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Journal of Chromatography A, 953 (2002) 207–214

JOURNAL OF CHROMATOGRAPHY A

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Evaluation of an extraction method in the determination of the 2,4,6-trichloroanisole content of tainted cork☆

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Received 12 June 2001; received in revised form 30 January 2002; accepted 8 February 2002

Abstract

A method based on solvent extraction and GC–electron-capture detection analysis for the determination of 2,4,6-trichloroanisole (TCA) from cork has been evaluated and optimised. Our sample treatment consists of an extraction stage with pentane while the sample and solvent are kept in contact in a mechanical shaker (shake-flask extraction). Different extraction conditions have been tested in order to find the best compromise between efficiency and time of analysis. Different columns were evaluated for use in the concentration and purification step. A silica column was found to give the best performance in terms of recovery of TCA and repeatability. Pentane and mixtures of pentane–diethyl ether at different ratios were tested as eluting agents. It was found that 10 ml pentane allowed the recovery of retained TCA. Finally, the eluate was concentrated and injected into the chromatograph for TCA determination. The optimised chromatographic conditions enabled the quantification of TCA and 2,6-dichloroanisole, which was assayed as the internal standard. The shake-flask extraction method was compared with Soxhlet and ultrasound assisted extraction procedures using pentane as a solvent. Similar results were obtained for the shake-flask and Soxhlet extraction methods, while sonication gave significantly lower recoveries. The optimised shake-flask method was applied to determine the distribution of TCA in naturally contaminated cork bark.

Keywords: Cork; Wine; Extraction methods; Trichloroanisole

1. Introduction

A serious problem for the wine and cork industries is that of a mouldy/musty off-flavour called ''cork taint'' that affects between 0.1 and 10% of European bottled wines [1]. It has been estimated that as much as US$10 billion is lost annually as a result of this effect [2]. Recent studies have concluded that the compound which is mainly responsible for this flavour is 2,4,6-trichloroanisole (TCA) [3–5], although it is thought that geosmin, guaiacol, 2-methyisoborneol, 1-octen-3-one, 1-octen-3-ol and chlorophenolic compounds may also contribute [6,7].

It has been found [1,8] that fungi may biosynthesise 2,4,6-TCA along with other chloroanisoles as

☆Presented at the 30th Meeting of the Spanish Group of Chromatography and Related Techniques/1st Meeting of the Spanish Society of Chromatography and Related Techniques, Valencia, 18–20 April 2001.

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00155-3

a detoxification mechanism in order to remove chlorophenols from their environment and these may migrate to wines from contaminated cork stoppers. The development of analytical methods for the determination of 2,4,6-TCA in cork stoppers is therefore of great interest to the cork and wine industries.

Gas chromatography with electron-capture detection (GC–ECD) and mass spectrometry (GC–MS) is used for the determination of TCA and other chloroanisoles. An internal standard calibration method, which can use lindane [9], dibromo-1,2-benzene [3], dimethyl-3,4-phenol [3] and *n*-pentadecane [10] for GC–ECD and polydeuterated TCA [11–13] for GC–MS, have been tested for TCA quantification.

However, in order to analyse TCA from cork a previous extraction step is necessary. Different approaches have been proposed for this extraction step, including solvent extraction, thermal desorption and solid-phase microextraction (SPME). In the case of solvent extraction, which is the most practical for industrial use, different methods have been tested [1,3,5,9,12,14–19]. Typically the whole cork, or a fraction of small pieces of cork material, is mixed, with or without mechanical shaking, with a solvent, normally either pentane or a hydroalcoholic solution. In only a few cases is the extract cleaned up before the determination step employing silica or Florisil [1,15]. Soxhlet extraction [10,20], simultaneous distillation extraction [21] and supercritical fluid extraction (SFE) [13] are other techniques which are applied for the solvent extraction of TCA from cork. The extraction of trichloroanisole from cork using supercritical fluids has the advantages that it is relatively fast, is selective, and only requires small volumes of organic solvents [13]. Other methods based on thermal desorption [22,23] and SPME [24] have been developed as alternatives to the use of solvents. Thermal desorption of the solid sample has been combined with an intermediate cryo-focusing step in the insert liner of a cooled injection system followed by temperature programmed sample transfer to the analytical column. In the case of SPME, TCA determination from cork material is carried out after moistening the ground cork with water and exposing the SPME fibre to the headspace for a fixed period of time followed by desorption in the GC injector. It is claimed that SPME is a rapid, solvent free, automatable and relatively economic method although there can be difficulties in reproducibility and quantification.

The simplicity of solvent extraction, well within the possibilities of most laboratories, has led us to choose this method for the extraction of 2,4,6-TCA from cork stoppers. The results of earlier studies have given conflicting data with regards to recovery levels and no definitive conclusion about the best procedure has been reached. In the present study we have four main objectives. Firstly, we attempt to optimise a solvent extraction method to recover 2,4,6-TCA from contaminated cork and compare the suitability of sonication, Soxhlet and shake-flash procedures. Secondly, we evaluate the efficiency of Florisil, activated silica and commercial cartridges in the clean-up step and the efficiency of different elution mixtures and volumes of solvent in eluting 2,4,6-TCA from the cartridges. The results obtained are discussed in terms of recovery and repeatability. Thirdly, we study the use of 2,6-dichloroanisole (DCA) both as a surrogate and an internal standard. Finally, we apply the optimised method to the determination of TCA distribution in a naturally contaminated cork bark.

2. Materials and methods

2.1. Reagents and chemicals

n-Pentane picograde (Promochem, Wesel, Germany), *n*-hexane pesticide residues grade (Carlo Erba, Milan, Italy), and diethyl ether for HPLC (Fluka, Buchs, Switzerland) were used as the solvents.

Stock solutions of 2,4,6-trichloroanisole and 2,6-dichloroanisole (Sigma–Aldrich, Madrid, Spain) were prepared in *n*-hexane.

Anhydrous sodium sulfate was of analytical-reagent grade (Panreac, Barcelona, Spain).

In the evaluation of the most efficient clean-up adsorbent we tested silica (SDS, Peypin, France), Florisil (Sigma–Aldrich) and Sep-pak Plus silica cartridges (Waters, Milford, MA, USA). Paper filters were obtained from La Paperera del Besós, S.A.

2.2. Cork material

Cork material in the form of both stoppers and slabs was supplied by Aecork (Associació d'Empresaris Surers de Catalunya, Palafrugell, Spain).

Since no reference material is available for the validation of the method, spiked cork was prepared by injecting 1 μl of a stock solution of TCA and/or DCA (8 mg/l TCA and 74.7 mg/l DCA, in hexane) in each of five different places of the interior of the cork stopper. In this way it was possible to calculate the recoveries of the two compounds as well as evaluate whether DCA can be used as a surrogate. Corks used for spiking were checked for background TCA by the analysis of several samples from the same lot and no contamination was found.

Naturally contaminated cork was used for the comparison of the three extraction techniques.

2.3. Apparatus

The cork material was milled with a conventional grinder Moulinette D56 (Moulinex España, Barcelona, Spain). The detachable parts of the grinder were carefully washed using the standard procedure for glassware. No TCA contamination carryover was observed.

An ultrasonic P Selecta bath (Barcelona, Spain) was used in the sonication experiments.

A rotary mixer (Dinko Instruments, Dinter, Barcelona, Spain) adapted to accommodate 100 ml bottles and an orbital mixer IKA Labortechnik KS250 basic (Staufen, Germany) were employed to shake the cork with the organic solvent.

Samples were concentrated with a rotary evaporator RE100 Bibby (Bibby Sterilin, Staffordshire, UK).

Gas chromatographic analysis was performed with a GC 8000 Series (8160) gas chromatograph equipped with an autosampler AS 800 and an electron-capture detector ECD 800 (Fisons Intruments, Milan, Italy).

2.4. Chromatographic conditions

A DB-5 capillary column (J&W Scientific, Folsom, CA, USA) (30 m×0.25 mm I.D., film thickness 0.25 μm) was used.

We modified the chromatographic conditions used for the determination of organochlorine pesticides [25] to obtain a good resolution for TCA and DCA peaks. The operating conditions were: injector temperature 270 °C; detector temperature 330 °C; carrier gas, He 5.0, 30 cm/s; make up gas, N_2 5.5, 43 cm/s; oven temperature programme, 3 min at 70 °C then 5 °C/min up to 180 °C and 10 °C/min up to 270 °C and finally 3 min at 270 °C. Splitless mode injections (1 μl) were performed with the purge valve opened at 1 min. The chromatographic data were analysed by Chrom-Card software.

A standard solution chromatogram (32.3 μg/l of TCA and 332.8 μg/l of DCA in *n*-hexane) is shown in Fig. 1. The repeatability of the chromatographic analysis was evaluated for a 20 μg/l TCA standard solution. We obtained an RSD of 0.04% ($n=12$) for the retention time and 3% for the peak area ($n=12$).

2.5. Extraction procedures

2.5.1. Shake-flask extraction

Approximately 4 g of ground cork was placed in a 100-ml glass bottle together with an appropriate volume of *n*-pentane. The sample was shaken in the rotary mixer for 60 min with 80 ml of solvent. Then the solvent was separated and 40 ml of pentane were added for a second extraction lasting 30 min. After filtering through a filter paper, the combined extracts were concentrated to about 1 ml first with a rotary evaporator operating at room temperature and then under a N_2 stream.

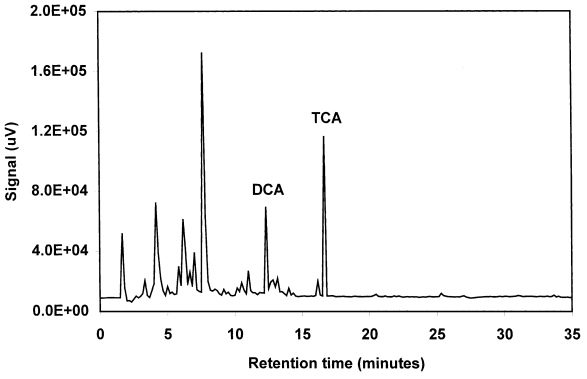

Fig. 1. Chromatogram of a standard solution of 32.3 μg/l TCA and 332.8 μg/l DCA in hexane.

2.5.2. Soxhlet extraction

Approximately 4 g of ground cork was placed in a Soxhlet apparatus together with 250 ml of *n*-pentane. The extraction procedure was conducted continuously over a 24-h period. The obtained extract was concentrated as in the shake-flask procedure described above.

2.5.3. Ultrasound assisted extraction

Approximately 4 g of ground cork was placed in a 100-ml glass bottle together with 40 ml of *n*-pentane. The bottles were immersed in an ultrasonic bath for 15 min and then for a further 15 min after separation of the solvent and the addition of 40 ml of *n*-pentane. The two fractions were combined and the same procedure as in the shake-flask experiment was followed.

2.6. Clean-up

The efficiency of Florisil, activated silica and commercial silica cartridges were evaluated for cleaning-up the extracted samples.

Self-made columns were prepared as follows: 0.69 g of either activated silica or Florisil (300 °C overnight and once cooled moistened with 2% water) were weighted and then a volume of pentane was added to form a gel. A thin layer of anhydrous sodium sulfate was placed at the bottom of a glass column that was filled with the gel mixture before finally adding anhydrous sodium sulfate to the top of the column. Sep-pak Plus silica cartridges also contained 0.69 g of silica.

After conditioning the columns or the cartridges with 10 ml of pentane, a 1 ml sample was applied.

We evaluated the capacity to elute TCA and DCA of different mixtures: pentane and pentane–diethyl ether. The efficiency of the eluting solution was established by considering the minimum volume of solvent needed for a higher recovery, i.e., the maximum amount of TCA in the eluted fraction.

For quantification by the internal standard method, 1 ml of a standard solution of 333 μg/l of DCA in hexane was added to the TCA solution eluted from the cartridge. Finally, the eluate was concentrated to ≤1 ml under a gentle N_2 stream.

2.7. Quantification

Quantitative determinations were obtained after calibration using either the internal or external standard methods. For the external standard method, standards of concentrations of TCA 4.08, 8.16, 12.24, 16.32, 20.40, 28.56, 32.64, 36.72, 40.8 and 48.96 μg/l were prepared by diluting with hexane a stock solution of 40.8 mg/l. Thus, the parameters of the calibration curve were: slope, $2.036 \cdot 10^5 \pm 2.807 \cdot 10^3$; intercept, $-3.012 \cdot 10^5 \pm 8.042 \cdot 10^4$; correlation coefficient, 0.9983; limit of detection, LOD (calculated as three times the standard deviation of the calibration curve), 1.19 μg/l; limit of quantification, LOQ (calculated as 10 times the standard deviation of the calibration curve), 3.95 μg/l; linearity 4–50 μg/l. Calibration curves with the internal standard method obtained from standard solutions containing DCA (333 μg/l) and variable amounts of TCA gave analytical parameters (i.e., LOD, LOQ and correlation coefficient) similar to those shown above.

The data corresponding to the efficiency of the three extraction techniques were treated with the multifactor analysis of variance (ANOVA) using Excel (Microsoft Office) software.

3. Results and discussion

3.1. Solvent extraction method (shake-flask) for the determination of TCA in cork material

When we conducted the preliminary extraction experiments using an orbital platform to mix the granulated cork and the solvent, we found that the cork remained floating on the surface of the liquid or stuck to the wall of the glassware used. To avoid this problem, we decided to use a rotary mixer and hence improve the extraction efficiency of TCA.

Pentane has frequently been used for the extraction of TCA and other chloroanisoles and chlorophenoles [1,3,16,21]. Different authors have employed varying volumes of solvent and mixing times for the quantitative extraction of trichloroanisole. For the optimisation of the solvent extraction method, we conducted some experiments to minimise the extraction time and avoid organic solvent wastage. To this end, four cork stoppers were previously spiked

by injecting 40 ng of TCA into each of five different places within the cork. Every cork stopper was ground separately and submitted to the solvent extraction procedure described in the Materials and methods section. Additionally, a third extraction employing 40 ml of pentane over a 30 min period (or 12 h in cork 4) was carried out. The 80+40 ml extract (extract A) and the 40 ml of the third extraction (extract B) were analysed for the TCA content using external standard calibration. The results obtained are shown in Table 1. As can be seen, the TCA concentration in extract B was, in corks 1, 2 and 3, below the LOQ of the calibration curve. In cork 4, the TCA content was below the LOD. We could not calculate the total recoveries in extract B due to the low concentration of TCA. Given these results, we discarded extract B and subsequently only conducted extraction with 80+40 ml pentane for a period of 60+30 min. These results agree with those obtained by Duncan et al. [16] who found, after the examination of the recoveries from three successive 40-ml pentane extractions of 18 cork samples, that the first two extracts had recovered 84% of the total TCA. Duncan et al. concluded that only two extractions were required.

Given the complexity of the cork matrix, a clean-up step was considered. When two chromatograms of a 1 ml pentane extract from a cork sample are compared (see Fig. 2), before and after passing the extract through a silica cartridge, we find that: there are compounds which co-elute with TCA affecting the response and complicating quantitative analysis and that there are others with higher boiling temperatures and with retention times (t_R)≥20 min (see chromatograms) which can contaminate the ECD system. Consequently, we considered the clean up step to be desirable to improve the analysis of TCA and the chromatographic conditions. Activated silica, Florisil and commercial Sep-Pak silica cartridges were employed as material for the solid-phase columns. TCA recovery from the column was evaluated taking into account both the volume and the polarity of the eluting solvent. The evaluation of the efficiency of the clean-up material was made by applying 1 ml of a standard solution of 1.6 mg/l of TCA in pentane. The results obtained (see Table 2) show that Sep-pak silica cartridges gave the highest recovery as well as offering good separation and a high degree of precision, which was determined the RSD.

As seen in Table 2, 93% of the total TCA eluted was obtained in the first 10 ml pentane (F1) applied to commercial silica cartridges. A second elution with 10 ml of a pentane–diethyl ether (8:2) mixture gives 6% of TCA, and in a third elution consisting of

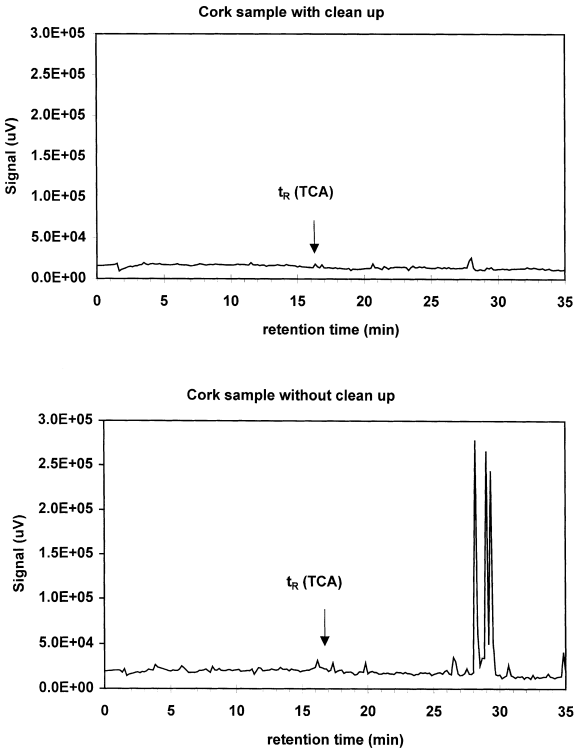

Fig. 2. Comparison of a cork chromatogram with and without clean up step.

Table 1
Results for the analysis of four cork stoppers spiked with 40 ng of TCA

	Extract A (μg TCA/l)	Extract B (μg TCA/l)
Cork 1	24.97	6.01
Cork 2	24.09	6.48
Cork 3	30.64	2.67
Cork 4	22.27	<LOD (*)
Mean value	25.5±3	
RSD (%)	14	

Extract A=80+40 ml of pentane during 60 and 30 min, respectively. Extract B=40 ml of pentane during 30 min (* or 24 h sample 4). LOD=2.2 μg/l.

Table 2
Comparison among three clean up systems: self-made activated silica columns, self-made Florisil columns and Sep-pak Plus silica commercial cartridges

TCA (1.6 μg)	Separation (% area in fraction 1)	Absolute recovery (%)	Precision (RSD, %)
Activated silica	94	85	6
Florisil	98	69	30
Sep-pak cartridge	93	110	10

10 ml pentane–diethyl ether (5:5) no TCA was found. Absolute recoveries of TCA are also shown in Table 2.

The elution profiles for TCA and DCA, obtained with pentane, pentane–diethyl ether (9:1) and pentane–diethyl ether (8:2) as eluting solutions, are presented in Fig. 3. In these experiments, the pentane solution applied to the cartridge contained 1.6 mg/l of TCA and 16.8 mg/l of DCA. The elution efficiency for both compounds was evaluated from the minimum volume of elution solution required to obtain the highest recoveries. Following this criterion, pentane gives the best efficiency although pentane–diethyl ether mixtures have much narrower elution profiles.

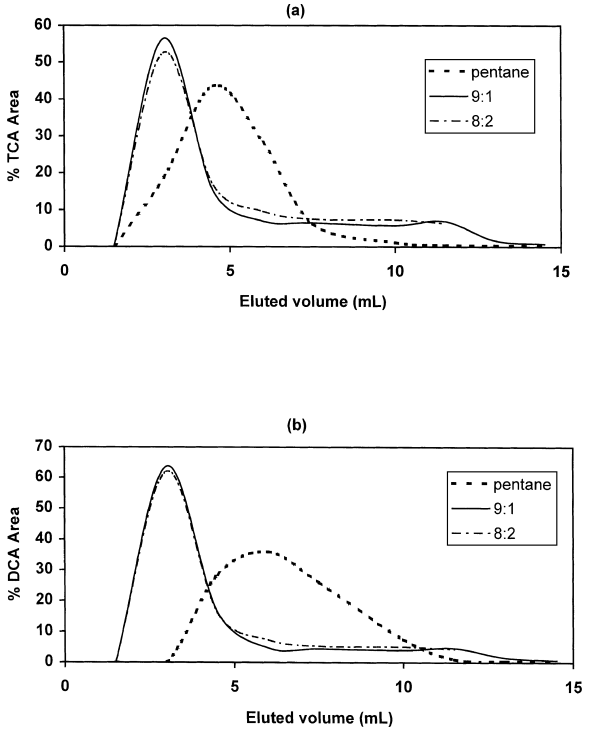

Fig. 3. Elution of retained TCA (a) and DCA (b).

The validation of the optimised method based on shake-flask extraction and a clean up with Sep-pak silica cartridges was carried out by using samples spiked with 40 ng of TCA, equivalent to 12.2 ng TCA/g cork. DCA was also added to these samples (373.5 ng or 111.7 ng DCA/g cork) in order to evaluate its use as a surrogate. The results obtained, 8.8±0.4 ng TCA/g of cork and 111.7 ng DCA/g of cork, show a lower recovery for DCA than for TCA. The recovery of 76.4% ($n=3$) found for TCA meets the requirements of the AOAC for a concentration level of 10 ng/g [26]. On the other hand, the lower value obtained for the recovery of DCA (43.8%) led us to reject its use as a surrogate. It can however be used as an internal standard as described in the Materials and methods section.

3.2. Comparison of shake-flask, Soxhlet and sonication as extraction techniques

Mechanical shaking, Soxhlet and sonication are three techniques commonly used for the extraction of organic compounds from different matrices. The first two have also been applied in the extraction of chlorophenols and chloroanisoles from cork [1,3,5,9,10,12,14–20]. Ultrasound assisted extraction has been employed by Cocito et al. [27] and Hernanz Vila et al. [28] for the extraction of aroma compounds from must and wine. Until now, it has not been used for the extraction of compounds from cork material.

In order to compare the efficiency of these techniques in the extraction of TCA from cork material, we conducted some experiments with naturally contaminated cork slabs. These slabs had not been accepted for use in the production process as they had either yellow stain or an anomalous odour; two

Table 3
Results (ng TCA/g cork) for the analysis of naturally contaminated cork material using different extraction techniques

	Replicates				Mean	Variance
	1	2	3	4		
Shake-flask	6.66	6.11	6.23	7.24	6.56	0.26
Soxhlet	6.53	7.11	6.56		6.73	0.11
Sonication	5.82	5.43	6	4.9	5.53	0.24

defects which are commonly related to the presence of TCA [1]. We ground one of these cork slabs into small pieces and mixed them thoroughly in order to homogenise the material. We took 12 4-g samples from this mixture and submitted four samples to each of the three techniques under consideration following the procedure described in the Materials and methods section. After the extraction and concentration of the extract, we determined the quantity of TCA using the internal standard method (Table 3). ANOVA was then carried out to compare the three sets of results: the one-tailed F-test showed that the means given by the three techniques differed significantly ($F=7.2884$, critical value$=4.4589$, $P=0.05$). The apparent lesser efficiency of ultrasound assisted extraction was confirmed by calculating the least significant difference [29] and comparing this with the gaps between the adjacent values of the means (previously arranged in ascending order).

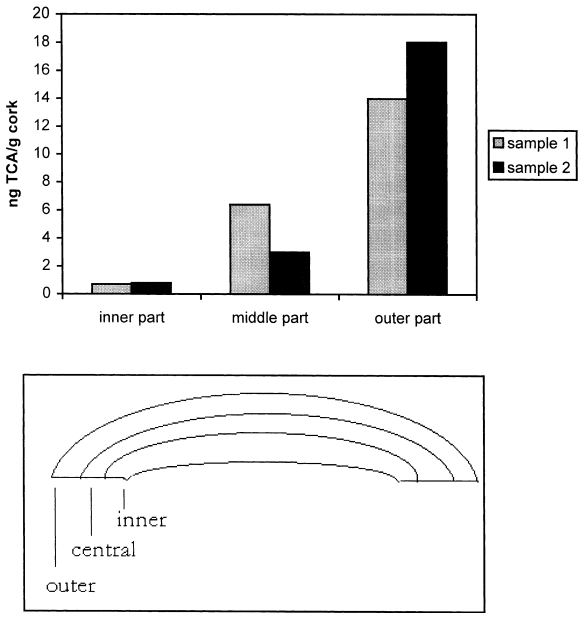

Fig. 4. TCA distribution in a contaminated cork bark. The bars show the results for the inner, middle and outer part: 0.7, 6.4 and >14 ng/g for sample 1 and 0.8, 3 and >18 ng/g for sample 2.

3.3. The distribution of TCA in cork bark

After dividing a naturally contaminated bark into three parts by age, we were able to map the distribution of TCA in the cork bark (Fig. 4).

We then used shake-flask extraction to establish the TCA profile (Fig. 4). A clear gradient can be observed running from the younger inner part of the core towards the outer section. These results agree with Hoffmann and Sponholz [23], who found the same gradient although they did not specify the TCA concentration for the different ages. Hoffmann and Sponholz [23] correctly point out that this gradient is an indication of the influence of the environment on the formation of tainted compounds.

4. Conclusions

In this study we have evaluated a solvent extraction method for the determination of TCA from cork material. Special emphasis was given to the optimisation of the extraction step as well as the clean up of the extract prior to the GC–ECD analysis.

We found that best efficiency for shake-flask extraction is obtained by treating 4 g of ground cork with 80 ml of pentane for 1 h and again with 40 ml for 30 min. A rotary mixer is found to be more effective than an orbital platform.

For the clean-up step, commercial silica cartridges gave better results in terms of recovery of TCA and reproducibility than self-made activated silica or Florisil columns. A quantitative elution of TCA was achieved with 10 ml of *n*-pentane.

A recovery of 76% was obtained when the whole shake-flask extraction procedure was applied to cork stoppers spiked with 40 ng of TCA.

2,6-Dichloroanisole was tested as a surrogate and as an internal standard for TCA quantification. The recovery of DCA by the shake-flash method was significantly lower than that of TCA (44 vs. 76%)

and is therefore ineffective as a surrogate although it can be used as internal standard.

A comparison of the effectiveness of our proposed shake-flask method with Soxhlet and sonication techniques for the extraction of TCA from naturally contaminated cork showed that shake-flash extraction and Soxhlet gave similar results whereas the efficiency of ultrasound assisted extraction was significantly worse. When the optimised shake-flask method was used to study the TCA distribution in cork bark, the higher TCA concentration found in the inner part of the bark made clear the influence of the environment on the formation of tainted compounds.

Acknowledgements

The study has been part-financed by the General Direction of INIA, project VIN00-020-C2-1. R.J. acknowledges the concession of a grant from Ministerio de Educación y Cultura (IN92 D40317889).

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