.

Table 4
Characteristics of bottled wines

No.	Wine type	Wine age	pH	Acidity	vol%
1	White	1	3.18 ± 0.8%	3595.8 ± 0.8%	11
2	Red	1	3.72 ± 0.5%	4270.8 ± 3.5%	11
3	White	2	3.50 ± 2.1%	3808.1 ± 2.4%	11
4	White	1	3.17 ± 0.3%	3716.7 ± 1.7%	11.5
5	Red	1	3.64 ± 0.4%	4606.9 ± 0.99%	15
6	Red	4	3.49	4166.3	12
7	White	3	3.21	4504.5	11.5
8	Red	3	3.62	3081.4	12

n.d., not determined.

Furthermore, an other significant factor affecting the transfer mechanism of 2,4,6-TCA between cork and bottled wine is the wine age. The production date (it is only known the year of wine making) of the wines tested during the current study was obtained from their label and the bottling date is completely unknown (Table 4).

In most of the cases, three different bottles of each wine were examined. The chemical analysis of obtained sample from the respective wine bottle was carried out in triplicate. An RSD% value of each wine was calculated in order to study the variation of 2,4,6-TCA concentration or other quality parameter from bottle to bottle of the respective wine.

As also observed by Amon et al. (1989) and Capone et al. (2002), there was significant variation in the distribution of 2,4,6-TCA between wines and cork stoppers (Table 5). In the majority of cases, a small amount of 2,4,6-TCA in the cork stoppers appears to have been absorbed by the corresponding wine after bottling. This can be concluded by the observation that releasable TCA in cork stoppers is generally much higher than the concentration of 2,4,6-TCA found in corresponding wines (Table 5). This is possibly due to different reasons. The location of 2,4,6-TCA in cork stoppers prior to bottling varies from one cork to another. The obtained results are in agreement with the conclusions of Amon and Simpson (1986) that transfer of chloroanisoles takes place, only when the contamination parts of the cork stopper are in direct contact with, or close to the wine or headspace above the wine. Moreover, the storage time of the wine after bottling, which was unfortunately unknown, is possibly less than the necessary time required for reaching equilibrium (regarding to 2,4,6-TCA) between cork stoppers and bottled wine. Hervé et al. (2000) found that the 2,4,6-TCA measured in bottled

Table 3
Recoveries, repeatability and detection limits of 2,4,6-TCA after extraction of 50 cork stoppers in 2 L of synthetic wine

	TCA spiked (ng/L)	TCA found (ng/L)	Recovery (%)	RSD (%)	Repeatability (RSD%)	Method detection limit (ng/L)	Limit of quantification (ng/L)
50 cork stoppers	27	25.13	93.10	2.72	3.240	0.366	1.258
50 cork stoppers	54	51.0	94.35	7.13			

Table 5
2,4,6-TCA analysis in bottled wines and their cork stoppers

No.	Wine type	No. of bottles	2,4,6-TCA in wine (ng/L)	No. of corks	Releasable TCA (ng/L)
1	White	3	0.41 ± 15.6%	3	0.54 ± 2.17%
2	Red	3	<LOD	3	0.73 ± 1.38%
3	White	3	60.8	1	123 ± 7.90%
			1 bottle found contaminated		
			n.d.	2	0.50 ± 2.37%
4	White	3	0.30 ± 3.9%	3	3.7 ± 7.8%
5	Red	3	13.5 ± 27.4%	3	<LOD
6	Red	2	7.18	2	30.19
7	White	1	1.70	–	–
8	Red	1	2.41	–	–

n.d., not determined.

wine after 14 months of storage was one half of the soak 2,4,6-TCA of the corresponding cork stopper. This is possibly explained by the smaller contact surface of wine with the cork stopper than in the case of determination of releasable TCA. However, one of the limitations of this study was the fact that the cork stoppers before bottling were coated with silicon/paraffin. This might have had a significant impact on the releasable TCA measurement. Moreover, the surface of the cylindrical base of each cork stopper was slightly destroyed during the extraction of the cork stopper from the bottle. Finally, it is possible that the wine could have been contaminated with 2,4,6-TCA before bottling. This seems to be the case for wine sample number 5, where although 2,4,6-TCA was detected in this wine, it seems that this defect is not due to the cork stopper of the bottle, as anticipated by the concentration determined by soaking of the cork stopper.

3.4.2. Cork stoppers and cork barks

Natural cork stoppers of different grades (1st, 4th, Superior), ready for bottling, were tested for the determination of releasable TCA. Repeated soaks of the same cork stoppers were carried out, resulting in the same TCA concentration in each cork soak, in agreement with the results presented by Hervé et al. (2000). Also three cork stoppers obtained from an intermediate stage of the cork production process, rejected by sensorial analysis during their screening for 2,4,6-TCA by the cork supplier, were analyzed in order to confirm that their releasable TCA was higher than the sensory threshold of this compound (Liacopoulos et al., 1999; Sefton & Simpson, 2005).

In addition, 10 g of cork barks, originating from the external layer of the cork tree, were chopped to small pieces, weighing 1–2 g a piece and were subjected to 2,4,6-TCA analysis. The results are shown in Table 6. According to the results presented in Table 6, the releasable TCA of cork stoppers, ready for bottling, is limited below the sensory threshold of about 5 ng/L (Liacopoulos et al., 1999; Sefton & Simpson, 2005). However, this is not happened for the case of the untreated cork stoppers rejected by sensorial analysis. In addition, the releasable TCA in the barks was quite high and ranged between 7 and 23 ng/L. These results are in agreement with the reported ones in the study of Juanola et al. (2002). It is also confirmed that the concentration levels of 2,4,6-TCA in cork barks, especially in the outer (older) part of the barks that were used in the current study, are very high and represent one of the most important origins of 2,4,6-TCA in cork stoppers.

Table 6
2,4,6-TCA in soaks from cork stoppers and cork barks

	No. of sample	ng/L releasable TCA
Cork barks	1	9.35 ± 1.00
	2	8.96 ± 0.82
	3	23.32 ± 3.10
	4	7.04 ± 0.38
	5	10.03 ± 1.44
	6	11.00 ± 1.58
Cork stoppers (ready for bottling)		
	Grade 1st	2.68 ± 0.52
	Grade 4th	2.81 ± 0.49
	Grade superior	2.51 ± 0.51
Cork stoppers (intermediate production stage)	1st	9.67 ± 1.43
	2nd	8.34 ± 0.03
	3rd	54.45 ± 0.67

The soak obtained from cork stoppers (one or three) extraction in synthetic wine or from cork bark extraction was analyzed in triplicate and the appropriate RSD% value was calculated.

In order to study the distribution of naturally contaminated cork stoppers in a lot, two bales of 50 untreated cork stoppers, which had been rejected by sensorial analysis, were analyzed in groups of three (except the last two cork stoppers of each bale). The obtained results are shown in Fig. 2a and b.

3.4.3. Statistical data analysis

The obtained results from the statistical analysis suggest that the majority of the cork stoppers are below the average releasable TCA of the population in both cases (1st bale: ranged from 0.40 to 30.0 ng/L and 2nd bale: ranged

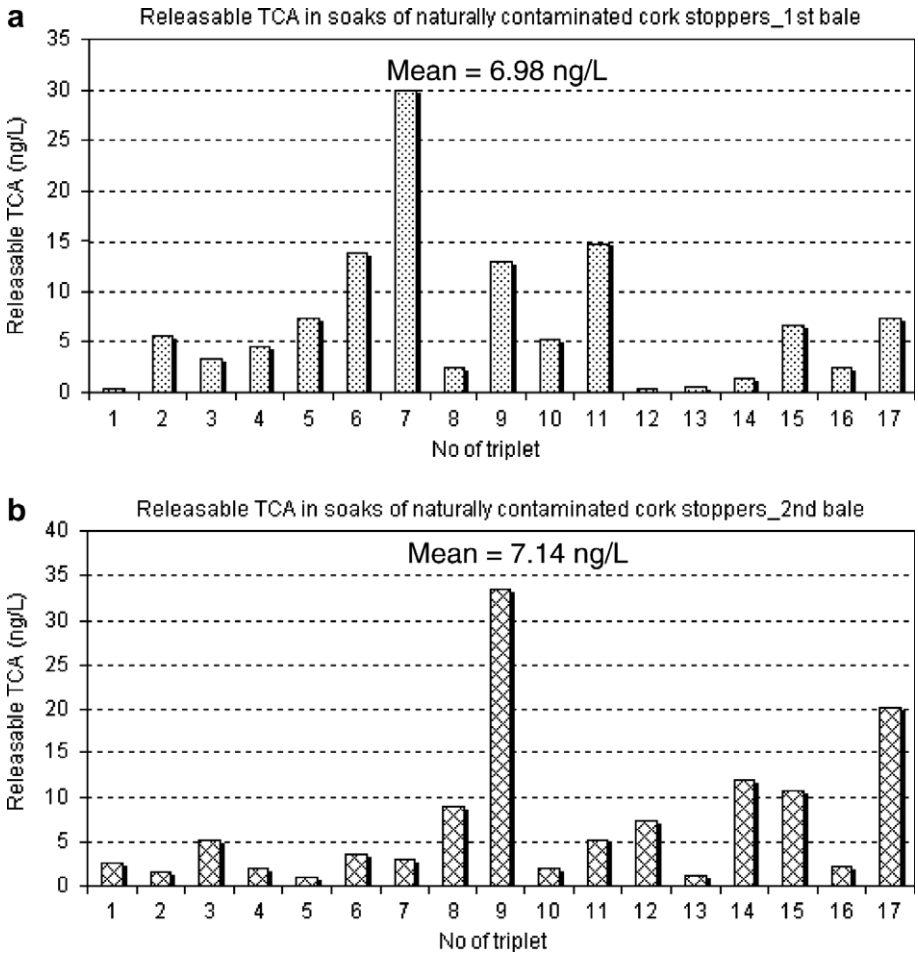

Fig. 2. Releasable TCA of 50 rejected cork stoppers by sensorial analysis, analyzed in triplets, in order to avoid matrix effects (a) 1st bale and (b) 2nd bale.

from 1.05 to 33.5 ng/L). Typically, 60% of the population had lower 2,4,6-TCA values than the mean. However, there were very few cork stoppers with extremely high 2,4,6-TCA content.

In order to determine the potential risk of causing cork taint by a cork stopper coming from a treated bale with SPP, the probability to find a tainted triple in the bale was estimated. The calculation of this probability was based on the simulation of the 2,4,6-TCA distribution in the cork population of the bale, according to the Gamma distribution, which is a staple distribution. The sample mean and standard deviation was estimated for each bale and using the Eqs. (6) and (7), the parameters γ and β were determined. For these parameters, the CDF was plotted (plots not shown) and the expected probability was estimated using Eq. (7), for $a = 5$ ng/L. The potential risk of causing cork taint by a cork stopper comes from a bale was expressed as the probability to find a tainted triple in the bale. Upon the results, the previous probability was 0.53 for the first bale and 0.55 for the second one.

Consequently, in bales with low and even accepted group soak 2,4,6-TCA levels, there might be very few corks with high 2,4,6-TCA levels. Same conclusion has been previously reached by Hervé et al. (2000).

4. Conclusions

The simplicity and accuracy of the SPME method combined with GC/ECD for the determination of 2,4,6-TCA in wine and cork soaks has been exemplified. However, during this study, matrix effects were observed when analyzing soaks obtained by the extraction of a high number of cork stoppers (>3). In order to overcome this difficulty and to obtain reliable and repeatable quantitative data, the standard addition calibration technique was successfully applied. The quantification of 2,4,6-TCA in wines and soaks obtained by the extraction of low number of cork stoppers (1–3) was carried out according to the internal calibration method. Thus the use of 2,3,6-TCT as an internal standard proved to be reliable and accurate. The method was also evaluated in real samples (wines and cork soaks). Variations in the distribution of 2,4,6-TCA between bottled wines and their cork stoppers were identified and the reasoning for this case was discussed. However, in most samples tested the 2,4,6-TCA measured in bottled wines was lower than the anticipated equilibrium concentration (releasable TCA). When the methodology was applied for 2,4,6-TCA measurement in cork barks, obtained by the outer part of bark, they were found contaminated with

significant levels of 2,4,6-TCA proving that they are one of the most important origins of 2,4,6-TCA found in cork stoppers. Finally, the industrial practice of testing cork stoppers, in bales of 50 or more, proved that even when low group soak 2,4,6-TCA levels are measured, that are even below the threshold limit for rejection (5 ng/L), this does not exclude the fact that there can be a appreciable number of cork stoppers with high enough 2,4,6-TCA levels, which could normally should be rejected if TCA testing could take place in lower numbered bales.

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