

Electrochemical insights on the hydrophobicity of cellulose substrates imparted by enzymatically-oxidized gallates with increasing alkyl chain length

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ABSTRACT

In this work, we studied the influence of the alkyl chain length in enzymatically-oxidized gallates on the development of hydrophobicity on paper-based materials, and further correlated the obtained effect to the redox mechanism of the enzymatic treatment. Laccase (Lac) enzyme was used to oxidize various members of the gallate homologous series in the presence or not of lignosulfonates (SL) to produce several functionalization solutions (FS), which were subsequently applied to cellulosic substrates. The hydrophobicity of the substrates was then assessed by means of water drop test (WDT) and contact angle (WCA) measurements. Hydrophobicity peaked reaching WDT and WCA values around 5000 seconds and 130° respectively, and then

decreased with increasing length of the hydrocarbon chain of gallate. Cyclic voltammetry (CV) was used to study the effect of SL on the redox reactions of several gallates. The intensity of the anodic peak in their voltammograms decreased increasing the chain length of the gallate. The electrochemical behavior of lauryl gallate (LG) differed from that of other gallates. The fact that the voltammetric curves for SL and LG intersected at a potential of 478 mV indicates an enhancing effect of SL on LG oxidation at high potentials (above 478 mV).

KEYWORDS: Laccase, alkyl gallates, cellulose, hydrophobicity, cyclic voltammetry

1. INTRODUCTION

In a previous study we demonstrated the efficiency of a functionalization solution (FS), obtained upon laccase-oxidized lauryl gallate (LG) and lignosulfonate (SL), to impart hydrophobicity onto cellulosic materials by the application of this FS on the paper surface ¹. The favorable effect of a heat treatment on the hydrophobicity of paper sheets previously subjected to the FS treatment was also discussed.

Lauryl gallate (LG) is the n-docecyl ester of gallic acid, which belongs to the gallates homologous series including a number of similar compounds of variable chain length that may be useful for enzymatic surface modification of cellulose materials. The hydrophobic nature of LG is related to its alkyl chain. Several studies have examined the influence of alkyl chains of variable length on the development of surface hydrophobicity on various materials ²⁻⁵. Studies, however, which focus on the effect of a long gallate alkyl chain on the development of hydrophobicity on cellulose sheets are lacking.

Alkyl gallates (viz. 3,4,5-trihydroxybenzoic acid alkyl esters) are widely used as food antioxidants⁶ though they are not natural compounds. Also, some alkyl gallates have proved useful as multifunctional food and pharmaceutical additives by virtue of their anti-browning⁷, antifungal⁸, and antibacterial effects^{9,10}. In addition, they have been shown to possess amphiphilic and anticancer properties¹¹⁻¹³. Several studies have employed gallates of variable alkyl chain length to modify the properties of materials such as wool¹⁴, cellulose¹⁵, chitosan¹⁶, titanium dioxide¹⁷, and ceramics¹⁸, conferring improved thermal and thermo-oxidative stability (polymers, foams) or dispersibility. However, little is known about the ability of gallates to modify the hydrophobicity of paper-based materials, and even less about the effect of the gallate chain length on their enzymatic oxidation reactions. Given the multiple inherent properties of alkyl gallates, one can hypothesize that, if they can be embedded onto the surface of various materials, they may be useful to confer them advanced properties. One of the most innovative aspects of the present work is that all the studied gallates can modify (or increase) the hydrophobicity of paper materials by using a biotechnical tool, e.g. laccase enzyme.

Biotechnical methods have the potential to provide substantial improvements in traditional manufacturing processes owing to their specificity and potential environmental advantages. Several studies have shown that cellulose fibers can be functionalized using enzymes. Surface modification of cellulose fibers to obtain hydrophobic paper sheets¹⁵, improve mechanical resistance¹⁹, or obtain antimicrobial properties²⁰, are among the many successful applications of enzyme-catalyzed cellulose functionalization. In contrast to what has been done so far, our recent studies were not aimed at directly modifying the surface of the fibers, but at functionalization of the

surface of paper sheets by a method in which the presence of the cellulosic substrate was not necessary during the enzymatic reaction ¹.

In the first part of the present work, we examined the effect of eight enzymatically-modified alkyl gallates on the hydrophobicity of cellulose materials. The study was conducted in view of elucidating the role of the hydrophobic moiety (alkyl chain) and the gallic acid moiety on this property. In the second part we investigated the electrochemical transformation of alkyl gallates, SL, and the combinations thereof upon laccase oxidation using cyclic voltammetry (CV). The choice of CV was dictated by the fact that laccases are oxidative enzymes and their catalytic effect on substrates can be simulated by using a working electrode to record, in a controlled manner, changes in the applied potential.

2. MATERIALS & METHODS

2.1 Paper, enzyme and chemicals. Filter paper from FILTERLAB[®] (Sant Pere de Riudebitlles, Barcelona, Spain) was used for functionalization. Laccase from *Trametes villosa*, 588 U/mL, was supplied by Novozymes (Denmark). Eight compounds from the gallate homologous series (Figure 1) were subjected to laccase-assisted treatments, namely: gallic acid (G), methyl gallate (MG), ethyl gallate (EG), propyl gallate (PG), butyl gallate (BG), octyl gallate (OG), lauryl gallate (LG) and stearyl gallate (SG). All compounds were purchased from Sigma–Aldrich (Spain).

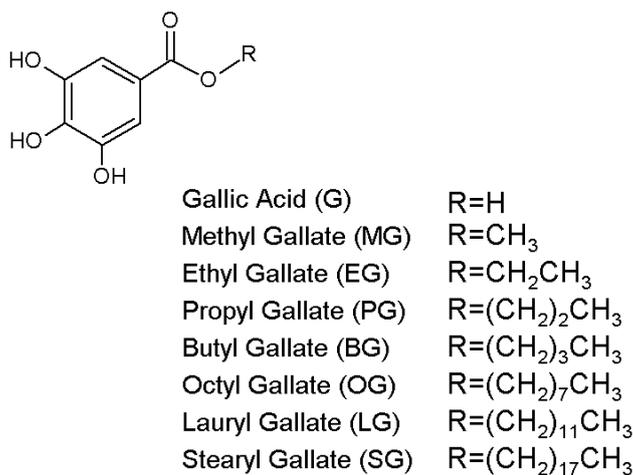


Figure 1. Chemical structure of gallic acid and its alkyl esters.

2.2 Preparation of the functionalization solutions (FS). The enzymatic oxidation of gallates were performed in an Ahiba Spectradye dyeing apparatus from Datacolor equipped with closed vessels of 250 mL with final concentrations equivalent to 50 mM sodium tartrate buffer (pH 4), gallate compound (1.2 g/L), and 1.2 U/mL laccase. If the gallate compound was hydrophobic and insoluble in water, then it was applied as a colloidal suspension achieving a reduction in the effective size ¹. In the case of using SL in the preparation of the FS it was introduced at 1.2 g/L. After adding the gallate compound, the beakers were maintained under agitation. Following, the enzyme was added and the beakers were kept stirred during 4 h reaction. The reaction was stopped by cooling the beakers.

2.3 Treatment of paper sheets with FS. The paper sheets were cut into circular pieces (4 cm in diameter) and soaked in the FS at room temperature. Thereafter the paper specimens were removed, spread onto blotting paper, and allowed to dry in a normalized atmosphere (23°C, 50% RH).

2.4 Assessment of paper hydrophobicity. Each gallate was oxidized by laccase under the reaction conditions described previously, and with no presence of SL during the reaction. The resulting liquid products were used to impregnate paper sheets that were then allowed to dry and assessed for hydrophobicity by water drop test (WDT) and contact angle (WCA) measurements. Additionally, the treated paper was subjected to a heat treatment in order to assess its effect on the development of hydrophobicity. WCA were measured with a Dataphysics[®] OCA15 contact angle goniometer, using a 4 μ L water drop in each measurement. WDT was applied on each treated paper specimen according to Tappi standard T835 om-08. Previously, the paper sheets were conditioned according to ISO 187.

2.5 Cyclic voltammetry. Voltammetric studies were performed using a μ Autolab Type III (EcoChemie, The Netherlands) potentiostat/galvanostat controlled by Autolab GPES software version 4.9. All experiments were carried out in a thermostatic, 20 mL, three-electrode configuration cell (Metrohm). The working electrode was a glassy carbon electrode with a surface diameter of 3 mm (Metrohm, The Netherlands). A platinum electrode and a silver–silver chloride (Ag/AgCl) electrode (Metrohm, The Netherlands) were used as counter and reference electrode, respectively. Voltammetric responses were recorded in 50 mL of 80/20 (v/v, %) aqueous-ethanol solution, containing 0.1 M tartrate buffer pH 4, and 0.5 mM of the compound to be studied (gallate compound) in the presence and absence of SL (0.17 mg/mL). Potential was scanned from 0 to 800 mV vs. Ag/AgCl at a scan rate of either 5 mV/s or 200 mV/s. In the cases where a specific compound was used as oxidation enhancer, the catalytic efficiency (CE) was determined. The

CE is the increase in anodic peak current of the compound acting as catalyst (or enhancer) in the presence of the compounds to be oxidized. The CE was calculated as $\Delta I/IE$, ΔI being the increase in anodic current at 700 mV of the enhancer-compound mixture, and IE the oxidation current of the enhancer alone at the same potential.

3. RESULTS AND DISCUSSION

3.1 Influence of the chain length of alkyl gallates on the hydrophobicity of FS-treated paper. Based on the curve for non heat-treated paper sheets, their WDT or WCA values showed increased hydrophobicity with increasing length of the gallate alkyl chain (Figure 2).

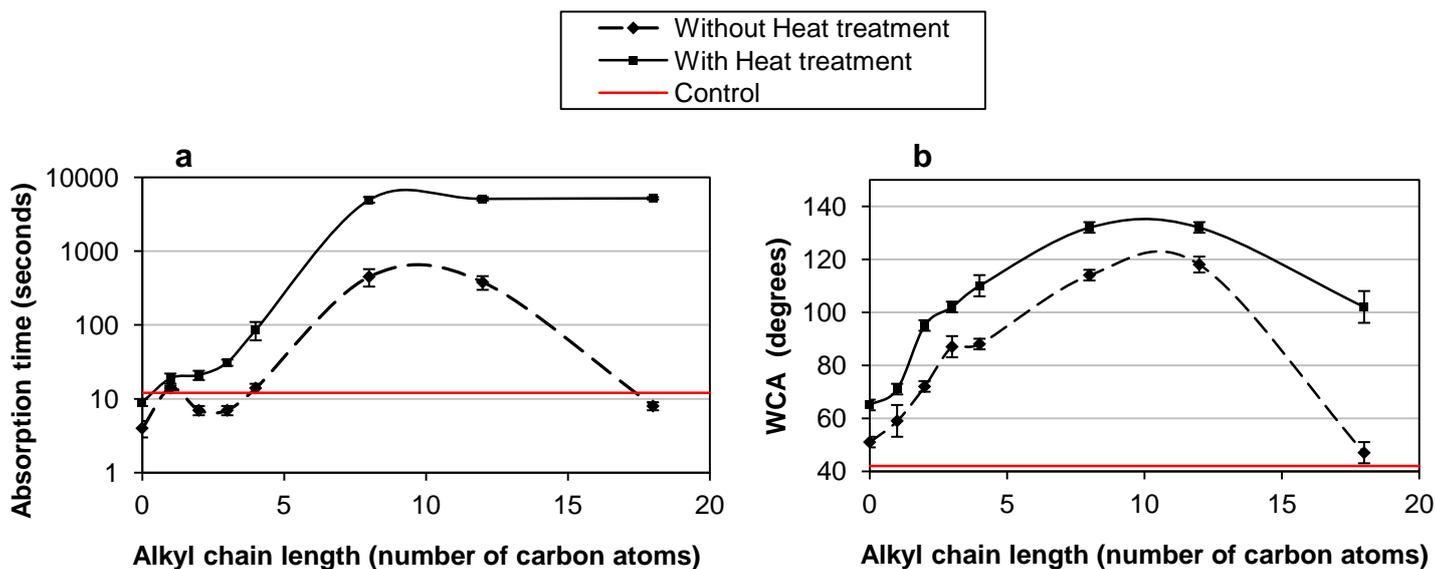


Figure 2. Hydrophobicity as determined from WDT (a) and contact angle measurements (b) of filter paper sheets treated with functionalization solutions (FS) containing gallates of variable alkyl chain length.

However, hydrophobicity peaked between OG and LG (i.e. a chain length of 8–12 carbon atoms) and then decreased markedly with the increasing further chain length. Therefore, an appropriate selection of alkyl chain lengths, based on past experience with LG compounds, allowed for optimal hydrophobicity on paper sheets. The hydrophobicity increased steadily from 0 to 5 carbon atoms, however, the effect was maximal for chains comprising 5–15 atoms.

We had previously found heat treatments to have a favorable effect on the development of hydrophobicity on paper sheets ²¹. Therefore, here, the paper samples treated with the FS of laccase-oxidized gallates of variable chain length, were subjected to a heat treatment in an oven at 150 °C for 30 min. As can be seen in Figure 2, all thermally-treated samples exhibited significantly increased hydrophobicity - in some cases by as much as one order of magnitude - irrespectively of the gallate alkyl chain. Unlike the non-heat-treated samples, which hydrophobicity considerably decreased when treated with long-chain gallates, the hydrophobicity of the heat-treated paper leveled off in terms of WDT (Figure 2a). Long-chain gallates also reduced WCA of the heat-treated sheets, albeit to a lesser extent than in the non-treated ones.

The FS obtained by reacting the enzyme with each gallate exhibited a marked color change from the initial product (before reaction) except for SG. The enzymatic reaction of SG yielded a FS with little signs of oxidation (viz. little color change). Also, SG did not form a homogeneous dispersion in the reaction medium, and showed an increased tendency to form large aggregates and precipitate. Most probably, the long alkyl chain of SG imposed considerable steric hindrance to the action of the enzyme by preventing the phenolic moiety from accessing its catalytic sites. In addition, the low solubility and dispersibility of the compound may have led to the formation of

aggregates further hindering the action of the enzyme. Since the enzymatic oxidation of the gallate is essential for effective hydrophobization effect on paper, partial or little oxidation of SG would be responsible for the low hydrophobicity of the samples treated with an SG-containing FS ¹.

3.2 Effect of heat treatments on LG arrangement. The increased, stable hydrophobicity obtained upon heat treatments can be ascribed to temperature-promoted chemical reactions between the enzymatically-modified gallates in FS and the cellulose surface, and also to the spatial distribution of the molecules over the substrate. Thus, functionalizing paper sheets at room temperature with FS involves physico-chemical adsorption of FS by positively-charged paper as reported previously ^{1,22,23}. This led to a cellulosic substrate with randomly-adsorbed FS molecules on the surface, which introduced substantial hydrophobicity (Figure 3a). However, the heat treatment may have triggered new coupling reactions only occurring at increased temperatures and causing the hydrophobic tail of the gallates to face outwards from the substrate, thereby further increasing its hydrophobicity (Figure 3b).

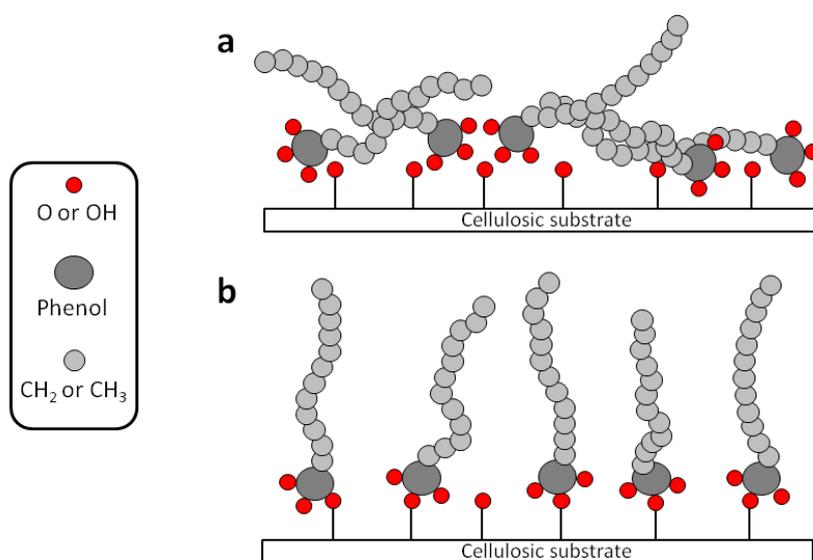


Figure 3. Schematic depiction of gallate molecules randomly adsorbed onto the cellulosic substrate upon treatment with FS at room temperature (a) and after heat treatment at 150°C for 30 min, which caused the alkyl chain to face outwards from the substrate (b).

In addition, small particles of enzymatically-modified gallate aggregates may have melted onto the surface by effect of the high temperatures, thereby increasing the nanoscale roughness of the cellulosic substrate and increasing its hydrophobicity as a result ²². Similarly to our results, the orientation of alkyl radicals upon grafting on different inorganic substrates has been reported by several authors ^{24,25}.

3.3 Cyclic voltammetry of gallates, SL, and their interactions. The chemistry of gallates and the effect of SL on the redox processes involved were studied by CV. In previous works we found that the presence of SL during enzymatic reactions boosted the oxidation of gallate compounds by the enzyme, and also affected the LG morphology ^{1,22}. For this reason, we introduce SL, in order to elucidate its effect on the gallate compounds electrochemically. Although the oxidation of alkyl gallates by laccase had previously been by CV ¹⁴, no specific information about the effect of SL on the oxidation of gallates of increasing chain length with this technique had been reported. In this section, various gallates of increasing chain length including gallic acid (G), ethyl gallate (EG), propyl gallate (PG), octyl gallate (OG) and lauryl gallate (LG) were combined with SL and their redox reactions studied by CV.

3.3.1 Electrochemical response of pure gallates. As can be seen from the cyclic voltammograms of gallates at a scan rate of 5 mV/s, all compounds exhibited only a

strong anodic peak, meaning that they underwent an irreversible oxidation in the potential range considered (Figure 4). The oxidized intermediates (phenoxy radicals) may have been rapidly removed by chemical reactions such as phenoxy radical coupling, formation of quinones or open-ring acid products, explaining the absence of a reduction cathodic signal. The intensity of the anodic peak, which was related to the current intensity supplied by the electron flow in the oxidation process, decreased steadily with the increasing chain length of the gallate. Such decrease of the current may have resulted from the high hydrophobicity and low solubility of long-chain gallates and their tendency to aggregate, hindering oxidation. Another reason might be the steric hindrance in the longer gallates (OG and LG) preventing them from approaching the surface of the electrode and increasing the resistivity of the medium.

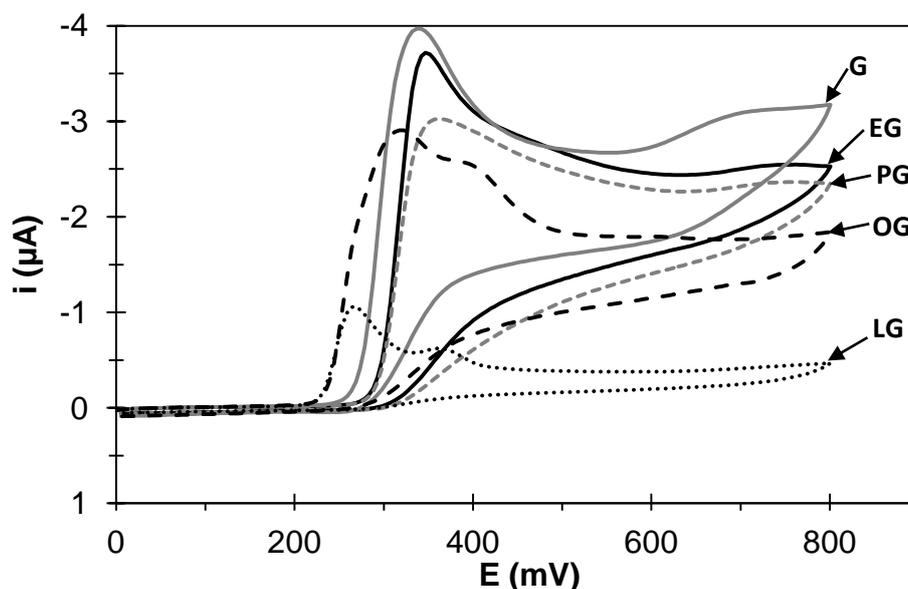


Figure 4. Cyclic voltammograms for 0.5 mM gallate solutions in a 80/20 (v/v) mixture of 0.1 M tartrate buffer and EtOH at pH 4 as obtained at a scan rate of 5 mV/s.

All studied gallates exhibited an anodic peak at 250-400 mV. However, after the decay of the initially oxidized species, all resulting compounds underwent further oxidation at potentials above 600 mV. G, EG and PG exhibited a sharp, well-defined anodic peak at 250-400 mV, while formation of a second oxidized species at higher potentials was only observed for OG and LG. With these two gallates, the initial species were oxidized at 268 and 313 mV, respectively, and these secondary species at 361 and 396 mV. As can also be seen from Figure 4, the first anodic peak was shifted to lower potentials as the length of the gallate chain was increased. This effect could be attributed to the fact that gallates have different pKa. The pKa value of gallates increase (positively) with the hydrocarbon chain length. Then, the acid-base equilibrium is shifted to less acidic, and this would affect the concentration of the active specie (phenolate). The concentration of the active specie was calculated, taking into account the pKa values, the pH of the buffered solution (pH=4), and the initial concentration of each gallate (0.5 mM). The results showed that as the hydrocarbon chain increased, the concentration of the active specie decreased. However, an important decrease on the concentration (by 4 orders of magnitude) was observed between GA and MG, while for the following gallates, the concentration was stabilized in the same range and under the same order of magnitude (the pKa values are also very similar). Therefore, the concentration of the active specie could not be the reason for the shift of the anodic peak potential to lower potentials. Also, if the shift would have been due to the pKa changes, the largest shift in Figure 4 should have been observed between GA and EG, and this is not the case. In Figure 4 the largest shift is observed between OG and LG. The results indicate that long-chain gallates are oxidized at lower potentials, but with less electron-flow as suggested by their lower peaks.

Additional voltammetric measurements of the studied gallates at higher scan rates (200 mV/s) were also made in order to ascertain whether some reversible processes could take place under such conditions and assess the stability of the resulting radicals. Based on the obtained results, all studied gallates underwent irreversible oxidation, even at increased scan rates, indicating poor stability of the oxidized species. OG and LG, however, which exhibited peaks for two different species at 5 mV/s, gave a single anodic peak (indicating formation of a single specie) at higher rates (results not shown).

3.3.2 Electrochemical interaction between SL and gallates. Figure 5 shows the cyclic voltammograms at slow scan rate (5 mV/s) for the target compounds in the absence and presence of SL. The oxidation current for SL alone was very weak; therefore, the SL was assumed to be electroinactive in the potential range of appearance of the anodic peaks of gallates. Therefore, the increased anodic peak current for PG, OG and LG observed in the presence of SL could be ascribed to regeneration of the gallate at the electrode surface, and oxidation of SL. As noted earlier, the phenoxy radicals produced by oxidation of the gallates exhibited very low stability. Nevertheless, the radicals were living long enough to oxidize SL. This effect of SL on the anodic current falls into the homogeneous redox catalysis class of electrochemical reactions²⁶ and was previously observed in studies on the effect of various types of lignin products in combination with laccase mediators/enhancers (Aracri et al., 2013; González-Arzola et al., 2009). The cyclic voltammogram for OG in the presence of SL exhibited a very slight increase in anodic current; by contrast, those for G and EG in the presence of SL exhibited no increase, but rather a decrease. A decreased anodic current of G in the

presence of a ligninolytic compound was previously observed by Díaz-González et al., (2011), and attributed to coupling reactions of the electrochemically generated phenoxy radicals in lignin structure.

The present voltammetric study was conducted with several representative gallate compounds with variably long alkyl chains in order to gain insight into the electrochemical behavior of these compounds in the presence of SL. The focus, however, was placed on LG and its interaction with SL because previous studies had indicated a favorable effect of SL on enzymatic treatments involving laccase and LG^{1,21}. In these studies, the product of the oxidation of LG by laccase was anchored on the surface of cellulosic materials, and the grafting reaction assumed to occur upon oxidation of LG. Thus, oxidation of the compound was essential in order to ensure grafting. Since introducing SL improved grafting, stability and particle size reduction²², and also helped preserve the enzyme activity, we expected that SL would act as an enhancer for the oxidation of LG. The electrochemical behavior of LG differed markedly from those of the other gallates, and gave lower currents due to its low solubility (Figures 4 and 5). As can be seen from Figure 5, the anodic currents for G, EG, PG and OG, and that for SL, differed in magnitude. Therefore, SL was assumed to be electroinactive towards the studied gallates. However, based on the curves for LG and SL in Figure 5, the anodic currents were of a similar magnitude.

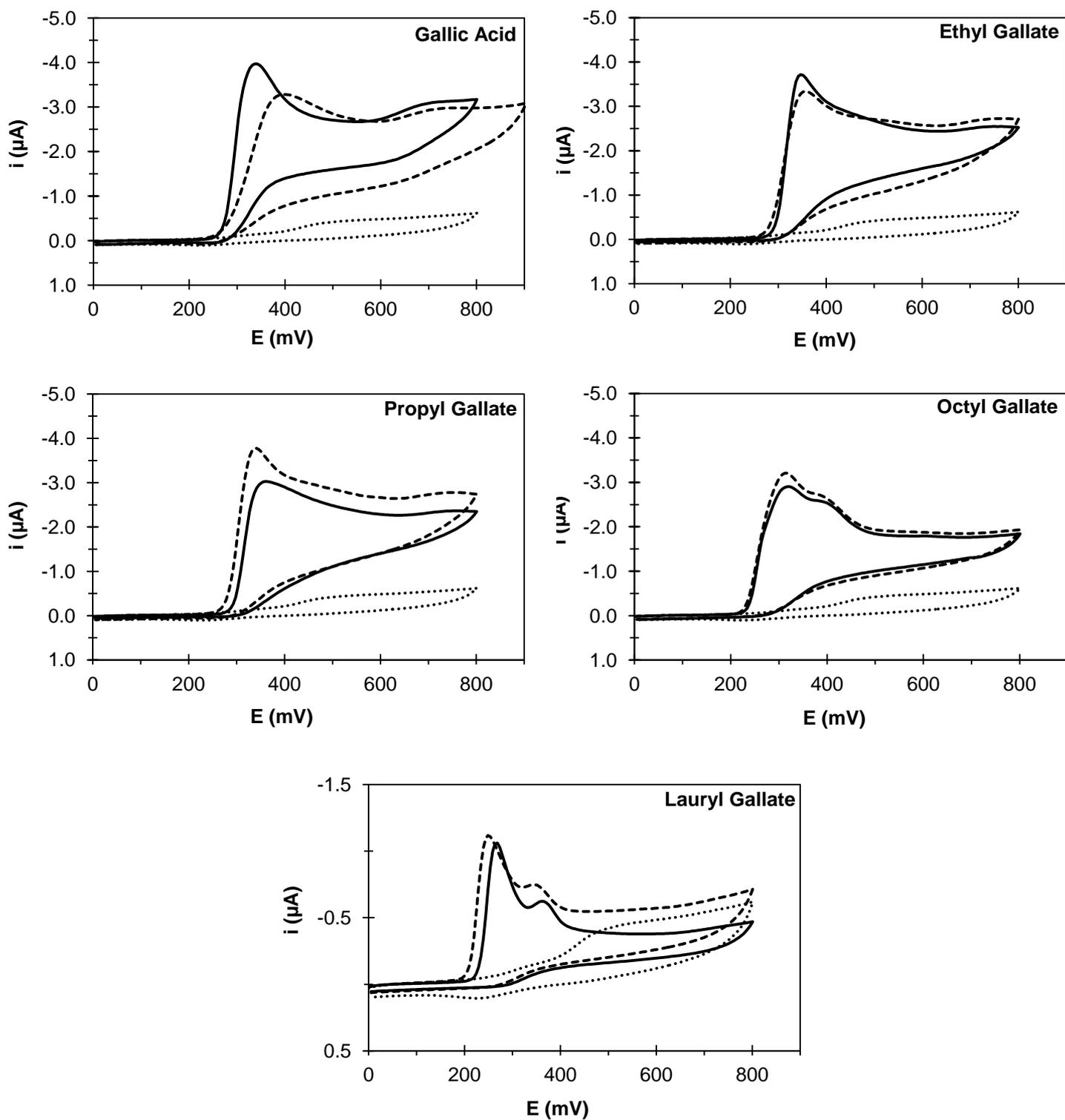


Figure 5. Electrode responses of 0.5 mM gallate solutions (solid lines —), a 0.17 mg/mL solution of SL (dotted lines •••), and gallates plus SL (dashed lines – –), all in tartrate buffer at pH 4 at room temperature, as obtained at a scan rate of 5 mV/s.

The key observation to understand the effect of SL on LG is the fact that the curves for the pure compounds alone (LG and SL) intersected at a potential of 478 mV. Below 478 mV, the anodic current of LG was above that of SL, while the opposite tendency was observed at higher potentials. This finding allows understanding the redox interactions between LG and SL (dashed curve). At potentials from 0 to 475 mV, SL is much less electroactive than LG and the main oxidation species at the electrode surface is provided by LG. The slightly increased anodic peak for the LG+SL system relative to LG alone was a result of the above-mentioned regeneration of LG –though to a small extent– at the electrode surface via oxidation of SL. Above 478 mV, the anodic current of SL surpassed that of LG, suggesting that in the couple (LG+SL), the increase in anodic current is due to the regeneration of SL and oxidation of LG. Increase in anodic charge at high potentials in the presence of lignin was previously observed by Aracri et al. (2013) and was assigned to the oxidation of non-phenolic lignin components. In the present experiments, the oxidation of lignin would have induced oxidation of LG at high redox potentials. The overall effect on LG in the LG+SL system was as follows: i) at potentials from 0 to 478 mV, LG was oxidized mainly by the electrode –with partial regeneration caused by lignin–, ii) at potentials above 478 mV the electrode was unable to oxidize the previously formed LG species, so the oxidant was SL. The overall effect was that SL enhanced the oxidation of LG by acting specifically in those potential ranges where the electrode was less active and could not carry on the oxidation of LG (Figure 6).

If the previous findings are extrapolated to laccase-LG interactions in the presence of SL, the role of the electrode in the voltammetric process is assumed by the laccase. Unlike the electrode, the redox potential of the enzyme did not initially increase

and then decreased steadily, but rather remained constant at a given potential. Some authors have reported redox potentials for laccases about 700 mV^{30,31}. Based on the voltammograms of LG in Figure 5, the anodic current of SL at the working potential for laccases exceeded that of LG, thus, the oxidation mechanism of LG by laccase in the presence of SL must be similar to that described above involving enzyme-oxidized LG, whose action is enhanced by the presence of SL.

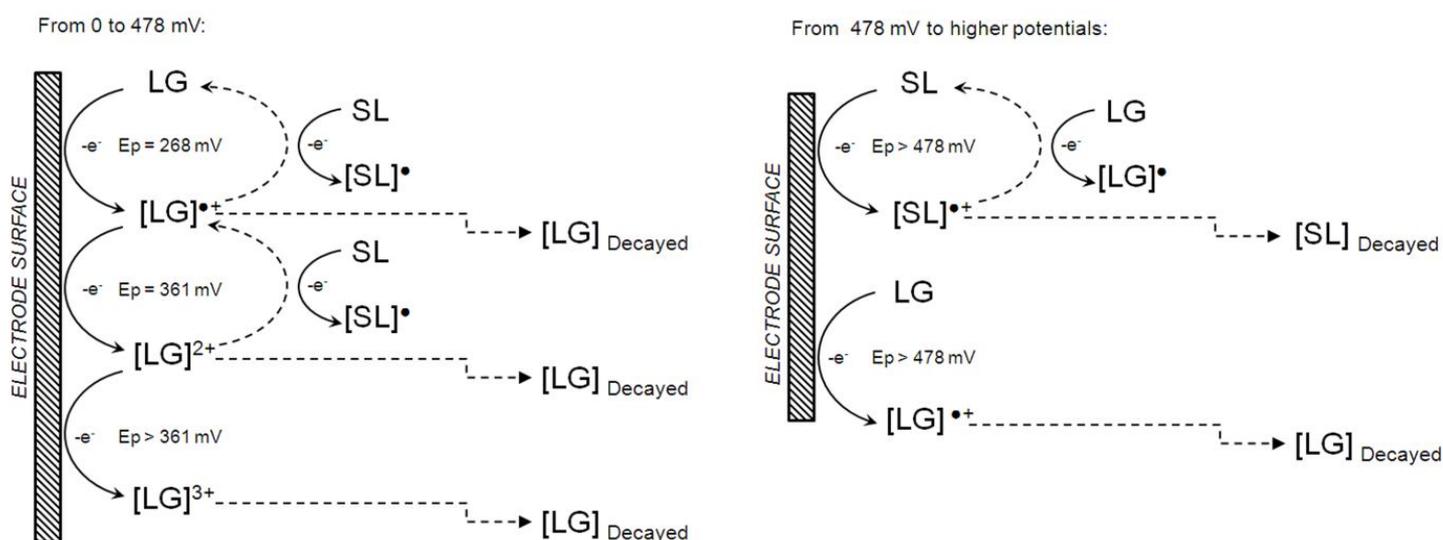
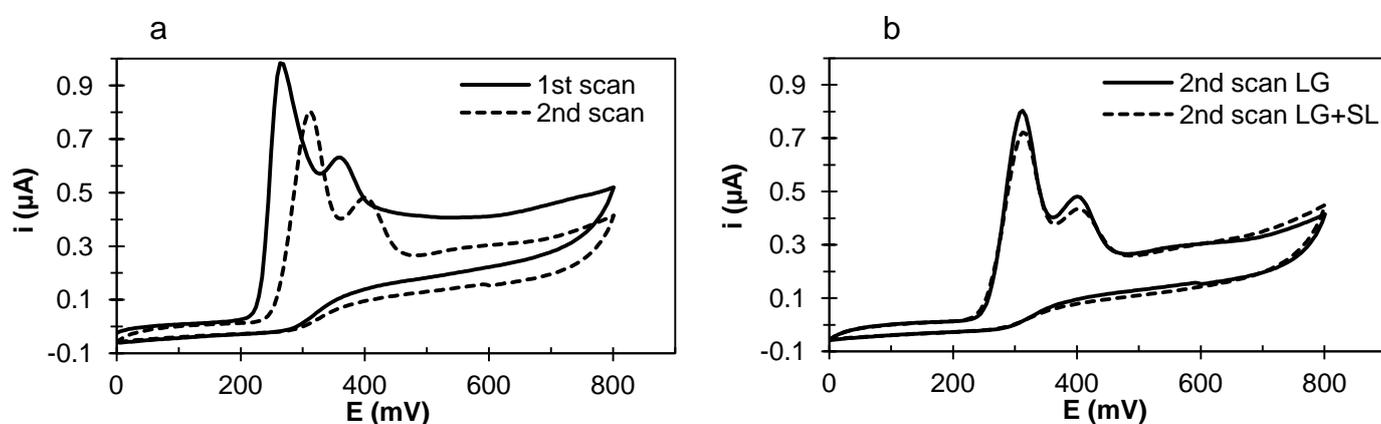


Figure 6. Schematic representation of redox catalysis for LG and SL.

3.3.3 Gallate polymerization and catalytic efficiency. The electrochemical behavior of the studied gallates, both alone and in combination with SL, was examined at a scan rate of 5 mV/s in two consecutive scans. All gallates and their combinations with SL exhibited an identical tendency in the first and second scans. Their behavior is summarized by the example in Figure 7a, which corresponds to LG+SL. The second scan revealed a marked decrease in oxidation current probably due to depletion of the analyte on the electrode surface, or polymerization of the species previously formed via

phenoxy radical coupling, as already observed by Hossain et al., 2010. Also, the anodic peaks in the second scan were shifted to the right, thus indicating that higher potentials were needed to oxidize LG owing to the presence of the previously-generated polymer layer on the electrode surface. Figure 7b compares the second scan curves for LG alone and in combination with the SL. As can be seen, the anodic peak for LG+SL was lower, which suggests that the presence of SL boosted the radical coupling of LG oxidized species and favored the formation of a thicker polymerized layer on the electrode



surface as a result.

Figure 7. Two-scan cyclic voltammogram for 0.5 mM LG+SL in 80/20 (V/V) 0.1 M tartrate buffer–EtOH mixture at pH 4 as obtained at a scan rate of 5mV/s (a); and comparison of the second scans for LG alone and LG+SL at the same scan rate (b).

The oxidation potentials for G, EG and PG increased slightly with increasing chain length (Table 1). By contrast, the one for OG exhibited a substantial decrease that was even more pronounced in LG - about one order of magnitude. Previous studies (Aracri et al., 2013; Bourbonnais et al., 1998; González-Arzola et al., 2009) examined the catalytic efficiency (CE) as the increase in anodic peak current of the compound

acting as catalyst in the presence of lignin or lignin model compounds. In such studies, lignin was assumed to be electroinactive and its oxidation to involve regeneration at the electrode surface of the catalyst. Based on the cyclic voltammograms for G, EG, PG and OG in the presence of SL (Figure 5), SL was less electroactive than the gallates. This suggests that the gallate was the specie acting as enhancer and SL the oxidized specie (CE at 700 mV, Table 1). By contrast, the main electroactive species in the LG+SL system at 700 mV was SL, which suggests that LG was oxidized through SL reduction at the electrode surface. The increase in anodic current upon reduction of the enhancer at the electrode surface was calculated as $\Delta I/IE$ (Table 1), ΔI being the increase in anodic current at 700 mV and IE the oxidation current of the enhancer at the same potential.

Table 1. Redox potential of the studied gallates and catalytic efficiency of G, EG, PG and OG against SL, and of SL against LG. (Substrate-to-enhancer weight ratio 1:1).

| Compound | $E_{p,a}$ (mV) | CE (at 700 mV) |
|----------|----------------|----------------|
| G | 337 | -0.05 |
| EG | 342 | 0.06 |
| PG | 361 | 0.18 |
| OG | 317 | 0.04 |
| LG | 259 | 0.15* |

* CE for LG was calculated by assuming SL to be the enhancer and LG the oxidized substrate.

As can be inferred from Table 1, G, EG and OG were ineffective, while PG exhibited a substantial positive effect on the oxidation of SL at 700 mV. On the other hand, the effect of SL as enhancer of the oxidation of LG was also significant (CE about 15%).

4. CONCLUSIONS

Based on the WDT and WCA results, the hydrophobicity of gallate-treated paper sheets depends on the length of the gallate alkyl chain. Thus, hydrophobicity increased with increasing chain length, peaked between octyl gallate (OG) and lauryl gallate (LG), and then decreased for longer alkyl chain gallates. A heat treatment substantially increased the hydrophobicity irrespectively of the gallate chain length. The oxidation of G, EG, PG, OG and LG by cyclic voltammetry demonstrated a well-defined relationship between the intensity of the anodic peak and the chain length of the gallate. The cyclic voltammograms for G, EG, PG and OG in the presence of SL revealed that SL is less electroactive than the gallates, and therefore, the gallates are the species acting as enhancers, while SL is the oxidized specie (CE at 700 mV). Nevertheless, the main electroactive specie in the LG+SL system at 700 mV was SL, which suggests that LG was oxidized through SL reduction at the electrode surface. The fact that the voltammetric curves for SL and LG intersected at 478 mV suggests that SL boosts the oxidation of LG at potentials above 478 mV.

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