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Volatile Compounds in Samples of Cork and also Produced by Selected Fungi

M. C. Barreto,[*,†,§] L. Vilas Boas,[†,§] L. C. Carneiro,[#] and M. V. San Romão[†,§,⊥]

[†]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (ITQB-UNL), Av. da República, Estação Agronómica Nacional, 2780-157 Oeiras, Portugal

[§]Instituto de Biologia Experimental e Tecnológica (IBET), Apartado 12, 2781-901 Oeiras, Portugal

[#]INRB I.P.-L-INIA, Oeiras, Portugal

[⊥]Ex Estação Vitivinícola Nacional, INRB I.P.-L-INIA, 2565-191 Quinta de Almoinha Dois Portos, Portugal

S Supporting Information

ABSTRACT: The production of volatile compounds by microbial communities of cork samples taken during the cork manufacturing process was investigated. The majority of volatiles were found in samples collected at two stages: resting after the first boiling and nontreated cork disks. Volatile profiles produced by microbiota in both stages are similar. The releasable volatile compounds and 2,4,6-trichloroanisole (TCA) produced in cork-based culture medium by five isolated fungal species in pure and mixed cultures were also analyzed by gas chromatography coupled with mass spectrometry (GC-MS).The results showed that 1-octen-3-ol and esters of fatty acids (medium chain length C8−C20) were the main volatile compounds produced by either pure fungal species or their mixture. Apparently,*Penicillium glabrum* is the main contributor to the overall volatile composition observed in the mixed culture. The production of releasable TCA on cork cannot be attributed to any of the assayed fungal isolates.

KEYWORDS: fungi, cork, 2,4,6-trichloroanisole, volatile compounds

INTRODUCTION

Cork is the material best-suited for sealing wine bottles, due to its unique physical and chemical properties.[1] During the manufacturing process of cork stoppers, a mycoflora develops, resulting from either cork colonization or factory environment.[2−5]

The germination of mycospora fungi can enable the metabolite production resulting from the available substrates' metabolism or produced as a response to environmental conditions.[6] The production of some volatile compounds by fungi in cork slabs, namely, chloroanisoles, is considered to be the most frequent cause of organoleptic defects of wines.[4,7,8] Although other compounds can contribute to a musty taint in wines, for example, 2,4,6-tribromoanisole (TBA)[9] and 2-methoxy-3,5-dimethylpyrazine,[10] 2,4,6-trichloroanisole (TCA) was recognized to be present in 80−85% of cork-tainted wines.[8] Due to its very low detection olfactory threshold (30−300 pg L^{-1} in water and 1.5−3 ng L^{-1} in alcoholic solution/wine)[11] and low perception threshold for humans, TCA was considered to be the main cause for unpleasant corky flavors.[12] The presence of these compounds can be at the origin of important losses in both wine and cork-stopper industries, endangering the sustainability of the cork stoppers industry because since the 1990s some alternative sealing devices have begun to be developed, especially in countries that are not producers of cork.

In this work, the volatile composition profile of cork samples taken during the manufacturing process of cork disks was investigated. There was also an attempt to establish a relationship with the released volatile compounds produced by a set of fungal species isolated during the process and inoculated (individually and as a mixture) in a cork-based medium. Moreover, releasable TCA was quantified in the same samples tested in the laboratory to assess the possible contribution of the selected fungi to produce TCA in conditions typical of cork-stopper factories.

MATERIALS AND METHODS

Determination of the Total Volatiles Released from Cork Samples Collected at Different Stages of the Cork Disk Manufacturing Process. *Sampling Plan.* The cork slabs are normally disposed in stacks inside the factory, each stack having three levels of slabs. A piece of cork was taken in the upper part of each level, and each sample is made of three cork pieces with approximately 20 cm side, taken diagonally across the stack. All of the samples were taken during the whole processing of the same batch of cork slabs. All of the cork samples used in this experiment were collected in the following manufacturing stages: nonboiled cork; immediately after the first boiling in water, which takes about 1 h; during the resting phase after boiling; immediately after the second boiling (normally 20−30 min); and nontreated cork disks.

Samples Preparation. Each cork sample was ground to powder of 0.1 mesh. Each sample (0.12 g) was weighed into a 20 mL GC screw-capped vial (20 mL La-Pha-Pack, Werner Reifferscherdt GmbH, Langerwehe, Germany). Four milliliters of a culture medium (containing 0.004 g of K_2HPO_4 previously autoclaved and added to 0.04 mL of a sterile salt solution containing, per 100 mL of distilled water, $NaNO_3$, 30 g; KCl, 5 g; $MgSO_4 \cdot 7H_2O$, 5 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 0.1 g; and $CuSO_4 \cdot 7H_2O$, 0.05 g) was poured in each vial. The different vials were

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incubated at 27 °C in the dark and agitated on an Innova 2300 rotary shaker (New Brunswick Scientific, Edison, NJ) during 3 months. Blanks containing only the culture medium and 0.12 g of cork sterilized by γ radiation (32 kGy)[13] were also run. All of the experiments were done in duplicate.

SPME-GC-MS Analysis of the Volatile Compounds. After the incubation period, the different samples were analyzed using a GC-MS system: autosampler AOC-5000 autoinjector, gas chromatograph—mass spectrometer Shimadzu GCMS-QP2010 (Shimadzu Corp., Kyoto, Japan), equipped with a capillary column DB-5MS (J&W Scientific, Folsom, CA), 28 m × 0.32 mm and 0.25 μm phase thickness.

A solid phase microextraction fiber DVD/CAR/PDMS (50/30 μm) (Supelco, Bellefonte, PA) was exposed to the sample headspace for 60 min at 45 °C with an agitation speed 250 rpm and then transferred to the GC injector at 250 °C to desorb during 2.5 min (injection in splitless mode).

The column temperature program started at 40 °C for 5 min, was raised at 5 °C min^{-1} to 170 °C and then at 30 °C min^{-1} to 250 °C, and held for 4 min. The carrier gas (helium) was kept at a constant flow (50 cm s^{-1}). Analyses were performed in full-scan mode in the range m/z 30—300 at a scan speed of 540 au s^{-1}.

Analyses of Releasable Volatile Compounds and Quantification of TCA Produced by Fungal Isolates Growing on Cork Culture Medium. *Fungal Isolates and Culture Conditions.* Five fungal strains previously isolated from cork slabs and identified using phenotypic and molecular techniques at CBS Fungal Biodiversity Centre (Utrecht, The Netherlands) were used, both in pure and mixed cultures. These strains are now deposited in international culture collections and designated *Chrysonilia sitophila* DSM 16514 (DSMZ, Germany), *Eurotium rubrum* CBS 126220 (CBS, The Netherlands), *Penicillium brevicompactum* CBS 126334, *Penicillium glabrum* CBS 126333, and *Penicillium paneum* CBS 126218.

A spore suspension containing 5 μL of 6 × 10^5 spores mL^{-1} of each species was used to inoculate a culture medium contained in 500 mL glass flasks.The fungal mixture was prepared using 1 μL of the same spore suspension of each of the five fungal species previously mentioned (5 μL total) and was inoculated in the culture medium. The medium was constituted by 7.5 g of sterile cork, 0.25 g of K_2HPO_4, and 2.5 mL of sterile salt solution to a final volume of 250 mL. The culture medium was prepared as previously mentioned. A blank assay containing only the culture medium was also prepared.

All of the inoculated media and blanks were incubated at 27 °C, agitated on the rotary shaker (60 rpm), in the dark. Two different culture periods were considered: 7 days and 4 weeks. All of the experiments were done in triplicate.

Analyses of Releasable Volatiles. *Sample Preparation.* After the fungal growth period, the sample preparation was done according to the procedure described in International Standard ISO 20752 for the determination of the releasable TCA. Briefly, after the incubation period, for each sample, the culture medium was discarded; the cork pieces were placed in a glass jar filled to the top with a 12% hydroalcoholic solution and maintained at room temperature (ca. 22 °C) during 24 h.

From each jar was taken 7 mL of the hydroalcoholic solution and transferred into a GC vial containing 3 g of NaCl. Each vial was agitated with a vortex shaker (type REAX 2000, Heidolph Instruments GmbH, Schwabach, Germany) for 2 min and transferred to the SPME-GC-MS system for analysis.

A blank containing the 12% hydroalcoholic solution was also analyzed.

SPME-GC-MS Analysis. The different samples were analyzed using the same GC-MS equipment and conditions referred to above, except that the column used was a Factor Four VF-5 m, 30 m × 0.25 mm and 0.25 μm phase thickness (Varian Inc., Lake Forest, CA).

Releasable TCA Quantification. The used method followed the International Standard ISO 20752 and was previously validated.[14]

Briefly, the sample was prepared as described before (under Sample Preparation), and the TCA analysis was done using the GC-MS equipment and column referred to above. A PDMS fiber (100 μm) (Supelco) was exposed to the sample headspace for 15 min at 40 °C with an agitation speed of 250 rpm and then transferred to the GC injector at 250 °C to desorb during 2 min (injection in splitless mode). The samples were analyzed according to the program and conditions described as follows: the column temperature started at 60 °C for 2 min, was raised at 25 °C min^{-1} to 205 °C and then at 30 °C min^{-1} until 265 °C, and held for 1 min. The carrier gas (helium) had a constant flow at 51 cm s^{-1}. Analyses were performed in the SIM mode for m/z 217, 215, 212, 210, 199, and 195. The acquisition data were taken every 0.20 s, and the limit threshold was 500.

The amount of released TCA in the various samples was calculated using a TCA calibration curve (0.5, 1, 2, 3, 4, 5, 6, 8, and 10 ng L^{-1} prepared from a stock solution of TCA 5 × 10^{-7} g L^{-1}. Pentadeuterated 2,4,6-TCA (d_5-TCA) (Cambridge Isotope Laboratories, Inc., Andover, MA) was used as internal standard: a solution 5 × 10^{-5} g L^{-1} was added to each vial containing calibration solutions or sample extracts to have a concentration close to 50 ng L^{-1}.

A blank containing the 12% (v/v) hydroalcoholic solution was analyzed under the same conditions.

The analyses of calibration standards were run as duplicates and the samples as triplicates.

Data Analysis. *Identification of the Compounds.* The identification of compounds from mass spectra obtained in scan mode was done by comparison of the mass spectra with spectra available in the data system libraries (NIST12, NIST27, NIST62, NIST147, and WILEY229). Shimadzu software GCMSsolution was used for chromatogram data acquisition, comparison of chromatograms, integration of peaks, and calculation of similarity indices on comparison of acquired mass spectra with those of the data system libraries.

The linear retention index (LRI) was calculated for each volatile compound detected in the samples and compared with published data.[15,16,17]

Principal Coordinates Analysis (PCOORDA). A qualitative table (presence/absence) of the identified volatile compounds produced by the studied fungi in pure and mixture cultures in both incubation periods was constructed. The Unweighted Pair-Group Method Using Arithmetic Averages (UPGMA) was applied to analyze the results. A similarity/dissimilarity matrix was obtained using Jaccard's similarity coefficient. A PCOORDA of the similarity matrix was computed, and the minimum spanning tree was calculated.

The system of programs NTSYS-pc[18] was used in all statistical treatments by multivariate analyses.

TCA Quantification. The validation for the releasable TCA quantification was done in two steps: qualification and quantification. The areas of the three TCA peaks (212, 210, and 195) and those of d_5-TCA (217, 215, and 199) were measured. The qualification of peaks for quantitative analysis is done by calculating the ratios between peak areas (A) and comparing with the expected values: A_{195}/A_{210} = 1.44, A_{195}/A_{212} = 1.51, and A_{210}/A_{212} = 1.06 for TCA; A_{215}/A_{217} = 1.0, A_{215}/A_{199} = 0.70, and A_{210}/A_{212} = 1.06 for d_5-TCA. Peak areas obtained for m/z 215 of d_5-TCA (internal standard) were found to be suitable for quantitative analysis because no interferences were observed.

A linear regression treatment was applied to the calibration curve; the linear equation and the correlation coefficients were determined for the ratio of measured area of each TCA peak in relation to the peak of the internal standard (m/z 215). A Grubbs test was used to establish if there were deviant values in the analysis of samples (triplicates).

RESULTS AND DISCUSSION

Analysis of Volatile Compounds Released from Cork Samples Collected during the Manufacturing Process. The volatile compounds detected in nonboiled cork were completely

Table 1. Qualitative Analysis (Presence/Absence) of Volatile Compounds of Cork Samples Collected during the Manufacturing Process of Cork Disks Incubated at 27 °C during 3 Months

	retention index	cork before boiling	cork after boiling	cork slabs resting stage after boiling	cork after second boiling	cork disks
Alcohols						
dodecanol	1042.52	−	−	+	−	+
tridecanol	1647.98	−	−	+	−	+
6,11-dimethyl-2,6,10-dodecatrien-1-ol	2064.20	−	−	+	−	+
Esters						
trans-methyldihydrojasmonate (like[a])	1631.60	−	−	+	−	+
isopropyl myristate	2026.45	−	−	+	−	+
Alkanes						
6-methyloctadecane	1639.91	−	−	+	−	+
2-methylnonadecane (like[a])	1691.04	−	−	+	−	+
3-methylheptadecane (like[a])	1693.93	−	−	+	−	+
1,1-bis-hexadecane (like[a])	2004.97	−	−	+	−	+
eicosane	2009.99	−	−	+	−	+
nonadecane	2014.11	−	−	+	−	+
1-(ethenyloxy)octadecane	2022.05	−	−	+	−	+
squalene	2485.47	−	−	−	+	+
hentriacontane	2514.08	−	−	−	+	+
Aldehydes						
hexadecanal	1613.36	−	−	+	−	+
3,7-dimethyloctanal (like[a])	1681.34	−	−	+	+	+
Chlorated Alkanes						
1-chloro-octadecane (like[a])	1661.88	−	−	+	−	+
1-chloro-8-heptadecene	2064.20	−	−	+	−	+
Terpenoid						
camphor	1076.92	−	+	+	−	+
Aromatic Hydrocarbon						
naphthalene-1,2,4a,6,8a-hexahydro-4,7-dimethyl-1 (1-methylethyl) (like[a])	1059.65	+	−	−	−	−
Ketone						
3-butyl-4,5-hexadien-2-one (like[a])	1041.88	+	−	−	−	−

[a] A homology below 85% corresponds to an identification of the compounds to be probable ("like").

different from those detected in cork samples from other processing stages. As expected, in the stages before boiling and immediately after the first and second boilings, few volatile compounds were detected. Under the conditions used in this study, volatile compounds were mostly detected in the cork resting stage after boiling and in cork disks without any treatment (Table1). This can be explained by fungal development over the humid cork slabs occurring during the resting stage after the boiling step.

Usually, cork slabs are rested for 4 days inside the factory until they attain adequate humidity to be processed. During this period the slabs become completely covered by fungal mycelium from several species, mainly *Penicillium*, *Aspergillus*, *Chrysonilia*, and *Trichoderma*.[4,19] At this stage, these species are active and consequently are able to produce volatile compounds and other exo-metabolites, as a result of the biodegradation of the cork constituents. Interactions between the microbial populations existing in the cork slabs can condition the metabolic processes and consequently the formed products.[20] Most of the detected volatile compounds mentioned in Table 1 can result from the substrate fatty acid oxidation or by microbial degradation of aliphatic alcohols (e.g., dodecanol, tridecanol), aliphatic aldehydes (e.g., hexadecanal), aliphatic ketones (e.g., 3-butyl-4,5-hexadien-2-one), and alkanes (e.g., nonadecane, 6-methyloctadecane) as previously reported.[16] Cork contains in its constitution suberin, which is a complex polymer of long-chain fatty acids and phenolic residues.[21] The degradation by fungi of suberin can be suggested by the occurrence of some compounds such as tridecanol. The isopropyl myristate, hexadecanal, dimethyloctane, dodecanol, tridecanol, and 6,11-dimethyl-2,6,10-dodecatrien-1-ol could result from the degradation of the fatty acid chains composing either the wax-like fraction of the extractives or the suberin layer. Furthermore, the produced alkanes could originate from the degradation of hydrocarbons of the aliphatic chains from both extractives and suberin layers.

Table 2. Qualitative Analysis (Presence/Absence) of the Releasable Volatile Compounds Produced by Five Isolated Fungi and a Fungal Mixture Grown during 7 Days and 4 Weeks

		C. sitophila		*P. glabrum*		*P. brevicompactum*		*P. paneum*		*E. rubrum*		fungal mixture	
	retention index	7 days	4 weeks	7 days	4 weeks	7 days	4 weeks	7 days	4 weeks	7 days	4 weeks	7 days	4 weeks
Alcohols													
1-octen-3-ol	884.37	+	+	+	+	−	+	+	+	−	+	+	+
2-octen-1-ol	965.20	−	−	+	−	−	−	−	−	−	−	−	−
2-methylhexadecan-1-ol	1849.88	−	−	−	−	−	−	−	−	+	−	−	−
9-hexadecen-1-ol	2044.04	−	−	−	−	+	−	−	−	−	−	−	−
Esters													
ethyl *n*-caproate	1000.99	+	−	−	−	−	−	−	−	−	−	−	−
ethyl nonanoate	1249.74	−	−	+	−	−	−	−	−	−	−	+	−
ethyl decanoate	1297.66	−	−	+	−	−	−	−	−	+	−	+	−
ethyl undecanoate	1448.75	−	−	+	−	−	−	−	−	−	−	+	+
ethyl laurate	1497.00	−	−	+	−	+	−	−	−	+	−	+	−
nethyl dihydrojasmonate	1634.24	+	−	+	−	+	−	−	−	−	−	+	−
ethyl pentadecanoate	1698.68	−	−	+	−	−	−	+	−	−	−	+	−
ethyl palmitate	1899.14	−	−	+	+	+	+	+	−	+	+	+	+
ethyl stearate	2099.06	−	−	−	+	−	−	−	−	−	−	+	+
Sesquiterpene Compounds													
unknown compound (like[a])	1442.05	−	−	−	−	−	−	−	+	−	−	−	−
sesquiterpenoid compound (like[a])	1477.60	−	−	−	−	−	−	−	+	−	−	−	−
Ether													
1,3-dimethoxybenzene	1085.46	−	−	−	+	−	−	−	−	−	+	−	+
Hydrocarbon													
3-eicosene	1849.65	−	−	−	−	−	−	+	−	−	−	−	−

[a] A homology below 85% corresponds to an identification of the compounds to be probable ("like").

Saturated hydrocarbons were also detected, which is in accordance with previous findings.[16,22] However, in the present work the detected hydrocarbons have much longer aliphatic chains (≥C18) than those previously found (≥C8). A possible explanation for the observed deeper degradation of cork tissues can be the constitution of the microbial population, a longer incubation time, or the combination of both factors.

Both cork after the second boiling and cork disks contained other volatile compounds. The presence of two hydrocarbons (squalene and hentriacontane) should be pointed out. These compounds are found in a variety of plants, the last one being involved in stimulation of fungal spore germination.[23]

Although many volatile compounds were detected during the resting stage after the first boiling of cork slabs and in nontreated cork discs, they will not influence negatively the cork stoppers final quality since none of these volatile compounds are known to contribute for the so-called wine cork taint. Furthermore, some of them like fatty acids and terpenes are volatile components of wine.[24]

Even if these volatile compounds were detected in the untreated cork disks, some of their contents would be reduced or even disappear due to the final treatment of the cork disks consisting of washing and drying. Therefore, the final product quality will not be impaired.

Analysis of Releasable Volatile Compounds Produced by Some Fungal Species. Qualitative results are presented in Table 2 showing the presence/absence of releasable volatiles produced by five fungal species in pure cultures and a mixture containing all of them. The samples were taken 7 days and 4 weeks after inoculation.

The results presented in Table 2 were used to construct a similarity/dissimilarity matrix using the Jaccard coefficient (r = 0.868), and a cluster analysis was performed. A PCOORDA was computed and Figure 1 shows the samples projected on the space defined by the three first principal coordinates that explain ∼50% of the accumulated variance, providing a representation with the greatest variability of the obtained results. The minimum spanning tree has been superimposed on the projections to show where distortion is more evident.

Figure 1a shows that the group of samples incubated during 7 days is separated from the isolates incubated during 4 weeks along the second axis. This apparent separation can be explained by the differences of volatile profiles produced by the samples in each incubation period. Moreover, with regard to the samples incubated during 7 days, they show a diverse volatile production. The majority of the studied fungi produced 1-octen-3-ol (except *P. brevicompactum* and *E. rubrum*, which produced 9-hexadecen-1-ol and 2-methylhexadecan-1-ol, respectively), and diverse esters, mainly with short and medium chains (e.g., ethyl caproate and laurate) were also formed. Besides those compounds, *C. sitophila*, *P. brevicompactum*, and *P. glabrum* also produced methyl dihydrojasmonate. Moreover, *P. paneum* was the only isolate to produce 3-eicosene, which, to our knowledge, had not been reported to be a fungal metabolite. However, the culture medium is mainly constituted by cork, and it is known that the

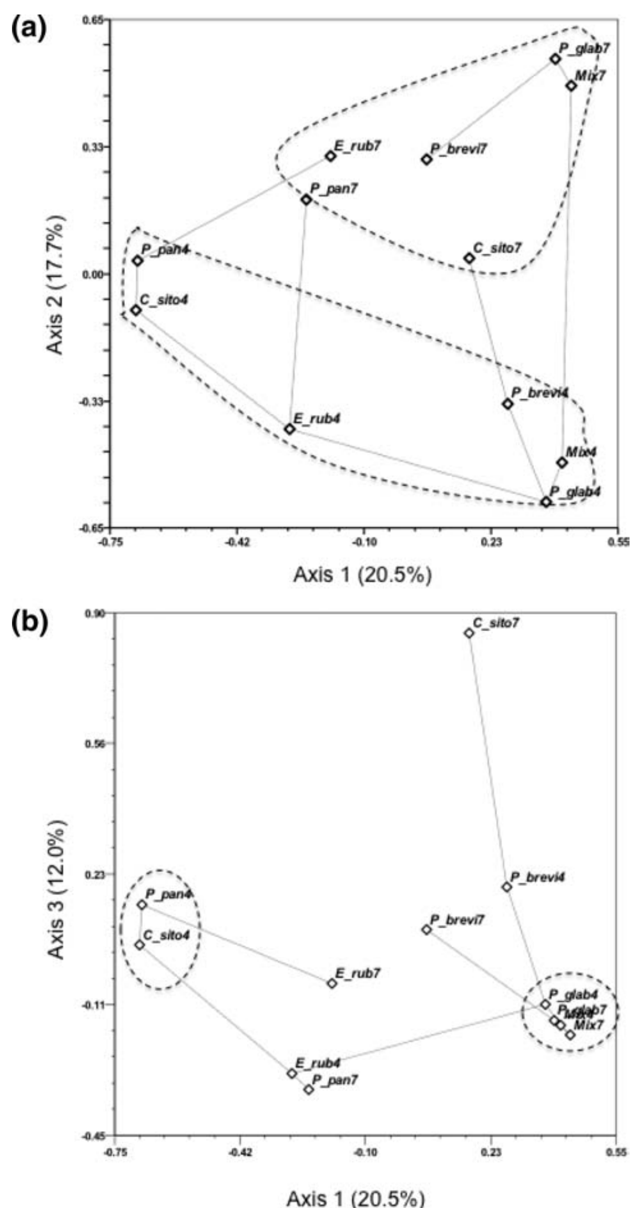

Figure 1. Plot of projections of 12 samples onto the principal coordinates axes: (a) plane of the first and second axes; (b) plane of the first and third axes. The minimum-length spanning tree is superimposed, and the variance (%) explained by the three first principal coordinates is displayed.

volatile compound production is highly influenced by the respective substrate composition and length of fungal growth.[25] These factors may reflect on the distribution of the samples along the third axis with *P. paneum* at the bottom and *C. sitophila* at the upper part of the graph (Figure 1b).

The samples incubated during 4 weeks show in general fewer volatile compounds. The five studied fungi consistently produced 1-octen-3-ol, in agreement with previous studies.[16,25] The esters formed by the fungal isolates were mostly ethyl palmitate (with exception of *C. sitophila* and *P. paneum*) and ethyl stearate (*P. glabrum* and fungal mixture), although the fungal mixture also produced ethyl undecanoate. Moreover, 1,3-dimethoxybenzene is a compound known to be an intermediate product of lignin degradation[22] and was produced by *E. rubrum*, *P. glabrum*, and the fungal mixture. The presence of this volatile compound suggests a more extensive degradation of cork by the fungal species incubated for 4 weeks.

These results may indicate that there is a greater similarity of volatile profiles for samples incubated during 4 weeks. This fact is shown by the distribution of the isolates along the third axis, where the isolates are located at the bottom and middle parts of the graph (Figure 1b). The fungal isolates *P. paneum* and *C. sitophila* seem to be exceptions, both producing the aliphatic alcohol 1-octen-3-ol, and *P. paneum* produced one unidentified sesquiterpene and one sesquiterpenoid-like compound. This fact is in accordance with earlier works that describe *P. paneum* as a terpenoid producer.[17] These fungal isolates are located at the left part of the graph.

The high similarity among the volatiles produced by *P. glabrum* and the mixed fungal cultures observed in both incubation periods (Figure 1) clearly suggests the greater contribution of that species to the overall volatile composition of fungal colonized cork. These observations support data collected over the years concerning the study of cork mycobiota[3] that the predominant fungal species during the cork stopper and disk manufacturing stages is *P. glabrum*.[19] Furthermore, *C. sitophila* samples produced one or two volatile compounds that will not contribute significantly to the overall volatile composition. It is known that *C. sitophila* mycelium completely covers the cork slabs immediately after the boiling stage,[3] and these results highlight again its innocuous presence on the cork substrate.[3,5,7]

Methyl dihydrojasmonate, a linoleic acid derived molecule, was the only compound detected in some chromatographic profiles obtained: both in the analysis of samples of cork with the natural microflora as in cork samples inoculated with previously isolated fungi. This volatile compound was described to be a signaling molecule, which mediated plant responses to environmental stress such as injury and insect or pathogen attack.[26] Its production by fungi has been reported earlier,[27] although its biological function in the fungal community is not clear yet. A rapid decline of this compound was observed in vivo, suggesting its fast metabolism.[28] Also in the present work, it was detected only in samples incubated for 7 days, being absent in the samples incubated during 4 weeks. However, this compound was also detected in samples of cork from two manufacturing stages incubated during 3 months.

Samples from some cork boiling waters were analyzed (Barreto et al., data not shown). In those samples many compounds usually described in the literature as plant-associated compounds were detected (e.g., sesquiterpenes, monoterpenes, and essential oil constituents). Some of the compounds detected in the boiling water were also found in the cork medium inoculated with some fungi: 1-octen-3-ol produced by all of the studied fungal species; 1,3-dimethoxybenzene produced by *P. glabrum*, *E. rubrum*, and the fungal mixture; and ethyl laurate produced by *P. glabrum*, *P. brevicompactum*, *E. rubrum*, and the fungal mixture. Moreover, some compounds detected in the cork samples incubated during 3 months were present in the boiling water: 1-tridecanol and eicosane produced during the cork resting stage after boiling and by cork disks.

This can be considered additional evidence that the fungal community is installed inside the cork structure[29] and is able to produce some volatile compounds, using cork constituents as substrates, which can be released into the water during the cork slab boiling process.

Quantification of Releasable TCA Produced by Fungal Isolates and a Fungal Mixture. The content of releasable TCA from samples inoculated with some fungal isolates in pure and mixed cultures was determined. The analysis conditions were very similar to those used in the analysis of cork stoppers in industrial quality control. The estimated detection limit (LD) and quantification limit (LQ) were, respectively, 1.6 and 5.4 ng L^{-1}.

The noninoculated cork media contained 1.90 and 1.78 ng L^{-1} of extractable TCA after 7 days and 4 weeks of incubation, respectively. TCA is formed by the *O*-methylation of the corresponding chlorophenol precursor.[6] Previous studies showed that at least some of the fungal species isolated from the cork possess the *S*-adenosyl-L-methionine (SAM)-dependent chlorophenol-*O*-methyltransferase (CPOMT) enzyme, which in the presence of TCP can metabolize TCA.[30] To understand the origin of theTCA values detected in our blank samples, the chlorophenol contents of the cork were determined. The analysis showed that cork contained an average of 2.7−3.3 ng g^{-1} of TCA, 5.2−6.7 ng g^{-1} of 2,4,6-trichlorophenol (TCP), 0.6−1.3 ng g^{-1} of 2,3,4,6-tetrachlorophenol and 0.7−1.1 ng g^{-1} of 2,4,6-tribromophenol. These results show that TCA was detected in noninoculated cork media and should have originated either from the corresponding trichlorophenol or from other chlorophenols also present in the cork.

Chlorophenols are common pollutants present in the environment due to earlier environmental contamination, and their presence was previously detected in cork.[31]

Under the conditions of our study, the TCA content determined in the cork samples inoculated with the fungal isolates was similar to that of the noninoculated samples. Moreover, the extracts of cultures, both of 7 days and 4 weeks incubation time, showed similar values of releasable TCA. Applying the variance analysis with 5% significance level (Supporting Information), in any case the TCA values obtained from the analysis of the inoculated samples could not be differentiated from those obtained from the noninoculated samples. It appears that under the conditions of analysis, the releasable TCA on cork cannot be attributed to any of the assayed fungal isolates.

To evaluate if cork dipped in the extracting medium could retain part of the TCA eventually produced by fungi, a hydroalcoholic solution (12% v/v) containing 800 ng L^{-1} of TCA and d_5-TCA was placed in contact with cork granules at ca. 22 °C. The relative concentrations of TCA present in the solution after different agitation times (5, 10, and 94 min) were evaluated by measurements of peak areas of TCA and d_5-TCA in total ion chromatograms (TIC) obtained by GC-MS analysis. The results showed that after 5 min of contact only ∼20% of the TCA content remained in the hydroalcoholic solution. After 94 min, only ∼11% of the initial TCA remained in the hydroalcoholic solution. No exchange of TCA between the extracting solution and the TCA originally present in cork was detected because no significant variation was observed in the ratio TCA/d_5-TCA. This experiment confirmed that cork had fixed most of the added TCA, which is in agreement with other studies that have shown that only ca. 3−5% of TCA contained in cork stoppers was released to the wine.[31,32] The amount of TCA adsorbed onto the cork granules depends on the cork surface, temperature, and time of exposure. In this study cork granules were used, which corresponds to a higher contact area between solution and cork than when entire cork stoppers are used in similar assays.

Chlorophenol precursors present in the cork tree can be converted into chloroanisoles by the existent colonizing fungal species in a chemical reaction catalyzed by the SAM-dependent *O*-methyltransferase. However, the levels of chlorophenols in the cork forests are not very high, as seen by the cork analyses done in our study. Under these studied conditions it is improbable that quantities of TCA produced by the fungal species present in cork can be released into the wine to produce a significant contamination.

To conclude, the levels of chlorophenols usually existing in cork slabs in an industrial environment are not high enough to induce biosynthesis of TCA by the existing fungi, even when grown in more favorable conditions provided by laboratory tests.

■ ASSOCIATED CONTENT

S Supporting Information. Tables of data for the quantification of releasable TCA. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +351 214469555. Fax: +351 214 421 161. E-mail: basilio@itqb.unl.pt.

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