JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Method for the Gas Chromatographic Assay with Mass Selective Detection of Trichloro Compounds in Corks and Wines Applied To Elucidate the Potential Cause of Cork Taint

GEORGE J. SOLEAS,†,‡ JOSEPH YAN,† TOM SEAVER,§ AND DAVID M. GOLDBERG*,‡

Quality Assurance Department, Liquor Control Board of Ontario, 55 Lakeshore Boulevard East, Toronto, Ontario, Canada M5E 1A4, and Department of Laboratory Medicine and Pathobiology, University of Toronto, 100 College Street, Toronto, Ontario, Canada M5G 1L5

To investigate the role of trichloro compounds as a potential cause of "cork taint" in wine, an assay for trichloroanisole (TCA) and trichlorophenol (TCP) in corks and wine was developed utilizing solid phase extraction on a C_{18} cartridge followed by gas chromatography with mass selective detection. Recovery and imprecision for TCA were 86−102 and 1.6−5.8%, respectively, and for TCP 82−103% and 1.7−3.9%, respectively. Limits of detection and quantitation were 0.1 and 2 ng/L, respectively, for TCA, and 0.7 and 4 ng/L, respectively for TCP. A survey of 2400 commercial wines revealed a higher incidence of cork taint in white wine than in red and in wines utilizing composite cork closures; wines from central Europe and Spain had higher overall rates of contamination and those from Canada and Italy the lowest. Significant but modest associations were found between the TCA and TCP contents of the wines and corks, but many wines exhibiting cork taint had low or undetectable concentrations of TCA. Over a 12-month period, experimentally bottled wines exhibited a slow increase in TCA and TCP content while cork closures manifested a decrease; most bottles showing cork taint contained low levels of TCA, and TCP concentrations were well below the sensory threshold. Neither compound was cytotoxic to human cell lines in culture up to final concentrations of 500 ng/mL. It was concluded that these two trichloro compounds are, at most, minor components of cork taint in commercial wines.

KEYWORDS: Trichloroanisole; trichlorophenol; analytical methods; wine; corks; cork taint; sensory perception

INTRODUCTION

The random presence of organoleptic taints in wines, perceived as "musty" or "moldy" aromas and flavors, represents a significant source of financial loss to the wine trade. Buser et al. (*1*) were the first to detect the compound 2,4,6-trichloroanisole (TCA) in wines characterized by this taint. It was not present in wines of good organoleptic quality but was found in the corks drawn from tainted wines. They suggested that TCA originated from chlorination of lignin-related substances during bleaching of the cork, the product(s) subsequently being leached into the wine during storage. This taint has come to be known as "corkiness" and is widely attributed to the interaction of bacteria and fungi with constituents of the cork. However, TCA can be formed during microbial contamination of soil (*2*), packaging materials (*3*), coffee (*4*), and raisins (*5*). One possible source is the degradation (methoxylation) by molds of 2,4,6-trichlorophenol (TCP), an industrial agent used to decontaminate wooden objects, including the floors, beams, and barrels present in wine cellars (*6*, *7*). These relationships are illustrated in **Figure 1**.

Many investigations into the origin of cork taint and the mechanisms (both chemical and microbiological) responsible for its production have been performed during the past two decades. These have been the subject of a recent review (*8*) in which the authors concluded that faulty corks were responsible for only a minority of contaminated wines. Direct experimental evidence bearing upon this issue has been slender, most reports being based upon retrospective analyses and on very few samples. In this paper, we describe our attempts to define the relationships between cork taint and the following variables based upon a survey of 2400 wines: concentrations of TCA and TCP in wines and corks; color and country of origin of the wines; type of closure employed.

Additionally, we sought to define the kinetics describing the leaching of TCA and TCP from the corks and the effect of different storage conditions. Finally, we examined the potential cytotoxicity of these compounds, as there is little information concerning this important matter. Implementation of these objectives required the development of ultrasensitive assays

* Author to whom correspondence should be addressed [fax (416) 978-5650; e-mail david.goldberg@utoronto.ca].
† Liquor Control Board of Ontario.
‡ University of Toronto.
§ Vincor International Inc.

10.1021/jf011149c CCC: $22.00
Published on Web 01/30/2002

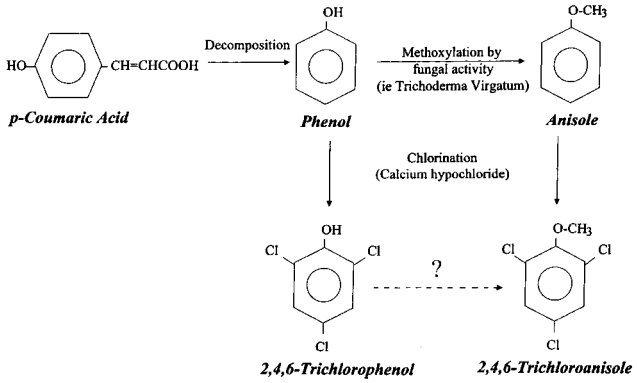

Figure 1. Structures, origin, and putative relationship between TCA and TCP.

utilizing a gas chromatographic (GC) method with mass-selective detection (MSD) offering more desirable characteristics than those that have been published so far (*1*, *9*−*11*).

MATERIALS AND METHODS

Reagents. The following solvents, all of HPLC grade, were purchased: acetonitrile 190 (catalog no. 1401-7-40), dichloromethane (catalog no. 3601-7-40), and ethyl acetate (catalog no. 4601-7-40), all from Caledon Laboratories (Georgetown, ON); ethanol (catalog no. 288142) from Rieder Distillery Ltd. (Grimsby, ON). The gases were nitrogen (medical grade, catalog no. NI-1146T) and helium (UHP, catalog no. SG 103143T), both from Praxair (Toronto, ON). The TCA (catalog no. 25,539-3) and TCP (catalog no. T1266) standards (>99% pure) were purchased from Sigma-Aldrich, Oakville, ON.

Cork Closures. The following cork closure types were purchased for comparison from two different suppliers: composite (natural cork particles from trimmings, cleaned of impurities and reconstituted into a uniform cork with plastomers); composite with disk (corks manufactured by laminating natural cork disks (4 mm thickness) to the two ends of a composite cork); natural grade 1 and natural grade 2 (natural corks of top to good visual appearance with no major flaws and with surface flaws of no depth or substance; holes or pores must not exceed 5 mm, cracks originating at the ends should be <18% of cork length, and no cracks in the body of the cork can exceed 25% of cork length); synthetic (proprietary synthetic polymer material); and Altec (similar to composite corks but free of lignins). By agreement, the names of the suppliers are being withheld. These were employed in a series of experiments that will be described subsequently under Results.

Toxicity Study. The following human cell lines were obtained from the American Type Culture Collection (ATCC), Rockville, MD: Colo-320 HSR (colon carcinoma); T-47D (breast carcinoma); SK-N-MC (neuroblastoma); BG-1 (ovarian carcinoma). They were grown to confluency in culture media in 24-well plates as recommended by the ATCC, at which point the number of cells per well approximated 0.2×10^6. TCA or TCP in final concentrations of 5−5000 ng/mL was added to various wells, and plates were incubated for a further 48 h. Cell viability was measured by means of the One Solution Cell Proliferation Assay (Promega Corp., Madison, WI) in which colorless MTS tetrazolium salt is converted to a formazan chromogen. Recording absorbance at 570 nm provides an index of cellular electron-transfer reactions and therefore of cell viability (*12*, *13*).

Cork Extraction. Corks were first carefully weighed and then placed in a 120 mL amber bottle with 50 mL of 40% (v/v) aqueous ethanol. After gentle shaking for 48 h using an orbit shaker (catalog no. 5520) from Lab-Line Instruments Inc., Melrose, IL, 50 mL of water was added to generate a final ethanol concentration of 20% (v/v); this solution is referred to as the *cork extract*.

Solid Phase Extraction. A C_{18} cartridge (3 mL/500 mg, Bakerbond Octadecyl Speedstick) from J. T. Baker, Phillipsburg, NJ, was conditioned with 2 mL of ethyl acetate, 2 mL of 96% (v/v) ethanol, and finally 2 mL of 10% (v/v) ethanol in water. A 50 mL aliquot of wine or of cork extract is then applied at a rate of 10 mL/min, after which the cartridge is dried under gradual suction on a Supelco Visiprep-24 vacuum manifold at a pressure of −65 kPa (Supelco, Mississauga, ON) for 35 min. TCA and TCP were eluted by adding 0.5 mL of dichloromethane; the first 200 μL of eluate containing all of the TCA and TCP applied was collected and mixed with 200 μL of acetonitrile, representing a 125-fold concentration of the original sample.

GC-MSD Procedure. The following instrumentation, all purchased from Hewlett-Packard (Agilent Technologies, Mississauga, ON) was employed: 5890 series II gas chromatograph; 5972A mass selective detector; Chem Station G1701BA (version B.02.05). Five microliters of sample was injected onto a DB-5MS column (30 m long, 0.25 mm i.d., and 0.25 μm film thickness; J&W Scientific, Folsom, CA) through a splitless injection port with a double-gooseneck glass insert and gold-plated injector seal by means of an HP7673B autoinjector and sampler tray capable of presenting 100 samples unattended. The injector and detector temperatures were set at 200 and 240 °C, respectively. The temperature program comprised two phases: initially the temperature was held at 50 °C for 5 min, ramped at a rate of 1.5 °C/min to 100 °C, and held for 3 min. It was then ramped at a rate of 30 °C/min to 250 °C and held for 5 min. The total run time was 51.3 min. The solvent delay was set at 25 min and the electron multiplier at 2600 V. Ultrahigh-purity helium with in-line Supelpure moisture trap and hydrocarbon trap (Supelco, Mississauga, ON) was used as carrier gas. The carrier gas line was set at 72 psi, column head pressure at 8 psi, and total flow at 22 mL/min. Detection was by selective ion monitoring (SIM), utilizing the sum of the ions at 195, 197, 199, 210, 212, and 214 amu for TCA and those at 196, 198, and 200 for TCP. The dwell time was set at 100 ms.

Statistics. Analysis of variance (ANOVAR) was used to evaluate differences between the TCA and TCP contents of corks and wine with respect to an array of variables including closure type, wine color, manufacturer, time of storage, and country of origin. The statistical significance of observed differences was measured by the Tukey−Kramer multiple comparisons test, where the method of Bartlett established that the standard deviations (SD) of the various columns in the same ANOVAR were not significantly different and the Kolmogorov−Smirnov test was consistent with the data being derived from populations following a Gaussian distribution. Otherwise, the Kruskal−Wallis test for nonparametric ANOVAR was performed and Dunn's multiple comparison test was used to evaluate the significance of observed differences. The associations between the levels of trichloro compounds in corks and wine were tested by nonparametric regression; Spearman's rho was calculated, and the values were corrected according to the Bonferroni procedure.

The data obtained in the 12-month investigation of the kinetics and mechanisms of the leaching of trichloro compounds from corks to wine and the impact upon sensory perception were analyzed in a distribution-free General Linear Model Multivariate Procedure providing regression analysis and ANOVAR for multiple dependent variables by one or more factor variables or covariates. Although the sums-of-squares and cross-products (SSCP) matrixes can generate the results of many statistical tests, we evaluated significance using Roy's largest root criterion (*F* statistic). For some non-numerical comparisons, frequency distributions were analyzed using the Wilcoxon test. These procedures were carried out using the SPSS10.0.7 statistical package (SPSS Inc., Chicago, IL).

RESULTS

Analytical Characteristics of Method. Total ion scans (**Figure 2**) show the spectra of TCA and TCP, justifying the choice of ions used for MSD. Total ion monitoring (**Figure 3**) demonstrates the excellent resolution achieved for TCA and TCP, eluting at 36.8 and 38.8 min, respectively. By measuring the sum of the selected ions for both compounds by SIM, we were able to achieve excellent sensitivities: for TCA, LOD and LOQ as previously defined (*14*), were 0.1 and 2 ng/L, respectively; for TCP, LOD and LOQ were 0.7 and 4 ng/L, respectively. Linearity was very satisfactory over the ranges of 2−100 μg/L for TCA ($r = 0.999$) and 4−400 μg/L for TCP ($r = 1.000$). For TCA, recovery ranged from 86 to 102% and

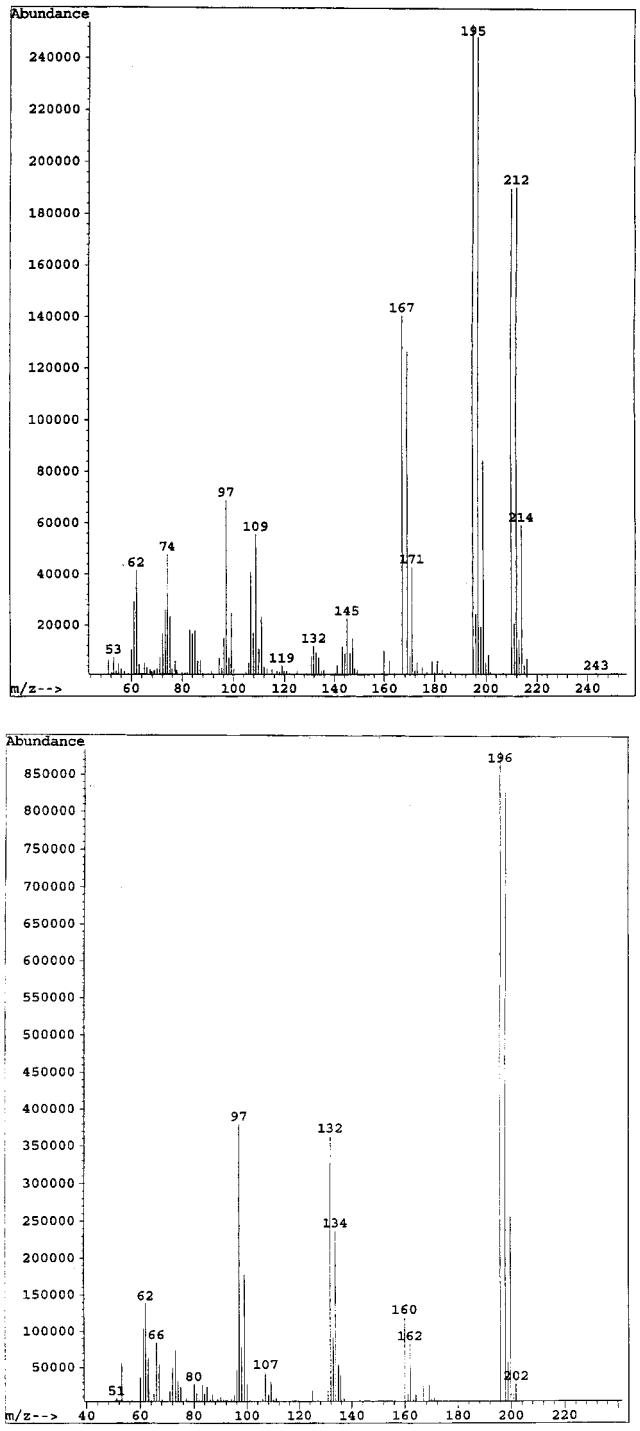

Figure 2. Total ion scan showing mass spectra of TCA (top) and TCP (bottom).

imprecision (measured as relative standard deviation, RSD) from 1.6 to 5.8% (**Table 1**). For TCP, the former ranged from 82 to 103% and the latter from 1.7 to 3.9%. Although **Table 1** presents data for wine, similar results were obtained for cork extracts, with virtually identical values for LOD, LOQ, and linearity (not shown).

Solubilization of TCA in Corks. Twenty composite corks from each of the two suppliers (A and B) were stored at 4 °C in three different white wines (Chardonnay, Riesling, and Sauvignon Blanc) and three different red wines (Cabernet Sauvignon, Merlot, and Pinot Noir) with aqueous ethanol concentrations between 11.5 and 12.5% (v/v). Triplicate analyses for TCA were carried out at 24 h, 48 h, and 7 days. As the

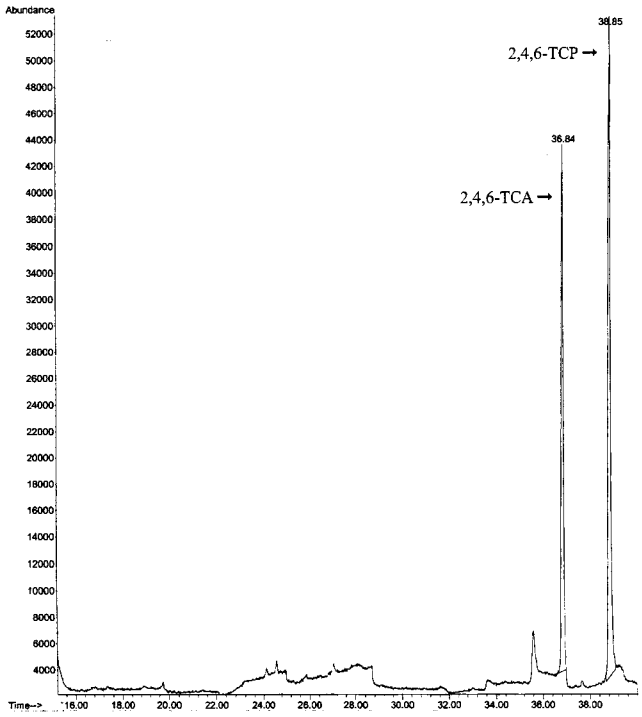

Figure 3. Total ion monitoring of chromatographic output of wine extract showing resolution and elution times of TCA and TCP.

Table 1. Recovery and Imprecision of 2,4,6-TCA and 2,4,6-TCP Added in White Wine at Three Concentrations[a]

compound	concn (ng/L)	recovery (%)	RSD[b] (%)
2,4,6-TCA	5	85.8 ± 5.6	1.6
	20	102.4 ± 0.4	4.5
	50	102.0 ± 1.7	5.8
2,4,6-TCP	12	81.9 ± 3.3	3.9
	48	103.0 ± 3.9	3.4
	120	82.9 ± 0.1	1.7

[a] All data are means ± SD of six independent assays. [b] Based on six replicate analyses.

Table 2. Effects of Red and White Wine on Solubilization of TCA (Nanograms per Cork) in Composite Corks from Two Manufacturers (A and B)

	time of leaching		
wine/cork	24 h	48 h	7 days
white wine ($n = 9$)			
cork A	8.2 ± 2.9	16.2 ± 4.5	11.0 ± 2.3
cork B	6.8 ± 1.8	7.6 ± 2.1	7.4 ± 1.3
red wine ($n = 9$)[a]			
cork A	8.6 ± 1.8	11.8 ± 2.2	13.4 ± 2.6
cork B	4.4 ± 1.1	6.2 ± 2.1	7.4 ± 1.1

[a] Each data point (mean ± SD) is based on immersion of 20 corks in each wine sample (three varietals of each color in triplicate).

three varietals in each color category gave very similar results, the values (nine for each category) were pooled, and the means are presented in **Table 2**. There were no significant differences (tested by ANOVAR) between the amounts of TCA solubilized by red or white wines or between the 48 h and 7 day time intervals. In other words, solubilization seemed to be complete within 48 h, justifying our use of this time interval in the analysis of cork TCA content. However, corks from the first manufacturer released more TCA than those of the second ($P < 0.01$)

Table 3. Incidence of Cork Taint Detected in Wine Related to Type of Closure in 2400 Consecutively Tested Wines

closure type	no. of samples	no. of tainted samples	% tainted
composite corks	94	30	31.9
composite with disk	255	12	4.7
natural corks	1720	97	5.6
synthetic	59	0	0
ROPP (screw cap)	185	0	0
sparkling wine corks	60	2	3.3
Altec	27	4[a]	14.8
all types	2400	145	6.1

[a] Tainted organoleptically but TCA not chemically detected.

In another experiment, natural and composite corks from both suppliers were stored in 1 L of white wine (Chardonnay), and TCA concentrations in the wine were analyzed in triplicate at weekly intervals up to 8 weeks. These did not increase beyond the first week. The amount of TCA solubilized from the natural corks was about half of that released by the composite corks ($P < 0.01$, tested by ANOVAR); the composite corks from the first supplier (A), reflecting the data of **Table 2**, released more TCA than those of the second supplier (B); $P < 0.008$, tested by ANOVAR.

Experiments To Explore the Relationship between Cork Taint and Trichloro Compounds. *Survey of Commercial Wines.* All wines submitted for routine testing at the Quality Assurance Laboratory of the Liquor Control Board of Ontario undergo organoleptic evaluation by a panel of experts. All are certified consultants possessing the Diploma (Level III) of the British Wine Guild. In addition to cork taint, faulty wines are classified for ethyl acetate, mercaptans, hydrogen sulfide, oxidation, excessive microbiological activity, SO_2, acetic acid, sorbic acid, and volatile acids among others. All panel members detected TCA in wines at concentrations ≥2 ng/L. Over a period of 12 consecutive months, 145 of a total of 2400 were judged to exhibit cork taint. The closures used for these wines are displayed in **Table 3**. The overall incidence of cork taint was 6.1%, but those closed by composite and by Altec corks demonstrated incidences of 31.9 and 14.8%, respectively ($P = 0.012$ and 0.042, respectively; Wilcoxon test). No wines utilizing ROPP (screw-cap) or synthetic cork were judged to be tainted. The TCA and TCP contents of all tainted wines and their respective corks were analyzed. **Figure 4A** illustrates the TCA concentrations of the 145 wines judged to be tainted; values <2 ng/L were found in 74 (51%), and indeed in as many as 51 (35%), TCA was below the detection limit of the method (<0.1 ng/L). For only 71 (49%) of the tainted bottles were TCA concentrations ≥2 ng/L recorded. TCA concentrations were also measured in 100 consecutive wines of all colors organoleptically free of taint. No values ≥2 ng/L were found (**Figure 4B**). On the basis of nonparametric correlation analysis employing Spearman's rho and the Bonferroni correction, the following manifested significant but modest association: TCA (wine) and TCA (cork) [rho = 0.332, $P < 0.01$]; TCP (wine) and TCA (cork) [rho = 0.389, $P < 0.01$]; TCA (cork) and TCP (cork) [rho = 0.379, $P < 0.01$]; TCA (wine) and TCP (cork)[rho = 0.197, $P < 0.05$]. No association was observed between TCP in the wine and TCP in the cork.

Table 4 presents data on the wines surveyed by color (red and white only) and by country or region. White wine displayed an overall cork taint incidence of 8.3% and red wines an incidence of 4.2% ($P = 0.029$, Wilcoxon test). Not shown are 81 rosé wines, of which 3 were tainted, an overall incidence of

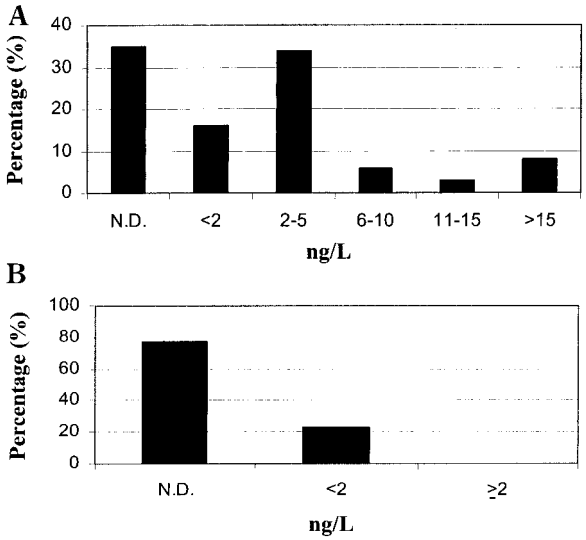

Figure 4. Frequency distribution of TCA concentrations in (A) 145 commercial wines judged to be tainted and (B) 100 commercial wines judged to be free of taint. (N.D. corresponds to <0.1 ng/L.)

Table 4. Incidence of Cork Taint by Country or Region

	white wines		red wines		combined
country or region	no. examined	% tainted	no. examined	% tainted	% tainted
Australia	99	18.2	125	3.2	9.8
Canada	556	4.2	262	3.4	3.9
central Europe[a]	46	19.6	41	2.4	11.5
France	67	14.9	175	3.9	7.2
Germany	36	8.3			
Italy	96	2.3	148	5.4	4.3
Portugal	22	4.5	60	6.7	6.1
South America[b]	90	12.2	119	4.2	7.7
Spain	20	25.0	92	6.5	9.8
United States	121	7.9	144	3.6	5.4
others[c]	38	10.7	43	3.6	7.1

[a] Austria, Bulgaria, Czech Republic, Germany, Greece, and Hungary. [b] Argentina and Chile. [c] Includes South Africa, New Zealand, Israel, Lebanon, Mexico, North Africa, India, and the United Kingdom.

3.7%. Among the white wines, those of Spain (25%), central Europe (20%), and Australia (18%) revealed the highest incidence, whereas wines of Italy (2%), Canada (4%), and Portugal (5%) revealed the lowest. Some of these trends were, surprisingly, reversed among the red wines, where the lowest incidence values were found for central Europe (2.4%), Australia (3.2%), and Canada (3.4%). Combining both red and white wines, Canada (3.9%) and Italy (4.3%) emerged as the countries with the lowest incidence of cork taint, whereas central Europe (11.5%) and Spain (9.8%) were the highest.

Factors Modulating Leaching of Trichloro Compounds into Wine. One bulk red wine and one bulk white wine produced at Vincor International Inc., free of TCA and TCP, were bottled under standard commercial conditions utilizing all five closures from both manufacturers, such that 24 bottles of each color were sealed with the same closure. For each closure, 12 bottles were stored erect (up) and 12 were inverted (down). At intervals of 1, 2, 3, 6, 9, and 12 months, two fresh bottles of each closure, manufacturer, color, and storage condition (i.e., 80 in total) were opened. TCA and TCP were analyzed in all corks and wines that were discarded after the chemical analyses and organoleptic evaluations were completed.

Table 5 displays the TCA content of the wines (means of duplicate individual bottles) after 12 months, although the

Table 5. Concentrations of TCA in Wines (Duplicate Bottles for Each Condition) at End of Experimental Storage Period and in Corks at Beginning and End of 12-Month Period as well as the Decrease over This Period[a]

closure and wine color	TCA in wine (ng/L)	TCA in cork (ng per cork) beginning	end	decrease	% decrease[b]
composite corks					
red	2.2	42.4	6.5	35.9	84.7
white	4.9	44.4	7.2	37.2	83.8
composite with disk					
red	1.1	18.2	3.1	15.1	83.0
white	3.5	39.5	8.8	30.7	77.7
natural grade 1					
red	0.2	21.6	1.0	20.6	95.4
white	0.3	8.4	1.5	6.9	82.1
natural grade 2					
red	0.3	10.2	1.5	8.7	85.3
white	0.5	11.0	5.2	5.8	52.7

[a] All data are based upon eight individual bottles using closures from two manufacturers; half of the bottles were stored erect and half inverted. [b] Expressed as percentage of initial value.

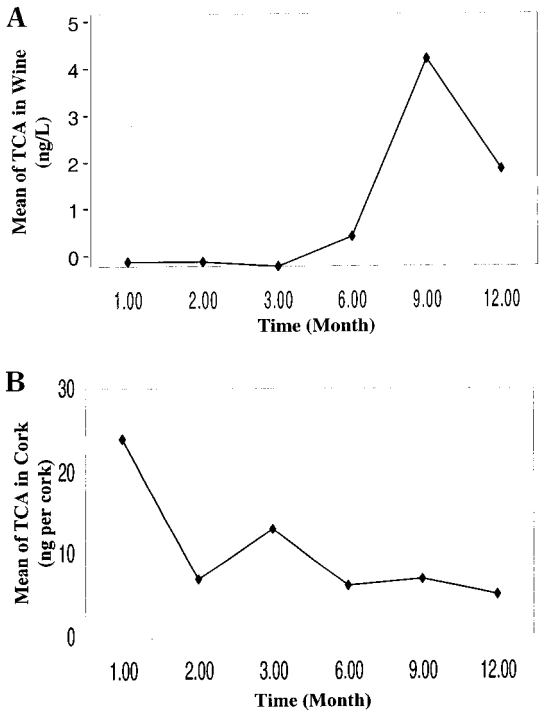

Figure 5. Composite time course of TCA changes in wines (A) and corks (B) in storage experiment as harmonic means derived from the General Linear Model.

highest concentration recorded over this period generally occurred at 9 months and decreased over the next 3 months (**Figure 5A**). **Table 5** also presents the mean TCA content of the corks after 1 and 12 months, the difference between the two, and the difference as a percentage of the initial value. It should be emphasized that these sequential changes with time are potentially distorted by the variance between individual corks from the same batch because each cork was analyzed at only a single point in time, that is, when the bottle was opened; nevertheless, a steady decrease with time was clearly evident (**Figure 5B**). Regrettably, we did not commence the TCP analyses until the third month, but the time courses showed similarities to those of TCA: its concentration in wine peaked at 9 months and then declined, whereas the cork TCP

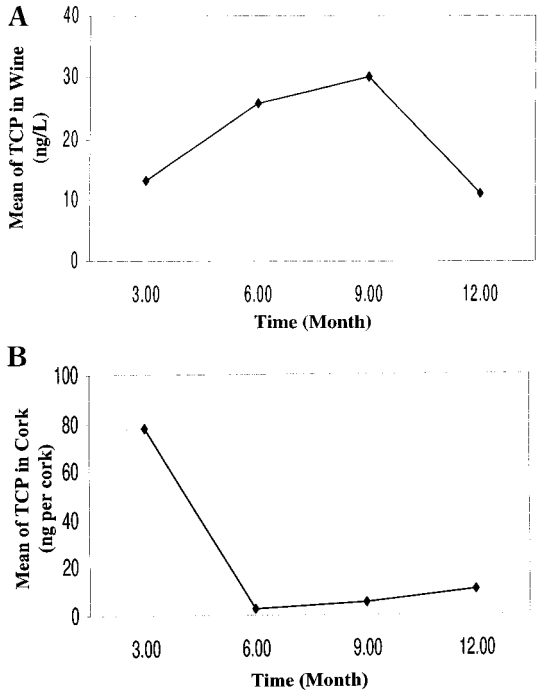

Figure 6. Composite time course of TCP changes in wines (A) and corks (B) in storage experiment as harmonic means derived from the General Linear Model.

Table 6. Matrix Illustrating the Variables Tested during the 12-Month Storage Experiment[a]

taster no.	wine color	cork type	position	manufacturer	taint score
1–4	white/red	1–5	up/down	A/B	0–4

[a] Duplicate bottles were prepared for each set of conditions.

content decreased sharply to a plateau between 3 and 6 months (**Figure 6**).

Each wine was evaluated organoleptically for cork taint immediately after opening by four expert tasters. A score of 0 (not tainted, <2 ng/L) to 4 (severely tainted, >15 ng/L) was used in this evaluation. The sensory perception of cork taint was quantified for each closure under each condition (color, manufacturer, and storage) by adding the total score for both duplicate bottles over all six tasting sessions corresponding to the times the wines were opened. The scoring matrix, illustrated in **Table 6**, reveals that each taster sampled 40 duplicate bottles in total at each session, that is, a sum of 80 bottles per taster. The General Linear Model established that the concentrations of TCA and TCP in the wines as well as quantitative sensory perception of cork taint were not affected by manufacturer or by storage position. Consequently, the sensory evaluations for all four tasters were pooled for closure type and wine color irrespective of the previous two variables over all six tasting periods (defined as "organoleptic scores") and are presented in **Table 7**. Each data point therefore represents a pooled value based upon 192 tasting "incidents" (i.e., 48 individual bottles each of which was tasted by 4 experts). Another index utilized was the number of bottles among the 48 in which a flaw was detected by any individual taster (defined as "flawed bottles") irrespective of the organoleptic score. These data are also compiled in **Table 7**.

The organoleptic scores revealed a significant effect of closure type in the development of cork taint, composite corks being

Table 7. Evaluation of Cork Taint in Experimentally Bottled Wines Compiled from Numerical Ratings of Four Independent Professional Tasters

closure and wine color	organoleptic scores[a]	flawed bottles[a] (% of total)
composite corks		
red	230	83 (43.2)
white	328	123 (64.1)
composite with disk		
red	58	27 (14.1)
white	83	32 (16.7)
natural grade 1		
red	31	14 (7.3)
white	37	22 (11.5)
natural grade 2		
red	72	29 (15.1)
white	62	30 (15.6)
synthetic corks		
red	4	3 (1.6)
white	29	14 (7.3)

[a] See text for definitions.

Table 8. Classification of TCA Concentrations in Wines Identified with Cork Taint by at Least One Expert during the 12-Month Storage Period

	TCA concn (ng/L)		
wine type (no.)	0[a]	<2[a,b]	≥2[a]
white (108)	65	14	29
red (72)	47	6	19

[a] Number of organoleptically tainted bottles in each class. [b] Above LOD but below LOQ.

by far the worst and synthetic closures the best when tested in the General Linear Model ($F = 3.58$; $P = 0.009$). The same conclusion could be drawn by analyzing the number of flawed bottles by closure type ($F = 3.26$; $P = 0.015$). Overall, the organoleptic scores and flawed bottles were higher in the white wines than in the red wines irrespective of closure type, but this trend was not significant.

Table 8 demonstrates a classification of TCA concentrations in white and red wines judged to be organoleptically tainted by at least one expert. There was a 3:2 ratio of white-to-red in the number of bottles deemed to be tainted. In 60% of the tainted white wines and 65% of the tainted red wines, TCA was <LOD. Indeed, in 73% of the tainted white and 74% of the tainted red wines the TCA concentration was <2 ng/L, the threshold for organoleptic taint perception by this group of experts. Thus, in only 26−27% of the total bottles with organoleptically detectable cork taint was TCA present in a concentration that these experts were capable of detecting, suggesting that constituents other than TCA were responsible for the perceived taint. These findings provide strong evidence for the assertion that TCA is responsible for this flaw in a minority of wines with perceptible cork taint.

In this study, there was no apparent relationship between wine TCP concentration and cork taint. This is best illustrated by **Table 9**, where the distributions of TCP in clean and tainted white wines are not significantly different.

Interestingly, the application of nonparametric correlation analysis to derive Spearman's rho (Bonferroni corrected) showed significant association between TCA (wine) and TCA (cork) [rho = 0.392, $P < 0.001$] and also between TCA (cork) and TCP (cork) [rho = 0.278; $P = 0.002$]. These observations are consistent with two of the findings described for corks from tainted commercial wines in the previous section. Again, there was no association between TCP in the wine and in the cork.

Table 9. Classification of TCP Concentrations in White Wines with and without Cork Taint between the 3rd and 12th Months of Storage

organoleptic evaluation	TCP concn (ng/L)		
	<20	>20–<50	≥50
clean[a]	24	34	14
tainted[b]	17	42	13

[a] As judged by all four experts. [b] As judged by at least one expert.

Cytotoxicity Experiments. Up to a concentration of 500 ng/mL (i.e., 3 orders of magnitude greater than the highest concentrations of these compounds measured in the 2400 wine samples), neither TCA nor TCP adversely affected the viability of any of the cell lines tested. At 5000 ng/mL, both reduced the viability of BG-1 ovarian cells by ∼30%, but even this concentration did not affect the other cell lines. We conclude that neither of these trichloro compounds represents a health hazard to human subjects through the consumption of commercial wines.

DISCUSSION

Our method offers very favorable analytical characteristics for the determination of TCA in wine and cork extracts (**Table 1**). Previous authors have reported an LOD of 2−5 ng/L (*15*), 20 ng/L (*5*), 0.5−2 ng/L (*16*), and 2.9 ng/L (*9*), compared with 0.1 ng/L in the present method. None of the above authors specify an LOQ; this was 5 ng/L in the method of Evans et al. (*10*) compared with our value of 2 ng/L. Imprecision with previous methods (expressed as RSD) was 18.2% (*16*) and 5−13% (*10*), whereas we experienced much lower values. Previous estimates of recovery include 43−72% (*1*) and 85−130% (*10*); our recovery was more consistent, being in the range of 86−102%. Thus, there are reasons to consider ours to be the method of choice for TCA analysis at the present time.

We have not been able to compare the TCP method developed in this work with others, as we were unable to identify a published method for wine and cork extracts, but it clearly offers analytical features equal to the best of current methods for the determination of TCA.

The notion that TCA is the main component contributing to the perception of cork taint in wine has gained wide currency. It is well established that this compound is produced in corks by the action of bacteria and yeasts, as well as by the use of hypochlorite in the bleaching process (*8*, *17*−*20*). Our data reveal that over a period of 12 months, there is a steady reduction in the TCA content of cork closures accompanied by a gradual increase in the TCA content of the bottled wine that reaches a peak at ∼9 months and then declines (**Figure 5**). However, the decrease in cork content is not matched by a corresponding increase in wine concentrations, especially in the first few months, and is not dependent upon whether the bottles are stored erect or inverted; and whereas there is a significant correlation between TCA content in wines and corks, this is rather modest and not supportive of a direct causal relationship. Rather than being fully leached into the wine, it seems likely that much of the TCA may escape peripherally through the cork into the atmosphere or undergo metabolic conversion within the cork.

The data in **Table 8** reveal that more often than not, wines perceived to be tainted have TCA levels that are either analytically undetectable or below the sensory threshold for cork taint. For the four experts forming our sensory panel, this was 2 ng/L. Other limits have been cited, for example, 10−30 ng/L (*1*), 4−50 ng/L (*9*), and 4.6 ng/L (*20*). Clearly, compounds as yet unidentified must be responsible for the taint in wines with low or undetectable TCA concentrations. Indeed, such compounds may even be more important than TCA in wines above the sensory threshold for the latter. Thus, TCA is sufficient but not necessary to generate cork taint in wines. Our results strongly support the assertion of Pereira et al. (*8*) that targeting TCA contamination of cork closures as the major cause of wine taint is not justified. A critical examination of the primary data on which the original assertions are based reveals small sample numbers (*1*, *9*) and a tendency to ignore anomalous results (e.g., wines with high taint and low TCA and vice versa) together with the absence of any statistical proof for the asserted relationship (*15*, *16*). The conundrum is that other candidate chlorinated compounds detected in cork are present even less frequently and in lesser concentrations in tainted wines (*15*−*17*). It is worth mentioning that alternative techniques to chlorine bleaching are now being introduced to minimize contamination of corks by chloro compounds, including the use of H_2O_2 (*20*).

An exception is TCP. This is commonly present in corks (*8*, *17*, *20*), where it is thought to be a precursor of TCA, and is present in much higher concentrations than the latter in hypochlorite-bleached corks (cited in ref *20*). We can find no record of any systematic investigation of this compound in wines. The present data shed some interesting light on this compound but appear to eliminate it as a serious contributor to cork taint in its own right. Concentrations >350 ng/L, established as the sensory threshold of our expert panel, were found in only 3 of the 145 tainted commercial wines, and in 2 of these TCA was >2 ng/L.

Of the corks drawn from the 145 tainted commercial wines in our survey of 2400, the TCP content was higher than that of TCA in only 38 (26%), yet the TCP concentrations were higher than those of TCA in 100 of the 125 wines in which one or the other was measurable (neither was measurable in 20 of these wines). In the 12-month storage experiment, TCA and TCP were each higher in about half the corks analyzed, yet the TCP concentration of the wines almost invariably exceeded those of TCA (all except 7 of the 268 bottles in which both were tested). Furthermore, the lack of any significant correlation between TCP in cork and wine in both the survey of commercial wines and the storage experiment suggests that the former is unlikely to be the source of the latter. Interestingly, wines stoppered with synthetic closures in the storage experiment almost invariably contained TCP, and in higher concentrations than with any other closure type, although the synthetic closures were devoid of TCP. However, TCA was never detected in any of these synthetically corked wines. Although microbial action may cause the methoxylation of TCP to form TCA in cork during its processing, this reaction does not appear to occur in bottled wines. The presence of TCP in the wine must be ascribed to sources arising from wine production prior to bottling, in line with earlier suggestions (*6*, *7*).

One notable feature in this experiment was that the TCP content of the natural grade 1 corks from both manufacturers was significantly lower than that of the corresponding grade 2 corks (General Linear Model, $P = 0.023$), suggesting that its presence may be inversely related to cork quality.

It is not surprising that red wines were less prone to be classified as tainted during organoleptic evaluation. Much of their highly regarded complexity is due to sensations (vegetal, leafy, earthy, barnyard) that come perilously close to the "corkiness" of tainted wines.

We have been able to identify only one paper that describes the effects of TCA upon living cells; this states that TCA was toxic to only 1 of 16 strains of fungi tested (*21*). The results of our cytotoxicity experiments demonstrate that, despite their possible economic damage to the wine industry, even the highest concentrations encountered in commercial wines are several orders of magnitude below those that may be harmful to human health.

ACKNOWLEDGMENT

We thank Len Franssen for help in organizing the data; Clair Anderson for his contribution to this study; Dr. D. E. C. Cole, who performed the statistical analyses; and Dr. E. P. Diamandis, in whose laboratory the cytotoxicity studies were performed. Finally, we are grateful to Sheila Acorn and Patricia Machado for skillful preparation of the manuscript.

LITERATURE CITED

(1) Buser, H.-R.; Zainer, C.; Tanner, H. Identification of 2,4,6-trichloroanisole as a potent compound causing cork taint in wine. *J. Agric. Food Chem.* **1982**, *30*, 359−362.

(2) Schmitzer, J.; Bin, C.; Scheunert, I.; Korte, F. Residues and metabolism of 2,4,6-trichlorophenol carbon-14 in soil. *Chemosphere* **1989**, *18*, 2383−2388.

(3) Tindale, C. R.; Whitfield, F. B.; Levingston, S. D.; Nguyen, T. H. L. Fungi isolated from packaging materials: Their role in the production of 2,4,6-trichloroanisole. *J. Sci. Food Agric.* **1989**, *49*, 437−448.

(4) Spadone, J.-C.; Takeoka, G.; Liardon, R. Analytical investigation of Rio off-flavor in green coffee. *J. Agric. Food Chem.* **1990**, *38*, 226−233.

(5) Aung, L. H.; Smilanick, J. L.; Vail, P. V.; Hartsell, P. L.; Gomez, E. Investigations into the origin of chloroanisoles causing musty off-flavor of raisins. *J. Agric. Food Chem.* **1996**, *44*, 3294−3296.

(6) Simpson, R. F.; Lee, T. H. The microbiology and taints of cork and oak. In *Abstracts of the 9th International Oenogical Symposium;* Lamperle, E., Figlestahler, E., Eds.; International Association for Modern Winery Technology and Management: Cascais, Portugal, 1990; p 653.

(7) Châtonnet, P.; Guimberteau, G.; Dubourdieu, D.; Boidron, J. N. Nature et origine des odeurs de "moisi" dans les caves. Incidences sur la contamination des vins. *J. Int. Sci. Vigne Vin.* **1994**, *28*, 131.

(8) Silva Pereira, C.; Figueiredo Marques, J. J.; San Ramão, M. V. Cork taint in wine: Scientific knowledge and public perception—a critical review. *Crit. Rev. Microbiol.* **2000**, *26*, 147−162.

(9) Fischer, C.; Fischer, U. Analysis of cork taint in wine and cork material at olfactory subthreshold levels by solid phase microextraction. *J. Agric. Food Chem.* **1997**, *45*, 1995−1997.

(10) Evans, T. J.; Butzke, C. E.; Ebeler, S. E. Analysis of 2,4,6-trichloroanisole in wines using solid-phase microextraction coupled to gas chromatography−mass spectrometry. *J. Chromatogr. A* **1997**, *786*, 293−298.

(11) Taylor, M. K.; Young, T. M.; Butzke, C. E.; Ebeler, S. E. Supercritical fluid extraction of 2,4,6-trichloroanisole from cork stoppers. *J. Agric. Food Chem.* **2000**, *48*, 2208−2211.

(12) Cory, A. H.; Owen, T. C.; Barltrop, J. A.; Cory, J. G. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun.* **1991**, *3*, 207−212.

(13) Berridge, M. V.; Tan, A. S. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch. Biochem. Biophys.* **1993**, *303*, 474−482.

(14) Soleas, G. J.; Yan, J.; Goldberg, D. M. Assay of ochratoxin A in wine and beer by high-pressure liquid chromatography photodiode array and gas chromatography mass selective detection. *J. Agric. Food Chem.* **2001**, *49*, 2733−2740.

(15) Amon, J. M.; Vandepeer, J. M.; Simpson, R. F. Compounds responsible for cork taint in wine. *Aust. N. Z. Wine Ind. J.* **1989**, *4*, 62−69.

(16) Pollinitz, A. P.; Pardon, K. H.; Liacopoulos, D.; Skouroumounis, G. K.; Sefton, M. A. The analysis of 2,4,6-trichloroanisole and other chloroanisoles in tainted wines and corks. *Aust. J. Grape Wine Res.* **1996**, *2*, 184−190.

(17) Lee, T. H.; Simpson, R. F. Microbiology and chemistry of cork taints in wine. In *Wine Microbiology and Biotechnology*; Fleet, G. H., Ed.; Harwood Academic Publishers: Chur, Switzerland, 1993; pp 353−372.

(18) Jäger, J.; Diekmann, J.; Lorenz, D.; Jakob, L. Cork-borne bacteria and yeasts as potential producers of off-flavours in wine. *Aust. J. Grape Wine Res.* **1996**, *2*, 35−41.

(19) Howland, P. R.; Pollinitz, A. P.; Liacopoulos, D.; McLean, H. J.; Sefton, M. A. The location of 2,4,6-trichloroanisole in a batch of contaminated wine corks. *Aust. J. Grape Wine Res.* **1997**, *3*, 141−145.

(20) Butzke, C. E.; Evans, T. J.; Ebeler, S. E. Detection of cork taint in wine using automated solid-phase microextraction in combination with GC/MS-SIM. In *Chemistry of Wine Flavor*; Waterhouse, A. L., Ebeler, S. E., Eds.; American Chemical Society: Washington, DC, 1999; pp 208−216.

(21) Ruckdeschel, G.; Renner, G. Effects of pentachlorophenol and some of its known and possible metabolites on fungi. *Appl. Environ. Microbiol.* **1986**, *51*, 1370−1372.

Received for review August 27, 2001. Revised manuscript received December 4, 2001. Accepted December 5, 2001. This work, forming part of the thesis submitted by G.J.S. in partial fulfillment of the requirements for the degree of Ph.D. at the University of Toronto, was generously supported by the National Research Council of Canada (IRAP).

JF011149C