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# Electrochemical Approach to Detection of Chlorophene in Water Catalyzed by a Laccase Modified Gold Electrode

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**Abstract:** Despite the increasing number of reports that relate antimicrobial chlorophene (CP) with health and environmental effects, few studies have addressed biosensing technologies to detect this threat. This work proposed an electrochemical approach for the detection of CP using laccase enzymes as an alternative recognition element immobilized onto thin-film gold electrodes. The electrochemical parameters of the detection method, under controlled conditions, resulted in a limit of detection (0.14 ± 0.06 mg $L^{-1}$) and quantification (0.48 ± 0.04 mg $L^{-1}$) that agreed with concentrations of CP that already had been measured in natural water samples. Nevertheless, during the analysis of natural river water samples, the provided method suffered a drawback due to matrix effects reflected in the obtained recovery percentage, the value of which was 62.0 ± 2.4% compared to the 101.3 ± 3.5% obtained by the HPLC reference method. These detrimental effects were mainly attributed to organic matter, SO4-2, and Cl- present in river samples.

**Keywords:** electrochemical biosensor; enzyme; laccase; chlorophene; emerging pollutant; water

## 1. Introduction

In recent years, the contamination of aquatic systems by emerging pollutants has become a significant problem, particularly in water supplies worldwide [1]. These pollutants, which include a wide range of recalcitrant compounds, their metabolites, and transformation products, enter the environment through anthropogenic pathways [2], primarily from personal-care products (e.g., disinfectants, cosmetics, perfumes) or industrial activities (e.g., pharmaceuticals, pesticides) [3]. Furthermore, since emerging pollutants are not commonly monitored in the environment, their fate, behavior, and ecotoxicological effects are not well understood. One such emerging pollutant is chlorophene (CP), an antimicrobial widely used in industrial and domestic cleaning activities [4,5]. Like other emerging pollutants, CP is known to cause serious health effects, such as fertility alterations, kidney damage [5], and cancer [6], even at trace concentrations. Efforts to monitor this emerging pollutant have been minimal, as it has been reported in water [3] and soil [7] from a backwater stream in Kerala (India) at a concentration of 0.13 mg/L [2] and 50 mg/L in activated sludge sewage [8]. Furthermore, CP at 7 µg/mL has been detected in male bream bile

specimens from the Dommel River (Europe) [9], suggesting a biomagnification effect of this compound in the food chain [4].

As with many other emerging pollutants, the detection of CP is commonly achieved by analytical techniques that are specialized in the analysis of trace concentrations, such as HPLC/MS [5] and GC/MS [4]. However, high operational costs, time-consuming sample pre-treatments, and laboratory-based instrumentation remain significant limitations to the use of these routine methods. Therefore, it is imperative to develop alternative methods for the direct detection and quantification of CP and other emerging pollutants. In this context, recent advances in the modification of several materials have been studied for their application in the development of sensitive and specific receptors to detect target molecules by electrochemical sensor devices [10]. Laccase enzymes, for example, have been widely used as the recognition element in biosensors due to their high stability and ability to catalyze the oxidation of a wide range of organic compounds [11]. These enzymes are known to catalyze organic compound oxidation with the concomitant reduction of oxygen [12]. Moreover, laccase enzymes are considered easily accessible because they are produced extracellularly by plants, fungi [13,14], and bacteria [15]. In this regard, it should be mentioned that there is a consensus for the laccase-catalyzed oxidative reaction pathway of ortho and para diphenols, polyphenols, aminophenols, and polyamines. This pathway involves the generation of free radicals through the transfer of an electron, which can lead to the formation of polymers by the coupling of radicals with each other, or the nucleophilic substitution of a halogen by a hydroxyl group, which is susceptible to being oxidized again [4,16]. For the particular case of the oxidation of chlorophenolic compounds (e.g., chlorophene and dichlorophen), Shi et al. have established that CP's main by-products are 2-benzyl-[1,4]benzoquinone and 2-benzyl-benzene-1,4-diol. However, the formation of dimeric species of CP and etherification reactions were also reported [16].

The current lack of studies that address the detection of chlorophenolic compounds using novel analytical tools has, in effect, limited routine monitoring of these contaminants to chromatographic techniques. Furthermore, the determination of chlorophene has only been studied using the surface plasmon resonance technique, employing laccase enzymes as a recognition element [17]. Moreover, few studies have addressed the detection of dichlorophen, a chlorophenolic-type molecule, where electrodes decorated with composites such as β–cyclodextrins [18] and coordination polymers with cerium ions [19] have been used as a recognition element. These composites are highly electroactive materials, but require a multi-step synthesis process [19]. Furthermore, these studies were based on a linear sweep and square-wave voltammetry, electrochemical techniques highly distinguished for their high analytical performance due to their speed and sensitivity [19]. However, no investigations have been presented regarding the electrochemical detection of chlorophene.

Thus, this work sought to establish an electrochemical approach as an analytical alternative for the detection of chlorophene in natural water, based on oxidation of the molecule by the laccase enzyme covalently immobilized on gold-coated electrodes. For this study, a cyclic voltammetry analysis was performed because it has demonstrated outstanding behavior during the characterization of an electrochemical system (e.g., reaction mechanisms), providing valuable data when there is a lack of information in the understanding of an electroactive species, such as CP [20]. The biosensor's analytical performance was compared with a standard HPLC method, establishing parameters such as the limit of detection, quantification, working range, and sensitivity. Finally, samples of fortified natural water were analyzed to establish possible matrix effects and evaluate the proposed method's recovery percentage.

## 2. Materials and Methods

### *2.1. Reagents*

The laccase enzymes from *Rhus vernicifera* and salts employed in buffer solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemical compounds 2,2′-azino-

di-(3-ethylbenzthiazoline sulfonic acid) (ABTS), ethanol 99%, 16-mercaptohexadecanoic acid (MHDA), 11-mercaptoundecanol (MUD), $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$ ethanolamine hydrochloride, N-hydroxysuccinimide (NHS), chlorophene (99%), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were supplied from Sigma-Aldrich (St. Louis, MO, USA). The stock solutions of chlorophene (10 mg/mL) were prepared in ethanol: water (90:10, $\%V/V$), and from them, dilutions were prepared in 0.1M phosphate buffer solution (PBS) (pH 7.3). The ultrapure water was obtained from a Milli-Q® water purification system (Millipore, Bedford, MA, USA). With a purity of 99.999%, chrome and gold pellets were purchased from Kurt J. Lesker Co. (Clairton, PA, USA) and employed in the thin-film process's evaporation. The acetonitrile and water for HPLC were purchased from Merck (Darmstadt, Germany), and column Zorbax ODS C18, 25 cm × 4.6 mm (size particle 5 µm) was obtained from SUPELCO Analytical (St. Louis, MO, USA).

### *2.2. Instrumentation*

The electrochemical analysis was performed using a three-electrode scheme, consisting of a laccase-gold working electrode (Lac-Au electrode), a platinum wire counter electrode, and Ag/AgCl (3.0 mol $L^{-1}$ KCl) reference electrode. Cyclic voltammetric tests were conducted from −0.6 V to 0.2 V with a scanning rate of 0.1 V/s, using a workstation CHI700E (CH Instruments, Inc.; Bee Cave, TX, USA). Electrochemical impedance spectroscopy (EIS) measurements were performed in 1 mM of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as a redox probe in PBS 0.1 M (pH 7.3) with a frequency range from 1 Hz to 100 kHz and alternate current amplitude set at 10 mV at the formal potential $E_{1/2}$ = 0.10 V (vs. Ag/AgCl), recorded in the CHI700E (CH Instruments, Inc.; Bee Cave, TX, USA) potentiostat. Obtained EIS spectra were fitted by the software Gamry Echem Analyst to model the data with equivalent circuits and obtain their components. The scanning electron microscope (SEM-JSM–7800F, JEOL Ltd., Tokyo, Japan) was employed to examine the gold thin-film electrode's surface before and after laccase immobilization. Finally, the immobilized electrode was evaluated by infrared analysis using a Spectrum 100 FTIR spectrometer (PerkinElmer Inc; Waltham, MA, USA) in the region between 4000 and 650 $cm^{-1}$ (resolution of 50 scans at 4 $cm^{-1}$), using a KBr window for solutions.

### *2.3. Fabrication of Working Electrode (Lac-Au Electrode)*

The working electrodes were fabricated on glass substrates (1 cm × 1 cm) coated with a chromium/gold thin film, following the method described by Luna-Moreno [21]. Since gold has very poor adherence to glass, an initial chromium layer between the glass and the gold was highly recommended to improve adherence [21]. Briefly, a chromium layer was evaporated up to 3 nm thickness by electron gun evaporation using a High Vacuum Coating Plant BA510 (Balzers High Vacuum Corp., Santa Ana, CA, USA) with a rate of 1.0 Å/s and an atmosphere of $8 \times 10^{-6}$ mbar. A gold film of 50 nm was then deposited by thermal evaporation at the rate of 5 Å/s and $8 \times 10^{-6}$ mbar. The thin films' thickness was evaluated employing a thickness monitor of quartz crystal microbalance (XTC/2 Depositions Controllers Leybold Inficon quartz monitor, San Jose, CA, USA). Before modification, the bare gold electrode was carefully polished with 0.05 mm alumina slurry, followed by subsequent sonication in ultrapure water and absolute ethanol (3 min in each solvent). Then, it was dried at room temperature.

Afterward, the clean electrodes were immersed overnight in a solution of 250 µM of alkanethiols in ethanol (MHDA/MUD at 25 and 225 µM, respectively), followed by a washing step with ethanol [21]. The sulfur atoms from alkanethiols were covalently attached to the gold, allowing the carboxylic groups of alkanethiols to immobilize the enzyme at the end of the chain. The carboxylic groups on the electrode's surface were activated by the addition of 200 µL of EDC/NHS crosslinkers solution (0.2 M/0.05 M) prepared in MES buffer (100 mM, 500 mM NaCl, pH 5.0) [21]. The activation step took 5 min of incubation. Finally, an enzymatic solution of 100 U $mg^{-1}$ (100 µL) is cast on the gold-coated electrode and mixed with the crosslinkers solution (previously added)

and incubated for 15 min, allowing the laccase immobilization through the formation of an amide bond. After the immobilization process, the electrode was rinsed thoroughly with ultrapure water. The schematic representation of the laccase-gold working electrode (Lac-Au electrode) and biosensing strategy for chlorophene detection is shown in Figure 1.

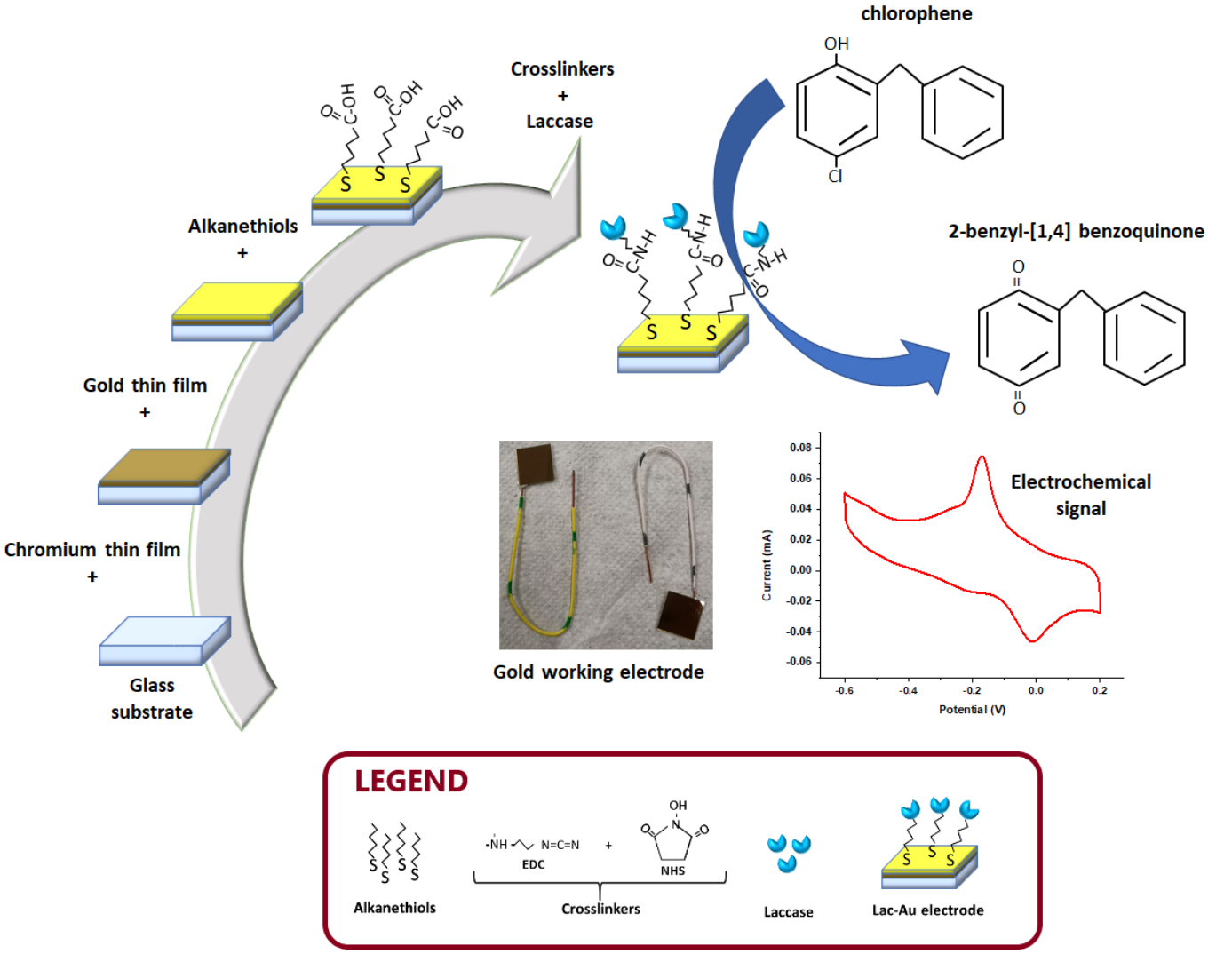



**Figure 1.** Fabrication process of the Lac-Au working electrode for chlorophene detection.

### *2.4. Enzymatic Activity*

The laccase activity was evaluated through the spectrophotometric assay adapted from Zhang et al. (2018) [22], where 200 µL of the enzyme was added to a solution containing 10 mM of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) in 0.1 M sodium acetate buffer (pH 4.5). The ABTS substrate's oxidation was recorded at 420 nm in a UV-Vis spectrophotometer (Cary 50, Varian Inc, Palo Alto, CA, USA). The evaluation of the enzymatic activity for immobilized enzymes was performed by immersion of the gold electrode in a solution containing 10 mM of ABTS in 0.1 M sodium acetate buffer (pH 4.5). Then, an aliquot of 200 µL was taken from the reaction solution and measured by UV-Vis at 420 nm. The activity units (U) were expressed as a function of the amount of enzyme necessary to produce 1 µM/min of product.

### *2.5. Electrochemical Measurements: Chlorophene Detection*

The electrochemical measurements were performed on standard solutions prepared with an appropriate amount of CP in 0.1 M phosphate buffer solution (PBS) (pH 7.3). The corresponding current increments recorded in the cyclic voltammetric analysis were studied from 2 to 10 mg $L^{-1}$; this procedure was performed in triplicate. Linear fitting of the CP concentration-dependent current response curve was then conducted to calculate the method's detection sensitivity. The detection limit was evaluated as 3 times the standard deviation of the baseline, while the limit of quantitation was 10 times the standard deviation. Recovery and reproducibility of the analytical procedure were established using chlorophene spiked in actual natural water to evaluate possible matrix effects.

### *2.6. Samples and HPLC Reference Method*

The samples from natural water were collected from a river located in León city, Guanajuato-México (21°09′54.0″ N 101°43′30.6″ W) according to the method presented by Quintanilla-Villanueva [17]. The water samples were spiked with chlorophene, following the recommendations of the surrogate addition method established by the IUPAC-AOAC [23]. The samples were then analyzed with the Lac-Au electrodes and the results compared to those of the HPLC reference analysis [3]. The HPLC methodology consisted of a mobile phase of acetonitrile:water (85:15) flowed at a rate of 1 mL/min in reversed-phase column and using a UV detector at 290 nm [3].

## 3. Results and Discussion

### *3.1. Fabrication of Lac-Au Electrode*

Working Lac-Au electrodes were fabricated in a step-by-step process, corroborating the materials' deposition by SEM images of cross-sectioned electrode. As observed in Figure 2a, the gold thin film deposited onto the substrate had a thickness of 49.7 (±1.3) nm with a chemical composition (in wt%) of 60.0% attributed to Au. The surface presented a smooth texture and homogeneous distribution across the material. Once the gold base material was deposited, the electrode was treated with alkanethiols to functionalize the gold-coated surface. After adding alkanethiols chains, the thickness layer was incremented to 54.1 (±1.6) nm (Figure 2b). The EDS spectra confirmed the deposition of alkanethiols by incrementing C and O concentrations in the material from 4.6 to 62.1 wt% of carbon and from 10.0 to 22.2 wt% of oxygen (present in carbonyl groups of alkanethiols). Finally, the results observed in Figure 2c suggested the enzymes were successfully added to the electrode's surface, with thickness increments in 64.6 (±9.3) nm with a co-increment of O and N at a concentration of 2.1 wt%. This supported the suggestion that the functionalized carboxylic groups were activated through the cross-linkers EDC/NHS, forming an amide bond that anchored the laccases. On the other hand, because no washing step was performed after activation of the electrode's surface (prior to enzyme immobilization), the irregularity observed on the final electrode's surface could be attributed to the formation of enzyme-enzyme conjugates (multilayer enzyme systems), which have been reported to form during the immobilization process when there is an excess of cross-linker agent (EDC/NHS), activating the carboxylic groups within the proteins [24].

The FTIR spectroscopy characterization of the immobilized gold surface (Figure 3) exhibited the characteristic bands of amide I at 1580 $cm^{-1}$ (NH, bending) and amide II at 1630 $cm^{-1}$ (C=O, stretching), and symmetric stretching vibrations of carboxylates at 1440 $cm^{-1}$ [25]. The presence of these bands has been previously reported for the proteins' immobilization through lysine side chains [26]. However, a signal at 2120 $cm^{-1}$ was also observed, and attributed to the carbodiimide group (N=C=N, stretching) of the EDC cross-linker.

After construction of the working Lac-Au electrode was completed, the enzymatic activity of immobilized enzymes was determined and compared against the free enzymes' initial activity. It was observed that the immobilized enzymes lost 48.5% of their oxidation capacity compared to the free enzymes. This decrease of activity has been attributed to

the presence of non-oriented covalent bonds between the enzyme and the alkanethiols present on the electrode's surface, resulting in the blockade of the enzyme's active site [27]. This observation was reported by Fan et al. (2017), who observed a 40% loss in activity of esterases after their covalent immobilization on silica [28].

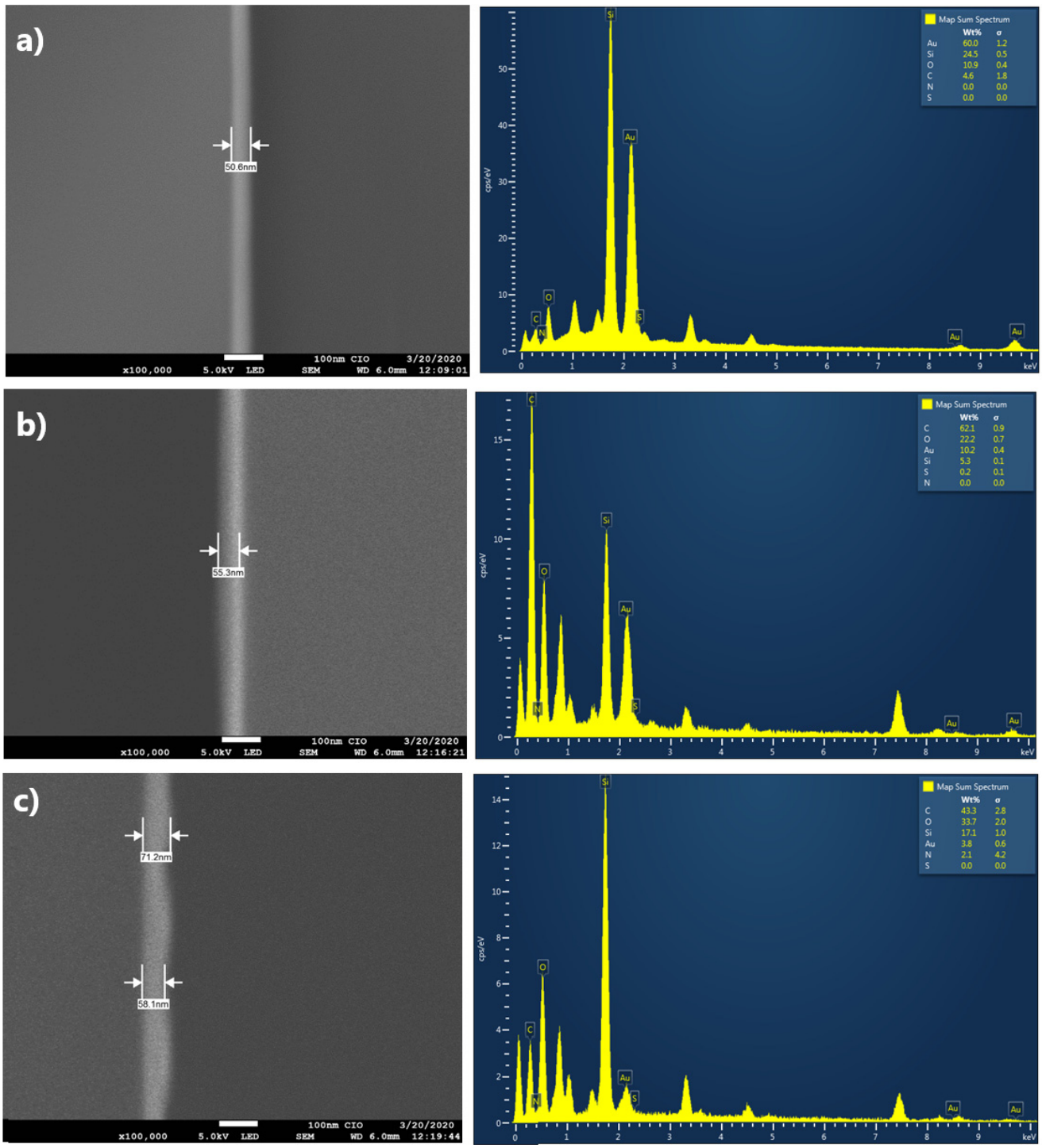



**Figure 2.** SEM images and EDS analysis of the cross-sectioned (**a**) bare gold-coated electrode, (**b**) functionalized gold surface, and (**c**) gold-coated electrode after enzyme immobilization.

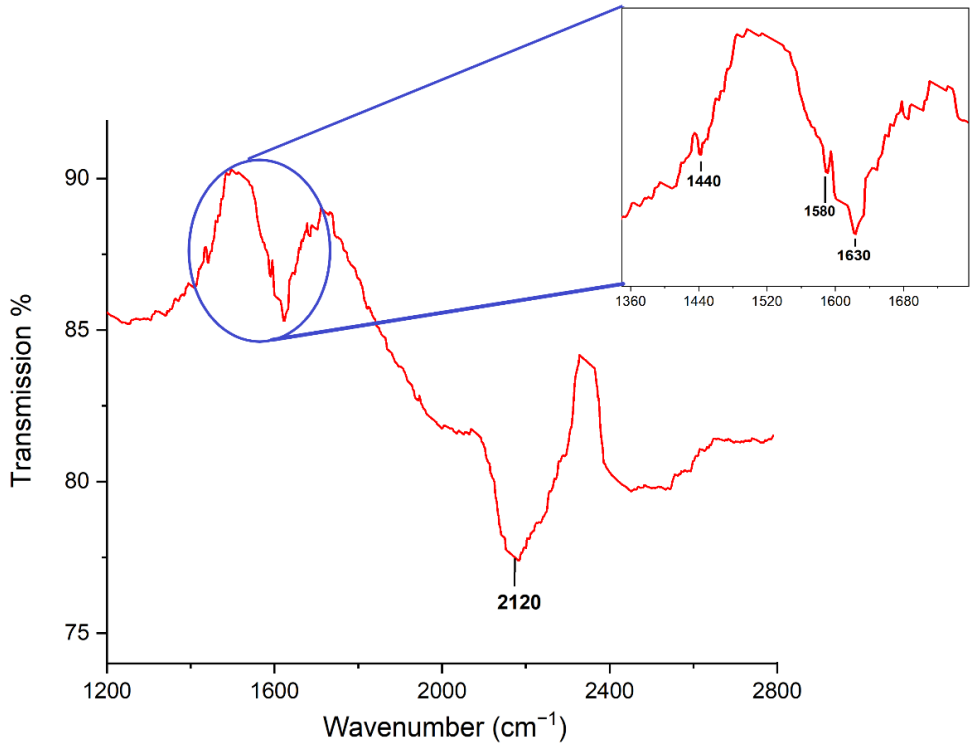



**Figure 3.** FT-IR spectrum of gold thin film immobilized with laccase. Inset: Zoom of FTIR region from 1360 to 1700 cm$^{-1}$.

Electrochemical impedance spectroscopy (EIS) analysis was performed to evaluate the changes in the modified electrode's conductivity. Figure 4a shows the impedance features of gold electrode at different modification steps: bare Au electrode, Au electrode modified with alkanethiols, and Lac-Au electrode. From the EIS spectra fitting, an equivalent circuit was simulated for the working electrode (Figure 4b). The equivalent circuit consisted of an electrolyte solution resistance (Rel) in series with a circuit branch containing in series a charge electron-transfer resistance Rct and Warburg impedance W (diffusion of the ionic species through the diffusion layer) components, which were in parallel with the double layer capacitance Cdl. The bulk properties of the electrolyte solution and diffusion of the redox species in solution are represented in *Rel* and *W* [19]. Additionally, the parallel combination of Rct and Cdl indicates the insulating and dielectric characteristics of the electrode/electrolyte interface, represented as a semicircle in the Nyquist plots (Figure 4).

The charge electron-transfer resistance Rct was obtained by measuring the diameter of the semicircle in the impedance spectrum [19]. The Rct for the bare Au electrode was estimated to be 100 Ω and increased to 140 Ω in the presence of alkanethiols. Finally, when the electrode was modified with laccase, the electron-transfer resistance incremented to 140 Ω, suggesting the generation of an insulating layer on the electrode surface as molecules were added during the modification steps. The barrier in the interfacial electron transfer, inferred from the Rct analysis, indicated assembly of the enzymes on the gold electrode's surface. The equivalent circuit data is listed in Table 1.

**Table 1.** Comparison of equivalent circuit components of gold electrode at different modification steps.

| Electrode | Rel (Ω) | Rct (Ω) | Cdl (F) | W (S * $\sqrt{s}$) |
|---|---|---|---|---|
| Bare Au | 20 | 100 | $2.8 \times 10^{-9}$ | 0.0012 |
| Au-Alkanethiols | 30 | 140 | $1.6 \times 10^{-9}$ | 0.0011 |
| Lac- Au | 40 | 160 | $1.4 \times 10^{-9}$ | 0.0015 |

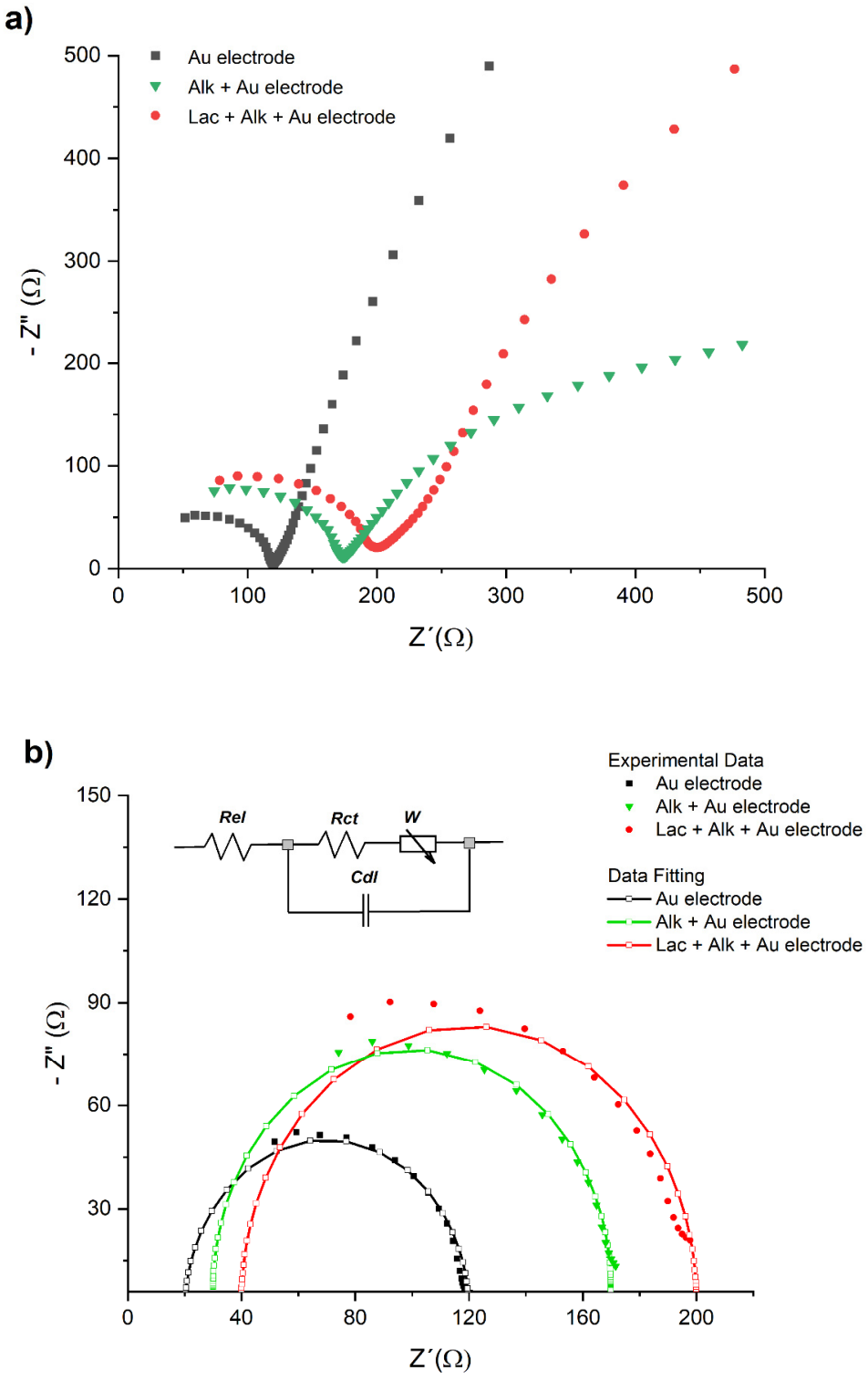



**Figure 4.** (**a**) Electrochemical impedance spectroscopy analysis (EIS) performed in 1 mM of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in PBS 0.1 M (pH 7.3) at bare Au electrode and different modification steps. (**b**) Zoom image of the EIS semicircle obtained from the data fitting to simulate the equivalent circuit (Rel, Rct, Cdl). Inset: equivalent circuit of the working electrode.

### *3.2. Chlorophene Detection*

Once the working Lac-Au electrode's enzymatic activity was confirmed, the electrochemical system was constructed and tested to detect the antimicrobial agent chlorophene. The cyclic voltammetry behavior of the biosensor in the presence of CP was recorded from

−0.6 V to 0.2 V with a scanning rate of 0.1 V/s. As shown in Figure 5a, the formation of an anodic peak occurred at −0.17 V, suggesting the oxidation of chlorophene by the enzyme, which could be used as an analytical signal. Furthermore, a reduction current was observed at −0.01 V, suggesting the reversibility of the reaction and regeneration of the chlorophene at the electrode's surface. It is worth noting that laccase has already been reported to present enzymatic activity on chlorophene and dichlorophen molecules [16]. According to Shi et al., laccase enzymes catalyze the oxidation of CP by generating a free radical through the transfer of one electron. This reaction mechanism leads to chlorine's nucleophilic substitution by the hydroxyl group, followed by further oxidation, producing 2-benzyl [1,4] benzoquinone [16]. After chlorophene's oxidation, the reduced form of laccase is reconstituted to its oxidized form by its interaction with oxygen, producing water along the way [16]. The redox electrochemical reaction mechanism is shown below:

$$\text{Chlorophene + Laccase(ox)} \leftrightarrows \text{2-benzyl-[1,4] benzoquinone + Laccase(red) + } 2H^+ + 2e^- \quad (1)$$

$$\text{Laccase(red)} + O_2 + 4H^+ \rightarrow \text{Laccase(oxy)} + 2H_2O \quad (2)$$

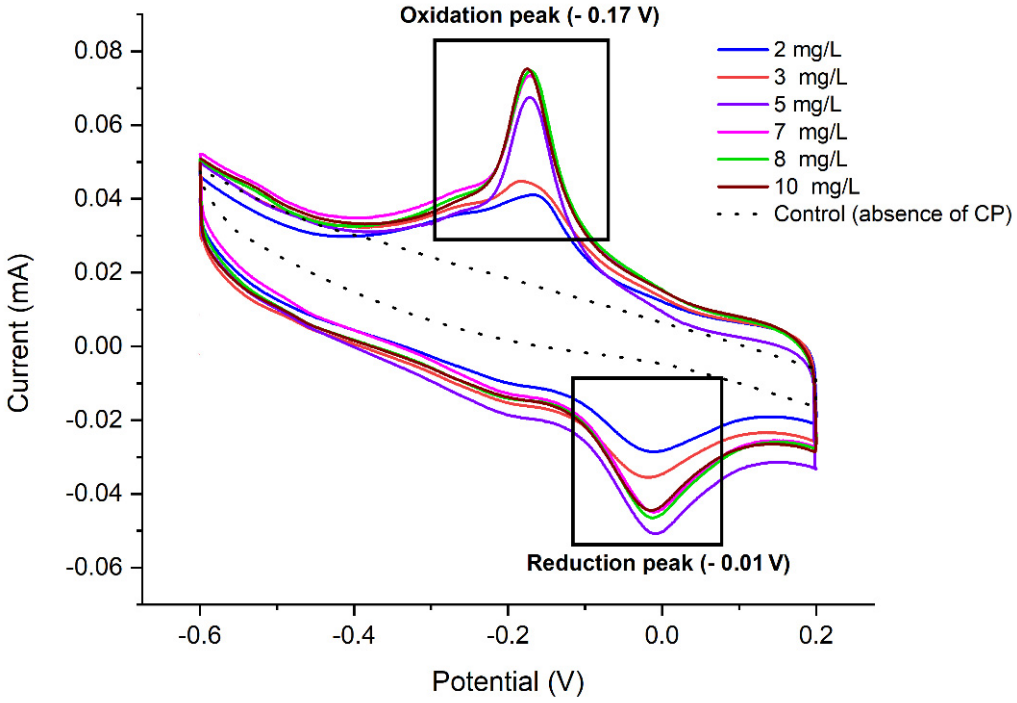
The increase in the CP concentration ranged from 2 to 10 mg $L^{-1}$, resulting in a linear and proportional increase in the oxidation peak current (Figure 5b). This behavior followed the equation $I_{(mA)} = 5.2 \times 10^{-3}\ c + 3.0 \times 10^{-3}$ [mg $L^{-1}$] with a correlation coefficient of 0.995. The resulting detection and quantification limits were 0.14 (±0.06) and 0.48 (±0.04) mg $L^{-1}$, respectively. With these results, the proposed method was in accordance with concentrations ranging from 0.13 to 50 mg $L^{-1}$ detected in real samples from a backwater stream in Kerala (India) [3] and activated sludge sewage [7].

Unfortunately, it should be noted that studies addressing the detection of chlorophenolic compounds remain scarce, which effectively limits the routine monitoring of this contaminant to chromatographic techniques. In this respect, most of the studies that have been carried out to detect chlorophene based on electrochemical techniques used electrodes decorated with composites comprising β–cyclodextrins [18] and coordination polymers with cerium ions [19]. Another study explored the determination of chlorophene using biosensors based on surface plasmon resonance, employing laccase enzymes as an element of recognition [17]. The analytical performance of the different strategies reported for the detection of chlorophenolic compounds is summarized in Table 2, showing outstanding behavior based on the linear sweep and square-wave voltammetry. In particular, these electrochemical techniques are distinguished for high analytical performance due to their speed and sensitivity, being able to detect micromolar concentrations of electroactive species [19]. In contrast, cyclic voltammetry offers excellent characterization of an electrochemical system (e.g., reversibility of reaction, reaction mechanisms, determination of the number of electrons transferred), but its detection is in the range of millimolar concentrations [20]. Thus, the combination of different electrochemical techniques would be ideal for biosensing applications when there is a lack of information on the behavior of an electroactive species.

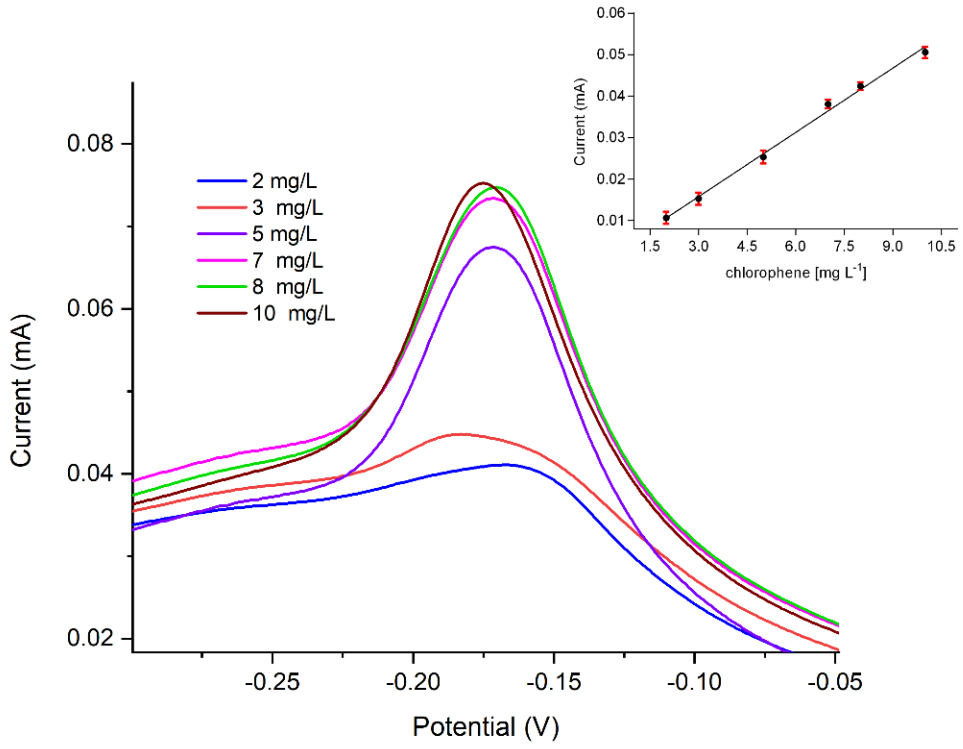
**Table 2.** Analytical strategies for detection of chlorophenolic compounds.

| Strategies | Element of Recognition | Analyte | LOD (μg $L^{-1}$) | LOQ (μg $L^{-1}$) | Reference |
|---|---|---|---|---|---|
| Surface plasmon resonance | Laccase enzyme | chlorophene | 330 | 1100 | [17] |
| Cyclic voltammetry | Laccase enzyme | chlorophene | 140 | 480 | This work |
| Linear sweep voltammetry | RGO@Ce-MOF composite | dichlorophen | 2 | - | [19] |
| Square-wave adsorptive stripping voltammetric | β–CDs/MWCNTs/GCE | dichlorophen | 3 | 12 | [18] |

RGO@Ce-MOF: reduced graphene oxide-encapsulated Ce- metal organic frameworks. β–CDs/MWCNTs/GCE: carbon electrode modified with β–cyclodextrins and multi–walled carbon nanotubes.




(**a**)




(**b**)

**Figure 5.** (**a**) Voltammetric profile of Lac-Au working electrode in the presence of CP in 0.1 M PBS buffer (pH = 7.3) at scan rate of 0.1 V/s (black dotted line: scan in PBS without CP). (**b**) Zoom image of oxidation peak current. Inset of calibration curve versus concentration of CP. The plotted signals correspond to the average measurements obtained in triplicate ($n$ = 3).

### 3.3. Analysis of Real Samples

Having detected the CP signal with the Lac-Au working electrode, we carried out tests to discard possible matrix effects caused by natural water while determining the contaminant in river samples. To do this, tests were performed on real samples spiked with a CP concentration of 3 mg $L^{-1}$. The results, shown in Figure 6, demonstrated that the current generated by the oxidation of CP in the spiked sample was less than the current obtained during the CP measurement test at the same concentration in a phosphate buffer solution.

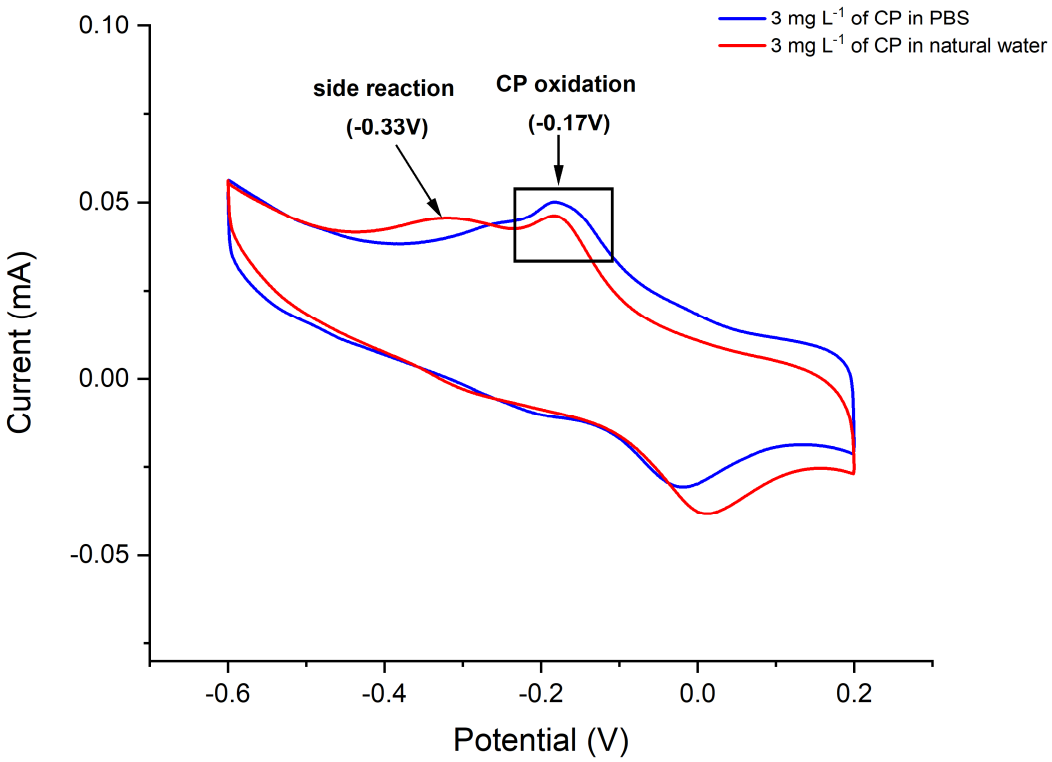



**Figure 6.** Voltammetric profile of Lac-Au working electrode in the presence of CP at 3 mg $L^{-1}$ in 0.1 M phosphate buffer solution (pH 7.3) (black line) and natural water from a river (red line) at a scan rate of 0.1 V/s. The plotted signals correspond to the average measurements obtained in triplicate).

Furthermore, during this analysis, an unwanted side reaction was observed at the electrode, which produced a second anodic peak at −0.33 V. This signal substantially reduced the current detection signal levels (see Table 3). Consequently, the recovery percentage obtained was lower (62 ± 2.4%; 1.86 mg $L^{-1}$) than that obtained by the HPLC reference method (101.3 ± 3.5%; 3.04 mg $L^{-1}$).

**Table 3.** Determination of spiked river samples by CV and HPLC methods ($n$ = 3).

| Fortification Level (mg $mL^{-1}$) | CV Method | | HPLC Method | |
|---|---|---|---|---|
| | Mean (mg $mL^{-1}$) | Recovery (%) | Mean (mg $L^{-1}$) | Recovery (%) |
| 3 | 1.86 ± 0.07 | 62.0 ± 2.4 | 3.04 ± 0.11 | 101.3 ± 3.5 |

Figure 7a presents the chromatographic peak of CP at different concentrations, showing an elution time of 3.98 min, which is in accordance with the literature [3]. The calibration curve followed the equation $I_{(mV)}$ = 8.9$c$ + 3.4 [mg $L^{-1}$] with a correlation coefficient of 0.994 (Figure 7c). However, the HPLC measurement carried out on real samples spiked

with a CP concentration of 3 mg $L^{-1}$ resulted in diverse chromatographic peaks from the impurities (possible interferences) present in the river sample (Figure 7d).

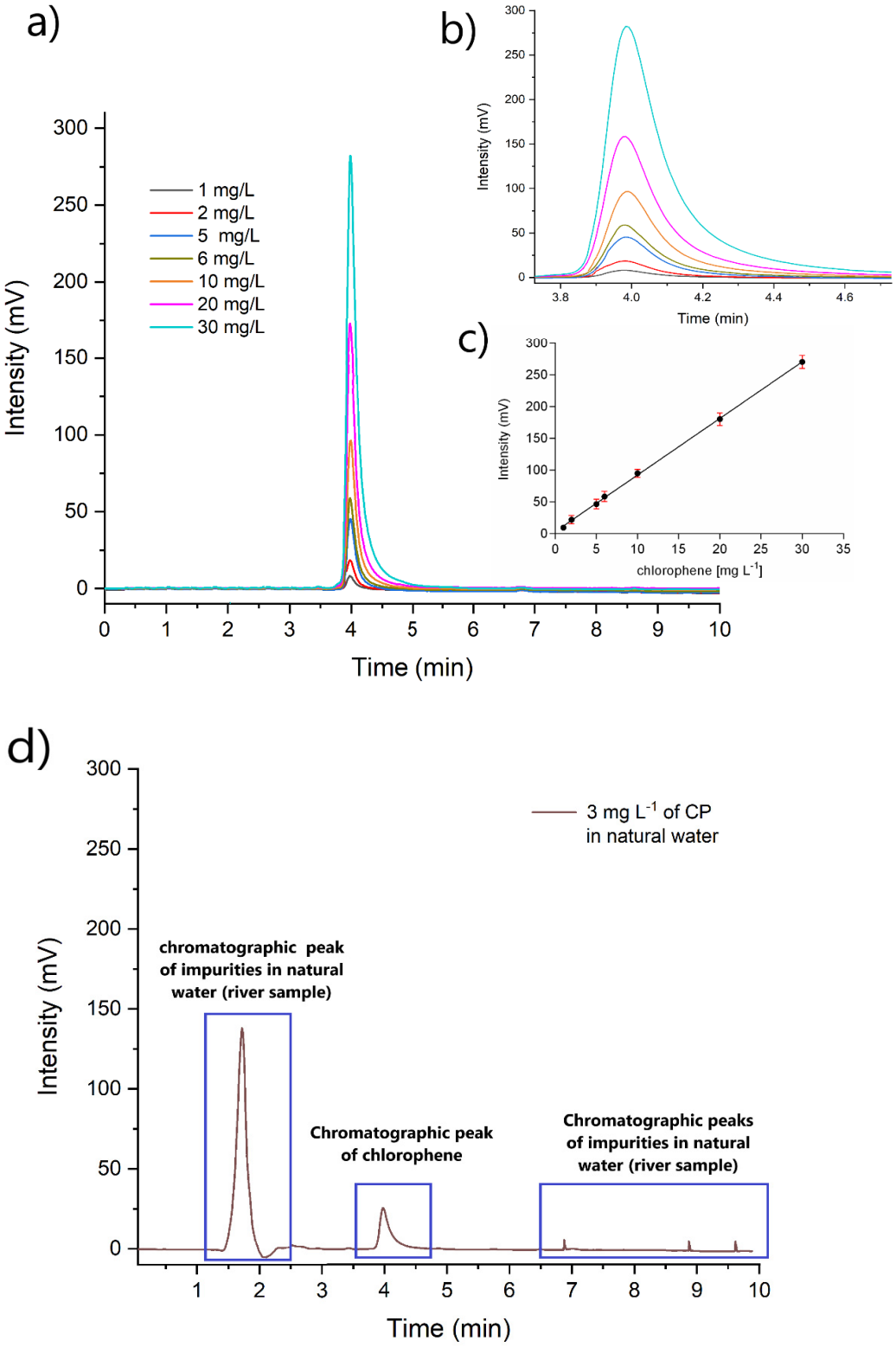



**Figure 7.** (**a**) Chromatographic peaks of CP al different concentrations in 0.1 M phosphate buffer solution (pH 7.3). Inset: (**b**) Zoom of CP peaks and (**c**) calibration curve obtained from the HPLC measurements using a UV detector at 290 nm and rate of 1 mL/min. (**d**) Chromatographic peaks of CP at 3 mg $L^{-1}$ in natural water from river. The plotted signals correspond to the average measurements obtained in triplicate).

These matrix effects can be attributed to the organic matter in the river sample, which reached a concentration of 23.66 mg $L^{-1}$ [17]. It has been reported that organic matter can interfere in electrochemical systems from concentrations as low as 10–15 mg $L^{-1}$ due to competitive reactions with the electroactive analyte (chlorophene) or scavenging of the reactive species [29]. Furthermore, it has been reported that the presence of high concentrations of ions present in the sample, such as $SO_4{}^{-2}$ (10.99 mg $L^{-1}$) and $Cl^-$ (32.11 mg $L^{-1}$) [17], leads to the formation of strong oxidants that have detrimental effects on the method's performance [29]. Thus, it is essential to perform a selectivity assay in the presence of related compounds (interferences), evaluated under realistic concentrations (typically present in natural samples) to appropriately determine the major responsibility of the matrix effect observed.

Overall, the laccase showed potential as an element of recognition in the detection of chlorophene using an electrochemical approach. Nevertheless, the provided method was treated as an initial stage toward further investigations into the optimization and validation of the electrochemical analysis. It will be crucial to evaluate non-specific interactions with non-related compounds and ions (selectivity) present in real samples, estimate the life cycle of the electrochemical biosensor, and assess the intra- and inter-day variability of the Lac-Au working electrode. Additionally, it will be important to establish different strategies that minimize the matrix effect under operational conditions, such as evaluating measurements at different pH, adding mediators, or applying N-fold dilutions of the real samples.

## 4. Conclusions

Currently, there is a scarcity of biosensors for the detection of chlorophene. Therefore, this study sought to contribute to this research area, establishing an electrochemical approach based on the fabrication of a working gold electrode immobilized with laccase enzymes. The results obtained were an initial step toward new investigations into the optimization and validation of the electrochemical analysis, which should include detailed evaluations of selectivity, sensitivity, and reproducibility. Under controlled conditions, the proposed method obtained analytical parameters with a limit of detection and quantification of 0.14 and 0.48 mg $L^{-1}$, respectively. These results shed light on the promising use of the laccase enzyme as a recognition element for chlorophene because its performance was in accordance with the CP concentrations that have been reported in natural water samples. However, the provided method suffered from a severe drawback due to matrix effects, limiting its feasibility and validation for direct use in analyzing actual samples. Consequently, future work must focus on testing different conditions (e.g., pH, use of mediators, N-fold dilution of the sample) that establish strategies to minimize detrimental effects from interference.

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