Journal of Chromatography A 1673 (2022) 463186

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development and validation of a method for the analysis of halophenols and haloanisoles in cork bark macerates by stir bar sorptive extraction heart-cutting two-dimensional gas chromatography negative chemical ionization mass spectrometry

A. Marsol-Vall*, S. Ainsa, R. Lopez, V. Ferreira

Department of Analytical Chemistry, Laboratory for Flavor Analysis and Enology (LAAE), Instituto Agroalimentario de Aragón (IA2), Faculty of Sciences, Universidad Zaragoza, Zaragoza E-50009, Spain

ARTICLE INFO

Article history:
Received 15 March 2022
Revised 30 May 2022
Accepted 30 May 2022
Available online 3 June 2022

Keywords:
Halophenols
Haloanisoles
Cork bark
Heart-cutting two-dimensional gas chromatography (GC-GC)
Negative chemical ionization (NCI)

ABSTRACT

A method has been developed for the quantitative determination in cork bark macerates of 7 halophenols and 5 haloanisoles with demonstrated or suspected contribution to the cork taint off-flavour. Macerates were extracted with stirbar (20 mm polydimethylsiloxane-coated) sorptive extraction under optimized conditions (pH 3.5, 20% NaCl and 60 min). The bars were analysed by automated thermal desorption, heart-cutting two-dimensional gas chromatography and negative chemical ionization-mass spectrometry. Matrix effects were compensated for by a "matrix matched" calibration curve. Limits of detection were in the range 0.03-0.24 ng L^{-1}, below the corresponding odor thresholds. Linearity ($0.983 \leq R^2 \leq 0.998$), intra- and inter-day precision (5.4-14.3%) and accuracy (89–126%) were satisfactory. The analysis of 48 natural cork bark samples affected/not-affected bya cryptogamic disease (yellow spot) revealed compositional differences in 2,4,6-trichloroanisole (2,4,6-TCA), 2,4,6-trichlorophenol (2,4,6-TCP) and also in 2,6-dibromophenol, 2,3,4- and 2,4,5-TCP, 2,3,4-TCA and 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP). In addition, the contents of 2,4,6-TCP and 2,4,6-TCA, and 2,3,4,6-TeCP and some TCPs presented strong linear correlations.

1. Introduction

Natural cork is the outer part of the cork oak tree (*Quercus suber* L.). The characteristics of this natural polymer include a low permeability to gases and liquids, good chemical and microbiological stabilities, low conductivity and some elasticity [1]. These properties make cork an excellent product for use as a stopper to seal wine bottles. Natural cork can suffer changes in its cell walls produced by microorganisms known in the cork manufacturing industry as "yellow spot" [2]. Following these structural changes, cork can accumulate precursors or odorants responsible for what is known in wine science as *cork taint*. This fault is commonly described as a musty/moldy off-flavor and it is known to be caused by some haloanisoles with very low odor thresholds (OTs), such as 2,4,6-trichloroanisole (2,4,6-TCA) with OT 1-3 ng L^{-1} [3], 2,3,4-TCA with OT 2-6 ng L^{-1} or 2,4,6-tribromoanisole (2,4,6-TBA) [4] with OT 2.7-9 ng L^{-1}, to name the most remarkable compounds. The sensory problem is not limited to the development of those unpleasant musty off-flavors, since some of these compounds, notably 2,4,6-TCA and 2,4,6-TBA, at extremely low concentrations can suppress the transduction of the odor signal elicited by the olfactory receptors, which results in the suppression of wine aroma [5].

The origin of the contamination of wines with haloanisoles is still unclear, as they can occasionally be formed in wooden items inside the cellar such as barrels or pallets, although most reports suggest that natural cork is the most frequent source of contamination. This explains the interest in the industry for robust analytical methods able to detect the presence of these compounds at very low levels. Compounds to target in cork should not only be haloanisoles but also the corresponding halophenols. In spite of the fact that they have higher OTs, they can form haloanisoles through *O*-methylation driven by chlorophenol-*O*-methyltransferase enzymes in filamentous fungi [6]. In addition to halophenols and haloanisoles, other aroma compounds such as alkylmethoxypyrazines or a few terpenoids can also contribute to the musty/moldy off-flavor; however, their origin is not the cork itself but the grapes or wines infected with a variable combination

* Corresponding author.
E-mail address: alexis.marsol@unizar.es (A. Marsol-Vall).

https://doi.org/10.1016/j.chroma.2022.463186

of fungi and/or bacteria [7]. The only exception is 3,5-dimethyl-2-methoxypyrazine for which a cork origin has been demonstrated [8,9].

Due to the relevance of halophenols and halohanisoles in cork, several research studies address their analysis. Sample preparation usually includes the extraction of ground cork with organic solvents such as dichloromethane [10], acetone [11] or with pressurized fluids [12] followed by injection of the organic extract. In addition, there is a publication in which a few chlorophenols and chloroanisoles are extracted with water (ethanol 15%) to make the extract compatible with the subsequent stir bar sorptive extraction (SBSE) [13]. Solventless extraction of ground cork employing headspace solid phase microextraction (HS-SPME) has been developed for the analysis of chloroanisoles but not for chlorophenols [14,15]. Derivatization of phenols by acetylation with acetic anhydride is sometimes performed to achieve a better extractability and analytical performance of the obtained acetylated halophenols [16]. In all cases, detection is carried out by gas chromatography (GC) coupled to electron impact mass spectrometry ((EI)MS) [7] or electron capture detection (ECD) [16].

In the present study, instead of an organic solvent extraction of the cork, we use a hydroethanolic (13% volume) solution to obtain a cork macerate as in the standardized International Organization of Vine and Wine (OIV) procedure for the analysis of "releasable" TCA. This is the only similarity with the current OIV method, as the former includes derivatization with acetic anhydride, extraction with ether-hexane and analysis by GC-ECD [17]. The maceration with a hydroethanolic solution, although it does not yield recoveries as high as organic solvents, provides a more realistic insight into the haloanisole content in cork that can effectively reach the wine when a given cork is used for the manufacturing of cork stoppers. The method is extensively used in the cork industry for quality control. Moreover, the aqueous-ethanolic extract obtained with this procedure is compatible with the polydimethylsiloxane (PDMS)-coated SBSE. Extraction is followed by thermal desorption (TD) and analysis by heart-cutting two-dimensional gas chromatography and mass spectrometry with negative chemical ionization (GC-GC-(NCI)MS). The hyphenation employed in the present work results in a novel analytical method developed and optimized to analyze halophenols and haloanisoles in cork macerates. While SPME contains up to 63 µL and it is normally used in headspace mode [18], SBSE is a high sample concentration capacity microextraction technique as it contains 126 µL of PDMS and it is conventionally used in the liquid immersion mode, which leads to a non-selective extraction, thus extracting many unwanted a compounds. Here lies the importance of GC-GC, which provides a high separation power and allows selecting the target analytes that reach the mass spectrometer, preventing the interferences to reaching the MS. To the best of our knowledge, the hyphenation including SBSE extraction of the cork macerate followed by TD and GC-GC-(NCI)MS is used for the first time to quantify these halogenated compounds in cork or wine matrices, with the aim of achieving an improved method for their analysis.

2. Material and methods

2.1. Reagents and standards

Water was purified in a Milli-Q water purification system (Millipore, Bedford, UK). Tartaric acid and sodium chloride were supplied by Panreac AppliChem (Barcelona, Spain) and ethanol was provided by Sigma-Aldrich (Steinheim, Germany). Analytical standards of 2,3,6-TCA, 2,4,6-TCA, 2,4,6-TBA, 2,6-dichlorophenol (2,6-DCP), 2,4,6-trichlorophenol (2,4,6-TCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP), 2,6-dibromophenol (2,6-DBP) and [$^2H_5$]2,4,6-TCA as internal standard were supplied by Sigma Aldrich. 2,3,6-TCP, 2,3,4-TCP and 2,4,5-TCP were obtained from Supelco (Steinheim, Germany). 2,3,4-TCA and pentachloroanisole (PCA) were purchased from Ehrenstorfer GmbH (Augburg, Germany).

A mixed stock solution containing all the analytes at a concentration of 100 µg L^{-1} was stored in ethanol in glass vials at -20 °C. Calibration curves were built from the mixed stock solution by consecutive dilutions with ethanol. Stir bars coated with 126 µL PDMS (20 mm length x 1.0 mm thickness) were obtained from Gerstel (Müllheim an der Ruhr, Germany). Before the first use, each stirbar was conditioned at 300 °C under constant nitrogen flow for 1 h according to the manufacturer's instructions.

2.2. Samples

Samples from natural cork barks directly obtained from cork oak trees (n = 48) were crushed and ground at room temperature at a particle size <200 µm, using a ZM 200 ultra centrifugal mill (Retsch, Haan, Germany). Samples were obtained from different locations in Spain and Italy. The cork samples were classified into affected and not affected with the "yellow spot" by visual inspection.

2.3. Maceration procedure

For the maceration procedure, 200 mg of cork per sample were weighed. The cork material was placed into Falcon tubes of 50 mL capacity, to which 40 mL of aqueous solution was added, containing 13% ethanol (pH 3.5 adjusted with tartaric acid). The maceration time was 24 h at room temperature, with gentle stirring (300 rpm) in a linear shaker. To remove the solid particles of cork, each sample was then filtered with filter paper (Scharlab, Spain) into new Falcon tubes, which were stored in a refrigerator at 3 °C until analysis.

2.4. Stirbar sorptive extraction procedure

For each sample, 10 mL of filtered extract and 2 g of NaCl were transferred into a clean 25 mL Erlenmeyer flask. The salt was dissolved by manual agitation and then 5 µL of [$^2H_5$]2,4,6-TCA (at a concentration of 50 µg L^{-1}) and a previously conditioned stir bar were added. The closed flask was placed onto a 20-position magnetic stirrer (Gerstel). Extraction conditions were stirring at room temperature and 500 rpm for 60 min. After sampling, the PDMS-coated stir bar was removed with a magnetic bar, rinsed in Milli-Q water and dried with a lint-free tissue. Each stir bar was put into a TD tube (60 mm length, 6 mm o.d. and 5 mm i.d.) and placed in the autosampler tray for analysis.

2.5. Thermal desorption

The stirbar was desorbed using an automated TD unit and a cryo-cooled injection system (CIS 4) with a programmable temperature vaporization inlet equipped with a MPS auto-sampler, all supplied by Gerstel. The TD was carried out in splitless mode. The desorption temperature program was: 30 °C (held for 0.2 min) at 120 °C min^{-1} to 300 °C (held for 5 min). The initial temperature of the CIS was set at −20 °C using liquid nitrogen. The CIS was heated to 150 °C at a rate of 16 °C s^{-1} and then ramped at 12 °C s^{-1} to 300 °C. This temperature was held for 30 min to transfer the sample into the capillary column in solvent vent mode.

2.6. Heart-cutting two-dimensional gas chromatography

The system for the GC-GC was an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a quadrupole MS (5975C, Agilent), equipped with a Deans switch

device (Agilent) allowing the selective transfer of heart cuts from the first column to the second. The oven temperature was held at 40 °C for 1 min, increased at 10 °C min^{-1} to 100 °C and then ramped at 8 °C min^{-1} to 250 °C (held for 10 min).

The first column was a weakly polar DB-5MS column (15 m x 250 µm i.d. x 0.25 µm film thickness, J&W Scientific, Folsom, CA) connected to a flame ionization detector (FID) and the Deans switch. An uncoated, deactivated column (6.7 m x 180 µm i.d.) from Agilent was used as a restrictor between the FID detector and the Deans switch. Helium was used as carrier gas with a constant pressure of 36 psi. The FID was kept at 280 °C and operated with 40 mL min^{-1} hydrogen and 450 mL min^{-1} air.

The second column was a polar DB-WAXETR (30 m x 250 µm i.d. x 0.5 µm film thickness, Agilent) directly connected to an Agilent 5975C mass spectrometer. The pressure was kept constantly at 31 psi. The single quadrupole mass detector was operated in selected ion monitoring mode (SIM) with NCI using methane as reagent gas. The temperatures of the ion source and quadrupole were set at 150 °C and the transfer line was kept at 250 °C.

2.7. Statistics

Statistical analysis was carried out with the IBM SPSS Statistics software (27 version). One-way analysis of variance (one-way ANOVA) with a level of 95% significance ($p \leq 0.05$) was used to find significantly different means and the *t*-Test ($p \leq 0.05$) to compare the means of two groups. The correlation matrix was evaluated using Pearson's correlation coefficient (associated with the *t*-Test, $p \leq 0.01$).

2.8. Method validation

Method performance was assessed by means of the following quality parameters. The linearity was investigated using the previously prepared working solution at six concentration levels in triplicate. Relative peak areas of A_0/A_i (A_0 is the peak area of the target compound and Ai is the peak area of the internal standard) were used for linear regression analysis. The limit of detection (LOD) was determined by 3 times the signal-to-noise ratio (S/N=3), and the limit of quantification (LOQ) as S/N=10. The matrix effect was assessed by comparing the slopes of the calibration curves obtained in the real matrix versus a hydroalcoholic solution by means of a *t*-test ($p<0.05$). The intra-day precision of the method was assessed by analysing a cork macerate spiked at two concentration levels within the same day for six repetitions. The inter-day precision was determined by measuring the cork macerate samples at the two concentration levels in triplicate on two consecutive days. The method accuracy was studied by spiking a blank cork macerate sample with the standard solution and calculating the concentration obtained with respect to the expected concentration.

3. Results and discussion

3.1. Heart-cutting two-dimensional gas chromatography separation

Initially, the target compounds were spiked in a cork macerate at a concentration *ca.* 10 mg L^{-1}. Identification of Selection of the chromatographic cuts was done in order to ensure full transfer of the target compounds into the second dimension. The analytes were eluted from the first dimension between 10.5 and 19.0 min, and consequently the Deans switch system was programmed for seven cuts as shown in Table 1. Retention times in the second dimension and *m/z* selected for each compound are indicated in Table 2. The chromatographic separation of the target compounds is shown in Fig. 2**A** for the first dimension (FID detection at *ca.* 10 mg L^{-1}) and Fig. 2**B** for an exemplary cork macerate sample spiked at 6.25 ng L^{-1}.

Table 1
Retention times (R_t) in the first dimension and chromatographic cuts.

Number	Compound	R_t (min)	Cut (min)
1	2,6-DCP	10.52	9.8 – 11.2
2	2,4,6-TCA	12.70	12.4 – 12.9
3	2,6-DBP	13.21	13.0 – 14.5
4	2,4,6-TCP	13.22	
5	2,4,6-TBA	13.44	
6	2,4,5-TCP	13.45	
7	2,3,4-TCP	13.53	
8	2,3,6-TCP	13.73	
9	2,3,4-TCA	15.66	15.3 – 16.0
10	2,3,4,6-TeCP	16.61	16.3 – 17.0
11	2,3,6-TCA	17.48	17.3 – 17.75
12	PCA	19.03	18.8 – 19.4

Table 2
Retention times (R_t) in the second dimension and selected *m/z*.

Number	Compound	R_t (min)	Selected *m/z* *
1	2,4,6-TCA-d_5	16.50	215 (64), 180 (68), **175**
2	2,4,6-TCA	16.58	**210**, 174 (82), 146 (28)
3	2,3,6-TCA	17.90	**210**, 174 (87), 146 (34)
4	2,6-DCP	18.80	164 (63), **162**, 126 (15)
5	2,3,4-TCA	21.33	**210**, 174 (47), 146 (20)
6	2,4,6-TCP	21.60	198 (97), **196**,162 (9)
7	2,4,6-TBA	22.37	264 (1), 81 (99)**, 79**
8	2,3,6-TCP	22.48	**198** (97), 196, 161 (14)
9	2,6-DBP	22.99	**252**, 250 (49), 79 (78)
10	PCA	23.44	**280**, 243 (83), 210 (38)
11	2,3,4-TCP + 2,4,5-TCP	23.63	**198** (97), 196,162 (6)
12	2,3,4,6-TeCP	25.47	232 (23), 198 (79), **196**

* **Quantifier ion**, qualifier ions. Values in brackets are relative proportions of abundance (%) to base peak.

3.2. Stirbar sorptive extraction and thermal desorption optimization

Currently, only two commercial phases with different extraction behavior exist for SBSE, namely PDMS and ethylene glycol–PDMS copolymer (EG–silicone). The former is optimal for the extraction of compounds with $\log(K_{ow})>4$, achieving for those compounds theoretical recoveries close to 100%. Hence, quantitative recoveries would be expected for haloanisoles with $\log(K_{ow})$ ranging from 3.74 to 5.10 for 2,3,4-TCA and PCA, respectively. For halophenols, the expected recoveries would be slightly lower, given that some of them have lower $\log(K_{ow})$ as in the cases of 2,6-DCP and 2,6-DBP ($\log(K_{ow})=2.98$ and 3.24, respectively). With the aim of improving the extraction efficiency of the phenols under study, the optimization of the extraction conditions was carried out studying the following parameters: pH, ionic strength of the solution and extraction time.

The pH of the extract was assayed at 5.4, the original pH of the macerate, and at pH=3.5. According to the acid-base properties of the target compounds, the pH is expected to have an effect on the halophenols as they have a weakly acidic proton (e.g. TeCP, pKa= 5.22). Fig. 1**a** shows an increase in the area for all the compounds at pH=3.5 compared to pH=5.4. These increments were especially noticeable for chlorophenols, being up to 56% for 2,4,6-TCP, while for haloanisoles the increments were generally lower, with the exception of 2,3,6-TCA which gave a 31% increase. The ionic strength of the macerate was modified by NaCl addition from 0 to 20%. This is conventionally known as the salting-out effect and aims to decrease the solubility of the analytes in the aqueous phase and facilitate the mass transfer from the aqueous solution to the

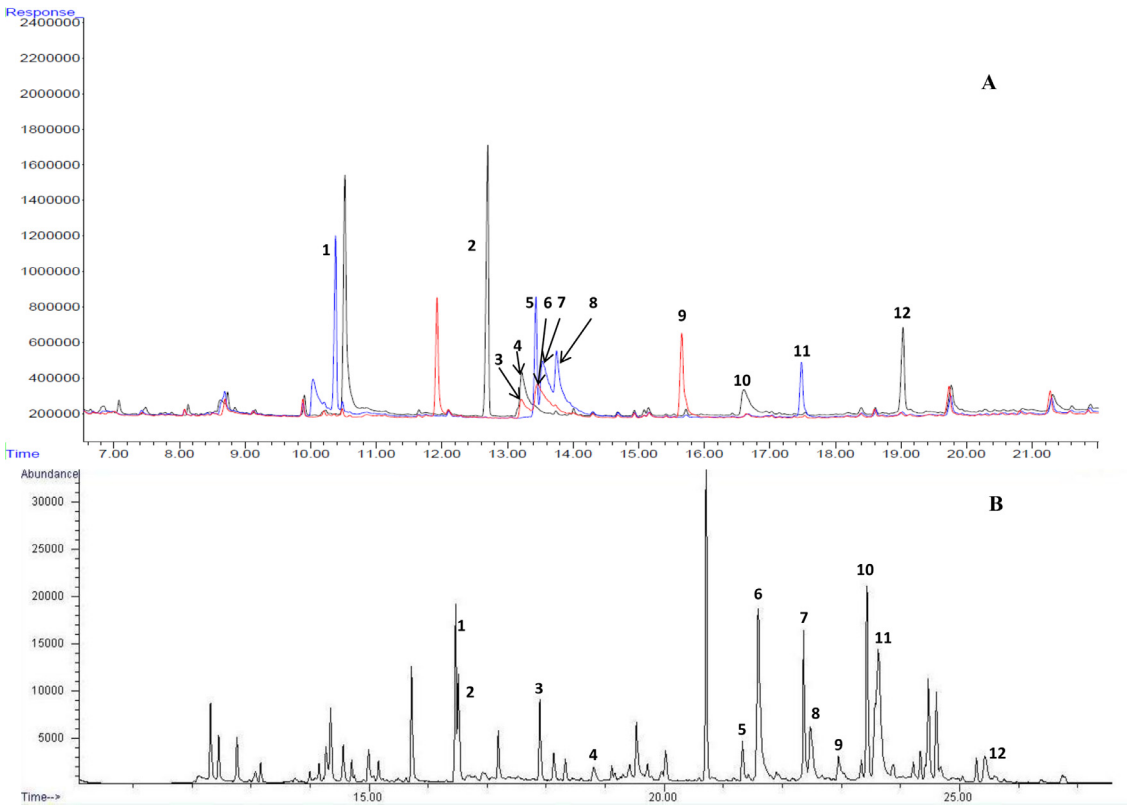

Fig. 1. (A) Overlay of 3 first dimension GC-FID traces corresponding to 3 mixtures of a cork macerate spiked at *ca.* 10 mg L^{-1}. Each mixture contains the compounds numbered as in Table 1B) Exemplary chromatogram obtained by GC-GC-(NCI)MS in SIM mode of a cork macerate spiked at 6.25 ng L^{-1}. Compounds numbered as in Table 2.

PDMS-coated stir bar. However, too high percentages of salts can increase the viscosity of the solution resulting in lower extraction efficiencies [19]. Fig. 1**b** shows that, in all cases, salt addition gave higher areas. Moreover, for all TCPs, 20% NaCl produced substantial differences compared to 5 and 10% NaCl. Consequently, 20% NaCl was chosen as the optimum percentage to maximize the analytes response. The extraction time was assayed from 15 to 120 min with the previously chosen conditions of pH=3.5 and 20% NaCl. As shown in Fig. 1**c,** the maximum extraction was achieved for 60 and 120 min. In order to reduce sample preparation time, a 60 min extraction at room temperature was chosen.

The main TD parameters considered for the optimizations were desorption time (Fig. 1**d**) at a temperature of 300 °C (the maximum allowed by the stir bar manufacturer), and the cryofocusing temperature in the CIS (Fig. 1E). For the desorption time, 10 min were chosen as 10, 15, and 20 min showed no differences, while 5 min resulted in lower responses for most of the compounds under study. In addition, no carry over effects were observed when the PDMS-coated stir bar was re-desorbed after analysis. Additionally, -20 °C was selected as the cryofocusing temperature given that a generally higher response was obtained. Although the cryogenic system here employed uses liquid N_2 and allows cryofocusing at temperatures as low as -150 °C, the fact that halophenols and haloanisoles are semi-volatile compounds explains that such low cryofocusing temperatures are not required, resulting in a lower consumption of liquid N_2.

3.3. Matrix effects

With the aim of assessing the possibility of using external calibration, calibration curves were prepared in an aqueous-ethanolic solution and in a cork macerate. Although linearity was satisfactory in both cases, significant differences ($p<0.05$) in the slopes were obtained for all compounds, thus revealing the existence of matrix effects (Table 3). To compensate for them, matrix-matched calibration curves were constructed employing a cork macerate with no initial content of any of the compounds under study.

3.4. Method performance

As shown in Table 4, the method gave LODs and LOQs ranging from 0.03 to 0.24 ng L^{-1} and from 0.10 to 0.78 ng L^{-1}, respectively. Additionally, it was observed that LODs were generally lower for haloanisoles, revealing a better performance of the analytical method for these compounds. Such behavior has a positive effect on the goal of reaching LODs below the corresponding OTs for the two most powerful aroma compounds, 2,4,6-TCA and 2,4,6-TBA, whose OTs are as low as 1 and 2.7 ng L^{-1} [3,4], respectively. A similar observation was made by Callejon et al. who reported LOD of 0.81 ng L^{-1} for 2,4,6-TCA and of 3.28 ng L^{-1} for 2,4,6-TCP [13]. As previously mentioned, as halophenols have lower log(K_{ow}), the extraction efficiency of the PDMS-coated stir bar is consequently lower. It is worth noting that in the present work LODs are one-fold lower than those reported by Callejon et al. This may be attributed to both the use of a more sensitive NCI compared to the conventional EI ionization source and to the reduced amount of interferences that reach the MS in a GC-GC system, as the chromatographic cuts selected in the first dimension only include the target compounds. In addition, in a similar work but employing a polar (EG-silicone)-SBSE, LODs for chlorophenols and chloroanisoles in wine were shown to be very similar, being 0.3-1.4 ng L^{-1} and 0.2 to 0.5 ng L^{-1}, respectively [20].

Good linearity and linear range were achieved employing the matrix-matched calibration approach and using [$^2H_5$]2,4,6-TCA as an internal standard, giving values of R^2 between 0.983 and 0.998. The intra-day precision was below 13.9% for the lower concentra-

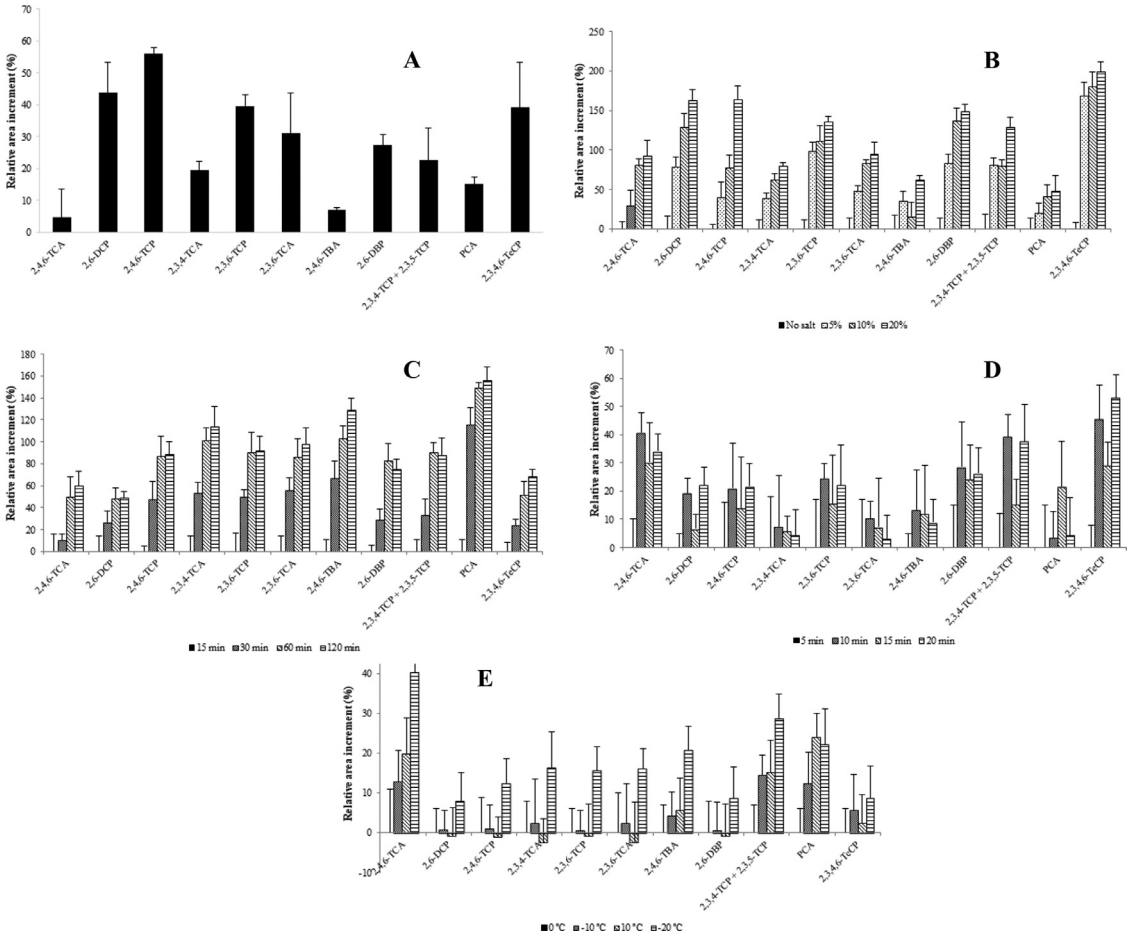

Fig. 2. Optimization of SBSE-TD employing a hydroalcoholic solution spiked at 20 ng L^{-1} with all compounds. (A) Effect of pH; (B) %NaCl (w/v) added; (C) Extraction time; (D) Desorption time and (E) Cryofocusing temperature.

Table 3
Calibration curves parameters (n = 3): (a) slope; (b) y-intercept.

Compound	In-solution calibration curve			In-matrix calibration curve		
	a ± SD	b ± SD	R^2	a ± SD	b ± SD	R^2
2,4,6-TCA	0.019 ± 0.001	0.023 ± 0.009	0.997	0.12 ± 0.02	0.078 ± 0.019	0.991
2,3,6-TCA	0.017 ± 0.001	0.013 ± 0.011	0.995	0.11 ± 0.02	0.057 ± 0.015	0.993
2,6-DCP	$7.3\ 10^{-3}$± $0.9\ 10^{-3}$	0.011 ± 0.007	0.994	0.034 ± 0.003	0.049 ± 0.041	0.994
2,3,4-TCA	0.020 ± $2\ 10^{-4}$	0.035 ± 0.008	0.992	0.13 ± 0.02	0.075 ± 0.029	0.994
2,4,6-TCP	0.16 ± 0.02	0.41 ± 0.06	0.987	0.80 ± 0.09	1.8 ± 0.4	0.996
2,4,6-TBA	0.12 ± 0.01	0.14 ± 0.04	0.995	0.53 ± 0.13	0.23 ± 0.21	0.988
2,3,6-TCP	0.054 ± 0.003	0.050 ± 0.026	0.992	0.24 ± 0.02	0.075 ± 0.085	0.998
2,6-DBP	0.022 ± 0.003	0.037 ± 0.030	0.978	0.10 ± 0.01	0.10 ± 0.05	0.992
PCA	0.13 ± 0.01	0.11 ± 0.05	0.996	0.60 ± 0.04	0.18 ± 0.13	0.983
2,3,4-TCP + 2,4,5-TCP	0.40 ± 0.12	0.84 ± 1.12	0.978	1.3 ± 0.2	1.03 ± 0.53	0.996
2,3,4,6-TeCP	0.028 ± 0.001	0.040 ± 0.018	0.989	0.086 ± 0.010	0.090 ± 0.077	0.989

tion level (0.78 ng L^{-1}) and less than 10.8% for the higher level (12.5 ng L^{-1}). Similarly, the inter-day-precision was slightly higher for most of the compounds with values between 6.3 and 14.3 % for 2,6-DBP. Accuracy was assessed by analyzing a blank cork macerate spiked at 15 ng L^{-1} with all the compounds under study, giving values around 100% (91–120%) with good %SD (1.3–7.3%).

Overall, the method performed better for haloanisoles than for halophenols. Although LODs at ng $L^{-1}$are not initially required for halophenols considering their OT is, for example, 350 ng L^{-1} for 2,4,6-TCP [3], we aimed for the lowest LOD for these compounds as it is accepted that halophenols are precursors of haloanisoles and the shelf-life of wine is expected to last for several years [21,22].

3.5. Comparison with other methods

In this section, an overview of analytical methods developed for the analysis of “releasable” halophenols and haloanisoles in cork macerate are presented, as shown in Table 5. SPE or SPME with chlorophenol derivatization with acetic anhydride resulted in LOD of 1 ng L^{-1} [23]. These values are comparable and even slightly higher than the ones obtained for the chlorophenols analyzed in the present method that ranged from 0.12 to 0.24 ng L^{-1}.

When HS-SPME-GC-ECD was applied for the analysis of 2,4,6-TCA, 1-fold higher LOD and LOQ were obtained, i.e. 0.4 ng L^{-1} for 2,4,6-TCA [24]. In a method with the same extraction methodology but employing GCGC in the separation, slightly lower LOD

Table 4
Linearity, LOD, LOQ, intra- and inter-day precision and accuracy for the SBSE-TD-GC-GC-(NCI)MS method.

Compound	Calibration curve			LOD (ng L^{-1})	LOQ (ng L^{-1})	Linear range (ng L^{-1})	Intra-day precision (%SD, n=6)		Inter-day precision (%SD, n=3 × 2)		Accuracy (15 ng L^{-1}, n=3)
	a ± SD	b ± SD	R^2				0.78 ng L^{-1}	12.5 ng L^{-1}	0.78 ng L^{-1}	12.5 ng L^{-1}	
2,4,6-TCA	0.12 ± 0.02	0.078 ± 0.019	0.991	0.06	0.19	0.19 – 25	5.7	5.9	6.3	6.1	99 ± 2
2,3,6-TCA	0.11 ± 0.02	0.057 ± 0.015	0.993	0.06	0.19	0.19 – 25	8.8	5.7	8.1	5.6	91 ± 3
2,6-DCP	0.034 ± 0.003	0.049 ± 0.041	0.994	0.12	0.39	0.39 – 25	13.6	9.3	13.9	10.7	120 ± 6
2,3,4-TCA	0.13 ± 0.02	0.075 ± 0.029	0.994	0.24	0.78	0.78 – 25	7.7	7.5	9.1	8.4	111 ± 3
2,4,6-TCP	0.80 ± 0.09	1.8 ± 0.4	0.996	0.12	0.39	0.39 – 25	8.8	5.4	10.9	7.9	103 ± 2
2,4,6-TBA	0.53 ± 0.13	0.23 ± 0.21	0.988	0.06	0.19	0.19 – 25	7.8	7.6	8.3	8.9	96 ± 5
2,3,6-TCP	0.24 ± 0.02	0.075 ± 0.085	0.998	0.12	0.39	0.39 – 25	6.0	7.0	9.2	9.0	113 ± 1
2,6-DBP	0.10 ± 0.01	0.10 ± 0.05	0.992	0.12	0.39	0.39 – 25	13.9	10.8	14.1	14.3	99 ± 7
PCA	0.60 ± 0.04	0.18 ± 0.13	0.983	0.03	0.10	0.10 – 25	7.2	8.6	13.1	9.6	101 ± 4
2,3,4-TCP + 2,4,5-TCP	1.3 ± 0.2	1.03 ± 0.53	0.996	0.24	0.78	0.78 – 25	9.6	8.8	9.5	7.4	108 ± 3
2,3,4,6-TeCP	0.086 ± 0.010	0.090 ± 0.077	0.989	0.12	0.39	0.39 – 25	10.2	8.0	11.3	8.1	107 ± 3

were obtained (0.1 vs. 0.4 ng L^{-1}) [25]. Similarly, HS-SPME-GC-MS analysis of 4 chloroanisoles (TCA, TeCA, TBA and PCA) resulted in LOD and LOQ of 0.01 ng L^{-1} and 0.05 ng L^{-1}, respectively [26]. A fast non-destructive analysis of TCA in cork suitable for individual quality control have been recently developed with satisfactory LOD and LOQ of 0.05 and 0.15 ng L^{-1} [27]. Callejon et al. developed a method employing the same extraction technique as in this work, e.g. PDMS-coated SBSE resulting in LOD of 0.81 ng L^{-1} for 2,4,6-TCA [13]. As mentioned previously, the lower LODs achieved in the present work were obtained by employing, in addition to SBSE, GC-GC for reducing the amounts of compounds reaching the spectrometer and NCI instead of EI, which provides an enhanced selectivity (as it only ionizes halogen-containing compounds) and improved sensitivity.

3.6. Sample analysis

The method was applied to the analysis of the 12 halophenols and haloanisoles in 48 cork bark samples. As shown in Table 6, 2,4,6-TCP and 2,4,6-TCA were the most abundant compounds in the cork samples affected with "yellow spot", with mean contents of 2245 and 629.5 ng kg^{-1}, respectively. Evidently, the cork affected with the "yellow spot" had in most cases contents of 2,4,6-TCA much higher than the OT (2-6 ng L^{-1} in wine), thus making this cork unsuitable for cork stopper manufacturing. It can also be observed that 2,3,4-TCA can also reach concentrations much higher than the threshold, both in wines affected and not affected by the "yellow spot" (Table 6). However, the other most aroma-powerful compound in the cork taint, 2,4,6-TBA, was only found at contents below the OT (2.7-9 ng L^{-1} in wine [4]). Some halophenols, namely 2,6-DCP, 2,3,6-TCP, 2,6-DBP, 2,3,4-TCP + 2,4,5-TCP, and 2,3,4,6-TeCP were also detected in some of the samples. Fig. 3 shows a histogram representing the most relevant compounds found in the sample analysis. Surprisingly, for some of the samples labeled as affected with the "yellow spot", no 2,4,6-TCA was found. On the contrary, 2,4,6-TCA was detected in samples not affected with the "yellow spot" (Fig. 3**a**), suggesting that the presence of the surface damage is not always associated with the presence of 2,4,6-TCA and that low or moderate contents of 2,4,6-TCA can be found even before the appearance of the visual damage on the cork surface. The 2,3,4-TCP and 2,4,5-TCP (Fig. 3**b**) contents were a bit higher in samples not affected, suggesting that these compounds do not result in 2,4,6-TCA, but probably evolve into 2,3,4-TCA given that higher contents were found in samples not affected with the "yellow spot" (Fig. 3**c**). The representation for 2,4,6-TCP (Fig. 3**e**) shows a similar pattern to that of 2,4,6-TCA, signifying higher contents of 2,4,6-TCA associated with high contents of 2,4,6-TCP. Finally, 2,3,4,6-TeCP suggests a similar pattern to that of 2,4,6-TCP but with overall lower contents, being almost negligible in samples not affected with the "yellow spot".

The correlation matrix at a significance level ($p<0.01$) revealed strong positive correlations between 2,4,6-TCA and 2,4,6-TCP ($r=0.725$), 2,3,6-TCP and 2,4,6-TCP ($r=0.607$), 2,6-DCP and 2,6-DBP ($r=0.625$), between 2,3,4,6-TeCP and 2,4,6-TCP ($r=0.779$), and between 2,3,4,6-TeCP and 2,3,6-TCP, with $r=0.617$. In addition, a moderate positive correlation was observed between 2,3,4,6-TeCP and 2,4,6-TCA ($r=0.491$). The correlation between 2,4,6-TCP and 2,4,6-TCA is in agreement with previous findings, confirming that the presence of chlorinated compounds in cork barks from oak trees is responsible for the formation of chloroanisoles [28]. Additionally, the reason for the correlations observed for TCPs and 2,3,4,6-TeCP are still uncertain, as there are several possible sources of chlorophenols. They might come from anthropogenic origin, as TCP is used as a biocide and pentachlorophenol as a wood preservative, but they might also have a natural origin, since

Table 5
Comparison with other analytical methods for the analysis of halophenols and haloanisoles in cork macerate.

Analytical method	Analytes	LOD (ng L^{-1})	LOQ (ng L^{-1})	Refs.
SPE(deriv.)[1]-GC-ECD	3 CP	1	n.d.	[23]
HS-SPME-GC-ECD	TCA	0.4	1.3	[24]
HS-SPME-GC-GC-ECD	3 CA	0.1	0.4	[25]
HS-SPME-GC-MS	4CA	0.01	0.05	[26]
TD-VOCUS	TCA	0.05	0.15	[27]
SBSE-GC-(EI)MS	5CA and 3CP	0.81-96	2.7-3188	[13]
SBSE-GC-GC-(NCI)MS	5 HA-7 HP	0.03-0.24	0.10-0.78	This method

[1] Derivatization with acetic anhydride.CP: chlorophenols, CA: chloroanisols, HA: haloanisols, HP: halophenols

Table 6
Sample analysis of cork bark samples affected and not affected with the "yellow spot". Halophenols and haloanisoles content expressed in ng kg^{-1} of natural cork.

Compound	Cork affected with yellow spot (n = 24)			Cork not affected with yellow spot (n = 24)		
	mean	minimum	maximum	mean	minimum	maximum
2,4,6-TCA	629.5	<LOQ[2]	30856	24.62	<LOQ	279.5
2,3,6-TCA	n.d.[1]			n.d.		
2,6-DCP	3.01	<LOQ	84.19	n.d.		
2,3,4-TCA	5.26	<LOQ	94.09	5.80	<LOQ	57.25
2,4,6-TCP	2245	1.00	16085	12.87	<LOQ	189.5
2,4,6-TBA	0.02	<LOQ	0.37	n.d.		
2,3,6-TCP	4.18	<LOQ	78.06	n.d.		
2,6-DBP	22.58	<LOQ	105.2	1.63	n.d.	24.07
PCA	n.d.			n.d.		
2,3,4-TCP + 2,4,5-TCP	2.72	<LOQ	30.29	34.42	n.d.	255.7
2,3,4,6-TeCP	8.60	<LOQ	44.49	n.d.		

[1] Compound not detected in any of the cork macerates.
[2] Compound detected in the macerate but below its LOQ.

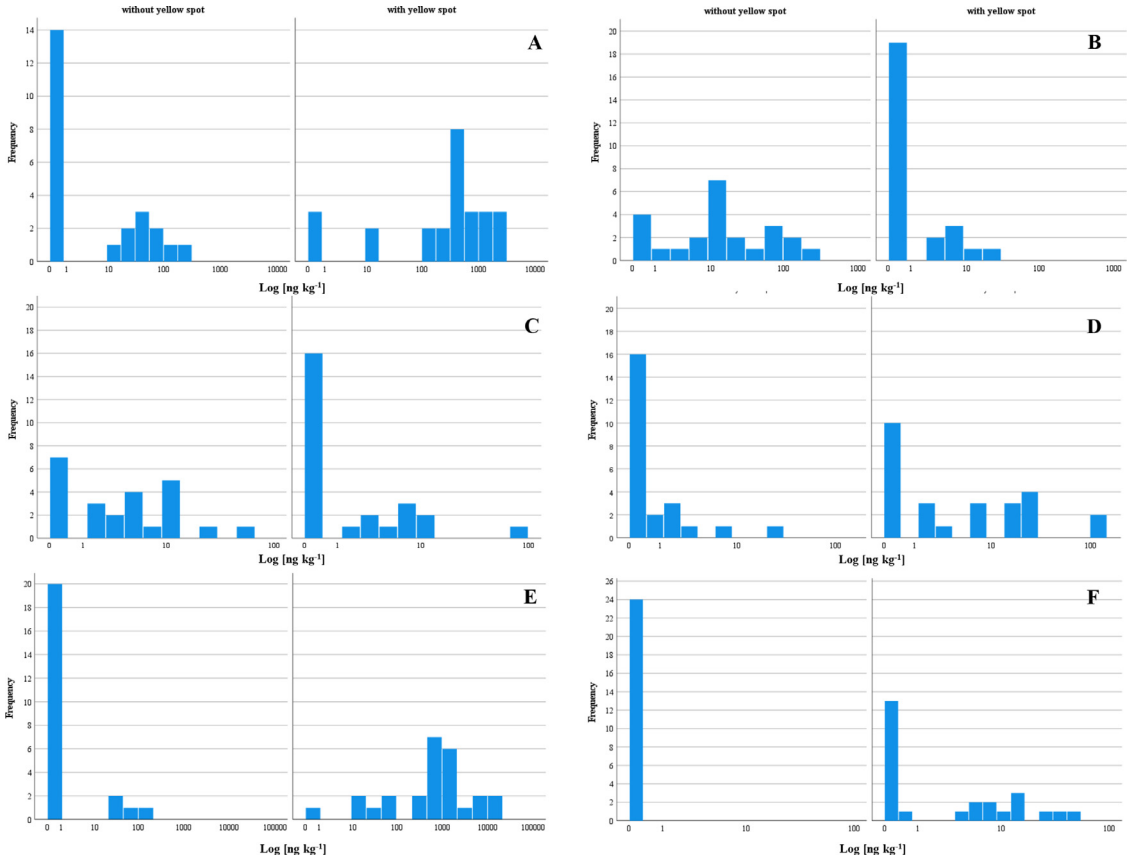

Fig. 3. Logarithmic histogram representation for samples affected and not affected with "yellow spot". (A) 2,4,6-TCA; (B) 2,3,4 + 2,4,5-TCP; (C) 2,3,4-TCA; (D) 2,6-DBP; (E) 2,4,6-TCP (F) 2,3,4,6-TeCP.

Basidiomycetes and certain *Penicillium* fungi can produce these molecules [28].

4. Conclusions

An analytical method based on the analysis of "releasable" halophenols and haloanisoles in cork has been developed and validated. The method is based on PDMS-coated SBSE of aqueous-ethanolic extracts. The coupling of GC-GC and MS analysis employing NCI as the ionization source allowed us to obtain LODs well below the OT for relevant compounds in the wine industry such as 2,4,6-TCA and 2,4,6-TBA. Other method parameters such as linearity ($0.983 \leq R^2 \leq 0.998$), intra- and inter-day precision (5.4–14.3%) and accuracy (89–126%) were considered satisfactory. Moreover, the method performed well for the analysis of halophenols which are considered to be precursors of haloanisoles through enzyme-catalyzed *O*-methylation, the latter compounds being ultimately responsible for the cork taint off-flavor in wine. Finally, the developed method was applied to the analysis of 48 samples of natural cork barks revealing clear differences in the contents of 2,4,6-TCP and 2,4,6-TCA between the samples affected and not affected with the "yellow spot" taint. In addition, high correlations were observed between the contents of 2,4,6-TCA and 2,4,6-TCP and the contents of 2,3,4,6-TeCP and 2,4,6-TCP, and 2,3,4,6-TeCP and 2,3,6-TCP.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

A. Marsol-Vall: Writing – original draft, Writing – review & editing, Conceptualization, Methodology, Investigation, Visualization. **S. Ainsa:** Writing – original draft, Investigation, Visualization. **R. Lopez:** Writing – review & editing, Conceptualization, Supervision. **V. Ferreira:** Writing – review & editing, Conceptualization, Project administration, Funding acquisition.

Acknowledgments

Research funded through the CORK2WINE project (Programa Estratégico CIEN). A.M-V. acknowledges support from the Agencia Estatal de Investigación (MICIU, Spain) through the Juan de la Cierva-Formación program (FJC2018-035486-I). Authors are grateful to Francisco Oller S.A. and Vigas S.A. (Spain) for supplying the cork barck samples. Special thanks to J. Roselló for coordinating cork sampling.

References

[1] A. Lagorce-Tachon, T. Karbowiak, D. Champion, R.D. Gougeon, J.P. Bellat, Mechanical properties of cork: effect of hydration, Mater. Des. 82 (2015) 148–154, doi:10.1016/j.matdes.2015.05.034.
[2] S.M. Rocha, M.A. Coimbra, I. Delgadillo, Demonstration of pectic polysaccharides in cork cell wall from Quercus suber L, J. Agric. Food Chem. 48 (2000) 2003–2007, doi:10.1021/jf991147g.
[3] G.J. Soleas, J. Yan, T. Seaver, D.M. Goldberg, Method for the gas chromatographic assay with mass selective detection of trichloro compounds in corks and wines applied to elucidate the potential cause of cork taint, J. Agric. Food Chem. 50 (2002) 1032–1039, doi:10.1021/jf011149c.
[4] A.R. Fontana, Analytical methods for determination of cork-taint compounds in wine, TrAC - Trends Anal. Chem. 37 (2012) 135–147, doi:10.1016/j.trac.2012.03.012.
[5] H. Takeuchi, H. Kato, T. Kurahashi, 2, 4, 6-trichloroanisole is a potent suppressor of olfactory signal transduction, Proc. Natl. Acad. Sci. USA 110 (2013) 16235–16240, doi:10.1073/pnas.1300764110.
[6] J. Rubio-Coque, L. Alvares, R.F. Martines, Causas y origenes de la contaminacion del vino por haloanisoles, Infoprint (2005) 11–42.
[7] A. Tarasov, D. Rauhut, R. Jung, "Cork taint" responsible compounds. Determination of haloanisoles and halophenols in cork matrix: a review, Talanta 175 (2017) 82–92, doi:10.1016/j.talanta.2017.07.029.
[8] R.F. Simpson, D.L. Capone, M.A. Sefton, Isolation and identification of 2-methoxy-3, 5-dimethylpyrazine, a potent musty compound from wine corks, J. Agric. Food Chem. 52 (2004) 5425–5430, doi:10.1021/jf049484z.
[9] P. Chatonnet, A. Fleury, S.p. Boutou, Origin and incidence of 2-methoxy-3, 5-dimethylpyrazine, a compound with a "fungal" and "corky" aroma found in cork stoppers and oak chips in contact with wines, J. Agric. Food Chem. 58 (2010) 12481–12490, doi:10.1021/jf102874f.
[10] P. Chatonnet, S. Bonnet, S. Boutou, M.D. Labadie, Identification and responsibility of 2, 4, 6-tribromoanisole in musty, corked odors in wine, J. Agric. Food Chem. 52 (2004) 1255–1262, doi:10.1021/jf030632f.
[11] J. Cacho, J. Nicolás, P. Viñas, N. Campillo, M. Hernández-Córdoba, Control of halophenol and haloanisole concentration in wine cellar environments, wines, corks and wood staves using gas chromatography with mass spectrometry, Aust. J. Grape Wine Res. 22 (2016) 391–398, doi:10.1111/ajgw.12231.
[12] Ó. Ezquerro, Á. Garrido-López, M.T. Tena, Determination of 2, 4, 6-trichloroanisole and guaiacol in cork stoppers by pressurised fluid extraction and gas chromatography–mass spectrometry, J. Chromatogr. A 1102 (2006) 18–24, doi:10.1016/j.chroma.2005.10.023.
[13] R. Callejon, A. Troncoso, M. Morales, Analysis for chloroanisoles and chlorophenols in cork by stir bar sorptive extraction and gas chromatography–mass spectrometry, Talanta 71 (2007) 2092–2097, doi:10.1016/j.talanta.2006.09.020.
[14] M. Riu, M. Mestres, O. Busto, J. Guasch, Determination of total chloroanisoles in different kinds of cork stoppers, Anal. Chim. Acta 563 (2006) 310–314, doi:10.1016/j.aca.2005.10.045.
[15] D. Özhan, R.E. Anli, N. Vural, M. Bayram, Determination of chloroanisoles and chlorophenols in cork and wine by using HS-SPME and GC-ECD detection, J. Inst. Brew. 115 (2009) 71–77, doi:10.1002/j.2050-0416.2009.tb00346.x.
[16] C. Pizarro, C. Sáenz-González, N. Pérez-del-Notario, J.M. González-Sáiz, Microwave assisted extraction combined with dispersive liquid–liquid microextraction as a sensitive sample preparation method for the determination of haloanisoles and halophenols in cork stoppers and oak barrel sawdust, Food Chem. 132 (2012) 2202–2210, doi:10.1016/j.foodchem.2011.12.063.
[17] OIV, Determination of releasable 2,4,6-trichloroanisole in wine by cork stoppers method OIV-MA-AS315-16 (Resolution OIV-Oeno 296/2009), (2009).
[18] F. David, N. Ochiai, P. Sandra, Two decades of stir bar sorptive extraction: a retrospective and future outlook, TrAC - Trends Anal. Chem. 112 (2019) 102–111, doi:10.1016/j.trac.2018.12.006.
[19] P.M. Dewick, Essentials of organic Chemistry: For Students of Pharmacy, Medicinal Chemistry and Biological Chemistry, John Wiley & Sons, 2006.
[20] J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba, Stir bar sorptive extraction polar coatings for the determination of chlorophenols and chloroanisoles in wines using gas chromatography and mass spectrometry, Talanta 118 (2014) 30–36, doi:10.1016/j.talanta.2013.09.047.
[21] M.L. Álvarez-Rodríguez, L. López-Ocana, J.M. López-Coronado, E. Rodríguez, M.J. Martínez, G. Larriba, J.-J.R. Coque, Cork taint of wines: role of the filamentous fungi isolated from cork in the formation of 2, 4, 6-trichloroanisole by O methylation of 2, 4, 6-trichlorophenol, Appl. Environ. Microbiol. 68 (2002) 5860–5869, doi:10.1128/AEM.68.12.5860-5869.2002.
[22] S. Prak, Z. Gunata, J.P. Guiraud, S. Schorr-Galindo, Fungal strains isolated from cork stoppers and the formation of 2, 4, 6-trichloroanisole involved in the cork taint of wine, Food Microbiol. 24 (2007) 271–280, doi:10.1016/j.fm.2006.05.002.
[23] S. Insa, V. Salvadó, E. Anticó, Development of solid-phase extraction and solid-phase microextraction methods for the determination of chlorophenols in cork macerate and wine samples, J. Chromatogr. A 1047 (2004) 15–20, doi:10.1016/j.chroma.2004.06.104.
[24] P. Vlachos, A. Kampioti, M. Kornaros, G. Lyberatos, Matrix effect during the application of a rapid method using HS-SPME followed by GC–ECD for the analysis of 2, 4, 6-TCA in wine and cork soaks, Food Chem. 105 (2007) 681–690, doi:10.1016/j.foodchem.2006.12.056.
[25] P. Slabizki, H.-G. Schmarr, Analysis of corky off-flavour compounds at ultra trace level with multidimensional gas chromatography-electron capture detection, J. Chromatogr. A 1271 (2013) 181–184, doi:10.1016/j.chroma.2012.11.020.
[26] P. Jové, A. Pareras, R. De Nadal, M. Verdum, Development and optimization of a quantitative analysis of main odorants causing off flavours in cork stoppers using headspace solid-phase microextraction gas chromatography tandem mass spectrometry, J. Mass Spectrom. 56 (2021) e4728, doi:10.1002/jms.4728.
[27] L. Cappellin, F.D. Lopez-Hilfiker, V. Pospisilova, L. Ciotti, P. Pastore, M. Gonin, M.A. Hutterli, Thermal desorption–vocus enables online nondestructive quantification of 2, 4, 6-trichloroanisole in cork stoppers below the perception threshold, Anal. Chem. 92 (2020) 9823–9829, doi:10.1021/acs.analchem.0c01326.
[28] R.F. Simpson, M.A. Sefton, Origin and fate of 2, 4, 6-trichloroanisole in cork bark and wine corks, Aust. J. Grape Wine Res. 13 (2007) 106–116, doi:10.1021/jf049484z.