Determination of 2,4,6-Trichloroanisole and 2,4,6-Tribromoanisole in Wine Using Microextraction in Packed Syringe and Gas Chromatography–Mass Spectrometry

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A selective and fast method for the quantitative determination of 2,4,6-trichloroanisole (TCA) and 2,4,6-tribromoanisole (TBA) in wine was developed. Microextraction in packed syringe (MEPS) was optimized for the extraction and preconcentration of the analytes using extremely small volume samples (0.1–1 mL). For GC-EI-MS, the limit of detection (LOD) for red and white wine was in the range 0.17–0.49 μg L^{-1} for TCA and TBA. In addition to GC-EI-MS both GC-NCI-MS and GC-HRMS were used to further improve both selectivity and sensitivity. The lowest LODs were achieved using GC-HRMS in the EI mode. In red and white wine samples the LODs were between 0.22–0.75 ng L^{-1} for TCA and TBA. The reproducibility and linearity for the GC-HRMS method was good, with RSD-values of 4–10% for spiked red wine samples at 1 ng L^{-1} and linearity with R^2 > 0.962 over a concentration range of 1 to 100 ng L^{-1}.

INTRODUCTION

Haloanisoles are known to cause off-flavor in food and beverages. One of the major organoleptic defects in wine is cork taint, which is associated with a musty or moldy aroma of wine. Several substances have been suggested to contribute to the cork taint, including chloroanisoles, chlorophenols, guaiacol, 1-octen-3-one, 1-octen-3-ol, and pyrazines (*1–3*). In a study by Soleas et al. (*4*), 2400 wines were tested and as much as 6.1% was affected by the organoleptic defect. Another study has been performed on European bottled wines, and it was found that between 0.1–10% were affected (*5*). 2,4,6-Trichloroanisole (TCA) has been found to be present in at least 80% of the cork defected wines studied (*6*). The origin of these off-flavor compounds is related to the microbial degradation of the corresponding halogenated phenols used as insecticides and fungicides in the enological industry (*7–10*). In addition to wine, TCA has been found in drinking and surface waters (*11*), coffee (*12, 13*) raisins (*14*) and freight containers (*15*). 2,4,6-Tribromoanisole (TBA) has also been identified as a potent source of cork taint (*7*) and has earlier been identified to provide unpleasant aromas in food products (*8*) and water (*16*).

Microextraction in packed syringe (MEPS) has recently been developed as a novel method for sample preparation. This technique has also been called microextraction in packed sorbent

(SGE). MEPS is a miniaturization of the conventional solid-phase extraction (SPE) packed bed techniques, but both the sample volume and volumes for extraction and washing solvents are reduced compared to SPE. MEPS consist of two parts, the MEPS syringe and the barrel insert and needle assembly (BIN) containing the SPE phase. The extraction phase of MEPS is based on a double pass system where the sample solvent both enters and exits from the bottom of the same bed volume. After extraction the bed volume can be washed with solvent before elution of the target compounds. MEPS has originally been developed for the analysis of local anesthetics in human plasma samples (*17, 18*), roscovitine in plasma and urine (*19*), and olomoucine in human plasma (*20*). Other applications are the analysis of polycyclic aromatic hydrocarbons in water (*21*) and recently the analysis of ochratoxin A in red wine (*22*).

Several studies on SPE based procedures for monitoring of different compounds in wine have been described in the literature (*23–25*) including methods for the determination of haloanisoles in wine. GC coupled to mass spectrometry or an electron-capture detector are often used for final detection. The sample preparation methods employed for the extraction and preconcentration of TCA are LLE (*26, 27*), LLE with a purge-and-trap (PT) system (*28*), stir bar sorptive extraction (SBSE) (*29, 30*), headspace mass spectrometry (HS-MS) (*31*), pervaporation (PV) (*32, 33*), perevaporation (PEV) (*32–34*), solid-phase microextraction (SPME) (*35–47*), and SPE. TBA has been preconcentrated with LLE (*7*), PV (*32, 33*), PEV (*34*), and

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headspace single-drop microextraction (HS-SDME) (*48*), SPME (*37, 38, 41*) and SPE (*4, 25*).

The development of MEPS opens the way for online coupling to GC or LC, allowing sample preparation and analysis without any modification of the chromatographic system. In contrast to SPE this miniaturized system does not require expensive robot like systems. In addition the bed volume in the syringe can be used several times after a washing stage, the consumption of solvents is minimal, and the cost is low. Compared to SPE and even to SPME, sample preparation time can be further reduced by using MEPS as a miniaturized combined extraction, clean up, concentration, and injection method.

Here we present the development of a MEPS based method in combination with GC-MS tested for analysis of TCA and TBA in wine. This method is compared to existing methods for the determination of TCA in wine. Spiked samples of red and white wine were used to evaluate the performance of the developed method. Different detection techniques (GC-EI-MS, GC-NCI-MS, or GC-HR-MS) were used not only to obtain the best sensitivity but also the best selectivity for the red and white wine extracts.

EXPERIMENTAL PROCEDURES

Solvents, Chemicals, and Standards. The internal standard (IS) 2,3,6-TCA was purchased from Ultra Scientific (north Kingston, RI). Both 2,4,6-TCA and 2,4,6-TBA were purchased from Sigma-Aldrich (Sleeze, Germany). Methanol and dichloromethane (Pestiscan grade) were purchased from LabScan (Dublin, Ireland) and toluene (Envisolv) from Riedel-de-Haën (Seelze, Germany). All stock solutions (1000 mg/L) were prepared in methanol, and all working solutions were prepared by dilution of the stock solution with methanol. Wine was spiked with the working solution to a final concentration of $0.001-100$ μg L^{-1}. The wine used in the study (Sangiovese and Sauvignon Blanc) was purchased from the Swedish Alcohol Retailing Monopoly. The wines were controlled to contain no TCA, TBA, or internal standard before usage in the experiments. The internal standard was added to the sample just before extraction to a final level of $0.01-100$ μg L^{-1}.

MEPS Conditions. MEPS was performed using a 100 μL gastight syringe filled with 4 mg of C18 (SGE, Ringwood, Australia) and it was conditioned using 30 μL of MeOH and 30 μL of water. Extraction was performed by drawing 100 μL or 10 × 100 μL of the sample through the syringe and the C18 solid phase. The C18 sorbent bed was dried by 3 × 80 μL of dry air. The analytes were then eluted with 10 μL of solvent into a GC-vial with a conical insert and injected using a standard GC autosampler. All samples have been manually extracted using MEPS and transferred to GC-vials for analysis in the GC-MS. Between sample extractions, the C18 adsorbent in the barrel insert and needle assembly was washed with methanol (5 × 80 μL) and water (4 × 80 μL). To control memory effects, blank samples of toluene were regularly eluted and analyzed between the extractions after the washing step. The same sorbent was used for more than 30 subsequent extractions.

GC-MS Conditions. The GC-MS system consisted of a Hewlett-Packard 6890 gas chromatograph coupled to a HP 5973 low-resolution-mass spectrometer using electron ionization (EI) at 70 eV or negative chemical ionization (NCI). Temperatures in the quadropole and ion source were 106 and 230 °C, respectively. The analytical column was a 30 m BPX5 (5% phenyl polysilphenylene) (0.25 mm 0.25 μm film thickness) with an integrated guard column (SilGuard) from SGE (Ringwood, Australia). The GC temperature program started with an initial oven temperature of 70 °C which was held for 3 min, heated to 180 at 5 °C/min and to 320 at 20 °C/min, and then held at 320 °C for 5 min. The total run time was 37 min. Splitless injection was used to inject 1 μL at 250 °C. Helium was used as carrier gas at 1.1 mL min^{-1}; when using NCI, methane was used as reagent gas at a pressure of 4.5 × 10^{-2} Pa. The mass spectrometer was run using single ion monitoring (SIM) mode, after a solvent delay of 9 min. In SIM mode, the most abundant mass of the chlorine or bromine cluster for each compound
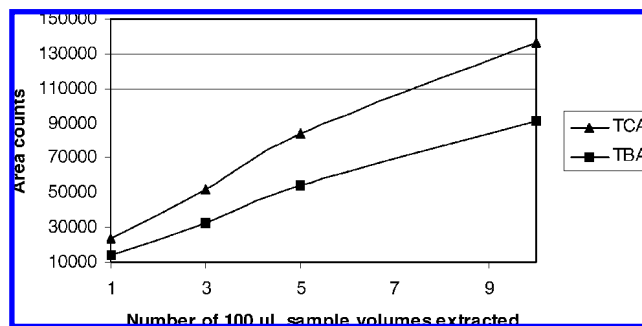

Figure 1. Extraction efficiency of different extraction volumes for analysis of TCA and TBA in spiked red wine using MEPS-GC-EI-MS.

Table 1. Repeatablity and Reproducibility (% RSD) for TCA and TBA in Samples of Spiked White and Red Wine Analyzed by MEPS-GC-EI-MS and MEPS-GC-HRMS

method	wine	sample (*n* = 3)	concentration	TCA RSD (%)	TBA RSD (%)
GC-EI-MS	white	different extractions	10 μgL^{-1}	2	3
GC-EI-MS	white	different extractions	1 μg L^{-1}	5	2
GC-EI-MS	red	different extractions	10 μgL^{-1}	4	4
GC-EI-MS	red	different extractions, different days	10 μgL^{-1}	11	3
GC-HRMS	red	different extractions	1 ng L^{-1}	10	4
GC-HRMS	red	same extract	1 ng L^{-1}	5	7

was monitored with a dwell time of 30 ms. Quantification was performed using *m/z* 195 for TCA, *m/z* 210 for the internal standard (2,3,6-TCA), and *m/z* 346 for TBA. When using NCI, quantification was performed using *m/z* 174 for IS and TCA and *m/z* 79 for TBA.

GC-high resolution (HR) MS was performed on a Micromass Autospec Ultima operating at 10 000 resolution using electron ionization at 35 eV. A 15 m SGE BPX5 (0.25 mm, 25 μm) GC column with SilGuard was used. The temperature program was started at 70 °C held for 3 min, heated to 160 at 5 °C/min and then to 300 at 32 °C/min, held at 300 °C for 5 min, and at last heated to 320 at 20 °C/min and held for 2 min. The injector temperature was at 280 °C. Solvent delay time was 7 min. In the SIM mode *m/z* 209.9406 and *m/z* 211.9377 were used for TCA and the internal standard. Mass 343.8780 and 345.7850 were used for TBA. For quantification, *m/z* 209.9406 was used for TCA and IS and mass 343.7870 for TBA.

RESULTS AND DISCUSSION

Method Optimization and Evaluation. All MEPS optimization experiments were conducted in duplicates of spiked red wine samples and analyzed by GC-EI-MS. Both a chlorinated solvent, dichloromethane, and an aromatic solvent, toluene, were tested for the elution of the target compounds from a standard MEPS containing 4 mg of C18 adsorbent. For optimizing the elution step, all experiments were conducted on samples containing 100 μL of spiked red wine to a concentration of 10 μg/L TCA and TBA and 100 μg/L IS. The recoveries were similar, 55% TCA and 77% TBA, using 10 μL of both solvents, compared to standard with analytes dissolved in toluene and directly injected into the GC. Toluene was used as eluant for the subsequent experiments mainly because of its lower volatility, which makes it more suitable when considering storage of samples. The elution efficiency was further tested by a second and third portion of toluene (10 μL). The amount of analytes eluted with the second portion toluene was 7% TCA and 11% TBA. The third elution volume contained 2% TCA and 3% TBA. To be able to use the method on line, the elution volume has to be reduced as much as possible, and the amount of elution solvent was limited to 10 μL of toluene. Because of the usage
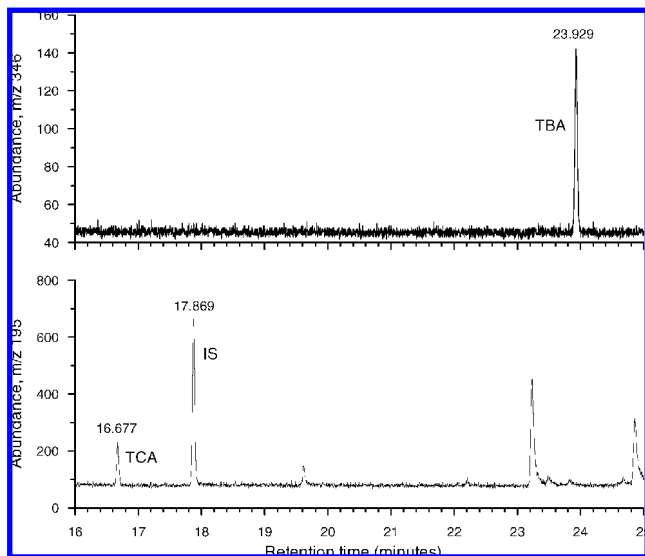
Figure 2. GC-MS chromatogram obtained from red wine spiked with 10 μg L^{-1} of TCA and TBA and 100 μg L^{-1} internal standard using MEPS-GC-EI-MS-SIM.

Figure 3. GC-MS chromatogram obtained from red wine spiked with 10 ng L^{-1} of TCA and TBA and 100 ng L^{-1} internal standard using MEPS-GC-NCI-MS-SIM.

Figure 4. GC-MS chromatogram obtained from red wine spiked with 1 ng L^{-1} of TCA and TBA and 10 ng L^{-1} internal standard using MEPS-GC-HRMS-SIM.

of an internal standard (2,3,6-TCA), recovery variations due to different wine matrices are compensated for during the quantification. The extraction efficiency was tested by pumping 100 μL of the same sample ten times through the C18 bed volume. These experiments showed a recovery of 55% TCA and 77% TBA after one extraction and 53% TCA and 75% TBA after 10 extractions (of the same volume of 100 μL sample). Multiple extractions of the same sample do not increase the extraction efficiency. The extraction efficiency was further tested using different sample volumes (1 × 100 μL, 3 × 100 μL, 5 × 100 μL, and 10 × 100 μL) of spiked red wine sample. The amount of analytes increased with the larger number of extraction volumes, but the extraction efficiency decreased after each extraction volume (**Figure 1**). By sampling more volumes of the wine, the competition for the active adsorption sites of the C18 sorbent seems to increase. The sorbent is very small, only 4 mg, and the capacity might be limited for sample volumes of 1 mL, as shown by the results of different extraction volumes (**Figure 1**). Ten extraction volumes resulted in 5.5 times the amount of TCA extracted and 6.8 times the amount TBA extracted, compared to one extraction volume. Also elution with a fresh second and third portion of toluene was tested and the amount eluted in the second extract was less than 11% TCA and TBA. The third eluate contained 9% TCA and 10% TBA. These results were similar to the values reported above for only one extraction volume of the sample.

None of the blank samples contained any TCA or TBA. MEPS proved to be very specific, and the eluted extracts were free of interference. No further clean up washing step of the MEPS solid phase was found to be necessary. Further sample clean up is normally necessary using 1% NaHCO$_3$ (*25, 49*) or a MeOH−water mixture (*25*) to remove interferences.

To clean the MEPS system between the extractions, it was washed with methanol and water to make it reusable. After the washing procedure between the extractions, the MEPS were completely clean for reuse. No memory effects or loss of performance was observed after more than 30 wine extractions.

Performance of the Method. The linearity of the method was studied using relative responses between the area of the internal standard and the area of the analytes, for samples of spiked red wine. For GC-EI-MS, a five-point calibration curve
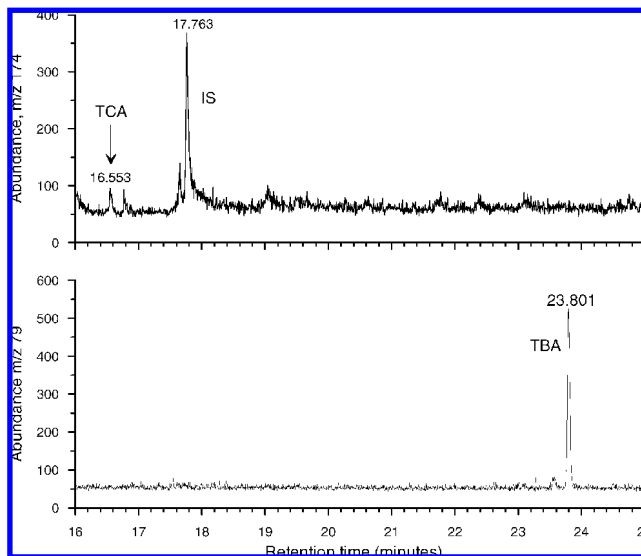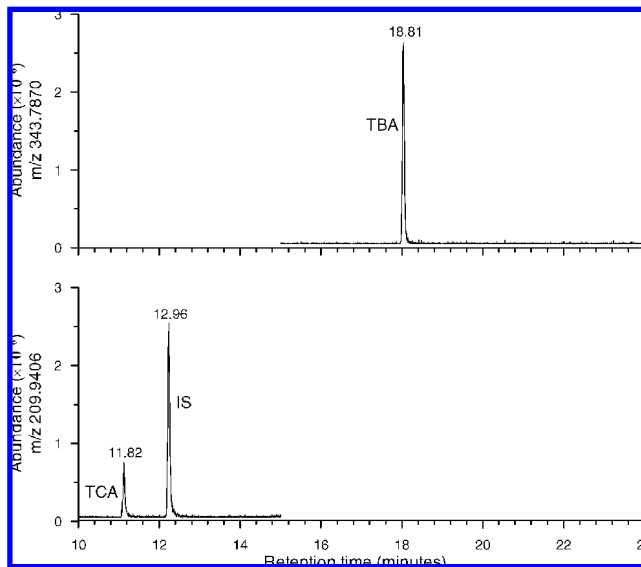
was measured in duplicates consisting of TCA and TBA at 1, 5, 10, 50, and 100 μg L^{-1} and the internal standard at 10 μg L^{-1}. Correlation coefficients were >0.999 for both TCA and TBA in the range 1−100 μg L^{-1}. For GC-HRMS, the linearity was tested in the range 1−100 ng L^{-1} (1, 10, 50, 100 ng L^{-1}) with correlation coefficients >0.962 for both TCA and TBA. Repeatability was studied using triplicate analysis at two levels of spiked white and one level of red wine at the low end of the calibration curve (**Table 1**) for GC-EI-MS. The RSD at 1 μg L^{-1} for white wine was 5% for TCA and 2% for TBA, and at 10 μg L^{-1} the RSDs were 2% and 3%, respectively. For red wine spiked at 10 μg L^{-1}, the RSD was 4% for both TCA and TBA. RSD for triplicates in red wine extracted and analyzed on two consecutive different days were 11% for TCA and 3% for TBA based on the internal standard method. RSD-values for the GC-HRMS method at 1 ng L^{-1} was 10% for TCA and 4% for TBA, respectively. TCA is more volatile than TBA, and this might be the reason for the slightly increased RSD

TCA and TBA in Wine Using MEPS and GC-MS

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Table 2. Comparison of Limits of Detection (S/N = 3) for TCA and TBA Using MEPS in Combination with GC-EI-MS, GC-NCI-MS, or GC-HRMS in Present Work and the Literature

method	extraction volume	limits of detection		reference
		TCA (ng L^{-1})	TBA (ng L^{-1})	
MEPS-GC-EI-MS (red wine)	100 μL	490	450	present study
MEPS-GC-EI-MS (white wine)	100 μL	270	170	present study
MEPS-GC-NCI-MS (red wine)	10 × 100 μL	20	5.0	present study
MEPS-GC-HRMS (red wine)	10 × 100 μL	0.75	0.23	present study
MEPS-GC-HRMS (white wine)	10 × 100 μL	0.67	0.22	present study
HS-SPME-GC-NCI-MS (red wine)	3 mL	0.3[a]	0.2[a]	50
HS-SPME-GC-HRMS (red wine)	3 mL	0.03[a]	0.03[a]	50
SPE-PTV-GC-ITMS (five wines)	50 mL	0.2	0.4	25
HS-SDME-GC-ECD (synthetic wine)	20 mL	8.1	6.1	48
MHS-SPME-GC-MS-MS	5 mL	10	10	38

[a] S/N = 10.

values for TCA. Repeatability was also studied for the GC-HRMS method, analyzing the same extract (spiked red wine, 1 ng L^{-1}) three times. The RSD was then 5% for TCA and 7% for TBA, respectively.

The influence of the sample matrix was tested using spiked red wine as a standard for the quantification of the anisoles in white wine, which was spiked in triplicate at two different concentrations, 1.0 μg L^{-1} and 10 μg L^{-1} of TCA and TBA. Both the red wine (the standard) and the white wine samples were extracted using MEPS as describe above. 2,3,6-TCA was used as the internal standard and was added to the red wine and the white wine samples prior to extraction. Quantification was performed by using the relative responses between the area of the internal standard and the area of the analytes in the standard and the samples. When employing red wine as a standard resembling the matrix, the concentration for white wine spiked at 1.0 μg L^{-1} was calculated as 1.2 μg L^{-1} for TCA and 1.17 μg L^{-1} for TBA. The white wine spiked at higher concentration (10 μg L^{-1}) was calculated to 9.62 μg L^{-1} and 9.27 μg L^{-1} for TCA and TBA, respectively. The recovery for the white wine was 64% for TCA and 95% for TBA, respectively, compared to analytes dissolved in toluene and directly injected into the GC. For extraction and preconcentration of TCA and TBA in these complex wine matrices, MEPS showed to be a robust technique with good linearity over a large concentration range and good reproducibility.

TCA can be sensorically noticed by humans in the range of 0.03−10.0 ng L^{-1}. The concentration of TCA considered to produce a defect in wine is somewhat higher, ranging from 10 to 40 ng L^{-1} (*36*). The sensory threshold in wine for TBA has been reported as 7.9 ng L^{-1} (*7*). TCA in sensorically tested cork defected wines has previously been found to be in the range 2−25 ng L^{-1} (*50*). Limits of detection (LOD) were calculated based on signal-to noise (S/N) 3/1 from spiked wine samples at low concentrations. LODs for TCA and TBA in the present study for red wine using GC-EI-MS was 490 and 450 ng L^{-1} for TCA and TBA, respectively. For white wine the LOD was 270 ng L^{-1} for TCA and 170 ng L^{-1} for TBA. In the previous study both NCI and GC-HRMS was found to be more sensitive than EI-MS (*50*). It was also found that while using GC-EI-MS the sensitivity between TCA and TBA was very similar, and for GC-NCI-MS the sensitivity for TBA was about four times better than for TCA. In addition the extraction volume was increased to 10 × 100 μL to enhance the LOD even further and to get closer to the earlier achieved LODs for SPME methods. The LODs for the MEPS method for red wine using GC-NCI MS was improved to 20 ng L^{-1} for TCA and 5 ng L^{-1} for TBA. Even further improvement was achieved by running the MEPS extracts on the high resolution GC-MS

system. LODs for TCA and TBA in red and white wine were 0.67−0.75 and 0.22−0.23 ng L^{-1} for TCA and TBA, respectively, which is clearly under the sensory threshold. Chromatograms of the wine samples run using the different MS detection techniques are presented in **Figures 2–4**. The LOD values for MEPS-NCI-MS and MEPS-GC-HRMS are similar to those found in the literature as presented in **Table 2**. The advantage of MEPS compared to SPME is the extraction time of only about 5 min per sample while SPME needs about 30 min (*50*). While the sensitivity of MEPS in combination with GC-HRMS is better, low resolution NCI is also an attractive alternative since high resolution instruments are less frequently available and more expensive to purchase and operate.

A selective and fast sample preparation method using MEPS in combination with different GC-MS techniques has been developed and validated for the determination of 2,4,6-trichloroanisole and 2,4,6-tribromoanisole in small volumes (0.1−1 mL) of wine. TCA and TBA were selectively extracted from complex wine matrices resulting in interference free and reproducible GC-MS chromatograms even without cleanup washing steps. The reproducibility of the method was increased by the usage of an internal standard (2,3,6-TCA) for quantification. LODs were extremely low for GC-NCI-MS and GC-HRMS and TCA and TBA can be detected in the wine before it is sensorically noticed as cork tainted. The MEPS based method is comparable to both SPME and SPE based methods, but the sample preparation time is reduced.

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