Supercritical Fluid Extraction of 2,4,6-Trichloroanisole from Cork Stoppers

Marisa K. Taylor,† Thomas M. Young,‡ Christian E. Butzke,† and Susan E. Ebeler*,†

Department of Viticulture and Enology and Department of Civil and Environmental Engineering, University of California, One Shields Avenue, Davis, California 95616

2,4,6-Trichloroanisole (TCA) is the compound most often associated with cork taint in wines and has been shown to have a very low sensory threshold (∼5 ng/L in wine). A supercritical fluid extraction (SFE) method for TCA in bark cork stoppers was developed with quantification via gas chromatography−mass spectrometry with selected ion monitoring. Supercritical carbon dioxide functioned as the extracting solvent, and temperature and pressure were optimized for the extraction. The method was validated using the stable isotope $^2H_5$-TCA as the internal standard. Recovery of TCA from spiked corks was found to be within 1−4% of the theoretical concentration with a coefficient of variation ranging from 2.6 to 9.7%. TCA levels in corks pulled from wines described as tainted by experienced judges ranged from 0.13 to 2.11 μg/g of cork. The SFE procedure offers a rapid, quantitative, nearly solvent-free, and automated method for the extraction of TCA from complex solid matrices such as cork.

Keywords: *2,4,6-Trichloroanisole; TCA; cork; supercritical fluid extraction; SFE; gas chromatography; cork taint; wine*

INTRODUCTION

Cork taint is a significant economic problem for the wine industry today, with an estimated annual loss of >$10 billion dollars worldwide (Fuller, 1995). The musty-mildew-like aroma associated with cork taint in wine has been attributed to the compound 2,4,6-trichloroanisole (TCA) (Buser et al., 1982). Although there are other aroma compounds that can be linked to corked wines, 2,4,6-TCA has been shown to occur in 60−100% of all tainted wines analyzed, and it has the lowest sensory threshold (Fuller, 1995; Amon et al., 1989; Pollnitz et al., 1996).

The source for the TCA in wines is linked to the cork stopper, and it is estimated that 2−5% of all corks are tainted (Fuller, 1995). In cork, TCA formation involves microbial methylation of a chlorophenol to an anisole (Lee and Simpson, 1993). The TCA then taints the wine through extraction from the contaminated cork stoppers. Other sources of TCA in wine have been suggested but not proven to contribute significantly to the extent of the problem. In isolated incidences, TCA contamination of wooden barrels, wine fining agents, or cellar materials may cause the taint of a particular batch of wine.

Previous methods of analysis of TCA in liquid media (wine) have included extractions with organic solvents, such as pentane and ethyl acetate, followed by concentration steps. Using spiked additions in the range of 30−100 ng/L, quantified recoveries were found to be 43−72% using an external standard procedure (Amon et al., 1989). Using solid-phase microextraction (SPME) and a polydeuterated TCA internal standard, recoveries of 107 ± 8% in wine were measured (Evans et al., 1997). However, quantitative measurements of TCA from cork closures are limited. In a common procedure, whole cork is soaked in absolute ethanol for 48 h. The ethanol is then extracted with pentane followed by concentration to 50 μL for gas chromatographic (GC) analysis. When TCA was spiked on the surface of whole cork using this procedure, Amon et al. (1989) reported recoveries of 90%. However, spiking the surface of the cork may not reflect true recoveries from corks in which the TCA has further penetrated into the cork material or lenticels. Pollnitz et al. (1996) soaked whole corks directly in pentane for 48 h, but no extraction recoveries were reported for this procedure. Fischer and Fischer (1997) utilized an SPME method for analyzing the amount of TCA in cork. They found that using the SPME fiber over moistened cork yielded very slow extraction kinetics, and they were unable to quantitatively determine the actual amount of TCA in the cork material.

Compared to the traditional liquid extraction procedures described above, supercritical fluid extraction (SFE) offers a rapid, selective extraction method that uses small volumes of organic solvents and results in concentrated extracts. Since the 1970s, SFE has been used in a variety of areas ranging from aroma recovery from spices and hops (Vitzthum and Hubert, 1980; Vollbrecht, 1982) to decaffeination of coffee beans and tea (Zosel, 1970). Currently, SFE is used on both commercial/industrial and analytical scales for the extraction of pesticides (Camel, 1998), refinery wastes, and flavors (including wine aroma volatiles) (Blanch et al., 1995).

Pentachlorophenol (PCP) in leather (Meyer and Kleibohmer, 1996) and organochlorine pesticides in eggs (Fiddler et al., 1999) are among a few of the examples that utilize supercritical fluids for the extraction of chlorinated compounds from solid matrices. Use of SFE

* Author to whom correspondence should be addressed [telephone (530) 752-0696; fax (530) 752-0382; e-mail seebeler@ucdavis.edu].

† Department of Viticulture and Enology.

‡ Department of Civil and Environmental Engineering.

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rather than traditional Soxhlet extraction decreased the analysis time from 2 days to 3 h for the extraction of PCP from leather. SFE of pesticides from eggs yielded higher recoveries (81−108%) than an Association of Official Analytical Chemists/Food and Drug Administration (AOAC/FDA) solvent extraction method (<40%).

For SFE, the solvent is in the supercritical state, having properties of both a gas (transport properties) and a liquid (density and solvating power). To optimize the extraction efficiency and selectivity, the pressure and temperature of the solvent are varied, which changes the density and thus the solvating power. Supercritical carbon dioxide is the most common solvent and is delivered by a pump to the extraction cell, which is contained in a temperature-controlled oven. As the CO_2 flows through the sample cell, the analyte is dissolved. The CO_2 and analyte then leave the oven and enter the restrictor, where a change in pressure from the set pressure to atmospheric pressure occurs. The change in pressure forces the analyte out of solution, where it is collected in a cooled trap. A solvent (e.g., methanol) is then used to flush the restrictor and trap to quantitatively collect the analyte in a small sample volume. The sample is normally directly analyzed via GC or high-performance liquid chromatography (HPLC).

Use of the deuterated stable isotope $^2H_5$-TCA as the internal standard and gas chromatography−mass spectrometry in the selected ion mode (GC-MS/SIM) provides a sensitive and selective method for the analysis of TCA in wine (Evans et al., 1997; Pollnitz et al., 1996). In this paper we describe the application of SFE combined with GC-MS/SIM for the rapid and quantitative determination of TCA in cork closures.

MATERIALS AND METHODS

Safety. All materials used during the SFE step must be capable of withstanding the high extraction pressures (150−350 atm) that may be obtained. Rupture disks must be provided to ensure that the system cannot exceed the design pressure.

SFE. A Suprex PrepMaster SFE instrument with an Accutrap automated variable stainless steel restrictor (Suprex Corp., Pittsburgh, PA) was used for the extractions. SFE grade carbon dioxide (Air Products, Folsom, CA) was used for all of the extractions. The cryogenic trap was cooled with industrial grade carbon dioxide (Puritan-Bennett Corp., Overland Park, KS). Nitrogen (instrumental grade, Puritan-Bennett Corp.) was used to purge the restrictor and trap. After the collection of the TCA on the trap, methanol (HPLC grade, Fisher Scientific, Fair Lawn, NJ) was used to flush the restrictor and trap. The trap was packed with C_{18} (Supelclean LC-18, Supelco Inc., Bellefonte, PA) and silanized glass beads (60/80, Alltech, Deerfield, IL) in a ratio of 60:40 (C_{18}/glass bead). Deactivated fused silica wool (Restek, Bellefonte, PA) was placed at either end of the trap and just before the 10 μm filter (Levy et al., 1998).

For extractions during the trap optimization, the sample chamber was filled with glass beads (0.5 mm diameter, 2.5 g/cm^3 density, Biospec Products Inc., Bartlesville, OK). 2,4,6-TCA (99%; Aldrich, Milwaukee, WI) was used to prepare standards for use during method optimization and for the preparation of standard curves. Polydeuterated 2,4,6-TCA ($^2H_5$-TCA; CDN Isotopes, Inc., Pointe Claire, PQ, Canada) was used as the internal standard (IS) for all extractions and calibration standards.

Optimized conditions for sample extractions were as follows. Trap temperatures of 0 and 25 °C were used for the trap collection and desorption, respectively. The oven temperature was set at 50 °C, and the pressure was 250 atm. The restrictor temperature was 100 °C. The solvent (methanol) volume was programmed to deliver 1.6 mL per collection vial. The absolute amount of solvent collected was measured each time. The solvent flow for the methanol was 1 mL/min. The CO_2 flow was 2 mL/min for each method step. The first step was an equilibrium/static mode extraction to allow the temperatures and pressure to reach the set values. This was followed by a 15-min dynamic extraction with a collection step and one flush. The third and fourth steps were two 5-min dynamic extractions with collection at the end of each step. The third and fourth steps were omitted after method validation because they did not significantly increase the total recovery of TCA.

Soxhlet Extractions. A microextraction Soxhlet apparatus with a 25-mL volumetric flask was used with single-thickness cellulose thimbles (50 mm × 10 mm i.d.; Whatman, Clifton, NJ). Methanol, 20 mL (HPLC grade, Fisher Scientific), was used as the extracting solvent with ~0.2 g of cork per thimble. A boiling chip was used to prevent bumping in the glass round-bottom flask. The extraction temperature was controlled by a rheostat (Staco, Inc.) and was adjusted to cycle solvent through the extraction vessel every 2−5 min. A water bath (Isotemp 10165 standard circulator, Fisher Scientific) was used to control the reflux condenser temperature at 5 °C. Extractions were performed for 24 h in duplicate.

GC-MSD. A Varian 8200cx autosampler (Walnut Creek, CA) with a 10-μL syringe mounted to a Hewlett-Packard 5890 gas chromatograph (Avondale, PA) paired with a Hewlett-Packard 5971 mass selective detector (MSD) with an electronics upgrade to a model 5972 was used for the analysis of the extracted samples. The software used with the system was MSD ChemStation by Hewlett-Packard.

The gas chromatograph was equipped with a DB-5MS (5% phenyl-methylpolysiloxane; 25 m × 0.25 mm i.d. × 0.25 μm film thickness) column (J&W Scientific, Folsom, CA). The oven parameters were as follows: initial temperature of 60 °C held for 2 min, ramp at 12 °C/min to a final temperature of 210 °C for 2 min (total cycle time including oven cool down was 20 min). Splitless injections (1 μL) were performed with the purge valve opened at 1 min and an injection temperature of 260 °C. The temperature of the transfer line from the GC to the MSD was 280 °C, and the helium carrier gas flow rate was 30 cm/s.

The MSD source was used at 1600 eV. A solvent delay of 8 min was used with a dwell time of 100 ms. The MSD was operated in the selected ion mode (SIM) and the two ions monitored were m/z 195 for TCA and m/z 215 for the $^2H_5$-TCA IS.

Standard Curves. A stock solution of TCA (100 mg/L) was prepared in methanol. The TCA stock solution was further diluted with methanol to give concentrations of 1.0, 5.0, 10.0, 25.0, and 50.0 mg/L and 1.0, 5.0, 10.0, 25.0, 50.0, 100 μg/L. A stock solution of 100 mg/L of IS was also prepared. A 1.0-mL aliquot of the IS stock was then diluted to 10.0 mL with each of the TCA solutions to yield final standard concentrations of TCA of 0.9, 4.5, 9.0, 22.5, 45.0, and 90.0 mg/L and 0.9, 4.5, 9.0, 22.5, 45.0, and 90.0 μg/L and a fixed IS concentration of 10.0 mg/L. The solutions were directly injected into the GC-MS. Integrated peak ratios (TCA peak area/IS peak area) were calculated and plotted versus the concentration ratio of the standards/IS to yield calibration curves. For TCA concentrations of 0.9−90.0 mg/L the resultant curves were linear with a coefficient of determination (r^2) of 0.99. Another curve for the concentration range of 0.9−90.0 μg/L yielded a coefficient of determination (r^2) of 0.98 (three replicate analyses at each concentration).

Spiked Recovery. A 3-mL extraction cell was filled with clean glass beads, 0.3 g (trap optimization only), or ground cork, 0.2 g, and spiked with 100 μL of a 100 mg/L IS solution (10 μg of IS) prepared in methanol and 100 μL of either a 300 mg/L TCA solution or a 100 μg/L TCA solution (30 μg or 10 ng TCA), also prepared in methanol. The spiked samples were extracted by SFE into a final volume of ~1 mL (absolute volume determined for each sample) and analyzed by GC-MS/SIM as described previously. Peak area ratios of TCA/IS were calculated and compared to unextracted standards of the same

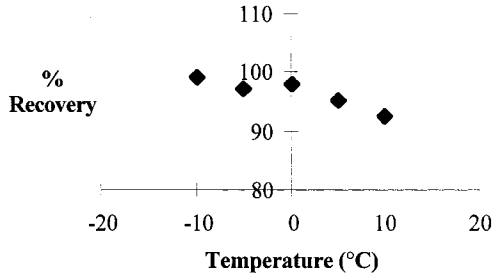

Figure 1. Optimization of trap temperature at constant oven temperature (50 °C) and pressure (250 atm). Optimization was conducted using 0.03 mg of TCA and 0.01 mg of IS spiked onto glass beads.

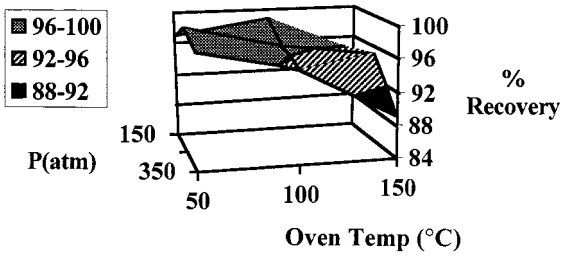

Figure 2. Optimization of oven temperature and pressure with the trap held at 0 °C. Optimization was conducted using 0.03 mg of TCA and 0.01 mg of IS spiked onto clean ground cork.

concentrations (30.0 mg/L and 10.0 μg/L) to determine the percent recovery.

Cork Dissection. Corks obtained from bottles of tainted retail wines were stored in sterile glass jars at 0 °C until dissection and extraction. The cork was cut into six sections, each section being 7 mm wide. Starting from the wine-exposed end of the cork, the sections were labeled A−F. A clean, sterile razor was used to thinly slice the cork into its six pieces. After sectioning, the slices were placed into separate 15-mL glass vials and stored at 5 °C without further preparation until SFE extraction.

RESULTS AND DISCUSSION

Trap Optimization. The oven temperature and the pressure were held constant at 50 °C and 250 atm, respectively, while the trap collection temperature was varied to determine the best temperature to trap the TCA. As Figure 1 shows, there was little variation among the recoveries for trap temperatures ranging from −10 to 10 °C. Because of the volatility of TCA and the performance of the SFE, a trap absorption temperature of 0 °C and a desorption temperature of 25 °C were chosen for the oven optimization step.

Oven Optimization. Effects of oven temperature (50, 100, 150 °C) and pressure (150, 250, 350 atm) were evaluated using clean ground cork (0.2 g) spiked with TCA. All parameters tested provided good recoveries (85−100%) (Figure 2). Therefore, we chose a temperature of 50 °C and pressure of 250 atm for all further extractions. Under these conditions, recovery was >96% (Figure 2). The density of CO_2 at 50 °C and 250 atm is 0.837 g/mL.

Limit of Detection (LOD) and Limit of Quantitation (LOQ). The absolute LOD (S/N = 3:1) and LOQ (S/N = 8:1) by GC-MS/SIM were 5 and 9 pg on column from extracted spiked cork samples.

Extraction Efficiency. After the addition of 100 μL of a 300.0 mg/L TCA solution (0.03 mg of TCA) to clean ground cork, a recovery of 97.3 ± 6.4% was obtained in the first 15-min dynamic extraction step (Table 1). Only one extraction step was used for all further analyses.

Table 1. Extraction Efficiency for Successive Extraction Steps from Cork Spiked with 0.03 mg of TCA (n = 6)

	steps 1 and 2 (15 min)	step 3 (5 min)	step 4 (5 min)
mass of TCA recovered (mg)	0.0292	0.006	0.0006
% TCA recovery	97.3	1.9	0.2
SD	6.5	0.5	0.02
CV%	7	26	10

Table 2. Accuracy and Precision Assay for Two Levels of TCA Spiked in Ground Cork

spiked TCA level	measured mean value	recovery (%)	SD	CV (%)
10 μg/L (n = 8)	10.3 μg/L	103.4	1.0	9.7
30 mg/L (n = 6)	29.8 mg/L	99.4	2.6	2.6

Table 3. Amount of TCA (Micrograms per Gram of Cork) Extracted from Tainted Retail Wines

	section						total
cork	A	B	C	D	E	F	(sum A−F)
1	1.21	0.47	0.26	0.11	0.06	0.03	2.11
2	0.03	0.02	0.02	0.02	0.02	0.02	0.13
3	0.02	0.02	0.03	0.03	0.06	0.04	0.20
4	1.21	0.24	0.11	0.07	0.05	0.16	1.84

SFE efficiency was further evaluated using whole corks that had been exposed to a saturated TCA vapor for 6 days in a closed container. The contaminated cork was sliced into 7-mm sections and extracted by SFE followed by Soxhlet extraction. TCA was not detected in any of the Soxhlet-extracted samples, again indicating nearly complete extraction by the SFE procedure. These corks may more closely simulate a naturally tainted cork matrix compared to spiking TCA on the cork surface. However, similar analyses with a large number of naturally tainted corks will be necessary to determine whether TCA can occur in a tightly bound or entrapped state within the cork matrix that could significantly decrease extraction efficiencies.

Accuracy and Precision. Precision assays were conducted at concentrations of 10.0 μg/L and 30.0 mg/L spiked into ground cork using the optimized trap and oven conditions. Measured values were within 1−4% of the theoretical concentration with a coefficient of variation (CV%) ranging from 2.6 to 9.7% (Table 2). The SFE recovery was comparable to the Soxhlet recovery of 98.8 ± 5% (data not shown). The SFE method, however, was much more rapid (20 min/sample) compared to the Soxhlet (24 h/sample) and utilized much lower volumes of methanol solvent (3 mL total for all SFE extraction steps versus 20 mL).

Extracted Corks from Tainted Retail Wines. Four corks pulled from wines described as tainted by experienced judges contained TCA amounts in the range of 0.13−2.11 μg/g of cork (Table 3). Previous studies performed by soaking whole corks in pentane or ethanol and analyzing the concentrated extract have also found a wide range of total TCA concentrations in the cork closure from tainted wines (Amon et al., 1989; Pollnitz et al., 1996; Howland et al., 1997). These previously reported levels were ∼10−400 ng/g of cork (estimated on the basis of an average cork weight of 3 g), which overlaps the range observed in the limited sampling of corks evaluated for this study.

Table 3 also reveals the concentrations of the TCA throughout the cork, with section A being the first 7 mm

of cork exposed to the wine. A detectable amount of TCA was found in each section throughout the cork. However, corks 1 and 4 showed a decrease in TCA concentration with increasing distance from the wine soaked end, whereas corks 2 and 3 showed approximately constant concentrations of TCA throughout the whole cork and location within the cork had no influence. Explanations for this difference may lie in the source of contamination, the quality and age of the cork, and the time of exposure to the wine. However, further studies are needed. Howland et al. (1997) observed that concentrations of TCA were greater in the outer 2 mm of the entire surface of the cork stopper regardless of the age of the cork. From their results, Howland et al. reasoned that the contamination of the corks occurred postpunching (including during storage and transportation) rather than during the conditioning and processing of the bark. TCA was also found in the older portions of the bark, which may be consistent with higher polychlorophenol pesticide (e.g., pentachlorophenol, 2,3,4,6-tetrachlorophenol, and 2,4,6-trichlorophenol) usage in years prior to harvesting or to lignin breakdown leading to larger phenol concentrations than in younger cork. The phenol may be chlorinated during further processing (e.g., chlorine bleaching) and then microbially methylated into the chlorinated anisoles (Lee and Simpson, 1993; Jäger et al., 1996; Howland et al., 1997).

Conclusion. The SFE method was found to quantitatively and reproducibly extract 97−103% of spiked TCA from the ground cork matrix, which is comparable to Soxhlet extractions (99%). However, the SFE procedure provided a more time efficient extraction that is readily automated and used minimal organic solvent. Using this procedure, detectable amounts (0.13−2.11 μg/g of cork) of TCA were found in several cork closures obtained from bottles of tainted wine. Utilization of the SFE method will now allow further investigations into the kinetics of transport of TCA from contaminated corks to wines, the identification of potential sources of cork contamination, and the effects of physical cork quality and age on the occurrence of cork taint in wines.

ABBREVIATIONS USED

SFE, supercritical fluid extraction; TCA, 2,4,6-trichloroanisole; $^2H_5$-TCA, polydeuterated 2,4,6-trichloroanisole; SPME, solid-phase microextraction; PCP, pentachlorophenol; CO_2, carbon dioxide; GC, gas chromatography; HPLC, high-performance liquid chromatography; GC-MS/SIM, gas chromatography−mass spectrometry/selected ion mode; IS, internal standard; CV, coefficient of variation; LOD, limit of detection; LOQ, limit of quantitation; SD, standard deviation.

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