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Urea enzymatic hydrolysis reaction: Optimization by response surface methodology based on potentiometric measurements

Farzad Deyhimi *, Maryam Bajalan

Department of Chemistry, Faculty of Sciences, Shahid Beheshti University, Evin-Tehran 1983963113, Iran

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ABSTRACT

The enzymatic hydrolysis reaction of urea by urease is optimized in this work by the chemometric response surface methodology (RSM), based on an initial rate potentiometric measurement using an NH_4^+ ion-selective electrode (ISE). In this investigation, the ranges of critical variables determined by a preliminary "one at a time" (OVAT) procedure were used as input for the subsequent RSM chemometric analysis. The RSM quadratic response was found to be quite appropriate for modeling and optimization of the hydrolysis reaction as illustrated by the relatively high value of the determination coefficient (R^2 = 90.1%), along with the satisfactory results obtained by the analysis of variance (ANOVA). All the evaluated analytical characteristics of the optimized method such as: the linear calibration curve, the upper and lower detection limits, the within-day precisions at low and at high levels, the assay recovery in pool serum media, along with the activation kinetic parameters, were also reported. Further, in order to check the quality of the optimization and the validity of the model, the assay of urea, both in aqueous laboratory and human serum samples, were performed. It has to be noted that the kinetic initial rate measurement method used in this work, permitted to overcome the general problem of NH_4^+ ISE low selectivity against Na^+ and K^+ interfering ions in real samples.

1. Introduction

The enzymatic hydrolysis reaction of urea by urease is of interest in many fields such as clinical chemistry, pharmaceutical industry, alcoholic beverages industry, environmental water, and soil sciences. Urease (urea amidohydrolase, EC. 3.5.1.5) occurs in many bacteria, several species of yeast and a number of higher plants. Urease, the only metallohydrolase that utilizes nickel in its active site, is known to be a highly efficient catalyst for the hydrolysis of urea [1–2] with a reaction rate enhancement over the uncatalyzed hydrolysis [3,4] that classifies it as the most proficient enzyme identified to date [4].

Urease catalyzed urea hydrolysis is schematically presented by the following reaction

$$(NH_2)_2CO + 2H_2O + H^+ \xrightarrow{\text{UREASE}} 2NH_4^+ + HCO_3^- \quad (1)$$

One of the most used methods of urea assay is a spectrophotometric method in which the NH_4^+ generated in the urea enzymatic hydrolysis reaction is coupled to the indicator glutamate dehydrogenase reaction [5]. However, the potentiometric assay of urea using conventional pH glass electrodes was also reported by many authors [6–12]. But, the response of pH electrode is found to be strongly dependent on the buffer capacity of the sample, where the used buffer reduces appreciably the resulting change of pH produced in the course of the urease catalyzed reaction. As a result this fact leads to a narrow dynamic range and a loss in sensor sensitivity. Katz and Rechnitz were the first to report the potentiometric assay of urea [13] and urease [14], using Beckman NH_4^+ ion-selective glass membrane electrode. The principle of measurement was based on the fact that the cell potential, after hydrolysis, is proportional to the amount of produced NH_4^+ and hence to the concentration of urea or urease. Later, Guilbault and coworkers [15] reported a thorough investigation of the response and selectivity of this Beckman electrode, along with a study of the kinetics of deaminase enzyme systems (urease, asparaginase, glutaminase, amino acid oxidase and amine oxidases). Nevertheless, the general problem of NH_4^+ selective electrodes is its low selectivity against Na^+ and K^+ interfering ions which are, for example, present in serum (about 4.5 mM K^+ and 140 mM Na^+) and urine. On the other hand, NH_4^+ selective solvent polymeric membrane electrode, based on nonactin ionophore, was as well used as alternative for this purpose. Although, these electrodes present higher selectivity against Na^+ and K^+ ions, however, the effect of interference by these ions are still important. A potentiometric measurement of urease activity with the use of an ammonium ion-selective electrode was also reported by Krajewska et al. [16]. These authors presented a detailed study, mainly, on the influence of different buffers and their pH profiles on the response of the NH_4^+ ion-selective electrode. In addition, the enzyme was also used as physically or chemically immobilized in various gels

* Corresponding author. Tel.: +98 21 22431661; fax: +98 21 22403041.
E-mail addresses: f-deyhimi@sbu.ac.ir, fddeyhimi@yahoo.com (F. Deyhimi).

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[17–21] or on fiber-optic sensor [22] although with the difficulty of laborious sample preparation steps and the time consuming determinations. Nevertheless, compared to the usual spectrophotometric method, the use of the alternative potentiometric approach for the assay of urea is attractive by its simplicity, rapidity and the fact that there is no need for a second coupling indicator reaction for the assay of urea. In view of the significant utility of this reaction in many applications, it seemed useful to optimize the experimental parameters involved in this potentiometric reaction by a suitable chemometric method such as response surface methodology (RSM). For this purpose, we have also used the NH_4^+ solvent polymeric ion-selective membrane electrode (NH_4^+ ISE), and for the validation this optimized potentiometric method, we have reported, as well, its detailed analytical capabilities for the assay of urea in aqueous, individual and pooled human serum samples. It should be mentioned that to overcome the general problem of low selectivity of NH_4^+ ISE against Na^+ and K^+ interfering ions in real samples, all measurements were performed, in this work, by a rapid kinetic initial rate (v_o)

$$v_o = -\left(\frac{d[Urea]}{dt}\right)_{t=0} = 2\left(\frac{d[NH_4^+]}{dt}\right)_{t=0} \quad (2)$$

method in which, the fixed amounts of interfering ions (e.g. Na^+ and K^+) were found to not perturb the rate of NH_4^+ ion production during the course of the reaction.

2. Experimental

2.1. Chemicals

All experiments were performed using solutions prepared from analytical grade chemicals and doubly distilled water. Urease (EC. 3.5.1.5, from jack bean, with specific activity ~100 U/mg) was obtained from Boehringer Mannheim (Germany), and all other compounds were obtained from Merck (Germany) or Fluka (Switzerland). Different tris(hydroxymethyl-aminomethane) solutions (Tris–HCl) were prepared in the concentration range 25–600 mM, and their pHs were adjusted, before use, according to the values indicated in the experimental design found in Table 1. A stock solution of 2.309 kU/cm^3 urease enzyme was obtained by dissolving the appropriate weight of initial enzyme in doubly distilled water. The laboratory made primary aqueous solutions of urea (116.55 mM), containing also NaCl, KCl and NH_4Cl electrolytes with background concentrations of 148.5, 4 and 0.25 mM, respectively, was used as primary standard solution. The activity of initial urease enzyme was determined (as 64.99 U/mg) by potentiometric measurement using NH_4^+ ISE and the Nernst equation. Human serum samples (obtained from Taleghani Hospital, Shahid Beheshti University) were used to check the quality of the improvement of the optimized enzymatic reaction for the assay of urea. The serum samples were stored at 253 K (−20 °C) until required for analysis.

2.2. Instrumentation

Both the used combined pH electrode and pH-meter (model 691) were from Metrohm (Switzerland). The reference Ag/AgCl electrode used in this work was from Fluka (Switzerland), and the solvent polymeric NH_4^+ selective membrane electrodes were fabricated from Selectophore grade compounds (Fluka), with the following membrane composition (mass %): 1.0% of the mixture of nonactin (72%) and monactin (28%) as ionophore, 0.6% sodium tetrakis[3,5-bis (trifluoromethyl)phenyl]borate, 65.7% bis(2-ethylhexyl)sebacate and 32.7% PVC, as previously reported [23–26]. NH_4Cl electrolyte (1 mM) was used as internal filling electrolyte solution. The experimental cell potentials were recorded using a high input impedance (>1 GΩ) Topward multimeter (model 1304, Taiwan, Korea) equipped with a GPIB interface Bus option (1304G) which was connected to a personal computer (Samsung, 386/32 MHz processor) via the GPIB interface card (IEEE488, Keithley, USA) for data acquisition and processing. A laboratory written Basic program combined with Microsoft Excel (XP-Office 2003) software were used for data acquisition and initial rate calculation. All measurements were performed under stirring conditions and the temperature was kept constant, employing a double-wall container enabling the circulation of thermostated water from a bath (Thelco, Precision Scientific Co., USA).

Table 1
Design table showing the randomized run order of experiment, and the value of the different factors in the experimental design of RSM

Run order	X_1 = pH	X_2 = Urease/(U/cm^3)	X_3 = [Tris–HCl](/mM)
1	4.2	17.2	146.6
2	4.2	64.8	146.6
3	7.8	17.2	503.4
4	7.8	64.8	503.4
5	4.2	64.8	503.4
6	7.8	64.8	146.6
7	6.0	41.0	325.0
8	4.2	17.2	503.4
9	6.0	41.0	625.0
10	6.0	41.0	325.0
11	6.0	41.0	325.0
12	6.0	41.0	325.0
13	3.0	41.0	325.0
14	6.0	41.0	325.0
15	9.0	41.0	325.0
16	6.0	41.0	25.0
17	7.8	17.2	146.6
18	6.0	1.0	325.0
19	6.0	41.0	325.0
20	6.0	81.0	325.0
21	6.0	41.0	625.0
22	3.0	41.0	325.0
23	6.0	41.0	325.0
24	6.0	41.0	325.0
25	6.0	81.0	325.0
26	9.0	41.0	325.0
27	4.2	64.8	503.4
28	4.2	64.8	146.6
29	4.2	17.2	146.6
30	6.0	41.0	325.0
31	7.8	64.8	146.6
32	6.0	41.0	325.0
33	7.8	64.8	503.4
34	4.2	17.2	503.4
35	6.0	1.0	325.0
36	7.8	17.2	503.4
37	7.8	17.2	146.6
38	6.0	41.0	25.0
39	6.0	41.0	325.0
40	6.0	41.0	325.0
41	9.0	41.0	325.0
42	4.2	17.2	503.4
43	6.0	41.0	25.0
44	7.8	17.2	503.4
45	6.0	41.0	325.0
46	6.0	41.0	625.0
47	4.2	17.2	146.6
48	7.8	64.8	503.4
49	6.0	41.0	325.0
50	4.2	64.8	146.6
51	6.0	41.0	325.0
52	7.8	64.8	146.6
53	6.0	41.0	325.0
54	6.0	41.0	325.0
55	7.8	17.2	146.6
56	3.0	41.0	325.0
57	6.0	1.0	325.0
58	6.0	41.0	325.0
59	6.0	81.0	325.0
60	4.2	64.8	503.4

3. Method

3.1. Principle

In the urea hydrolysis reaction catalyzed by urease, as schematically presented by reaction (1), the quantity of urea is determined via the amount of generated NH_4^+ produced in the reaction. Using a galvanic cell, containing both NH_4^+ ISE and reference electrode, the production of NH_4^+ was monitored during the course of reaction by the increase of cell potential according to the Nernst equation

$$E = E' + \frac{2.303RT}{F}\log([NH_4^+]/M) \quad (3)$$

where E is the experimental cell potential, E' is the cell constant potential, F and R are Faraday and gas constants, respectively, T is the Kelvin temperature, $s = \frac{2.303RT}{F}$ is the Nernstian slope of the NH_4^+ ISE. The data collection started after the start of the reaction with a sampling time of 0.1 s and maximum during 2 min. The initial rate was each time determined by linear regression performed on the reaction progress curve, mainly between 10 and 20 s (but up to 60 s in worst case during optimization steps), after the start of the 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 urease enzyme into the reagent mixture. Clearly, as it turns out well in highly diluted solution, concentration is used instead of activity for the calculation of NH_4^+ ion concentrations in the above equation.

3.2. Chemometric procedure

The indicator reaction was first analyzed, using initial rate measurements by OVAT method, by monitoring the production of NH_4^+ ions via potentiometric detection using NH_4^+ ISE and Ag/AgCl reference electrode. Typical reaction progress curves (expressed in terms of urea concentrations versus time) are presented in Fig. 1.

The critical variables of the urea hydrolysis reaction along with their variation ranges were, therefore, first determined based on a preliminary OVAT procedure, and further optimization was then carried out by the chemometric response surface methodology (RSM) in order to optimize properly the experimental conditions for the potentiometric assay of urea. The RSM procedure consisted of a nearly rotatable central composite design (CCD) with three factors at 2 levels, including full factorial with 8 cube points, 6 center points, and 6 axial points (α=1.682) with 3 blocks. The design was nearly rotatable; this means that the design had points which were almost equidistant from the centre. The selected runs were also randomized. This procedure leads to 60 experiments, where the experimental response data were analyzed by a regression procedure based on the response surface methodology (RSM) [27].

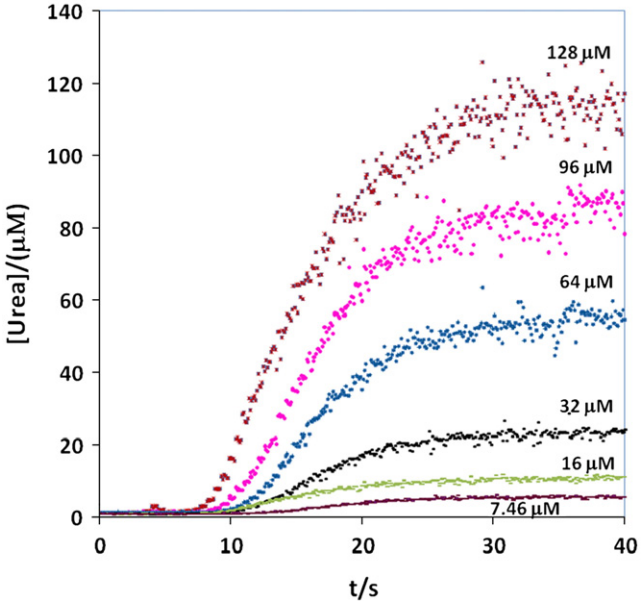


Fig. 1. Typical enzymatic reaction progress curves, illustrating the amount of hydrolyzed urea versus time, for different assays of urea at T=298 K (25 °C).

Table 2
Statistical evaluation of the regression coefficients for the quadratic response model (Eq. (4)) by RSM

Term	Coef	SE Coef	T	P
a_0	−5.14209	0.497509	−10.336	0
Block 1	0.07578	0.034363	2.205	0.032
Block 2	−0.03472	0.034363	−1.01	0.317
a_1	1.39643	0.121832	11.462	0
a_2	0.05393	0.007545	7.148	0
a_3	0.00517	0.001016	5.094	0
a_{11}	−0.10245	0.008996	−11.389	0
a_{22}	−0.00046	0.000051	−9.063	0
a_{33}	0	0.000001	−1.142	0.259
a_{12}	0.00205	0.000906	2.263	0.028
a_{13}	−0.00051	0.000121	−4.225	0
a_{23}	−0.00004	0.000009	−4.239	0

Terms and other column headings designate: block effect (repeated 2 series of experiments), coefficient related to different terms in (Eq. (4)), standard error of coefficients (SE Coef), Student's T-values (T), and P=P-values, respectively.
S=0.1882.
R^2=90.1%.
R^2(adj)=87.8%.

It is known that the composite design is a useful design capable to describe curvature, which is needed to explain a non-linear variation behavior property (i.e. the variation of enzyme activity upon changing the pH value). The model that can be fitted to a composite design is an empirical function, determined from the statistical correlation suitability of the observed responses and the experimental factors. For this purpose, a second order polynomial model equation is usually used [27–29]

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 \quad (4)$$

where Y is the predicted response (e.g. initial rate of NH_4^+ production), and X_1 (pH), X_2 (urease enzyme activity), and X_3 (Tris–HCl buffer concentration) are the independent variables or the experimental factors. The linear coefficients a_1, a_2, and a_3, express the linear effect of each variable; the a_{11}, a_{22}, and a_{33}, coefficients express the quadratic effects; a_{12}, a_{13}, and a_{23}, coefficients express interactive effects between the variables and a_0 is a constant corresponding to the central point of experimental variables. The statistical design, data analysis and various plots were obtained by using Minitab Statistical Software (Release 14).

4. Results and discussion

4.1. Chemometric procedure

Based on potentiometric initial rate measurements, the OVAT preliminary optimization procedure for the determination of urea was performed at T=298 K (25 °C), as a first step, in the presence of the following background electrolytes NaCl, KCl, NH_4Cl, with initial concentrations 148.5, 4, and 0.25 mM, respectively, similar to those found in normal human serum samples. Accordingly, the following optimized parameters and concentration ranges were selected for further optimization by RSM procedure: pH (6–9), buffer concentration (50–450 mM), and enzyme activity (2–60 U/cm^3). In all these

Table 3
Statistical analysis of variance (ANOVA) for the evaluated urea assay model by RSM

Source	*df*	Seq SS	Adj SS	Adj MS	*F*	*P*
Blocks	2	0.1727	0.1727	0.08634	2.44	0.098
Regression	9	15.3266	15.3266	1.70296	48.1	0
Linear	3	7.0129	5.3699	1.78998	50.5	0
Square	3	6.8636	6.8636	2.28787	64.6	0
Interaction	3	1.4502	1.4502	0.48338	13.7	0
Residual Error	48	1.7003	1.7003	0.03542		
Lack of Fit	33	1.3426	1.3426	0.04068	1.71	0.136
Pure Error	15	0.3577	0.3577	0.02385		
Total	59	17.1997				

The column headings designate: degrees of freedom (*df*), sequential sums of squares (Seq SS), adjusted sums of squares (Adj SS), adjusted mean squares (Adj MS), *F*-test value (*F*), and *P*-value (*P*), respectively.

tests, the final fixed concentration of the urea in the chemometric experiments was 0.186 mM. In the RSM procedure, the range of the used variables, their respective levels and the randomized experimental design are presented in Table 1. Table 2 shows the regressed value of the coefficients for the empirical quadratic model Eq. (4), and their evaluated statistical characteristics.

The results show the importance of the factors that affect the response are in the following order: pH(X_1)>>urease Enzyme activity (X_2)>Tris–HCl Buffer concentration(X_3). The estimated relatively high value of the determination coefficient, expressed as a percentage (R^2=90.1%), indicates that the model fits 90.1% of the experimental raw data. The fact that the R^2(adj)=87.8% value is also very close to the R^2 value confirm also that there is not a necessity for a significant correction, due to the sample size and the number of terms in the model. The quality of the regression, estimated by the analysis of variance (ANOVA), is shown in Table 3.

The Fisher variance ratio (*F*-value) is the ratio of the mean square due to regression, divided to the mean square due to error. The mean squares are obtained by dividing the sum of squares of each of the two sources of variation (the model and the error variance) by the respective degrees of freedom. If the model is a good predictor of the experimental data the computed *F*-value would be higher than the tabular *F*-value. The evaluated values of ANOVA (Table 3) for the quadratic response function, demonstrates that the model is highly significant, as the computed *F*-value (=48.1) is much greater than the tabular *F*-value (=2.76) at the 5% level, for the regression, linear, square and interaction terms. Table 3 shows also that, in the model, both the resulting "Lack of Fit" (*F*-value=1.71) and "block effect" (*F*-value=2.44) are not significant. Generally, the *P*-levels can also be used as a tool to check the significance of each of the regression coefficients. This information is necessary to explain the correlation of the mutual interactions between the factors. The smaller the

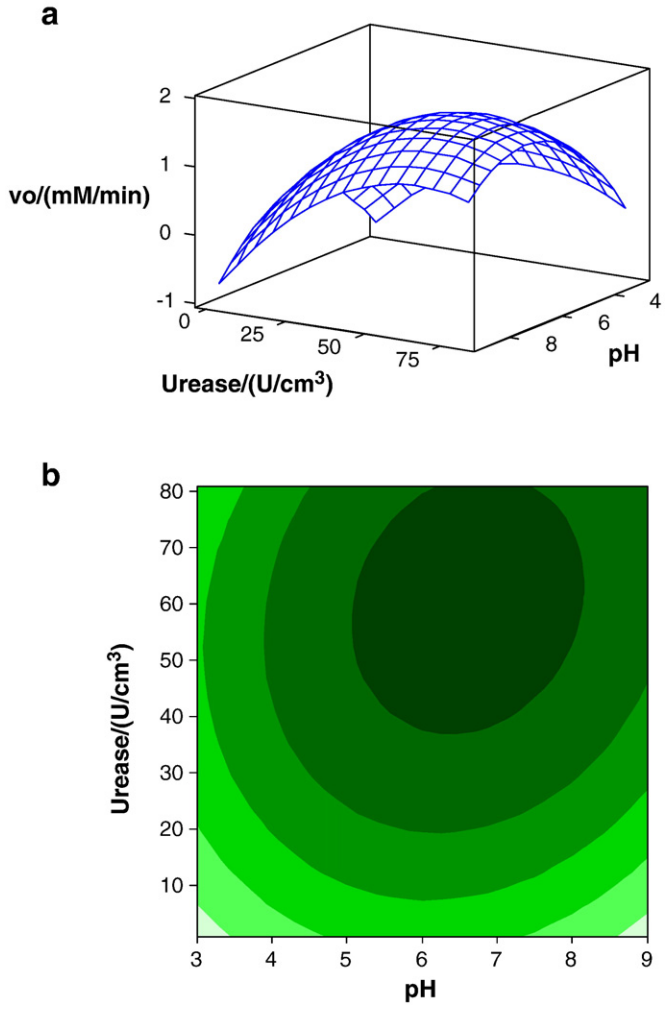


Fig. 2. Different 3- and 2-dimensional (contour) response surface plots versus experimental variables (enzyme activity and pH), at *T*=298 K (25 °C).

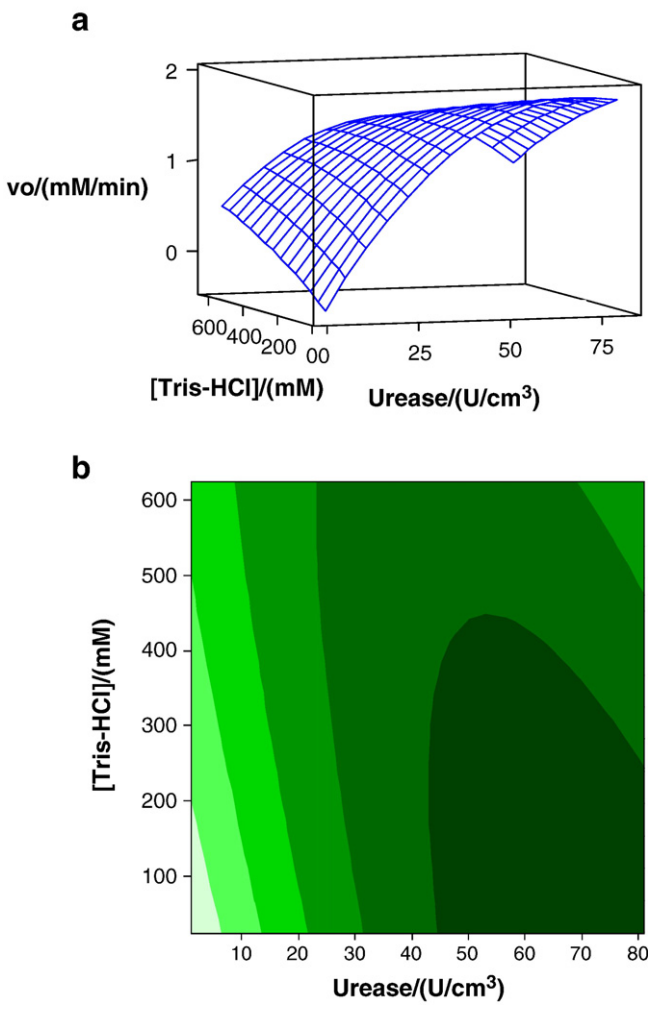
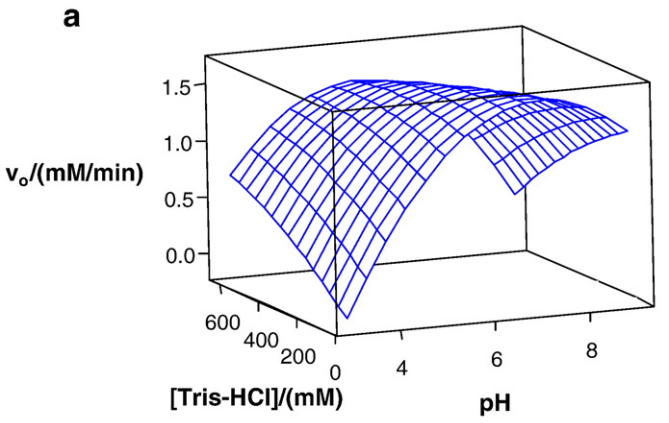


Fig. 3. Different 3- and 2-dimensional (contour) response surface plots versus experimental variables (buffer concentration and enzyme activity), at *T*=298 K (25 °C).

magnitude of the *P*, the more significant is the corresponding coefficient. In particular, the *P*-values in Table 3 reveal that all linear, square (except for X_3^2, where X_3=buffer concentration, with P=0.259), and interaction terms are significant at α=0.05 level. Therefore, the model confirms the presence of curvature in the response surface. The *P*-values for the regression (Table 3) confirm, once again, the adequacy of the model. The plot of the residuals (difference between the observed and fitted values) versus the randomized run order presented a completely random pattern and did not show any systematic effects or unusual observations. Also, the plot of residuals versus the fitted values confirmed a reasonable random distribution of the residuals around the zero line. The linear trend of the normal probability plot and the bell-shaped tendency of the residuals confirmed as well a fairly normal character of the residuals. Figs. 2a,b–4a,b show the various 3-dimensional plots of the response surface model. These plots are useful to visualize the generated response surfaces by the model based and based on potentiometric measurements. Accordingly, the point of maximum response was found to be at X_1=pH=7, X_2=urease Enzyme activity=60 U/cm^3, and X_3=Tris–HCl Buffer concentration=100 mM.

4.2. Evaluation of kinetic parameters

Using the previously obtained optimum parameters (X_1=pH=7, X_2=urease Enzyme activity=60 U/cm^3, and X_3=Tris–HCl Buffer concentration=100 mM), the kinetic parameters (V_{max}, K_m) were evaluated by the reciprocal graphical Lineweaver–Burk method (see Fig. 5), with the final urea concentration range of 100 to 1.63 10^3 μM. Accordingly, from the plot of 1/[*v*/(mM /min)] versus 1/([C_{urea}]/μM) (Fig. 5), V_{max} and K_m values were found to be 5.33 mM/min and 2.59 mM respectively, based on the corresponding regressed line (y=0.4668+0.1877, R^2=0.9966). The obtained K_m value (2.59 mM) was found to be consistent with the majority of the K_m values, reported for enzyme in free state [30–34] and particularly with jack bean urease [35–40], which fall in the range 1 to 4 mM.



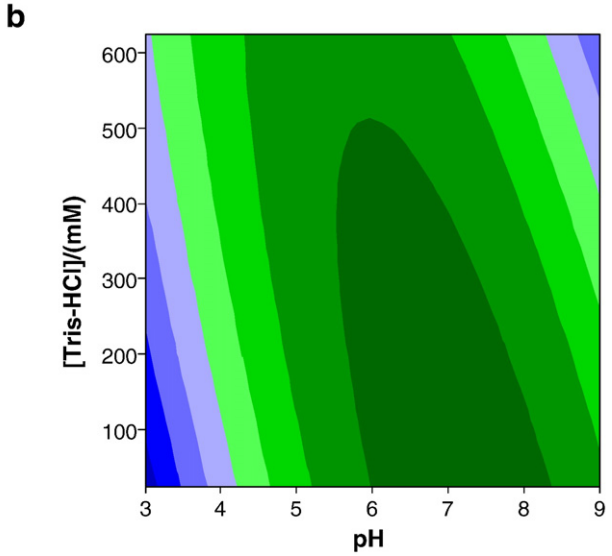


Fig. 4. Different 3- and 2-dimensional (contour) response surface plots versus experimental variables (buffer concentration and pH), at T=298 K (25 °C).

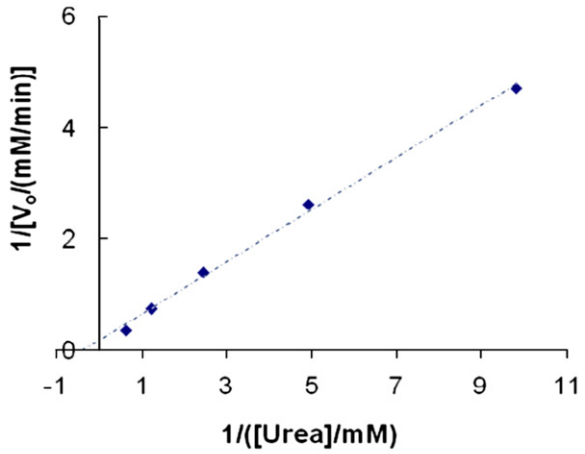


Fig. 5. Plot of 1/[*v*/(mM/min)] versus 1/([C_{urea}]/mM) for the determination of V_{max} and K_m according to the Lineweaver–Burk method.

4.3. Analytical performances and application to real sample assays

The analytical characteristics of the indicator reaction were determined in the optimized RSM conditions (pH, enzyme activity, buffer concentration). Accordingly, using urea standard solutions, the linear range of the method (y=0.0549+1.1179, R^2=0.9995) was determined by plotting initial rate versus concentration (see Fig. 6).

From the intersection of the linear portions of the urea calibration curve, initial rate (Y=v_o/(μM/s)) versus urea concentration (x=[Urea]/(mM)), the upper and lower detection limits were also evaluated by graphical method. The extension of the linearity range for the urea assay, from x=[Urea]/(mM)=8.10^{-4} to x=[Urea]/(mM)=10^{-1}

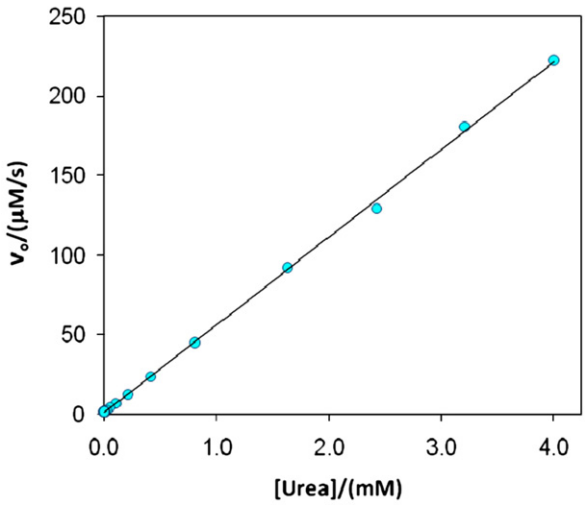


Fig. 6. Linearity of the initial rate potentiometric measurement of urea assay versus urea concentration in enzymatic reaction at T=298 K (25 °C).

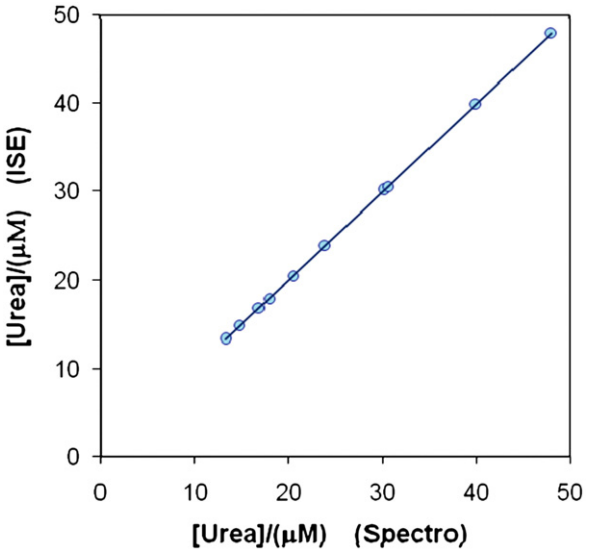


Fig. 7. Comparison of the methods of urea assay in real serum samples: the investigated initial rate potentiometric method (using NH_4^+ ISE) versus the usual spectrophotometric method (using commercial urea assay kit), at T=298 K (25 °C).

(with y=0.13725 x+4.4716, R^2=0.999), before optimization, to x=[Urea]/(mM)=8.10^{-4} up to x=[Urea]/(mM)=4 (with y=54.9345 x+1.1179, R^2=0.9995) after RSM optimization confirms the resulting response improvement. Within-day precisions of the method, expressed as the relative standard deviation (%RSD), were also determined for 14 replica using aqueous urea aqueous standard solutions at low level (x=[Urea]/mM=8.10^{-2}) as 2.34%, at high level (x=[Urea]/mM=8.10^{-1}) as 1.01%, and also for human pool serum (x=[Urea]/mM=$8.9\cdot10^{-2}$) as 1.65%. Analytical recovery of this initial rate-potentiometric method was as well determined using human pool serum samples and standard addition technique. The amount of urea recovered in pool serum after addition of 20%, 40%, 60%, 80% and 100% urea were: 19.63%, 40.07%, 60.65%, 80.13%, and 100.98%, respectively. The exactitude of the optimized potentiometric assay of urea was further compared to the usual spectrophotometric method using commercial urea kit (Pars Azmoun, Iran) on human serum samples. Fig. 7 illustrates the satisfactory correlation (y=1.004 x−0.008, R^2=0.9974) obtained between the results by the present method versus those obtained by using the commercial spectrophotometric kit at 303 K (30 °C) for the urea assay in individual serum samples. In the used commercial kit (Pars Azmoun Co, Iran), the formation of NH_4^+ generated by the urease catalyzed urea hydrolysis reaction, is determined via the NADH-glutamate dehydrogenase coupled reaction system.

5. Conclusion

The combined OVAT and RSM procedures used in this work proved to be quite adequate for modeling urease catalyzed hydrolysis reaction of urea as a potentiometric indicator reaction using both initial rate measurement and an NH_4^+ ISE. The RSM analysis permitted to explain the impact of the experimental factors, their interactions and also their optimum ranges. As a result, the non-linear nature of the obtained response was conveniently described by a quadratic polynomial equation obtained with a satisfactory determination coefficient (R^2=90.1%). Satisfactory analytical characteristics including: linearity (with low and high detection limits), within-day precisions (at low and high levels and also in real sample) and recovery (in pool serum sample) were found and the validity of the model was also tested by the assay of urea both in aqueous and in human serum matrix. The exactitude of the optimized potentiometric assay of urea was further confirmed by its comparison to the usual spectrophotometric method using human serum samples, and the activation and kinetic parameter values corresponding to the optimized conditions were also reported. It has to be noted that the potentiometric initial rate measurement method used in this work, permitted conveniently to overcome the previously reported general problem of low selectivity of NH_4^+ ISE against Na^+ and K^+ interfering ions in real samples [41]. In conclusion, this optimized method offers, at once, the satisfactory analytical simplicity of the potentiometric method (precision, recovery, insensitivity to color or turbid samples) and those associated with the initial rate kinetic method (rapidity and selectivity). The obtained quadratic response equation would be particularly useful for predicting the expected response in any desired experimental conditions, particularly, in clinical, industrial, or environmental applications, for the assay of different urea real samples.

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