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APPLICATION OF STATISTICAL OPTIMIZATION METHODS TO THE SEPARATION OF MORPHINE, CODEINE, NOSCAPINE AND PAPAVERINE IN REVERSED-PHASE ION-PAIR CHROMATOGRAPHY

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SUMMARY

The separation of morphine, codeine, noscapine and papaverine in reversed-phase ion-pair chromatography has been investigated by means of statistical optimization methods. The value of the capacity factor, as a function of the methanol–water ratio, pH and the concentrations of buffer and ion-pair reagent, including their interactions, was studied for each compound. It was shown that, by using such methods, conditions could be chosen which essentially improved the separation and that a quantitative characterization of the optimum region was facilitated.

INTRODUCTION

In this paper we will discuss the application of factorial design and response surface calculations to the optimization of a reversed-phase ion-pair chromatographic system. This analytical technique, pioneered by Schill and co-workers[1–3], has been shown to be a powerful tool for ionizable organic substances. During the last few years the reversed-phase mode has become the separation method of choice for many pharmaceutical products. Much interest has been focused on the mechanisms involved[4–6] and on the influences of various additives in the mobile phase, counter ions, buffers, pH and ionic strength on the selectivity[7–10]. One difficulty in evaluating the retention mechanisms arises from the variations in chromatographic behaviour due to the different properties of the reversed-phase materials. A thorough discussion of ion-pair high-performance liquid chromatography (HPLC) has recently been given by Bidlingmeyer[11].

Our analytical problem was to find a method for use in routine work which would accomplish a separation good enough for quantification of the four alkaloids

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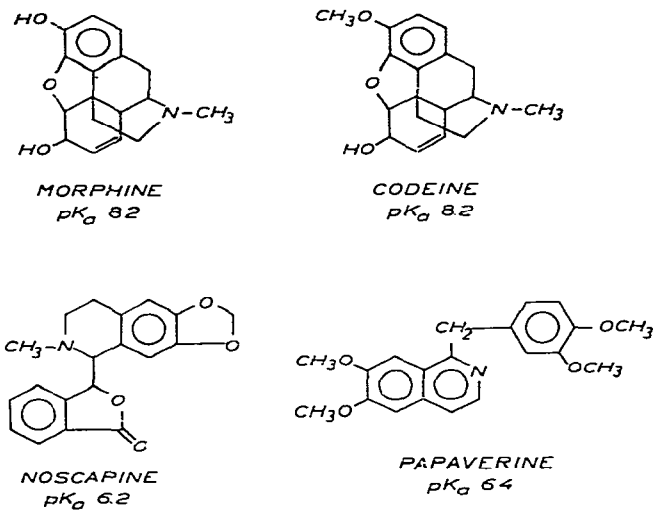

Fig. 1. Structures and pK_a values of the compounds.

morphine, codeine, noscapine and papaverine (Fig. 1) in a commercial solution. Similar problems have been dealt with by Knox and Pryde[12] who separated morphine, codeine and papaverine on a 125 × 5 mm, 6-μm, Hypersil SAS silica column with methanol–water (1:1), containing 25 mM ammonia as the eluent. Wu and Wittick[13] separated all these four alkaloids using two 300 × 4 mm μBondapak C_{18} columns in series and an aqueous mixture containing 25% v/v acetonitrile and 100 mM NaH_2PO_4, pH 4.8, as the eluent. However, with this system it was not possible to quantify morphine since this compound was eluted too close to the solvent front. Besides, the time of analysis was very long, about 40 min for the last compound eluted. For routine analyses the toxic and expensive acetonitrile is also less suitable.

Optimization of chromatographic conditions can be achieved by use of two principally different approaches. The most usual, but perhaps not always the most successful, is to base the choice of chromatographic conditions on knowledge and experience with the sample and the available chromatographic methods. This approach is useful if all relevant factors are known and their importance in the actual case is well understood. The other possible route, which is useful when all circumstances are not fully known, is to seek the optimum via a mathematical-statistical method. The great advantage of the latter approach is that no extensive chemical assumptions have to be made and therefore the results are not subject to chemical model errors. However, chemical knowledge must still be used in the consideration of the choice of variables, in order to reduce the number of experiments. The application of statistical optimization methods, *e.g.*, factorial designs and simplex, has been reviewed[14].

A number of optimization methods has been proposed for use in chromatography. Watson and Carr[15] applied the simplex algorithm to optimize a gradient elution separation, and used the chromatographic response function (CRF), originally proposed by Morgan and Deming[16], as a measure of the quality of the sepa-

ration. The CRF is a function of the experimental peak separation for each pair of peaks (originally defined by Kaiser[17]), the desired peak separation, and the actual and acceptable time of analysis. Smits and co-workers[18,19] optimized an ion-exchange separation using the simplex method and chose "the information content", P_{inf}

$$P_{inf} = \sum_{i=1}^{n} {}^{2}\log S_i$$

as a criterion. Here n is the number of peaks in a fixed period of time and S_i expresses the overlap between neighbouring peaks. These authors also emphasized the difficulties in the choice of the quality criterion. Gant *et al.*[20] developed an optimization method based on semiempirical estimates of the capacity factor and the selectivity. These values are then combined with theoretical values of plate number for the Knox equation to allow calculation of the resolution as a function of all experimental parameters. The consistency of theoretical and experimental values is excellent. However, the general validity of the theoretical assumptions in this approach is doubtful for more complex LC systems. Another question is the applicability of this approach when dealing with incompletely gaussian peaks. As discussed by Christophe[21], this markedly affects the reliability of resolution and plate number calculations. Recently, some authors[22–24] have suggested that, instead of reducing the chromatograms to one single figure, the optimization can be carried out by means of values obtained from each pair of peaks. In this way the risk of losing significant information is diminished.

EXPERIMENTAL

Apparatus

A Laboratory Data Control Constametric II pump, an Altex 152 UV-detector operated at 254 nm, a Rheodyne 7010 injection valve equipped with a 20-μl loop and a Vitatron recorder were used. One 300 × 4 mm μBondapak C_{18} column (Waters Assoc.) was used throughout the experiments.

Reagents and solutions

Methanol, p.a. quality, was from May & Baker (Dagenham, Great Britain); water was obtained from a Millipore Super-Q system. Camphorsulphonic acid (CSA), synthesis quality, from E. Merck (Darmstadt, G.F.R.), was used without purification. Morphine, codeine, noscapine and papaverine were of pharmacopoeial grade. All other chemicals used were of reagent grade and used as received. The water–methanol solutions were prepared by weighing, and the volume ratios given were calculated from the apparent densities. pH values were measured directly in the eluent because reproducible results were given priority over formally correct measurements.

Procedure

The sample was injected and the hold-up volume was determined by the first detector signal deflection from the baseline. The resulting mean for all injections was

2.71 ml (S.D. 0.08 ml). This corresponds to a total column porosity of 0.72 which is in good agreement with the literature value[25].

All measurements were made on equipment contained in a thermostatted room at 21.5°C. Approximately twenty column volumes were pumped through after each change in eluent. Each capacity ratio, k', is the mean from three measurements.

To avoid biasing the results, all experiments were performed randomly. The experiment numbering in Table I is thus only for clarity and does not represent the order in which the experiments were made.

Factorial design

In order to investigate the effect of each component (variable) in the eluent as well as their possible interactions, a full factorial design (FD) was adopted as the optimization strategy. In a FD each variable, v, is given two values, denoted by + or −, defining the experimental domain, see Fig. 2. For M variables, 2^M experiments have to be performed to allow calculation of the magnitudes of the effects and their interactions ($M = 3$ in Fig. 2). The numerical values for the effect of one variable are obtained by subtracting the response at the minus level from another experiment on the plus level. The magnitude of the mean effect of one variable, *e.g.*, v_1, is then equal to the mean of these response differences. The interaction effect between two variables, *e.g.*, v_1 and v_2, is calculated as the difference between those response differences where the variable v_2 has a high value and those where v_2 has a low value. Thus if the effect of v_1 is different at the two levels of v_2 this is seen from the numerical value for the calculated interaction $v_1 \times v_2$. A comprehensive treatment of the method has been given previously[26,27].

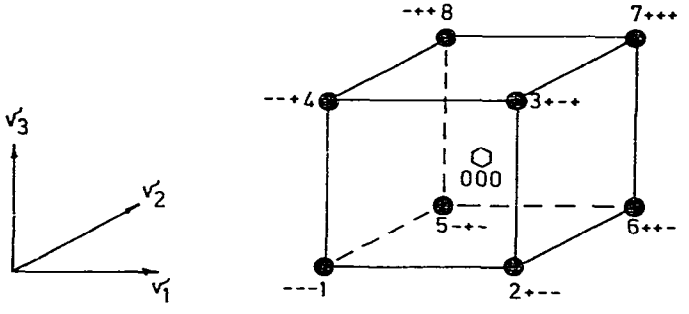

Fig. 2. Schematic representation of a full factorial design for three variables.

Response surface

A response surface was generated in order to get a better picture of the probable optimum[28]. Our approach has three basic steps. (1) The experiments were carried out according to a "central composite design". In this design the experiments are situated at the centre and on the perimeter of a circle. All peripheral experiments are placed with the same distance from each other. This design was later expanded with four experiments because the first evaluation of the data indicated that the optimum

was located at the border of the experimental domain. (2) The data were fitted to a mathematical model for the capacity factor

$$k' = a_0 + a_1 x_1 + a_2 x_2 + a_3 x_1^2 + a_4 x_2^2 + a_5 x_1 x_2$$

where x_1 = concentration (mM) of ion-pair reagent and

$$x_2 = 10^{V_{\text{methanol}}}/(V_{\text{methanol}} + V_{\text{water}}) \text{ (where } V = \text{volume)}$$

(3) This model was used to generate a response surface contour plot.

RESULTS AND DISCUSSION

Screening experiments

As mentioned in the Introduction, our analytical problem was to find a method for use in routine work which would achieve a separation good enough to enable quantification of the four alkaloids morphine, codeine, noscapine and papaverine in a commercial solution. A 300 × 4 mm μBondapak C_{18} column was the natural choice, not least in view of the promising results reported by Wu and Wittick[13]. Besides, most pharmaceutical analyses are performed using C_{18} columns.

The screening experiments showed that it was difficult to separate morphine from codeine without obtaining excessively high k' values for noscapine and papaverine. Camphorsulphonic acid (CSA) was chosen as counter ion and pH of the eluent was kept between 2.0 and 7.5 to avoid destruction of the column. Bad tailing was noted when approaching pH values close to the pK_a, and therefore we chose to avoid this situation when both protonated and unprotonated alkaloids were present in the mobile phase. The values of pK_a in Fig. 1 are for aqueous solutions; the corresponding values in methanol–water mixtures with different autoprotolysis are probably somewhat higher[29].

The best situation achieved after the screening experiments is shown in Fig. 3. Our problem now is to look for conditions for which the time of analysis is reduced without causing any significant loss in resolution. It is also desirable to increase the retention of the first compound eluted to avoid possible interference effects due to the solvent front.

The optimization

The choice of general quality criteria is fraught with problems since the requirements are often ambiguous and difficult to express in quantitative terms. In order to solve the optimization problem we adopted a somewhat different strategy to that used by some previous authors[15,16,19]. It was convenient to study the influence of the various variables on the k' values of the compounds since this quantity is well-defined and simple to evaluate. Moreover, a measure of the time of analysis is obtained from the k' value. These values can then be compared with each other in order to establish the conditions which best correspond to the set requirements. However, by using k' no measure of the separation between adjacent peaks is obtained. This is not a serious drawback because, for a given separation system, there is a correlation between the resolution and the differences in the capacity factors of consecutive peaks.

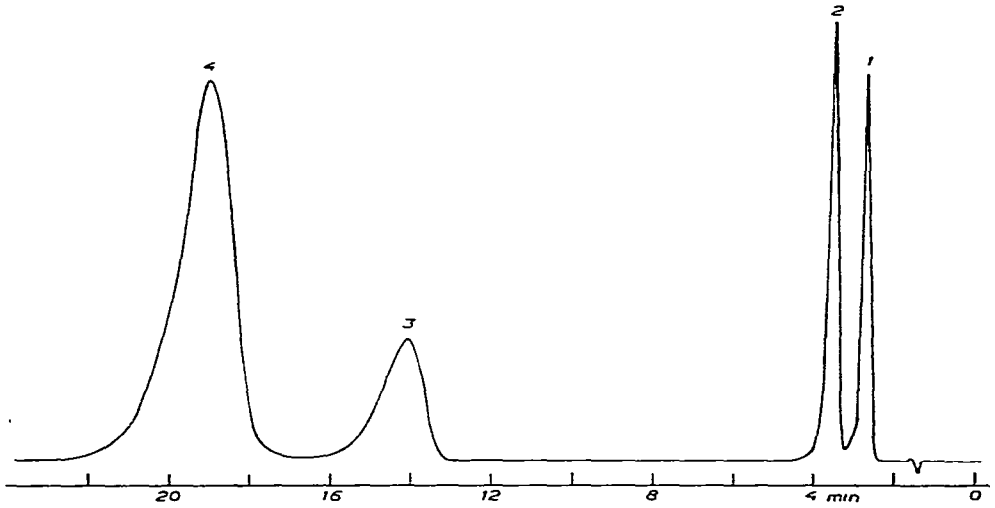

Fig. 3. Separation achieved after screening experiments. Eluent: methanol–water (35:65), 0.050 *M*, phosphate buffer, 0.005 *M* CSA, pH 3.0. Column: μBondapak C_{18}. Flow-rate: 1.50 ml min^{-1}. Elution order with k' values in parentheses: 1 = morphine (0.5); 2 = codeine (1.0); 3 = noscapine (6.8); 4 = papaverine (9.6).

The selection of variables and their values is a critical part of the optimization procedure and here chromatographic experience has to be used. If too many variables are included there will be too many experiments to perform; on the other hand, if too few or the wrong variables are chosen, valuable information will be lost. Our choice of variables included the eluent strength, the pH and the concentrations of phosphate buffer and camphorsulphonic acid. There are also practical limits to the variable range. For example, the buffer concentration must be sufficiently high to maintain a constant pH. The pH range was chosen so that the ion-pair mode should prevail during the experiments. The methanol and camphorsulphonic acid concentrations were selected to give a reasonable change in retention from each variable.

The best eluent from the screening experiments was taken as the origin of a four-dimensional cube spanned by the coordinates (values) of the four variables. In Table I the coordinates for each variable in all the eluents are shown together with the resulting values of the capacity factor for each alkaloid. The effects of the variables on the k' values of the alkaloids were calculated as described in *Factorial design*, and the results are given in Table II. A positive value indicates that the compound has a higher retention at the positive level and a negative value shows higher retention at the negative level. It is seen that the effect of a variable is greater for a substance having a higher capacity factor, and that the methanol–water ratio and the CSA concentration have the greatest numerical values and thereby importance. The effects of the other variables are smaller and can be neglected from an optimization point of view. The interaction effects were also calculated and the two-variable interactions are shown in Table II. Higher order interactions were very small and are therefore omitted. It is noted that the interaction between solvent strength and the CSA concentration is of some importance, which means that the alteration in k' caused by the change in counter ion concentration is dependent on the methanol–water ratio. Fur-

TABLE I

ELUENT COMPOSITIONS AND k' VALUES OBTAINED FROM THE FACTORIAL DESIGN EXPERIMENTS

Expt. no.	*Methanol–water ratio (v/v)*	*pH*	*Buffer concn. (M)*	*CSA concn. (M)*	*k'*			
					Morphine	*Codeine*	*Noscapine*	*Papaverine*
1	38:62	4.0	0.09	0.010	0.44	0.81	5.19	6.79
2	38:62	4.0	0.09	0.000	0.30	0.58	3.71	5.11
3	38:62	4.0	0.01	0.010	0.65	1.16	7.32	9.34
4	38:62	4.0	0.01	0.000	0.27	0.54	3.53	4.83
5	38:62	2.0	0.09	0.010	0.57	1.02	6.05	7.82
6	38:62	2.0	0.09	0.000	0.24	0.48	2.29	4.13
7	38:62	2.0	0.01	0.010	0.73	1.28	7.42	9.55
8	38:62	2.0	0.01	0.000	0.24	0.48	2.89	4.10
9	32:68	4.0	0.09	0.010	0.64	1.35	12.4	17.8
10	32:68	4.0	0.09	0.000	0.42	0.86	7.62	11.3
11	32:68	4.0	0.01	0.010	0.95	1.88	16.4	22.5
12	32:68	4.0	0.01	0.000	0.35	0.78	7.37	10.8
13	32:68	2.0	0.09	0.010	0.88	1.78	16.0	22.3
14	32:68	2.0	0.09	0.000	0.32	0.69	6.01	9.33
15	32:68	2.0	0.01	0.010	1.08	2.16	19.3	26.5
16	32:68	2.0	0.01	0.000	0.29	0.66	6.18	9.42

TABLE II

CALCULATED EFFECTS FROM THE FACTORIAL DESIGN SHOWING THE VARIATION IN k' CAUSED BY THE CHANGE IN THE ELUENT (CALCULATED FROM TABLE I)

Variable	*Morphine*	*Codeine*	*Noscapine*	*Papaverine*
1 Methanol–water ratio	−0.19	−0.48	−6.6	−9.8
2 pH	−0.42	−0.072	−0.31	−0.58
3 Buffer concn.	−0.096	−0.17	−1.4	−1.6
4 CSA concn.	0.44	0.79	6.3	7.9
Interaction 1/2	0.011	0.033	0.59	0.69
Interaction 1/3	0.011	0.029	0.41	0.57
Interaction 1/4	−0.11	−0.25	−2.9	−4.1
Interaction 2/3	–	–	–	–
Interaction 2/4	−0.11	−0.19	−1.5	−1.8
Interaction 3/4	−0.13	−0.21	−1.3	−1.7

thermore, for all compounds the k' values increase with increasing CSA concentration and decrease with increasing methanol–water ratio, as expected. If, for each compound, the magnitudes of these two effects are compared, it is seen that for noscapine and papaverine they are roughly the same. It should be noted, however, that for morphine and codeine the CSA-concentration effect is approximately twice as large which means that an increase in the CSA concentration will have a greater influence on the first compounds eluted.

These results show that it should be possible to change selectively the capacity factors by appropriate modification of the eluent. Our initial separation problem was to decrease the k' values of noscapine and papaverine without affecting the values for morphine and codeine. This cannot be accomplished simply by increasing the solvent

strength since the capacity factors for morphine and codeine would still decrease. However, the latter effect can be compensated for by a corresponding increase in the CSA concentration. According to Table II, an increase in the CSA concentration will have a smaller influence on the later compounds eluted, the net effect being an improvement in the separation.

When the coordinates from the screening experiment [methanol–water (35:65), 5 m*M* CSA] were reflected through the best point from the factorial design [methanol–water (38:62), 10 m*M* CSA] new coordinates were obtained at [methanol–water (41:59), 15 m*M* CSA]. As is seen in Fig. 4, the experimental results and the calculations are in good agreement. The k' values for noscapine and papaverine are 4.6 and 5.7, respectively, compared with 6.8 and 9.6 in Fig. 3. The resolution, as well as the distance to the solvent front, are still good enough to allow quantification of all the peaks.

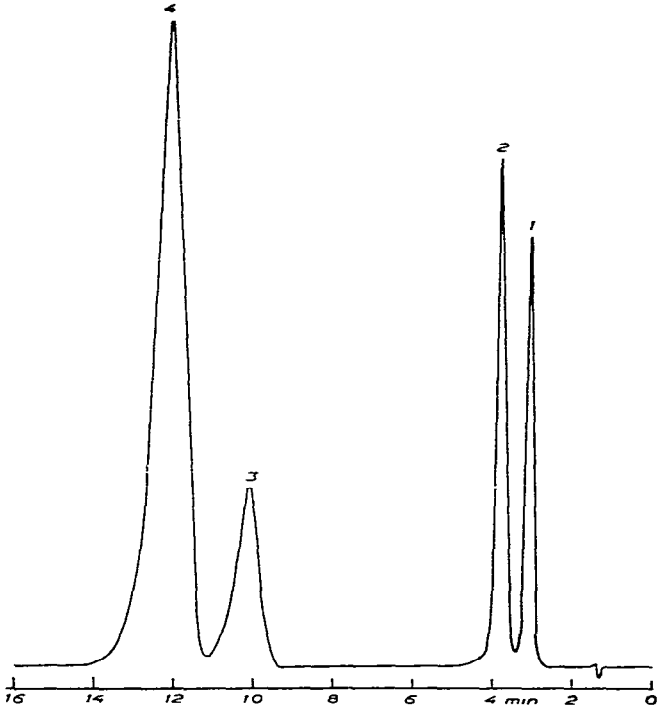

Fig. 4. Separation obtained by use of the results from the factorial design. Eluent: methanol–water (41:59), 0.010 *M* phosphate buffer, 0.015 *M* CSA, pH 2.0. Other details as in Fig. 3. k' values: 1, 0.6; 2, 1.1; 3, 4.6; 4, 5.7.

The response surface

In order to test the validity of the optimum found in the factorial design a response surface was generated with respect to the two most significant variables. The buffer concentration and pH were set at favourable levels (10 m*M* phosphate, pH 2) and the methanol–water ratio and the CSA concentration were varied in the area where the optimum was assumed to be situated. To avoid masking of the optimum, the increments of methanol and CSA were reduced to 1.5 v/v-part and 2.5 m*M*, respectively. Since the optimum was not in the centre of the experimental design, this

was enlarged with four additional experiments. Multiple regression analysis was carried out with k' as the dependent variable. The independent variables were the methanol–water ratio and the CSA concentration, their interaction term and their quadratic terms.

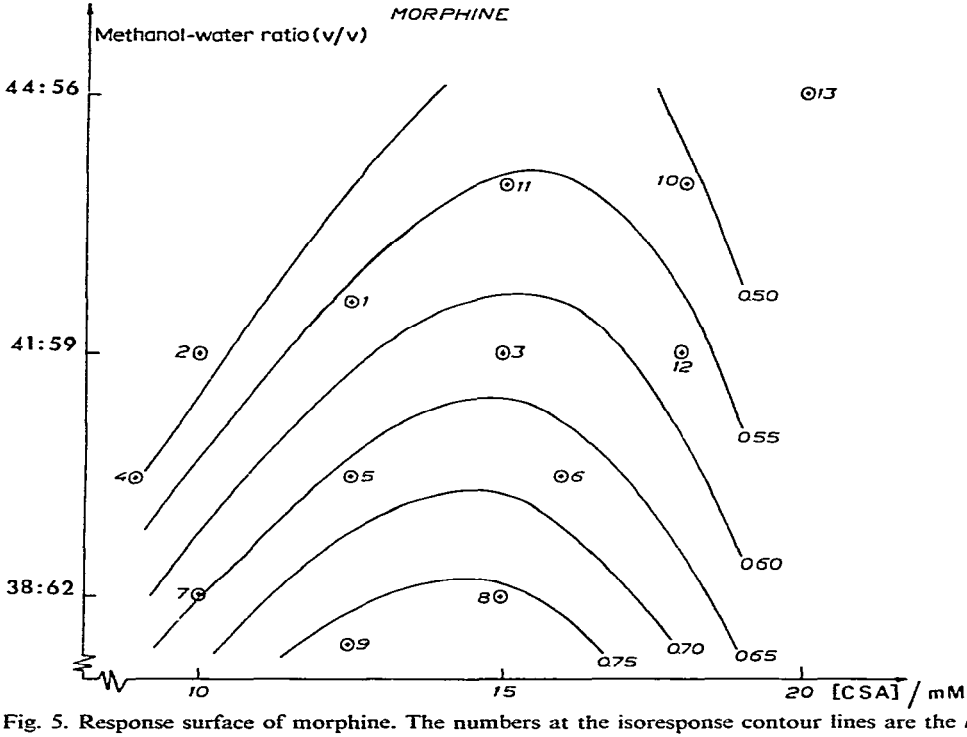

Fig. 5. Response surface of morphine. The numbers at the isoresponse contour lines are the k' values. Experimental points are marked with numbered rings.

From the morphine response surface in Fig. 5 it is seen that, for a fixed methanol–water ratio, k' first increases and then decreases with increasing CSA concentration. This behaviour has been noted before in ion-pair reversed-phase systems and has been studied by Horváth *et al.*[9]. It should be noted that the scale on the ordinate axis is exponential. Also, that the surface has a narrow ridge-like feature which means that morphine is comparatively more sensitive to the CSA concentration than the other alkaloids (see Figs. 6–8). These results are in agreement with those obtained from the factorial design (see Table II). The increasingly shallower curves in Figs. 6–8 demonstrate the diminishing effect of the counter-ion concentration in the order: morphine, codeine, noscapine and papaverine. Maximum k' values are obtained for all four alkaloids at *ca.* 15 mM CSA. For this concentration, changes in the methanol–water ratio have less effect on k' than at, *e.g.*, 10 or 18 mM.

By superimposing the plots from the first and last peaks eluted as shown in Fig. 9, it is apparent that strong retention of morphine and at the same time a weak retention of papaverine will be achieved somewhere in the shaded area. Outside this, k' for morphine decreases faster than for papaverine. We note that the eluent composition suggested by the factorial design [methanol–water (41:59 v/v), 15 mM CSA] lies within this area.

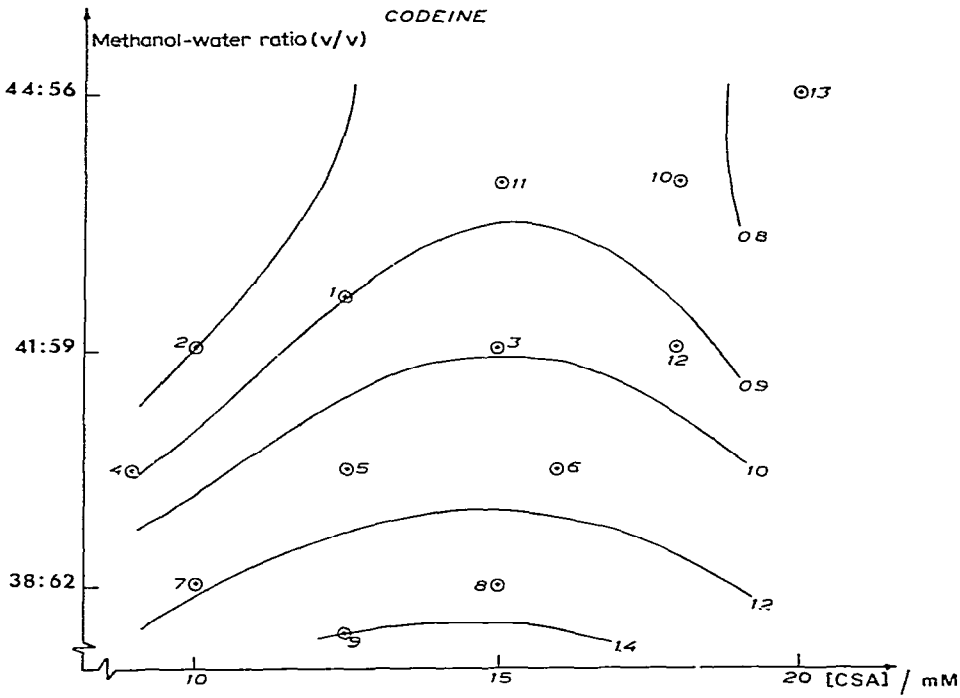

Fig. 6. Response surface of codeine.

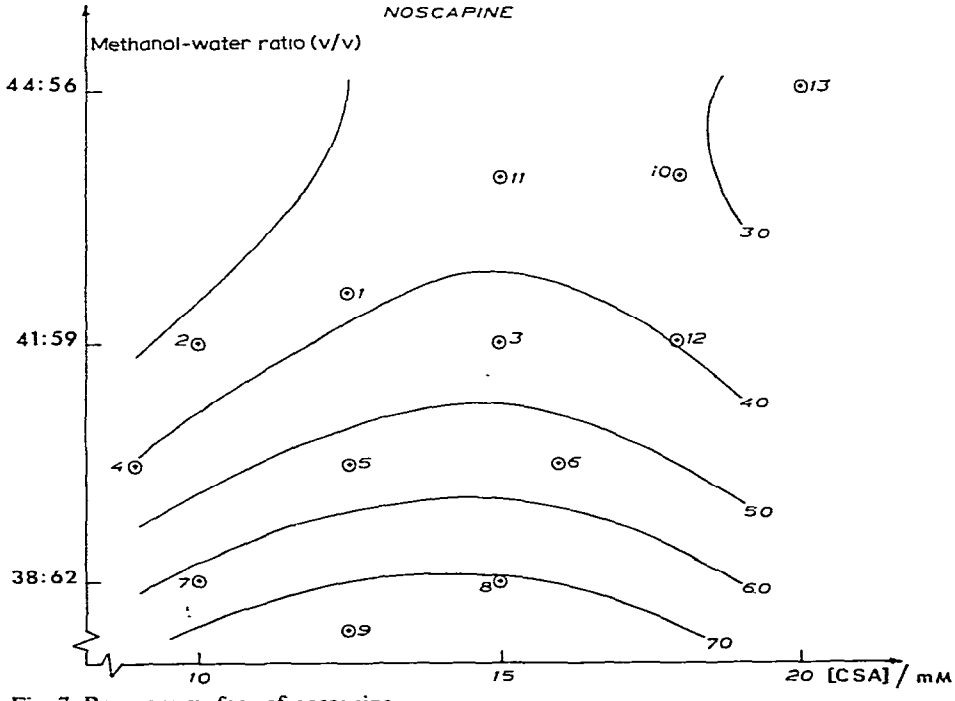

Fig. 7. Response surface of noscapine.

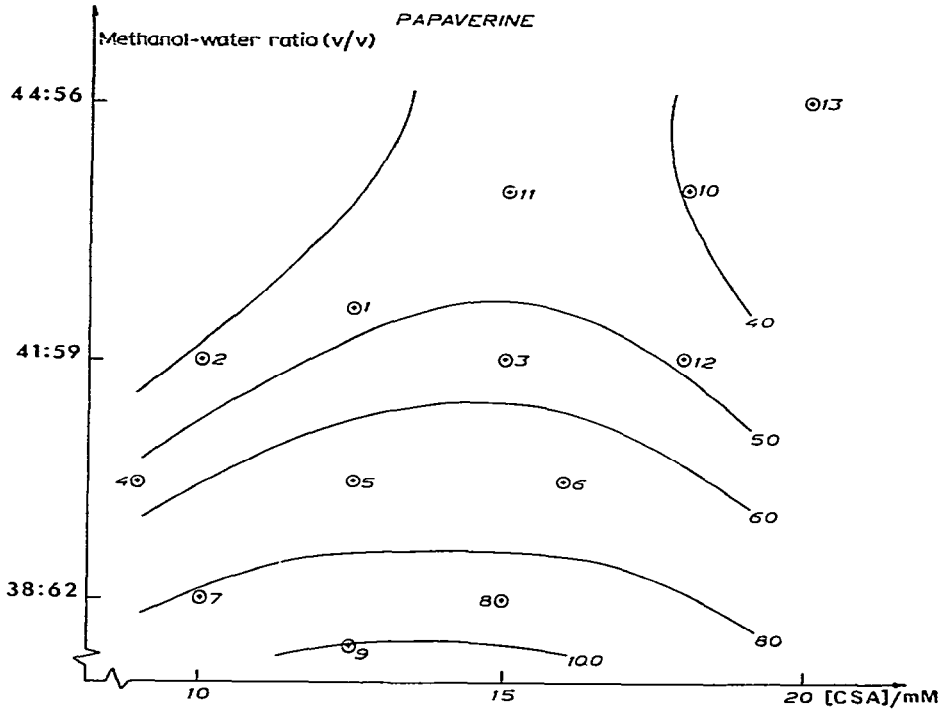

Fig. 8. Response surface of papaverine.

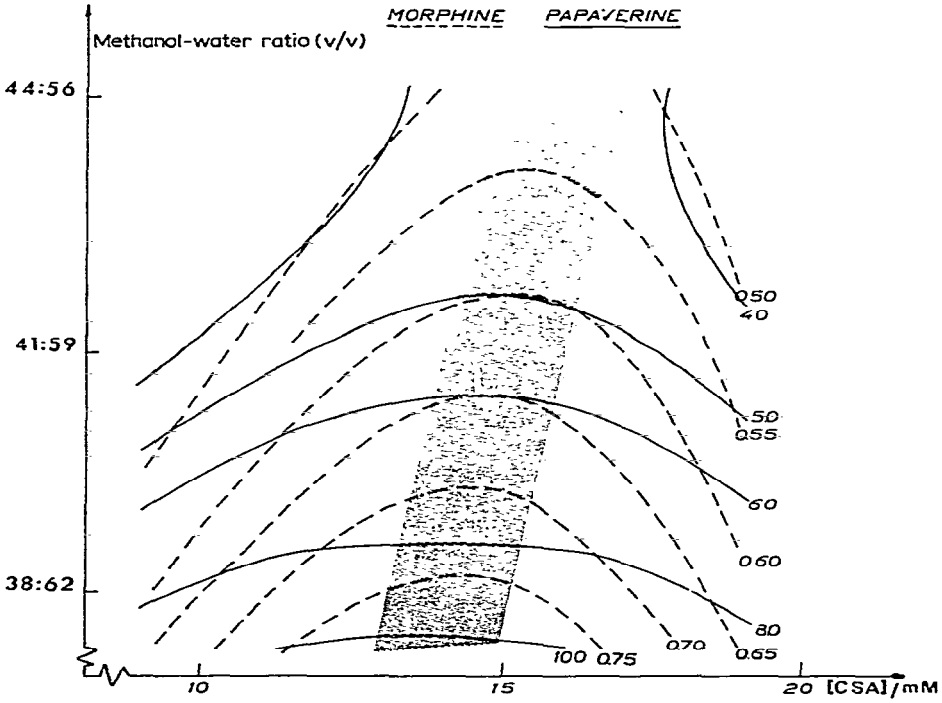

Fig. 9. Overlapping response surfaces of morphine and papaverine.

The plot shown in Fig. 9 also illustrates that the concept of "optimum conditions" depends on the purpose of the separation. Chromatographers requiring high speed of analysis and/or low detection limit should concentrate on the upper part of the shaded area. On the other hand, accurate measurements are favoured by higher resolution and thus higher k' values might be advantageous, as found in the lower shaded parts. Additionally, with increasing retention the probability of separating and detecting possible impurities or decomposition products is higher.

CONCLUSIONS

The utility of statistical methods for optimization purposes in reversed-phase ion-pair chromatography has been illustrated. Such methods offer unique possibilities for judging the qualitative importance of the considered variables, as well as for providing a quantitative picture of the optimum region.

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