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Modeling and optimization of the bi-substrate peroxidase-enzyme catalyzed potentiometric assay of hydrogen peroxide by response surface methodology with a central composite rotatable design

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ABSTRACT

In view of the great importance of determination of hydrogen peroxide in many chemical, biological, clinical, environmental processes, and the attractive operational simplicity of potentiometric approach for the enzymatic assay, after preliminary optimization, the efficient response surface methodology (RSM) with a central composite rotatable design (CCRD), was used for modeling and optimization of the initial-rate potentiometric method of HRP enzyme catalyzed the assay of H_2O_2. The combined "OVAT" (one-variable-a-time), full factorial and RSM analysis was able to explain the importance of the factors, their interactions, along with their optimum values (i.e. acetate buffer concentration 300 mM, 4-fluorophenol concentration = 3 mM, pH = 6.15, enzyme activity = 0.95 U/ml and T = 41 °C). The obtained results showed that the determined second-order polynomial equation explains adequately the non-linear nature of the modeled response, as confirmed by a reasonable coefficient of determination (R^2 = 78.3%). Accordingly, the ANOVA (analysis of variance) indicated, particularly, that the terms x_1 = pH and $x_2{}^2$ = pH * pH are highly significant (with $p_{value} < 0.05$) in this model. The performance of the optimized method including its linearity range (1–200 μM), within-day reproducibility of H_2O_2 detection in aqueous samples at 2 levels (RSD% = 1.06 at 17 μmol l^{-1}, and RSD% = 1.81 at 34 μmol l^{-1}, N = 10 replica), along with recovery (or matrix effect) of the method (97.71–03.84%) in hair bleaching real samples, were all determined. The optimized method was also successfully validated by the assay of H_2O_2 in commercial hair bleaching samples and compared to the corresponding values obtained by the reference spectrophotometric method.
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1. Introduction

The determination of hydrogen peroxide is of great importance in many chemical, biological, clinical, and environmental processes. Among the various methods developed for this purpose, the enzymatic method is the most attractive one due to its high selectivity, sensitivity and rapidity. The peroxidase catalyzed H_2O_2 oxidation reaction takes place in the presence of a suitable peroxidase substrate electron donor. The survey of literature shows that this enzymatic reaction was exploited as detection system in many colorimetric, fluorimetric, luminescence, amperometric or potentiometric methods [1–5].

Although the usual measurement of H_2O_2 is based on spectrophotometric method, the use of the alternative potentiometric approach for the enzymatic assay is attractive by its simplicity, and rapidity. In this context, fluoride ion-selective electrode (FISE) was for the first time used by Siddiqi [4,6] for the assay of horseradish peroxidase enzyme (HRP), glucose and cholesterol. This potentiometric method was based on the bi-substrates reaction reported previously by Hughes and Sanders [7], in which the heme protein enzyme peroxidase catalyzes the oxidation of halogenated aromatic H-donor compounds (e.g. 4-fluorophenol) and liberation of fluoride ions:

$$H_2O_2 + \text{Fluoroaromatic compound} \xrightarrow{HRP} H_2O + \text{Polymerized Aromatic Products} + F^-$$

Scheme 1.

The mechanism of this reaction related to polymerization processes was considered by Pirzad et al. [8,9]. Effectively, according to this mechanism, the produced free radical intermediates can disproportionate or initiate a variety of nonenzymatic reactions

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including degradation and many polymerization processes. The involvement of free radicals (on the ring of aromatic compounds), has also been confirmed experimentally, by electron spin resonance studies of Kobayashi et al. [10] and Mottley and Mason [11].

In view of the impact of this indicator reaction and its potential application utility, it seemed useful to improve the performance of this potentiometric HRP enzyme catalyzed oxidation reaction by a combined “OVAT” (one-variable-a-time), full factorial and RSM analysis.

It should also be mentioned that all measurements were performed, in this work, by a rapid kinetic initial rate method. For the validation of the optimized potentiometric method, the range of linearity, upper and lower detection limits, the reproducibility at two levels were all determined in optimized conditions. Subsequently, the content of H_2O_2 in two commercial hair bleaching products were determined and compared to the similar results obtained following to the usual spectrophotometric method. Finally, the recovery of the optimized method was as well determined in the commercial hair bleaching product.

2. Experimental

2.1. Chemicals

All used chemicals were of analytical and obtained from Merck (Germany) or Fluka (Switzerland), except the enzyme hydrogen peroxide oxidoreductase (HRP, E.C.1.11.1.7) which was purchased from Boehringer-Manheim Company (Germany). All aqueous solutions were prepared in doubly distilled water. Concentration of hydrogen peroxide solutions were determined many times using its molar absorption coefficient ($\varepsilon = 43.6\,\text{cm}^{-1}\,\text{M}^{-1}$) at $\lambda = 240\,\text{nm}$ [12] by dilution of the supplied H_2O_2 (30%, V/V) solution. Standard solutions of 4-fluorophenol (4-FP), NaF and hydrogen peroxide were then prepared by appropriate dilutions in doubly distilled water. Different Tris–HCl (or tris(hydroxymethyl-aminomethane)), phosphate and acetate buffer solutions were prepared according to their corresponding standard procedures in the concentration range 50–500 mM and by adjusting their pHs before use.

A stock solution of HRP enzyme 10 mg/ml was prepared by dissolving the appropriate weight of initial enzyme in appropriate buffer. The activity of initial HRP enzyme was also determined (as 214.68 U/mg) following the reference procedure [13]. This reference method is based on the well known Trinder assay, in which a quinonimine dye is produced by coupling of 4-aminoantipyrine with the produced compound from the oxidation reaction of H_2O_2 with phenol, in the presence of HRP. One unit of enzyme activity (U) results in the decomposition of one micromole of hydrogen peroxide per minute at 25 °C and pH 7.0 under the specified conditions.

The real samples consisted of two different peroxide oxidant hair-bleaching lotion and cream used for validation of the optimized reaction performances. The manufacturer declared contents of these oxidants were:

- Sample 1 (RS1): H_2O_2 (6%), stearyl alcohol, sodium stearyl sulphate, PEG-40 Castor oil, disodiumpyrophosphate, disodium EDTA, sodium benzoate, phosphoric acid, perfume and H_2O. Manufactured by Igora, Oxygenta lotion produced under license by Hans Schwarzkopf GmbH & Co., KG, Hamburg, Germany.
- Sample 2 (RS2): H_2O_2 (9%, w/w), stearyl alcohol, herbal essences, additives. Manufactured by Welloxon, oxidant cream produced under the license by Wella AG, Darmstadt, Germany.

2.2. Instrumentation

The F^- ion-selective and the reference Ag/AgCl electrodes were obtained from Fluka (Switzerland), and the pH combined electrode and ion-meter/pH meter (model 6.0238.000) used in this work were from Metrohm (Switzerland). The experimental cell potentials were recorded via an automated potentiometric data acquisition setup using a GPIB bus in the Topward multimeter (model 1304, Taiwan) interfaced with a personal computer. The reagent mixture was placed in into a mini double-wall glass container enabling the circulation of thermostated water from a bath (kept at the desired temperature ±0.05 K). The experimental absorption data were also obtained by using quartz cells ($l = 1$ cm) and a Shimadzu UV–Vis spectrophotometer (model 2100, Japan) equipped with a thermostat and interfaced with a personal computer for data acquisition and processing.

3. Method

3.1. Principle

In the enzymatic reaction, as schematically presented by the Scheme 1, the amount of H_2O_2 is proportional to the amount of generated F^- ion produced in the reaction. Using a galvanic cell, containing both a F^- ion-selective and an Ag/AgCl reference electrodes, the production of F^- was monitored during the course of reaction by the decrease of cell potential according to the Nernst equation

$$E = E' - s \cdot \log[a_{F^-}] \quad (1)$$

where E is the experimental cell potential, E' is the cell constant potential, $s = 2.303\,RT/F$ is the F^- ion-selective electrode ideal Nernstian slope, F is the Faraday constant, R is the gas constant and T is the Kelvin temperature, and a_{F-} the activity of the fluoride ions, respectively.

In a preliminary step, the response of F^- ion-selective electrode versus the reference Ag/AgCl electrode was determined using laboratory standard F^- solutions in the concentration range of 10^{-1}–10^{-5} M. Then, by linear regression on the response data of the F^- ion-selective electrode (E) versus the logarithm of the activity of F^-, the intercept (E') and the slope ($s = 57.5$ mV) of the electrode were obtained (with $R^2 = 0.996$), according to the Eq. (1).

For the investigation of the enzymatic reaction, the data were collected after the start of reaction with a sampling time of 0.2 s. The initial rate was each time determined by linear regression performed on the reaction progress curve, mainly between 10 and 40 s (but up to 90 s in worst case during optimization steps), after the start of reaction. The initial rate, which is proportional to the slope of the reaction progress curve, at the beginning of reaction, was measured after addition of the enzyme into the reagent mixture. Clearly, as it turns out well in highly diluted solution, concentration was used instead of activity for the calculation of F^- ion concentrations in the above equation. Typical reaction progress curves (expressed in terms of F^- concentration versus time) are presented in Fig. 1.

3.2. Experimental design

As a first optimization step, the critical variables of the HRP catalyzed H_2O_2 oxidation reaction along with their variation ranges were estimated based on a classical “one-variable-a-time” (OVAT) procedure. For this purpose, three different buffers were tested at fixed buffer concentration (200 mM) in the following pH ranges: phosphate (pH = 5–7), Tris–HCl (pH = 7–8.2), and acetate (pH = 4–7). Among the three tested buffers, acetate buffer was found to be more efficient than phosphate or Tris–HCl buffers. The tested experimental parameters were: T (10–50 °C), 4-FP (0.012–12 mM), enzyme activity (0.01–0.66 U/ml), and acetate buffer concentration (50–500 mM). The OVAT procedure showed that among enzyme

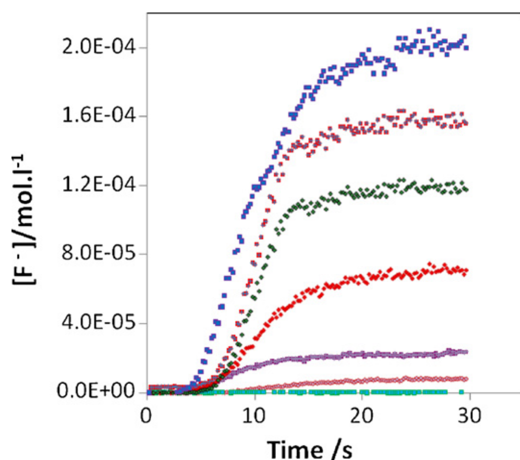

Fig. 1. Typical reaction progress curves for F^- production during the course of the enzymatic reactions (F^- concentration versus time). The different progress curves correspond to different level of H_2O_2 which are: (–) 1 μM; (×) 3 μM; (△) 6 μM; (◯) 16 μM; (◊) 24 μM; (+) 32 μM; (□) 32 μM;.

activity, pH value, 4-fluorophenol concentration, buffer concentration, and temperature, the critical variables are enzyme activity, pH, T, and buffer concentration. In fact, 4-fluorophenol concentration showed a maximum constant response in the range of 1–9 mM, therefore, it was excluded in next optimization steps. The remaining variables (enzyme activity, pH, buffer concentration, and temperature) were then used as input variables in a full factorial analysis, designed at two-level (4^2), with 2 replicates, 8 center points, and 2 blocks. The input levels of different factors, including center points, for the full factorial procedure were: pH (3, 5.5, and 8), buffer (50, 250 and 450 mM), enzyme activity (0.06, 0.36 and 0.66 U/ml), and T (20, 35, and 50 °C). The full factorial analysis showed that the significant terms (with p-values < 0.05) are related to enzyme activity, temperature, and 2nd order interaction terms (enzyme activity * temperature and pH * temperature). Consequently, enzyme activity, temperature, and pH variables were selected for final optimization step. Accordingly, in order to optimize efficiently the potentiometric enzymatic assay of H_2O_2, further optimization was performed by the more efficient chemometric RSM with a central composite rotatable design (CCRD). From the results obtained by OVAT and full factorial procedures, the concentration of acetate buffer (300 mM) and 4-FP (3 mM) were fixed in the RSM procedure, and the range of variation of other selected variables were pH = 2.05–6.95, enzyme activity (E) = 0.01–0.99 U ml^{-1}, and T = 13.7–46.3 °C.

The response surface methodology consists of a group of statistical techniques for developing empirical model and its exploitation. Through a careful design and analysis of experiments, RSM try to relate a response (or output variable), to the levels of a number of parameters (or input variables) that affect such response. RSM has been used in many fields to improve the efficiency of many processes and to find optimized design variables [14]. In this work, the following quadratic equation was considered for modeling the enzymatic reaction:

$$y = \text{Const.} + \sum_{i=1}^{3} b_i x_i + \sum_{i<j}\sum_{j=1}^{3} b_{ij} x_i x_j + \sum_{i=1}^{3} b_{ii} {x_i}^2 \quad (2)$$

where y, x_1, x_2 and x_3 refer to the response (or initial reaction rate), pH, enzyme activity and temperature, respectively. For estimation of the parameters in the above quadratic model, the designed RSM procedure was a central composite rotatable design (CCRD) for the three design variables at two-level in two blocks, which consisted of 40 runs including 16 full factorial (cube) points, 8 center points

Table 1
Design table showing the randomized run order of experiment based on the different values of variables designed for the determination of modeled response (Eq. (2)).

Run order	Blocks	pH	E/(U ml^{-1})	T/°C
1	1	6	0.8	40
2	1	3	0.2	20
3	1	3	0.8	20
4	1	6	0.2	40
5	1	4.5	0.5	30
6	1	4.5	0.5	30
7	1	6	0.8	20
8	1	3	0.8	40
9	1	3	0.2	40
10	1	6	0.2	40
11	1	4.5	0.5	30
12	1	4.5	0.5	30
13	1	4.5	0.5	30
14	1	4.5	0.5	30
15	1	4.5	0.5	30
16	1	3	0.2	20
17	1	3	0.2	40
18	1	6	0.2	20
19	1	6	0.2	20
20	1	6	0.8	40
21	1	6	0.8	20
22	1	4.5	0.5	30
23	1	3	0.8	40
24	1	3	0.8	20
25	2	4.5	0.5	30
26	2	4.5	0.01	30
27	2	2.05	0.5	30
28	2	4.5	0.5	30
29	2	4.5	0.01	30
30	2	2.05	0.5	30
31	2	4.5	0.5	30
32	2	4.5	0.99	30
33	2	4.5	0.5	46.3
34	2	4.5	0.5	30
35	2	4.5	0.5	13.7
36	2	6.95	0.5	30
37	2	4.5	0.5	46.3
38	2	6.95	0.5	30
39	2	4.5	0.99	30
40	2	4.5	0.5	13.7

in cube, 12 axial points and 4 axial center points on the axis of each design variables. The order of the experimental runs was randomized in order to avoid any trends in the settings of variables (see Table 1). A particular aspect of this design is that it is orthogonal and rotatable. Accordingly the variance of the predicted response remains constant at all points that are equidistant from the design center. The factorial points contribute to the estimation of linear effects (b_i) and interaction effects (b_{ij}). The axial points contribute to the estimation of square effects (b_{ii}). The center runs are necessary for the estimation of quadratic terms and provide an internal estimation of measurement error.

4. Results

The coefficients in quadratic polynomial model Eq. (2) were determined by multiple regressions using the method of least squares

$$y = -5.413 + 1.632x_1 + 1.921x_2 + 0.078x_3 - 0.186x_1^2 - 0.891x_2^2 - 0.002x_3^2 + 0.225x_1x_2 + 0.011x_1x_3 - 0.016x_2x_3 \quad (3)$$

The regression analysis indicated that the terms x_1 = pH and $x_1^2 = \text{pH} * \text{pH}$ are highly significant (p < 0.05) in this model (Table 2). The positive coefficients for x_1 = pH, x_2 = enzyme activity and x_3 = temperature, indicate that the linear effects increase the response, while the square coefficients (for $x_1^2 = \text{pH} * \text{pH}$, $x_2^2 = E * E$ and $x_3^2 =$

Table 2
Statistical evaluation of regression coefficients for the 2nd order response (Eq. (2)).

Term	Coef	SE Coef	T	P
Constant	−5.4132	1.8278	−2.962	0.006
Block	0.0664	0.0762	0.871	0.391
pH	1.6323	0.4601	3.548	0.001
E	1.9209	1.9803	0.97	0.34
T	0.0778	0.069	1.127	0.269
pH * pH	−0.1861	0.0408	−4.557	0
E * E	−0.891	1.0208	−0.873	0.39
T * T	−0.0015	0.0009	−1.638	0.112
pH * E	0.225	0.2623	0.858	0.398
pH * T	0.0109	0.0079	1.387	0.176
E * T	−0.0163	0.0394	−0.413	0.683

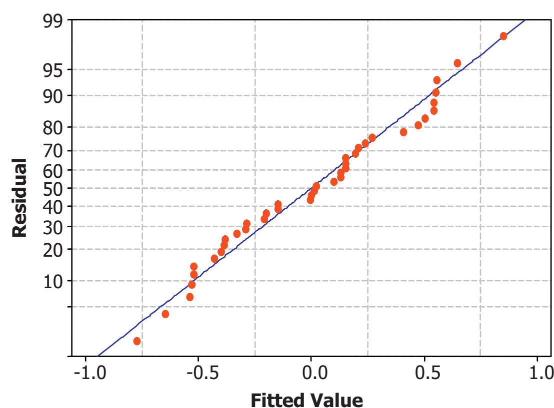

Fig. 2. . Normal probability plot of the residuals.

$T * T$) decrease the responses. The ANOVA (analysis of variance) (Table 3) shows that at a significance level ≥ 95% (with a confidence interval p-value ≤ 0.05), this model (although with a lack-of-fit) is a reasonably good representation of the data including significant contribution of linear (pH) and square (pH * pH) effects. This fact is also confirmed by a reasonable coefficient of determination (R^2 = 78.3%). The resulting residuals versus both observation order and versus fitted values (not shown) do not exhibit any trend and all data stay well within the range of ±1 standard deviations, with a normal distribution (see Fig. 2).

A point of maximum response could mathematically be determined by partial derivatives of the generated response model equation with respect of each of the corresponding experimental variable (factor). In matrix notation, the derivative of the quadratic model equation (y) with respect to the elements of the vector $\mathbf{x}$ equated to zero is

$$\left(\frac{\partial y}{\partial \mathbf{x}}\right) = \mathbf{a} + 2\mathbf{A}\mathbf{x} = 0 \quad (4)$$

Table 4
Comparison of the H_2O_2 content in two hear bleaching (real samples) determined by initial rate potentiometric and spectrophotometric reference methods, in optimized conditions.

Method	Real sample	H_2O_2	
		(g/g) %	±SD
Potentiometric			
	RS1	4.75	0.02
	RS2	8.51	0.04
Spectrophotometric (Reference Method)	RS1	4.68	0.04
	RS2	8.52	0.05
Manufacturer values	RS1	6	
	RS2	9	

where $\mathbf{a}$ is a vector of the first-order coefficients, $\mathbf{A}$ is a symmetric matrix whose main diagonal elements are the pure quadratic coefficients and whose off-diagonal elements are half the mixed quadratic coefficients, and all coefficients correspond to the generated model equation, respectively. The point of maximum response ($\mathbf{x}_s$), also called the stationary point, is the solution to Eq. (3) [14], or

$$\mathbf{x}_s = -\frac{1}{2}\mathbf{A}^{-1}\mathbf{a} \quad (5)$$

Using the fitted polynomial quadratic model, response the related three-dimensional surface and contour plots (Fig. 3a–f) can be generated to illustrate the relationships between the main and interactive effects of the independent variables on the dependent one.

Accordingly, the point of maximum response was estimated to be located at pH = 6.15, enzyme activity = 0.95 U/ml, T = 41 °C (see Fig. 3a–f), 4-fluorophenol (3 mM), acetate buffer concentration (300 mM), and NaF (10^{-5} M). The addition of initial NaF in the reagent mixture (with a total volume of 2.5 ml) was to permit the initial stabilization of the cell potential at the start of reaction, before measuring the produced F^- ion during the progress of reaction.

The optimum parameter values were then used for the subsequent validation of the potentiometric method of enzymatic assay of H_2O_2. For this purpose, the linearity range of the method (1–200 μM), lower and upper detection limits (0.8 μM and 200 μM) were first determined as illustrated in Fig. 4. The reproducibility at two levels was also determined in optimized conditions with a relative standard deviation (RSD) % = 1.06 at 17 μmol l^{-1}, and RSD% = 1.81 at 34 μmol l^{-1}, with N = 10 replica). Subsequently, the content of H_2O_2 in two commercial hair bleaching products were determined and compared to the similar results obtained by the usual spectrophotometric method. The corresponding experimental level of H_2O_2 content for these two real samples, determined by the presented optimized potentiometric method, was compared to

Table 3
Statistical analysis of variance (ANOVA) for the evaluated response.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	0.1691	0.1691	0.16906	0.76	0.391
Regression	9	23.1455	23.1455	2.57172	11.54	0
Linear	3	17.4518	2.8218	0.94059	4.22	0.014
Square	3	5.0626	5.0626	1.68753	7.57	0.001
Interaction	3	0.6311	0.6311	0.21036	0.94	0.432
Residual Error	29	6.4651	6.4651	0.22294		
Lack-of-Fit	5	4.1419	4.1419	0.82838	8.56	0
Pure Error	24	2.3232	2.3232	0.0968		
Total	39	29.7797				

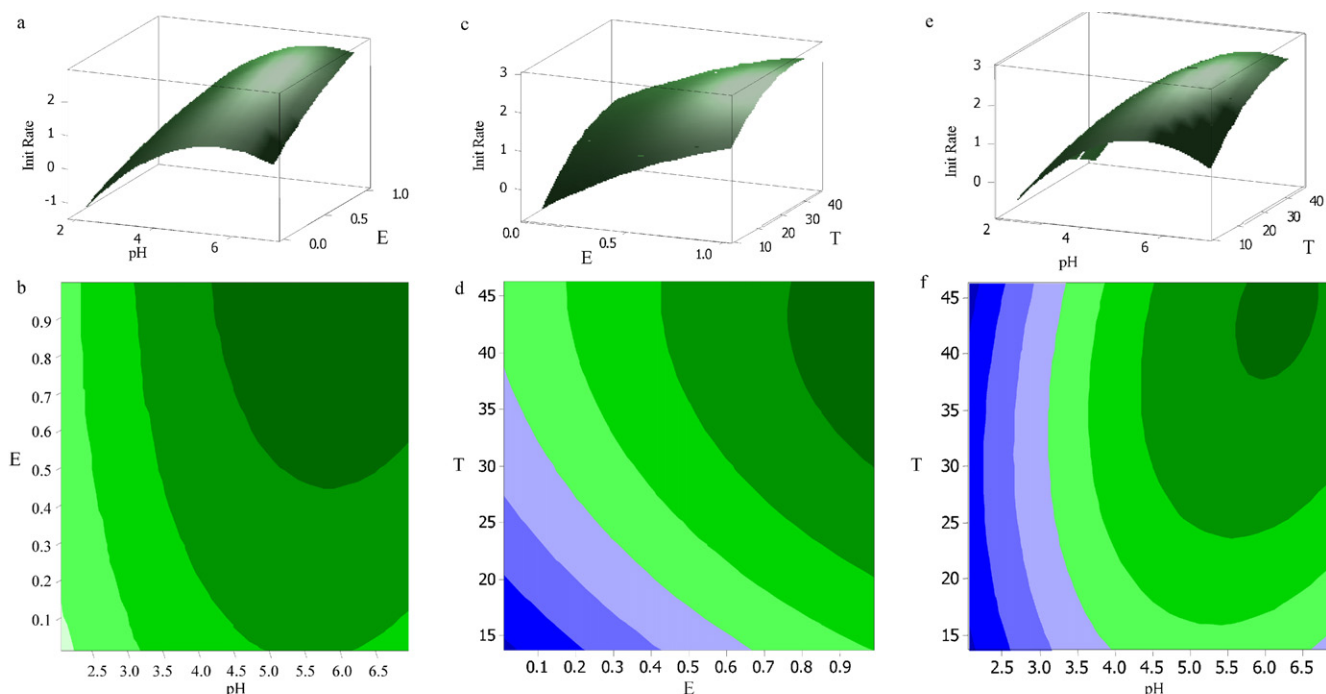

Fig. 3. (a)–(f) Different three and two-dimensional response surface plots. The response is the initial rate (μmol l^{-1} s^{-1}) and the used variables are pH, enzyme activity (E/(U ml^{-1}), and temperature (T/°C).

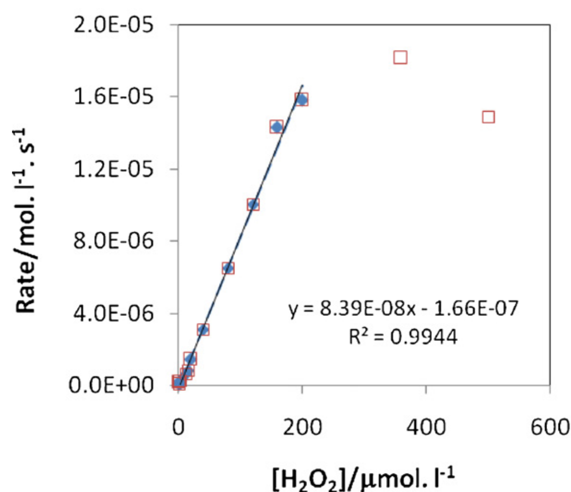

Fig. 4. Linearity curve (initial rate vs. concentration) using standard aqueous H_2O_2 solutions in optimized experimental conditions.

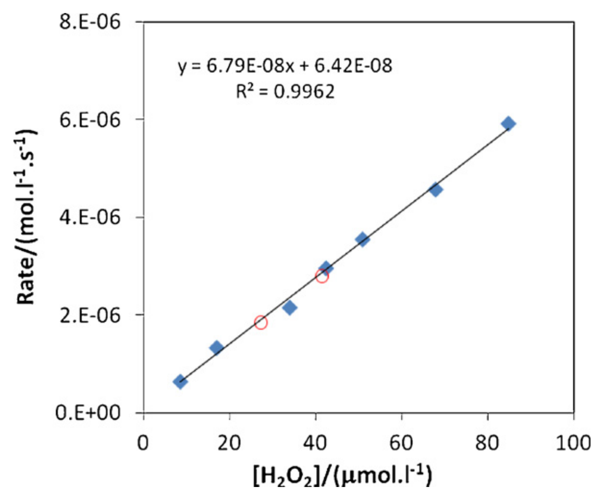

Fig. 5. Calibration curve (initial rate vs. concentration) using H_2O_2 standard aqueous solutions (♦) for the determination of H_2O_2 in real samples (○) in optimized experimental conditions.

the corresponding values obtained by the reference spectrophotometric method (see Table 4 and Fig. 5) and to the declared manufacturer values. Although, due to the instable character of H_2O_2, the determined H_2O_2 values are lower than those declared

Table 5
Recovery results in the determination of H_2O_2 content in two hear bleaching samples by initial rate potentiometric method in optimized conditions.

Added %	Expected Rate (mol/l s)	Observed Rate (mol/l s)	Recovery %
0	2.37E−06	2.37E−06	–
20	2.84E−06	2.80E−06	101.86
40	3.32E−06	3.34E−06	99.07
60	3.79E−06	3.70E−06	103.88
80	4.27E−06	4.32E−06	97.72
100	4.74E−06	4.72E−06	100.84

for these commercial samples, the correlation between the results obtained by the optimized potentiometric and by the reference spectrophotometric methods is satisfactory. Finally, the recovery of the optimized method was as well determined in the commercial hair bleaching product. The recovery expresses the relative difference between the expected (added) and observed (measured) concentrations of H_2O_2 in the real sample. The reported data in Table 5 confirm that the recovery of the optimized reaction for the assay of H_2O_2 is also satisfactory in the real matrix media.

5. Conclusion

Modeling and optimization of the initial-rate potentiometric method of HRP enzyme catalyzed the assay of H_2O_2 was performed

in this work by the combined "OVAT" (one-variable-a-time), full factorial and response surface methodology. The non-linear nature of the modeled response was explained by a second-order polynomial equation. This methodology is considered to be quite adequate for the design and optimization of process, and is able to explain the importance of the factors, their interactions, along with their optimum values. In order to check the performance of the optimized method, assays of H_2O_2 were performed on commercial hair bleaching samples. The satisfactory analytical characteristics of the optimized procedure such as: (1) the linear range of the method, (2) within-day precisions contents of H_2O_2 in aqueous samples at 2 levels, (3) within-day reproducibility in typical cosmetic samples, (4) recovery of the method (or the matrix effect) in real samples, (5) the determination of H_2O_2 in real samples in optimized experimental conditions were also determined. This optimized method benefits also from the analytical advantages of the combined potentiometric and the kinetics initial-rate methods which are: instrumental simplicity, precision, sensitivity, rapidity and selectivity (absence of interference from dissociated or undissociated organic compounds and inorganic ions). In addition, compared to optical based methods, the reported potentiometric method does not suffer from signal overlapping, and offer the possibility of measurement in turbid real samples.

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