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Experimental design strategies in the optimization and robustness testing of adsorptive stripping voltammetric conditions for kynurenic acid determination[1]

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Abstract

Experimental design was used for the optimization and robustness testing of an adsorptive stripping voltammetric procedure for kynurenic acid determination. The optimization of the peak height response proceeded through a screening phase (D-optimal design strategy) followed by a response surface study (Doehlert design) applied to the variables pH, pulse amplitude and stirring rate. An interaction between pH and stirring rate was pointed out. The optimized method was validated and the variation of factors that was expected to occur in practice was simulated in a robustness test. A composite fractional matrix for the evaluation of method robustness was used and pH emerged as the only critical factor. The linear range found applying the optimized conditions was 2.5×10^{-9} to 2.5×10^{-7} M and the calculated limit of detection was 1.72×10^{-9} M. © 1997 Published by Elsevier Science B.V.

Keywords: Adsorptive stripping voltammetry; Chemometrics; Experimental design; Kynurenic acid determination; Robustness testing; Validation

1. Introduction

Method development and optimization require the exploration of a space defined by independent parameters which are changed during experimentation in order to produce desirable values of the response. To achieve this aim, the experimental design selects the best location of experimental points in the predictor space and collects these points in a design matrix [1,2]. Some strategies of experimental design were developed in our laboratory to optimize sensitive adsorptive stripping voltammetric procedures for assaying different electroreducible drugs in dosage forms and biological fluids [3–5]. An interesting application of experimental design is robustness testing, which provides information on those critical parameters affecting the response. Robustness testing is an important part of method validation and it should

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show the reliability of an analysis with respect to deliberate small variations in method parameters [6–8].

The present paper describes the development and validation of a highly sensitive procedure based on adsorptive stripping voltammetry (AdSV) for kynurenic acid (KYNA) determination. KYNA (Fig. 1) is a triptophan metabolite of considerable biological and clinical significance, present in mammalian biological fluids and in the central nervous system [9]. KYNA has an antagonist effect on the ionotropic glutamate receptors and a significant increase in its brain concentration could be useful in pathological situations [10]. In pharmacological studies, the determination of KYNA in dialysate is important in order to understand how the functional activity changes with respect to variations in concentration. Methods for determining KYNA are generally based on a gas chromatography/mass spectrometry (GC/MS) approach or high-performance liquid chromatography (HPLC) with fluorometric, coulometric or spectrophotometric detection [11–16].

In the present work, an AdSV procedure, characterized by a large number of factors likely to affect the peak height response, was optimized for KYNA determination. Experimental design strategies were used for the optimization and robustness testing. The chemometric approach described is of general interest for application to other analytical procedures and gives practical guidelines for robustness testing.

2. Experimental

2.1. Equipment and materials

Voltammetric experiments were performed with an AMEL 433 polarographic analyzer (Amel, Milan, Italy) incorporating a magnetic stirrer and a three-electrode system consisting of a hanging mercury drop working electrode coupled with an Ag/AgCl reference electrode and a platinum wire as auxiliary electrode. The AMEL analyzer was connected with a personal computer and the data handling, storage, print-out and graphics were obtained by means of a special AMEL software. A Metrohm 691 pH meter (Metrohm, Herisau, Switzerland) was used to obtain pH measurements. The experimental design was generated, and statistical analysis of the data was performed, using Nemrod software (LPRAI, Université de Marseille III, France).

Kynurenic acid was purchased from Sigma Chemical (St. Louis, MO, USA). All chemicals used were of analytical–reagent grade with no further purification. All solutions were prepared with water obtained by means of a Milli-Q system (Millipore). KCl supporting electrolyte (0.01 M) was prepared by diluting 3 M KCl (Metrohm, Herisau, Switzerland) with water and adjusting the pH (2.0–4.0) with 0.1 M HCl. An accurately weighed amount of KYNA (3 mg) was dissolved in 100 ml H_2O and the resulting standard solution was stable for at least 4 days at 4°C. Working solutions were prepared daily by diluting the standard solution 20-fold (experimental design), or 100-fold (calibration curve) with water.

2.2. Adsorptive stripping procedure

The adsorptive stripping procedure was carried out as follows: 10 ml KCl (0.01 M, pH 2.6) were transferred into the voltammetric cell and deaerated by bubbling nitrogen-free oxygen for 10 min in the first cycle and 30 s for each successive cycle. The accumulation step on the hanging mercury drop electrode was performed at 0 V for 50 s, stirring the solution at 200 rev min^{-1} with a magnetic stirrer. After an equilibrium time (10 s), a cathodic differential pulse potential scan was applied between −0.5 V and −1.1 V with the following optimized settings: scan rate 40 mV s^{-1}; pulse amplitude −60 mV; drop size 40 arbitrary units (a.u.). The voltammetric cycle was repeated twice with a new drop for each analyzed solution; the mean of these voltammograms was

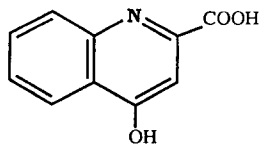

Fig. 1. Kynurenic acid chemical structure.

obtained and, after blank subtraction, the peak height was measured.

3. Results and discussion

3.1. Method development using experimental design

Many variables have to be optimized when developing an AdSV method, such as pH, type and ionic strength of supporting electrolyte, accumulation time, accumulation potential, pulse amplitude, scan rate, stirring rate, drop size and temperature. Due to the high number of the variables, a complete response surface would be a complicated multidimensional structure requiring a strong experimental effort to be fully determined. Hence, a screening phase that allowed the key factors to be established was advisable.

The optimization process for KYNA, concerning the maximization of the response peak height, involved the following seven variables: accumulation potential, E_{acc} (x_1); pH of supporting electrolyte (x_2); accumulation time, t_{acc} (x_3); scan rate, v_{scan} (x_4); stirring rate, stir (x_5); pulse amplitude, ΔE (x_6); drop size, ds (x_7). As regards the best background electrolyte, preliminary experiments allowed acidic 0.01 M KCl to be selected.

3.1.1. Screening phase: D-optimal design

The true relationship between the response y and the factors x is usually unknown. Therefore, the model chosen during the screening phase was an approximating function (Eq. (1)) which was likely to follow the response closely over the region of interest and included, according to our previous experience, the seven main effects and nine first-order interactions:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3 + x_3 + b_4x_4 + b_5x_5 + b_6x_6 + b_7x_7 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{25}x_2x_5 + b_{26}x_2x_6 + b_{34}x_3x_4 + b_{35}x_3x_5 + b_{46}x_4x_6 + b_{56}x_5x_6 + b_{57}x_5x_7 \quad (1)$$

In a screening phase, where one cannot afford a lot of experiments, it is necessary to select a minimum set of experiments to estimate the model coefficients. The selection of an appropriate set is very important; in fact, the accuracy of parameter estimate increases with the number of experiments, but it also depends upon the location of the design points [17]. For the KYNA screening phase, the experiments to carry out were selected by a D-optimal design strategy.

The optimal design is aimed at minimizing the variance of estimates of the effects [1]. There are several criteria for defining design optimality; D-optimal design, in particular, minimizes the determinant D of the dispersion matrix. To establish a design, the model, the desired number of experimental runs and an arbitrary initial set of experiments must be specified. By an iterative procedure, the initial set of candidate experiments is modified by adding and deleting experiments so that the determinant D of the dispersion matrix is minimized. Several matrices with different number of experiments and different parameters (determinant of dispersion matrix, leverage, matrix trace and inflaction factors) are obtained and it is possible to select the matrix with the best compromise between the quality of information obtained and the number of experiments to be performed [17,18].

In the present case, we had to estimate 17 coefficients (constant term b_0, seven main effects and nine first-order interactions) and the experimental matrix containing the candidate points was a 2^7 full factorial design. Between the different optimal matrices obtained, a matrix with 21 experiments (Table 1) was selected as the best for this screening phase. Each variable was varied at two levels reported in Table 2 and the experiments were carried out in a randomized order with a KYNA concentration of 4.0×10^{-8} M.

The analysis of variance (ANOVA) indicated that the regression model assumed was significant (Table 3) [18]. The null hypothesis H_0, i.e., the hypothesis that all coefficients β_i are equal to zero, was to be disproved and the alternative hypothesis that one or more of the coefficients is not equal to zero was considered adequate [2]. In particular, the coefficient statistic analysis showed that pH (x_2), scan rate (x_4), stirring rate (x_5) and some first-order interactions, such as pH–pulse

Table 1
D-optimal design matrix

Expt. no.[a]	E_{acc} (mV)	pH	t_{acc} (s)	v_{scan} (mV s^{-1})	Stir (rev min^{-1})	ΔE (mV)	ds (a.u.)
4	−	+	+	−	−	−	−
8	+	−	−	+	−	−	−
17	+	−	+	+	−	−	−
12	−	−	−	−	+	−	−
18	+	+	+	+	+	−	−
6	−	+	−	−	−	+	−
9	−	−	+	−	−	+	−
16	+	−	−	+	−	+	−
13	+	+	+	+	−	+	−
21	−	+	+	−	+	+	−
2	+	+	−	+	+	+	−
1	+	−	+	+	+	+	−
7	+	−	−	−	−	−	+
14	−	+	−	+	−	−	+
20	+	+	−	−	+	−	+
11	+	−	+	−	+	−	+
15	−	−	−	+	+	−	+
5	+	+	+	−	−	+	+
19	−	−	+	+	−	+	+
10	+	−	−	−	+	+	+
3	−	+	+	+	+	+	+

[a] Randomized order.

amplitude (b_{26}), were important for the response. The analysis of effects also showed that the variables accumulation potential, accumulation time, pulse amplitude and drop size did not have a statistically significant main effect (Table 4), thus indicating that their main effect estimates could be generated by the noise.

The same conclusions on the 'active' effects were obtained (Fig. 2) by means of the graphic analysis of effects [19]. The advantage of this plot is that the numerical values of the effects are displayed. This analysis requires the construction of a bar graph in which the length of each bar is proportional to the absolute effect value. The effects that exceed the reference lines, corresponding to the 95% confidence interval, are those significant for the response. If there are no degrees of freedom, Lenth's approach can be used to obtain an estimate of the pseudo-standard deviation (PSE) in order to define the confidence interval [19]. From Fig. 2 it is also possible to decide the new experimental domain to be explored in order to obtain the maximization of the peak height response. In fact, in the right panel of Fig. 2, the positive effects are represented while the left panel reports the negative effects. Combining the information obtained from the screening phase, it was possible to fix four variables. Due to the absence of its significant interaction effects and its important positive linear effect (indicating an increase in the response as the parameter was varied from the low to the high level), scan rate was fixed at its high level (40 mV s^{-1}). The other fixed variables were those the effect of which was not

Table 2
Variables and their levels during the screening phase

Variable	Low level	High level
E_{acc} (mV)	0	−300
pH	2.0	4.0
t_{acc} (s)	50	70
v_{scan} (mV s^{-1})	20	40
Stir (rev min^{-1})	200	400
ΔE (mV)	30	50
ds (a.u.)	20	40

Table 3
ANOVA for the linear model assumed during the screening phase (Eq. (1) in text)

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-ratio
Regression	6.97×10^4	16	4.36×10^3	9.1[a]
Residuals	1.91×10^3	4	4.76×10^2	—
Total	7.17×10^4	20	—	—

[a] $9.1 > F^{crit} = 5.80$ (with 16 and 4 degrees of freedom and $\alpha = 0.05$).

statistically significant on the response: accumulation potential (0 mV), accumulation time (50 s) and drop size (40 a.u.). As regards pulse amplitude (x_6), owing to the important interactions with pH (x_2) and stirring rate (x_5), this variable was maintained in the study, even if its main effect was negligible for the response.

After this simplifying approach, reducing reasonably the number of factors, a response surface study near to the maximum of the response was performed, respectively, with the variables pH (x_1), pulse amplitude (x_2) and stirring rate (x_3) in the corresponding experimental domain: 2–3; 40–60 (mV); 100–300 (rev min^{-1}).

3.1.2. Response surface study: Doehlert design

The response surface was approximated by second-order polynomial and a Doehlert design was used to estimate the coefficients.

The points of a Doehlert design are generated from a regular simplex by taking differences among its vertices. This operation led to a geometric figure in which each vertex corresponds to one experiment and, in general, the number of experiments is equal to $k^2 + k + n$, where k is the number of the factors and n the number of centre points; in this design one variable must be studied at three levels, one at seven levels and the other $k - 2$ variables at five levels [1,20]. Due to the instrumental settings, it was impossible to study stirring rate at more than three levels in the experimental domain studied. As regards pH, a variation smaller than 0.2 units was considered inappropriate, and it was therefore decided to study pH at five levels, stirring rate at three levels and pulse amplitude at seven levels in a 17-experiment sequence including five replicates at the centre points. The ANOVA revealed that the regression was significant and the optimized conditions were selected by means of response surface plots from which it was possible to predict any point within factor space, even if that point had not been included in the design [21]. Fig. 3(a)–(c) shows the response surfaces for pH against stirring rate, maintaining pulse amplitude at its highest level (60 mV (Fig. 3(a)), at its centre level (50 mV, Fig. 3(b)) and at its lowest level (40 mV, Fig. 3(c)); from this figure it is evident that the pulse amplitude value for which we have the maximum response value is 60 mV (Fig. 3(a)). The interaction existing between pH and stirring rate is evident (Fig. 3(a)): the effect of pH is much higher when the stirring rate is at its higher levels and the effect of stirring rate is more important when pH has a codified positive value. In these conditions, the optimum values were 2.6 for pH, 200 rev min^{-1} for stirring rate, and 60 mV for pulse amplitude.

The good prediction quality of the model was verified by means of the good agreement observed between the experimental and predicted response obtained using the optimized conditions [5].

Table 4
D-optimal design: estimates of effects

Effect	Estimate	Effect	Estimate
b_0	62.16	b_{24}	5.51
b_1	−0.30	b_{25} *	14.56
b_2 *	−14.24	b_{26} *	−30.14
b_3	−5.24	b_{34}	0.54
b_4 *	19.54	b_{35} *	22.33
b_5 *	−15.79	b_{46}	0.16
b_6	−5.49	b_{56} *	−19.07
b_7	1.30	b_{57} *	−20.22
b_{23}	10.28	—	—

[a] Significant effect.

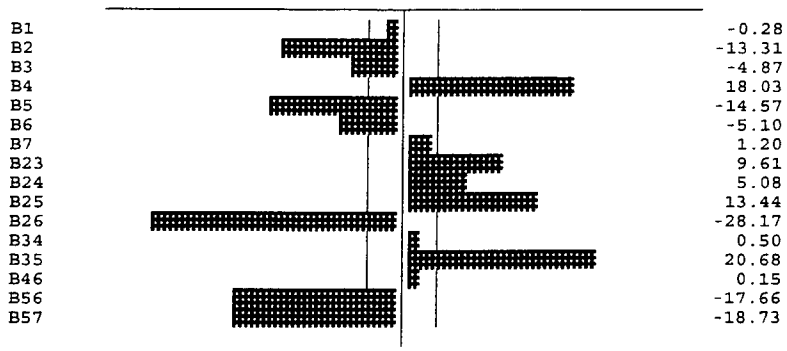

Fig. 2. Graphic analysis of effects. The lines define the 95% confidence interval. Right panel, positive effects; left panel, negative effects.

3.2. Analytical performance parameters

3.2.1. Robustness testing

Robustness testing involves producing small deliberate changes to the optimized operating conditions and measuring the effect upon a measured response such as the voltammetric peak height. A robustness test thus requires the selection of the factors and the levels at which to test them, followed by the selection of a suitable experimental design. The choice of the design depends on the number of the factors to test and on the postulated model. In general, linear models are usually sufficient because of the small experimental domain. However, to maximize information regarding method robustness around the best conditions, a quadratic model can be used. For each controlled factor, it is necessary to know its optimized value in order to define the interval within which it can be controlled.

In the assessment of the AdSV method for KYNA, five of the seven selected parameters (pH, x_1; t_{acc}, x_2; E_{acc}, x_3; ΔE, x_4; ds, x_5) were considered, due to the instrumental settings that did not allow small variations of the parameters scan rate and stirring rate. The experimental domain of selected variables is given in Table 5. The ranges examined were small deviations from the method settings which would normally occur.

At the beginning of robustness testing, a linear model was postulated and an eight-run Plackett–Burman design was chosen for the evaluation of coefficients. To test the model linearity, four experiments with the optimized conditions, corresponding to the centre of experimental domain, were carried out.

The linear model postulated did not fit well with the results obtained at the centre points, thus indicating that a curvature was present. Since the model was wrong, the results obtained from the Plackett–Burman matrix could not be used. In fact, in robustness testing, it is necessary to find a model that fits the data well in order to evaluate the simultaneous effect of small variations of all factors on the response. Hence, the following quadratic model for the five critical parameters was postulated:

$$\begin{aligned} y = {} & b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_{11}x_1^2 \\ & + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{55}x_5^2 + b_{12}x_1x_2 \\ & + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{15}x_1x_5 + b_{23}x_2x_3 \\ & + b_{24}x_2x_4 + b_{25}x_2x_5 + b_{34}x_3x_4 + b_{35}x_3x_5 \\ & + b_{45}x_4x_5 \end{aligned} \quad (2)$$

To estimate the coefficients of a second-order model, the variables have to be studied at more than two levels. Since it is difficult to divide a small experimental domain into a number of levels, it was decided to study the critical factors at three levels: -1, 0 and $+1$ and a 30-run composite fractional matrix was used.

Composite fractional design consists of the points of a fractional factorial design which have been augmented with some extra points at the centre of the design and 2m star points, where m

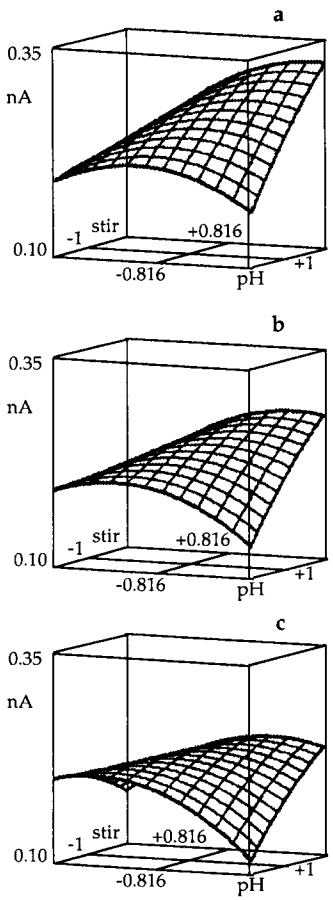

Fig. 3. Response surface for pH against stirring rate (stir) at fixed values of pulse amplitude: (a) 60 mV; (b) 50 mV; (c) 40 mV.

is the number of variables. These star points are located at $+\alpha$ or $-\alpha$ from the centre of the experimental domain and the value of α as well as the number of experiments at the centre depend upon the design criterion (orthogonality and rotatability). When the design region is spherical, the variables are studied at five levels: $-\alpha$, -1, $+1$, $+\alpha$; while, if the experimental domain is cubic, $|\alpha|$ is equal to $|1|$ and the variables are studied at three levels [17,22]. In our case, a cubic experimental region was studied and the matrix employed is reported in Table 6. The experiments were carried out in a randomized order with a KYNA concentration in cell of 3.24×10^{-8} M.

Table 5
Method settings and range investigated during robustness testing

Variable	Optimized value	Range investigated
pH	2.6	2.4–2.8
t_{acc} (s)	50	48–52
E_{acc} (mV)	0	+5–−5
ΔE (mV)	60	58–62
ds (a.u.)	40	38–42

The regression model assumed was found significant by means of ANOVA and the only factor causing the largest variation of the response was pH. The variation in pH was seen to exert a critical effect on the response (Fig. 2(a)–(c)). In fact (Fig. 4(a)), the response surface obtained for pH against drop size, maintaining the other variables at its centre value, shows that a change in pH value produces a great change in the response. The same behaviour can be observed in the surface where pH is plotted against accumulation time (Fig. 4(b)). However, for example, the plot of accumulation time against drop size, maintaining the other variables at the centre level (Fig. 4(c)), is markedly different from the above response surfaces. The shape of this response surface resembles an upside-down saddle. As the drop size or accumulation time were varied over the factor space, there was only a relatively small change in the observed response; this suggests that the two factors, like the other factors not shown, exert a small effect on the response compared with that of pH. The central point of the saddle region clearly corresponds to the robust conditions required for voltammetric analysis.

3.2.2. Linearity and range

Using the optimized conditions (pH, 2.6; accumulation time, 50 s; accumulation potential, 0 mV; pulse amplitude, 60 mV; scan rate, 40 mV s^{-1}; stirring rate, 200 rev min^{-1}; drop size, 40 a.u.), a well-defined signal with a peak potential of -840 mV was obtained and a linear relationship was found between peak height and KYNA in-cell concentration in a nine-level range, 2.5×10^{-9} to 2.5×10^{-7} M. Fig. 5 reports typical voltammograms (with blank subtracted) for in-

Table 6
Composite fraction matrix

Expt. no.[a]	pH	t_{acc} (s)	E_{acc} (mV s^{-1})	ΔE (mV)	ds (a.u)
4	−	−	−	−	+
3	+	−	−	−	−
11	−	+	−	−	−
18	+	+	−	−	+
13	−	−	+	−	−
2	+	−	+	−	+
22	−	+	+	−	+
19	+	+	+	−	−
6	−	−	−	+	−
20	+	−	−	+	+
14	−	+	−	+	+
23	+	+	−	+	−
27	−	−	+	+	+
8	+	−	+	+	−
25	−	+	+	+	−
15	+	+	+	+	+
26	−	0	0	0	0
9	+	0	0	0	0
24	−	−	0	0	0
1	+	+	0	0	0
12	0	0	−	0	0
17	0	0	+	0	0
5	0	0	0	−	0
28	0	0	0	+	0
16	0	0	0	0	−
21	0	0	0	0	+
10	0	0	0	0	0
7	0	0	0	0	0
29	0	0	0	0	0
30	0	0	0	0	0

[a] Randomized order.

creasing KYNA in-cell concentration in the range 2.67×10^{-9} to 2.52×10^{-7} M. The linear relationship found was $y = 2.62 \times 10^{-2}$ ($\mu A \cdot l\ \mu g^{-1}$) $- 4.93 \times 10^{-3}$ (μA). The square of correlation coefficient R^2 of the regression line was 0.9992 and the cross-validated R^2, in which the residual sum of squares is substituted by the predictive residual sum of squares [1], was 0.9987.

3.2.3. Accuracy and repeatability

The ranges used to assess accuracy and repeatability (with blank subtraction) were 2.59×10^{-9} to 1.27×10^{-8} M and 1.28×10^{-8} to 6.37×10^{-8} M that can be useful for analytical applications [11]. Using a calibration line obtained in the first range, recovery was $97.8 \pm 2.7\%$ (RSD, $n = 3$) at the lower level, $98.3 \pm 2.8\%$ at the central level (7.74×10^{-9} M) and $102.2 \pm 2.3\%$ at the upper level [7].

3.2.4. Detection limit

The limit of detection (DL) was calculated as 3(S.D.)/S, where S was the slope of the calibration curve in the range 2.59×10^{-9} to 1.27×10^{-8} M and S.D. was the residual standard deviation of the regression line [7,23]. The DL was found to be 1.72×10^{-9} M and was validated by independent analyses of KYNA samples prepared at the DL.

4. Conclusions

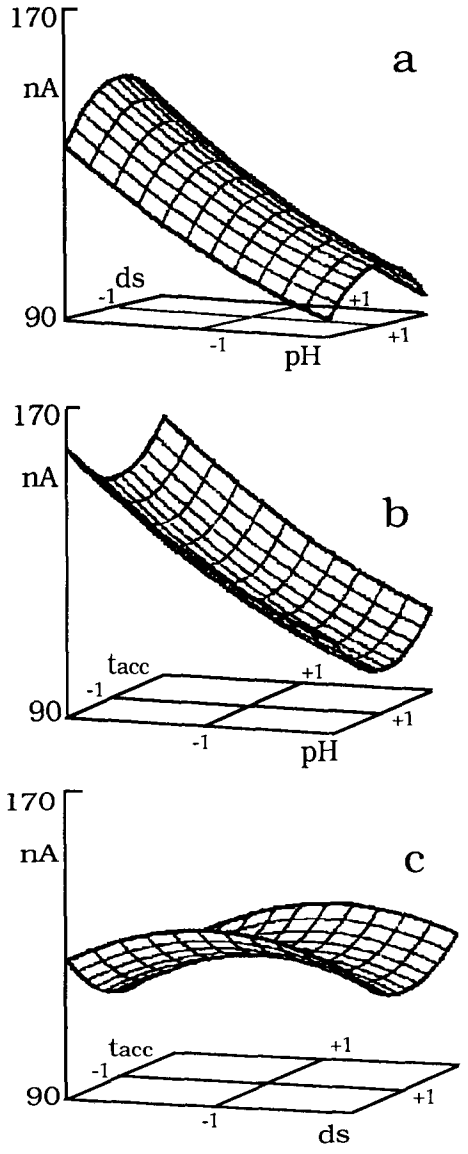

Fig. 4. Robustness testing response surfaces: (a) pH against drop size (ds), maintaining pulse amplitude at 60 mV, accumulation potential at 0 V and accumulation time at 50 s; (b) pH against accumulation time (t_{acc}), maintaining pulse amplitude at 60 mV, accumulation potential at 0 V and drop size at 40 a.u.; (c) drop size (ds) against accumulation time (t_{acc}), maintaining pulse amplitude at 60 mV, accumulation potential at 0 V and pH at 2.6.

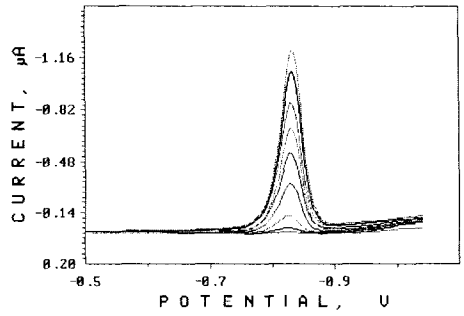

Fig. 5. Adsorptive stripping voltammograms of kynurenic acid for successive additions of working solution (0.313 μg ml^{-1}): the first addition of 15 μl; the second of 35 μl; the third of 100 μl; and successive additions of 250 μl.

The use of experimental design strategies for optimization and robustness testing allowed a sensitive AdSV procedure to be developed and validated. Due to the mean KYNA human daily urine excretion of about 8.4 μmol [11], the optimized method seems suitable for the potential AdSV determination of KYNA in urine. The method was found to be robust with respect to those parameters evaluated apart from the critical factor pH for which a precautionary statement should be included in the procedure. The approach described is of general use and it is to be encouraged for the set-up and validation of an analytical method.

Acknowledgements

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References

[1] I.E. Frank and R. Todeschini (Eds.), The Data Analysis Handbook, Elsevier, Amsterdam, 1994.
[2] S.N. Deming and S.L. Morgan (Eds.), Experimental Design: A Chemometric Approach, Elsevier, Amsterdam, 1993.
[3] S. Furlanetto, P. Gratteri, S. Pinzauti, R. Leardi, E. Dreassi and G. Santoni, J. Pharm. Biomed. Anal., 13 (1995) 431–438.
[4] P. Gratteri, S. Furlanetto, S. Pinzauti, R. Leardi and P. Corti, Electroanalysis., 7 (1995) 1161–1164.

[5] S. Pinzauti, P. Gratteri, S. Furlanetto, P. Mura, E. Dreassi and R. Phan-Tan-Luu, J. Pharm. Biomed. Anal., 14 (1996) 881–889.
[6] US Pharmacopeia 23, US Pharmocopeial Convention, Rockville, MD, 1995, pp. 1982–1984.
[7] ICH Topic Q2B, Validation of analytical methods: methodology, 3rd Int. Conf. Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Yokohama, Japan, 29 Nov.–1 Dec., 1995.
[8] K.D. Altria and S.D. Filbey, Chromatographia, 39 (1994) 306–310.
[9] F. Moroni, P. Russi, G. Lombardi, M. Beni and V. Carlà, J. Neurochem., 51 (1988) 177–180.
[10] R. Carpenedo, A. Chiarugi, P. Russi, G. Lombardi, V. Carlà, R. Pellicciari, L. Mattioli and F. Moroni, Neuroscience, 61 (1994) 237–244.
[11] K. Shibata, J. Chromatogr., 430 (1988) 376–380.
[12] M.P. Heyes and B.J. Quearry, J. Chromatogr., 530 (1990) 108–115.
[13] K.J. Swartz, W.R. Matson, U. MacGarvey, E.A. Rian and M.F. Beal, Anal. Biochem., 185 (1990) 363–376.
[14] M. Bizzarri, A. Catizone, M. Pompei, L. Chiappini, L. Curini and A. Lagana, Biomed. Chromatogr., 4 (1990) 24–27.
[15] K. Mawatari, F. Iinuma and M. Watanabe, Anal. Sci., 7 (1991) 783–784.
[16] V. Carlà, G. Lombardi, M. Beni, P. Russi, G. Moneti and F. Moroni, Anal. Biochem., 169 (1988) 89–94.
[17] A.C. Atkinson and A.N. Donev, Optimum Experimental Designs, Oxford Science Publications, Oxford, 1992.
[18] R. Carlson, Design and Optimization in Organic Synthesis, Elsevier, Amsterdam, 1992.
[19] R.V. Lenth, Technometrics, 3 (1989) 469–473.
[20] P. Gratteri, S. Furlanetto, E. La Porta, S. Pinzauti and R. Leardi, Farmaco, 51 (1996) 231–246.
[21] N. Kettaneh-Wold, J. Pharm. Biomed. Anal., 9 (1991) 605–610.
[22] E. Morgan (Ed.), Chemometrics: Experimental Design, Wiley, Chichester, 1991, pp. 238–245.
[23] J.C. Miller and J.N. Miller (Eds.), Statistics for Analytical Chemistry, 3rd ed., Ellis Horwood, Chichester, 1993, pp. 110–117.