STUDY OF CHLOROANISOLES ASSAY AND TCA ASSAY VALIDATION IN WINE AND CORK STOPPERS SOAKED IN DILUTE ALCOHOL SOLUTION USING SIDA-HSSPME-GC-MS/EI-SIM*

ÉTUDE ET VALIDATION DU DOSAGE DES CHLOROANISOLES ET DU TCA DANS DU VIN ET DES MACÉRATIONS HYDROALCOOLIQUES DE BOUCHONS EN LIÈGE PAR SIDA-HSSPME-GC-MS/EI/SIM*

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Abstract : The work we present in this article aims at optimizing and validating the assay technique based on SIDA-HSSPME-GC-MS/EI-SIM by means of intra-laboratory trials (NF V03-110 AFNOR 1998 and MA-F-AS1-06-PROVAL (OIV)) on several chloroanisoles (2,4,6-trichloroanisole, 2,3,4,6-tetrachloroanisole, 2,3,4,5,6-pentachloroanisole) likely to contaminate wine by direct or indirect contact, in order to define the precise characteristics and limitations of the method.
We showed this technique to be a specific, accurate, linear, and repeatable method. Moreover, seeing the increasing reliance on this type of approach in scientific research and industrial monitoring laboratories (ISO/TC87/GT10) to assess the quality of cork stoppers and because of the important quantity of 2,4,6-Trichloroanisole present in this kind of material, we deemed appropriate to carry out an inter-laboratory study (ISO 5725-1,2,3,4 and 5) of TCA assays on dilute alcohol soaks of corks using the same method. Our results should make it easier to understand and interpret assay results obtained in different laboratories using the same technique.

Résumé : Dans ce travail, nous présentons un travail d'optimisation et de validation intra-laboratoire (NF V03-110 AFNOR 1998 et MA-F-AS1-06-PROVAL (OIV) du dosage des différents chloroanisoles (2,4,6-trichloroanisole, 2,3,4,6-tetrachloroanisole, 2,3,4,5,6-Pentachloroanisole) susceptibles de contaminer le vin par contact direct ou indirect, afin de préciser exactement les caractéristiques et les limites de la technique de dosage par SIDA-HSSPME-GC-MS/EI-SIM. La méthode validée est spécifique, juste, linéaire et répétable avec une limite de détection comprise entre 0.2 et 0.6 ng/L ainsi qu'une incertitude de 0.6 à 1.0 ng/L suivant le composé. De même, compte tenu de la généralisation de ce type d'approche dans les laboratoires d'analyse et dans les laboratoires de contrôle industriels pour le contrôle de la qualité des lots de bouchons neufs (ICO/TC87/GT10) et compte tenu de d'importance du 2,4,6-trichloroanisole dans ce type de matériau, une étude inter-laboratoires (selon ISO 5725-1,2,3,4 et 5) du dosage du 2,4,6-trichloroanisole dans des macérations hydro-alcooliques de bouchons a été réalisée.
La méthode ainsi que les laboratoires paraissent posséder un léger biais lorsque la concentration en 2,4,6-trichloroanisole augmente au-delà de 6 ng/L avec tendance à une légère sous-estimation. Les résultats présentés doivent faciliter la compréhension et l'interprétation des résultats de dosages obtenus entre différents laboratoires utilisant une méthodologie identique.

Mots clés : chloroanisoles, TCA, vin, bouchons en liège, validation statistique
Key words : chloroanisoles, TCA, wine, cork-stoppers, statistical validation

* : Stable Isotope Dilution Assay - Headspace Solid Phase Microextraction - Gas Chromatography - Mass Spectrometry / Electron Impact / Selected Ion Monitoring.
Dilution isotopique - Micro-Extraction en Phase Solide en espace de tête - Chromatographie Gaz - Spectrométrie de Masse / Impact Electronique / Sélection d'ions

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INTRODUCTION

The contamination and spoilage potential of chloroanisoles makes it crucial to develop efficient techniques to identify and assay these pollutants in wines and materials with which they come into contact. In a previous article (CHATONNET *et al.*, 2003), we presented a technique to assay chloroanisoles and chlorophenols both in wines and in corks, and we showed its practical value for the identification of contaminating sources in bottled wines. Among the organochloride contaminants measured in wines, in relation with cork, only chloroanisoles (2,4,6-trichloroanisole TCA, 2,3,4,6-tetrachloroanisole TeCA, and, to a lesser extent, pentachloroanisole PCA) are detectable by tasting.

TCA assays in wine have been made by solid phase microextraction (or SPME) in headspace (or HS) mode (EVANS *et al.*, 1917), using deuterated analogs (POLLNITZ *et al.*, 1996) as standard for the Stable Isotope Dilution Assay (or SIDA). However, this technique is totally inadequate to analyse chlorophenols (which are not volatile enough) or contaminants inside solids. Nevertheless, it is a very easy method to estimate the TCA contamination potential of wines (BUTZKE *et al.*, 1999) and dilute alcohol cork soaks (HERVE *et al.*, 1999).

In recent years, several methods have been proposed to measure the contaminant potential of batches of new corks. The concept of «releasable TCA», i.e. TCA that migrates easily when in contact with a dilute alcohol solution, was proposed as a model to simulate the behavior of a stopper in a bottle (HERVE *et al.*, 1999) in order to study the localization of TCA in the surface layers and the lenticels of corks (HOWLAND *et al.*, 1997).

The adsorption capacity of cork (CAPONE *et al.*, 1999) and the absence of migration through thick layers of cork tissue (CAPONE *et al.*, 2002) confirm that this is a realistic approach. Whole corks are soaked in a dilute alcohol solution and the amount of TCA that has migrated is assayed after a given interval of time. Several researchers (BUTZKE *et al.*, 1999; HERVE *et al.*, 1999;RIBOULET *et al.*, 2003) have proposed other approaches to assay TCA by SIDA-HSSPME-GC-MS/EI-SIM, based on the principle originally developed by EVANS *et al.* (1997).

The work we present in this article aims at optimizing and validating the assay technique based on SIDA-HSSPME-GC-MS/EI-SIM by means of intra-laboratory trials on several chloroanisoles (TCA, TeCA, PCA) likely to contaminate wine by direct or indirect contact, in order to define the precise characteristics and limitations of the method. Moreover, seeing the increasing reliance on this type of approach in scientific research and industrial monitoring laboratories (ISO/TC87/GT10), we decided to carry out an inter-laboratory study of TCA assays on dilute alcohol soaks of corks using the same method. Our results should make it easier to understand and interpret assay results obtained in different laboratories using the same technique.

MATERIAL AND METHODS

I - CHEMICAL STANDARDS

2,4,6-trichoroanisole (TCA) [87-40-1] 99 % Aldrich (L'Isle d'Abeau, France), 2,4,6-trichlorophenol (TCP) [88-06-2] > 98 % Aldrich, 2,3,4,6-tetrachloroanisole (TeCA) [938-22-7] 99 % CIL Cluzeau (Ste Foy La Grande, France), 2,3,4,6-tetrachlorophenol (TeCP) [58-90-2] > 95 % CIL Cluzeau, 2,3,4,5,6-pentachloroanisole (PCA) [1825-21-4] 98 % Aldrich, and 2,3,4,5,6-pentachlorophenol (PCP) [87-86-5] 98 % Aldrich.

II - SYNTHESIS OF DEUTERATED ANALOGS OF 2,4,6-TRICHLOROANISOLE, 2,3,4,6-TETRACHLOROANISOLE AND 2,3,4,5,6-PENTACHLOROANISOLE

For each tested deuterated chloroanisole analog, the corresponding chlorophenol (6 mmoles) was mixed with anhydrous potassium carbonate (5 mmoles) (> 98 %, Fisher Scientific, Elancourt, France) and with deuterated iodomethane CD_3I ([865-50-9], isotope enrichment > 99.5 %, CDN Isotopes, Pointe-Non-polar, Canada) in 10 mL of 99 % dimethylsulfoxide (Fisher Scientific). The solution was stirred at room temperature for three hours. The mixture was then poured into 50 mL of dilute hydrochloric acid N (Fisher Scientific). This solution was extracted three times with 50 mL of diethyl ether (Pestipur®, SDS, Peypin, France). The ether phases were static-settled, then combined, and rinsed with 250 mL purified distilled water (Simplicity, Millipore), then with sodium hydroxide N (Fisher Scientific), then with water again. The mixture was dried from anhydrous sodium sulphate (Rectapur™, Fisher Scientific) and the ether was slowly evaporated at 38 °C in a Kuderna-Danish concentrator equipped with a ten-plate reflux column and a nickel helix. The solid residue was recrystallized in 99 % absolute ethanol (ALDRICH). The chloroanisole OCD_3 molecule thus obtained was purified by adsorption chromatography using a 50 x 0.5 cm flash-chromatography column filled with activated silica (80-100 mesh, activity V, Fisher Scientific, Elancourt, France). The synthesized product was rinsed with n-pentane (SDS Pestipur™ > 99.5 %) then eluted with the following mixture: 96: 4, pentane: diethyl ether (SDS, Pestipur ™ > 99 %).

The solvent was evaporated in a cold vacuum rotary evaporator and the purity of the solid diluted in n-pentane was measured using a gas phase chromatography (DB5-MS™ J&W column, 30 m x 0.25 mm, phase thickness 0.25 µm, J&W, Interchim, Montluçon , France) coupled

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to an HP 5973-II mass spectrometer (Agilent Technologies, Palo Alto, California, USA) operating in electron impact (70 KeV, 150 °C) and in scan mode (SCAN = 40-400 uma). As expected, the mass spectrum we obtained for each synthesized molecule showed a purity of over 99 % of the total signal for 2,4,6-TCA-OCD_3, 2,3,4,6-TeCA-OCD_3, and 2,3,4,5,6-PCA-OCD_3.

III - ASSAYING CHLOROANISOLES IN WINE OR DILUTE ALCOHOL SOAKS OF NEW CORKS

1) Preparation and extraction of wine samples

10 mL of wine were put into a 20 mL flask adapted to fit a CombiPAL™ sampler (CTC analytical, Zwingen, Switzerland). With the help of a syringe, exactly 20 µL were added of a solution containing 3 g +/- 0.1 sodium chloride and 10.0 µg/L of the various deuterated chloroanisoles (internal standards) in absolute ethanol, stored in a borosilicate glass flask to prevent adsorption (Rectapur™, Fisher Scientific, Elancourt, France). The flask was then crimped using an aluminium capsule over a perfectly airtight, disposable elastomer septum. For alcohol contents of less than 15 % vol, spirit samples can be analyzed in the same way, after dilution in distilled purified water (Simplicity, Millipore). Sparkling wines should first be degassed in an ultrasound bath for 30 s.

2) Preparation and analysis of corks or cork-based stoppers

First, we prepared a dilute alcohol solution containing 12 % +/- 0.2 ethanol by volume (prepared from absolute alcohol stored in a glass flask) and 5 g/L tartaric acid, neutralized to pH 3.7 +/- 0.05 using sodium hydroxide N. This solution was prepared each day and stored in glass flasks. Each batch was then analyzed to check for contamination.

In the case of corks, the sample to be analyzed (10 or 25 corks) was soaked in a 1 000 ml glass flask containing 200 to 500 ml solution, depending on the number of corks. The flask was closed with a metal screw cap to prevent the adsorption of chloroanisoles (thermosetting epoxy coating). The contaminants were extracted by completely submerging all the corks in the solution at 22 +/- 4 °C for 24 h without stirring, as described by HERVE *et al*. (1999). An extraction plateau was reached after 24 h, irrespective of the type of cork or cork-based stopper, indicating that it had reached equilibrium. It proved useless and even unadvisable to prolong the soaking time since the chloroanisole concentration in the solution tended either to decrease, or to increase sharply, due to re-adsorption and microbial development (results not shown).

Individual natural corks were soaked separately in wide-mouthed (48 mm), approximately 100 mL glass flasks containing 50 mL of dilute alcohol solution. The cork was kept completely immersed by means of a stainless steel insert fixed on the flask stopper, that prevented it from floating to the surface. Composite Champagne corks were soaked under the same conditions, but the cork was held in a vertical position in only 20 mL of solution, so as to ensure that only the cork disks and approximately 10 mm of the agglomerate cork were immersed.

After soaking, for 24 hours, a 10 mL sample was taken from the soaking solution and put in an analysis flask, to which 20 µL internal standard solution, as well as 3 g +/-0.1 sodium chloride were added so as to proceed with the assay.

3) Chromatographic analysis and chloroanisole assay

The flask containing the internal standards and the sample solution were then placed on a Combi PAL™ auto-sampler (CTC Analyticals Inc., Zwingen, Switzerland) equipped with a solid phase microextraction (SPME) system using polydimethylsiloxane fibre (PDMS, 100 µm, Supelco, L'Isle d'Aneau, France). The chloroanisoles were adsorbed in the stationary phase in headspace mode during 15 min in a temperature-controlled chamber at 45 °C, with shaking (HSSPME).

The SPME fiber, prepared according to the manufacturer's recommendations, was desorbed thermally directly in the splitless injector at the column head for 5 min (260 °C, purge flow: 25.0 mL/min, purge time: 5 min, specific 0.78 mm internal diameter SPME insert), coupled to a Fast-GC® DB5-MS J&W capillary column (5 % phenylmethylsiloxanes) 10 m x 0.10 mm (phase thickness: 0.10 µm), installed on an HP 6890 II chromatograph. The balance gas (Helium N55, Air Product, Toulouse, France) was used at constant flow-rate (initial column head pressure: 37.63 psi, flow-rate: 0.4 mL/min, linear velocity: 40 cm/s). The temperature was programmed to increase from 60 °C (initial isotherm: 0.50 min) to 100 °C at a rate of 20 °C/min, then to 135 °C at a rate of 3 °C/min, then a 2 min pause, to finally increase to 300 °C at a rate of 60 °C/min . For the detection, we used an HP5973 II quadrupole mass spectrometer, operating in electron impact mode (El, source temperature = 230 °C, quadrupole temperature = 150 °C, constant ionization potential = 70 Kev, electron multiplier = 1600 V) and in fragmentometry mode tuned to the (selected) ion characteristic of each molecule (TCA: 197, 210, 212; TeCA = 244, 246, PCA: 278, 280, internal standards: TCA-d3 = 213, TeCA-d3 = 249, PCA-d3 = 283 uma, Dwell time: 10 ms). For the quantification, the following ratios were used: m/z 212/213 (TCA); 246/249 (TeCA) and 280/283 (PCA). Figure 1 shows a graph of the signal measured for each of the chloroanisoles and their respective deuterated analogs. Total injection to injection analysis time: 28 min.

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The system was calibrated using a range of known concentrations, prepared from pure standard products at concentrations of 0, 1, 2, 5, 10, 20, and 50 ng/L diluted in an uncontaminated dilute alcohol solution, and analyzed under the same conditions as the samples. The range of standard substances was injected every 15 samples, every 24 h, and at least once a week, to ensure constant proper calibration of the system.

IV- STATISTICAL INTRA-LABORATORY VALIDATION OF THE CHLOROANISOLE ASSAY METHOD:

The proposed analysis method was assessed using the validation protocol for a standard method and the calculation methods described in the OIV repository (MA-F-AS1-06-PROVAL, 2001) and the ISO 5725-1 to 4 (AFNOR, 1994 a,b,c,d) and NF V 03-110 (AFNOR, 1998) standards. A rosé table wine (11.8 % vol. ethanol, total acidity: 5 g/L tartaric acid, pH: 3.67, 2 g/L glucose + fructose, and 10 g/L glycerol) was used to validate the assay in wine.

V - INTER-LABORATORY STUDY OF THE CHARACTERISTICS OF A 2,4,6-TRICHLOROANISOLE ASSAY IN DILUTE ALCOHOL CORK SOAKS

This group study was aimed at quantifying the accuracy of an analysis method as expressed by its repeatability r and reproducibility R. Repeatability, r, represents the value below which the absolute difference between two single test results obtained under the same conditions (same operator, same laboratory, and same apparatus, within a short period of time) may be expected to lie with a given probability (95 % in our study). Reproducibility, R, represents the value below which the absolute difference between two single test results obtained under different conditions may be expected to lie with a given probability. On the basis of these results, it is possible to calculate the uncertainty of repeatability or reproducibility, as well as the overall uncertainty of the method. Furthermore, the difference between the mathematical expectation of the test results of a particular laboratory and an accepted conventional value is used to estimate the laboratory bias, if any. The difference between the mathematical expectation of test results from all the laboratories and an accepted conventional value gives the bias of the measurement method used.

The statistical methods used are those described in ISO 5725-2 standard (AFNOR, 1994b). The experimental values obtained by each laboratory were subjected to a series of Mandel and Grubbs tests for the mean values, and to Mandel and Cochran tests for the standard deviations so as to identify any outliers (test statistics at or below the critical value at 5 %), so that these measurements could be repeated, if necessary. Isolated values were always kept, but were marked (test statistics at or below the critical value at 1 % and over 5 %).

ISO 5725-2 (ISO, 1994b) recommends carrying out two tests to find outliers (Cochran and Grubbs) and eliminate all data that exceed the critical value at a significant level of 1 %. This procedure involves eliminating specific data, or only data from a particular level, or all data from a particular laboratory. However, since eliminating these data has a substantial impact on the values calculated for the standard deviations of repeatability and reproducibility, it is not necessarily very satisfactory to do so. Data analysts should not make decisions that affect the results of their calculations. Thus, if there are reasons to expect that the experimental results in a reliable study includes outliers or marginal data, which is *a priori* true in our case, it is preferable to use the robust data processing methods described in the ISO 5725-5 standard (ISO, 1996). With these methods, outliers do not affect the calculation of standard deviations. If the participants in the study do not produce too many poor quality data, the robust processing methods give valid standard deviation values based on the good quality data and are unaffected by any poor quality data.

We studied the performance of the method for assaying extractable 2,4,6-trichloroanisole (TCA) using the same principle and the main operating techniques described above, asking each laboratory to analyze four model dilute alcohol solutions containing approximately 1, 2, 5, and 8 ng/L TCA, and three natural cork soaks in dilute alcohol at 12 % vol. ethanol in distilled water stabilized with 100 mg/L sodium fluoride, containing approximately 6 and 9 ng/L TCA, i.e. 7 levels, and to repeat all measurements five times (thus yielding 35 analysis results per laboratory).

The number of laboratories required was estimated according to the recommendations of ISO 5725-4 (1994) to calculate the accuracy of a method. The minimum number of laboratories was calculated by estimating a true standard error, S, for a certain probability, P. From this, it was possible to draw conclusions concerning the interval around S in which we could expect to find the estimated value, s. The formula frequently used for this purpose is as follows:

$$P\,[-A < (s-S)/S < +A] = P$$

A is given as a percentage, i.e. the estimated standard deviation; s is a number lying within an interval of +/-*A* around the true standard deviation, S, with a specific probability P (95 % in our case).

Thus, for a true standard deviation, S, of 0.25 around the target value of 1.0 ng/l, the concentration that needs

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to be measured correctly (i.e. *Ar* = 0.25), at least eight laboratories must repeat each measurement five times in order to obtain an accurate estimate of repeatability, r, using the equation :

$$Ar = 1{,}96\sqrt{1/(2p(n-1))}$$

with *p* the number of laboratories and *n* the number of test results for each level.

To calculate the standard deviation of the reproducibility, SR, an additional factor γ, is introduced, which represents the ratio of the standard deviation of the reproducibility, SR, to the standard deviation of the repeatability, Sr. In our case γ is equal to 1.34 (γ= 0.224/0.300) for a measured value of 3.35 ng/l. Thus, since *AR* = 0.50, at least four laboratories had to repeat the measurements five times to estimate reproducibility, *R*, correctly according to the equation:

$$AR = 1{,}96\sqrt{\left[p\cdot\left(1+n\cdot(\gamma^2-1)\right)^2+(n-1)\cdot(p-1)\right]/(2\gamma^4\cdot n^2\cdot(p-1)\cdot p)}$$

We therefore worked with eight laboratories in various locations on different continents in order to obtain a correct estimation of the accuracy of the method generally used for this type of analysis. The results were calculated using the methods described in the OIV standard MA-F-AS1-07 ETCOL (2001), and in the NF ISO standard for intra and inter-laboratory validation methods (ISO 5725-1 à 4, AFNOR, 1994 a,b,c,d ; ISO 5725-5, ISO, 1996 and NF V 03-110, AFNOR, 1998).

RESULTS AND DISCUSSION

I - SELECTING THE TYPE OF SPME FIBRE

Since the time it was devised by ARTHUR and PAWLISZYN (1990), the SPME technique benefited from the development of different types of adsorbent stationary phases with different deposit thicknesses. Considering the specific physicochemical properties of the chloroanisoles studied here, in particular their molecular weight, their volatility and the concentrations involved, the increase in phase thickness only affects the loading capacity of the fiber and has relatively little impact on the recovery rate of the analyte. The significant increase in the adsorption of volatile molecules with low molecular weights is mainly due to the high microporosity of carbon (Carboxen™, Supelco), but the use of this carbon considerably weakens the fibers.

For the liquid polymer phases used in SPME, the quantity of analyte adsorbed (n) at equilibrium is related to its concentration in the sample according to the following formula:

$$n = K_{fs}V_fC_oV_s / K_{fs}V_r + V_s$$

Where n = mass of adsorbed analyte

C_0 = initial concentration

K_{fs}= distribution coefficient of the analyte between the sample matrix and the stationary phase

V_f = phase volume

V_s = sample volume

Therefore, in order to maximize the quantity adsorbed, the phase used must have a good affinity with the analyte. In any case, K_{fs} is never large enough to permit total extraction of the analyte, so that SPME always remains an equilibrium sampling method.

Among the many physicochemical properties involved in the affinity of the phase for chloroanisoles, the hydrophobic character indicated by log P (octanol/water partition coefficient) is one of the main parameters (table I). By comparing log P of the different chloroanisoles, we found they were most similar to those of PDMS and DVB polymers, which therefore induced the highest Kfs coefficients .

We tested the various available formula of PDMS and DVB (results not shown) and, in agreement with Butzke *et al.* (1999), we selected a standard SPME fiber coated with 100 µm PDMS (reference: 57342-U, Supelco, L'Isle d'Abeau, France). The injection of a solution containing a high concentration of chloroanisoles (100 ng/L) after adsorption did not show any detectable memory effect, provided the desorption times were longer than 5 min at 260 °C.

II - ADSORPTION BALANCES ACCORDING TO TIME AND TEMPERATURE

We studied the effects of adsorption time/temperature ratios so as to optimize the analysis conditions for all three chloroanisole molecules, which have quite different properties in terms of volatility (figures 1 and 2). Thus, with a 100 µm PDMS fiber, the optimal conditions to analyze a mixture of the three chloroanisoles corresponds to a headspace adsorption time of at least 15 min at a maximum temperature of 55 °C and a minimum temperature of 35 °C (at lower temperatures, repeatability was poor). An adsorption time of 15 min is a good compromise between adsorption quality and total analysis time (the time needed to do the analysis and to stabilize the chromatography chamber).

III - STATISTICAL INTRA-LABORATORY VALIDATION OF THE SIDA-HSSPME-GC-MS/EI-SIM ASSAYING METHOD OF CHLOROANISOLES IN WINES AND IN MODEL DILUTE ALCOHOL SOLUTIONS

1) Linearity range and limitations of the assay

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To check the linearity range and to obtain the calibration characteristics of the method, we analyzed a blank solution (concentration zero) and 5 different concentrations (1,5,10,15, and 20 ng/L) in the assumed linearity range of each substance so as to detect any non-linearity with a risk of 1 % (FEINBERG, 1997). For each concentration, we prepared 5 (theoretical) standard solutions from a standard solution of known purity, and we took the instrument reading without repeated dilutions. Table II summarizes the statistical results thus obtained.

In all cases, linear regression was satisfactory (Fisher test FI was above the critical value F at risk ± = 1 % for 1 and *p*(n-1) degrees of freedom). We also checked that

Table I

Physicochemical characteristics of chloroanisoles and traditional polymers used as stationary phases for SPME from PhysProp Database Syrres Inc. (2003)

Caractéristiques physico-chimiques des chloroanisoles ainsi que des différentes phases en polymères traditionnellement utilisées en SPME extrait de PhysProp Database Syrres Inc. (2003)

Molecule [CAS number]	Boiling point °C 760 mm Hg	Melting point °C 760 mm Hg	Vapor pressure 10-3.mm Hg	Log P octanol/water	Water solubility mg L^{-1}	Water solubility mg L^{-1}
TCA [87-40-1]	241	61	0.228	4.11	10.0	130.0
TeCA [938-86-3]	-	84	3.190	4.65	1.4	96.1
PCA [1825-21-4]	-	108	0.592	5.29	0.4	1930.0
Polydimethylsiloxane (PDMS)				4.44*		
Divinylbenzene (DVB)				4.14*		
Polyacrylate (PA)				0.77*		
Carbon				0.78*		

* calculated log P from TEKTO *et al*. (2001)

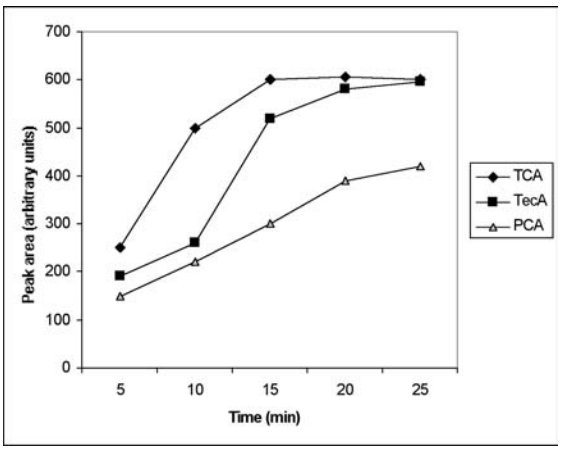


Figure 1 - Influence of the HSSPME adsorption time on the intensity of the chloroanisole (5 ng/L in wine) signal at a given temperature (45 °C)

Influence du temps d'adsorption en HSSPME sur l'intensité du signal des chloroanisoles (dans du vin à 5 ng/l) à une température donnée (45 °C).

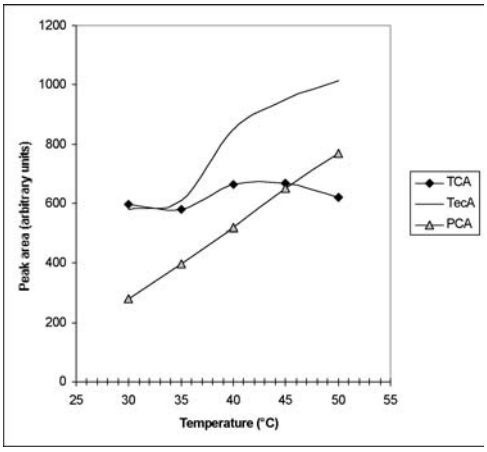


Figure 2 - Influence of the temperature on the intensity of the chloroanisole (5 ng/L in wine) signal for a given HSSPME adsorption time (15 min).

Influence de la température sur l'intensité du signal des chloroanisoles (dans du vin à 5 ng/l) pour un temps donné d'adsorption en HSSPME (15 min).

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Table II - Results of the Intra-laboratory validation

Résultats de la validation intra-laboratoire

	TCA	TeCA	PCA
Calibration			
Number of levels	5	5	5
Linearity range studied (ng/L)	0-21	0-21	0-21
Total number of measurements	25	25	25
Sensitivity	0,983	1,037	1,002
Control	0,242	-0,366	-0,009
Standard deviation of sensitivity	0,010	0,018	0,012
Standard deviation of control	0,116	0,186	0,148
Linearity			
Fi of regression model	10391,18*	4351,90*	8750,70*
Fnl of model error	0,98**	3,11**	2,47**
Detection and Quantification limits			
Experimental standard deviation	0,32	0,51	0,41
Detection limit (ng/L)	0,60	0,18	0,44
Quantification limit (ng/L)	1,43	1,44	1,47
Specificity			
Slope of the linear regression	0,984	0,962	0,985
Standard deviation of the slope	0,0049	0,0027	0,0026
Value at intersection with the origin***	0,436	0,217	0,321
Standard deviation of the intersection	0,1547	0,0629	0,0890
Student test regression curve****	0,232	0,739	0,301
Student test intersection with the origin****	1,108	0,866	1,073
Accuracy (number of samples for each compond=15 , number of trials=30)			
	Mesured / Theoretical	Mesured / Theoretical	Mesured / Theoretical
Mean (ng/L)	8,093 / 6,851	8,487 / 5,953	10,463 / 7,204
Repeatability standard deviation	11,604 / 0	0 / 11,989	11,989 / 14,757
Variance of repeatability	134,665	0	143,742
Difference of means d	1,243	2,535	3,259
Standard deviation of differences sd	0,557	1,365	1,533
Egality test Id I/sd	2,232	1,857	2,126
Reproducibility (number of operators=2, number of different days=10, total number of trials=20)			
Mean (ng/L)	3,35	3	3,38
Variance of internal repeatability Sr_	0,05	0,21	0,25
Standard deviation of internal repeatability SCElr	1,09	1,36	2,51
Variance of samples SL_	0,03	0,03	0,02
Variance of internal reproducibility SR_	0,09	0,18	0,26
Standard deviation of internal reproducibility SR	0,29	0,42	0,51
Variation of reproducibility CV %	8,7%	14,0%	15,1%
Uncertainty of the assay (risk = 0,05) (ng/L)	0,58	0,84	1,02

*: Fi>F theoritical at risk 1% : linear regression can be accepted
**: Fnl>F theoritical at risk 1% : regression is linear in all the range studied
***: critical value=0
**** Student t (p (n-2)=8 ; =0,05) = 3,355

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there were no model errors and that a straight line capable of modeling the relationship throughout the selected area (Fnl) was lower than or equal to the Fisher value F at a risk of 1 % for p-2 and p(n-1) degrees of freedom. Those measurements which determined the linearity limits were used to calculate the sensitivity, the detection threshold (3 times the background noise plus blank/sensitivity), and the quantification threshold (10 times the background noise plus blank/sensitivity); the blank consisted of the matrix without any added analyte.

2) Specificity

The specificity of a method is its capacity to deal exclusively with the analyte under consideration and it guarantees that the assay results are not due to interference. Specificity was evaluated globally by looking for non-significant matrix effects after adding given quantities of analyte. To check whether the slope of the regression line was equal to 1, the Student Factor T was calculated with p-2 degrees of freedom at risk threshold ± and compared with critical value t (table II-specificity). In all cases, the calculated factors Tobs were lower than the critical Student Factor. This allowed us to conclude that there was no interference and that the specificity level was acceptable (slope = 1). We also concluded that the regression line intersects the coordinate axes at the origin, since the added concentrations (figure 3) clearly indicate a linear relationship without displaying any statistically significant bias.

3) Accuracy

Accuracy was defined as a close agreement between the mean value calculated from a large series of measurements (in the 0-20 ng/L range, 15 levels, 2 repeats) and an accepted conventional value. In our case, accuracy was calculated for the value of added analyte, taking into account its purity factor (zero variance). In the absence of a standard method and material, the precision of the method could not be studied. Accuracy was estimated by comparing the mean values for the series of analyses and calculating the correlation w between the absolute value d for the differences in mean values, and sd, the standard deviation of the mean values obtained for each sample (table II-accuracy). In all cases, w was less than 3.0 (NF ISO 5725-2) . We therefore concluded that the method was accurate with a 1% risk of error.

4) Internal reproducibility and assay uncertainty

The internal reproducibility of a method indicates its accuracy when used by several operators at long intervals of time. A wine sample was selected among those used for experiment C and measurements were made by two different operators at a 10-day interval. Table II - reproducibility and uncertainty - shows intra-laboratory reproducibility and related assay uncertainties.

IV- AN INTER-LABORATORY STUDY TO DETERMINE THE CHARACTERISTICS AND UNCERTAINTY OF THE SIDA-HSSPME-GC-MS/EI-SIM ASSAYING METHOD OF 2,4,6-TRICHLOROANISOLE IN MODEL DILUTE ALCOHOL SOLUTIONS

1) Processing the results with the robust method

Initially, the analysis data were processed without distinction, using a robust method based on the analysis of medians rather than of mean values.

We studied the correlation between the standard deviation for repeatability (Sr) and reproducibility (SR) and the mean value (m) for each level (table III). We found a linear relationship between SR and Sr and the value of m: $Sr = ar+br \, . \, m$ and $SR = aR + bR \, . \, m$. Coefficients a and b were calculated according to ISO 5725-2, which is used to calculate the coefficients of a straight line by weighted linear regression. The uncertainty of the assay (I) was calculated using $I = +/- \, T \, . \, (SR^2+Sr^2)$; when T =2 we found that uncertainty depended on concentration levels (table IV).

2) Processing with optimization of the results

Once the raw results had been processed using the Mandel, Cochran, and Grubbs tests, we eliminated the results of laboratory H, which had repeated the analyses only three times instead of five. Furthermore, this laboratory was the only one where analysis of the means produced outliers. Two levels from laboratory F were eliminated as they were considered too low. Finally, a few values on the rejection threshold for the statistical tests were also eliminated from processing (laboratory B: an isolated value on level 1; laboratory G: two isolated values on levels 3 and 5). After the elimination of these data, SR and Sr depended linearly on m (figure 4). At concentrations greater than 2 ng/L, the uncertainty of the assay was slightly lower when calculated for the concentration levels than by using the robust method (table V).

3) Analysis of possible bias in the method

The possibility of bias in the method was checked using the first four known TCA concentration levels. The method was found to have significant bias for concentrations higher than approximately 6 ng/L, with a slight tendency to underestimate values (table VI).

4) Analysis of laboratory bias

Laboratory-related bias was also evaluated, using the first four TCA levels. As shown in table VI, a majority

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Table III - Mean values and standard-deviation of the inter-laboratory study of TCA assay
Moyennes et écart-types de l'étude inter-laboratoire du TCA

Level / Laboratory	Mean value (ng/L)						
	1	2	3	4	5	6	7
A	1.33	2.17	5.80	9.17	3.84	7.15	10.47
B	1.14	2.01	5.05	7.95	4.23	6.17	9.54
C	**0.77**	1.69	**4.20**	7.24	3.38	5.42	7.96
D	1.32	2.18	5.21	7.92	4.36	6.74	9.70
E	0.98	1.72	5.06	8.10	3.70	5.24	8.08
F	0.74	1.70	4.58	**6.12**	3.24	**4.52**	**6.58**
G			**3.29**	**6.11**	2.31	**4.14**	7.85
H	1.57	2.53	**6.84**	**10.98**	**7.08**	**9.62**	**12.66**
Mean value /level	1.12	2.00	5.00	7.95	4.02	6.13	9.11
Standard deviation of mean/level	0.31	0.32	1.06	1.60	1.40	1.75	1.90
	Standard deviation						
A	0.07	0.03	0.22	0.37	0.15	0.22	0.46
B	0.19	0.19	0.16	**0.74**	0.12	0.29	0.23
C	0.06	0.12	0.25	0.21	0.14	0.29	0.48
D	0.17	0.04	0.11	0.22	0.10	0.22	0.27
E	0.08	0.16	0.25	0.37	0.23	0.23	0.36
F	0.05	0.16	**0.49**	0.34	0.24	**1.41**	**1.00**
G			0.17	0.23	0.28	0.14	0.24
H	0.12	0.02	0.06	**0.52**	0.15	**0.44**	0.15

results in bold : non-conform result

of the laboratories showed a bias at or above the third level (i.e. at approximately 6 ng/L), with a slight tendency to underestimate values. However, laboratory A had biased results for the two lowest concentrations (1 and 2 ng/L) but not at higher levels.

CONCLUSIONS

The proposed SIDA-HSSPME-GC-MS/EI-SIM assay method for chloroanisoles complies with the following standards: NF ISO 5725-1/2, NF V 03110 (AFNOR), and MA-F-AS1-06-PROVAL (OIV). This method is specific, accurate, linear and repeatable in the 0-20 ng/L range. However, the method continues to be linear, even above this concentration limit (Results not shown). The detection threshold varies from 0.18 to 0.60 ng/L, depending on the compound, and the quantification threshold lies in the vicinity of 1.45 ng/L for all the molecules studied. These limits are compatible with the sensitivity required to identify this type of contamination (assay in ppt) with an acceptable reproducibility rate, varying from 8.7 to 15.1 %, depending on the exact chloroanisole to be assayed. We therfore conclude that this method provides an accurate, objective analysis of the quantity of tri-, tetra and pentachloroanisole present in a wine or a cork soak.

The inter-laboratory study provided a better calculation of the uncertainty involved in assaying 2,4,6-trichloroanisole (TCA) in wine and above all, in dilute alcohol soaks used for cork quality control. The uncertainty calculated with either a standard or the robust method gave quite similar values, comparable to those determined on an intra-laboratory scale. The assay method apparently has a slight bias at TCA concentrations above 6 ng/L, showing a slight tendency to underestimate. This is attributable to the principle behind the method used, based on an adsorption balance depending on analyte concentration and time. It probably requires more time than the 15 min generally used by the laboratories to reach the adsorption equilibrium at higher concentrations. However, considering the usual range of concentrations analyzed and knowing the uncertainty of the assay, the observed differences are not large enough to justify changing the analysis protocol. Likewise, the often observed laboratory bias at values higher than approximately 6 ng/L was probably due to the same reason. However, one of the laboratories in the study showed significant bias at lower rather than at higher levels. The minor differences which were observed could be due to the method and, most of all, to the set of calibration solutions used by each laboratory (range of concentrations and standards).

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Table IV - Robust treatment of the inter-laboratory comparison of TCA assay

Méthode robuste de comparaison des essais inter-laboratoire du TCA

Algorithm A

Number of laboratories	7*	7*	8	8	8	8	8
Level	1	2	3	4	5	6	7
Mean value	1.12	2.00	5.00	7.84	3.79	5.96	8.90
Standard deviation	0.35	0.36	0.98	1.56	0.80	1.58	1.64

Algorithm S

Mean value	1.12	2.00	5.00	7.84	3.79	5.96	8.90
Sr	0.09	0.12	0.20	0.35	0.17	0.27	0.33
Sd	0.35	0.36	0.98	1.56	0.80	1.58	1.64
Sl	0.35	0.36	0.98	1.55	0.79	1.57	1.64
SR	0.36	0.38	1.00	1.59	0.81	1.60	1.67

* : one laboratory missing for levels 1 and 2

Table V - Uncertainty levels of the TCA assays of the inter-laboratory comparison, calculated with various methods

Comparaison des incertitudes à différents niveaux des essais inter-laboratoire du TCA par deux méthodes.

Concentration	Concentration range in ng/L						
	0.1	0.5	1	2	5	8	10
Uncertainty after elimination of the suspicious values	0.34	0.45	0.59	0.89	1.79	2.69	3.30
Uncertainty from the raw data using robust methodology	0.27	0.43	0.62	1.01	2.17	3.33	4.11

To conclude, the performance of the SIDA-GC-MS/EI-SIM method for assaying chloroanisoles in a dilute alcohol matrix, such as wine or the solution used for cork quality control, was considered to be satisfactory. The main variations observed, particularly in the sensitivity at low concentrations, were mostly due to differences in laboratory performance.

Acknowledgements: We would like to thank the following for participating in the comparative inter-laboratory study of 2,4,6-trichloroanisole assays: Amorim & Irmaos (Portugal), Australian Wine Research Institute (South-Australia), Corchos de Merida (Spain), ETS (USA), Faculté d'Œnologie de Bordeaux (Prof. Alain Bertrand, France), Relvas (Portugal) and Sabaté (France).

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Table VI - Search for bias in the TCA assays due to methodology and to individual laboratories, using the inter-laboratory comparison

Recherche des biais dus à la méthode ou au laboratoire dans les essais inter-laboratoire du TCA

Level	1	2	3	4
n repetition	5	5	5	5
Target value (ng/l)	0,953	1,906	5,720	9,533
Methodology				
Mean value	1,046	1,913	4,769	7,488
Interpretation of bias	NS	NS	S	S
Laboratory A				
Mean value	1,330	2,174	5,804	9,174
Interpretation of bias	S	S	NS	NS
Laboratory B				
Mean value	1,142	2,010	5,052	7,630
Interpretation of bias	NS	NS	NS	S
Laboratory C				
Mean value	0,767	1,694	4,196	7,240
Interpretation of bias	NS	NS	S	S
Laboratory D				
Mean value	1,322	2,180	5,208	7,916
Interpretation of bias	-	S	NS	S
Laboratory E				
Mean value	0,980	1,720	5,060	8,100
Interpretation of bias	NS	NS	NS	S
Laboratory F				
Mean value	0,740	1,700	-	6,250
Interpretation of bias	NS	NS	-	S
Laboratory G				
Mean value	-	-	3,294	6,106
Interpretation of bias	-	-	S	S

NS : not significant for 5 % risk
S : significant

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Manuscrit reçu le 12 mai 2004 ; accepté pour publication, après modifications le 16 juin 2005

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