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Enzymatic electrochemical detection coupled to multivariate calibration for the determination of phenolic compounds in environmental samples

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ABSTRACT

An approach based on the electrochemical detection of the horseradish peroxidase enzymatic reaction by means of square wave voltammetry was developed for the determination of phenolic compounds in environmental samples. First, a systematic optimization procedure of three factors involved in the enzymatic reaction was carried out using response surface methodology through a central composite design. Second, the enzymatic electrochemical detection coupled with a multivariate calibration method based in the partial least-squares technique was optimized for the determination of a mixture of five phenolic compounds, i.e. phenol, p-aminophenol, p-chlorophenol, hydroquinone and pyrocatechol. The calibration and validation sets were built and assessed. In the calibration model, the LODs for phenolic compounds oscillated from 0.6 to 1.4×10^{-6} mol L^{-1}. Recoveries for prediction samples were higher than 85%. These compounds were analyzed simultaneously in spiked samples and in water samples collected close to tanneries and landfills.

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1. Introduction

The phenolic compounds are characterized by having at least one aromatic ring with one or more hydroxyl groups or other atoms and/or functional groups attached. They are widely present in the environment due to their application in the production of drugs, fragrances, polymeric materials, synthetic detergents, plasticizers, dyes, papers, pesticides, among others [1]. They have significant detrimental effects on water quality or animals as well as on some plants, even at very low levels, due to their toxicity and carcinogenic activity. For these reasons, some of them have been included in the lists of priority pollutants [2]. Many analytical techniques have been used for monitoring phenols, such as colorimetry [3], gas chromatography [4], liquid chromatography [5], and capillary electrophoresis [6]. However, some of these techniques are expensive, time consuming and sometimes require complex sample pre-treatment such as pre-concentration and extraction steps that increase the risk of sample loss. Therefore, the development of sensitive and fast methods for simultaneous determination of phenol and its derivatives is very important in environmental control and in the control of phenolic compound biodegradation in industrial wastewater [7]. Grosso et al. [8] reported that the initial concentration and the kind of compound have great influence on the efficiency of this biodegradation, at high concentrations (>435 mg L^{-1}) these compounds are rapidly reduced, while at low concentrations and in the presence of substituted phenols, the process becomes slower [9]. On the other hand, according to local regulations, the maximum allowed levels for phenol in drinking water should not exceed 2 μg L^{-1}, while other phenolic compounds should be absent [10].

Phenolic compounds can be easily analyzed by using electrochemical detection, due to the fact that they can generate electroactive products in the presence of horseradish peroxidase enzyme (HRP). The operating principle is based on a ping-pong mechanism (see Fig. 1). The first stage involves the oxidation of a ferric native enzyme to a ferryl-oxo porphyrin radical intermediary (compound I or CI) by H_2O_2. In the second stage, the compound I is reduced to the ferryl-oxo intermediary (compound II or CII) by a phenolic molecule. In the third stage, another phenolic molecule is necessary to reduce the compound II to the native enzyme. The second and third stages also involve the oxidation of phenolic compounds to quinines or free radicals. The oxidation products can be electrochemically reduced on the surface of the electrode and so an electrochemical signal directly proportional to the concentration of the phenolic compound is obtained when the H_2O_2 is present in excess [11–14].

The methods based on univariate calibrations determine one compound by using only one analytical response; while methods that use the multivariate regressions assess simultaneously several compounds from various responses. For the environmental

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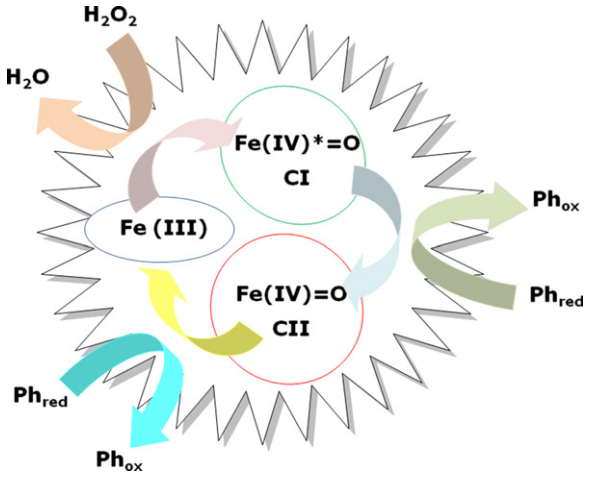

Fig. 1. Schematic representation of HRP enzymatic behavior with H_2O_2 substrate and phenolic compounds (Ph).

samples analysis the multivariate calibration would be the most appropriate method, since it can give information about many analytes in shorter times.

The partial least-squares (PLS) technique is often used for the analysis in multivariate calibration methods [15–18]. There are two types of PLS techniques: on the one hand, PLS1 analyzes one analyte at a time and optimizes working conditions for each analyte independently; on the other hand, PLS2 evaluates and predicts the concentrations of several simultaneously analytes [19]. The PLS calibration can be performed by ignoring the concentrations of all other components except the analyte of interest; this is an additional advantage of such multivariate methods. Therefore, these methods are especially interesting for the determination of the analytes in complex samples, whose matrix may show analytical signals, which are severely overlapped with those from the analytes.

This work is focused on the development of methods based on the electrochemical detection of electroactive products generated by the reaction of HRP and phenolic compounds. On the one hand, a systematic optimization procedure of three factors involved in the enzymatic reaction was carried out using response surface methodology through a central composite design. An enzymatic electrochemical detection coupled with univariate calibration was performed and then evaluated to phenol analysis in natural water samples. Finally, a method based on the enzymatic electrochemical detection coupled with multivariate calibration was developed for the quantification of five phenolic compounds and then was applied to the analysis of water samples recollected close to tanneries and landfills.

2. Materials and methods

2.1. Instrumentation

Cyclic voltammetry, square wave voltammetry (SWV), amperometry and chronoamperometry were performed with a voltammetric analyzer Epsilon BAS, Bioanalytical Systems Inc (West Lafayette Indiana—USA) with a three electrode system based on graphite-epoxy composite (GEC) as working electrodes [20–22]; platinum as auxiliary electrode and a (Ag/AgCl) silver/silver chloride electrode in 3 mol L^{-1} NaCl solution as reference electrode (Orion 92-02-00). The effective areas of electrodes were 0.22 cm^2 (RSD%=14%, n=9) by cyclic voltammetry and chronoamperometry with potassium ferricyanide.

2.2. Reagents

Horseradish peroxidase (1310 U mg^{-1}), hydroquinone and pyrocatechol were purchased from Sigma. All other reagents such as hydrogen peroxide, phenol, p-chlorophenol and p-aminophenol were also analytical grade. Phenol, hydroquinone, H_2O_2 and pyrocatechol solutions were prepared with Millipore water, while p-chlorophenol and p-aminophenol solutions were prepared in ethanol:millipore water (50:50). The working buffer solutions were phosphate 0.1 mol L^{-1} and KCl 0.1 mol L^{-1} (for buffering from pH 5.0–7.0) and acetate/phosphate/borate 0.05 mol L^{-1} and KCl 0.1 mol L^{-1} (for buffering from pH 6.0–9.0).

Water samples were collected according to 29012 IRAM norms from different localities close to tanneries and public landfills of the geographic regions of The Littoral and the Mesopotamia in Argentine (see Table 1) and were stored in plastic containers at 4 °C in the dark.

2.3. Enzymatic reaction and electrochemical detection

The cyclic voltammetry (with a scan rate 0.1 V s^{-1}) was independently performed for phenol, hydroquinone, p-chlorophenol, pyrocatechol and p-aminophenol in the range from 1200 to −400 mV. Phenolic compound solutions 2×10^{-3} mol L^{-1} in phosphate buffer 0.1 mol L^{-1} and KCl 0.1 mol L^{-1} at pH 6.00 were used in these assays.

In a 5.0 mL reaction cell, the enzymatic electrochemical detection was carried out with 4.0 mL of buffer and KCl 0.1 mol L^{-1} where small volumes of HRP enzyme, phenolic compound standards and the H_2O_2 solutions were added at different concentration ranges. Then, the analytical signals were obtained by SWV (whose parameters: step width=4 mV, amplitude=25 mV and frequency=15 Hz were not optimized and were set to default values by instrument).

In univariate calibration for phenol, the reduction current peak was obtained by SWV when it was swept from 500 to 50 mV vs. reference electrode.

In multivariate calibration for five phenolic compounds, the analytical signal was the sum of two voltammograms by SWV.

Table 1
Water samples analyzed by enzymatic electrochemical detection coupled to univariate and multivariate calibration.

Samples	Calibration[a]	Type of water	Source nearby to	Location[b]
-1-	UV	Groundwater	At 100 m of tannery	Castellanos, SF
-2-	UV	Groundwater	At 200 m of tannery	Castellanos, SF
-3-	MV	Groundwater	At 100 m of tannery	Las Colonias, SF
-4-	MV	Groundwater	At 200 m of tannery	Las Colonias, SF
-5-	MV	Groundwater	At 100 m of tannery	General Obligado SF
-6-	MV	Groundwater	At 200 m of tannery	General Obligado SF
-7-	MV	Stream water	At 300 m of tannery	General Obligado SF
-8-	MV	Superficial water	At 100 m of tannery	La Capital, SF
-9-	MV	Stream water	At 100 m of tannery	La Capital, SF
-10-	MV	Stream water	At 50 m of landfill	Nogoyá, ER
-11-	MV	Superficial water	At 50 m of landfill	Nogoyá, ER

[a] univariate calibration=UV and multivariate calibration=MV.
[b] Santa Fe, Argentine=SF and Entre Ríos, Argentine=ER.

The first was swept from 500 to 250 mV and then the second was swept from 0 to −400 mV vs. reference electrode.

2.4. Univariate calibration for phenol

The univariate calibration of enzymatic electrochemical response as current density ($\mu A\ cm^{-2}$) vs. phenol concentration ($mol\ L^{-1}$) was performed. In order to do that the buffer pH, the H_2O_2 concentration and enzymatic concentration were kept at 6.0, $1.0\times10^{-3}\ mol\ L^{-1}$ and $4.8\times10^{-8}\ mol\ L^{-1}$ respectively while that the phenol concentration was ranged from 0.05 to $5\times10^{-6}\ mol\ L^{-1}$. Then, the analytical signal was obtained by SWV at 5 min.

A homocedasticity test was assayed to determine if an ordinary least-square (OLS) or a weighted least-squares (WLS) calibration should be applied. In order to do that, F-test was performed with a confidence interval of 95%. Due to that the data presented a heterocedastic distribution, they were analyzed by WLS calibration utilized MATLAB version 7.6.0. (R2008a) [23,19].

2.5. Phenol univariate calibration in groundwater samples

Two groundwater samples without any pretreatment were evaluated; only the ionic strength and the pH in the samples were regulated to achieve $0.1\ mol\ L^{-1}$ and 6, respectively. After $4.8\times10^{-8}\ mol\ L^{-1}$ of HRP and $1.0\times10^{-3}\ mol\ L^{-1}$ of H_2O_2 were incorporated to the sample and then the analytic signal by SWV was obtained.

In addition, repeatability tests and recovery experiments were performed by spiking of $1.7\times10^{-6}\ mol\ L^{-1}$ and $2.5\times10^{-6}\ mol\ L^{-1}$ of phenol standards in the samples.

2.6. Multivariate calibration for phenolic compound determinations

The multivariate calibration was performed by the PLS1 using MATLAB version 7.6.0. (R2008a). The calibration and prediction sets were designed by central composite design (CCD) and 2-level factorial design (2-LFD), respectively by Design-Expert 7.1.6.

The concentration levels for each design were established within the linear range obtained by univariate calibration for each compound (see Table 2). But the superior concentrations were preset at 80% of superior limit of linear range of each compound. In addition, the sums of the concentration of five compounds corresponding to the "high star" level of CCD and the "high cube" level of 2-LFD were maintained at one value lower than $125\times10^{-6}\ mol\ L^{-1}$ to avoid the response saturation.

In the calibration stage, a design of 53 experiments was blocked in three blocks according to the used electrode. In the prediction stage, a design of 16 experiments was blocked in two blocks according to used electrode. In the analysis of data by PLS, concentration vectors (in $mol\ L^{-1}$) for each compound and the response matrix (in $\mu A\ cm^{-2}$) for calibration and prediction were built.

The matrix was designed with the current densities obtained in the first potential range from 500 to 250 mV (sensor numbers from 1 to 62) followed of the current densities obtained in the second potential range from 0 to −400 mV (sensor numbers from 63 to 162), resulting in a total sensor range from 1 to 162 for each sample. Before applying of this procedure, to obtain the blank signal, the actual and artificial samples should be analyzed by SWV in both ranges of tested potential, but without adding of the enzyme. In all cases, the absence of response suggested the absence of electrochemical interferences in the tested ranges.

Moreover, the elliptical joint confidence regions (EJCR) to determine the accuracy and precision of the method were performed by MATLAB 7.6.0. (R2008a) [23].

2.7. Phenolic compounds multivariate calibration in water samples

Water samples were analyzed without any pretreatment (idem Section 2.5) and were evaluated by SWV in two ranges of potentials (according to Section 2.3). Then the matrix of the current densities for the samples was analyzed by PLS1. Posteriori, the samples were spiked with a known amount of each phenolic compound for recovery studies.

3. Results and discussion

3.1. Univariate calibration for phenol

The electrochemical behavior of phenol was studied by cyclic voltammetry. A typical voltammogram for phenol is shown in the Fig. 2A. As can be seen in this figure, the reduction and oxidation peaks of phenol are observed at potentials of 308 and 758 mV, respectively. The difference between these two potentials was almost 450 mV. This value is much larger than the value expected of 59 mV from a classical reversible system, indicating an irreversible electrode process.

Table 2
Concentration levels for each phenolic compound used in calibration and validation sets.

Compound	Concentration levels ($\times10^{-6}\ mol\ L^{-1}$) Central Composite Design CCD					Concentration levels ($\times10^{-6}\ mol\ L^{-1}$) 2-Level Factorial Design 2-LFD	
	Low star	Low cube	Center	High cube	High star	Low cube	High cube
[a] Phenol	1.9	2.0	2.7	3.4	3.5	1.7	3.0
[b] p-Chlorophenol	0.8	2.5	10.0	18.0	21.2	5.0	20.0
[c] p-Aminophenol	0.5	2.0	10.0	18.0	21.2	4.0	20.0
[d] Pyrocatechol	1.5	5.0	20.0	35.0	40.0	10.0	38.0
[e] Hydroquinone	0.5	2.5	19.0	35.0	38.0	5.0	37.0

The linear regression curve for each compound was obtained under the experimental condition following: the buffer pH=6.0; $[H_2O_2]=1.0\times10^{-3}\ mol\ L^{-1}$ and [HRP] $4.8\times10^{-8}\ mol\ L^{-1}$.

[a] Linear regression curve for phenol: $y[\mu A]=0.045[\mu A]+3.60\times10^{5}\left[\frac{\mu A}{(mol/L)}\right]X\left[10^{-6}mol/L\right]$ and $r^2: 0.99_6$.

[b] Linear regression curve for p-chlorophenol: $y[\mu A]=0.42[\mu A]+1.83\times10^{5}\left[\frac{\mu A}{(mol/L)}\right]\cdot X\left[10^{-6}mol/L\right]$ and $r^2: 0.999_2$.

[c] Linear regression curve for p-aminophenol: $y[\mu A]=0.006[\mu A]+1.87\times10^{5}\left[\frac{\mu A}{(mol/L)}\right]X\left[10^{-6}mol/L\right]$ and $r^2: 0.999_7$.

[d] Linear regression curve for pyrocatechol: $y[\mu A]=0.008[\mu A]+6.97\times10^{5}\left[\frac{\mu A}{(mol/L)}\right]X\left[10^{-6}mol/L\right]$ and $r^2: 0.99_7$

[e] Linear regression curve for hydroquinone: $y[\mu A]=0.022[\mu A]+9.17\times10^{5}\left[\frac{\mu A}{(mol/L)}\right]X\left[10^{-6}mol/L\right]$ and $r^2: 0.99_8$ where r^2: Coefficient of determination.

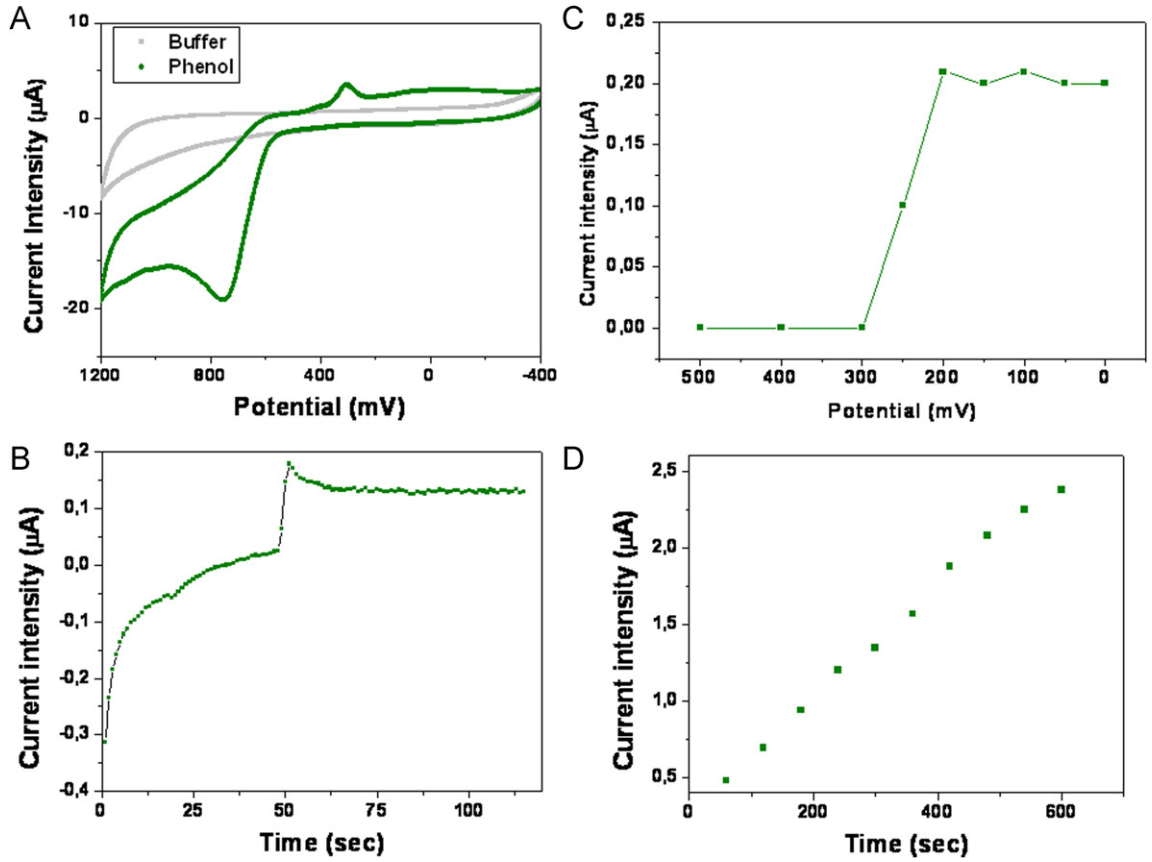

Fig. 2. (A) Cyclic voltammogram of phenol and supporting electrolyte solution pH 6.0 in range from 1200 to −400 mV. Scan rate: 100 mV s^{-1}, [Phenol]$=2\times10^{-3}$ mol L^{-1}; (B) Amperometry of phenol in 3.8×10^{-8} mol L^{-1} HRP, 0.6×10^{-3} mol $L^{-1}H_2O_2$, 2.5×10^{-6} mol L^{-1} phenol at pH 7.0; (C) Hydrodynamic voltammetry (the same conditions that for amperometry); (D) Kinetic studies of the enzymatic reaction. Graphic of current intensity (mA) obtained by SWV vs. the reaction time (s) in 4.8×10^{-8} mol L^{-1} HRP, 1.0×10^{-3} mol $L^{-1}H_2O_2$, 2.5×10^{-6} mol L^{-1} phenol at pH 6.0.

First, the most sensitive electrochemical technique for phenol detection was chosen between SWV and amperometry. As in the quantification of phenol and its derivatives, the oxidation products generated by the reaction of HRP are reduced electrochemically therefore both techniques must be utilized in cathodic branch. Then, the applied potential range for SWV was ascertained by observing the cathodic branch of cyclic voltammogram of phenol therefore the potential range from 500 to 50 mV was selected for this technique. On the other hand, the applied potential in amperometry was ascertained by hydrodynamic voltammetry. A typical amperogram for phenol is shown in the Fig. 2B. The hydrodynamic voltammetry was performed in stirred enzymatic solutions by applying decreasing potentials at the working electrode in steps of 50 mV in the cathodic branch (see Fig. 2C). Since the maxima electrochemical signal was achieved at potential values lesser than 180 mV, a potential enough low of 50 mV was selected as applied potential in amperometry for phenol quantification. Next, the enzymatic response at enzymatic concentration of 3.8×10^{-8} mol L^{-1}, H_2O_2 concentration of 0.6×10^{-3} mol L^{-1} and phenol concentration of 2.5×10^{-6} mol L^{-1} in phosphate buffer 0.1 mol L^{-1} and KCl 0.1 mol L^{-1}, pH$=$7.0 were measured by SWV and amperometry. The current densities of 3,7 μA cm^{-2} (RSD%$=$12%, $n=7$) at 3 min of reaction and of 0.40 μA cm^{-2} (RSD%$=$14%, $n=8$) were obtained by SWV and amperometry, respectively. Based on these data, SWV showed higher sensitivity than amperometry therefore SWV was selected as electrochemical technique for the univariate calibration of phenol and for multivariate calibration of phenolic compounds (in Section 3.3.).

Second, in order to select the best experimental conditions of the enzymatic reaction, a systematic optimization procedure of three factors involved in the enzymatic reaction was carried out using response surface methodology through a central composite design [24–26] by Design-Expert 7.1.6. The evaluated factors were A$=H_2O_2$ concentration $(0.9–1.7)\times10^{-3}$ mol L^{-1}, B$=$enzymatic concentration $(2.9–4.8)\times10^{-8}$ mol L^{-1}, C$=$Buffer pH (pH from 6.0 to 9.0, acetate/phosphate/borate 0.05 mol L^{-1} and KCl 0.1 mol L^{-1}), and the phenol concentration was kept constant at 2.5×10^{-6} mol L^{-1}. In all cases, the conditions for the enzymatic reaction were kept in order 0 towards the hydrogen peroxide and in order 1 towards phenol. A two-factor interaction linear model was chosen by the ANOVA test for fitting this system. Only the interaction between pH and enzymatic concentration was significant. The numeric optimization both of the factors and of the response was performed by obtaining the best value of desirable response or desirability. The maximum desirability reached was 0.85 and under this condition, the theoretical optimum values for the buffer pH, the H_2O_2 concentration and enzymatic concentration were 6.0, 1.0×10^{-3} mol L^{-1} and 4.8×10^{-8} mol L^{-1} respectively. In order to corroborate experimentally these parameters, five SW voltammograms were performed and the reduction current intensity peak of phenol was recollected as response at 325 mV. The response predicted by model and the response obtained experimentally were 8.8 μA cm^{-2} and 7.9 ± 0.9 μA cm^{-2} ($n=5$), respectively. These responses were not significantly different when were compared by a mean comparison test with alpha level of 0.05 [27]. Based on this evidence, the values optimized of three factors were selected for the univariate calibration of phenol and for multivariate calibration of phenolic compounds (in Section 3.3).

Third, the enzymatic reaction time was selected by a kinetic study under optimum conditions. The enzymatic reaction time, which is the

time of sampling of the electrochemical signal or reduction current peak obtained by SWV, was evaluated for 10 min by performing a voltammograms each minute. Due to a continuous increment of 0.29 μA of current intensity was obtained for each cycle, a reaction time of 5 min was selected to guarantee an appropriate level of sensitivity (see Fig. 2D). Therefore, the reduction current density at 325 mV obtained at 5 min was 6.7 μA cm^{-2} (RSD%=12%, n=3).

Finally, the univariate calibration for phenol was performed by WLS, since the errors did not exhibit constant variance. The experimental data showed heterocedastic distribution where the variances increase proportionally with the concentration and show a conic or funnel shape along the concentration. In order to calculate the figures of merit, the statistical parameters were obtained from the weighted regression [23]. The figures of merit obtained were a linear range of $(1.95–5.50)\times 10^{-6}$ mol L^{-1}, an analytic sensitivity of 7.5×10^{6} L mol^{-1} and a relative standard deviation (RSD%) of 8.7 (n=3). In this case, the RSD% was calculated as (SD/m)×100, where SD was standard deviation of slope and m was the slope or sensitivity. The limit of detection (LOD) of 6.5×10^{-7} mol L^{-1} and the limit of quantification (LOQ) of 1.95×10^{-6} mol L^{-1} were lower than the reported values by Jun-ping et al. [28]. The LOD and LOQ were calculated as follows: LOD=3×SD/sensitivity; LOQ=10×SD/sensitivity, where SD is the standard deviation of the blank signal. On the other hand, our limits were lower lightly than the reported values by Azevedo et al. [29].

3.2. Phenol univariate calibration in groundwater samples

Samples (1 and 2) collected in locations close to a tannery from Castellanos Department (Santa Fe, Argentine) were assessed by enzymatic electrochemical detection coupled with univariate calibration. Before quantifying of phenol by this procedure, these samples should be analyzed by SWV in the range of tested potential. The absence of response suggested absence of the electrochemical interferences in the tested range. Despite the fact that the samples 1 and 2 were collected at 100 m and at 200 m of the tannery, no response of phenol was obtained, indicating its absence or a lower phenol concentration than the LOD (i.e. 6.5×10^{-7} mol L^{-1}).

The recovery and repeatability tests were also studied by spiking of phenol at two concentration levels in the groundwater samples. The average recovery values obtained for sample 1 were 102 (RSD%=11%, n=8) and 98 (RSD%=9%, n=8) and the values obtained for sample 2 were 99 (RSD%=7%, n=8) and 101 (RSD%=11%, n=8). These recovery values demonstrate absence of matrix effect and the RSD% values satisfy the regulation for the concentration levels analyzed [30,31].

3.3. Multivariate calibration for phenolic compound determinations

The first step in multivariate calibration analysis by PLS is the selection of an appropriate sensor range and of the optimum number of factors (latent variables), which can improve the predictive ability and robustness of calibration model.

In our case, an appropriate potential range of SWV should be selected in analogy to spectral regions in spectroscopic analysis coupled with a multivariate calibration [23,19]. For that reason, the applied potential ranges in SWV were ascertained by observing of cyclic voltammograms for the different phenolic compounds (see Fig. 3).

From cyclic voltammograms of pyrocatechol, p-aminophenol and hydroquinone can be observed well-defined electrochemical couples. On the contrary for phenol and p-chlorophenol, the oxidation peak currents were much larger than those corresponding to the reduction peaks, indicating irreversible electrode processes. After observing these voltammograms, for each compound, the selection

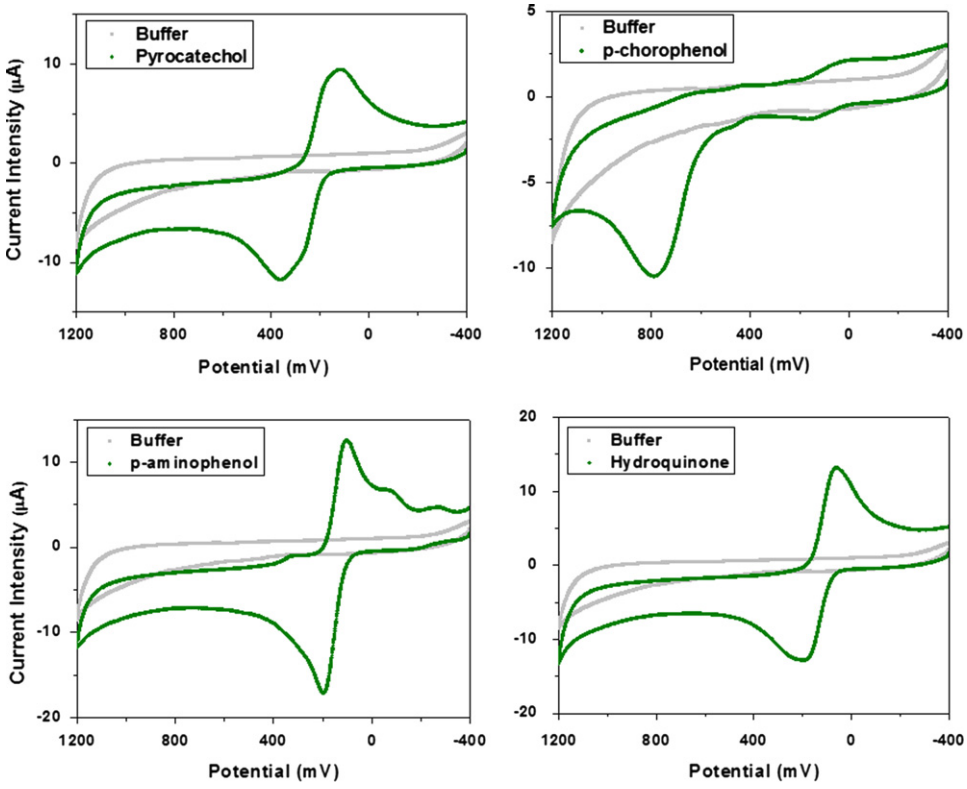

Fig. 3. Cyclic voltammograms of p-chlorophenol, pyrocatechol, p-aminophenol, hydroquinone and supporting electrolyte solution pH 6 in range from 1200 to −400 mV. Scan rate: 100 mV s^{-1}, [Phenolic Compound]=2.0×10^{-3} mol L^{-1}.

of appropriate potential range in the cathodic branch was performed by avoiding the electrochemical interference of the other branch.

In order to confirm experimentally the potential ranges selected, SW voltammograms for each compounds were performed. For each voltammogram, the buffer pH, H_2O_2 concentration and enzymatic concentration were kept at the optimal conditions obtained by the composite central design for phenol while that the concentration of each phenolic compound was kept constant at 2.5×10^{-6} mol L^{-1}. Thus, the reduction peak potentials obtained in SWV were 325, 430, −110, −50 and −150 mV for phenol, p-chlorophenol, p-aminophenol, pyrocatechol and hydroquinone, respectively.

Due to the fact that these potentials were appreciably different, we selected regions of voltammograms where components of the mixture showed significant electrochemical information. Therefore, the potential range to construct the calibration and prediction matrix was constituted by sum of two ranges: the first from 500 to 250 mV for phenol and p-chlorophenol, plus the second from 0 to −400 mV for p-aminophenol, pyrocatechol and hydroquinone (see Figs. 4 and 5).

A kinetic study was performed to select the time of sampling of the voltammograms. In order to do that the buffer pH, H_2O_2 concentration and enzymatic concentration were applied according to Section 3.1, while that a mixture of five phenolic compounds was kept constant at concentration level minimum for each compound according to Table 2. Then, this enzymatic reaction time was evaluated in 10 cycles during 10 min by SWV in both potential ranges. A time of sampling of 4 min was chosen because in this time, the voltammograms reached current intensity levels that guaranteed an appropriate sensitivity for the method.

Then the multivariate calibration was applied for five phenolic compounds and the electrochemical data analysis was performed by PLS1. To fulfill with this algorithm, in the calibration and prediction steps, several concentration levels (according to Table 2) were employed to obtain an adequate model. Estimating the number of factors in the calibration stage was done by cross validation according to criterion of Haaland and Thomas [32]. Previously at the PLS regression, the pre-processing of data of actual and artificial samples should be performed. There are several pre-processings of data and are frequently utilized for spectral data but there are not so much bibliography related to their applications to electrochemical data. The mean centering preprocessing is the most employed. Multiplicative signal correction (MSC) and standard normal variate (SNV) are designed to remove additive and multiplicative effects related with spectral data [23,33–35].

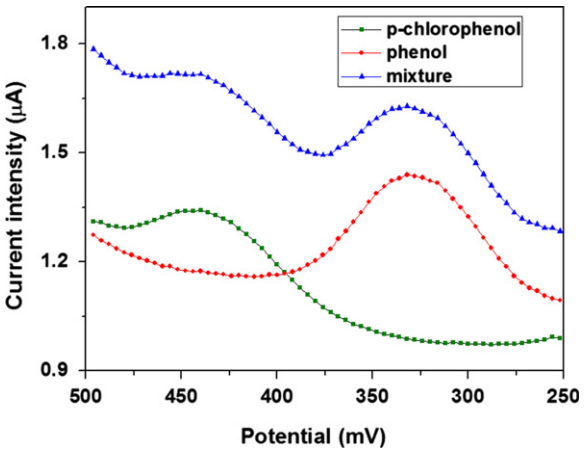

Fig. 4. SW voltammograms of phenol, p-chlorophenol and (phenol/p-chlorophenol) mixture in range from 500 to 250 mV, [Phenolic Compound]= 1.0×10^{-6} mol L^{-1}.

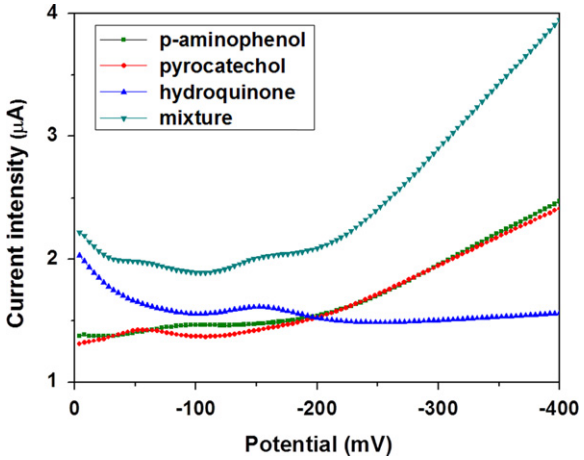

Fig. 5. SW voltammograms of p-aminophenol, hydroquinone, pyrocatechol and (p-aminophenol/hydroquinone/pyrocatechol) mixture in range from 0 to −400 mV, [Phenolic Compound]= 1.0×10^{-6} mol L^{-1}.

For the preprocessing of our data, SNV and MSC could be the most adequate methods, due to the resemblance between our data and spectral ones. In the present work, the selection of data preprocessing was based on recovery values obtained with the prediction samples. When applying SNV, the highest recoveries for all the compounds were obtained. Therefore, SNV was chosen as the preprocessing method. In addition, it was applied a moving window strategy to the calibration set, in order to find the most informative ranges in voltammograms by localizing the minimum calibration variance [36,37] but this optimization did not improvement the predictive performance with regard to the use of the previously selected ranges of sensors.

Therefore, after SNV preprocessing, the calibration was performed by PLS-1 and the statistical parameters were calculated. The root mean square error (RMSE) measures the average error in the analysis and evaluate the goodness of fit of the calibration data [19,23,38–40]. Consequently, as can be seen in Table 3, the calibration parameters are acceptable for the five compounds and these parameters would indicate that the least squares adjustment is appropriate to model the complex system under study. Apparently factor numbers (latent variables) used and the current densities matrix constructed with two ranges of potentials were adequate for the model.

On the other hand, the employment of an experimental design blocked according to the used electrode could contribute to improve the system precision. The overall effectiveness of the PLS model for predicting of phenolic compounds in the validation set was ascertained by calculating the relative error of prediction (REP) and percentage average recoveries. The calculated values were acceptable according to the concentration levels analyzed and the bibliography [30,31].

For five phenolic compounds, the LODs oscillated from 0.6 to 1.4×10^{-6} mol L^{-1}. Although these limits were higher than other mentioned values in literature for the electrochemical detection coupled with univariate calibration of phenol [28], hydroquinone [41], p-aminophenol [42], p-chorophenol [43] and pyrocatechol [7,44]; they were similar to obtained values by spectrophotometric methods coupled with artificial neural network for multicomponent analysis of some phenolic compounds [45,46]. In addition, in the present work, it should be taken into account that it was analyzed a major number of phenolic compounds.

Table 3
Statistical parameters when applying PLS1 analyses for phenol, p-chlorophenol, p-aminophenol, pyrocatechol and hydroquinone.

Statistic parameters	Phenol	p-chlorophenol	p-aminophenol	pyrocatechol	hydroquinone
Sensor range	1–62	1–62	63–162	63–162	63–62
Number of factors	2	2	3	3	3
[a]RMSECV ($\times 10^{-6}$ mol L^{-1})	0.1_0	0.4_2	0.3_7	0.2_9	0.3_8
[b]RMSEP ($\times 10^{-6}$ mol L^{-1})	0.1_7	0.7_3	0.5_5	0.6_2	0.4_6
[c]REP (%)	8.5	6.6	13.5	7.9	3.6
Average recovery (%) of prediction set	89	86	95	94	96
[d]γ ($\times 10^6$ L mol^{-1})	2.9	2.4	4.7	4.1	5.5
[e]LOD ($\times 10^{-6}$ mol L^{-1})	1.1	1.4	0.7	0.8	0.6

RMSE=root mean square error, $RMSE=[\left(1/n\right)\ \sum_1^n (C_{act}-C_{pred})^2]^{1/2}$.

[a] RMSECV=root mean square error of calibration when cross-validation is applied where n: is the number of calibration samples and C_{act} and C_{pred} are the actual and predicted concentrations, respectively.

[b] RMSEP=root mean square error of prediction where n: is the number of validation samples and C_{act} and C_{pred} are the actual and predicted concentrations, respectively.

[c] REP (%)=relative error of prediction, $REP(\%)=(RMSEP/C_{mean})\times 100$ where C_{mean} is the average phenolic compound concentration for n samples.

[d] (γ) is the analytical sensitivity,$\gamma=SEN_n/Sy$, where Sy is the instrumental noise and SEN_n is the sensitivity. The Sy and SEN_n values are averages of the values corresponding to n validation samples, Ref. [38].

[e] LOD=limit of detection, calculated as $3.3\times\gamma^{-1}$, reference [39].

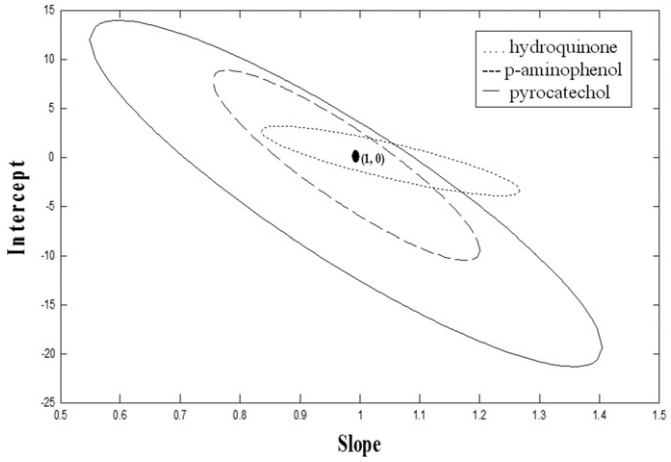

Fig. 6. Comparison of elliptical joint regions (at 95% confidence level) for hydroquinone (dotted line), p-aminophenol (dash line) and pyrocatechol (solid line) system. The central point indicates the theoretical point (intercept: 0, slope:1).

Finally, in order to determine the accuracy and precision of the method, only the compounds that showed greater degree of overlapping in their voltammograms and lower LODs (p-aminophenol, pyrocatechol and hydroquinone) were analyzed by EJCR (see Fig. 6). In this test, the size of the ellipses and the including of the ideal point (1,0) were analyzed for evaluating the precision and accuracy, respectively. The ideal point was included within the ellipses for all compounds. The imprecision order increased in the sequence from hydroquinone, p-aminophenol, to pyrocatechol.

3.4. Phenolic compounds multivariate calibration in water samples

In order to evaluate the applicability of the proposed method, the five phenolic compounds were analyzed simultaneously in different water samples. Thus, nine water samples (from 3 to 11) without pretreatment were analyzed by the optimized enzymatic electrochemical detection coupled with multivariate calibration. The matrix constructions for PLS1 analysis were performed the same way that calibration and prediction stages. Table 4 summarizes the information provided of PLS1 about quantification. The water samples were collected close to tanneries and landfills, such samples were four groundwater samples, two superficial samples and three stream samples. The tanneries from departments: Castellanos, Las Colonias and La Capital are still in activity while the tannery of the General Obligado department was closed ten years ago.

In the quantification assays, phenol was not found in any sample; this evidence suggests its possible degradation or interaction in the environment. Hydroquinone used commonly as a bleaching product was only detected in stream water near public landfill. Unfortunately, the degradation products (p-benzoquinone, etc.) of this compound [47] in environmental cannot be detected by this method. P-chlorophenol, a pesticide residue, was detected in all samples. Therefore, its presence can be correlated with the intensive use of pesticide in the regions where the water samples were recollected. Pyrocatechol and p-aminophenol, two highly reactive compounds, were only found in the samples collected at 100 m from the tanneries.

Since these compounds in water samples were found in very low concentrations, the performance of the method was assessed by recovery studies therefore the samples without pretreatment were spiked with five compounds at single concentration level (an intermediate concentration for each compound "center level" according to CCD in Table 2). As can be seen in Table 4 about the recovery studies, the average values ranged from 50 to 100%, indicating a great variability in the matrix effects between samples. The higher recoveries were found for groundwater samples (3–6), with higher values than 80%. On the contrary, lower recoveries were observed for the samples (7–11) with a high number of particles in suspension.

P-aminophenol and hydroquinone could not be recovered or showed very low recovery values. These low values could be explained by a high reactivity of these amines with the environmental matrix. In the most turbid sample, the stream of La Capital, phenolic compounds were not recovered. These results strongly suggest that the presence of humic substances (dissolved organic matter at low concentrations) in these samples would act as complexing reagents of phenolic compounds, transforming them into less bioavailable for the enzymatic reaction. It well knows that the exposure to an ecotoxic compound of an organism or an active biological molecule (in our case, an enzyme) in environmental medium is not related to the total concentration of that substance in the medium but rather to the amount that is actually available or bioavailable [48,49].

For this reason, after a more complete validation, the developed method could be included in the protocol for the determination of bioavailability and biokinetics of ecotoxic compounds. It well knows that the knowledge of biodegradation kinetics and

Table 4
Quantification and recovery studies for each phenolic compound in different water samples.

Samples ($n=3$)	Quantification[a] (Concentration $\times 10^{-6}$ mol L^{-1})					% Average recovery[b,c]				
	phenol	p-chlorophenol	hydroquinone	pyrocatechol	p-aminophenol	phenol	p-chlorophenol	hydroquinone	pyrocatechol	p-aminophenol
-3-	ND	6.0	ND	18.6	18.6	93 (11)	90 (9)	84 (11)	98 (10)	95 (7)
-4-	ND	5.9	ND	ND	ND	90 (8)	92 (11)	94 (5)	92 (18)	89 (12)
-5-	ND	10.7	ND	21.0	20.0	94 (12)	102 (11)	91 (12)	93 (9)	85 (5)
-6-	ND	9.0	ND	ND	ND	90 (14)	90 (4)	96 (14)	90 (6)	90 (10)
-7-	ND	10.5	ND	ND	ND	83 (7)	93 (13)	55 (11)	87 (14)	U
-8-	ND	11.9	ND	22.4	22.0	81 (12)	97 (15)	63 (9)	91 (7)	54 (9)
-9-	ND	10.6	ND	ND	ND	U	U	U	U	U
-10-	ND	9.4	11.5	ND	ND	69 (11)	53 (15)	62 (6)	78 (9)	U
-11-	ND	10.5	ND	ND	6.0	87 (9)	87 (10)	85 (9)	72 (7)	77 (10)

[a] ND=not detected.
[b] U=unrecovered.
[c] Values between parenthesis correspond to standard deviation (SD) for $n=3$.

the bioavailability of organic pollutants can facilitate decisions on bioremediation treatments of contaminated areas [50]. In addition, the evidences obtained make us also rethink about new strategies for quantification analysis, for example, to address this problem in future approaches by using other algorithms that have the second-order advantage could be assayed [51].

4. Conclusions

First, the enzymatic electrochemical detection coupled with a univariate calibration method with limit of detection of 6.5×10^{-7} mol L^{-1} for phenol was optimized and applied to the determination of phenol in environmental samples. After a more complete validation, this method could be used for the fast and simple phenol quantification in this kind of environmental water samples.

Second, five phenolic compounds (phenol, p-chlorophenol, p-aminophenol, pyrocatechol and hydroquinone) in water samples were simultaneously evaluated by enzymatic electrochemical detection coupled to multivariate calibrations. In the optimization stage, two potential ranges were utilized for evaluating them by PLS1 as chemometric tool. Although the LODs for phenolic compounds (from 0.6 to 1.4×10^{-6} mol L^{-1}) were higher than the maximum permitted level in drinking water, but in the context of assessing the bioavailability, the proposed methods could be useful as screening tests for environmental samples as well as for control of the wastewater and industrial effluent treatment.

We were able to demonstrate the applicability of an inexpensive alternative analytical method and the obtained results were good, taking into account of the simple-pretreatment of the samples and the matrix complexity. However, these results are only preliminary and they should be confirmed in additional studies by applying chromatographic separations where phenolic compounds should be assessed against a standard curve of corresponding compounds.

In future, the approach proposed could be improved by utilizing screen-printed electrodes with the immobilized enzyme, and the electrochemical measures could be also done on field by using a portable potentiostat.

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