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Evaluation of seven cosubstrates in the quantification of horseradish peroxidase enzyme by square wave voltammetry

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ABSTRACT

The electrochemical detection for horseradish peroxidase–cosubstrate–H_2O_2 systems was optimized. o-Phenilendiamine, phenol, hydroquinone, pyrocatechol, p-chlorophenol, p-aminophenol and 3,3′-5,5′-tetramethylbenzidine were evaluated as cosubstrates of horseradish peroxidase (HRP) enzyme. Therefore, the reaction time, the addition sequence of the substrates, the cosubstrate:H_2O_2 ratio and the electrochemical techniques were elected by one-factor optimization assays while the buffer pH, the enzymatic activity and cosubstrate and H_2O_2 concentrations for each system were selected simultaneously by response surface methodology. Then, the calibration curves for seven horseradish peroxidase–cosubstrate–H_2O_2 systems were built and the analytic parameters were analyzed. o-Phenilendiamine was selected as the best cosubstrate for the HRP enzyme. For this system the reaction time of 60 s, the phosphate buffer pH 6.0, and the concentrations of 2.5×10^{-4} mol L^{-1} o-phenilendiamine and of 1.25×10^{-4} mol L^{-1} H_2O_2 were chosen as the optimal conditions. In these conditions, the calibration curve of horseradish peroxidase by square wave voltammetry showed a linearity range from 9.5×10^{-11} to 1.9×10^{-8} mol L^{-1} and the limit of detection of 3.8×10^{-11} mol L^{-1} with RSD% of 0.03% ($n = 3$).

1. Introduction

In most immunoassays, the HRP and alkaline phosphatase enzymes are utilized as tracer in detection systems. HRP is ideal for these applications because it is smaller, more stable and less expensive than alkaline phosphatase. It also has a high turnover rate that allows generation of strong signals in a relatively short time [1]. Although the literature is very wide about these enzymes, there is not any agreement in the ratio more appropriate between the H_2O_2 and cosubstrate concentrations, especially for electrochemical detection systems. For example, for the cosubstrate more widely used, TMB (3,3′,5,5′-tetramethylbenzidine), the TMB:H_2O_2 ratios were 1:1 (4×10^{-4} mol L^{-1}); 2:1 ($2.5{:}1.3 \times 10^{-4}$ mol L^{-1}); 1:2 ($2{:}4 \times 10^{-4}$ mol L^{-1}); 1.5:1 ($4.2{:}2.7 \times 10^{-4}$ mol L^{-1}) according to references [2–5] respectively.

The enzymatic reaction of HRP follows a ping-pong mechanism [6–9]. In the first stage, a peroxide molecule is bound to a free coordination site of iron (Fe^{III}) in the HRP and is reduced to water in a rapid two-electron process, whereby the compound I is generated as the stable primary intermediary. The compound I is the oxyferryl species ((Fe^{IV} = O)P^{*}) constituting by one oxygen atom from a molecule of peroxide, one electron comes from iron and other electron is withdrawn from the heme group to generate a porphyrin π cation radical. In the next stage the porphyrin π cation radical is reduced by one reduced cosubstrate molecule to compound II species ((Fe^{IV} = O)P) which is subsequently reduced to the remaining native enzyme (Fe^{III}) by other reduced cosubstrate molecule (see Fig. 1a). The reduced cosubstrate molecules could be aromatic amines, phenols, hexacyanoferrates, ascorbates and iodine [10–12]. Some examples of HRP electron donor cosubstrates are phenol [13], o-phenilendiamine [14,15], TMB [16–18], hydroquinone [19,20], p-chlorophenol [21], pyrocatechol [22] and p-aminophenol [23] (see Fig. 1b). As it is well known, the routine methods for the HRP activity determination involve colorimetry and fluorimetry technique. In the last decades the electrochemical-device technology seeks the miniaturization and generation of potentially automatic devices with more sensitivity and faster analytical signals. These electrochemical devices are potentially useful for the determination of different nature analytes.

Numerous papers describe the amperometric analysis for products of the catalyzed reaction by HRP [11,16,20]. Despite the fact that the amperometry is the electrochemical technique more often used; it is less sensitivity and slower than the pulse techniques such as differential pulse voltammetry (DPV) and square wave voltammetry (SWV) [24].

The optimization of an analytical system searches the improvement of its performance to obtain its maximum response with economy of resources. Traditionally, the optimization of an

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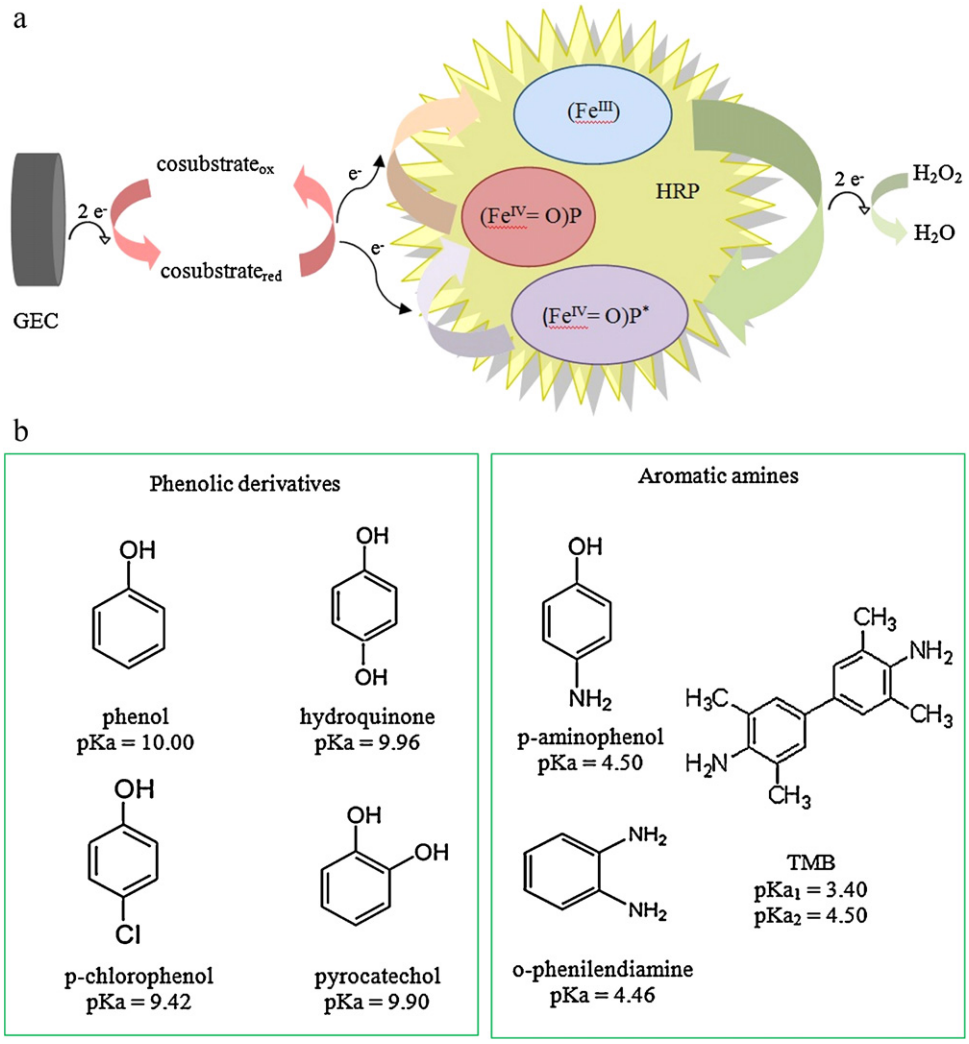

Fig. 1. (a) Enzymatic mechanism of HRP over electrode surface and (b) the chemical structures of the HRP cosubstrates.

experimental response in analytical chemistry is carried out with the analysis of one factor at a time. In this case, one parameter or factor is changed while the others are at constant level. This optimization technique is called one-variable analyzed at-a-time or one-factor optimization. Its major disadvantage is that it does not include effects by the interaction among the studied factors. As a consequence, this technique does not describe the complete effects of the parameters about the response. Another disadvantage is the increase in the number of experiments, which causes the enhancement in time and reagents. Moreover these pre-selected working conditions can be distant of "optimal conditions" in the system. To overcome this problem, multivariate statistical techniques can be used in the optimization of analytic procedures, being one of the most important the response surface methodology (RSM) [25,26]. The RSM, being a set of mathematical and statistical techniques, is useful in the systematic optimization and is used to explore, analyze and optimize a system whose response depends on several factors. Before applying the RSM, the selection of an experimental design should be carried out for defining experiments in the studied experimental region. The central composite design (CCD) is widely used in the construction and estimation of second-order response surfaces [27–29].

Consequentially, this work presents the study about the enzymatic behavior of HRP with H_2O_2 as substrate and phenol, o-phenilendiamine, TMB, hydroquinone, p-chlorophenol, pyrocatechol and p-aminophenol as cosubstrates. Although the phenol is a pollutant, it was included as model cosubstrate because it has a simple chemical structure. On the one hand, the time of reaction, the addition sequence of substrate, the cosubstrate:H_2O_2 ratio and the electrochemical technique were selected by the one-factor optimization. On the other hand, the cosubstrate and substrate concentrations, the enzymatic activity and buffer pH for each HRP–cosubstrate–H_2O_2 system were chosen by response surface methodology. Then, calibration curves for the determination of the enzymatic concentration were built for seven HRP–cosubstrate–H_2O_2 systems in optimal conditions.

2. Materials and methods

2.1. Instrumentation

Cyclic voltammetry, square wave voltammetry, 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 [30–32]; platinum as auxiliary electrode and Ag/AgCl as reference electrode (Orion 92-02-00). The effective areas of electrodes were 0.21 cm^2 (RSD% = 14%, n = 10) by chronoamperometry with 2 mM potassium ferricyanide.

Table 1
Conditions of seven HRP–cosubstrate–H_2O_2 systems in hydrodinamic voltammetry and SWV.

Systems	Hydrodinamic voltammetry[a]				SWV[a]			
	[HRP] (mol L^{-1})	[Cosubstrate] (mol L^{-1})	[H_2O_2] (mol L^{-1})	pH	[HRP] (mol L^{-1})	[Cosubstrate] (mol L^{-1})	[H_2O_2] (mol L^{-1})	pH
HRP–H_2O_2–phenol	1.9×10^{-8}	0.1×10^{-3}	0.25×10^{-3}	7.0	3.8×10^{-8}	0.5×10^{-3}	0.25×10^{-3}	7.5
HRP–o-phenilendiamine–H_2O_2	9.5×10^{-9}	0.25×10^{-3}	0.125×10^{-3}	6.0	9.5×10^{-9}	2.5×10^{-3}	1.25×10^{-3}	6.0
HRP–p-chlorophenol–H_2O_2	1.9×10^{-8}	2.0×10^{-3}	2.0×10^{-3}	6.0	1.9×10^{-8}	2.0×10^{-3}	2.0×10^{-3}	6.0
HRP–hydroquinone–H_2O_2	1.9×10^{-8}	2.0×10^{-3}	2.0×10^{-3}	7.5	1.9×10^{-8}	2.0×10^{-3}	2.0×10^{-3}	7.5
HRP–TMB–H_2O_2	1.9×10^{-8}	2.0×10^{-3}	2.0×10^{-3}	6.0	3.8×10^{-8}	1.0×10^{-3}	4.0×10^{-3}	7.0
HRP–pyrocatechol–H_2O_2	9.5×10^{-9}	0.5×10^{-3}	0.25×10^{-3}	6.0	9.5×10^{-9}	2.0×10^{-3}	0.5×10^{-3}	6.0
HRP–p-aminophenol–H_2O_2	3.8×10^{-8}	0.25×10^{-3}	0.125×10^{-3}	5.0	3.8×10^{-8}	2.5×10^{-3}	2.5×10^{-3}	5.0

[a] In phosphate buffer solution 0.1 mol L^{-1} and KCl 0.1 mol L^{-1}.

2.2. Reagents and solutions

Horseradish peroxidase (1310 U mg^{-1}), hydroquinone, 3,3′,5,5′-tetramethylbenzidine and pyrocatechol were purchased from Sigma. All other reagents, such as hydrogen peroxide, phenol, p-chlorophenol, o-phenilendiamine, p-benzoquinone and p-aminophenol were also analytical grade. Phenol, hydroquinone, H_2O_2 and pyrocatechol solutions were prepared with Millipore water. o-Phenilendiamine, p-chlorophenol, p-aminophenol and p-benzoquinone solutions were prepared in ethanol:Millipore water (50:50), while 3,3′,5,5′-tetramethylbenzidine solution was dissolved in dimethylsulphoxide. Working buffer solutions were phosphate 0.1 mol L^{-1} and KCl 0.1 mol L^{-1} (for buffering from pH 5.0 to 7.0) and acetate/phosphate/borate 0.05 mol L^{-1} and KCl 0.1 mol L^{-1} (for buffering from pH 4.0 to 9.0).

2.3. Electrochemical characterization of cosubstrates

The electrochemical behaviors of phenol, o-phenilendiamine, TMB, hydroquinone, p-chlorophenol, pyrocatechol and p-aminophenol were analyzed by cyclic voltammetry (scan rate 0.1 V s^{-1}) in the range from 800 to −800 mV. Cosubstrate 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.

2.4. Optimization of experimental conditions

The enzymatic assays were performed in a reaction cell of 5.0 mL; with 4.0 mL of buffer and KCl 0.1 mol L^{-1} to variable pH where small volumes (μL) of HRP enzyme, cosubstrate and H_2O_2 at different concentrations were added.

On the one hand, a systematic optimization procedure of four factors involved in the enzymatic reaction was performed by using response surface methodology through a central composite design by Design-Expert 7.1.6. The evaluated factors were A = buffer pH (pH from 4.0 to 9.0, acetate/phosphate/borate 0.05 mol L^{-1} and KCl 0.1 mol L^{-1}), B = enzymatic concentration (9.5–38.2 nM), C = cosubstrates concentration ($0.5–10\times10^{-3}$ mol L^{-1}) and D = H_2O_2 concentration ($2–10\times10^{-3}$ mol L^{-1}).

On the other hand, the selection of the electrochemical technique between square wave voltammetry and amperometry were performed for each HRP–cosubstrate–H_2O_2 system. In order to do that, cosubstrate, H_2O_2 and enzyme concentrations were kept according to the central composite design.

2.5. Electrochemical detection

2.5.1. Amperometry

The applied optimum voltage in amperometry for each system was ascertained by hydrodynamic voltammetries. These were performed by amperometry in stirred enzymatic solutions by applying decreasing potentials at the working electrode in steps of 50 mV in the cathodic branch. Then, the steady-state reduction current responses were plotted vs. applied potential. Each experiment was performed in triplicate with a fresh electrode and the results quoted represent the average reduction current values. Table 1 shows experimental conditions of seven HRP–cosubstrate–H_2O_2 systems analyzed by hydrodynamic voltammetry.

2.5.2. Square wave voltammetry

Square wave voltammetries (step height = 4 mV, amplitude = 25 mV and square-wave frequency = 15 Hz) were carried out after 3 min of enzymatic reaction, sweeping the voltage in the cathodic branch for each enzymatic system. Table 1 shows experimental conditions of seven HRP–cosubstrate–H_2O_2 systems analyzed by SWV.

2.6. Determination of the apparent Michaelis constants

The apparent Michaelis constant (K'_m) and the apparent maximal velocity (V'_{max}) in the reaction of various cosubstrates and hydrogen peroxide with HRP were evaluated. In all assays the enzymatic activity and the buffer pH were kept at optimal condition of central composite design. On the one hand, the H_2O_2 concentration was constant and the cosubstrate concentration was varied in the range from 0.5 to 10×10^{-3} mol L^{-1} for each system. On the other hand, the cosubstrate concentration was constant and the H_2O_2 concentration was varied in the range from 0.5 to 10×10^{-3} mol L^{-1}.

The apparent constants of cosubstrates and H_2O_2 in each system were obtained using the Lineweaver–Burk method for the two-substrate ping-pong mechanism followed by the peroxidase [6–8]. Moreover, the turnover numbers (K_{cat}) and the catalytic efficiency (E_{ff}) were calculated.

2.7. Calibration curves for the determination of the enzymatic concentration

The calibration curves of the enzymatic response as current density (μA cm^{-2}) vs. the enzymatic concentration (mol L^{-1}) were performed. The buffer pHs were kept at optimal condition of central composite design while that the enzymatic concentration was ranged from 1.9×10^{-11} to 5.7×10^{-8} mol L^{-1}. 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) [33,34].

Table 2
Electrochemical parameters of each cosubstrate by cyclic voltammetry (scan rate = 0.1 V s^{-1}).

Cosubstrate[a]	Cyclic voltammetry		
	$E_{cathodic}$ (mV)	$I_{cathodic}/I_{anodic}$	ΔE (V)
Quasi-irreversible systems			
Phenol	500	0.04	0.157
o-Phenolendiamine	−47	0.1	0.470
p-Chlorophenol	−200	0.01	0.262
Quasi-reversible systems			
Hydroquinone	−78	1	0.133
TMB	−84	1	0.301
Pyrocatechol	188	1	0.193
p-Aminophenol	200	0.95	0.084

[a] [Cosubstrate] = 2×10^{-3} mol L^{-1} in phosphate buffer 0.1 mol L^{-1} and KCl 0.1 mol L^{-1}, pH 6.00.

3. Results and discussion

3.1. Electrochemical characterization of cosubstrates

The electrochemical behaviors of phenol, o-phenilendiamine, TMB, hydroquinone, p-chlorophenol, pyrocatechol and p-aminophenol were analyzed by cyclic voltammetry. The separations between peak potentials were higher than 0.059 V/n in all cases, evidencing slow electronic transferences. In addition the ratio of the reverse-to-forward peak currents was less than 0.1 for phenol, o-phenilendiamine, p-chlorophenol and was close to 1 for hydroquinone, TMB, pyrocatechol, p-aminophenol (see Table 2). Considering the experimental conditions, the nature of the working electrode and the observed electrochemical behaviors, we classified these compounds into quasi-irreversible and quasi-reversible systems whose current ratios were less than 0.1 and close to 1, respectively [24].

3.2. Electrochemical detection

As was mentioned above the enzymatic reaction of the peroxidase is based on the double displacement or "ping-pong" mechanism, in which H_2O_2 and the electron-donor cosubstrate are involved. In the enzymatic reaction, cosubstrates such as phenolic compounds or aromatic amines are converted mainly into quinones or free radical products, which are electroactives and can be electrochemically reduced on the electrode surface. Two electrochemical techniques such as amperometry and square wave voltammetry, were utilized as detection techniques of the enzymatic reaction. The hydrodynamic voltammetry was performed for each enzymatic system in stirred solutions to ascertain the applied potential in amperometry. The hydrodynamic voltammetries and the potentials that caused the maxima electrochemical signals are shown in Fig. 2. Then, potentials of 200, −125, −350, −210, −50, 50 and 50 mV were selected as applied potentials in the amperometric analysis for the systems of phenol, o-phenilendiamine, p-chlorophenol, hydroquinone, pyrocatechol, TMB and p-aminophenol, respectively. Systems with o-phenilendiamine, pyrocatechol, TMB and p-aminophenol as cosubstrates whose potentials are close to zero would be the most advantageous for future applications, since they would have less probability of electrochemical interferences. Moreover, the poor reversibility of cited compounds is an advantageous characteristic in this work for the application of the sweep techniques. This characteristic allowed the selection of an appropriated potential range in the cathodic branch without the electrochemical interference of the other branch for each system. Fig. 3 shows the voltammograms for each enzymatic system by SWV, whose potentials of reduction were displaced to zero with respect to obtained potentials in cyclic voltammetry. In addition, this figure shows the amperometric analysis for TMB. The cathodic current intensity peaks at potentials of 265, −140, −280, −150, 50, and 100 mV were observed and selected as electrochemical signals for the systems with phenol, o-phenilendiamine, p-chlorophenol, hydroquinone, pyrocatechol and p-aminophenol as cosubstrates, respectively. All cosubstrates presented typical voltammograms with well-defined peaks except for the pyrocatechol whose peak was much wider, evidencing that its electrode process can involve electro-polymerization.

On the other hand, the enzymatic system with TMB as cosubstrate could not be analyzed by SWV. Due to the oxidized TMB was generated at the initial potential in the SWV, an undesirable reduction current was obtained. Therefore, amperometry was applied as detection technique for the system of TMB.

3.3. Optimization of experimental conditions

The addition sequence of the substrates, the reaction time, the cosubstrate:H_2O_2 ratio and the electrochemical technique were

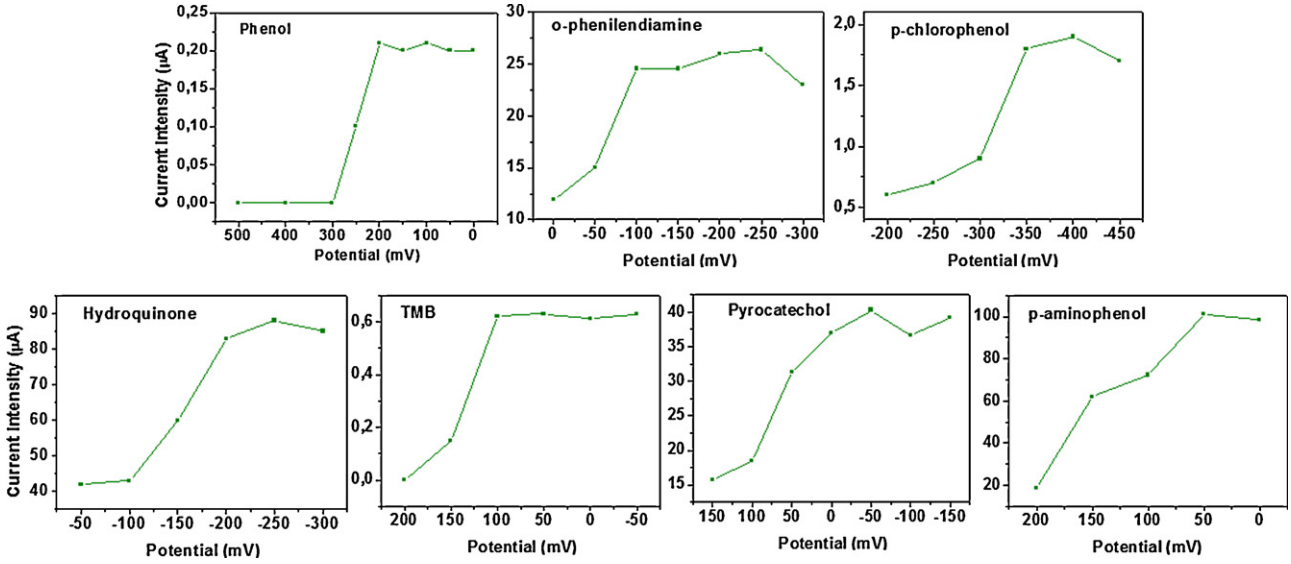

Fig. 2. Hydrodynamic voltammetry. Table 1 shows experimental conditions for seven HRP–cosubstrate–H_2O_2 systems.

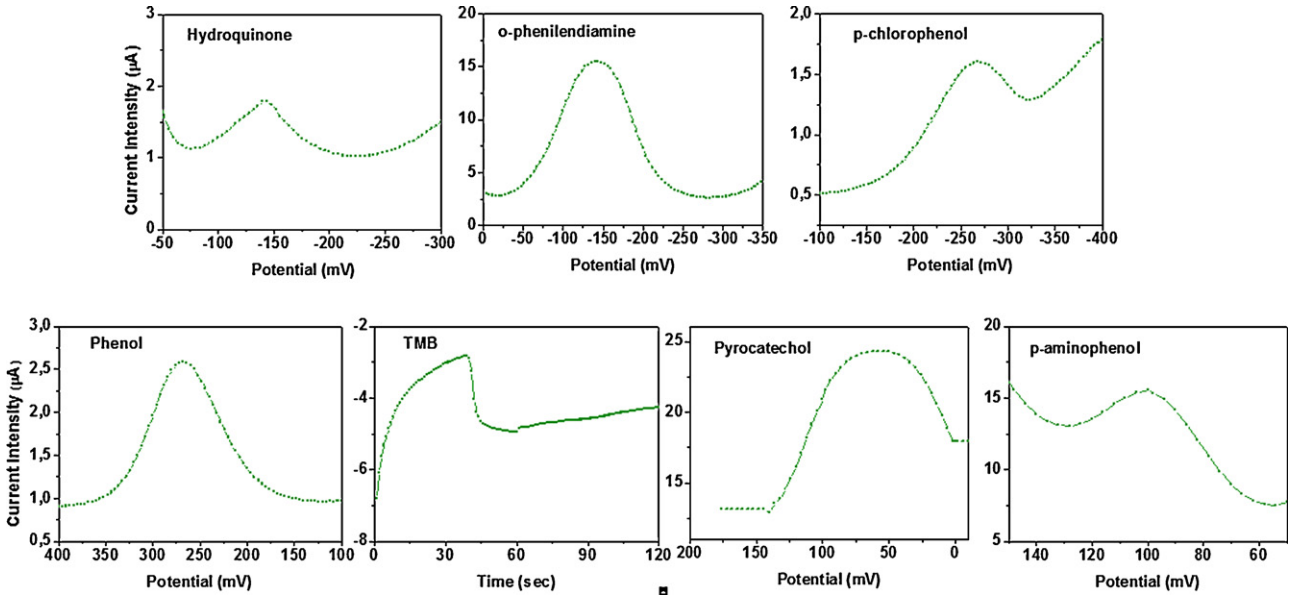

Fig. 3. Square wave voltammetry and amperometry. Table 1 shows experimental conditions for seven HRP–cosubstrate–H_2O_2 systems.

opted by one-factor optimization assays, while the buffer pH, the enzymatic activity and cosubstrate and H_2O_2 concentrations for each system were selected simultaneously by response surface methodology.

First, the experiments were performed in two ways by adding of the cosubstrate and afterward H_2O_2 to the enzymatic reaction medium, and vice versa, to ascertain the addition sequence of substrates that guarantees a maxima electrochemical signal at 3 min of the reaction. Cuadrado et al. [35] suggested that the binding between the enzyme and its substrates would proceed via random and that the binding of the peroxide with the enzyme before the binding with its cosubstrate is not necessary [35]. Our experiments demonstrated that the cosubstrate–H_2O_2 addition order produced maxima electrochemical signals for all cosubstrates except for the cosubstrate with the simpler molecule, i.e. phenol. This suggests that the cosubstrates with more complex molecules than the H_2O_2 molecule would diffuse slowly toward the active site of the enzyme and consequently they would be the restrictive reagents for the generation of a gradient of appropriate diffusion to originate the enzymatic reaction.

Second, a kinetic study for each system was performed by SWVs to select the reaction time, which is the time of sampling of the electrochemical signal. Fig. 4 shows the kinetics of the system with hydroquinone as cosubstrate. A first range from 0 to 10 min was performed in 10 cycles. Since a maximum response was obtained before to 1 min, a second range from 0 and 3 min was evaluated, in which electrochemical signals every 20 s were recollected (see insert in figure). The selected time for the HRP–HQ–H_2O_2 system was 20 s and for other systems can be seen in Table 3. A contrasting kinetic behavior can be observed among the studied compounds, since the systems with hydroquinone and p-aminophenol as cosubstrates were the fastest (20 s), while the systems with phenol and pyrocatechol required the higher reaction time (300 s). Reaction times of other systems varied within 60 s.

Third, an optimal combination of experimental factors was researched for seven systems to optimize the enzymatic response coupled to electrochemical detection. The studied factors were the buffer pH (A), enzymatic concentration (B), cosubstrate concentration (C) and H_2O_2 concentration (D). Therefore a central composite design of 30 experiments with six center points was performed. This is often used as useful tool to reduce the number

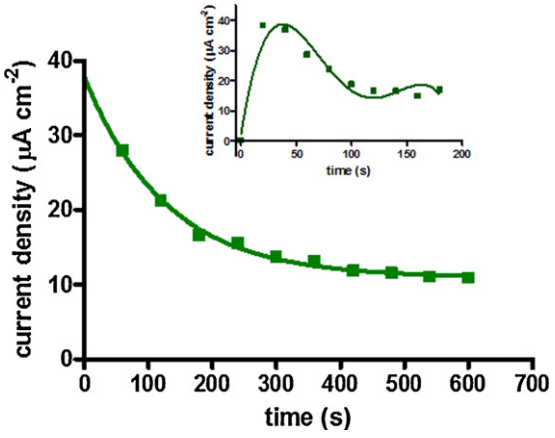

Fig. 4. Kinetic studies of the enzymatic reaction with hydroquinone–H_2O_2 as substrates. Graphic of current density ($\mu A\,cm^{-2}$) obtained for SWV vs. the reaction time (s) in 1.9×10^{-8} mol L^{-1} HRP, 2×10^{-3} mol L^{-1} HQ, 2×10^{-3} mol L^{-1} H_2O_2 and phosphate buffer 0.1 mol L^{-1} and KCl 0.1 mol L^{-1}, pH 7.50 as reaction medium. First, 10 consecutive cycles each 60 s during 10 min were evaluated. In inserted graph, the electrochemical signal was recollected each 20 s during 3 min.

of experiments and also to allow delimiting of working ranges for each factor. In this work, very wide working ranges of substrate and cosubstrate concentrations were evaluated to determinate the kinetics of enzyme catalyzed reaction. In this design, an ANOVA test was applied to each HRP–cosubstrate–H_2O_2 system, to obtain a significant fitted model and not significant lack of fit (p-values must be minor and major to 0.05, respectively). After obtaining the fitted model the factor combination that provides the best "*values of desirable response*" should be investigated. In the optimization stage the maximum electrochemical response directly related with the enzymatic concentration (or activity) was searched therefore substrate concentrations must be present in an excess amount, i.e. the reaction must be independent of substrate concentrations (zero-order kinetics). The global desirability function, calculated from importance and weight criteria for all factors and responses, was ranged between 0 (where the combinations does not fulfil any requirement) and 1 (where all responses have a simultaneously desirable value) [36]. Table 3 shows the global desirability function, the

Table 3
Central composite design results and the experimentally obtained response for each system.

System	Reaction time (s)	Optimal conditions factors[a]				Global desirability function	Predicted response ($\mu A\,cm^{-2}$)	Obtained response ($\mu A\,cm^{-2}$)
		A	B	C	D			
HRP–H_2O_2–phenol	300	7.5	38	0.5	5.0	0.83	22.5	26 ± 4 (n = 3)
HRP–o-phenilendiamine–H_2O_2	60	6.0	38	2.5	6.0	0.81	295	305 ± 60 (n = 4)
HRP–p-chlorophenol–H_2O_2	40	6.0	19	2.0	2.0	0.86	27.5	30 ± 4 (n = 4)
HRP–hydroquinone–H_2O_2	20	7.5	38	5.0	2.5	0.73	350	360 ± 70 (n = 3)
HRP–TMB–H_2O_2	40	6.0	38	2.0	4.0	0.62	12.8	13 ± 2 (n = 3)
HRP–pyrocatechol–H_2O_2	300	6.0	9.5	2.0	2.0	0.97	102	105 ± 10 (n = 4)
HRP–p-aminophenol–H_2O_2	20	5.0	9.5	2.0	5.0	0.96	27.8	31 ± 2 (n = 3)

[a] Factors: A, buffer pH; B, enzymatic concentration ($\times 10^{-9}$ mol L^{-1}); C, cosubstrate concentration ($\times 10^{-3}$ mol L^{-1}); D, H_2O_2 concentration ($\times 10^{-3}$ mol L^{-1}).

predicted response and the experimentally obtained response for each system. The predicted and experimental responses in all cases were not significantly different when were compared by a mean comparison test with alpha level of 0.05 [37,38]. In this context, the global desirability function could be considered as an efficiency measure of the enzymatic activity and the electrochemical transference. Percentage global desirability function values higher than 70 were obtained in all enzymatic systems except for the system with TMB as cosubstrate and amperometric detection (D = 62%). We supposed that the low-sensitive detection technique used for this system and the complex molecular structure of TMB that delay the electronic transference within the enzyme active site by steric hindrance would cause the poor performance.

The optimal values of factors are also shown in Table 3. The buffer pHs were set between 5 and 7.5. Phenol, p-chlorophenol, pyrocatechol and hydroquinone have their pK_a above 7.5, while o-phenilendiamine, p-aminophenol and TMB have their pK_a below 5. This suggests that the enzyme would have more affinity for no-charged aromatic amines and phenolic compounds and these pH ranges would also favor the reduction of the products of the enzymatic reaction on the electrode surface.

Next, the cosubtrate:H_2O_2 ratios were optimized to restrict obtained results by central composite design and to avoid the enzymatic inactivation. It is well known that the hydrogen peroxide at high concentrations is the suicide substrate, which converts the compound II species ((Fe^{IV} = O)P) to a highly reactive peroxy iron (III)–porphyrin free radical, called compound III. When another hydrogen peroxide molecule reaches this free radical, a hydroxyl radical is produced, which may attack the tetrapyrrol structure of the heme group leading to the irreversible inactivation of the hemoenzyme [39]. This risk is higher when the concentration of the cosubstrate is much lower than hydrogen peroxide concentration.

In consequence, the maxima enzymatic responses were searched for each system in the enzymatic saturation region. In order to do that, cosubtrate:H_2O_2 ratios were evaluated in the range 1/10 and 10/1 while the enzymatic concentrations and the buffer pHs were kept constant in their optimum values of central composite design. The cosubstrate:H_2O_2 ratio of 2:1 was chosen for the systems with phenol, o-phenilendiamine and pyrocatechol as cosubstrates, while the ratio of 1:1 was enough for the systems with para-substituted cosubstrates such as hydroquinone and p-aminophenol. On the other hand, optimal ratios of 1:4 were obtained for the systems with TMB and p-chlorophenol as cosubstrates.

Finally, square wave voltammetric and amperometric techniques were evaluated to select the electrochemical technique for each HRP–cosubstrate–H_2O_2 system. Fig. 5 shows the relative responses (as current density/enzymatic concentration) vs. HRP–cosubstrate–H_2O_2 systems for each detection technique. These relative responses allowed the comparison of the system sensitivity independently of the enzymatic concentration. The sensitivity depends on the nature of electron-donor cosubstrate, the stability of the free radical compounds produced in the enzymatic reaction, the electrode material, the parameters of the electrochemical technique (applied potential and the potential sweeping rate, etc.). A free radical compound is stabilized by charge dissipation through its conjugation within the molecule; therefore, substituents that increase the electron density are very important in this function. A stabilized free radical compound retards polymerization process or dismutation reactions. On the other hand, if the reduction technique is not fast enough, the free radical compounds can polymerize. This causes passivation of electrode surface and therefore produces a loss of sensitivity. From Fig. 5 it can be observed that for both techniques, the systems with phenol and p-chlorophenol as cosubstrates presented the lowest sensitivity. We suppose that the molecules of these cosubstrates do not have appropriate substituents therefore they do not have the capacity to stabilize to free radical compounds. In consequence, unstable free radical compounds would generate no-electroactive compounds and this would cause a low sensitivity in the detection.

The performances of the amperometric systems with o-phenilendiamine and p-aminophenol as cosubstrates were similar. In addition they were more sensitive than those that utilized

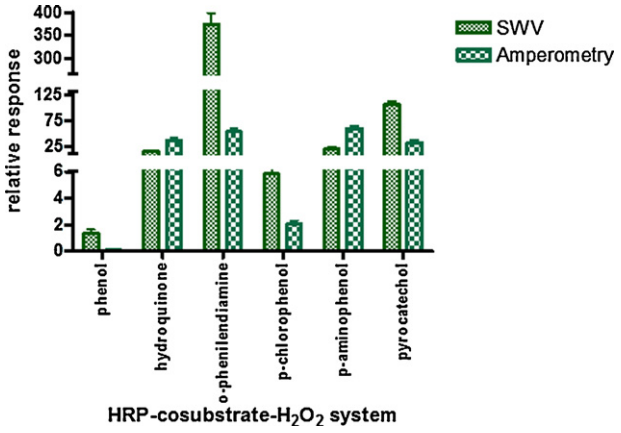

Fig. 5. Graphic of relative response (current density/enzymatic concentration) in $\mu A\,cm^{-2}/10^{-9}$ mol L^{-1} vs. HRP–cosubstrate–H_2O_2 system for square wave voltammetry and amperometry. The reaction condition for HRP–phenol–H_2O_2 system were [HRP] = 3.8×10^{-8} mol L^{-1}, [phenol] = 2.5×10^{-3} mol L^{-1}, [H_2O_2] = 1.25×10^{-3} mol L^{-1} at pH 7.5; for HRP–o-phenilendiamine–H_2O_2 system were [HRP] = 3.8×10^{-8} mol L^{-1}, [o-phenilendiamine] = 2.5×10^{-3} mol L^{-1}, [H_2O_2] = 1.25×10^{-3} mol L^{-1} at pH 6.0; for HRP–p-chlorophenol–H_2O_2 system were [HRP] = 1.9×10^{-8} mol L^{-1}, [p-chlorophenol] = 7.5×10^{-3} mol L^{-1}, [H_2O_2] = 1.9×10^{-3} mol L^{-1} at pH 6.0; for HRP–hydroquinone–H_2O_2 system were [HRP] = 3.8×10^{-8} mol L^{-1}, [hydroquinone] = 2.5×10^{-3} mol L^{-1}, [H_2O_2] = 2.5×10^{-3} mol L^{-1} at pH 7.5; for HRP–pyrocatechol–H_2O_2 system were [HRP] = 9.5×10^{-9} mol L^{-1}, [pyrocatechol] = 2.0×10^{-3} mol L^{-1}, [H_2O_2] = 1.0×10^{-3} mol L^{-1} at pH 6.0; for HRP–p-aminophenol–H_2O_2 system were [HRP] = 9.5×10^{-9} mol L^{-1}, [p-aminophenol] = 2.0×10^{-3} mol L^{-1}, [H_2O_2] = 2.0×10^{-3} at pH 5.0. For all system the working buffer was phosphate 0.1 mol L^{-1} and KCl 0.1 mol L^{-1}.

hydroxyl-substituted phenolic compounds as cosubstrates. These results were in according to results reported by Ruzgas et al. [11], Skládal et al. [40] and Lagrimini et al. [41].

The HRP–o-phenilendiamine–H_2O_2 system presented the highest response $375 \pm 25\ \mu A\ cm^{-2}\ 10^9\ mol^{-1}\ L$ by square wave voltammetry and it was approximately four times more sensitivity than the next system (with pyrocatechol as cosubstrate). On the other hand, it showed the higher square wave voltammetry/amperometry ratio with an approximate value of 7 followed by systems with pyrocatechol, phenol, p-chlorophenol, hydroquinone and p-aminophenol, as cosubstrates, respectively. According to the bibliography the square wave voltammetry should be more sensitivity than the amperometry [24]. This assertion is fulfilled in all cases except for the systems with hydroquinone and p-aminophenol as cosubstrates; probably the rate of SWV is not enough to avoid the loss of sensitivity by the electrode fouling due to the fast polymerization and/or dimerisation of these compounds. For most systems under optimal experimental conditions, the square wave voltammetry was the most sensitive technique and six times faster than amperometry. Consequently, square wave voltammetry was selected as electrochemical technique for all systems except for the system with TMB as cosubstrate that can only be analyzed by amperometry as it was explained in Section 3.2.

3.4. Determination of Apparent Michaelis constants

The V'_{max} and K'_m were calculated for H_2O_2 and cosubstrates in each system with the Lineweaver–Burk method. In order to do that, the substrate concentration reciprocal was plotted against the initial velocity reciprocal. This velocity was expressed as the oxidized substrate concentration in the time ($mol\ L^{-1}\ s^{-1}$). The oxidized substrate of hydroquinone (p-benzoquinone) is commercially available only. Therefore, the curves of p-benzoquinone and of electrochemically oxidized hydroquinone in the range of potential from −100 to 300 mV were separately performed. These curves showed similar analytical sensitivity (data not showed). Consequently, curves of electrochemically oxidized compound were performed for the other cosubstrates whose oxidized forms are not commercially available. The oxidations were achieved by sweep in SWV for all cosubstrates except to TMB that was oxidized by amperometry at 350 mV. The ranges of potentials in SWV were from 0 to 500, from 300 to 900, from −100 to 200, from 100 to 400 and from 0 to 300 for o-phenilendiamine, phenol, p-chlorophenol, pyrocatechol and p-aminophenol, respectively.

The plot of the o-phenilendiamine concentration vs. the initial velocity is shown in Fig. 6. First, the kinetic parameters of o-phenilendiamine in the range from 0.6 to $6.0 \times 10^{-3}\ mol\ L^{-1}$ at $6 \times 10^{-3}\ mol\ L^{-1}$ H_2O_2 concentration were performed (Fig. 6a). Second, a similar approach was taken to determine the parameters of H_2O_2 in the range from 0.5 to $6 \times 10^{-3}\ mol\ L^{-1}$ at $2.5 \times 10^{-3}\ mol\ L^{-1}$ o-phenilendiamine concentration (Fig. 6b). The other cosubstrates were similarly analyzed.

V'_{max}, K'_m, K_{cat} and E_{ff} for each cosubstrates and H_2O_2 in each enzymatic system are quoted in Table 4. It is well-known that high V'_{max} and low K'_m indicate high affinity of the substrates for the HRP enzyme. On the one hand, the turnover numbers ($K_{cat} = V'_{max}/[Enzyme]$) decreased in the sequence of o-phenilendiamine > p-aminophenol > pyrocatechol > p-chlorophenol > phenol > TMB > hydroquinone. On the other hand, the catalytic efficiencies ($E_{ff} = K_{cat}/K'_m$) decreased in the sequence of o-phenilendiamine > p-aminophenol ∼ pyrocatechol > phenol > p-chlorophenol ∼ hydroquinone > TMB. Shivakumar et al. [42], Bólado et al. [43], Zapata et al. [44] and Gómez et al. [45] reported similar trends and kinetic parameters with orders of magnitude in according with our obtained data. However, the reported works

Table 4
Kinetic parameters of HRP with variable cosubstrate concentrations at fixed H_2O_2 concentration and variable H_2O_2 concentrations at fixed cosubstrate concentration.

Systems	Cosubstrate[a]				H_2O_2[a]			
	V'_{max} ($\times 10^{-6}\ mol\ L^{-1}\ s^{-1}$)	K'_m ($\times 10^{-3}\ mol\ L^{-1}$)	K_{cat} (s^{-1})	E ($\times 10^4\ L\ mol^{-1}\ s^{-1}$)	V'_{max} ($\times 10^{-6}\ mol\ L^{-1}\ s^{-1}$)	K'_m ($\times 10^{-3}\ mol\ L^{-1}$)	K_{cat} (s^{-1})	E ($\times 10^4\ L\ mol^{-1}\ s^{-1}$)
HRP–H_2O_2–phenol	2.0 ± 0.2	1.9 ± 0.1	52 ± 2	2.7 ± 0.1	30.8 ± 0.5	0.031 ± 0.003	805 ± 40	2590 ± 700
HRP–o-phenilendiamine–H_2O_2	12.0 ± 0.8	1.8 ± 0.3	315 ± 15	17.4 ± 0.8	7.7 ± 0.2	0.15 ± 0.02	200 ± 30	134 ± 25
HRP–p-chlorophenol–H_2O_2	1.5 ± 0.8	6.5 ± 0.9	79 ± 12	1.2 ± 0.3	8.1 ± 0.8	0.8 ± 0.1	845 ± 80	106 ± 36
HRP–hydroquinone–H_2O_2	0.7 ± 0.1	1.7 ± 0.2	18 ± 5	1.2 ± 0.6	0.5 ± 0.1	1.1 ± 0.2	26 ± 3	2.4 ± 0.1
HRP–TMB–H_2O_2	0.90 ± 0.06	2.5 ± 0.01	23 ± 4	0.9 ± 0.1	0.87 ± 0.06	0.7 ± 0.2	23 ± 5	3.2 ± 0.6
HRP–pyrocatechol–H_2O_2	1.4 ± 0.1	1.1 ± 0.3	146 ± 20	13.3 ± 0.9	0.70 ± 0.06	0.10 ± 0.08	73 ± 10	73 ± 20
HRP–p-aminophenol–H_2O_2	1.6 ± 0.6	1.2 ± 0.4	168 ± 10	14.0 ± 0.4	1.2 ± 0.2	1.5 ± 0.7	31 ± 8	2.1 ± 0.7

[a] The Lineweaver–Burk reciprocal plots were build with five points for triplicate.

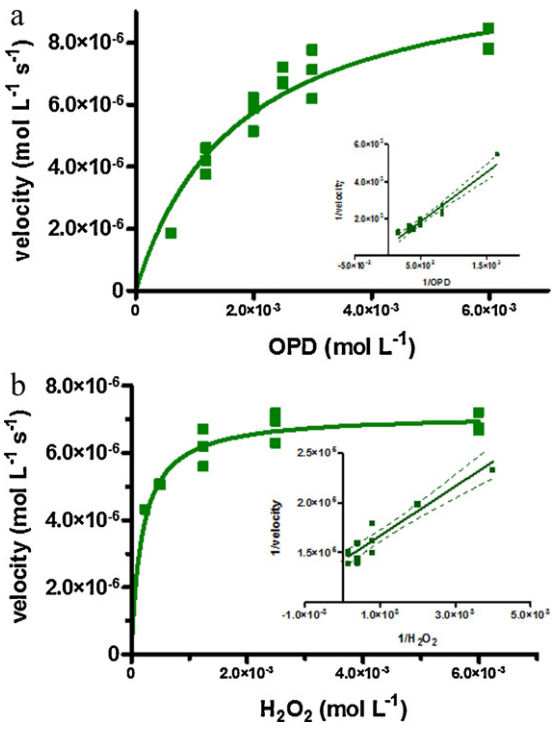

Fig. 6. Enzymatic kinetic curve for the HRP–o-phenilendiamine–H_2O_2 system and Lineweaver–Burk reciprocal plot in the insert graphic. Reaction conditions: phosphate buffer 0.1 mol L^{-1} and KCl 0.1 mol L^{-1}, pH 6.0; HRP concentration = 3.8×10^{-8} mol L^{-1}. (a) H_2O_2 concentration constant = 6×10^{-3} mol L^{-1}; (b) o-phenilendiamine concentration constant = 2.5×10^{-3} mol L^{-1}.

were performed by other detection techniques in the different experimental conditions. After evaluating both the affinity and efficiency for each compound, o-phenilendiamine demonstrated the best kinetic characteristics and therefore it can be considered as the most efficient cosubstrate.

3.5. Calibration curves for the determination of the enzymatic concentration

The calibration curves for determination of the enzymatic concentration in each system were performed. A wide linearity range of these curves would be advantageous for the determination of target analytes in the future immunoassays, since the HRP response is related with the analyte concentration. As the dates presented heterocedastic distribution, the curves were analyzed by WLS. Table 5 shows the analytic parameters for each enzymatic system. The analytic sensitivities were ranged from 0.3 to 1.3 nM^{-1} of the HRP concentration. The standard deviation of the slope (RSD%) in all cases were minor than 10% except for phenol (RSD% of 12.9%, $n = 3$). The system with one of cosubstrates more efficient, the o-phenilendiamine, presented the widest linearity range with the lowest limit of detection (LOD). These values are similar to the reported by Fornera et al. [46], Guo et al. [47] and Fanjul Bolado et al. [48]. In the first and the second work a HRP system with o-phenilendiamine and bromopyrogallol as cosubstrate are described, respectively. In both cases spectrophotometric detection was applied. In the third work a system with TMB as cosubstrate using amperometric detection is detailed. Although p-aminophenol was the second best cosubstrate with wide linearity range, HRP concentrations were higher than the cited by Sun et al. [49]. However, a fixed potential of −0.56 V (vs. Ag/AgCl) is performed in this work. This potential negative could be disadvantageous, since some interference in the sample would be reduced. The performances of the systems with pyrocatechol and

Table 5
Analytical parameters of the HRP regression curves by WLS.

System	System experimental conditions			Analytic sensibility ($\times 10^9$ L mol^{-1})	RSD%[a] ($n = 3$)	Linearity range ($\times 10^{-9}$ mol L^{-1})	LOQ ($\times 10^{-9}$ mol L^{-1})	LOD ($\times 10^{-9}$ mol L^{-1})	*E* peak (mV)	Detection
	[Cosubstrate] ($\times 10^{-3}$ mol L^{-1})	[H_2O_2] ($\times 10^{-3}$ mol L^{-1})	pH[b]							
HRP–H_2O_2–phenol	2.5	1.25	7.5	0.34	12.9	35–38	35	12	265	SWV[c]
HRP–o-phenilendiamine–H_2O_2	2.5	1.25	6.0	0.75	0.060	0.096–19	0.096	0.032	−140	SWV
HRP–p-chlorophenol–H_2O_2	7.5	1.9	6.0	1.2	6.1	7.4–19	7.4	2.4	−280	SWV
HRP–hydroquinone–H_2O_2	2.5	2.5	7.5	1.1	6.9	4.9–9.7	4.9	1.6	−150	SWV
HRP–TMB–H_2O_2	3.0	0.75	6.0	0.37	8.8	24–38	24	8.1	50	A[d]
HRP–pyrocatechol–H_2O_2	2.0	1.0	6.0	1.3	5.8	7.1–19	7.1	2.3	50	SWV
HRP–p-aminophenol–H_2O_2	2.0	2.0	5.0	1.0	1.4	3.9–38	3.9	1.3	100	SWV

[a] RSD% were calculated as $(SD/m) \times 100$, where SD was standard deviation of slope and m was a slope.
[b] In phosphate buffer 0.1 mol L^{-1} and KCl 0.1 mol L^{-1}.
[c] Square wave voltammetry.
[d] Amperometry.

p-chlorophenol as cosubstrates were similar but higher LOD and lower linearity range than systems with o-phenilendiamine and p-aminophenol as cosubstrates. Finally, systems with phenol, TMB and hydroquinone as cosubstrates presented a very narrow linearity range.

4. Conclusions

The electrochemical signals of seven cosubstrates in the HRP–cosubstrate–H_2O_2 systems were optimized by one-factor optimization assays and systematic optimization procedure with chemometric techniques. The selected electrochemical technique was square wave voltammetry for all systems except for the system with TMB as cosubstrate that was analyzed by amperometry. The o-phenilendiamine showed the highest electrochemical efficiency and good characteristics in its role as HRP cosubstrate (high affinity and efficiency for the enzyme) in comparison with the other cosubstrates.

Then, the calibration curves for the quantification of HRP were built from these optimized systems. The analytical sensitivities of the different systems were ranged from 3.0×10^{10} to $1.3 \times 10^{9}\,\mathrm{mol\,L^{-1}}$ of the HRP concentration. The HRP–o-phenilendiamine–H_2O_2 system presented the highest linearity range (9.6×10^{-11} to $1.9 \times 10^{-8}\,\mathrm{mol\,L^{-1}}$) with the lowest LOD ($3.0 \times 10^{-11}\,\mathrm{mol\,L^{-1}}$). The reaction time was very short (60 s) for this system, which could be useful for their subsequent applications in detection systems of immunoassays.

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