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Use of a Doehlert design in optimizing the analysis of selected tropane alkaloids by micellar electrokinetic capillary chromatography

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Abstract

The Doehlert design was used in optimizing the analysis of selected tropane alkaloids (including hyoscyamine, scopolamine and littorine) by micellar electrokinetic capillary chromatography. Three variables, i.e., pH, SDS concentration and organic modifier percentage were investigated. Resolutions as well as analysis time, generated power and current were established as responses. A model was obtained by linear multiple regression of a second-degree mathematical expression. The Doehlert design structure allows to study the response surfaces with a good quality of the parameters estimation of the quadratic model. Thus, it is possible to obtain the region in which the optimum values of such variables are simultaneously achieved. From the models, the most favorable conditions were determined by optimizing the resolution between hyoscyamine and littorine –two positional isomers– and by setting the other responses at threshold values. Successful results were obtained using a 30 m*M* borate–phosphate buffer at basic pH (8.7) in the presence of 40 m*M* sodium dodecyl sulfate and 16.5% acetonitrile. Results were compared with a previous study in which a systematic investigation of the operating parameters was carried out. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Chemometrics; Experimental design; Response surfaces; Optimization; Doehlert design; Alkaloids; Hyoscyamine; Scopolamine

1. Introduction

Atropine [(±)-hyoscyamine] and scopolamine, which are obtained from various solanaceous species [1], are among the principal tropane alkaloids of medicinal interest in this group. They are extensively used in ophthalmic diagnosis as mydriatic, and also as anticholinergic, antispasmodic and pre-anaesthetic agents. Thus, there is a great need to develop rapid and sensitive analytical methods for the assay of alkaloids, both in pharmaceutical preparations and in plant extracts.

Thin-layer chromatography [2], gas chromatography [3,4] and high-performance liquid chromatography [5–8] have been widely used in the analysis and the quantitation of tropane alkaloids. Recently, capillary electrophoresis has evolved as an interesting alternative for the analysis of complex matrices because of its high efficiency, flexibility, accuracy and very high resolution [9]. However, few reports [10–15] have been dedicated to tropane alkaloid analysis by capillary electrophoresis. Two validated capillary zone electrophoresis methods have been

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developed for the determination of atropine, scopolamine and homatropine in ophthalmic solutions [12] and for the determination of atropine and scopolamine derivatives in pharmaceutical formulations [14]. However, in plant material, hyoscyamine and scopolamine are generally present together with other tropane alkaloids presenting similar structure and charge to mass ratio. Therefore, capillary electrophoresis under the micellar mode was found to be more appropriate for the analysis of such compounds in plant complex extracts, as reported previously [13]. Different experimental parameters, such as buffer and micellar concentration, buffer pH and organic modifier type and percentage, were systematically investigated. This classical approach involves the systematic alteration of a single variable whilst maintaining the others constant, with no straightforward prediction of the separation conditions. It ensures that such a univariate optimization generates a large number of experiments whereas experimental designs allow a high quality in optimization processes with a smaller number of experiments [16–21].

The Doehlert designs [22], which have already been used in optimizing processes [23,24] and analytical methods [25–28], offer a uniform distribution of points over the whole experimental region, arranged in a rhomboidal figure. In the case of three variable designs, a cuboctahedron is produced geometrically (Fig. 1).

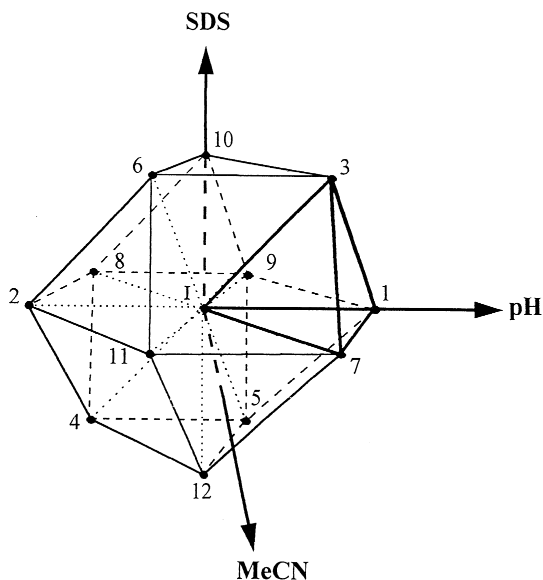

Fig. 1. Working parameters for the buffer pH, the SDS concentration and the acetonitrile percentage represented by the Doehlert experimental design.

The prediction quality of the model is verified by means of the good agreement observed between experimental and predicted responses.

In this paper, a Doehlert design was carried out in order to find the optimized electrophoretic conditions for the analysis of selected tropane alkaloids using micellar electrokinetic capillary chromatography. The method was validated and successfully applied to the determination of hyoscyamine in a Belladonna extract.

2. Experimental

2.1. Chemicals and samples

Hyoscyamine sulfate and scopolamine hydrobromide were purchased from Sigma (St Louis, MO, USA). Tropic acid was supplied by Fluka (Buchs, Switzerland). Homatropine hydrobromide was obtained from Merck (Darmstadt, Germany). Littorine was a gift of Dr. K. Shimomura (Tsukuba Medicinal Plant Research Station, Japan). Belladonna extract was obtained from Siegfried (Zofingen, Switzerland). All chemicals were of analytical grade: sodium dodecyl sulfate (SDS), sodium dihydrogenphosphate, sodium tetraborate, sodium hydroxide, methanol and acetonitrile (MeCN) were obtained from Fluka (Buchs, Switzerland). Ultrapure water, provided by a Milli-Q RG unit from Millipore (Bedford, MA, USA), was used for standard and sample preparation. Electrolyte solutions were filtered through a 0.20 μm microfilter (Supelco, Bellefonte, PA, USA) before use.

2.2. Instrumentation and electrophoretic procedure

Electrophoresis was carried out on a HP^{3D} capillary electrophoresis (CE) system (Hewlett–Packard, Waldbronn, Germany) equipped with an on-column diode-array detector (DAD). The capillary (Hewlett–Packard) was 64.5 cm long (56 cm effective length)×75 μm I.D. An alignment interface con-

taining an optical slit matched to the internal diameter was used. Detection at 56 cm from the point of sample introduction was set at 195 nm with a bandwidth of 10 nm. A capillary electrophoresis Chemstation (Hewlett–Packard) was used for instrument control, data acquisition and data handling.

All experiments were carried out in cationic mode (anode at the inlet and cathode at the outlet). The capillary was thermostated at 25°C and a constant voltage of 30 kV, with an initial ramping of 500 $V s^{-1}$, was applied during analysis. Sample injections (ca 18 nl injection volume) were achieved using the pressure mode for 5 s at 30 mbar.

The carrier buffer was obtained by dissolving a suitable amount of SDS and organic modifier in a solution prepared by mixing sodium dihydrogenphosphate, sodium tetraborate and sodium hydroxide solutions in appropriate ratio to give suitable pH value between 8.5 and 10.5. Each day, the capillary was rinsed with 0.1 *M* sodium hydroxide for 20 min followed by water for 10 min. To achieve high migration time reproducibility and avoid solute adsorption, a washing method was performed before each run. It included flushing the capillary with 0.1 *M* NaOH for 2 min, followed by water for 2 min and then equilibrated with the running buffer for 3.5 min. Before its first use, the capillary was flushed with 0.1 *M* sodium hydroxide for 30 min followed by water for 15 min.

As electrolysis can alter the running buffer and subsequently change the electroosmotic flow (EOF), a replenishment system was also used to maintain a high reproducibility. Prior to each sequence, two blank injections were performed to stabilize the capillary wall surface, and allow the buffer and sample solutions to reach a constant temperature on the autosampler tray. For each experimental condition, triplicate injections of the solutions were performed.

2.3. Standard and sample solutions

Stock standard solutions were prepared by dissolving each compound in methanol (1.0 $mg\,ml^{-1}$) and were suitably diluted in water to obtain standard solutions at a final concentration of 0.1 $mg\,ml^{-1}$. The use of water as a dissolving solvent allowed sample stacking which was effective in enhancing sensitivity (increasing peak height) by on-column preconcentration of the sample within the capillary. A peak area calibration curve for hyoscyamine and scopolamine was established over the sample concentration range of 50–125 $\mu g\,ml^{-1}$ and in the presence of 100 $\mu g\,ml^{-1}$ homatropine, used as internal standard. The Belladonna extract was dissolved in water prior injection.

2.4. Buffers

Buffers (pH 8.5–10.5) were prepared using the Phoebus software 1.0 (Centre Analyse, Orleans, France). Buffer solutions were always freshly prepared and filtered immediately before use.

2.5. Computation

The coefficients for the regression models and the optimized conditions were calculated using the NEMROD (LPRAI, Marseille, France) and the LUMIERE (SIER, Enghien-les-Bains, France) software packages. Response surfaces were drawn with Microsoft Excel (version 7.0).

3. Results and discussion

3.1. Procedure

The analysis of hyoscyamine and scopolamine was performed by the use of experimental design in order to optimize several electrophoretic parameters. Homatropine, structurally related to hyoscyamine and scopolamine, is used as internal standard. Littorine, considered as a biosynthetic precursor of hyoscyamine is difficult to separate from the latter compound. Therefore, the resolution between littorine and hyoscyamine, which are two positional isomers, is the primordial response taken into account in this analytical optimization. Furthermore, tropic acid, an hydrolysis product of hyoscyamine and scopolamine, is added to the alkaloidal mixture (Fig. 2).

3.2. Design of experiments

According to the results previously obtained [13],

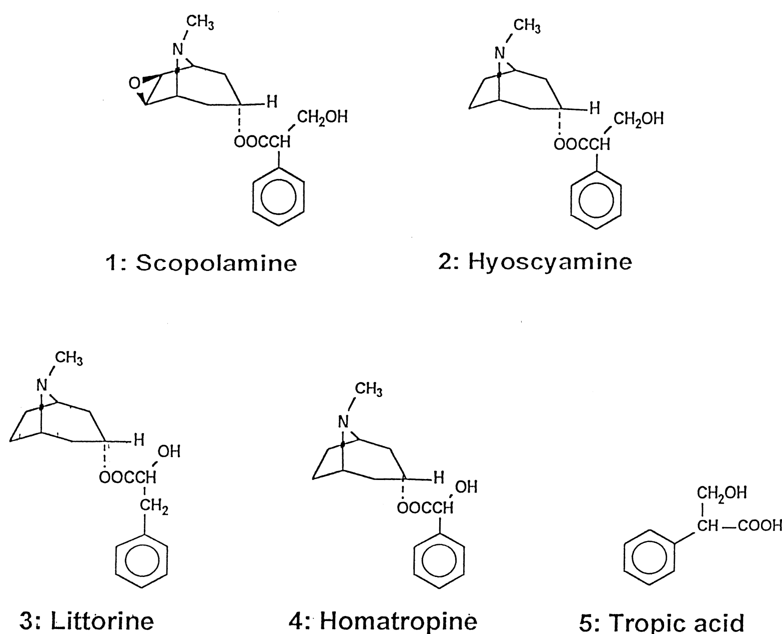

Fig. 2. Structure of the investigated alkaloids.

three factors were investigated: the buffer pH (X_1), the micelle concentration (X_2) and the acetonitrile percentage (X_3). Indeed, it has been demonstrated that the resolution between hyoscyamine and littorine can only be achieved in the presence of an organic modifier such as acetonitrile or methanol. The levels of each factor are listed in Table 1.

The experimental conditions for the fifteen experiments are given in Table 2. The experiment at the center was carried out three times in order to obtain an estimation of the experimental error. The fifteen experiments were performed randomly.

The effect of each factor was examined by means of four responses. The resolution (R_s) between hyoscyamine and littorine was calculated with the following equation:

Table 1
Coded values of experimental factors

Level	X_1 pH	X_2 SDS (mM)	X_3 Acetonitrile (%)
−1	8.5	10	10
0	9.5	30	15
+1	10.5	50	20

$$R_s = 2[(t_2 - t_1)/(W_{b(1)} + W_{b(2)})]$$

were t_1 and t_2 are the migration times of hyoscyamine and littorine, respectively; $W_{b(1)}$ and $W_{b(2)}$ are the peak widths measured at the baseline. In order to represent the quality of the separation, the analysis time (*Time*), measured by the migration time (t_m) of the last compound (littorine), as well as the power (*Power*) and current (*Current*) generated during electrophoresis were also examined.

In order to obtain a global response reflecting criteria of different and often conflicting nature, such as separation quality and analysis time, an individual response (R_s) was optimized, taking the other responses into account as threshold values. Then, response surfaces were studied by estimating the parameters of the quadratic model. It was possible to determine the region in which the optimum values of such variables are simultaneously obtained.

3.3. Regression modelling

Multiple regression enables the mathematical relationship between the responses and the independent variables. A Doehlert design provides sufficient data

Table 2
Three-factors Doehlert design with the corresponding responses

Trial	Run order	Experimental factors			Measured responses			
		X_1	X_2	X_3	R_s	Time (min)	Power (W)	Current (μA)
1	6	1.000	0.000	0.000	3.67	7.67	2.75	91.5
2	8	−1.000	0.000	0.000	1.85	10.81	2.25	75.0
3	1	0.500	0.866	0.000	3.41	9.65	3.65	123.0
4	5	−0.500	−0.866	0.000	1.92	5.72	1.50	51.5
5	12	0.500	−0.866	0.000	1.45	5.05	1.80	62.0
6	10	−0.500	0.866	0.000	2.71	11.68	3.05	102.0
7	7	0.500	0.289	0.816	3.23	6.98	2.25	100.0
8	4	−0.500	−0.289	−0.816	1.16	10.45	2.00	66.0
9	14	0.500	−0.289	−0.816	1.68	7.57	2.40	79.0
10	15	0.000	0.577	−0.816	1.79	10.59	2.80	93.0
11	2	−0.500	0.289	0.816	3.39	7.55	2.60	86.5
12	9	0.000	−0.577	0.816	1.37	5.25	1.90	63.5
13	11	0.000	0.000	0.000	1.99	8.55	2.40	79.5
14	13	0.000	0.000	0.000	1.90	8.29	2.40	80.0
15	3	0.000	0.000	0.000	2.59	8.13	2.40	80.5

for the fitting of a second-degree expression, such as given below:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + B_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3$$

where Y represents the experimental response, X_i the independently evaluated factors (in coded variables), b_0 the intercept and b_{ij} the parametric coefficients of the model obtained by multiple regression. Table 3 shows the regression parameters calculated for each response.

3.4. Optimization of the analysis

The quadratic regressions allowed to determine the optimal conditions by maximizing the resolution between hyoscyamine and littorine (R_s), setting the other responses as threshold values. The analysis time was set at a value superior to 10 min in order to obtain a sufficient migration window available for the analysis of complex plant extracts. The power and the current were set at values below 3 W and 100 μA, respectively, to avoid excessive Joule effect. Values of each factor resulting from the optimal conditions were 30 m*M* buffer at pH 8.7, 40 m*M* SDS and 16.5% acetonitrile. Experiments were carried out at 30 kV and at 25°C.

A comparison was made between predicted and observed responses (Table 4). The residual error value of each experiment was contained within a range of ±2 $S.D._{exp}$, where $S.D._{exp}$ is the experimental standard deviation obtained through the experiments at the center ($n=3$), and it can be concluded

Table 3
Regression coefficients estimated for each response

Coefficient	R_s	Time	Power	Current
b_0	2.1600	8.3233	2.4000	80.000
b_1	0.5288	−1.5538	0.2438	11.375
b_2	0.9288	2.8052	0.8985	31.393
b_3	0.6859	−1.8024	−0.0919	2.449
b_{11}	0.6000	0.9167	0.1000	3.250
b_{22}	0.0833	−0.7033	0.1000	5.083
b_{33}	−0.2558	−0.4408	−0.1625	−0.083
b_{12}	0.6755	−0.7852	0.1732	6.062
b_{13}	−0.6552	1.6922	−0.5205	−1.837
b_{23}	0.9275	−0.2652	−0.0530	7.189

Table 4
Comparison of predicted and measured results ($n=3$) under optimal conditions

	R_s	Time (min)	Power (W)	Current (μA)
S.D. [a]	0.38	0.41	0.00	0.5
Predicted	2.81	10.61	2.74	89.8
Measured	3.51	11.37	2.70	89.8

[a] S.D. represents the standard deviation obtained by performing three times the point at the center.

that each response is sufficiently explained by the regression models. Moreover, the good prediction quality of the model was experimentally verified by means of the good agreement observed between the experimental and the predicted response using the optimized conditions.

In Fig. 3, the electropherogram obtained under optimal conditions (B) was compared to that achieved in the previous univariate study (A). All compounds were resolved in less than 12 min.

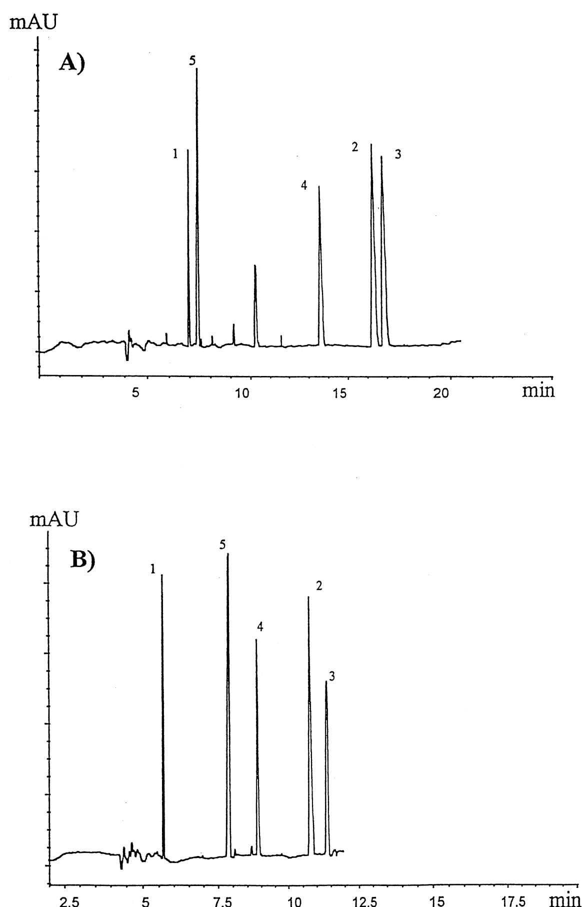

Fig. 3. Comparison between (A) the univariate study and (B) the optimized conditions obtained by maximization of the resolution between hyoscyamine and littorine. *Conditions*: (A) 30 mM phosphate–borate buffer, pH 8.5, 50 mM SDS and 10% acetonitrile. Applied voltage 30 kV (i=115 μA). (B) 30 mM phosphate–borate buffer, pH 8.7, 40 mM SDS and 16.5% acetonitrile. Applied voltage 30 kV (i=89.8 μA). Uncoated fused-silica capillary: 64.5 cm (56 cm effective length)×75 μm I.D. Peak numbering is the same as in Fig. 2.

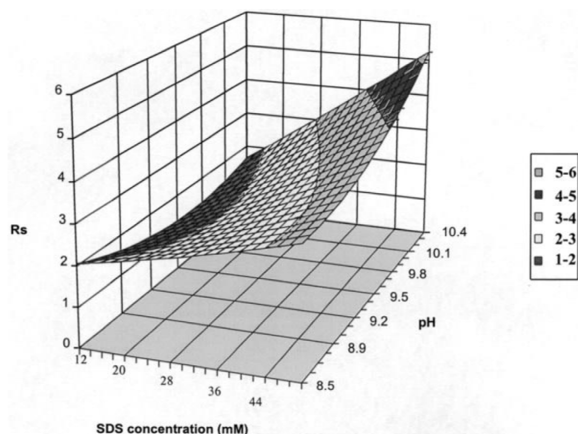

Fig. 4. Surface response plot for R_s as a function of the pH and the SDS concentration. The acetonitrile percentage is set at its optimal level (MeCN=16.5%).

It is possible to visualize the surface responses as a three-dimensional plot of two factors, while keeping the other constant at its optimal value (Fig. 4). For the sake of simplicity, other surface responses are not reported. These response surfaces allow the determination of an optimal zone, where a good quality of the separation can be established for further validation.

3.5. Method validation

Once the optimized conditions selected, the method was validated for the determination of hyoscyamine and scopolamine using homatropine as internal standard. The validation was carried out in a similar way to that generally adopted for HPLC and now employed to validate CE methods [29]. The procedure requires the assessment of migration time and peak area precision, detector response linearity with sample concentration, sensitivity and accuracy.

3.5.1. Precision

Method precision was determined by measuring repeatability and intermediate precision (between-day precision) of migration times and normalized peak areas for each alkaloid.

In order to determine the repeatability of the method, replicate injections (n=6) of a 100 μg ml^{-1} solution containing hyoscyamine, scopolamine and homatropine were carried out. In Table 5, relative

Table 5
Precision of CE expressed as R.S.D. values for migration time and peak area ratio

	Hyoscyamine	Scopolamine
Repeatability		
Migration time	0.51%	0.50%
Peak area ratio [a]	3.10%	3.56%
Intermediate precision		
Migration time	2.17%	1.09%
Peak area ratio [a]	2.41%	3.94%

[a]The peak area ratio is defined as the analyte peak area divided by the internal standard (homatropine) peak area.

standard deviations (R.S.D.s) are given for migration time and peak area ratio. In all cases, repeatability was better than 1% for the migration time and 4% for the peak area ratio.

The intermediate precision was evaluated over 3 days by performing six successive injections daily. Results (Table 5) show that R.S.D. values were in the same order of magnitude than those obtained for repeatability. The use of an internal standard is, in this case, necessary in order to compensate the poor precision observed with the hydrodynamic injection, and hence to achieve a good method precision [29].

3.5.2. Linearity

Detector response linearities were assessed by preparing calibration standards covering the sample concentration range 50–125 $\mu g\ ml^{-1}$. Each sample was injected in triplicate. Regression curves were obtained by plotting peak area ratios (analyte peak area divided by internal standard area) versus concentration, using the least squares method. In all cases, the correlation coefficient (Table 6) was improved by using peak area ratios. The intercept is not significantly different from zero (Student t test).

Table 6
Regression data, LOD and LOQ for hyoscyamine and scopolamine

Regression parameters		Compound	
		Hyoscyamine	Scopolamine
Regression equation			
($y=ax+b$)	a	0.0177±0.0006	0.0070±0.0002
	b	−0.1172±0.0590	−0.0117±0.0160
r^2		0.9916	0.9960
LOD (μg/ml)		0.6	1.0
LOQ (μg/ml)		1.8	3.0

3.5.3. Limits of detection (LOD) and quantification (LOQ)

The limit of detection, defined as the lowest concentration of analyte that can be detected above the baseline signal, is estimated as three times the signal-to-noise ratio. The LOD was determined by injecting test mixture solutions of various concentrations. The estimated limit of detection (Table 6) was determined as less or equal to 1 $\mu g\ ml^{-1}$, giving a limit of quantification value of less or equal to 3 $\mu g\ ml^{-1}$ for each compound.

3.5.4. Accuracy

The method accuracy was determined by performing the dosage of hyoscyamine in a Belladonna extract. A typical electropherogram of such extract is given in Fig. 5. According to the Swiss Pharmacopeia, the belladonna extract is labelled as containing 1.00±0.05% (w/w) of total alkaloids of which hyoscyamine represents ca. 90%. Therefore, in a 100 mg powdered sample (completely soluble in water), it is expected to obtain between 85.50% and 94.50% of hyoscyamine. Accuracy was also confirmed by recovery experiments, using the standard

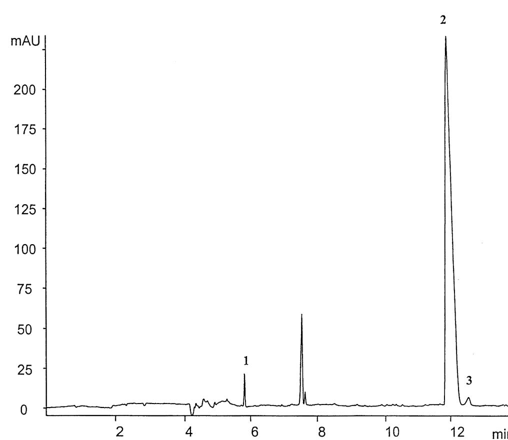

Fig. 5. Electropherogram of a Belladonna extract analyzed under optimized conditions: 30 m*M* borate–phosphate buffer, pH 8.7, 40 m*M* SDS and 16.5% acetonitrile. Applied voltage 30 kV (i=89.8 μA). Uncoated fused-silica capillary: 64.5 cm (56 cm effective length)×75 μm I.D. Peak numbering is the same as in Fig. 2.

Table 7
Data for accuracy

Dosage	Component	Label claim (%, w/w)	Amount of hyoscyamine added (%)	Amount found (%, w/w)	Recovery (%)
Belladonna extract	Hyoscyamine	90	0	89.87	99.86
			10	100.08	100.08
			20	108.82	98.93
			30	117.57	97.98
			40	127.39	97.99
				Mean:	8.97
				S.D.:	1.00

addition technique by adding four different levels of hyoscyamine to the preanalyzed sample. From the amount of hyoscyamine found, the percent recovery was calculated (Table 7). The recoveries obtained show that there is no interference from the extract matrix. The average recovery obtained by the developed CE method is 98.97%, which demonstrates the good accuracy of the method (Table 7). Hence, the described method represents a valuable tool for the dosage of hyoscyamine and scopolamine in various plant extracts.

4. Conclusion

A Doehlert design with a quadratic model has been used for the simultaneous optimization of three experimental parameters in micellar electrokinetic capillary chromatography (MEKC), the buffer pH, the SDS concentration and the acetonitrile percentage. Four responses, the resolution between hyoscyamine and littorine, the analysis time, the power and the current generated during electrophoresis, were evaluated. By studying response surfaces of the quadratic model and by building sequential designs, it was possible to determine the optimal region. It is obvious that the experimental design allows a better and faster prediction of the optimal conditions than the univariate development.

Finally, the optimized conditions were selected and the method was validated showing satisfactory validation data for sensitivity, linearity, precision and accuracy. The results obtained for hyoscyamine determination in a Belladonna extract attest the precision and the accuracy of the method. Owing to its simplicity and rapidity, the validated method is now applied to the dosage of hyoscyamine and scopolamine in plant extracts (results under publication).

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