SIMULTANEOUS ASSAY OF CHLOROPHENOLS AND CHLOROANISOLES IN WINES AND CORKS OR CORK-BASED STOPPERS

APPLICATION IN DETERMINING THE ORIGIN OF POLLUTION IN BOTTLED WINES

DOSAGE SIMULTANÉ DES CHLOROPHÉNOLS ET DES CHLOROANISOLES DANS LES VINS ET DANS LES OBTURATEURS EN LIÈGE OU À BASE DE LIÈGE

APPLICATION À LA DÉTERMINATION DE L'ORIGINE DE LA POLLUTION DES VINS EN BOUTEILLES

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Abstract: The contamination of wines and, more rarely, of brandies by corks or cork-based stoppers used as bottles closures has been a recurring issue for many years. The relative frequency of this type of spoilage, characterized by the distinctive, unpleasant odor of «moldy cork taint», has led to a considerable body of research on this subject. In wine, 2,4,6-trichloroanisole (TCA), produced by the methylation of its direct precursor, 2,4,6-trichlorophenol (TCP), has clearly been identified as the main compound responsible for the "moldy off-flavors" produced by defective corks. In this study, we present a method for assaying the main chloroanisoles and chlorophenols identified as potential contaminants in wines and corks or cork-based stoppers (2,4,6-trichloroanisole and -phenol, 2,3,4,6-tetrachloroansiole and -phenol, pentachloroanisole and -phenol). An extraction method using dichloromethane for cork and a dichloromethane/-pentane mixture for wine isolates almost the entire quantity of organochlorine contaminants from these matrices. The chloroanisoles and precursors are identified simultaneously by gas-phase chromatography coupled with electron impact mass spectrometry with selective ion monitoring. This method provides an accurate analysis of the contamination with a view to establishing the type and origin of the "musty, moldy, corky" characteristics in bottled wines by studying the migration of contaminants from the stoppers into the contents of the bottles. This method for the simultaneous assay of chlorophenols and chloroanisoles was defined in compliance with the following standards: NF ISO 5725-1/2, NF V 03110 (AFNOR), and MA-F-AS1-06-PROVAL (OIV). The method is specific, accurate, linear, repeatable, and has been validated. The detection and quantification ranges are compatible with the sensitivity requirements for this type of contaminant.

Résumé : Dans ce travail, nous présentons les caractéristiques d'une méthode de dosage simultané des principaux chloroanisoles et chlorophénols identifiés comme contaminants potentiels des vins et des obturateurs en liège ou à base de liège. La mise en œuvre d'une extraction à l'aide de solvants organiques permet d'obtenir la quantité totale de contaminants organo-chlorés présent dans ces matrices. La détermination simultanée de l'ensemble des chloroanisoles et de leurs précurseurs par chromatographie en phase gazeuse couplée à la spectrométrie de masse en mode multi-fragmentométrique permet de réaliser des bilans de contamination précis en vue d'établir l'origine des défauts organoleptiques décelés dans les vins embouteillés par l'étude de la migration des contaminants des obturateurs vers le contenu des bouteilles. Les caractéristiques de la méthode de dosage simultané des chlorophénols et des chloroanisoles proposée ont été précisées en respectant les exigences des référentiels NF ISO 5725-1/2, NF V 03110 de l'AFNOR et MA-F-AS1-06-PROVAL de l'OIV. La méthode validée est spécifique, juste, linéaire et répétable avec des limites de détection et de quantification compatibles avec les exigences de sensibilité imposées par ce type de contaminant.

Key words : chlorophenols, chloroanisoles, wine, cork stoppers, statistical validation.

Mot clés : chlorophénols, chloroanisoles, vins, bouchons en liège, validation statistique.

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INTRODUCTION

The contamination of wines and, more rarely, of brandies by corks or cork-based stoppers used as bottle closures has been a recurring issue for many years. The relative frequency of this type of spoilage, characterized by the distinctive, unpleasant odor of «musty cork taint», has led to a considerable body of research on this subject. The proportion of wines spoiled by corks or cork-based stoppers traditionally used for wine bottles varies from 0.5 to 6 % for still wines (HEIMANN *et al.*, 1983), and 0.5 to 2 % for champagnes (PANAÏOTIS *et al.*, 1993). However, a considerable quantity of contradicting data has been published on this problem, but no truly representative statistics.

Chloroanisoles, particularly 2.3.4.6-tetrachloranisole (TeCA) and pentachloroanisole (PCA), have been known to cause off-odors in eggs and chicken carcasses since 1966 (ENGEL *et al.*, 1966). WURDIG (1975), WURDIG and TANNER (1982), and DUBOIS and RIGAUD (1981), were the first to link the «musty» odor of certain wineries and the use of pentachlorophenol (PCP), containing tetrachlorophenol (TeCP) as an impurity, to treat wooden vats. It was not, however, until research by CHATONNET *et al.* (1994) that the various means of remote contamination by TeCA and PCA, produced by the biomethylation of organochlorine pesticides used in or near wineries, were identified and explained.

In wine, 2,4,6-trichloroanisole (TCA), produced by methylation of its direct precursor, 2,4,6-trichlorophenol (TCP), has been clearly identified as the main culprit in the «musty odors» transmitted by defective corks (TANNER *et al.*, 1981, BUSER *et al.*, 1982, TANNER and ZANNIER, 1983). RIBOULET (1982) contested the role of cork in transmitting TCA to wines. However, AMON *et al.* (1989) investigated the various molecules identified and quantified in wines with off-odors and their corks and concluded that 61 % of the wines involved contained TCA and 82 % of the cases of contamination could be connected with the cork. The results of the *Quercus* European research program also showed that 80 % of the bottled wines with «musty» off-odors contained sufficient quantities of TCA to explain this tasting defect (BUTZKE *et al.*, 1998). However, according to SOLEAS *et al.* (2002), only 27 % of the wines diagnosed as having «musty» off-odors on tasting that they tested contained over 2 ng/L TCA. This indicated that other molecules must be capable of causing or contributing to this defect.

Chloroanisoles are very strong-smelling chlorophenol derivatives. CURTIS (1972) measured the following perception thresholds in water: TCA 0.3 ng/L, TeCA 4 ng/L, and PCA over 4 000 ng/L. GRIFFITHS (1974) confirmed the perception threshold of TCA in water. DUERR (1985) estimated the detection threshold of TCA in red wine at 1.4 ng/L. Thresholds for TeCA in wines were initially considerably over-estimated (CHATONNET *et al.*, 1994, CHATONNET and LABADIE, 1995), as the detection of chloroanisole-related defects in wines must take into account not only direct olfaction but also retro-olfaction (aromas perceived on the palate). TeCA, a less volatile molecule than TCA, may thus be detected at concentrations in the vicinity of 12 ng/L in red wines and under 5 ng/L in sparkling wines. PCA cannot be solely responsible for off-odors at the concentrations assayed in wines, but it may act in synergy with other chloroanisoles (CHATONNET *et al.*, 1994).

Accurate quantification of these contaminants in wines and cork is generally carried out by extraction and concentration followed by gas-phase chromatography analysis. TANNER and ZANNIER (1983), and AMON *et al.* (1989) extracted the volatile substances from wine using n-pentane and from cork by maceration in ethanol diluted with water to 20 % alcohol by volume, followed by extraction using n-pentane. The organic extracts were analyzed directly by gas-phase chromatography on a non-polar phase (polymethylsiloxanes) coupled with a mass spectrometer (GC/MS) or an electron capture detector (GC/ECD), using 3,5-dimethyll-2,4,6-trichloroanisole (DMTC) as an internal standard. RIGAUD *et al.* (1984) extracted chlorophenols and chloroanisoles from wine and cork using n-pentane then analyzed the extract directly by gas-phase chromatography with electron capture detection using an external standard (dibromo-1,2-benzene), while the chlorophenols were previously methylated using diazomethane. Derivatization was used to facilitate analysis of the chlorophenols, which are susceptible to adsorption and less volatile than the corresponding anisoles. Acetylation with a blend of pyridine/acetic anhydride (1:0.5) is an easier process than methylation, but the extract must be suspended in water and extracted again using a solvent before it can be analyzed. WITHFIELD *et al.* (1986) quantified chloroanisoles from several matrices at 0.01 ppt using vapor distillation and co-extraction with organic solvents (pentane-diethyl ether-toluene) on a continuous Lickens-Nickerson system, with DMTC as an internal standard, followed by GC/MS and mass fragmentometry (SIM). CANTAGREL and VIDAL (1990) analyzed chloroanisoles and chlorophenols in Cognac and cork, using n-pentane extraction with DBB and 3,4-dimethyl-phenol (DMP) as internal standards, followed by direct GC/MS/SIM analysis. In order to obtain total extraction from solid matrices of varying complexity such as cork, CHATONNET *et al.* (1994) devised a low-temperature continuous extraction process

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in a soxhlet using dichloromethane as a solvent. The wines were extracted with n-pentane and analyzed by GC/MS/SIM. This special equipment made it possible to analyze phenol contaminants and halogenated anisoles very efficiently by direct high-temperature desorption coupled with GC/MS/SIM (HOFFMANN and SPONHOLZ, 1994). POLLNITZ *et al.*(1996) used n-pentane extraction and GC/MS/SIM with $TCAd_5$ as an internal standard to assay chloroanisoles in wine and corks. Finally, SOLEAS *et al.* (2002) assayed TCA (limit of detection - LD - 0.1 ppt, limit of quantification - LQ - 2 ng/L) and TCP (LD 0.7, LQ 4 ppt) in wine using non-polar solid phase extraction (SPE - C_{18} bonded silica) then GC/MS/SIM. Cork was macerated in ethanol, then the solution was diluted in water (20 % ethanol/vol.), extracted by SPE, and analyzed using GC/MS/SIM.

Solid phase micro-extraction (SPME) in headspace mode has been suggested for assaying TCA in wine (EVANS *et al.*, 1997). However, this technique is totally inadequate for analyzing chlorophenols (insufficiently volatile) or contaminants inside solids. It is also possible to assay TCA by stir bar sorptive extraction (SBSE), using a stir bar impregnated with polydimethyl-siloxanes in the water or wine to be analyzed. Analytes were then subjected to direct thermal desorption on the head of the column coupled on-line with GC/MS/SIM (HOFFMANN *et al.*, 2000). Unfortunately, this technique is not really any more effective for assaying chloroanisoles However, the use of other phase types makes it possible to envisage extending the application of SBSE to contaminants in liquid media, but it is still not applicable to those in solids.

This article presents a method for simultaneously assaying the main chloroanisoles and chlorophenols identified as potential contaminants in wines, corks, and cork-based stoppers. Organic solvents make it possible to extract the total quantity of organochlorine contaminants from these matrices. Simultaneous assay of all the chloroanisoles and their precursors gives an accurate contamination balance, which makes it possible to identify the causes of the organoleptic faults found in bottled wines by studying the migration of contaminants from the stoppers into the bottle contents.

MATERIALS AND METHODS

I - CHEMICAL STANDARDS

2,4,6-trichoroanisole [87-40-1] 99 % Aldrich, 2,4,6-trichlorophenol [88-06-2] > 98 % Aldrich, 2,3,4,5-tetrachloroanisole [938-86-3] 98 % (in equivalent 2.3.4.6-TeCA), 2,3,4,6-tetrachlorophenol [58-90-2] 95 % Lancaster, 2,3,4,5,6-pentachloroanisole [1825-21-4] 98 % Aldrich, pentachloro-phenol [87-86-5] 98 % Aldrich, 2,4,6-tribromophenol-3,5-d2. [118-79-6] 99 % CDN Isotopes Inc.

II - PREPARING AND EXTRACTING WINE SAMPLES

The wine (200 ml) was centrifuged (15 min at 5 000 g) and put into a 200 ml borosilicate glass graduated vial, then exactly 1 ml of a 2,4,6-tribromophenol-3,5-d2 (internal standard) solution at 10 mg/L in absolute ethanol stored in a PTFE flask was added. The contents of the vial were homogenized and transferred to a 250 ml Erlenmeyer flask then approximately 1 ml 1/3 sulfuric acid was added using a graduated pipette to acidify the medium, as well as a PFTE stir bar (6 g, 25 mm) previously rinsed with ethanol. Then 10 ml of a 1:1 dichloromethane/n-pentane blend (Pestipur™, SDS) was added and stirred for 5 min (250 rpm). The contents of the Erlenmeyer flask were then static settled in a separating funnel rinsed with dichloromethane. The organic phase was collected carefully in a sealed 50 ml flask. The solution was extracted twice more using 5 ml solvent. The organic phases were combined, the emulsion broken by asymmetrical agitation, and the aqueous phase eliminated by means of a disposable Pasteur pipette. The organic phase was dried on anhydrous sodium sulfate (Aldrich) then transferred into a 100 ml Zymark™ concentrating tube, and the flask was rinsed carefully twice with 1 ml dichloromethane/pentane.

The solution was concentrated automatically to 0.5 ml using a Zymark™ Turbovap II, operating at 25°C +/- 2 in a stream of nitrogen (quality I, Air Products™) at 1 bar, which took approximately 15 minutes. The sample was transferred to a 500 µl disposable injection flask and kept at –20 °C in a dark place prior to analysis.

Spirits samples may be analyzed in the same way, provided that the samples are diluted with purified distilled water (MilliQ™) to an alcohol content below 20 % vol.

III - Analyzing corks or cork-based stoppers

The cork was weighed (+/- 0,01 g) and its humidity measured using a capacity measure Gahn™ moisture meter, calibrated in relation to the reference method (weighed after 48 h drying in an oven at 105 °C) to determine the relative humidity. The results may thus be expressed at a constant rate of humidity or as dry weight.

The upper quarter of the stopper in contact with the external environment was cut off to avoid bias from contaminants outside the cork (CHATONNET *et al.*, 1994) then grated manually with a metal grater. The

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grater was decontaminated between samples by soaking it in a detergent solution (5 % Decon 90™) with added ethanol (50 % vol.) and drying overnight in an oven at 105°C. The cork was grated onto a sheet of aluminum foil. It would have been possible to divide the cork differently, but we recommend that the upper quarter of each stopper be analyzed separately.

1 - Extraction by maceration with stirring

A 0.5 g cork sample (+/- 0.01 g) was put in an 150 ml Erlenmeyer flask stopped with emery and 100 µl internal standard (2,4,6-tribromophenol-3,5-d2 at 1.182 mg/L absolute ethanol) was added, together with 70 ml dichloromethane and 0.1 ml glacial acetic acid (Rectapur™, Prolabo) to facilitate extraction of the chlorophenols. It was stirred for 120 min at room temperature with a stir bar (25 mm, PFTE, 6 g, 250 rpm). The extract was rapidly filtered through purified cellulose paper to eliminate any cork dust, concentrated to 0.5 ml then transferred to a flask for storage as described above.

Several maceration times (60 to 300 minutes) were compared using the same sample of naturally contaminated cork to determine the optimum extraction time under these conditions.

2- Continuous extraction in a soxhlet

Cork (0.5 g) was placed in a cellulose paper capsule that had previously been purified by continous extraction in a soxhlet with ethanol during 24 h. The internal standard was added to the cork in the capsule then placed in the boat of a 75 ml continuous soxhlet extractor. The evaporator flask was filled with 70 ml dichloromethane and 0.1 ml glacial acetic acid was added. The condenser was fed with water at 12°C +/- 5.The flask was heated to produce a reflux of condensed solvent at a rate of approximately 3 ml/min for 120 min. The extractor was then removed from the heating system and cooled. The solvent was transferred from the extractor and flask directly into a 100 ml concentration tube and the flask was rinsed out twice with approximately 2 ml hexane.

Several times of extraction (60 to 300 minutes) were compared using the same sample of naturally contaminated cork to determine the optimum conditions.

IV - CHROMATOGRAPHIC ANALYSIS AND ASSAY OF THE CHLOROPHENOLS AND CHLOROANISOLES

1- Chromatographic separation

A Combi PAL™ automatic system (10 µl syringe, CTC Analyticals Inc.) was used to inject 1 µl (cork) or 2 µl (wine) extract into a 30 m x 0.25 mm HP5-MS capillary column (5 % phenyl-methyl-siloxane) (phase thickness: 0.25 µm) installed on an HP 5890 chromatograph equipped with an injector operating in splitless mode (250°C, initial pressure: 7.1 psi, pulsed splitless: 25.0 psi, pulse duration: 1.5 min, bleed: 50 ml/min, bleed time: 1.5 min) and an Agilent 5183 4711 insert. The balance gas (Helium N55, Air Product™) was used at constant flow-rate (initial pressure at column head: 7.1 psi, flow-rate: 1.0 ml/min, linear velocity: 36 cm/s). The temperature was programmed from 40 °C (initial isotherm: 3 min) to 110 °C at a rate of 25 °C/min, then up to 230 °C at a rate of 5 °C/min, and up to 310 °C at a rate of 25 °C/min (final isotherm: 5 min). Analysis system inertness was checked by weekly injection of a column test (pentadecane, decylamine, octanol-3, dichloro-2,4-phenol). Analysis of chlorophenols requires a perfectly inert system, assessed by the retention time, surface area, and width of the decylamine and 2.4-dichlorophenol peaks, as well as changes in the response factors of the chlorophenols in relation to the internal standard.

2- Mass spectrometry and multi-fragmentometry detection

An HP5973 quadripole mass detector operating in electron impact mode was used for detection (source temperature = 150 °C, constant ionization potential = 70 Kev, electron multiplier = 1 500 V) and fragmentometry mode on selected ions characteristic of each molecule (TCA 197, 210, 212 ; TCP 196, 198 ; TeCA TeCP 230, 232, PCA 278, 280, PCP 264, 266, internal standard 332, 334, 336 uma, Dwell time 100 ms).

Table I – Numbers of assays used for the varous experimental plans for the intralaboratory validation

Nombre de mesures effectuées pour les différents plans d'expérience de validation intra-laboratoire des méthodes de dosage

Experimental plan	Mesured characteristic	Samples	Repetitions	Assays
A	Linearity, limits of determination	5	5	25 à 35
B	Specificity	10	1	10
C	Fidelity and accuracy	15	2	30
D	Internal reproducibility	10	2	20
	Total			100

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The following ratios were used for quantification: m/z 210/334 (TCA 196/334 (TCP 246/334 (TeCA 232/334 (TeCP 280/334 (PCA) and 266/334 (PCP), where 334 was one of the ions in the molecular makeup of the internal standard.

3 – Calibration

The system was calibrated using a range of known concentrations, prepared from pure standard products at concentrations of 0, 2, 5, 10 , 20, and 50 ng/L diluted in uncontaminated wine, and 0, 5, 10, 20, 50, and 100 ng/g impregnated in uncontaminated cork using continuous extraction (24 h) in a soxhlet by methanol (dried for 24 h in an oven at 105°C then stored in glass) and analyzed under the same conditions as the contaminated samples.

The pure chlorophenols were stored in PFTE flasks and the chloroanisoles in borosilicate glass. The range of standard substances was injected every 15 samples, every 24 h, and at least once per week, to ensure that the analysis system was always properly calibrated.

V - VALIDATING ASSAY METHODS

The proposed analysis method was assessed using the validation protocol for a standard method and the calculation methods described in the OIV repository (2001) and the French standards for intra-laboratory validation: NF ISO 5725-1 and NF V 03-110 (AFNOR, 1998). Table I below summarizes the experiments used, i.e. the number of samples, repetitions, and measurements used to validate the methods.

The unpolluted red wine used had the following characteristics: alcohol content 12.5 % vol., total acidity 3.50 g/L H_2SO_4, volatile acidity 0.68 g/L acetic acid, and total polyphenol index (A280 = 38) and a series of sample corks was selected for low contamination by preliminary analysis (crushed and combined to produce a standard sample).

6- Applying simultaneous assay of chlorophenols and chloroanisoles to expert examinations

Following claims by several primary distributors, legal expert examinations were carried out to determine the origin of the organoleptic faults known as «musty» off-odors in bottled wines. We report here on several examples where the analysis method developed was used to identify the contaminants and measure the concentrations in wines and stoppers in order to identify the origin of the pollution and thus, the liabilities involved.

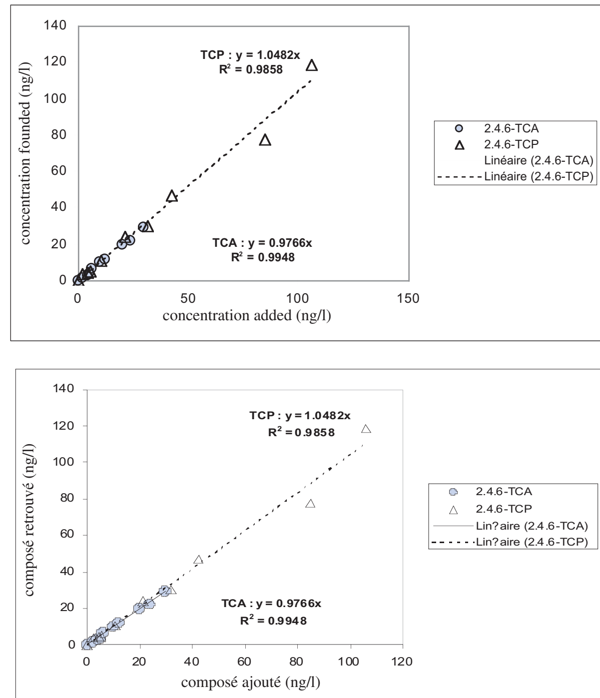

Fig. 1 – Exemple de spécificité de la méthode de dosage simultané des chloroanisoles et des chlorophénols dans les vins (exemple de rajouts dosés de 2.4.6-TCA et de 2.4.6-TCP)

Example of the specificity of the simultaneous assay for chloroanisoles and chlorophenols in wine (know adducts of 2,4,6-TCA and 2,4,6-TCP)

RESULTS AND DISCUSSION

I - ASSAYING CHLOROPHENOLS AND CHLOROANISOLES IN WINE

1- Linearity range and limitations of the assay

Experiment A was used to check the linearity range and deduce the calibration characteristics. The experiment required a blank (zero concentration) and 5 (2, 5, 10, 25, 50 ng/L) to 7 (2, 5, 10, 25, 50, 150, 250 ng/L) concentrations located in the assumed linearity range for each substance, in order to detect any non-linearity with a risk of 1 % (FEINBERG, 1997). For each concentration, we prepared 5 or 7 (theoretical) standard solutions from a standard of known purity and measured the instrument reading without repeated dilutions. Table II summarizes the statistical results obtained.

Linear regression was satisfactory in all cases (Fisher test FI was above critical F at risk a = 1 % for 1 and $p(n^{-1})$ degrees of freedom). We also checked that 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 F at a risk of 1 % for p-2 and $p(n^{-1})$ degrees of freedom).

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Table II – Linearity and limits of the simultaneous assay for chlorophenols and chloroanisoles in the wines (following experimental plan A)

Linéarité et limites de la méthode de dosage simultané des chlorophénols et des chloroanisoles dans les vins (selon plan d'expérience A)

Characteristics	TCA	TCP	TeCA	TeCP	PCA	PCP
Calibration						
Number of levels	5	5	5	6	5	7
Linerauty domain test (ng/L)	0-49	0-53	0-43	0-142	0-52	0-257
Total number of assays	25	25	25	30	25	35
Sensibility	0,998	0,95	0,904	0,991	0,881	0,99
Blank	0,145	0,911	0,551	1,112	1,634	0,827
Standard divation of sensibility	0,011	0,014	0,011	0,008	0,01	0,007
Blank standard deviation	0,282	0,367	0,247	0,485	0,277	0,782
Linearity						
Fi of the regression test	9012.779*	7294.201*	9613.465*	15772.058*	6816.294*	24607.958*
Fnl of the model error	2.064**	4.808**	4.634**	0.854**	0.685**	2.020**
Limits of detection and quantification						
Experimental standard deviation	0,971	1,274	0,85	2,087	0,965	3,611
Limit of detection (ng/l)	1,0	2,1	1,4	2,6	2,8	3,2
Limit of quantification (ng/l)	3,0	4,8	3,3	6,0	5,0	8,7

* : Fi > F theoretical at the risk threshold of 1 % : the linear regression is acceptable ; ** Fnl < theoretically risk at the thresholds of 1 % : the linear regression is acceptable

Table III – Specifity study of the simultaneous assay of chlorophenols and chloroanisoles in the wines (following experimental plan B)

Étude de la spécificité du dosage simultané des chlorophénols et des chloroanisoles dans les vins (selon plan d'expérience B)

					T_{obs}**	T_{obs}**
Molecule	Slope of the regression curve	Standard deviation of the slope	Value of crossing of the curve*	Standard deviation of the cross value	Student test of the curve	Student test of the cross
TCA	0,98907	0,000608	0	0,15327	0,443	0,611
TCP	1,04864	0,001985	0	5,34389	1,087	0,007
TeCA	0,95293	0,00088	1	1,55302	1,373	0,495
TeCP	0,97100	0,000605	1	1,07023	1,179	1,433
PCA	0,97669	0,000144	0	0,37312	1,941	0,415
PCP	0,99417	0,000499	-1	1,27338	0,261	0,643

*:critical value = 0 ** T_{obs} < t de Student p(n-2) = 8, α = 0.0001, 1-α =4.501, 1-α/2 =5.041

The measurements collected to define the limits of linearity were used to calculate sensitivity, detection threshold (3 times background noise plus blank/sensitivity), and quantification threshold (10 times background noise plus blank/sensitivity). The blank was carried out on a matrix with no added analytes, rather than on no matrix (table II). It is easy to increase the injection volume from 2 to 4 µl and reduce the detection thresholds by approximately 30 % (results not shown).

2- Specificity

The specificity of a method is its capacity to deal exclusively with the analyte under consideration and guarantee that the assay results are not due to interference. It was assessed globally by looking for non-significant matrix effects following the addition of measured quantities. To check whether the regression line was equal to 1, the Student T factor was calculated with p-2 degrees of freedom at risk threshold a and compared with critical value t.

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Tableau IV – Estimation of accuracy of the simultaneous chlorophenols and chloranisoles assay in the wines (following experimental plan C) (number of samples for each analyte = 15, number of assays = 30)

Estimation de la justesse du dosage simultané des chlorophénols et des chloroanisoles dans les vins (selon plan d'expérience C) (nombre d'échantillons par analyte= 15, nombre de mesures = 30)

Molecule	Value	Mean	Standard deviation of repetability	Variance of repetability	Difference of the means d	Stand deviation of the differences sd	w Egality test ld l/sd
TCA	mesured value	43,487	68,996	4760,42			
					-4,383	8,015	0,547
	theoretical value	47,871	0	0			
TCP	mesured value	5,0405	83,276	6934,92			
					2,084	2,484	0,839
	theoretical value	51,322	0	0			
TeCA	mesured value	41,59	65,097	4237,67			
	theoretical value	41,553	0	0	0,037	3,129	0,673
	mesured value	44,551	70,525	4973,78			
TeCP					2,95	4,338	0,673
	theoretical value	41,602	0	0			
	mesured value	48,815	75,827	5749,676			
PCA					-1,486	2,589	0,538
	theoretical value	50,301	0	0			
	mesured value	47,276	77,743	6043,93			
PCP					-2,685	3,819	0,703
	theoretical value	49,961	0	0			

The T_{obs} factors calculated were lower in all cases than the Student critical value. It was, therefore, possible to conclude that there was no interference and that the specificity level was acceptable (slope = 1). It was also possible to conclude that the intercept of the regression line was not different from 0 as the added concentrations (figure 1) were sufficiently clearly indicated in an unbiased, linear relationship.

3- Accuracy

Accuracy was defined as a close agreement between the mean value obtained from a large series of measurements (in the 0-200 ng/L range, 15 levels, 2 repetitions) and an accepted conventional value. In our case, it corresponded to the value of added analyte, taking its purity factor into account (zero variance). The precision of the method could not be studied in the absence of a standard method and material.

Accuracy was estimated by comparing the mean values for the series of analyses and calculating the relationship w between the absolute value d for the differences in mean values and sd, the standard deviation of the means obtained for each sample (table IV).

The value of w was below 3.0 in all cases (NF ISO 5725-2). It was therefore possible to conclude that the method was accurate with a 1 % risk of error.

4- Internal reproducibility and assay uncertainty

Internal reproducibility of the method, i.e. its accuracy when used by several operators at long intervals. A wine sample was selected among those used for experiment C and measurements were made by three different operators over a 10-day period.

Table V shows intra-laboratory reproducibility and related assay uncertainties.

II - ASSAYING CHLOROPHENOLS AND CHLOROANISOLES IN CORKS OR CORK-BASED STOPPERS

1- Choice of method and extraction conditions

Relatively powerful solvents are required for total extraction of the organochlorine pollutants from a micro-porous matrix, as cork, which contains high percentages of fats and complex fatty acid polyesters. Chloroanisoles may easily be extracted using a variety of organic solvents, but are susceptible to losses through volatilization. Chlorophenols, in view of their polarity, the possibility of salts (chlorophenates), and combi-

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Tableau V – Study of the internal reproducibility of the simultaneous chlorophenols and chloroanisoles assay in the wines

(following experimental plan D, number of operators = 2, number of days = 10, total number of assays = 20)

Étude de la reproductibilité interne du dosage simultané des chlorophénols et des chloroanisoles dans les vins

(selon plan d'expérience D, nombre d'opérateurs = 2, nombre de jours différents = 10, nombre total de mesures = 20)

Parameters	TCA	TCP	TeCA	TeCP	PCA	PCP
Mean (ng/l)	38	55	39	31	42	28
Standard deviation of internal repetability Sr2	1,66	6,35	0,81	1,9	3,44	2,86
Variance of internal repetability SCElr	52,55	64,28	39,13	95,03	58,13	83,63
Variance of internal repetabilitye SL_	2,09	0,4	1,77	4,33	1,51	3,21
Variance internal reproducibility SR_	3,75	6,75	2,58	6,23	4,95	6,08
Standard deviation of internal reproducibility SR	1,94	2,60	1,61	2,50	2,22	2,47
Variation dof reproducibility CV %	5,10	4,70	4,10	8,10	5,30	8,80
Incertitudes of the assay (ng/l) [i = 2 x SR for a =0.05]	3,9	5,2	3,2	5,0	4,4	4,9

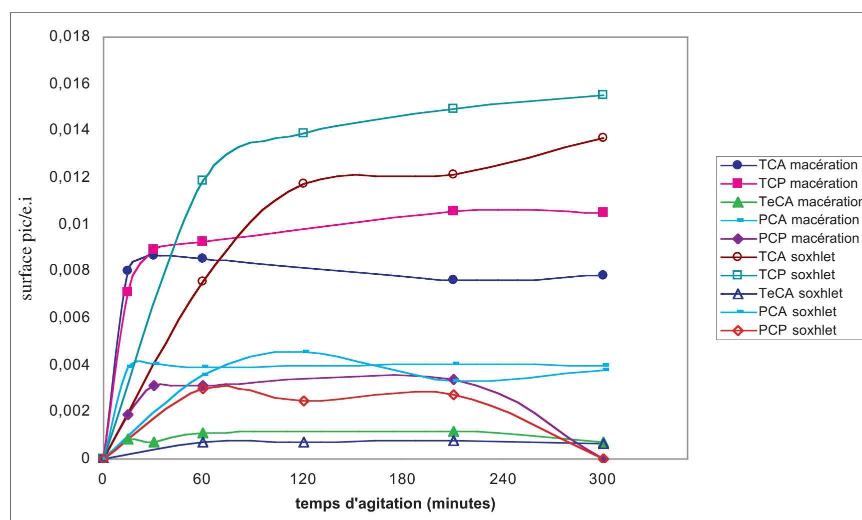

Fig. 2 – Kinetic of chloroanisoles and chlorophenols extraction from a cork matrix slightly contaminated with agitation/soaking or continous solvent extractions

Cinétiques d'extraction de chloroanisoles et de chlorophénols wà l'intérieur d'une matrice de liège faiblement polluée par macération agitation ou extraction continu

nations with certain polymer binders used in cork-based stoppers, were plus difficult to extract and also more susceptible to losses through adsorption.

An extraction carried out under the same conditions with an identical solid matrix, an external standard, and several types of solvent showed that n-pentane, diethyl ether, and dichloromethane gave similar results with chloroanisoles. Polar solvents were also slightly more effective in extracting chlorophenols. Dichloromethane, slightly acidified with acetic acid, enhanced extraction of pentachlorophenol from certain matrices (results not shown).

Extraction by maceration with magnetic stirring was compared with continuous extraction under solvent in a soxhlet extractor using a moderately polluted natural sample (TCA 22, TCP 19, TeCA 5, TeCP not assayable, PCA 11, PCP 5 ng/g) to determine which method was most appropriate for analyzing contamination.

The extraction curves for the various molecules under consideration (figure 2) were found to be similar with both methods. Extraction using maceration/stirring reached a plateau relatively rapidly, after approximately 60 min. When excessive heating was avoided, continuous extraction required approximately 60 min to reach a constant reflux rate and produced slightly more extraction than maceration. The differences, however, remained small, in comparison with the results obtained using both methods after 60 min (figure 3): TCP was quantified at a slightly higher level (24.8 %) with the soxhlet and TCA at a

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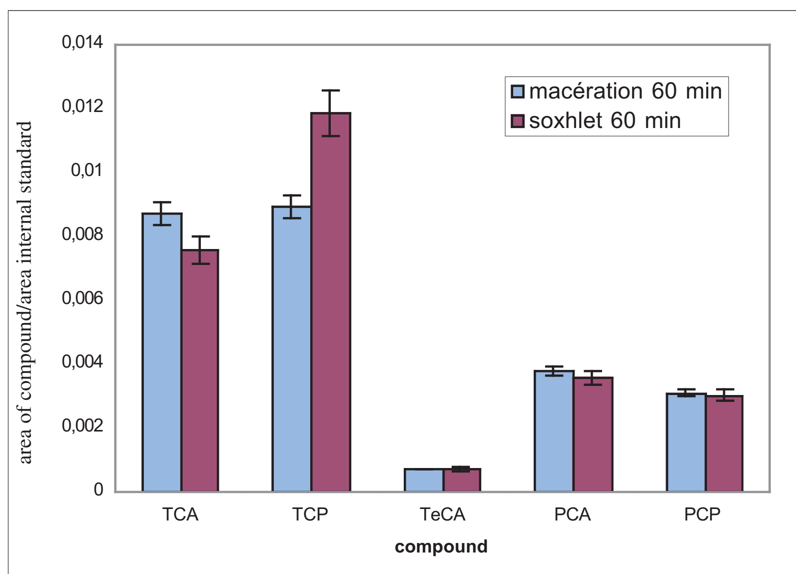

Fig. 3 – Estimation of the comparative intensity of extraction for various chlrophenols and chloroanisoles depending the way of extraction after 60 minutes

Estimation de l'intensité d'extraction comparative de différents chlorophénols et chloroanisoles présents dans du liège selon la méthode employée après 60 min d'extraction

Tableau VI - Linearity and limits of the simultaneous assay of chlorophenols and chloroanisoles in the cork (following experimental plan A)

Linéarité et limites de la méthode de dosage simultané des chlorophénols et des chloroanisoles dans le liège (selon plan d'expérience A)

Characteristics	TCA	TCP	TeCA	TeCP	PCA	PCP
Calibration						
Number of levels	5	5	5	5	5	5
Linearity domain test (ng/L)	0-49	0-53	0-43	0-43	0-52	0-52
Total number of assays	25	25	25	25	25	25
Sensibility	1,006	0,923	0,99	1,175	1,002	0,943
Blank	-0,342	0,294	-0,107	-1,137	0,543	-0,496
Standard divation of sensibility	0,011	0,659	0,016	0,028	0,018	0,024
Blank standard deviation	0,273	0,367	0,326	0,595	0,464	0,061
Linearity						
Fi of the regression test	8047.79*	1197.25*	4414.55*	1549.107*	3447.171*	1773.08*
Fnl of the model error	1.099**	0.259**	1.637**	0.213**	2.093**	2.124**
Limits of detection and quantification						
Experimental standard deviation	0,967	2,33	1,154	2,104	1,644	2,145
Limit of detection (ng/l)	0,5	2,5	0,9	0,6	1,9	1,4
Limit of quantification (ng/l)	2,4	7,5	3,2	4,1	5,2	5,9

* : Fi > F theoretical at the risk threshold of 1 % : the linear regression is acceptable ; ** Fnl

slightly lower level (12.5 %), while the results for the other analytes were not significantly different. Extraction by maceration with stirring for at least 60 minutes is, therefore, an effective method for quantitative extraction of the organochlorine contaminants inside corks.

2- Linearity range and assay limits

To validate the assay method on cork, we used the same procedure as in wine. Table VI summarizes the results obtained. The assay proposed was perfectly linear in the 0-50 ng/g range.

3- Specificity

The T_{obs} factors calculated were lower than the Student critical value for these experimental conditions

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Table VII - Specifity study of the simultaneous assay of chlorophenols and chloroanisoles in the wines (following experimental plan B)

Étude de la spécificité du dosage simultané des chlorophénols et des chloroanisoles dans le liège (selon plan d'expérience B)

Molecule	Slope of the regression curve	Santard deviation of the slope	Value of crossing of the curve*	Standard deviation of the cross value	T_{obs}** Student test of the curve	T_{obs}** Student test of the cross
TCA	1,04539	0,000826	-0,139	0,06307	1,579	0,554
TCP	1,01326	0,002519	-0,106	0,22096	0,264	0,225
TeCA	0,9856	0,001245	0,008	0,07178	0,407	0,028
TeCP	0,9745	0,000982	0,024	0,05661	0,813	0,100
PCA	1,04063	0,001066	0,357	0,08979	1,244	1,192
PCP	1,06797	0,001639	-0,24	0,13627	1,678	0,649

Table VIII - Estimation of accuracy of the simultaneous chlorophenols and chloranisoles assay in the wines (following experimental plan C) (number of samples for each analyte = 15, number of assays = 30)

Estimation de la justesse du dosage simultané des chlorophénols et des chloroanisoles dans le liège (selon plan d'expérience C)

Molecule	Value	Mean	Standard deviation of repetability	Variance of repetability	Difference of the means	Standard deviation of the differences sd	w Egality test Id I/sd
	mesured value	10,842	14,775	219,29			
TCA					-0,344	1,385	0,249
	theoretical value	11,187	0	0			
	mesured value	12,034	17,659	311,842			
TCP					0,041	0,933	0,041
	theoretical value	11,993	0	0			
	mesured value	10,134	14,475	209,514			
TeCA					0,423	1,061	0,399
	theoretical value	9,711	0	0			
	mesured value	9,778	14,303	204,587			
TeCP					0,056	0,627	0,089
	theoretical value	9,722	0	0			
	mesured value	12,467	17,639	311,121			
PCA					0,713	0,551	1,295
	theoretical value	11,755	0	0			
	mesured value	11,748	16,236	263,604			
PCP					0,0073	0,703	0,104
	theoretical value	11,675	0	0			

(table VII). It was therefore possible to conclude that there was no interference and that specificity was acceptable (slope = 1). It was likewise possible to conclude in all cases that the intercept of the regression line was not different from 0; the added concentrations were sufficiently reflected in the results with a linear relationship throughout the range.

4- Accuracy

In all cases, w was less than 3.0 (NF ISO 5725-2). It was therefore possible to conclude that the method was accurate with a 1 % risk of error (table VIII).

5- Internal reproducibility and assay uncertainty

A cork sample was selected from those used in experiment C as representative of the moderate pollution frequently observed in practice and measurements were made by two operators over a 10-day period. Table IX presents the intra-laboratory reproducibility and associated assay uncertainties.

III - EXAMPLE OF THE APPLICATION OF THE SIMULTANEOUS ASSAY OF CHLOROPHENOLS AND CHLOROANISOLES IN WINES AND CORKS OR CORK-BASED STOPPERS

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Table IX - Study of the internal reproducibility of the simultaneous chlorophenols and chloroanisoles assay in the wines (following experimental plan D, number of operators = 2, number of days = 10, total number of assays = 20)

Étude de la reproductibilité interne du dosage simultané des chlorophénols et des chloroanisoles dans le liège (selon plan d'expérience D, nombre d'opérateurs = 2, nombre de jours différents = 10, nombre total de mesures = 20)

Parameters	TCA	TCP	TeCA	TeCP	PCA	PCP
Mean (ng/l)	8,0	17,0	7,0	3,0	13,0	10,0
Standard deviation of internal repetability Sr2	0,75	0,89	0,65	0,41	1,96	2,98
Variance of internal repetability SCElr	11,27	47,07	5,98	10,59	5,44	39,03
Variance of internal repetabilitye SL_	0,25	2,17	0,01	0,38	0,68	0,68
Variance internal reproducibility SR_	1,00	3,06	0,66	0,79	1,28	3,66
Standard deviation of internal reproducibility SR	1,00	1,75	0,81	0,81	1,13	1,91
Variation of reproducibility CV %	12,50%	10,30%	11,60%	27,00%	8,70%	19,10%
Incertitudes of the assay (ng/l)	2,0	3,5	1,6	1,6	2,3	3,8

Tableau X - Simultaneous assay of chorophenols and chloroanisols in bottled wines and their respective cork stoppers

Dosages simultanés des chlorophénols et des chloroanisoles dans des vins en bouteilles et dans leurs bouchons respectifs

ng/l wine								
N° bouteille	TCA	TCP	TeCA	TeCP	PCA	PCP	Musty taint*	Relative intensity of cork taint**
T1 (plastique)	n.d	13	5	n.d	9	n.d	-	0
15	traces	24	4	n.d	14	n.d	-	0
27	traces	11	nd	traces	3	traces	-	0
4	3	14	2	n.d	3	n.d	+/-	1
21	traces	5	traces	traces	4	n.d	+/-	0,5
11	67	107	4	n.d	3	n.d	+	4
14	14	47	0	traces	n.d	n.d	+	2
23	22	136	traces	n.d	5	traces	+	2
29	14	64	n.d	n.d	3	n.d	+	2
Detection limit (ng/l)	0,99	2,12	1,43	2,59	2,8	3,2		
Quantification limit (ng/l)	2,97	4,8	3,34	6	5	8,74		
ng/cork								
N° bouteille	TCA	TCP	TeCA	TeCP	PCA	PCP	Cork weight (g)	
T1 (plastic)	58	n.d.	traces	n.d.	traces	traces	6,3	
15	18	64	n.d.	traces	n.d.	n.d.	7,8	
27	27	94	n.d.	traces	n.d.	95	8,2	
4	33	128	n.d.	traces	n.d.	traces	6,7	
21	34	95	n.d.	n.d.	n.d.	n.d.	7,9	
11	223	750	n.d.	traces	n.d.	n.d.	8,2	
14	96	470	n.d.	traces	n.d.	n.d.	6,1	
23	216	517	n.d.	n.d.	n.d.	n.d.	9	
29	654	1886	traces	traces	37	n.d.	6,2	
Detection limit (ng/l)	0,47	2,46	0,88	0,55	1,93	1,4		
Quantification limit (ng/l)	2,38	7,46	3,19	4,09	5,18	5,9		

** : on a relative scale from 0 to 5, * - : absence ; n.d.= no detection < detection limit ; traces = < quantification limit

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A high frequency (37.5 %) of «musty» off-odors was detected on tasting representative samples of rosé wine from bottles corked with cork-based agglomerated stoppers. There was considerable bottle variation in the intensity of the fault. Part of the same batch of wine was stopped with synthetic plastic corks and showed no spoilage. The cork was, therefore, strongly suspected to be responsible for the defect, but the type and origin of the contamination needed to be determined as the wine had been stored in wooden pallet-boxes that were massively impregnated with disgusting-smelling chlorophenols.

Table X summarizes the assays carried out on a selection of wines found spoiled to varying degrees by tasting and their respective corks. Wine from an «unspoiled» bottle did not contain any detectable traces of TCA and only a tiny quantity of TeCA, well below the spoilage threshold (< 10 ng/L). Wines with suspect stoppers that did not shown any tasting faults contained traces of TCA > 0.99 ng/L (LD: detection threshold) but less than 2.97 ng/L (LQ: quantification threshold), while TeCA was present in trace amounts or undetectable. Wines that were obviously spoiled all contained large quantities of assayable TCA (> LQ), while TeCA was not detectable in most samples. The constant presence of low levels of PCA confirmed the existence of a source of environmental pollution, but the concentrations found did not account for the spoilage. The intensity of the «musty» odor correlated perfectly with the quantity of TCA assayed in the wine above a limit (# < 3 ng/L) corresponding to the perception threshold for TCA in this wine (figure 4).

In this case, identifying and assaying the contaminants present and drawing up a contamination balance for the wine and stoppers made it possible to conclude irrefutably that the corks used were responsible for spoiling the wine.

CONCLUSIONS

The proposed simultaneous assay method for chlorophenols and 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 with detection and quantification limits compatible with those required for this type of contaminant (assay range: ppt). It provides an accurate, objective analysis of the type and origins of pollution in bottled wines with corks or cork-based stoppers due to phenols and, in particular, chloroanisoles.

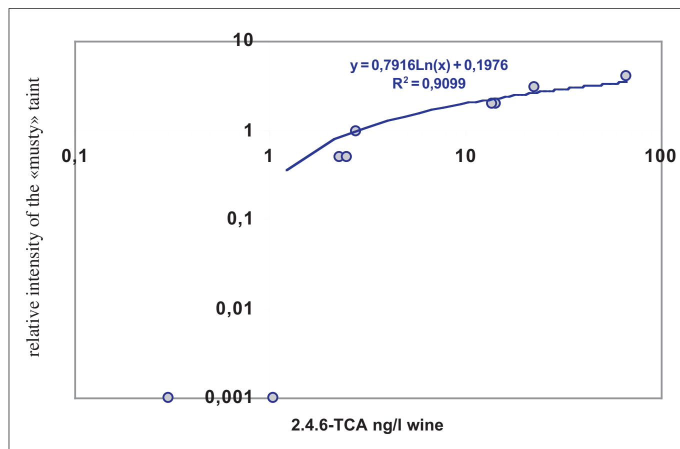

Fig. 4 – Relation between the «musty» odor intensity judeged by sensorial analysis and the TCA amount of the wine analysed

Relation entre intensité de l'odeur « moisie » appréciée à la dégustation et teneur en TCA du vin analysé

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