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Destruction of Chloroanisoles by Using a Hydrogen Peroxide Activated Method and Its Application To Remove Chloroanisoles from Cork Stoppers

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ABSTRACT: A chemical method for the efficient destruction of 2,4,6-trichloroanisole (TCA) and pentachloroanisole (PCA) in aqueous solutions by using hydrogen peroxide as an oxidant catalyzed by molybdate ions in alkaline conditions was developed. Under optimal conditions, more than 80.0% TCA and 75.8% PCA were degraded within the first 60 min of reaction. Chloroanisoles destruction was followed by a concomitant release of up to 2.9 chloride ions per TCA molecule and 4.6 chloride ions per PCA molecule, indicating an almost complete dehalogenation of chloroanisoles. This method was modified to be adapted to chloroanisoles removal from the surface of cork materials including natural cork stoppers (86.0% decrease in releasable TCA content), agglomerated corks (78.2%), and granulated cork (51.3%). This method has proved to be efficient and inexpensive with practical application in the cork industry to lower TCA levels in cork materials.

KEYWORDS: Cork stopper, 2,4,6-trichloroanisole, pentachloroanisole, wine, moldy-musty taint, hydrogen peroxide

INTRODUCTION

Contamination with fungal (musty or moldy) odors and flavors is one of the most unpleasant organoleptic defects in wines, and it adversely affects consumer acceptability and represents a significant source of economic losses to the wine industry in the whole world,[1,2] which have been estimated in the order of U.S. $10 billion in annual loss.[3]

Chloroanisoles (CAs), especially 2,4,6-trichloroanisole (TCA), and to a lesser extent, pentachloroanisole (PCA), and 2,3,4,6-tetrachloroanisole (TeCA) have been identified as the major compounds responsible for the musty taint of wines. TCA is regarded as the greatest contributory compound to this defect due to its particularly low sensory threshold in the ng/L range,[4] being responsible for about 80% of the taint cases detected.[4−6]

Although the origin of the TCA tainting of wine can be diverse,[1,2] cork stoppers have been frequently blamed as the most likely origin of the pollution. Accordingly, this phenomenon is known as cork taint. In fact, as stated by Duncan et al.,[7] cork taint affects between 0.1 and 10% of the European bottled wine. More recently, Soleas et al.[8] reported a 6.1% incidence of cork taint in a screening of 2400 tested wines. Once TCA is located on cork closures, it can migrate through the wine.[9−12] A high variation has been found in the amount of TCA that a cork stopper can yield to wine. Thus, Hervé et al.[13] described that as much as 50% of the TCA present in a cork could migrate into wine. However, other studies reported migration rates of less than 4% of the total TCA located in a tainted cork,[9,14] or in the range 0.7−2.7%,[8] after a 12 month bottling period.

TCA is formed in cork by the O-methylation of the pesticide 2,4,6-trichlorophenol (TCP). This reaction is mainly carried out by many filamentous fungi species[15−17] that are usually found in cork samples. The O-methylation reaction is catalyzed by an inducible chlorophenol-*O*-methyl transferase enzyme (CPOMT),[18] which can catalyze the O-methylation of a wide spectrum of chlorophenols (CPs) to produce the corresponding CAs.

Surprisingly, in spite of the great significance of TCA for both the cork industry and the wineries, very few studies can be found in the literature regarding the degradation or destruction of CAs, although the biodegradation of TCA by bacterial soil isolates[19] or white-rot fungi[20] has been recently reported by our group.

On the contrary, because of their great significance as environmental pollutants (in fact, five CPs are listed as priority pollutants by the U.S. Environmental Protection Agency),[21] many different studies regarding the biodegradation of the chlorophenolic precursors of CAs have been conducted.[22,23] In a similar way, several chemical degradation methods for the destruction of TCP and pentachlorophenol (PCP) have been extensively studied, like sonolysis,[24] photodegradation,[25] or the use of activated hydrogen peroxide,[26,27] among several others. Hydrogen peroxide can be activated by using many different catalysts, including Fe^{2+} in the Fenton reaction,[28,29] or other ions like MoO_4^{2-},[27] Mn^{2+}, Cu^{2+}, or Co^{2+}.[29] The activation of hydrogen peroxide generates high amounts of oxidant radicals, like hydroxyl groups and/or singlet oxygen,[27] which can react and destroy the phenolic compounds.

Bearing in mind that the chemical structure of CAs and CPs is very similar, we decided to check whether several of the chemical

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methods used to destruct CPs could also be effective in the destruction of CAs. We mainly focused our attention on the methods based on the use of activated hydrogen peroxide, first because hydrogen peroxide is an environmentally acceptable oxidant for the treatment of many substances[27] and second because hydrogen peroxide is routinely employed by many cork companies to clean or bleach the closures as a washing step during the manufacture of cork stoppers.[1] The major goal of this study was to examine the effectiveness of a method based on peroxide hydrogen activation by molybdate or other metallic ions under basic conditions to destroy CAs in both aqueous solution and cork materials (natural cork stoppers, agglomerated corks, and granulated cork). The developed method is a tool that could be easily applied during cork stopper manufacture to produce effective diminishing of the TCA content of corks and, accordingly, to lower the TCA contamination rate of wine.

MATERIALS AND METHODS

Reagents. TCA was obtained from Aldrich (Steinheim, Germany). PCA (1825-21-4) was purchased from Supelco (Bellefonte, PA). TCA-d_5 (catalog no. D-6084) and 2,4,6-tribromoanisole-d_5 (TBA-d_5) (catalog no. D-5678) were purchased from CDN Isotopes Inc. (Cluzeau Info Lab, Sainte Foy La Grande, France). Sodium molybdate dihydrate (10102-40-6), potassium chromate (7789-00-6), potassium dichromate (7778-50-9), potassium permanganate (7722-64-7), Fe^{2+} sulfate heptahydrate (7782-63-0), Cu^{2+} sulfate pentahydrate (7758-99-8), and Co^{2+} chloride hexahydrate (7791-13-1) were purchased from Panreac (Barcelona, Spain). Hydrogen peroxide (7722-84-1) was obtained from Merck KGaA (Darmstadt, Germany). Glucaric acid (potassium salt) (576-42-1), ascorbic acid (50-81-7), sodium bicarbonate (144-55-8), and formic acid were supplied by Sigma (St. Louis, MO). All of the solvents used (*n*-hexane, ethyl acetate, ethanol, methanol, acetonitrile, and dichloromethane) and also formic acid (64-18-6) were high-performance liquid chromatography (HPLC) grade, and they were obtained from Scharlab (Barcelona, Spain).

Cork Materials. Natural cork stoppers (49 mm × 24 mm), agglomerated corks (24 mm × 45 mm), and granulated cork (2−4 mm in diameter) were provided by Trefinos S.L. (Palafrugell, Spain).

Reactions of Hydrogen Peroxide Activation Catalyzed by Metallic Cofactors. Fenton reactions were basically performed as described by Chu and Law,[29] with some minor modifications, in a volume of 5 mL in Milli-Q water. The typical reaction mixture contained in a 5 mL volume: TCA (1 mM), Fe^{2+} (4 mM), and H_2O_2 (10 mM). The pH was adjusted to 2.0 to prevent the formation of a precipitate of $Fe(OH)_2$. Reactions were conducted in the dark until a maximum time of 3 h at different temperatures (25, 30, and 37 °C). TCA destruction mediated by hydrogen peroxide activated by Co^{2+} and Cu^{2+} was checked as indicated by Gabriel et al.[30] with minor modifications. The reactions in a final volume of 5 mL in Milli-Q water contained TCA (1 mM), $CoCl_2$ (2 mM), ascorbic acid (60 mM), and H_2O_2 (100 mM) or TCA (1 mM), $CuSO_4$ (5 mM), glucaric acid (18.75 mM), and H_2O_2 (200 mM). The reaction was carried out in the dark at pH 2.0 from 30 to 180 min and at 25, 30, or 37 °C. In all cases, the reactions were stopped by freezing at −20 °C. Next, PCA (50 μg/mL) was added as an internal standard. The CAs were extracted twice from 0.5 mL samples by using the same volume of ethyl acetate. The organic phases were combined and evaporated under reduced pressure and finally analyzed by HPLC.

Conditions for the in Vitro Assay by Using the Oxidant Molybdate. A typical reaction mixture contained in a final volume of 5 mL in Milli-Q water 1 mM TCA or PCA and 2 mM sodium molybdate. The assay mixture was adjusted to pH 10.0 by using NaOH, and the reaction was initiated by addition of 30 mM (final concentration) H_2O_2. Negative controls were run in parallel by omitting sodium molybdate to quantify the putative loss of TCA by evaporation. Reactions were incubated at 30 °C for up to 180 min, and then, they were stopped by acidification to pH 2.0 with 4 N HCl. Next, TCA (50 μg/mL in the PCA degradation reactions) and PCA (same concentration in the case of TCA degradation reactions) were added as internal standards. The CAs from the samples (0.5 mL) were extracted twice with the same volume of ethyl acetate. The organic phases were combined and evaporated under reduced pressure and finally analyzed by HPLC.

Conditions of the Assays with Cork Samples. Natural cork stoppers, agglomerated corks, and also granulated cork samples, naturally tainted by TCA, were used in the assays to test the efficiency of the degradation of 2,4,6-TCA on cork materials. Alternatively, cork stoppers were artificially tainted with TCA-d_5 as described next: Briefly, 24 cork stoppers were held vertically in a cylindrical glass jar (24 cm in diameter and 18 cm high) on a platform of wire mesh to avoid the direct contact of the stoppers with the floor of the jar. The corks were arranged so that a gap of approximately 15 mm separated each cork from those around it. In the center of the jar, a glass vial was deposited containing 1 mL of a TCA-d_5 solution (2 mg/mL) in methanol. The jar was hermetically closed and maintained at 25 °C for 2 months, until all of the TCA-d_5 solution had been completely evaporated.

The cork stoppers (naturally contaminated or artificially tainted) were individually introduced in a 100 mL Pyrex glass bottle, which was filled by adding 100 mL of mixture reaction. For the assays with granulated cork, a 3 g sample was introduced into the same type of recipient, and 80 mL of mixture reaction was added. Reactions were conducted at different temperatures for 60 min in a water bath with rotatory shaking (75 rpm) (JP Selecta, Barcelona, Spain). After that, the CAs in the cork samples (stopper or granulated cork) were extracted in 100 mL Pyrex bottles containing 50 mL of 40% (v/v) ethanol in water according to the methodology described by Soleas and colleagues.[8] Briefly, bottles were incubated for 20 h at 4 °C and 150 rpm in an orbital shaker (New Brunswick Scientific, Edison, NJ). The resulting hydroalcoholic solution was named cork extract. Every cork extract was spiked with 25 ng of TBA-d_5 as an internal standard. CPs and CAs from cork extracts or wines were extracted by solid phase extraction (SPE) by using 3 mL/500 mg C18 cartridges (Bakerbond Octadecyl Speedstick from J. T. Baker, Philipsburg, NJ). Cartridges were conditioned with 1 mL of ethyl acetate, 1 mL of 96% (v/v) ethanol, and 2 mL of 40% (v/v) ethanol in water. The 50 mL cork extracts were applied to the columns at a rate of 5 mL/min, after which cartridges were dried by gradual suction carried out on a Visiprep SPE Vacuum Manifold (Sigma-Aldrich). CPs and CAs were eluted by adding 2 mL of hexane. Finally, the samples were concentrated until a final volume of 50 μL remained (representing a 1000-fold concentration of the original sample) by using a 5301 concentrator (Eppendorf AG, Hamburg, Germany).

Determination of Physical Characteristics of Cork Stoppers. To check if the chemical treatment had some effect on the physical properties of the stoppers, several parameters were tested for a batch of 30 corks and compared with untreated stoppers. The parameters were checked according to ISO normative. The tested properties were dimensions (length and diameter; ISO 9727-1), density (by using the length and diameter data and estimating the weight in a precision balance), liquid seal capability (ISO 9727-6), dimensional recovery after compression (ISO 9727-4), and extraction force (ISO 9727-5).

Analytical Methods. The extent of TCA and PCA degradation at aqueous solution assays was determined by HPLC as previously described.[19,20] Briefly, chromatography was performed in a Waters 600 Unit coupled to a PDA 996 detector and an Autosampler System 717 equipped with a Reverse phase LiChrospher 100RP-C18 (250 mm × 4 mm) 5 μm pore column (Merck). PCA (16.5 min) and TCA (13.1 min) were eluted by using a mobile phase consisting of a linear gradient of acetonitrile

(solvent A) and formic acid (0.1%) in water (solvent B) developed from 30:70 (v/v) at time 0 to 1:99 (v/v) at 30 min. The area under each peak was referenced to calibration curves in the range 0−1.25 mM obtained with authentic products (TCA calibration curve: $y = 4.2927x + 21.3619$; $r = 0.996$; PCA calibration curve: $1.2312x + 7.1810$; $r = 0.989$). Products were identified according to their retention times and their absorption spectra at the maximum of absorption by comparison with those of standards. The quantification of chloride ion (Cl^-) release during TCA and PCA chemical degradation at in vitro assays was estimated by using a chloride ion electrode (Cole-Parmer, Vernon Hills, IL), as described previously,[19,20] being the electrode calibrated against NaCl solutions of known concentrations according to the manufacturer's instructions. Residual H_2O_2 levels in the finished reactions were measured by using a specific H_2O_2 electrochemical sensor (Russell Mainstream Supply Ltd., Ladybank, Fife, United Kingdom) following the manufacturer's instructions. Gas chromatography−mass spectrometry (GC-MS) was performed as previously described[14] at a Hewlett-Packard 5890 series II gas chromatograph (GC) equipped with a 5972A mass selective detector. Samples (5 μL) were injected onto a TC-Wax (60 m long × 0.25 mm i.d., 0,25 μm film thickness; GL Sciences, Saitama, Japan) column through a splitless injection port by means of a HP7637B autoinjector, the split flow being set to 30 mL/min. The injector and detector temperatures were set at 250 and 300 °C, respectively. The temperature program consisted of 40 °C for 2 min, heated to 150 at 4 °C/min, and maintained for 1 min; heated to 200 at 4 °C/min, maintained for 1 min, and raised to 220 at 15 °C/min and held for 5 min. The carrier gas was high-purity helium flowing through the column at 0.8 mL/min (head pressure, 175 kPa). Positive ion electron impact spectra at 70 eV were recorded, and the detection was in selective ion monitoring (SIM) mode, with the following characteristic ions (m/z): 197, 210, 212 (TCA); 278, 280 (PCA); and 351 (TBA-d_5). Quantification was achieved on the sum of m/z ions: 197 + 210 + 212 for TCA and 278 + 280 for PCA.

Identification of Final Degradation Products of TCA. The final products from TCA degradation were identified by using two different approaches. Initially, the reaction mixture (60 min) was evaporated under vacuum conditions. The residue was then dissolved in 2.5 M HCl (pH 2.0). The resulting solution was saturated with NaCl and extracted three times with diethyl ether. The combined ether extracts were dried under vacuum conditions after adding anhydrous sodium sulfate. The residue was dissolved in methanol, and degradation products were derivatized with trimethylsulfonium hydroxide and analyzed by GC-MS as described by Sorokin et al.[31]

Second, the identification of those products that could not be identified by GC-MS was performed by NMR as follows: A reaction mixture was frozen in liquid nitrogen and then lyophilized in a Telstar CRYODOS lyophilizer (Terrasa, Spain). Different fractions were obtained after successive extractions of the dried reaction mixture with dichloromethane, methanol, and water. The identification of putative degradation products in every fraction was carried out by H NMR, although only relevant signal products were detected in the dichloromethane fraction. NMR spectra were recorded in deuterated chloroform ($CDCl_3$) at room temperature using a Bruker WM 500 spectrometer [500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR)]. Chemical shifts are given on the δ-scale and were referenced to the solvent (δc = 77.0 ppm) and to tetramethylsilane (TMS) as an internal standard. The pulse programs of the following 2D experiments were taken from the Bruker software library, and the parameters were as follows: 500/125 MHz gradient-selected HMQC spectra;[32] relaxation delay D_1 = 1.5 s; 500/125 MHz gradient-selected HMBC spectra;[32] relaxation delay D_1 = 1.5 s; evolution delay D_2 = 3.33 ms; delay for evolution of long-range coupling D_6 = 60 ms. 500 MHz gradient-selected ^{1}H,^{1}H correlation spectroscopy (COSY) spectra:[33] relaxation delay D_1 = 1.5 s; 90° pulse for ^{1}H. 500 MHz NOESY spectra:[34,35] relaxation delay D_1 = 1.5 s, mixing time D_8 = 500 ms. The L-SIMS spectrum was recorded on a VG Autospectrum instrument using m-nitrobenzyl alcohol as the matrix and cesium as ion bombardment at 35 KV.

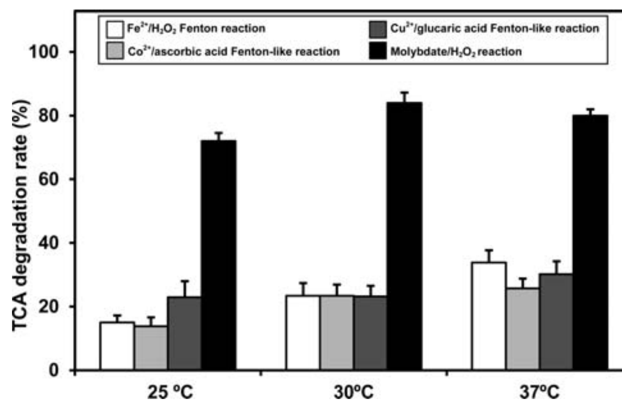


Figure 1. Comparison of the efficiency of different chemical reactions to achieve TCA destruction in aqueous solution mediated by different activated hydrogen peroxide methods like Fenton reaction, other Fenton-like reactions, and molybdate activation under alkaline conditions.

RESULTS AND DISCUSSION

TCA Degradation by Hydrogen Peroxide Activated by Several Cofactors. A comparative test was carried out to check the efficiency of different chemical reactions to achieve TCA destruction in aqueous solution mediated by activated hydrogen peroxide (Figure 1). The Fenton reaction (Fe^{2+}/H_2O_2) did not support good yields in TCA degradation. The best degradation rates were obtained by using 1 mM TCA, 4 mM Fe^{2+}, and 10 mM H_2O_2 concentrations in the assay reaction. Under these assay conditions, the higher TCA destruction percentages observed ranged between 15.0 (±2.2)% (reaction performed at 25 °C), 23.4 (±4.0)% (30 °C), and 33.8 (±3.9)% (when the assay was carried out at 37 °C) in 3 h reactions. We also tested the efficiency of TCA destruction by other Fenton-like reactions (Figure 1) when Fe^{2+} was substituted by a mix of Co^{2+}/ascorbic acid or Cu^{2+}/glucaric acid.[30] The efficiency of the Co^{2+}/ascorbic acid/H_2O_2 system in the TCA removal was poor, ranging between 13.8 (±2.8)% (3 h reactions conducted at 25 °C), 23.4 (±3.5)% (30 °C), and 25.7 (±3.1)% (37 °C), neither could significantly better results be obtained by using a Cu^{2+}/glucaric acid/H_2O_2 system. In this case, the yield of the reaction ranged from 22.9 (±5.1)% (25 °C) to 23.2 (±3.3)% (30 °C) and 30.2 (±4.0)% (37 °C). These results indicated that Fenton or Fenton-like reactions that had proven to be suitable for the degradation of CPs and others xenobiotics under acid pH in aqueous solution[28−30] are not effective to destroy TCA in similar conditions. On the contrary, high levels of TCA degradation [average of 86.6 (±6.3)%] were routinely obtained when this compound was oxidized by H_2O_2 in the presence of molybdate (MoO_4^{2-}) ion under alkaline conditions and to a lesser extent by using chromate [70.6 (±5.5)%], dichromate [72.3 (±8.5)%], or permanganate ions [66.3 (±9.3)%]. In this case, the effectiveness of the process was similar to that showed in the destruction of TCP and PCP.[27] In these reaction conditions, the addition of sodium azide to the reaction mix efficiently inhibited the reaction (data not shown). These data, as previously reported, suggested that the degradation of CAs mainly proceeded via the pathway

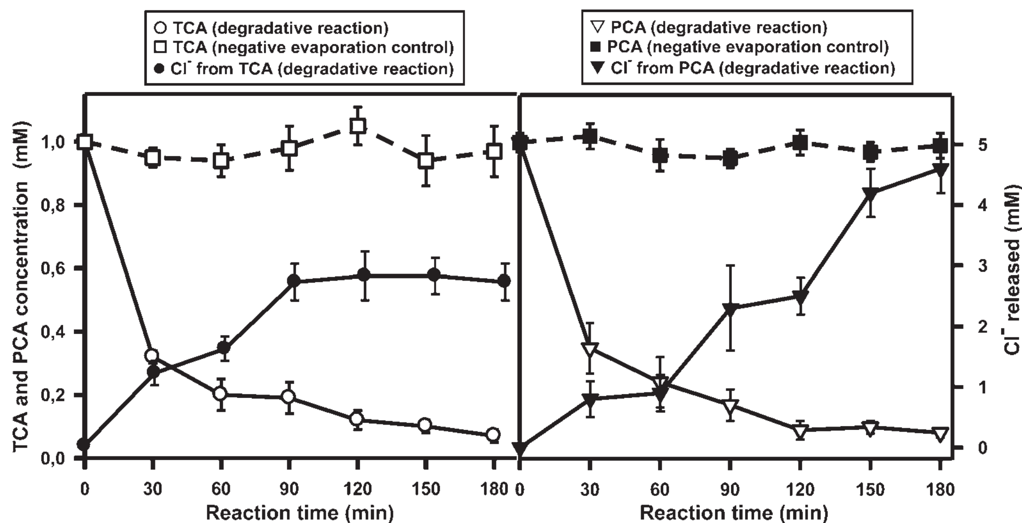


Figure 2. Time course of the degradation of TCA and PCA and concomitant chloride ion release from the degradation of TCA and PCA in a standard degradative reaction (reaction time, 60 min; temperature, 30 °C; pH 10.0; TCA, 1 mM; PCA, 1 mM; sodium molybdate, 2 mM; and H_2O_2, 30 mM) performed in an aqueous solution. The results shown correspond to the average of duplicate samples obtained in three independent experiments.

with singlet oxygen.[27] The effectiveness of this reaction for degrading CAs is very significant from an environmental point of view since CAs are detected as contaminants in any material or ecosystem tainted with CPs; therefore, this method might be used to effectively remove CAs contaminating aquatic environments.

TCA and PCA Time−Course Degradation. Taking into account that the higher TCA destruction were obtained by using molybdate, all subsequent assays were performed with this chemical. Accordingly, we decided to continue improving this method as a tool for obtaining high yields in the destruction of CAs in aqueous solution. Figure 2 shows the time course of the degradation for TCA and PCA in a standard in vitro assay (containing 2 mM molybdate and 30 mM H_2O_2 at pH 10.0 and 30 °C). The results obtained indicated that H_2O_2, when activated by MoO_4^{2-}, can be used to effectively destroy TCA and PCA in aqueous solution at room temperature (25 °C). Most of the TCA (80.0%) and PCA (75.8%) were degraded within the first 60 min of reaction, although higher degradation of 93.0 and 91.8% were, respectively, obtained when the incubation time was extended to 180 min. No significant loss of TCA and PCA by evaporation was detected by running in parallel evaporation controls consisting in reaction mixes lacking of molybdate. The extent of degradation of TCA and PCA was also quantified by measuring the release of chloride ions. Up to 2.9 chloride ions per molecule of 2,4,6-TCA (93.3% of the total chlorine) and 4.6 chloride ions per molecule of PCA (92.0%) were released at the end of the experiment. The amount of chloride released almost matched exactly the amount of CAs degraded, indicating an almost total dehalogenation of both compounds under the experimental conditions tested. This highly efficient dechlorination observed is very important, since chloroaromatic compounds are more readily biodegraded in the environment when they contain a lower content of chlorine atoms in their structure.[21,22]

Determination of Optimal H_2O_2 and MnO_4^{2-} Concentrations. To establish the best experimental condition for the in vitro assay, we tested the influence of molybdate and H_2O_2 concentrations on the time−course degradation of TCA and PCA. The effect of molybdate and H_2O_2 concentration was studied at a pH of 10.0 with a 1 mM concentration of CAs at 25 °C. Figure 3 shows the effect of testing different molybdate concentrations in the reaction mixture on TCA and PCA degradation and dechlorination. As can be seen in Figure 3, a 2 mM concentration of molybdate supported the highest degradation rates for both TCA (80.0%) and PCA (85.0%). Lowering the molybdate concentration to 1 and 1.5 mM did not significantly affect the degradation rate of PCA since degradation levels of 80.1 and 82.2% were obtained. In the case of TCA, the effect of molybdate concentration in the range from 1 to 2 mM was more evident: 67.0% of TCA was destroyed by using a 1 mM concentration, whereas the degradation rate increased up to 78.1 and 80.0% by using a 1.5 and 2 mM concentration, respectively. These results lead us to conclude that an optimal 2 mM concentration of molybdate supported the maximum TCA degradation rate. However, from an industrial (economical) point of view, a 1.5 mM concentration could be effectively used, given the

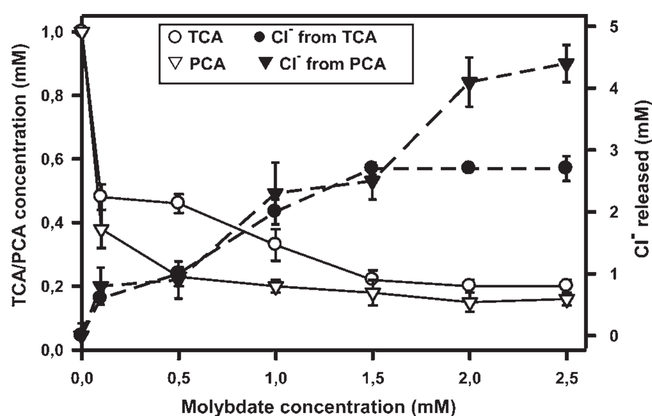


Figure 3. Influence of molybdate on TCA and PCA degradation and chloride ion release from the degradation of TCA and PCA. Data were obtained from duplicate samples corresponding to three different experiments. The reactions conditions were as follows: reaction time, 60 min; temperature, 30 °C; pH 10.0; TCA, 1 mM; PCA, 1 mM; and H_2O_2, 30 mM.

similarity of the degradation levels obtained. In the case of PCA, a 1−2 mM molybdate concentration could be used without a significant loss of effectiveness in the process.

The effect of peroxide hydrogen on TCA and PCA degradation is shown in Figure 4. An optimal concentration of 25 mM H_2O_2 was established for TCA degradation since 81.3% of this compound was destroyed. The increase of H_2O_2 concentration up to 50 mM did not support an improvement in the degradation level. Analytical data showed that most of the H_2O_2 was consumed in the reaction since the determination of residual H_2O_2 levels, by using an electrochemical specific sensor, showed that an average of 0.45 (±0.15) mM H_2O_2 remained after the completion of the reaction when the initial H_2O_2 concentration used was 25 mM. The residual H_2O_2 levels increased on average up to 4.15 (±0.53) mM when the initial H_2O_2 concentration was 50 mM. In the case of PCA, the optimal H_2O_2 concentration ranged between 30 and 50 mM: 87.2 and 88.0% of PCA was degraded, respectively, at those concentrations. Again, the data for chloride release indicated an almost total dehalogenation of CAs at the optimal molybdate and H_2O_2 concentrations determined.

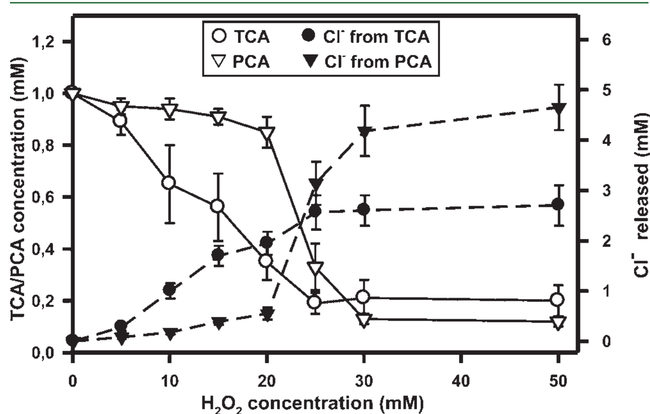


Figure 4. Influence of peroxide hydrogen concentration on TCA and PCA degradation and chloride ion release from the degradation of TCA and PCA. The results shown correspond to the average of duplicate samples obtained in three independent experiments performed by using the next reaction conditions: reaction time, 60 min; temperature, 30 °C; pH 10.0; TCA, 1 mM; PCA, 1 mM; and sodium molybdate, 2 mM.

Effect of pH and Temperature on TCA and PCA Degradation. The pH and temperature dependence on the degradation of TCA and PCA were checked (Figure 5A,B, respectively). The highest destruction rates for both TCA (82.1%) and PCA (78.9%) were obtained by adjusting the initial pH of the reaction mix to 10.0. The efficiency of the reaction continued to be high in the pH range from 9 to 11, with only a slight reduction at pH values 9 and 11. On the contrary, the efficiency of the reaction dramatically dropped at pH values lower than 9 (in fact, at pH 8.0, the destruction rate was reduced to 41.0% for TCA and 58.8% for PCA) and at pH values higher than 11. In this last case, the poor destruction rates observed were probably due to the H_2O_2 dissociation that occurs in very alkaline aqueous solutions.[27]

The reaction showed a clear dependence on temperature (Figure 5B). The best degradation rates were obtained when the reaction was performed at 30−40 °C, with a slight decrease in the reaction efficiency at lower temperatures (20 and 25 °C). In fact, at 40 °C, about 80.5 and 78.9% of TCA and PCA were degraded. Surprisingly, the efficiency of the reaction dramatically dropped at 50 and 60 °C. This fact is intriguing since the destruction of CPs in similar conditions showed a clear correlation between CPs degradation and temperature in the 15−75 °C range.[27] These analyses let us establish the next optimal conditions for the destruction of CAs (TCA and PCA) in aqueous solutions: 120 min of reaction performed at pH 10 and 30−40 °C by using 2 mM molybdate and 30 mM H_2O_2.

Identification of Degradation Products from TCA. The data for chloride release from TCA during the reaction indicated that an almost complete dehalogenation of TCA was happening. Accordingly, the analysis by GC-MS led to identify oxalic acid and fumaric acid as the main degradation products (identified according their next characteristic *m/z* ions: 29, 31, 45, 59, and 118 for oxalic acid and 53, 59, 85, 113, and 114 for fumaric acid). These products had also been previously detected from the degradation of TCP.[26,27] The structure of another abundant compound was elucidated by NMR. In this case, the typical methoxy group of TCA was not detected in the NMR (^1H and ^{13}C) spectra, suggesting that complete TCA demethylation had taken place during TCA degradation. The ^1H NMR spectrum of

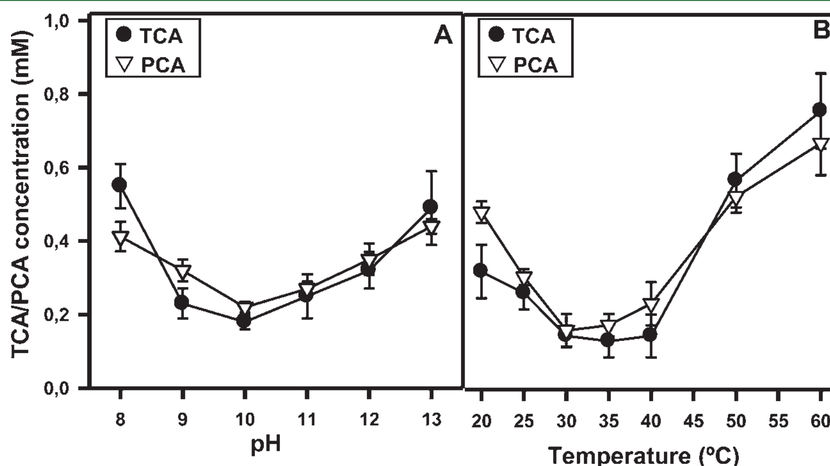
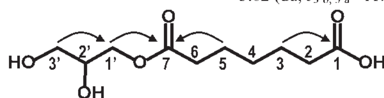


Figure 5. Influence of pH (at 30 °C) (A) and temperature (at pH 10.0) (B) on the degradation of TCA and PCA. Data corresponding to three independent experiments made by duplicate. Reactions were performed by using standard conditions: reaction time, 60 min; TCA, 1 mM; PCA, 1 mM; sodium molybdate, 2 mM; and H_2O_2, 30 mM.

Table 1. ^{1}H and ^{13}C NMR Chemical Shifts Assignments for the Majority Compound Identified as Product from the TCA Degradation (Compound 1; Its Structure Can Be Seen at the Lower Part of the Table) in $CDCl_3$

Position number	DEPT	$\delta^{13}C^{a}$ (ppm)	$\delta^{1}H^{b}$(ppm)
1	C	174.37	
2	CH_2	34.14	2.27 (t, $J_{2,3}$ = 7.0 Hz)
3	CH_2	24.92	1.57 (m)
4	CH_2	29.00	1.21 (m)
5	CH_2	24.95	1.55 (m)
6	CH_2	34.18	2.25 (t, $J_{6,5}$ =7.0 Hz)
7	C	173.95	
1′a	CH_2	63.37	4.08 (dd, $J_{1'a,1'b}$ =11.7; $J_{1'a,2'}$ =6.5 Hz)
1′b			4.12 (dd, $J_{1'b,1'a}$ =11.7; $J_{1'b,2'}$ =3.9 Hz)
2′	CH	70.31	3.86 (m)
3′a	CH_2	65.21	3.52 (dd, $J_{3'a,3'b}$ =11.7; $J_{3'a,2'}$ =6.5 Hz)
3′b			3.62 (dd, $J_{3'b,3'a}$ =11.7; $J_{3'b,2'}$ =3.9 Hz)



[a] Recorded at 125 MHz. [b] Recorded at 500 MHz.

the compounds mixture extracted with dichlorometane showed groups of lines in the three dense regions (0.6−1.8, 2.0−4.5, and 6.5−7.5 ppm). The signals of the region (δ 0.6−1.8 ppm) were not possible to resolve the individual lines even with a 500 MHz spectrometer. The signals of this aliphatic area suggested that the aromatic ring had been totally destroyed in the TCA degradation reaction.

However, the region (δ 2.0−4.5 ppm) that showed a major compound **1** (see Table 1 for the structure) derived from the opening aromatic ring was detected, and its structure was established by NMR analysis: (i) the H COSY spectrum, which is used for determining proton connectivity on the basis of geminal and vicinal couplings, and (ii) a TOCSY experiment, which can provide evidence for two-, three-, four-, and even five-bond H−H correlations. The determination of the chemicals shifts of the protons and protonated carbons was made on the basis of the study of the HSQC−HMQC spectrum. The complete structure was established by the HMBC spectrum, which shows correlations between protons and carbons for two- and three-bond. In this way, the H COSY spectrum showed connectivities between the two protons H-2 at δ 2.27 (t, J = 7.0 Hz) and the two protons H-3 at δ 1.57 (m). The TOCSY experiment was used to establish the signal corresponding to the protons H-4 at δ 1.21 (m). In addition, the protons H-4 showed connectivities with the protons H-5 at δ 1.55 (m) and the protons H-6 at δ 2.25 (t, J = 7.0 Hz), with H COSY and TOCSY spectra. The assignments of the carbon signals were made from the HSQC−HMQC spectrum, δ (ppm) 34.14 (C-2), 24.92 (C-3), 29.00 (C-4), 24.95 (C-5), and 34.18 (C-6). The HMBC spectrum showed linkages between the protons H-3 and the carbon atom C-1 (δ 174.37) and also between the protons H-5 and the carbon atom C-7 (δ 173.95). The signals corresponding to the ester group (CH_2-1′, CH-2′, and CH_2-3′) were assigned by using the HMBC spectrum. In this spectrum, the carbon atom C-7 showed correlation with the protons H-1′at δ 4.08 (dd, J = 11.7 and 6.5 Hz) and at δ 4.12 (dd, J = 11.7 and 3.9 Hz). In addition, the protons H-1′ showed connectivities with C-3′. The assignment of the remaining signals were carried out with the H COSY, TOCSY, and HSQC−HMQC spectra. In this way, signals were assigned corresponding to C-1′ (δ_C 63.37), C-2′ (δ_C 70.31), and CH_2-3′ (δ_H 3.52 and 3.62, δ_C 65.21). The results obtained are summarized in Table 1. During chemical TCP and PCP degradation by using activated hydrogen peroxide methods, several final nonhalogenated degradation products like oxalic acid, fumaric acid (also produced from TCA degradations as indicated above), malonic acid, and formic acid[26,27] were detected. Compound **1** might be formed as a result of intermolecular reactions among some of these compounds or among any other similar nondetected compounds produced during TCA degradation.

Finally, the region (δ 6.5−7.5) of the H NMR spectrum showed a very minor compound obtained from the partial dechlorination of the aromatic ring (2,4-dichlorophenol). In this spectrum, the doublets at δ 7.20 ($J_{6,5}$ = 8.0 Hz) and 7.28 ($J_{3,5}$ = 2.7 Hz) could be attributed to H-6 and H-3, respectively, and the signal at δ 7.01 (dd, $J_{5,6}$ = 8.0 and $J_{5,3}$ = 2.7 Hz) is assigned to H-5. The assignment of the protonated carbon signals was accomplished by using HSQC−HMQC spectrum δ (ppm): 118.9 (C-6), 123.5 (C-5), and 124.4 (C-3). On the other hand, the HMBC spectrum showed linkages between the protons H-3, H-5, and H-6 with C-1 (δ 149.20). The chemical shift of C-1 suggested the presence of an oxygenated group at C-1. All of these data were compatible with the structure of 2,4-dichlorophenol, which obviously could be easily generated from TCA by dechlorination of the 6-position of the aromatic ring. Otherwise, the levels of this compound in the reaction mix were very low, which obviously matched the results previously shown, indicating that during the reaction CAs undergo an almost complete dehalogenation. All of these data confirmed that by using the described method, TCA is almost completely degraded to produce oxalic acid, fumaric acid, compound **1**, and to a much lower extent 2,4-DCP. This later chlorinated compound had not been previously detected in TCP and PCP degradation reactions by activated hydrogen peroxide,[26,27] although some other chlorinated compounds like chloromaleic, dichloromaleic acid, and chloromalonic acid had been described.

Optimization of Conditions for TCA Removal from an Artificially Tainted Batch of Natural Cork Stoppers. Once the optimal conditions to destroy TCA in in vitro aqueous reactions were identified, we checked whether the methodology described could be applicable to remove TCA from cork materials. A typical characteristic of batches of cork stoppers is the great variability that they exhibit in their TCA content.[2,13,14] In fact, in cork stopper lots identified as problematic, it is very usual to find corks with very different levels of TCA and even a high proportion of noncontaminated stoppers. This is the reason why the TCA content analysis of corks frequently yields very high standard deviation values. To avoid this problem and to establish the optimal conditions for cleaning cork stoppers by using the hydrogen peroxide-activated method developed, we artificially spiked a batch of natural cork stoppers by exposure to an atmosphere containing TCA-d_5 as indicated in the Materials and Methods. This methodology allowed us to obtain a batch of natural cork stoppers uniformly tainted, whose releasable TCA content was estimated to be 12.8 (±2.3) ng/L.

The treatment of these artificially tainted natural corks with a reaction mixture by using the conditions previously optimized for TCA destruction in aqueous solution yielded an average low reduction of 25 (±4.3)% in the values of releasable TCA levels. Several attempts were then made to optimize the conditions for TCA destruction including changes in the molybdate and H_2O_2

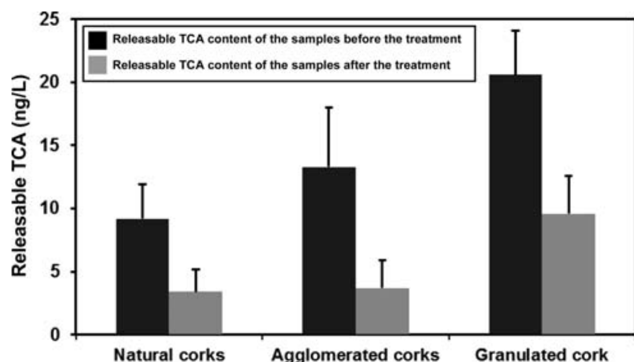


Figure 6. Average values in releasable TCA content of batches of natural cork stoppers, agglomerated corks, and granulated cork before (black bars) and after the treatment (gray bars). The data shown were obtained from duplicate samples corresponding to three independent experiments.

concentrations, pH, and reaction temperature (data not shown). In this way, the optimal conditions for TCA destruction in cork materials were determined to be 60 min of reaction at pH 11.0 by using 2 mM molybdate and 30 mM H_2O_2 at 65 °C with agitation. By using these conditions, the releasable TCA content of the batch was lowered by average 81.2 (±9.3)%. Two factors had a higher influence on TCA destruction on cork: pH and temperature. When using cork materials, an adjustment of the initial pH of the reaction mixture should be done to pH 11.0−11.5 to avoid (due to the acid character of cork) that its pH dropped below 9.0, thus compromising the efficiency of the reaction. Also, the temperature of the reaction should be increased to 65 °C. This is probably due to the fact that in cork TCA is trapped inside the cork cells, and perhaps by increasing the temperature, we can achieve a greater accessibility of the reaction mixture to the TCA trapped inside the cells. The performed treatment did not significantly affect the physical properties of the stoppers like dimensions, density, liquid seal capability, dimensional recovery after compression, and extraction force. In fact, a washing step with H_2O_2 is very often used by many manufacturers to bleach corks without the mechanical properties of the stoppers being affected. Only a slight darkening of the corks could be noticed after the treatment, which in no way would affect its putative commercialization.

Finally, we applied these optimized conditions to the destruction of TCA in batches of naturally tainted natural and agglomerated cork stoppers and also in granulated cork. As can be seen in Figure 6, a significant reduction in the releasable TCA content of cork was observed in all of the cork materials treated showing an average reduction of 64.3% for natural corks stoppers, 71.8% for agglomerated corks, and 53.6% for granulated cork. The lower effectiveness of the treatment for granulated cork might be due to the larger area that a determined amount of granulated cork has as compared to the surface of a cork stopper of the same weight. This could imply that no sufficient levels of reactive forms of oxygen are produced during the reaction to cover such a wide surface. The data obtained by treatment of cork materials confirm that their washing with aqueous solution containing peroxide hydrogen in the presence of molybdate ions efficiently removes TCA from corks, resulting in a significant reduction of releasable TCA content of corks. Summarizing, we can conclude that the developed oxidative chemical treatment is able to efficiently destroy TCA present in both aqueous solutions and cork materials. Although other methods have been recently developed to remove TCA from drinking and contaminated waters, mainly based on ozonation mediated by different catalysts,[36−38] to our knowledge, this is the first report of a system developed to remove CAs from aqueous solution that can be applied for cleaning cork stoppers.

Comparison with Other Methods To Remove TCA from Cork Stoppers. Several different methods have been developed in recent years to diminish the TCA content on cork stoppers. Some of them are based on the extraction of TCA from cork granules by using water steam in an autoclave, like the ROSA technology developed by the Amorim Group (the largest world corks producer), which is able to remove 75−80% of releasable TCA.[2,39] In a similar way, Alvaro Coelho Irmaos (another major Portuguese cork producer) have developed the NBS System, which consists of a totally automated process with a continuous extraction of volatiles, by using a boiler with a controlled opening. Unfortunately, these methods are not fully suitable to be applied on natural corks and natural cork disks since they slightly modify the cork cells, disturbing the physical properties of the cork, mainly affecting the final dimensions of the stoppers, requiring costly and time-consuming rectification. Other companies (like the Portuguese producer Cork Supply) have developed the technology INNOCORK.[40] It basically consists of steam distillation with ethyl alcohol: The vapor mixture contacts the cork stopper efficiently extracting TCA (more than 50% reduction of contaminant levels). According to the authors, this is the first developed technology on the extracting TCA strategy that could be applied to natural wine cork stoppers without affecting the cork structure. The main drawback of this technology is precisely the use of an organic solvent, such as ethanol, that could produce problems in the handling of industrial waste and environmental pollution. An alternative strategy is the supercritical carbon dioxide extraction. This technology is almost completely efficient to remove TCA from microgranulated cork[4] but requires high cost investments, and many consumers and wine cellars do not like the visual aspect of the treated corks. Other technologies are based on the use of radiations. In this way, the DELFIN (Direct Environmental Load Focused Inactivation) System employs microwave technology:[41] Electromagnetic waves penetrate to the center of the stopper producing the heating and evaporation of the water and also many volatiles, including TCA. Alternatively, the application of γ-radiation to partially or totally destroy the TCA, depending on the dose used, has been proposed.[42] The main disadvantage of both methods is the high price, increasing excessively the final price of the stoppers. Finally, the Symbios technology (developed by the Cork Technology Centre, CTCOR, Santa Ma. de Lamas, Portugal) is a preventive biological process that tackles the root causes of the problem during the boiling of cork planks. It is based on the use of an additive for the water, which is fixed to the walls of cork cells and to the back of the cork planks, inhibiting both the microbial development of microorganisms at the postboiling stabilization and the TCA formation. CTCOR reports that by using this technology, a significant decrease from 1.2 to 0.08% of tainted corks can be achieved. However, to our knowledge, this system is not currently being industrially applied.

The main advantage of the described method in this work is that it is a low cost, simple, and easy technology to perform, and it could be applied in two different steps of the productive process. First, it could be used to treat cork planks when they are introduced into the hot water bath. This treatment could destroy any

CP or CA present in the aqueous solution and the surface of the cork planks, obviously reducing the levels of CAs on the cork planks, as well as avoiding the formation of TCA in subsequent steps of the production process due to the additional reduction of the levels of chlorophenolic precursors. Second, the treatment could also be applied to treat the final product (natural or agglomerated corks) to diminish its releasable TCA content, without affecting the physical properties of the corks. Finally, we would like to emphasize that the low cost, simplicity, and the medium-high effectiveness of this method could represent a good alternative, once its application is optimized on an industrial scale, and in combination with other methods, to reduce the TCA level of cork materials to values below levels considered as thresholds or dangerous, to avoid wine taint by TCA originally located on cork stoppers.

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