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ISOCHRONAL OPTIMIZATION

III. EXPERIMENTAL VERIFICATION

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SUMMARY

The theoretical aspects of isochronal optimization (time normalization) are verified experimentally in the present communication. The variant examined here utilizes simultaneous changes in mobile phase composition and velocity. Two empirical relationships for the capacity factors were examined. From these relationships, predicted resolution surfaces were generated and these were compared with the experimental ones. It was found that quadratic relationships between the natural logarithm of k' and the mobile phase composition yielded surfaces that resembled the experimental ones most closely. Moreover, it was found that only three experiments, with three different mobile phases, were sufficient to calculate the resolution surface with adequate accuracy. Isochronal lines (resolution lines with constant analysis times) were generated from the predicted data and compared with experimental isochrons. The agreement between the two sets of lines was very good. Based on three experiments it is possible to predict experimental conditions that will yield better resolution without increasing the analysis time.

INTRODUCTION

Chromatography in general, and reversed-phase liquid chromatography (RPLC) in particular, are at the forefront of analytical separation techniques. Still, the issue of optimization of the resolution is the topic of active research. For successful optimization, a clear understanding of the underlying theories of liquid chromatography is needed. The selection of satisfactory separation conditions is frequently based on an intuitive approach. Several researchers have proposed schemes for establishing a framework for predicting the resolution and its dependence on experimental parameters[1–10]. Similarly, many approaches were taken in trying to improve the resolution. D'Agostino *et al.*[11] and Berridge[12] have recently reviewed most of the methods used for the optimizing the resolution.

The majority of the methods used for optimizing the resolution hinge on the manipulation of the selectivity of the mobile phase. However, while the nature of the mobile phase is frequently the dominating factor controlling the resolution, it is only

one of several parameters that can influence separation. When other considerations besides resolution must be taken into account, then different approaches to optimization must by sought.

Recently, isochronal optimization (time normalization) was reintroduced into high-performance liquid chromatography (HPLC)[13,14]. In this approach, cognizance is taken of the fact that the analysis time can be an important parameter. In isochronal analysis, the optimization of the resolution proceeds in such a way as to keep the analysis time constant throughout the process, *i.e.*

$$t_{R,A} = t_{R,B} \tag{1}$$

where the subscripts A and B indicate two different sets of experimental conditions. In other words, the retention time of the last peak remains the same during the optimization process. This approach can have important implications for the optimization of on-line process control, or in kinetic studies where a predetermined analysis time is desired. From the definition of the retention time, eqn. 1 can be re-written as

$$L_A(1 + k'_A)/u_A = L_B(1 + k'_B)/u_B \tag{2}$$

where L represents the column length, u the mobile phase velocity and k' the capacity factor of the solute of which the analysis time is kept constant. Eqn. 2 is the fundamental expression of isochronal optimization. It points to the fact that two experimental parameters must be changed in this approach. One parameter is altered in order to improve the resolution, while the other is varied to compensate the effect of changing the first parameter on the retention time. Thus, in isochronal analysis two experimental variables are changed simultaneously during the optimization procedure.

With the constraint of constant analysis time, the resolution equation can be written as

$$\begin{aligned} R_S = \tfrac{1}{4}L^{1/2}\{&2D(1 + k'_A)/[u_A(1 + k')] + \\ &Ad_p^{1.33}u_A^{0.33}(1 + k')^{0.33}/[D^{0.33}(1 + k'_A)^{0.33}] + \\ &Cd_p^2 u_A(1 + k')/[D(1 + k'_A)]\}^{-1/2} \cdot [1 - (k'_1 + 1)/(1+k')] \end{aligned} \tag{3}$$

where D is the diffusion coefficient of the solute in the mobile phase, d_p is the particle size, A and C are the coefficients in the Knox equation and subscript 1 indicates the first eluted peak in the pair to be separated. Eqn. 3 provides a general framework within which the isochronal optimization can be pursued.

In the previous papers on isochronal analysis[13,14] the theoretical foundation of the approach was laid out. It was shown that from a practical point of view, there are only three pairs of experimental variables that can easily be manipulated. These are (1) the mobile phase composition and the mobile phase velocity, (2) the column temperature and the mobile phase velocity, and (3) the mobile phase composition and the column temperature. For each of these pairs, a resolution surface was plotted and discussed. It was shown that an optimum can be found on the surface, both globally and locally. The present communication will provide the experimental verification, showing that the method can be applied in practice.

EXPERIMENTAL

Instrumental

The liquid chromatograph consisted of a Spectra Physics (Santa Clara, CA, U.S.A.) 8700 delivery system and SP 8300 UV detector. The detector was operated at 254 nm. The column used was a C_{18} RP cartridge (Merck, Darmstadt, F.R.G.). The data were recorded with the aid of a Yokogawa (Tokyo, Japan) recorder. Two columns were used, both with 4 mm I.D. One was 19 mm long. When necessary, a column of 44 mm length was used.

Materials

The solutes studied were benzene, toluene, propylbenzene and 1,2,4-trichlorobenzene. All solutes were of analytical grade and were used without additional purification. The methanol in the mobile phase was of HPLC grade (Bio-Lab, Jerusalem, Israel). The water was purified in our laboratory with the aid of a Seral (Munich, F.R.G.) purifying system (resistivity > 18 MΩ).

Procedures

All solutes were dissolved in the mobile phase prior to injection. The flow-rate of the mobile phase was varied to cover the velocity range from 0.1 to 0.55 cm/s. The column temperature was maintained constant at $35 \pm 2°C$ with the aid of a thermostated water-bath.

All analyses were performed in triplicate. After the column reached equilibrium at a certain mobile phase composition and velocity, the sample was injected. The dead time, t_0, was estimated by injecting a $2 \cdot 10^{-3}$ M solution of sodium nitrate dissolved in the mobile phase.

RESULTS AND DISCUSSION

The isochronal optimization approach takes advantage of the fact that two experimental variables can be changed simultaneously. One is altered in order to improve the resolution, while the other is changed to maintain constant analysis time. Therefore, the ability to predict the effects of these changes over a wide range of experimental conditions is essential. The prediction is crucial because frequently the change of one experimental variable will influence several chromatographic parameters. For example, changing the temperature will alter the values of the capacity ratio, the selectivity and the efficiency. Together, these three parameters determine the resolution. The simultaneous variation of two experimental variables increases the complexity of the present approach. In this paper, we shall study several empirical (or at best quasi-theoretical) relationships between experimental conditions and chromatographic parameters. The effect of these relationships on the ability to predict theoretical resolution surfaces will be examined by a comparison with experimental surfaces. This also allows a comparison of theoretical isochrons (lines of constant analysis time on the resolution surface) with the experimental ones. The case of changing the mobile phase composition (fraction of methanol in a binary mixture with water) and the velocity will be described first.

Optimization by changing the methanol content and the mobile phase velocity

In this variant of isochronal analysis the mobile phase composition and the velocity are changed simultaneously. The composition of the mobile phase may affect the resolution through changes in the capacity factor (k'), the selectivity (α), and the plate number. Altering the mobile phase velocity can influence the resolution only through variations in the plate number. Changing the mobile phase composition affects mainly the values of the capacity factors of the solutes. There are quite a few papers dealing with the dependence of k' on the modifier concentration[15–20]. It is now recognized that the relationship between the capacity factor and the amount of modifier is exponential in nature, although the exact dependence is still a contested issue. The two most frequently used relationships are a linear function:

$$\ln k' = Ax + B \tag{4}$$

and a quadratic function:

$$\ln k' = A'x^2 + B'x + C \tag{5}$$

A, A', B, B' and C are coefficients, the values of which depend on the solute. In the case of methanol, it is thought that the linear relationship is most appropriate. Fig. 1 shows the variation of the k' values of three solutes as a function of the methanol concentration, in the range between 0.165 (or 0.345 for trichlorobenzene) and 1 for the mass fraction of methanol in water. The lines in the figure represent the best possible straight lines. The data show clearly that over narrow and intermediately large ranges of methanol concentrations a straight line may provide an accurate representation of the experimental observations. However, over a wide range of methanol concentrations the linear behaviour fails to describe the data. Fig. 2 shows the same data as in Fig. 1, but here the lines represent a quadratic fit. Obviously, these lines describe the behaviour much more accurately. The results given here sup-
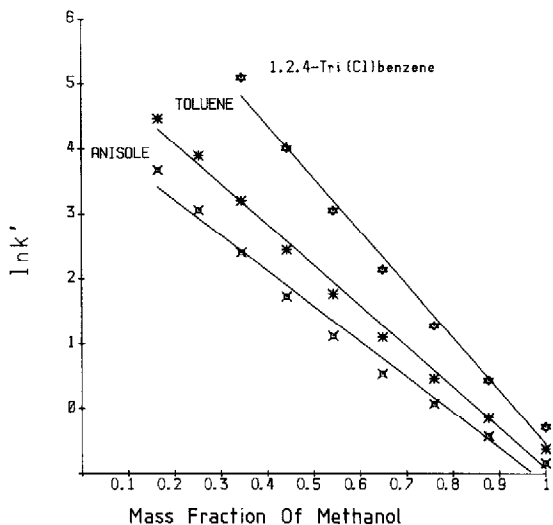

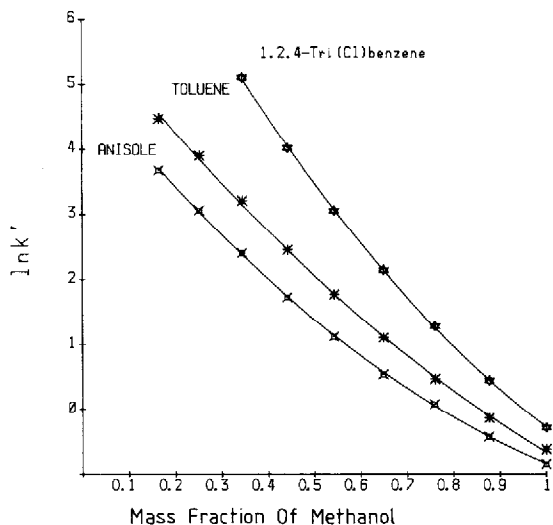Fig. 1. Linear relationship between ln k' and the mass fraction of methanol.

Fig. 2. Quadratic relationship between ln k' and the mass fraction of methanol.

port the work of Wells and Clark[15], Schoenmakers *et al.*[18], and others, who found a non-linear behaviour of ln k' over a wide range of methanol concentrations in the mobile phase. It will be shown below that the accuracy of the predictions made during isochronal analysis depends on the equation used for describing the variation of the capacity factor with the modifier concentration.

In the discussion that follows we will examine two separate cases. First, the separation of benzene from toluene will be described. Thereafter, the behaviour of propylbenzene and 1,2,4-trichlorobenzene will be analyzed. The first case, although trivial from a practical point of view, will be described rather thoroughly, whereas the second case will be summarized to emphasize the salient points of the method in the present variant of isochronal optimization.

Optimization of the separation of benzene from toluene

Comparison between experimental and calculated resolution surfaces. In the previous papers dealing with the theoretical aspects of isochronal analysis[13,14], we made use of resolution surfaces in order to examine the variation of the resolution with varying experimental conditions. A similar approach should be followed for experimental data. For this purpose, the resolution between several pairs of solutes was measured over a wide range of methanol concentrations and mobile phase velocities.

Fig. 3 shows the experimental surface for the resolution between benzene and toluene. Toluene is the isochronal solute, *i.e.*, the solute for which the retention time is maintained constant. Each point of the grid represents an experimental determination. The surface extends from 0.1 to 0.55 cm/s on the velocity axis, and form 0.165 to 1.00 mass fraction units on the methanol concentration axis.

The most prominent feature of the surface in Fig. 3 is the sharp drop in resolution with increasing methanol concentration. The main reason for this is the decrease in selectivity, which varied from 3.0 at a methanol mass fraction of 0.165 to
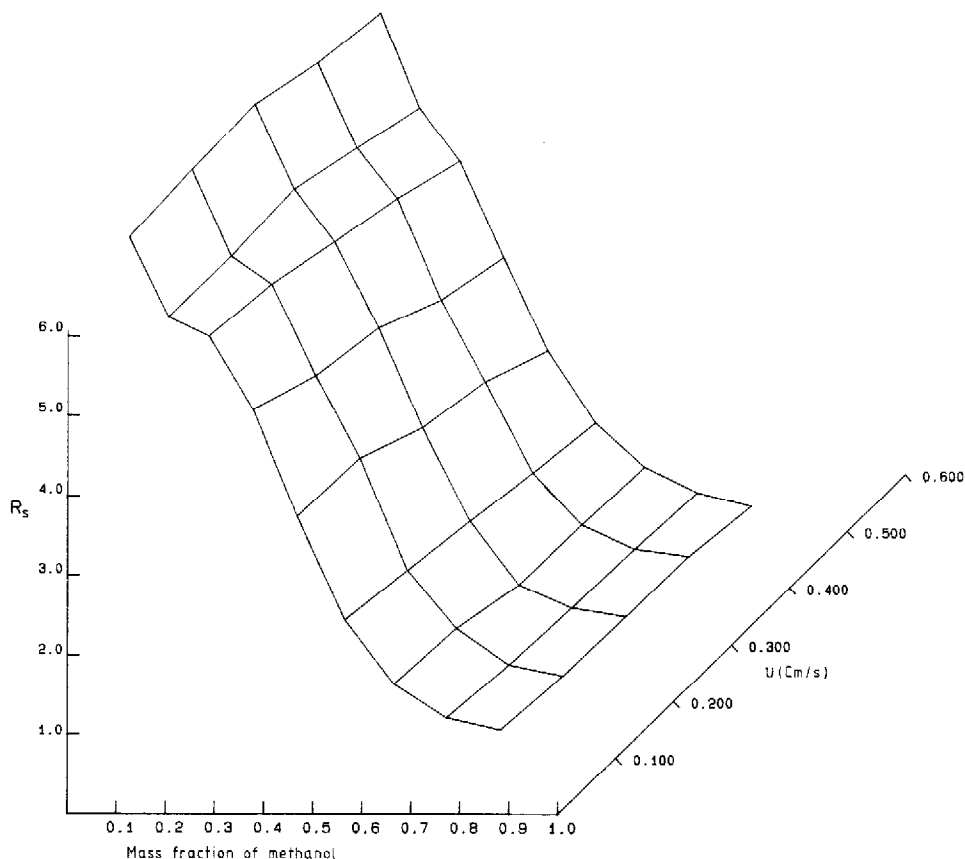
Fig. 3. Experimental resolution surface for benzene and toluene in the mobile phase velocity and composition space.

1.28 at 100% methanol. Another reason for the behaviour shown in the figure is the decrease in the capacity factors of the two solutes with increasing methanol concentration. The experimental plate height increased continuously with increasing modifier concentration. However, due to the square root dependence of the resolution on the plate height, this effect is not a major one.

The velocity axis in Fig. 3 is featureless, at least at high methanol concentrations. At the water-rich end of the figure, there seems to be a shallow maximum in the resolution at a velocity of about 0.3 cm/s. This is due to the variation of the plate height with the mobile phase velocity.

The resolution surface in Fig. 3 indicates that for isochronal analysis the function of changing the velocity is mainly to keep the time constant. Changes in the mobile phase composition are responsible for any variations in the resolution. Thus, the role played by each experimental parameter in the optimization is quite clear. However, this observation is true only for the two solutes under discussion. For two other solutes the effect of the velocity on the resolution (through the plate height) might be more pronounced, and then both the velocity and the mobile phase com-

position will control the resolution and the retention time of the isochronal solute.

The experimental surface can be compared with calculated surfaces, which are based on the experimental variation of the capacity factor as a function of the mobile phase composition. Fig. 4 shows a resolution surface for benzene and toluene, calculated on the basis of the assumption that the dependence of the logarithm of the capacity factor on the methanol concentration is linear (*e.g.* eqn. 4). The coefficients A and B were obtained from linear regression using all data points, as shown in Fig. 1. Fig. 5 shows the resolution surface which was obtained assuming that ln k' is a quadratic function of the methanol concentration (eqn. 5). The coefficients A', B' and C were obtained by fitting a quadratic line to all the experimental points as in Fig. 2. For both calculated resolution surfaces it was assumed that the Knox coefficients were $A = 1$ and $C = 0.05$ and that the dependence of the diffusion coefficients of the solutes on the mobile phase composition could be described by the Perkins-Geankoplis approximations (see the discussion in Refs. 13 and 14 for the implications of these assumptions). The values used for the column length and the particle diameter were the actual experimental values ($L = 1.9$ cm, $d_p = 7 \cdot 10^{-4}$ cm). The surfaces were computed over a wider range of conditions than the experimental surface. The velocity range was from 0.02 to 0.6 cm/s, while the methanol concentration axis spanned the whole range from 0.0 to 1 mass fraction units.

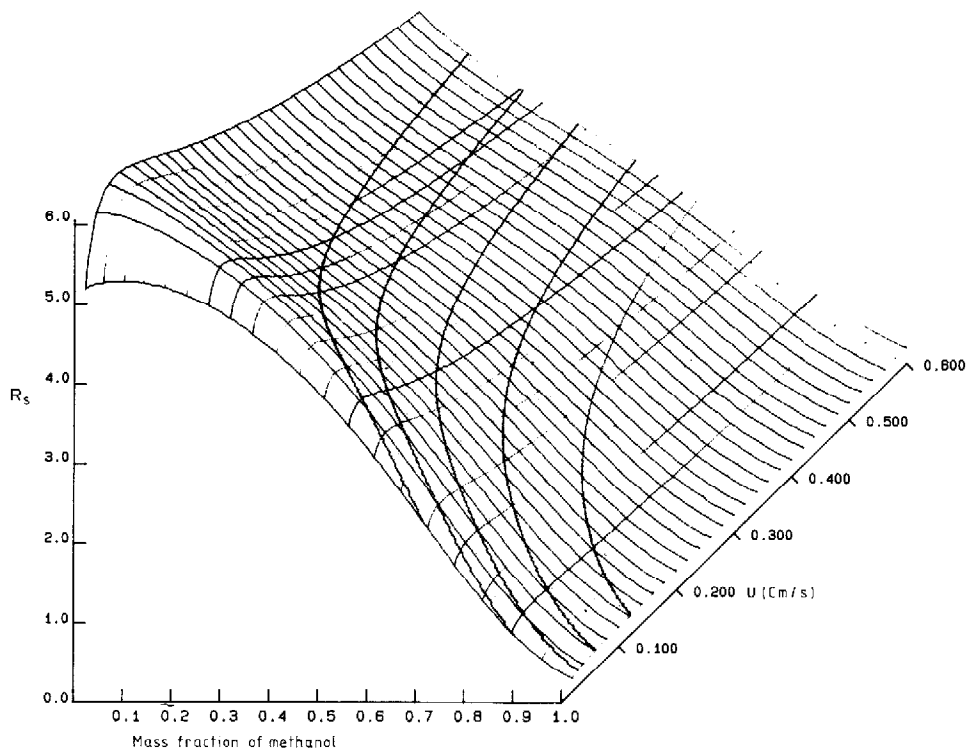Over the ranges of velocities and mobile phase compositions used in the ex-

Fig. 4. Calculated resolution surface for benzene and toluene in the same space as in Fig. 2. Surface generated using a linear relationship between ln k' and mobile phase composition.

TABLE I

COMPARISON BETWEEN EXPERIMENTAL AND CALCULATED VALUES FOR THE RESOLUTION AND OTHER CHROMATOGRAPHIC PARAMETERS AT SEVERAL MOBILE PHASE COMPOSITIONS

Mobile phase velocity, 0.21 cm/s. Solutes, benzene and toluene; only the capacity factors of toluene are given in the table. pre(1) = calculated parameters based on a linear ln k' relationship. pre(2) = calculated parameters based on a quadratic ln k' relationship.

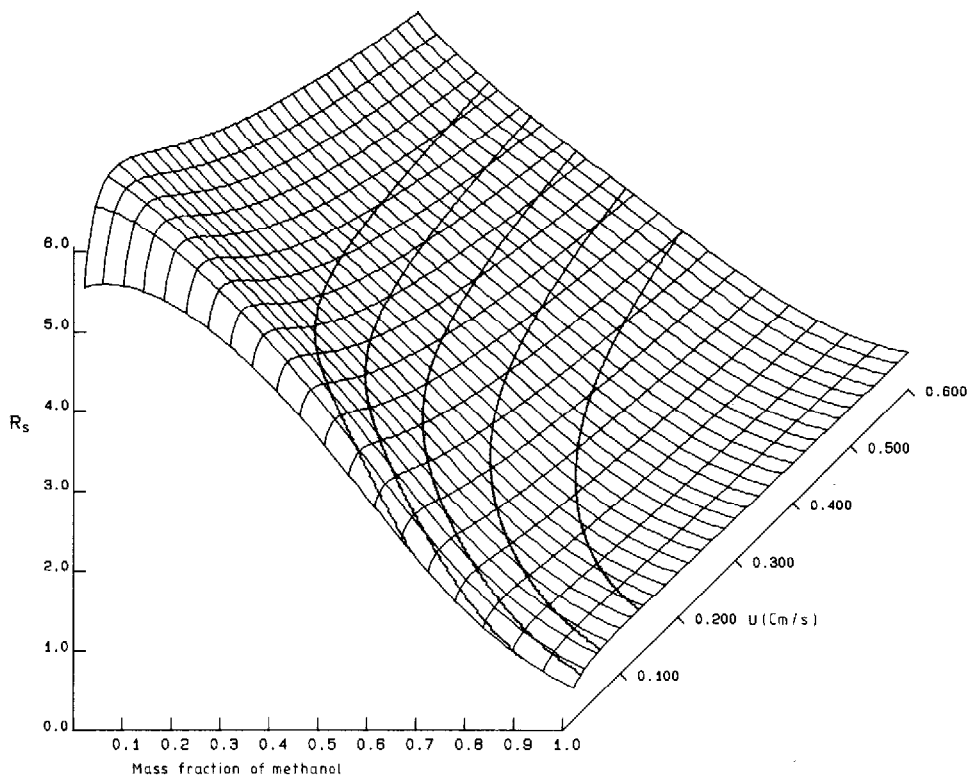| Methanol content[*]	$R_{s\,pre(1)}$	$R_{s\,pre(2)}$	$R_{s\,exp}$	$k'_{pre(1)}$	$k'_{pre(2)}$	k'_{exp}	$\alpha_{pre(1)}$	$\alpha_{pre(2)}$	α_{exp}
0.165	4.68	4.85	6.56	72.71	91.68	84.75	2.86	3.04	3.00
0.345	3.84	3.79	5.13	23.45	22.28	24.43	2.33	2.29	2.32
0.542	2.83	2.60	2.94	6.84	5.71	5.71	1.87	1.78	1.81
0.760	1.47	1.37	0.81	1.76	1.61	1.53	1.47	1.44	1.40
1.00	0.25	0.56	0.22	0.39	0.52	0.53	1.12	1.24	1.20

[*] Mass fraction of methanol.

Fig. 5. Calculated resolution surface for benzene and toluene in the same space as in Fig. 2. Surface generated using a quadratic relationship between ln k' and mobile phase composition.

periments, a comparison of all three surfaces reveals that they are all very similar. The downward curvature of the predicted surfaces at the very low velocities is due to increasing H values. The experimental surface does not show this feature, since very low velocities were not investigated. At velocities higher than 0.1 cm/s, the calculated surfaces behave very similar to the experimental ones. The dependence of the resolution on the velocity is very small. The surface generated using the quadratic dependence of ln k' generally resembles the experimental surface to a better degree. This is demonstrated in Table I, which shows a comparison between some experimental values and those calculated by the two relationships for the capacity factors. The data indicate that, as expected, the prediction of the the capacity factors is better when the quadratic relationship is used. Individual resolutions are predicted fairly well with both relationships. However, the overall surface is described more accurately with the quadratic relationship.

The surfaces discussed above are very important from a theroretical point of view. From a practical point of view, it is not desirable to generate the entire resolution surface each time that an isochronal optimization is to be performed. Instead, the user should be able to predict a resolution surface from a minimum number of experiments, and to follow an isochronal line to find the optimum resolution. Assuming that the plate height is independent of the capacity factor, the absolute mini-
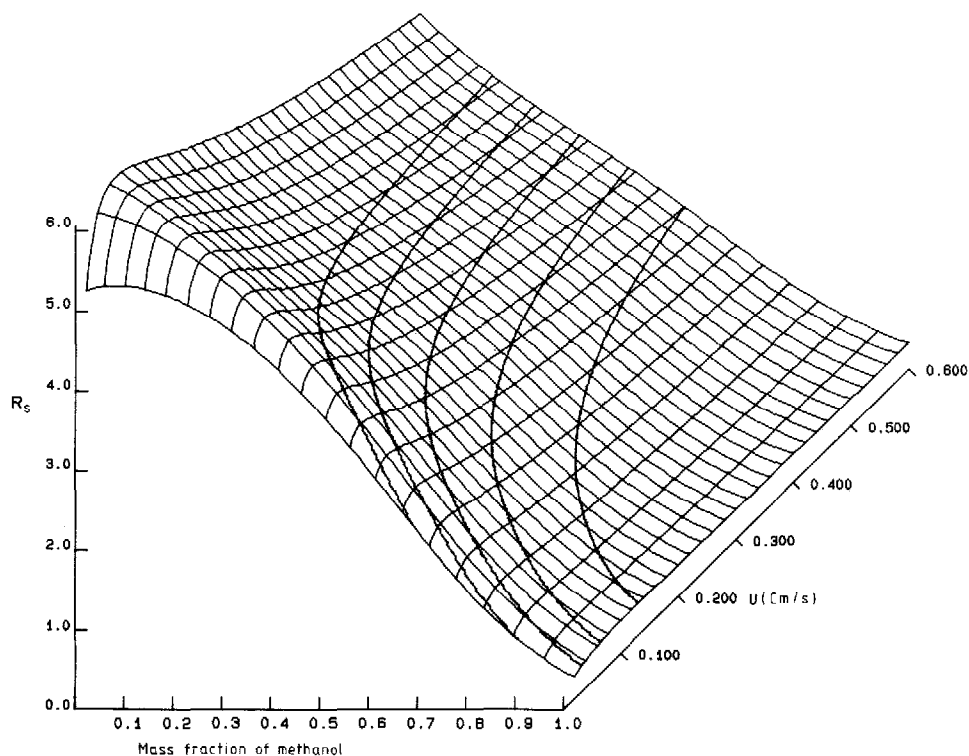
Fig. 6. Calculated resolution surface for benzene and toluene in the same space as in Fig. 2. Surface generated using three experimental data points and a quadratic relationship for the capacity factor.

TABLE II

COMPARISON BETWEEN CALCULATED AND EXPERIMENTAL ISOCHRONS

Solutes were benzene and toluene. The retention of toluene was constant. Only k' values for toluene are given in the table. (p) = calculated values. (e) = experimental values. The numbers 1 and 2 in the titles of the columns represent the first and second (isochronal) solutes.

Methanol content*	u (cm/s)	$R_{s(p)}$	$R_{s(e)}$	$t_{R(1p)}$	$t_{R(1e)}$	$t_{R(2e)}$	$k'_{(p)}$	$k'_{(e)}$
Isochronal time = 305 s								
0.441	0.171	5.23	4.95	161.1	165.0	314.0	10.86	11.02
0.542	0.097	4.70	4.49	188.3	186.0	304.2	5.73	5.95
0.648	0.057	3.69	3.69	219.1	216.8	300.8	2.97	3.02
0.760	0.036	2.37	2.26	249.8	253.3	310.3	1.51	1.61
Isochronal time = 170 s								
0.441	0.307	4.57	4.69	89.6	92.1	175.1	10.86	11.13
0.542	0.174	4.23	4.31	104.8	102.5	167.4	5.73	5.83
0.648	0.103	3.47	3.64	121.8	118.8	166.8	2.97	3.09
0.760	0.065	2.36	2.27	139.1	139.0	170.2	1.52	1.58

* Mass fraction of methanol.

mum number of experiments needed for the calculation is either 2 or 3, depending on whether ln k' is a linear or a quadratic function of the methanol concentration. The above discussion indicates that when the predicted surface is based on the quadratic ln k' relationship, there is good agreement with the experimental results. For that reason, an attempt was made to generate a resolution surface based on only three experimental k' values. Fig. 6 shows the resulting surface. A comparison with the surface in Fig. 3 shows the similarities between the two surfaces. In principle it is therefore sufficient to perform only three experiments, with three different mobile phase compositions, in order to be able to predict the isochronal optimum. This contention will be examined next.

Isochronal considerations. Eqn. 3 can be used to calculate the resolution under the conditions of isochronal analysis. For each analysis time, a line can be drawn on the resolution surface. This line, which we call an isochron, shows the resolution that can be obtained for the two solutes in a given time. The L-shaped lines on the resolution surfaces in Figs. 4–6 are predicted isochrons. These were calculated from eqn. 3 and the appropriate ln k' relationships. Each line on the surfaces represent a different analysis time. The times were chosen arbitrarily to correspond to a wide range of analysis times. Starting with the line on the right (the one next to the velocity axis) these times were 50, 100, 200, 400 and 800 s.

The experimental surface (Fig. 3) presents a problem, because it is difficult to draw the isochrons directly on the surface. For that reason, the experimental data will be compared numerically with the calculated values (see Table II). Table II describes two isochrons, one with an analysis time of 305 s and the other one at about 170 s. Given in the table are the experimental values of the resolution, of the retention times of both solutes and of the capacity factor of the second solute. Also given in
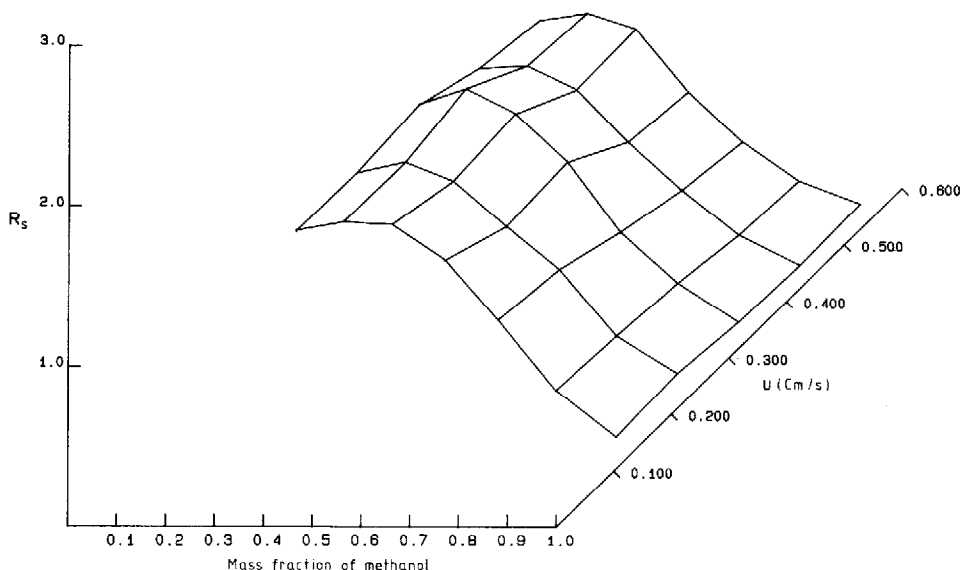

Fig. 7. Experimental resolution surface for propylbenzene and 1,2,4-trichlorobenzene in the mobile phase velocity and composition space.
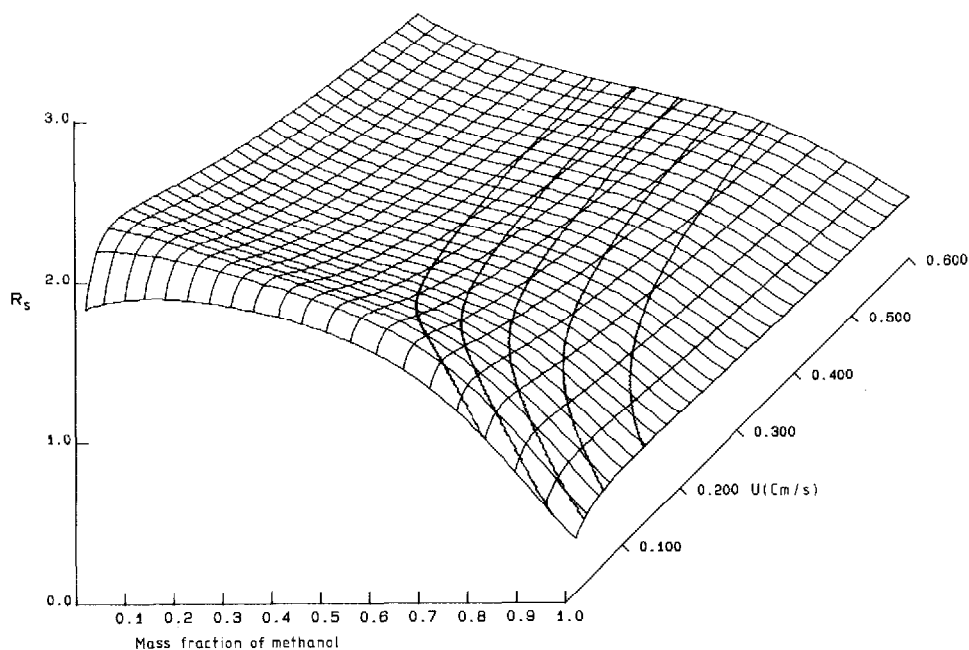
Fig. 8. Calculated resolution surface for propylbenzene and 1,2,4-trichlorobenzene in the same space as in Fig. 7. Surface generated using a linear relationship between ln k' and the mobile phase composition.

the table are the calculated values. These calculated values are based on three retention measurements at different mobile phase compositions, and on the assumption of a quadratic relationship for ln k' (Fig. 2). The experimental resolution values were obtained after correcting for the asymmetries of the peaks. This correction was made by measuring the back half of the first peak and the front half of the second peak, and using these data in the resolution expression.

The agreement between the calculated and experimental values is very good. In both cases a maximum in the resolution is suggested to occur at the low methanol end of the surface. Thus, the chromatographer can predict with a fair degree of accuracy, based on three experiments, the conditions that will yield a maximum resolution at a given analysis time.

The accuracy of the calculated isochron is rather insensitive to the mobile phase compositions used for the experiments. This is true, provided that a fairly wide range of mobile phase compositions is covered. Given the fact that three points define a quadratic equation, the agreement between the experimental and calculated results is quite amazing.

Optimization of the separation of propylbenzene from 1,2,4-trichlorobenzene

Resolution surfaces. The experimental surface for the separation between these two solutes is shown in Fig. 7. Again, each point on the grid represents the result of one experiment. On the whole, the surface is similar to the one in Fig. 3, except that the resolution does not change as much. Due to the long retention times of these solutes at low methanol concentrations, the surface was constructed starting at a

mass fraction of 0.34 (40%, v/v) of methanol. In analogy with the previous case, the dominating factor for the resolution behaviour is the strong decrease in selectivity with increasing methanol concentration.

The surface shown in Fig. 8 is the calculated resolution surface for propylbenzene and 1,2,4-trichlorobenzene. It was calculated by assuming that ln k' is a linear function of the methanol concentration (*i.e.* eqn. 4). The constants A and B were calculated by fitting the ln k' points over the entire range of mobile-phase compositions, as shown in Fig. 1. The surface was generated over the entire range of methanol concentrations. Over the experimental range of the methanol concentrations, the resemblance between the calculated and the experimental surface is not too good. The main reason for the disagreement is the relatively poor fit of the linear ln k' expression to the experimental values. Fig. 9 shows the surface generated using the quadratic relationship for ln k'. Again, the entire experimental range was used to obtain the fitting coefficients A', B' and C, and the surface was calculated to cover the entire range of methanol concentrations. In the region of the experimental data, this surface represents the experimental one much more closely. The only exception is at the top of the experimental surface, which is seen to level off. A closer resemblance of the surfaces could be expected based on the better fit of the quadratic ln k' equation (Fig. 2). The surface generated from three experimental k' values is shown in Fig. 10. Again, the resemblance between the experimental and the calculated surface is quite satisfactory. Thus, isochrons can be calculated with a good degree of accuracy from three experiments.
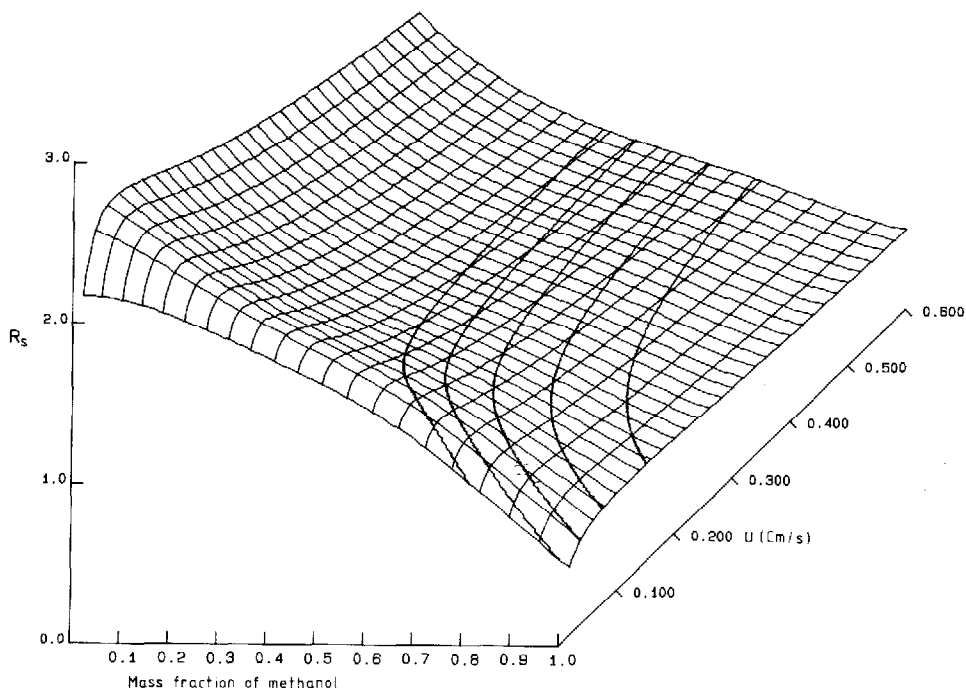

Fig. 9. Calculated resolution surface for propylbenzene and 1,2,4-trichlorobenzene in the same space as in Fig. 7. Surface generated using a quadratic relationship between ln k' and mobile phase composition.
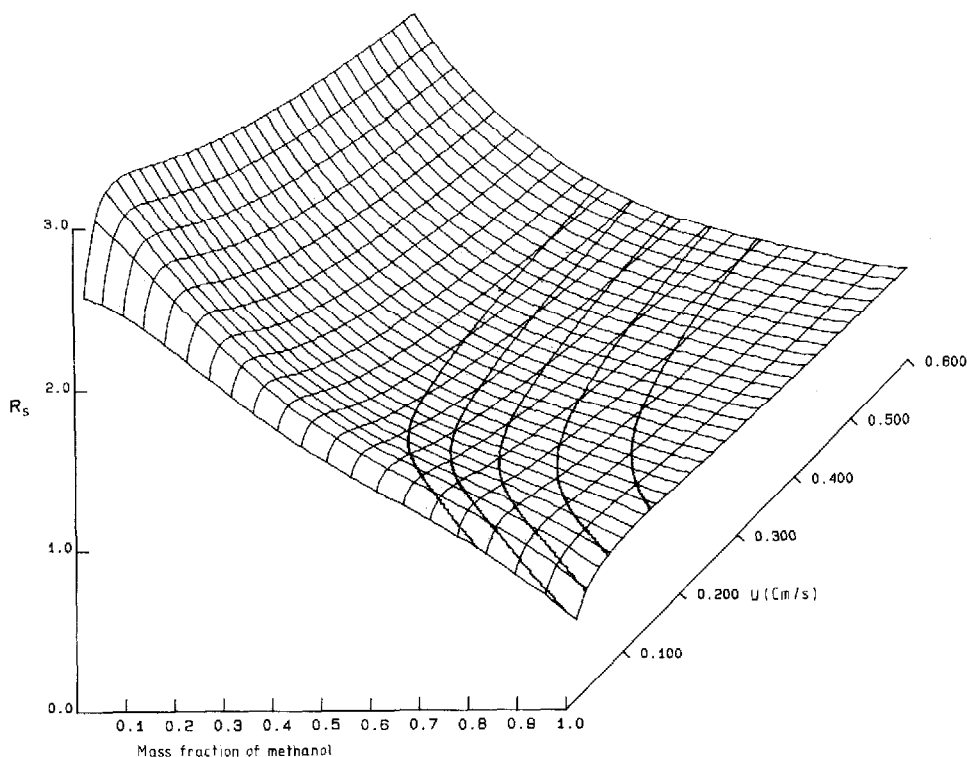
Fig. 10. Calculated resolution surface for propylbenzene and 1,2,4-trichlorobenzene in the same space as in Fig. 7. Surface generated using three experimental data points and a quadratic relationship for the capacity factor.

TABLE III

COMPARISON BETWEEN CALCULATED AND EXPERIMENTAL ISOCHRONS

Solutes, propylbenzene and 1,2,4-trichlorobenzene. The retention of 1,2,4-trichlorobenzene was constant. Capacity factors are give only for the isochronal solute. (p) = calculated values. (e) = experimental values. The numbers 1 and 2 in the titles of the columns represent the first and second (isochronal) solutes.

Methanol content*	u (cm/s)	$R_{s(p)}$	$R_{S(e)}$	$t_{R(1p)}$	$t_{R(1e)}$	$t_{R(2e)}$	$k'_{(p)}$	$k'_{(e)}$
Isochronal time = 445 s								
0.441	0.551	1.82	1.80	347.1	351.0	451.4	54.76	55.75
0.542	0.222	1.88	1.92	365.1	363.4	438.4	21.42	21.56
0.648	0.094	1.87	1.91	378.5	374.6	441.8	8.48	8.70
0.760	0.044	1.72	1.68	387.4	376.0	441.2	3.42	3.47
Isochronal time = 179 s								
0.542	0.551	1.49	1.49	146.8	144.6	175.3	21.41	21.46
0.648	0.234	1.58	1.61	152.1	150.0	177.4	8.48	8.63
0.760	0.109	1.62	1.66	155.6	152.0	176.6	3.42	3.44

* Mass fraction of methanol.

Isochronal considerations. The L-shaped lines on the surfaces in Figs. 8 and 9 are the isochrons for these two solutes. As in the previous case, they were chosen somewhat arbitrarily at 50, 100, 200, 400 and 800 s. The isochrons again show that the resolution can be varied by changing two experimental variables simultaneously, without increasing the analysis time.

Isochrons were also calculated from the experimental data at only three mobile phase compositions to define the relationship for ln k'. These calculated isochrons are compared with experimentally obtained isochrons in Table III. This table shows the calculated and experimental resolution under isochronal conditions, the capacity factors, and the retention times for two different isochrons. In one case, at an analysis time of about 445 s, both the calculated and the experimental data show that the resolution can be optimized. The second case in Table III represents an analysis time of about 179 s. Both the calculated and experimental data indicate that an increase in the resolution can be obtained when the mass fraction of methanol is increased from 0.54 to 0.76 under isochronal conditions. The general agreement between the calculated and the experimental values is excellent.

CONCLUSIONS

The work described here demonstrates that isochronal optimization can be applied in practice. Moreover, instead of following a trial-and-error approach to accomplish optimization, the present treatment shows that the optimum can be predicted with a very high accuracy. The number of initial experiments that must be carried out for the prediction is surprisingly small —three experiments at three different mobile-phase compositions seem to be sufficient to estimate the isochronal conditions of optimum resolution. This may provide a "quick-and-dirty" approach to optimization, which does not require an exhaustive search for an appropriate mobile phase.

The fact that only three experiments are needed for a fairly accurate description of the isochronal optimization is surprising. The possible error in the calculated values for the capacity factors can be quite large. However, it was found that as long as the data are collected over a relatively wide range of methanol concentrations and as long as a quadratic equation is used to describe the ln k' relationship, the differences between experimental and calculated resolutions are small.

Isochronal optimization can also be performed varying other pairs of experimental parameters. Forthcoming publications will deal with the case in which the temperature is varied along with either the mobile phase velocity or the composition. It will be shown that simultaneous changes can again be utilized for isochronal optimization. All three methods will be compared and critically evaluated.

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