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Robustness testing for a capillary electrophoresis method using the ''short-end injection'' technique☆

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Abstract

A multivariate approach for testing the robustness of a capillary electrophoresis method using the ''short-end injection'' technique is presented. Firstly, a Plackett and Burman (PB) design with 11 factors (eight real factors and three dummies) was used to identify the critical factors on resolution, plate number, plate count, asymmetry and assay. Then, the factors which were found to be significant were studied in a central composite design to predict the variation of resolution inside the area investigated in the PB design. PB and central composite designs yielded conclusions that were in good agreement with one another. They showed that the separation could be considered as robust, notwithstanding the fact that some factors where found to be statistically significant and should be controlled (injected volume and electrolyte concentration). Using the factor values which gave the worst-case situation for R_s still led to acceptable values for this parameter. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Robustness test; Short-end injection; Injection methods; Multivariate methods; Central composite design; Resolution; Capillary electrophoresis

1. Introduction

We recently developed a capillary electrophoresis (CE) method using the short-end injection technique for monitoring the dissolution kinetics of calcium acamprosate from enteric-coated tablets [1]. We showed that the short-end injection technique in combination with high electrolyte concentration, low operating voltage, and sample dilution in an aqueous internal standard solution, overcomes the problem of the high ionic strength of dissolution test samples. As the method is intended for routine use in quality control, it was decided to investigate its robustness, i.e. its capacity to remain unaffected by small but deliberate variations introduced into the method parameters [2,3] before performing extensive validation. In this paper, the experimental and statistical approach which was developed for testing the robustness of the CE method developed for calcium acamprosate is reported.

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2. Experimental

2.1. Apparatus

Analysis was performed using Beckman P/ACE MDQ (Fullerton, CA) CE instrument equipped with a photodiode array detector. A fused-silica capillary 31.2 cm long (21 cm to the detector)×50 μm internal diameter purchased from Composite Metal Services (Hallow, UK) was used for the separations. Prior to its first use, the capillary was pre-conditioned by washing for 20 min with 0.1 *M* sodium hydroxide, and then for 5 min with water.

2.2. Chemicals

Calcium acamprosate reference standard and calcium acamprosate tablets (500 mg of calcium acamprosate per tablet) were kindly provided by Lipha (Lyon, France). Sodium tetraborate·10H_2O and potassium sorbate were from Merck (Darmstadt, Germany). Other chemicals were of analytical grade.

2.3. Method tested for robustness

2.3.1. Solutions

The electrolyte was an aqueous solution of borate buffer 50 m*M* (natural pH of 9.2). A stock solution of citrate buffer (100 m*M*, adjusted to pH 6.8 with sodium hydroxide) simulating intestinal fluid, a standard solution of calcium acamprosate (500 mg/l) in the citrate buffer, and a potassium sorbate solution (90 mg/l) in water were prepared. The working standard solution of acamprosate was prepared by dilution of the stock standard solution of acamprosate 4-fold with the potassium sorbate solution (internal standard). The tablet test sample solution in the citrate buffer was centrifuged then diluted 4-fold with the potassium sorbate solution (internal standard).

2.3.2. Operating conditions

The operating conditions are given in Table 1.

Each solution was injected in duplicate. Two blank injections were performed prior to initiation of any analyses. The electrolyte solution of the separation vials (2 ml) was changed after 40 injections. Relative corrected peak areas (area/migration time) of acamprosate/sorbate were used for quantitation.

3. Results and discussion

For testing the robustness of an analytical method, a multivariate approach in which the variations in the factor levels of the method are simultaneously introduced into a matrix of experiments is recommended since the influence of each factor is calculated from several experiments, and factor interactions can be taken into account. Because in capillary electrophoresis there are a large number of factors which are potentially critical for the separation and/or assay, factorial fractional designs are often used as screening designs (see Ref. [3] and references cited therein). A Plackett and Burman (PB) design which requires the minimum number of experiments, was initially used as a screening design to select a small number of significant factors in the method. This allowed a response design to be used in a further step, which gives more information and allows the prediction of the responses around the nominal values of the factors.

3.1. Screening design

Firstly, a screening design was used to identify the critical factors in the method.

Table 1
Operating conditions of the CE method under investigation for robustness

Capillary rinsing	1 min, 0.138 MPa (20 p.s.i.) with 50 m*M* borate buffer
Sample injection, detector side, 25°C	5 s, 0.0041 MPa (0.6 p.s.i.); 1.5 nl
Water co-injection, detector side, 25°C	1 s, 0.00069 MPa (0.1 p.s.i.)
Separation	−15 kV (−500 V/cm), 25°C for 2.5 min
Detection	UV 200 nm, spectral bandwith 10 nm, Acquisition rate 4 Hz; filter normal; <16 points

3.1.1. Selection of critical factors, factor levels and level number.

Eight potentially critical factors were selected which were believed to affect the results:

1. the concentrations of the active ingredient (A) and
2. internal standard (C), which may affect the resolution between the analytes
3. the separation voltage (B) which may affect the plate count and resolution
4. the injection time (D), at a fixed injection pressure, which determines the injected volume and may influence the plate number and resolution
5. the detection wavelength (E) as wavelength variations, in particular around 200 nm, may affect analyte peak areas and hence resolution
6. the rinse time (F) which may affect capillary equilibration
7. the separation temperature (G) which affects the mobility of the species
8. the electrolyte concentration (H) which has an impact on the electroosmotic flow and on the plate number by a stacking or destacking effect.

Symmetrical values around the nominal level were selected for factor levels, which were assumed to reflect the variations which could be encountered in laboratories due to the use of different types of instruments which may have different injection systems, different lengths of capillary from the injection point to the detection window, different temperature regulation systems, different detector types, etc.

3.1.2. Design and response selection

A saturated Plackett and Burman (PB) matrix was employed because of the large number of parameters to be tested. The matrix of experiments is presented in Table 2.

A matrix with 11 factors (eight real factors A–H and three fictitious factors or dummies I–K) was used because PB designs do not exist for eight to ten factors. In this matrix, the main effect of each factor is estimated independently, but two-factors and higher order interactions are confounded with the main effects. In this design, the estimation of an effect is real if interactions are insignificant. However, it was assumed that interactions between factors were negligible as variations in factor levels were small. The three dummies included in the design were used to evaluate the variability of the procedure throughout experiments. This approach was found to be appropriate in the identification of highly significant factors in high-performance liquid chromatography (HPLC) [4]. The experiments were carried out in random order generated by a software program to take into account non-controlled factors likely to produce a bias in the responses. In each experiment, a standard solution of calcium acamprosate diluted in the internal standard solution at the required concentration was injected, in addition to a test solution of tablet corresponding to complete release of acamprosate in the dissolution bath. Each solution was injected twice and the average response was used for calculation. The first experiment in the

Table 2
Plackett–Burman matrix of experiments with 11 factors ($N=12$ experiments) used for screening

Exp.	Order of Exp.	Factors										
		A	B	C	D	E	F	G	H	I	J	K
1	9	550	−16	80	6	205	65	23	45	−	+	−
2	3	450	−16	100	4	205	65	27	45	−	−	+
3	2	550	−14	100	6	195	65	27	55	−	−	−
4	6	450	−16	80	6	205	55	27	55	+	−	−
5	7	450	−14	100	4	205	65	23	55	+	+	−
6	10	450	−14	80	6	195	65	27	45	+	+	+
7	1	550	−14	80	4	205	55	27	55	−	+	+
8	4	550	−16	80	4	195	65	23	55	+	−	+
9	5	550	−16	100	4	195	55	27	45	+	+	−
10	12	450	−16	100	6	195	55	23	55		+	+
11	8	550	−14	100	6	205	55	23	45	+	−	+
12	11	450	−14	80	4	195	55	23	45	−	−	−

randomised matrix was repeated at the end of the test to assess that there was no drift. The responses analysed were migration time (t_m) resolution (R_s), plate count (N), tailing factor (T) [5] and tablet content. The same set of large volume (20 ml) separation vials was used throughout the test to avoid buffer depletion effects [1].

3.1.3. Analysis of the results

In this study, the average responses of duplicate runs were used for calculations. This was considered to be a reasonable approach in view of the highly repeatable results obtained for 10 successive injections at the nominal value. The relative standard deviation was 1.39% for R_s, 3.68% for N, 1.93% for T and 0.91% for the assay ($n=10$). In addition, the average t_m values of duplicate injections of analytes in each experiment were very similar, which showed that the capillary equilibration with the electrolyte was obtained after the first injection. A Cochran's test performed on each of the responses showed that variances for R_s, N and assay results were homogeneous ($P=0.05$) in each case. In each experiment, analyte peaks were separated with baseline resolution in less than 2.3 min.

The effects of each of the factors on R_s, N and assay result and their statistical interpretation are given in Tables 3–5.

In these tables, E_x represents the effect of the factor on the response when the factor is changed from a low to a high level [Eq. (1)]:

$$E_x = \frac{\sum Y_{(+)}}{n_{(+)}} - \frac{\sum Y_{(-)}}{n_{(-)}} = \frac{\sum Y_{(+)}}{N/2} - \frac{\sum Y_{(-)}}{N/2} \quad (1)$$

where $n_{(+)}=6$, number of experiments where the factor X is at a high level (+1); $n_{(-)}=6$, number of experiments where X is at a low level (−1); $N=12$, total number of experiments; Y=response; and E_x% the percentage of effect (Eq. (2)):

$$E_x(\%) = 100\frac{E_x}{Y_{nom}} \quad (2)$$

where Y_{nom} is the response at the nominal level.

If we consider the effect from the changed level to the nominal level, this effect should be divided by two.

The experimental Student's variable $t_{obs}=E_x/(SE)_e$ was calculated using the dummy variables to evaluate the standard error $(SE)_e$ given by Eq. (3):

$$(SE)_e = \sqrt{\frac{\sum E^2_{dummy}}{n_{dummy}}} \quad (3)$$

Corresponding standardised Pareto plots (Figs. 1–4) which represent the absolute value of t_{obs} on R_s, N and assay for each factor, give rapid visual information on the magnitude of the effect. The length

Table 3
Effects of factors on resolution and statistical results[a]

Factor	Effect E_x	E_X (%) $(R_s)_{nom}=2.278$	t_{obs}
[Acamprosate]	0.041	1.814	0.871
Voltage	−0.113	−4.975	−2.389
[Sorbate]	−0.072	−3.146	−1.510
Injection time	−0.613	−26.924	−12.927[b]
Wavelength	0.015	0.658	0.316
Rinse time	0.046	2.005	0.962
Temperature	−0.121	−5.297	−2.543
[Borate]	0.461	20.222	9.709[b]
Dummy 1	0.072	3.146	1.510
Dummy 2	0.034	1.478	0.710
Dummy 3	0.022	0.966	0.464
	Critical values	$t_{(0.01;\ 3)}$	5.841
	Estimation	$(SE)_e$	0.047

[a] Estimation $(SE)_e$ 690; critical values $t_{(0.01;3)}$ 5.841.
[b] Significant at $P=0.01$.

Table 4
Effects of factors on plate count for acamprosate and statistical results[a]

Factor	Effect E_x	E_x (%) $(N_A)_{nom}=19638$	t_{obs}
[Acamprosate]	559	2.847	0.809
Voltage	−4423	−22.523	−6.414[b]
[Sorbate]	−2041	−10.393	−2.958
Injection time	−20714	−105.479	−30.017[b]
Wavelength	1095	5.576	1.587
Rinse time	891	4.537	1.291
Temperature	−5666	−28.852	−8.211[b]
[Borate]	4205	21.413	6.094[b]
Dummy 1	1000	5.092	1.449
Dummy 2	540	2.750	0.782
Dummy 3	−371	−1.889	−0.537
	Critical values	$t_{(0.01;\,3)}$	5.841
	Estimation	$(SE)_e$	690

[a] Estimation $(SE)_e$ 690; critical values $t_{(0.01;3)}$ 5.841.
[b] Significant at $P=0.01$.

of the bar is proportional to the magnitude of the effect and a negative sign means that changing the factor from a low to high level has a negative effect on R_s and vice versa.

The effects were also estimated by the regression coefficients of a first order polynomial model and confirmed the results previously obtained. These coefficients correspond to the variations with respect to the nominal level (half-values of those previously obtained).

Table 5
Effects of factors on assay and statistical results[a]

Factor	Effect E_x (mg)	E_x(%) $y_{nom}=508.8$ mg	t_{obs}
[Acamprosate]	−0.898	−0.18	−3.403
Voltage	1.475	0.29	5.587
[Sorbate]	−1.592	−0.31	−6.029[b]
Injection time	0.395	0.08	1.496
Wavelength	1.355	0.27	5.132
Rinse time	−0.245	−0.05	−0.928
Temperature	−0.438	−0.09	−1.660
[Borate]	−1.522	−0.30	−5.763
Dummy 1	−0.055	−0.01	−0.208
Dummy 2	−0.442	−0.09	−1.673
Dummy 3	0.105	0.02	0.398
	Critical values	$t_{(0.01;3)}$	5.841
	Estimation	$(SE)_e$	0.264

[a] Estimation $(SE)_e$ 0.264; critical values $t_{(0.01;3)}$ 5.841.
[b] Significant at $P=0.01$.

The factors that most influenced R_s were injected volume and electrolyte concentration, injected volume, temperature, electrolyte concentration and voltage for N, and borate concentration for assay. The sign of these effects was as expected: increasing the injected volume resulted in a decrease of R_s due to a decrease of the plate number; increasing the borate concentration resulted in a stacking effect which increased the plate count. However, because factors influencing the responses could be statistically significant but responses can still comply with the method requirements, the responses and size and percentage of effects obtained in each experiment were carefully examined to decide if the method should be accepted or rejected. Baseline resolution was obtained in each experiment (R_s values 1.5–2.6) and the percentage of effect for the assay (Table 4) was very low in each experiment – maximum variation of 0.3% from low to high level which represents a variation of 0.15% with respect to the nominal value, which is within acceptable limits.

3.1.4. Conclusion of screening experiments

PB design allowed, in a small number of experiments, the evaluation of the effect of changing the level of eight potential critical factors on different responses. Assay result and resolution, which were considered as the most important responses to consider, were in all cases within the acceptance criteria

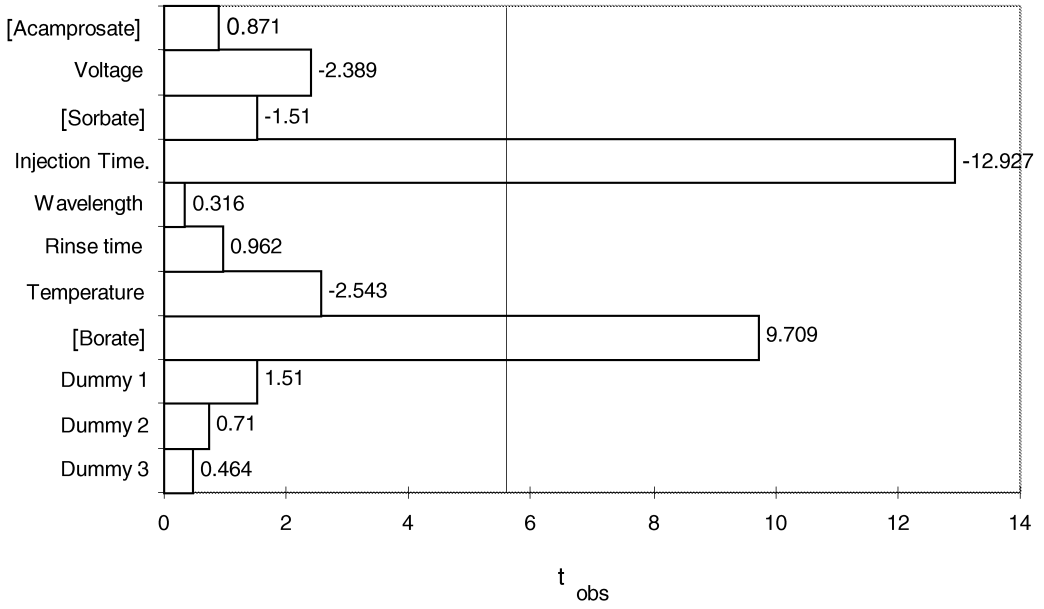

Fig. 1. Standardised Pareto plot for resolution (vertical bar: $t_{crit}=0.01$).

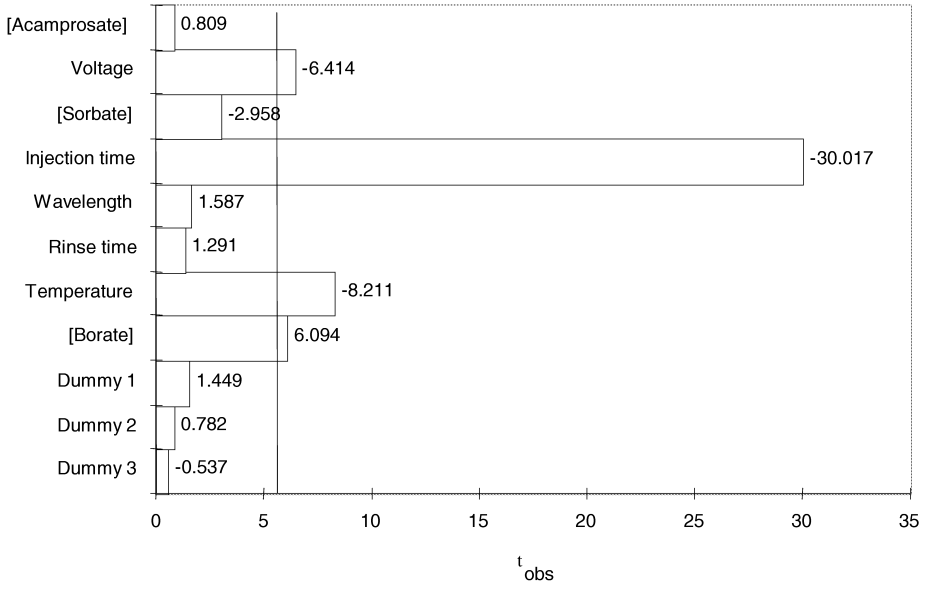

Fig. 2. Standardised Pareto plot for plate count of acamprosate (vertical bar: $t_{crit}=0.01$).

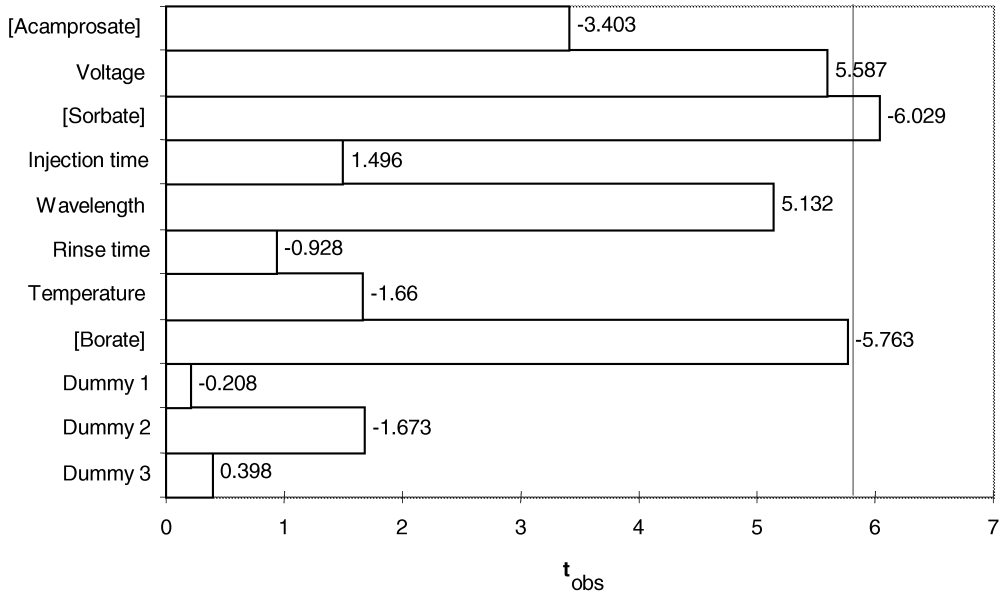

Fig. 3. Standardised Pareto plot for acamprosate assay (vertical bar: $t_{crit}=0.01$).

and the screening design permitted the conclusion that the method was sufficiently robust for its intended use. However, further experiments were carried out to investigate the behaviour of R_s around the nominal values of the factors and gain more insight in the method.

3.2. Response surface design

The goal here was to predict the variation of resolution inside the area investigated in the PB design.

3.2.1. Selection of critical factors, factor levels and level number

Four factors (electrolyte concentration, temperature, injected volume and applied voltage) which were found to produce relatively large effect on R_s and N in the screening design, were investigated at five levels.

3.2.2. Design and response selection

A central composite design was selected as it is well suited to robustness testing. Thirty experiments were performed including six central points (Table 6). The experiments were randomised and in each experiment duplicate injections of a standard solution of calcium acamprosate diluted in internal standard solution was carried out. Separation vials of 2 ml were used, which were changed every 20 injections. The responses analysed were R_s and plate count.

3.2.3. Analysis of the results

The low relative standard variation of t_m values for the central point (0.38% for acamprosate and 0.45% for sorbate) confirmed the absence of drift in the system throughout experiments. t_m values of duplicate injections were repeatable and variances of responses were homogeneous. Experimental results were computed using Nemrod software. An analysis of variance (ANOVA) was used to test the fit of the data to a polynomial to the power of two. The coefficients of the model confirmed that electrolyte concentration and injected volume were the factors that most influenced R_s. Because the goal of response surface design was to predict R_s, the factor values corresponding to a minimum R_s in the domain investigated were calculated by an iterative method from the equation of the model using S-Plus software It was found that a 45 mM electrolyte concentration, a 27°C separation temperature with a 6 s injection time and a −16 kV voltage gave the lowest R_s with a

Table 6
Central composite matrix of experiment used for robustness testing of the method

Exp.	Order of exp.	A = [Borate]	B = temperature	C = injection time	D = voltage
1	11	47.5	24	4.5	−14.5
2	13	52.5	24	4.5	−14.5
3	7	47.5	26	4.5	−14.5
4	5	52.5	26	4.5	−14.5
5	6	47.5	24	5.5	−14.5
6	9	52.5	24	5.5	−14.5
7	4	47.5	26	5.5	−14.5
8	8	52.5	26	5.5	−14.5
9	1	47.5	24	4.5	−15.5
10	24	52.5	24	4.5	−15.5
11	3	47.5	26	4.5	−15.5
12	10	52.5	26	4.5	−15.5
13	16	47.5	24	5.5	−15.5
14	23	52.5	24	5.5	−15.5
15	21	47.5	26	5.5	−15.5
16	27[a]	52.5	26	5.5	−15.5
17	15	45	25	5	−15
18	25[a]	55	25	5	−15
19	19	50	23	5	−15
20	12	50	27	5	−15
21	18	50	25	4	−15
22	2	50	25	6	−15
23	29[a]	50	25	5	−14
24	22	50	25	5	−16
25[a]	14	50	25	5	−15
26[a]	17	50	25	5	−15
27[a]	30[a]	50	25	5	−15
28[a]	20	50	25	5	−15
29[a]	28[a]	50	25	5	−15
30[a]	26[a]	50	25	5	−15

[a] Refers to the experiments at the nominal level (central point).

value of 1.485. The response surface plot as a function of injection time and borate concentration with temperature and voltage fixed at the least favourable level (27°C and −16 kV, respectively) and at the nominal level level (25°C and −15 kV) are given in Figs. 4 and 5, respectively. The regular curvature and lack of inflexion point in the response surface shows there is no factor interaction. An acceptable R_s can be predicted in the domain investigated even using the worst-case factor-level combinations.

4. Conclusion

PB and central composite designs used for robustness testing of a CE method for calcium acamprosate using short-end injection technique yielded conclusions that were in good agreement with one another. They showed that the separation could be considered as robust notwithstanding the fact that some factors where found to be statistically significant and should be controlled (injected volume and borate concentration). Using the factor values which gave the worst-case situation for R_s still led to acceptable values for this parameter. Since this robustness test was performed, the method was successfully validated and good agreement was found between CE and HPLC results in tablet dissolution testing. The method was also successfully applied by different operators on different instruments and on different days and with capillaries from different suppliers.

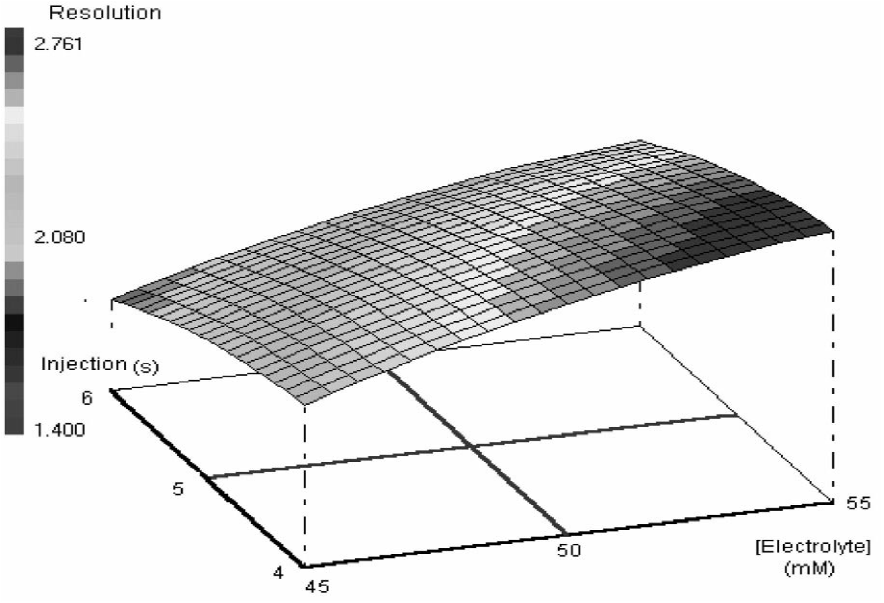

Fig. 4. Response surface for resolution as a function of injection time and electrolyte concentration with temperature set at 27°C and voltage at −16 kV (worst conditions).

Although robustness testing is not required in the Guidelines of the International Conference on Harmonization [2], it is recommended to include this test for acceptation of CE methods in the validation dossiers because the robustness of this relatively new technique is sometimes questioned. Robustness testing in CE is now often included in the validation procedure of CE methods of pharmaceutical companies [6]. It is more readily performed in CE than in HPLC because of the short equilibration time when changing the composition of electrolyte and the fact that instrumentation is automated. In addition, sepa-

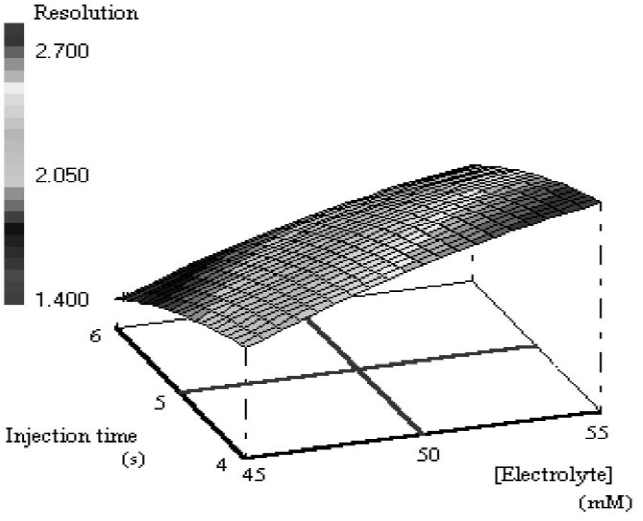

Fig. 5. Response surface for resolution as a function of injection time and electrolyte concentration with temperature set at 25°C and voltage at −15 kV (nominal conditions).

rations are generally rapid, which means that response surface designs may be obtained within a short time. The 30 experiments (60 injections) of the central composite design used to test the robustness of the separation between calcium acamprosate and its internal standard were carried out in only 5 h.

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