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Spherical coordinate representations of solvent composition for liquid chromatography method development using experimental design

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Abstract

One of the major techniques used for the method development of ternary and quaternary high performance liquid chromatography (HPLC) systems has been to use mixture designs, often referred to as ''Glajch's Triangle''. This technique does not allow for the systematic and simultaneous optimization of other factors such as gradient time, pH and temperature that affect the quality of separations. An alternative approach is to use experimental designs. The condition, however, that the composition of all components of the mobile phase must total 100% presents a problem when trying to mathematically represent ranges of each mobile phase constituent of a ternary or quaternary system. A method is described here, based on spherical coordinate representations, that adheres to the constraints of the mobile phase composition and allows experimental designs, such as central composite and factorial designs, to be applied to the simultaneous optimization of the mobile phase composition. Other factors, in particular temperature and gradient time, can then be included in the design. As a result of applying these designs to the HPLC separation of phenols and corticosteroids, it was found necessary to include three-way interactions between experimental factors in the model. The significance of these interactions shows that they need to be considered in HPLC method development.

Keywords: Experimental design; Ternary gradient systems; Isocratic quaternary systems; Mixture designs; Gradient elution

1. Introduction

One of the most common approaches to optimizing reversed-phase high performance liquid chromatography (HPLC) is outlined in the text by Snyder et al. [1]. For this approach, once the column type has been selected on the basis of chemical information of the sample type to be separated, the HPLC method development begins with either a gradient run of 5–100% of an organic solvent such as acetonitrile, or an isocratic run. The gradient run will indicate whether isocratic or gradient elution is required to separate the compounds within an acceptable time frame. The next step is to determine the percent organic for an isocratic separation or the gradient time (time to go from 5 to 100% organic modifier) for a gradient elution. If the instrument does not have gradient elution capability, then an isocratic elution of 100% organic solvent (i.e. the strongest eluent) is run first. The level of organic composition of the solvent phase is decreased until a suitable elution

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time is found, where generally an acceptable retention factor range of $1<k<10$ is sought. Once this is achieved, other binary eluant systems such as methanol/water and THF/water, which alter solute selectivity (relative retentions), can be tried until an acceptable separation is achieved

The ''triangle'' method (mixture design) [2] approach can be adopted if separation is not achieved using any of the above binary systems. This method is used with the overlapping resolution mapping technique, ORM. This can be used for a ternary system; for example, water/ACN/MeOH, or a quaternary system, i.e. water/ACN/MeOH/THF. The quaternary system is treated as three binary systems, water/MeOH, ACN/water and water/THF. The three binary systems are of equal solvent strengths within a suitable retention factor, *k*, range. Seven experiments are performed; three are binary systems, three are ternary systems and one is a quaternary system. In the ORM method the resolution of each peak pair is calculated in each of the seven experiments. The resolution of each peak pair is then overlapped in the triangle space to locate the region where a suitable separation occurs. The use of the three binary systems at the same solvent strength limits this optimization to selectivity optimization and not solvent strength optimization. There could very well be an optimum (or better) separation at a different solvent strength of this system.

A problem with the triangle method is that other factors such as temperature, gradient and pH cannot be included in the design process. To overcome this Glajch et al. [2] describe a gradient method that uses the triangle method. The method considers four factors: three solvents for the mobile phase and another factor, such as gradient, pH or temperature. This method was used to optimize the separation of phenylthiohydantoin amino acids [3]. This is an isoselective multi-solvent gradient elution (IMGE) [4], method that alters the ratio of organic modifier to water changes but not the ratio of the organic modifiers to each other. Kirkland also describes a method that is an extension of the triangle method that alters the selectivity as well as the solvent strength, called selectivity multi-solvent gradient elution (SMGE). This approach does not generate a systematic set of experiments, apart from the initial choice of mobile phase composition. The gradient is stepped depending on the previous set of results from the IMGE method.

Snyder et al. also developed the software program Drylab [5], which can predict the separation as a function of mobile phase composition from two runs, gradient composition and column conditions. However, the Drylab software is limited in that it cannot simultaneously optimize combinations of mobile phase, pH, temperature and gradient compositions. In Drylab version 1.9B1 the ternary and quaternary systems are treated as binary systems where two solvent mixtures have different ratios of the mobile phase solvents, including water.

Experimental design methods have been commonly applied to optimization problems but designs such as the central composite design and factorial designs (e.g. [6–10]) are difficult to apply to ternary and quaternary solvent systems. This is due to these two designs having combinations of low and high levels, and the interdependency of the solvent volume fractions such that the composition of the solvents must add up to 100%. This makes it difficult to apply the designs to the solvent composition. For example, consider a tertiary system where the ranges are as follows: water (40–80%), methanol (10–60%), acetonitrile (10–50%). If the first two factors are set at high levels the sum is already 140% and even if the first two are at their mid-range levels that still sums to 100% so does not allow for any acetonitrile. This restricts the range of mixtures that can be used in the above experimental designs.

Another approach for a ternary system is to let the organic composition be one factor, and the ratio of acetonitrile to methanol be another factor. This would allow more control of the solvent strength, although it would not work for a quaternary system. Clearly, a more flexible method of representing the solvent composition is required.

A novel approach to the optimization of a ternary system using a central composite design is described by Rouberty et al. [8]. This group used a central composite design to test the three factors injection volume, eluent flow and composition of a ternary eluent mixture for an isocratic separation of simazin and atrazin. An arbitrary polarity coefficient was used to express the composition of the ternary eluent

mixture as described in Eq. (1), hence a composition of 45:45:10 of water/methanol/acetonitrile has a relative polarity, *P*, of 0.765.

$$P = 1.0\phi_{\text{water}} + 0.9\phi_{\text{MeOH}} + 0.5\phi_{\text{ACN}} \quad (1)$$

A critical problem with this method is that more than one combination of ternary compositions will give the same polarity value, i.e. there is no unique mathematical solution to derive the values of ϕ_{water}, ϕ_{MeOH} and ϕ_{ACN} for a given polarity value, *P*. For example, a ternary mixture of 50% water, 30% methanol and 20% acetonitrile and a ternary mixture of 58% water, 20% methanol and 22% acetonitrile will both give the same polarity value of 0.87. Therefore, once the conditions for the best separation are determined by the model in respect of optimum *P* value, it is not possible to determine from the polarity what the actual appropriate ternary composition is. It is incorrect to assume that all compositions with the same polarity would produce the same result. Clearly, an increase from 50 to 58% water will have a significant effect on the retention of all the solutes, but to varying degrees for each; the 2% increase in ACN is unlikely to compensate for this difference in water composition.

2. Spherical coordinate representation of solvent composition in experimental design methods

In our work a novel approach to describing the composition of the mobile phase has been used. This approach uses spherical coordinates rather than rectangular coordinates to represent the mobile phase composition. This solves the problem of representing the mobile phase composition for HPLC when the amount of one solvent is dependent on another. The amount and composition of organic modifiers in the mobile phase can be expressed by Eq. (2). Each term in this equation represents one of the organic solvents (A, B and C) in the mobile phase and ρ^2 represents the total amount of organic solvent in the mobile phase.

$$\rho^2 = \rho^2 \sin^2 \phi + \rho^2 \cos^2 \phi \sin^2\theta + \rho^2 \cos^2 \phi \cos^2\theta \quad (2)$$

Eq. (2) is the equation of a sphere of radius ρ. The spherical coordinates ρ, ϕ and θ give the position of a point on the surface of the sphere in a three-dimensional space (Fig. 1). Note that the correct notation for spherical coordinates would measure the angle of ϕ from the positive *z*-axis down not from the *xy* plane towards the positive *z*-axis. The reason for measuring the angle of ϕ from the *xy*-plane to the positive *z*-axis is that when ϕ is zero so is the composition of solvent A. On the diagram the composition of the mobile phase in a quaternary system is equal to z^2, y^2, x^2 and $1-\rho^2$, where *z*, *y* and *x* represent one of the organic solvents, A, B, and C, and $1-\rho^2$ is the volume fraction of water.

Using Eqs. (3)–(6), the composition of the mobile phase for a quaternary system can be determined from ρ, ϕ and θ. The ranges of the angles θ and ϕ are restricted to 0–90° (0 to $\pi/2$ radians) so that the composition of each solvent is zero or positive. If the range of ρ^2 is restricted to being between 0 and 1 inclusive, then the sum of Eqs. (3)–(6) will always be 1, i.e. it sums to 100% of the total solvent composition. For example, if the water composition of the mobile phase is 55% and ϕ is 30° and θ is 70° then $0.55 = 1-\rho^2$, and ρ^2 is equal to 0.45, and solvent A is ($0.45 \sin^2 30°$) 11.25%, solvent B is ($0.45 \cos^2 30° \sin^2 70°$) 29.80%, and solvent C is

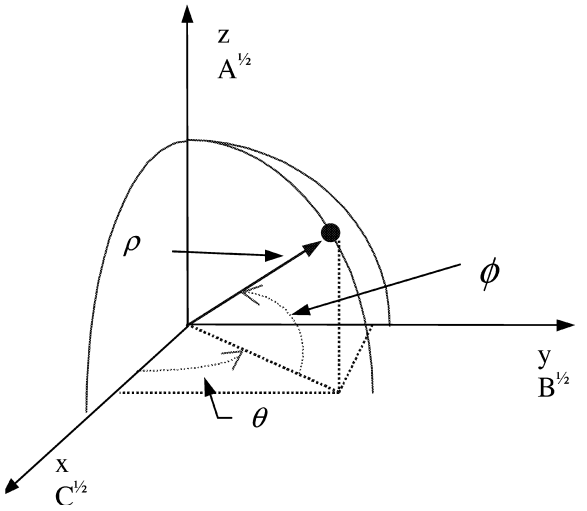


Fig. 1. Spherical coordinate representation of solvent composition.

(0.45 $\cos^2$ 30° $\cos^2$ 70°) 3.95%, totaling 55% + 11.25% + 29.80% + 3.95% = 100%.

$$\text{Water} = 1 - \rho^2 \quad (3)$$

$$A = \rho^2 \sin^2\phi \quad (4)$$

$$B = \rho^2 \cos^2\phi \sin^2\theta \quad (5)$$

$$C = \rho^2 \cos^2\phi \cos^2\theta \quad (6)$$

The use of a geometric transformation does mean that a design that is uniform in the coded form will lose some of this uniformity when translated back to real mixture compositions, however, we believe the greater flexibility of this approach more than compensates for this disadvantage.

For a ternary system the approach can be simplified. Eq. (7) describes the relationship between the solvents in the mobile phase using two parameters, r and θ. The terms in the equation represent the composition of the two organic solvents and r^2 is the total organic composition of the mobile phase. This equation describes a circle in the xy plane. The domain used by this strategy uses the first quadrant as shown in Fig. 2

$$r^2 = r^2 \sin^2\theta + r^2 \cos^2\theta \quad (7)$$

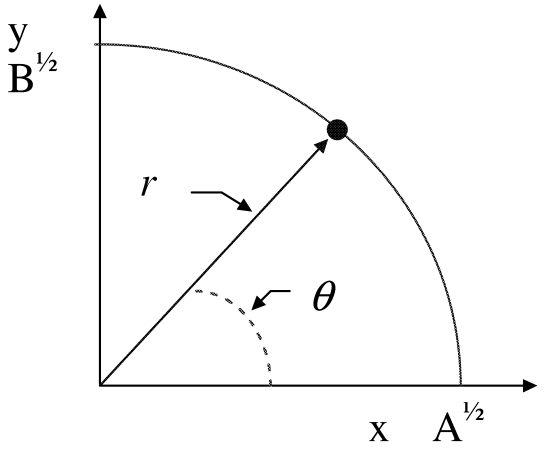


Fig. 2. Solvent domain for a ternary system using polar coordinates.

Eqs. (8)–(10) are used to calculate the composition of the ternary phase.

$$\text{Water} = 1 - r^2 \quad (8)$$

$$A = r^2 \cos^2\theta \quad (9)$$

$$B = r^2 \sin^2\theta \quad (10)$$

The coordinates ρ, ϕ and θ for a quaternary system and the coordinates θ and r for a ternary system can be used as factors for the mobile phase composition in central composite, factorial and Plackett–Burman experimental designs. The use of polar or spherical coordinates overcomes the ''volume interdependency'' of the solvents in the mobile phase, allowing the composition and other factors, such as pH, flow-rate, temperature and gradient time, to be included in the design. This approach again allows the full range of solvents to be explored in the experimental design.

A central composite experimental design (CCD), employing the spherical or polar coordinates, was used to optimize the separation of a phenol mixture, using gradient elution, in reversed-phase HPLC. The CCD is a popular multi-level design, used to generate response surfaces in optimization studies [12,13].

3. Materials and methods

A Shimadzu Class LC10A HPLC system (Shimadzu Corporation, Kyoto, Japan) with a photo diode array detector, quaternary solvent delivery and auto injector was used. Shimadzu Class-LC10 software was used to control the HPLC system, and for data acquisition.

A Merck LiChrocart 125-4 Lichrospher 100 RP-18 (5 μm) (Merck, Kilsyth, Australia) column was used throughout. The flow-rate was set at 1 ml/min, and all the conditions were adjusted according to the experimental design. Adequate time was allowed for column equilibration where temperature of analysis was varied. Solvents used were acetonitrile, and Hipersolv grade methanol and tetrahydrofuran (all solvents from BDH, Melbourne). Water was obtained from a Milli-Q ultrapure water system (Millipore, North Ryde, Australia).

3.1. Samples

A 17-component phenol sample mixture, PHM-804 in methanol, was obtained from Ultra Scientific (North Kingstown, USA). The mixture was diluted 1:4 with Hipersolv grade methanol.

For the experimental design runs a 5-ml mixture of corticosteroids in Milli-Q water was prepared from compounds dissolved in Hipersolv grade methanol as follows: 0.4 ml dexamethasone (265 μg/ml), 0.3 ml prednisone (335 μg/ml), 1 ml prednisolone (100 μg/ml), 0.5 ml hydrocortisone (200 μg/ml), 0.2 ml Reichstein's substance (cortexolone; 470 μg/ml), 0.3 ml cortisone (360 μg/ml), and 0.75 ml corticosterone (195 μg/ml). All standards were from Sigma–Aldrich (Castle Hill, NSW, Australia).

The generation of the central composite designs and the multi-linear regression analysis were performed on Minitab for Windows version 10 (Minitab Inc, State College, PA, USA). Statistica version 4.5 (Statsoft, Hamburg, Germany) was used to generate response surfaces. Target analysis was performed using Microsoft Excel 5 (Microsoft Corporation, USA)

4. Results and discussion

4.1. Optimization of the HPLC separation of a phenol mixture

The factors evaluated for the optimization of the HPLC separation of the phenol mixture were gradient time, t_g, initial organic solvent composition, θ_i, final organic solvent composition, θ_f, and temperature. All runs started at 10% total organic solvent and finished at 100% total organic composition. The composition of the organic solvents methanol and acetonitrile were calculated using Eqs. (9) and (10), respectively. For the initial composition $r^2 = 0.1$ and for the final the composition $r^2 = 1$. For example, for a final organic composition $r^2 = 1$ and $\theta = 22.5°$ then from Eqs. (9) and (10) the organic composition of acetonitrile, solvent A and methanol, solvent B, is (100*(1 $\cos^2$ 22.5°)) 85.4% and (100*(1 $\sin^2$ 22.5°)) 14.6%, respectively. The CEF value [11] of a chromatogram was used as the response with $R_{opt} = 1.5$, $a = 3$ and $t_{max} = 40$ min. The CEF is an objective function designed to express quantitatively the quality of a chromatogram with regard to resolution and time. The function includes adjustable parameters so the relative importance of resolution and maximum retention time can be set. A chromatogram with all peaks resolved and a short maximum retention time will have a low CEF value so the object of the optimization is thus to minimize this function. Details of the method of calculation of this function are given by Morris et al. [11]. The central composite experimental design and responses expressed as CEF values are given in Table 1.

It was proposed that the starting and finishing total organic compositions, *r*, could be adjusted after the selection of the best conditions without altering the resolutions between components. This is similar to the approach described by Snyder et al. [1] in which the retention times of the final and initial components from a 5 to 100% ACN 20-min gradient run were used to estimate a suitable starting and finishing percent organic composition. Taking this approach eliminates two factors, starting and finishing organic composition, thereby not only simplifying the optimization process but also reducing the number of experiments necessary to optimize the separation. It was also decided that flow-rate need not be a variable since flow will have a predictable effect on efficiency and its effect can be verified after optimization using the CCD design.

The CEF responses for the central composite design are given in Table 1. The response model obtained from the design using multi-linear regression has a poor fit. Removing runs 1, 4 and 12 improves the fit from an adjusted R^2 (adjusted for degrees of freedom) value of 14.9 to 69.9%, which is still unacceptable. It is expected that the two-way interactions between the gradient time, initial organic composition and final composition will be significant and possibly the three-way interaction between these factors will also be significant. Inclusion of the three- and four-way interactions and all runs in the regression analysis resulted in an adjusted R^2 value of 62.1%, which is still a poor fit. The run with the greatest residual in this model was run 26, which had a fast gradient time (low t_g). Sometimes an unusual experimental result is due to the inadequacy of the

Table 1
Central composite experimental design for the HPLC separation of phenol mixture PHM804

Run name	Block	Coded variables				Uncoded variables				Initial % ACN	Initial % MeOH	Final % ACN	Final % MeOH	CEF
		t_g	θ_i	θ_f	T	t_g	θ_i	θ_f	T					
1	1	1	−1	1	1	68	22.5	67.5	40	1.5	8.5	85.4	14.6	33 042
2	1	0	0	0	0	60	45.0	45.0	35	5.0	5.0	50.0	50.0	200
3	1	1	−1	−1	−1	68	22.5	22.5	30	1.5	8.5	14.6	85.4	37 515
4	1	−1	−1	−1	1	52	22.5	22.5	40	1.5	8.5	14.6	85.4	16 459
5	1	−1	1	1	1	52	67.5	67.5	40	8.5	1.5	85.4	14.6	716
6	1	0	0	0	0	60	45.0	45.0	35	5.0	5.0	50.0	50.0	188
7	1	1	1	1	−1	68	67.5	67.5	30	8.5	1.5	85.4	14.6	16 563
8	1	1	1	−1	1	68	67.5	22.5	40	8.5	1.5	14.6	85.4	18 219
9	1	−1	−1	1	−1	52	22.5	67.5	30	1.5	8.5	85.4	14.6	224
10	1	−1	1	−1	−1	52	67.5	22.5	30	8.5	1.5	14.6	85.4	86
11	2	1	1	−1	−1	68	67.5	22.5	30	8.5	1.5	14.6	85.4	279
12	2	1	1	−1	1	68	22.5	22.5	40	1.5	8.5	14.6	85.4	18 387
13	2	1	1	1	1	68	67.5	67.5	40	8.5	1.5	85.4	14.6	137
14	2	−1	1	−1	1	52	67.5	22.5	40	8.5	1.5	14.6	85.4	16 315
15	2	0	0	0	0	60	45.0	45.0	35	5.0	5.0	50.0	50.0	259
16	2	−1	−1	−1	−1	52	22.5	22.5	30	1.5	8.5	14.6	85.4	118 179
17	2	−1	−1	1	1	52	22.5	67.5	40	1.5	8.5	85.4	14.6	15 107
18	2	−1	1	1	−1	52	67.5	67.5	30	8.5	1.5	85.4	14.6	15 097
19	2	1	−1	1	−1	68	22.5	67.5	30	1.5	8.5	85.4	14.6	77
20	2	0	0	0	0	60	45.0	45.0	35	5.0	5.0	50.0	50.0	259
21	3	0	−2	0	0	60	0.0	45.0	35	0.0	10.0	50.0	50.0	33 698
22	3	0	0	0	−2	60	45.0	45.0	25	5.0	5.0	50.0	50.0	18 208
23	3	0	0	0	0	60	45.0	45.0	35	5.0	5.0	50.0	50.0	154
24	3	0	0	2	0	60	45.0	90.0	35	5.0	5.0	100.0	0.0	1014
25	3	0	0	−2	0	60	45.0	0.0	35	5.0	5.0	0.0	100.0	35 634
26	3	−2	0	0	0	44	45.0	45.0	35	5.0	5.0	50.0	50.0	15 273
27	3	2	0	0	0	76	45.0	45.0	35	5.0	5.0	50.0	50.0	131
28	3	0	0	0	2	60	45.0	45.0	45	5.0	5.0	50.0	50.0	16 490
29	3	0	2	0	0	60	90.0	45.0	35	10.0	0.0	50.0	50.0	16 926
30	3	0	0	0	0	60	45.0	45.0	35	5.0	5.0	50.0	50.0	161

quadratic model in not being able to model the sharp changes observed, as is the case of run 26. Removal of the run can allow the model to fit the rest of the data adequately. If the unusual run was at the edge of the tested domain, as in this case, then the domain would change. With the removal of run 26 the tested domain for t_g is now changed at the lower end from 44 to 52 min. The resulting regression analysis had a more acceptable adjusted R^2 value of 80.1%. The coefficients for this model are given in Table 2.

The factors that were indicated as significant (P-values below 0.05) when all runs were included were still significant with the removal of run 26. The only change was the degree to which the factors affected the response, that is a change in the value of the coefficients. The factors found to be significant were the main effect and second order effects of the initial and final organic compositions, the second order effect of temperature and the three-way interaction between temperature and the initial and final organic compositions (Table 2). Fig. 3a–e show the relationship between θ_i and θ_f.

The diagonal contours on the surface diagrams for Fig. 3a,b,d,e show that interactions occur between θ_i and θ_f. This shows that the best setting for one parameter is dependent on the other. The three-way relationship between temperature, θ_i and θ_f is shown by the significant change in the response surface and the optimum θ_i and θ_f conditions with a change in temperature in Fig. 3a–e.

Apart from the center point, these points are located near the edge of the region, resulting in a

Table 2
Regression coefficients, *t*-ratio and *P*-values for optimization of the phenol mixture separation

Predictor	Coefficient	*t*-ratio	*P*
Constant	203	0.09	0.931
t_g	2663	1.94	0.094
θ_i	−4380	−3.87	0.006
θ_f	−4738	−4.19	0.004
Temperature	1122	0.99	0.354
$t_{g_2}^2$	−1554	−1.08	0.316
θ_i^2	6109	5.68	0.000
θ_f^2	4362	4.06	0.005
Temperature^2	4118	3.83	0.006
$t_g \times \theta_i$	−2254	−1.63	0.148
$t_g \times \theta_f$	−293	−0.21	0.838
$t_g \times$ Temperature	21	0.02	0.988
$\theta_i \times \theta_f$	2481	1.79	0.116
$\theta_i \times$ Temperature	−1477	−1.07	0.321
$\theta_f \times$ Temperature	232	0.17	0.871
$t_g \times \theta_i \times \theta_f$	142	0.10	0.921
$t_g \times \theta_i \times$ Temperature	−63	−0.05	0.965
$t_g \times \theta_f \times$ Temperature	1984	1.43	0.195
$\theta_i \times \theta_f \times$ Temperature	−8355	−6.03	0.000
$t_g \times \theta_i \times \theta_f \times$ Temperature	−2485	−0.80	0.449
Block 1	−298	−0.10	0.921
Block 2	−233	−0.08	0.938

distribution of points with respect to actual percent ACN, that do not form a uniform distribution of points across the design region as would be desired. To achieve the desired distribution of points $\sin^2\theta$ (or the cosine^2 of the angle, whichever is appropriate) rather than θ will be used as a factor.

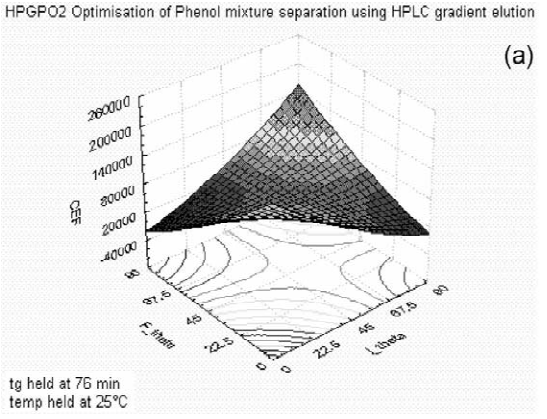
The optimum conditions for separation were predicted to be a gradient time of 76 min, an initial organic composition (10%) of methanol, a final organic composition (100%) of acetonitrile and a temperature of 25 °C. This prediction was performed by using the Microsoft Excel Solver macro to find the optimum response using the regression model.

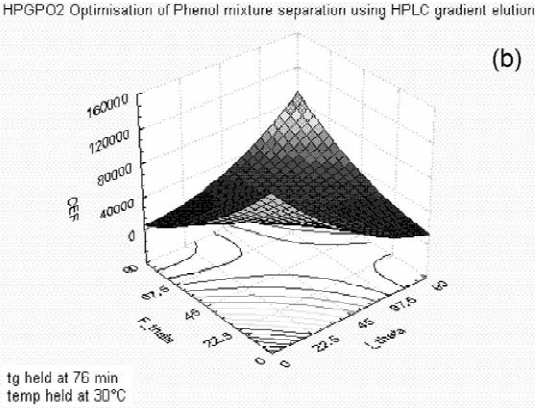
In the chromatogram run under the optimal conditions, the time to the first peak was over 10 min and the time of the final peak is less than 60 min. This final peak time is considerably less than the 76 min it takes for the mobile phase composition to change from 10% MeOH to 100% ACN. The excessive time before the first peak and after the final peak is a waste. To decrease the total elution time of the optimized separation the initial and final percent organic composition was adjusted by setting the initial solvent composition to the composition at the time of the first peak minus the dwell time. This is done by using Eq. (11). The dwell volume is 4 ml, the flow-rate is 1 ml/min, and the time of the first peak is 17.8 min. Therefore, the starting conditions can be set to the mobile phase composition at 13.8 min giving a chromatogram with the same resolution but without the initial 10 min before the first peak.

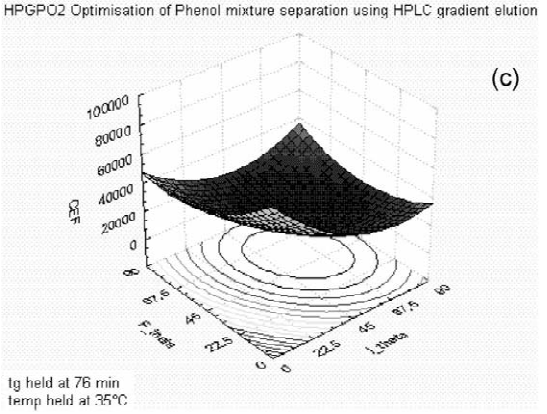
$$\%\text{solvent} = \frac{\%\text{initial} - \%\text{final}}{t_g} \times (\text{time of first peak} - \text{dwell time}) \quad (11)$$

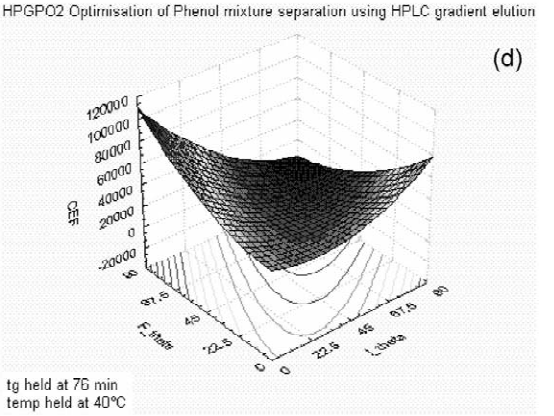
The adjusted initial solvent compositions are 18.2% ACN and 8.2% MeOH. For the final conditions the run is stopped after the final peak is eluted. The gradient time, t_g, and the final mobile phase composition must be changed so the rate of change of each solvent is the same as for Fig. 4, the optimized chromatogram. The conditions for this adjusted optimum chromatogram were a temperature of 25 °C, initial mobile phase composition of 18.2% ACN, 8.2% MeOH and 73.6% water to a final mobile phase composition of 65% ACN, 3.5% MeOH and 31.5% water, with a t_g of 35.6 min.

The components of the phenol mixture were identified by injecting available pure samples separately under the same conditions as the optimized chromatogram. The disadvantage of not using *r*, the percent of organic in the mobile phase, as a factor is the lack of flexibility in the starting organic composition. With a different starting percent organic than the 5% in the experimental design a better chromatogram may have been obtained from a different combination of solvents in the mobile phase. However, increasing the number of factors to include *r* will increase the number of experiments to 41 plus center points. It will also make it difficult to visually inspect the response surface for the optimum, since only three dimensions can be viewed at once on a response surface diagram. One of the dimensions is used for the response and the other two dimensions are used for two of the factors; all other factors are held constant. The more factors included in the optimization process the more that are held constant, thus visually inspecting the response surface to locate the optimum is increasingly more difficult.









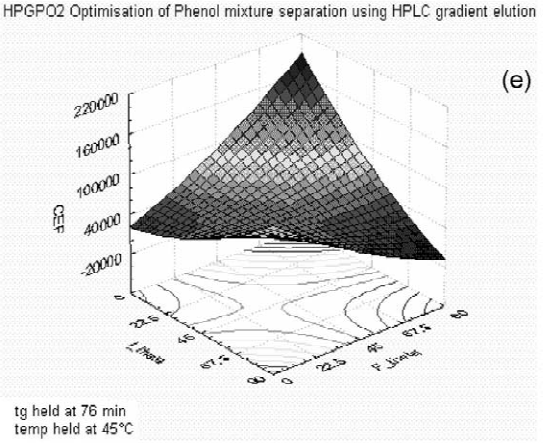


Fig. 3. (a) Response surface model for the HPLC separation of phenol mixture, CEF vs. θ_i and θ_f, for a gradient time of 76 min and temperature of 25 °C. (b) Response surface model for the HPLC separation of phenol mixture, CEF vs. θ_i and θ_f, for a gradient time of 76 min and temperature of 30 °C. (c) Response surface model for the HPLC separation of phenol mixture, CEF vs. θ_i and θ_f, for a gradient time of 76 min and temperature of 35 °C. (d) Response surface model for the HPLC separation of phenol mixture, CEF vs. θ_i and θ_f, for a gradient time of 76 min and temperature of 40 °C. (e) Response surface model for the HPLC separation of phenol mixture, CEF vs. θ_i and θ_f, for a gradient time of 76 min and temperature of 45 °C.

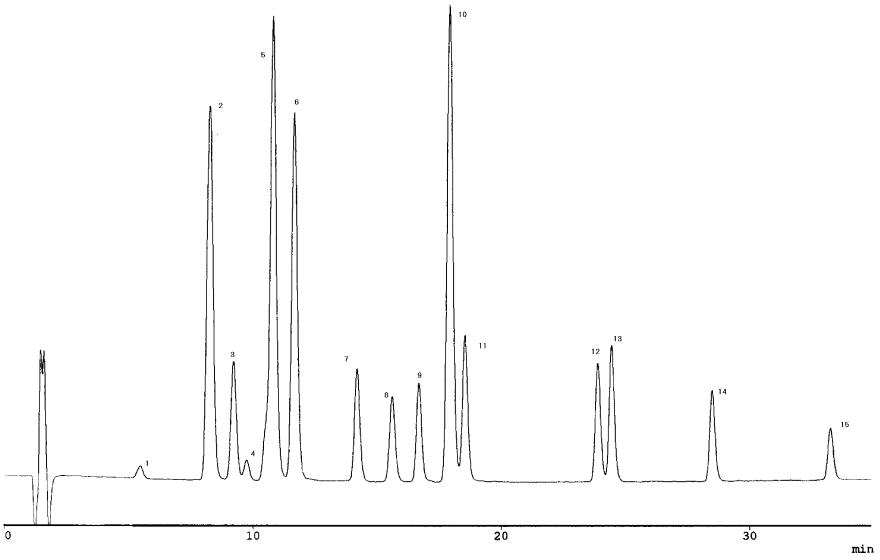


Fig. 4. Adjusted optimized separation of Phenol mixture. Peaks identified as follows: (1) phenol; (2) 4-nitrophenol; (3) *p*-cresol and *m*-cresol; (4) *o*-cresol; (5) *o*-chlorophenol and 2,4-dinitrophenol; (6) 2-nitorphenol; (7) and (8) either 2,6-dichlorophenol or 2,4-dimethylphenol; (9) 4-chloro-*m*-cresol; (10) 2-methyl-4,6-dinitrophenol; (11) 2,4-dichlorophenol; (12) 2,4,6-trichlorophenol; (13) 2,4,5-trichlorophenol; (14) tetrachlorophenol; (15) pentachlorophenol.

4.2. Optimization of the isocratic HPLC separation of a mixture of corticosteroids: initial considerations

An optimization strategy requiring the use of spherical coordinates for inter-dependent factors of the mobile phase, i.e. ACN, MeOH, THF and water, was used to develop a method for the separation of seven corticosteroids. A study on six of these corticosteroids [1] indicated that different selectivities would be found with different eluent compositions, hence presenting a challenging study for this application.

The first stage is to determine the solvent strength range that will give a retention factor, *k*, range of between 1 and 20 ($0 < \log k < 1.30$). This was done by eluting the corticosteroids at varying percent methanol in the mobile phase at 25 °C. To keep within the *k* range the mobile phase needs to be between 43 and 55% MeOH. The solvent strength range of methanol transfers to an approximate equivalent solvent strength range using ACN of between ~30 and 50%, and between 20 and 40% for THF according to Snyder et al. [1]. A temperature of 25 °C was used when determining a suitable percent organic range. The design covers a temperature range between 25 and 45 °C. At a higher temperature there is likely to be a reduction in the retention time, enabling a lower organic content that will still be within the *k* range. Therefore, the total organic composition was expanded to a range from 10 to 54%.

The factors evaluated were temperature, percent organic in mobile phase and ratio of THF/ACN/MeOH in the mobile phase. Using the spherical coordinates the experimental design factors and ranges were set as follows: temperature (25–45 °C), % organic (10–54%), $\sin^2\theta$ (0–1) and $\sin^2\phi$ (0–1). θ and ϕ thus range from 0 to 90°. However, from the investigation of the previous study on the HPLC separation of the phenols, $\sin^2\theta$ and $\sin^2\phi$, respectively, were used in this study to ensure rotatability and a uniform distribution of points with respect to the actual solvent proportions. The experimental design is given in Table 3.

The resulting CEF values for each chromatogram are given in Table 3. A regression analysis on these results gave an adjusted R^2 value of 52.5% indicating that the model is inadequate for predicting the optimum. The removal of run 25, which has the

Table 3
Experimental design for the optimization of the separation of corticosteroids

Run no.	Uncoded variables							Corresponding organic composition				%THF	%ACN	%MeOH	CEF*
	Block	Temp	%Organic	$Sin^2\theta$	$Sin^2\phi$	Temp	%Organic	$Sin^2\theta$	$Sin^2\phi$	θ	ϕ				
1	1	0	0	0	0	35	32	0.5	0.5	0.785	0.785	16	8	8	10 644
2	1	0	0	0	0	35	32	0.5	0.5	0.785	0.785	16	8	8	10 619
3	1	−1	−1	−1	1	30	21	0.25	0.75	0.523	1.047	15.75	1.313	3.938	134
4	1	−1	1	1	1	30	43	0.75	0.75	1.047	1.047	32.25	8.063	2.688	27 337
5	1	−1	1	−1	−1	30	43	0.25	0.25	0.524	0.524	10.75	8.063	24.19	1490
6	1	1	−1	1	1	40	21	0.75	0.75	1.047	1.047	15.75	3.938	1.313	553
7	1	1	1	−1	1	40	43	0.25	0.75	0.524	1.047	32.25	2.688	8.063	19 208
8	1	−1	−1	1	−1	30	21	0.75	0.25	1.047	0.524	5.25	11.82	3.938	45 345
9	1	1	1	1	−1	40	43	0.75	0.25	1.047	0.524	10.75	24.19	8.063	19 381
10	1	1	−1	−1	−1	40	21	0.25	0.25	0.524	0.524	5.25	3.938	11.82	68 231
11	2	−1	1	1	−1	30	43	0.75	0.25	1.047	0.524	10.75	24.19	8.063	19 545
12	2	0	0	0	0	35	32	0.5	0.5	0.785	0.785	16	8	8	10 666
13	2	1	−1	−1	1	40	21	0.25	0.75	0.524	1.047	15.75	1.313	3.938	35
14	2	−1	1	−1	1	30	43	0.25	0.75	0.524	1.047	32.25	2.688	8.063	18 465
15	2	−1	−1	−1	−1	30	21	0.25	0.25	0.524	0.524	5.25	3.938	11.82	29 571
16	2	1	1	1	1	40	43	0.75	0.75	1.047	1.047	32.25	8.063	2.688	27 626
17	2	0	0	0	0	35	32	0.5	0.5	0.785	0.785	16	8	8	10 715
18	2	1	1	−1	−1	40	43	0.25	0.25	0.524	0.524	10.75	8.063	24.19	1472
19	2	1	−1	1	−1	40	21	0.75	0.25	1.047	0.524	5.25	11.82	3.938	2109
20	2	−1	−1	1	1	30	21	0.75	0.75	1.047	1.047	15.75	3.938	1.313	159
21	3	0	−2	0	0	35	10	0.5	0.5	0.785	0.785	5	2.5	2.5	171 132
22	3	0	0	0	0	35	32	0.5	0.5	0.785	0.785	16	8	8	10 681
23	3	0	0	−2	0	35	32	0	0.5	0	0.785	16	0	16	113
24	3	0	0	2	0	35	32	1	0.5	1.571	0.785	16	16	0	10 355
25	3	0	2	0	0	35	54	0.5	0.5	0.785	0.785	27	13.5	13.5	43 612
26	3	2	0	0	0	45	32	0.5	0.5	0.785	0.785	16	8	8	1061
27	3	0	0	0	2	35	32	0.5	1	0.785	1.571	32	0	0	10 102
28	3	0	0	0	−2	35	32	0.5	0	0.785	0	0	16	16	2498
29	3	−2	0	0	0	25	32	0.5	0.5	0.785	0.785	16	8	8	585
30	3	0	0	0	0	35	32	0.5	0.5	0.785	0.785	16	8	8	10 719

For CEF calculations $R_{opt}=1.5$, $a=3$ and $t_{max}=20$ min.

Table 4
Coefficients for the optimization of HPLC separation of corticosteroids

Factor	Model with run 25 removed	
	Coefficient	*P*-value
Constant	11 474	0.039
Temperature	−103	0.968
%Organic	−1651	0.598
$\sin^2\theta$	997	0.698
$\sin^2\phi$	−3268	0.223
Temperature^2	−7093	0.016
%Organic^2	33 735	0.000
$\sin^2\theta^2$	−5990	0.032
$\sin^2\phi^2$	−5723	0.039
Temperature×%organic	321	0.918
Temperature×$\sin^2\theta$	−5125	0.129
Temperature×$\sin^2\phi$	380	0.903
%Organic×$\sin^2\theta$	6441	0.066
%Organic×$\sin^2\phi$	12 195	0.004
$\sin^2\theta\times\sin^2\phi$	2014	0.525
Temp.×%org×$\sin^2\theta$	5050	0.134
Temp.×%org×$\sin^2\phi$	−228	0.942
Temp.×$\sin^2\theta\times\sin^2\phi$	5130	0.129
%Org.×$\sin^2\theta\times\sin^2\phi$	−4348	0.189
Temp.×$\sin^2\theta\times\sin^2\phi$×%org	−6396	0.339
Block 1	8238	0.197
Block 2	−5784	0.338
Rsq	96.2%	
Rsq adjusted	86.1%	

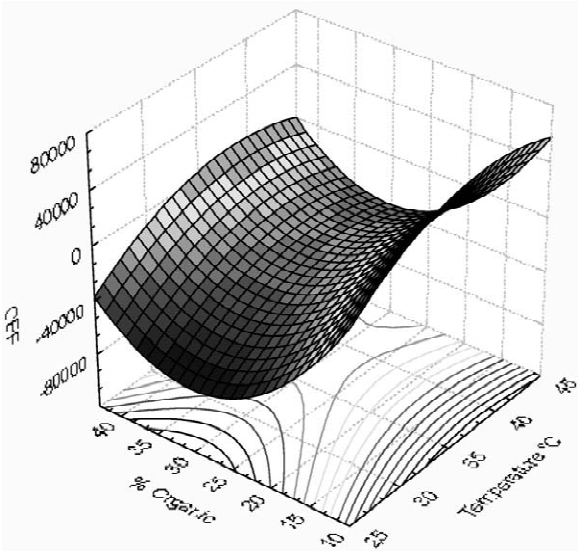


Fig. 6. Response surface for the two-way interaction model for the HPLC separation of seven corticosteroids, CEF vs. %organic and temperature. $\sin^2\theta$ is held at 0 and $\sin^2\phi$ is held at 1, corresponding to the organic component of the mobile phase consisting of only THF.

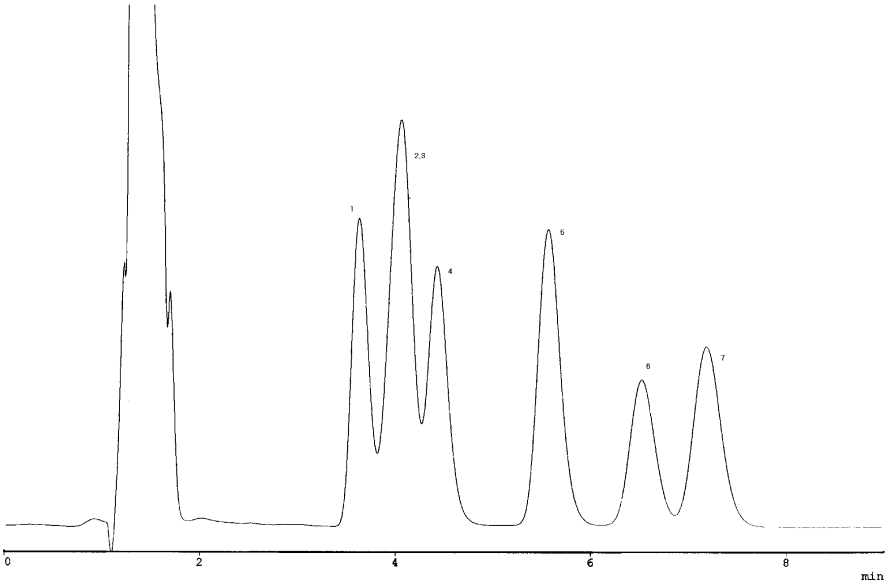


Fig. 5. Mixture of seven corticosteroids, temperature set at 25 °C and mobile phase composition 30.5% THF. CEF = 972.

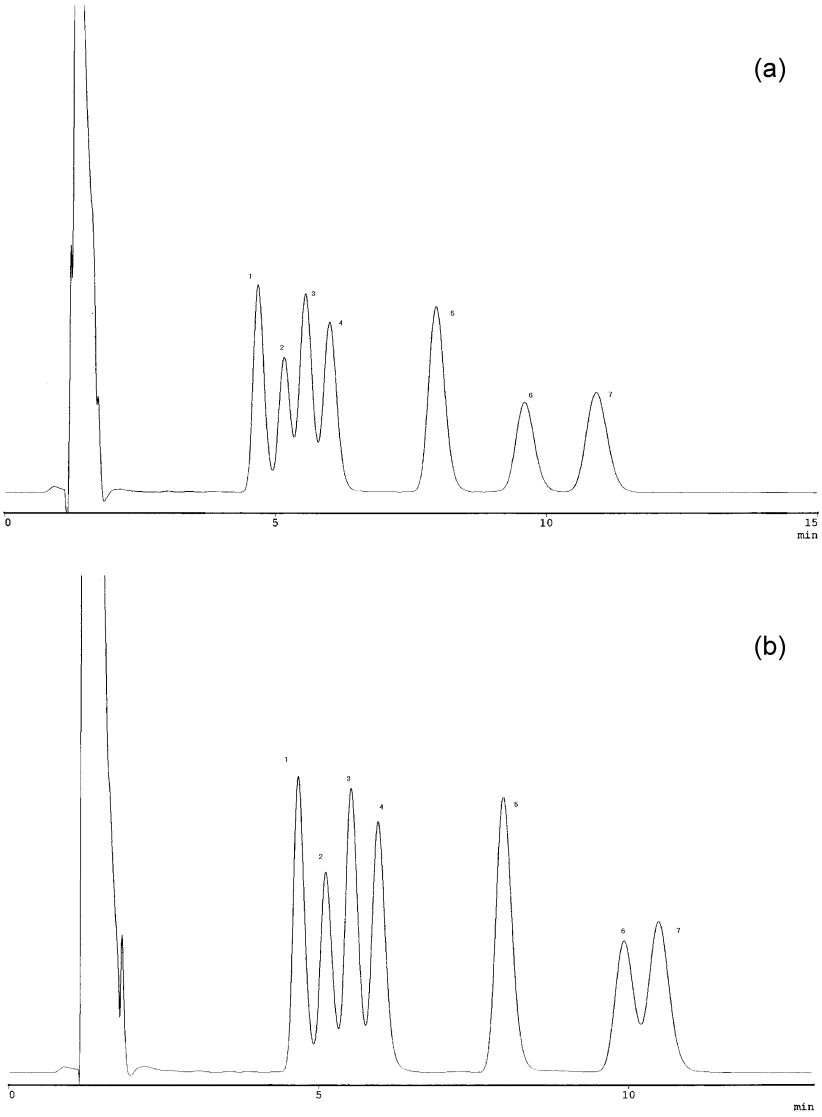


Fig. 7. Mixture of seven corticosteroids, temperature set at 25 °C and mobile phase composition 26% THF. CEF = 59. (b) Mixture of seven corticosteroids, temperature set at 45 °C and mobile phase composition 22.6% THF. CEF = 55.

highest level of organic in the mobile phase of 54%, improves the fit giving an adjusted R^2 value of 86.1%. This gives the coefficients shown in Table 4. A comparison of the center points replicated in both blocks indicates no significant differences between the blocks.

The predicted conditions for optimum separation for the two-way interaction model with run 25 removed were 25 °C and 30.5% THF. Fig. 5 shows the chromatogram at these conditions. For these conditions two components coeluted. A local optimum at 45° and 26% THF can be seen in the surface diagram of percent organic (100% THF) versus temperature for the two-way interaction model in Fig. 6. This set of conditions is on the opposite side of the saddle point to the surface's global optimum. The chromatogram run under these conditions still has compounds 2 and 3 unresolved

and the resolution between compounds 6 and 7 is lessened. Changing the temperature to 25 °C and using a mobile phase composition of 26% THF improves the separation significantly (Fig. 7a) highlighting the sensitivity of the separation to temperature. This set of conditions is just slightly off the optimum as shown in Fig. 6. These results indicate the model is weak at the edges of the domain where not many experimental points have been taken, making it difficult to locate the optimum.

The experimental data were also fitted to a model including three- and four-way interaction terms. The three- and four-way interaction model with run 25 removed indicated the conditions for an optimum separation to be 19.4% ACN and 45 °C. Separation takes too long, the last two components have not eluted after 70 min and are therefore outside the acceptable k range of 1–20.

However, target analysis selected a local optimum at 22.6% THF and 45 °C. The chromatogram obtained at these conditions is shown in Fig. 7b. This chromatogram has the best overall resolution of components in an acceptable time.

5. Conclusions

Spherical coordinates have been used to represent the mobile phase composition. This solves the problem of applying experimental designs such as central composite designs to the simultaneous optimization of ternary or quaternary mobile phase composition with other factors such as pH, temperature and gradient in HPLC. The use of spherical coordinates allows the total mobile phase composition to be restricted to 100% while the composition of the solvents in the mobile phase can be freely varied, including total organic composition, at the same time without violating this restriction.

For the quaternary mobile phase optimization using spherical coordinates, the edges of the model for two of the three binary conditions are inaccurate in estimating the actual responses. This is most likely because no experimental results were taken at the points where $\sin^2\phi = 0$ and $\sin^2\theta = 1$ and where $\sin^2\phi = 0$ and $\sin^2\theta = 0$. These points correspond to the binary conditions of acetonitrile/water and methanol/water, respectively.

It has been found that three-way interactions need to be considered in the simultaneous optimization of mobile-phase composition and other HPLC factors such as temperature, pH and gradient. In the models obtained for the gradient HPLC optimization of the phenol separation and the isocratic HPLC separation of the corticosteroids, some three-way interactions were found to be significant. For the phenols separation the three-way interaction between temperature and the initial and final organic composition was significant (P-value = 0.000). For the corticosteroid separation the three-way interaction between temperature and $\sin^2\phi$ and $\sin^2\theta$ was found to be significant (P-value = 0.016) as well as the three-way interaction between percent organic, $\sin^2\phi$ and $\sin^2$. The significance of two of these three-way interactions seems to be inferring that there is a dependent relationship between the temperature of the column and the type of organic composition on the separation of both mixtures of compounds. This dependent relationship between temperature and organic type is quite logical in that an increase in methanol and a decrease in acetonitrile content of the organic composition will increase the elution time due to the differences in viscosity of the two solvents. The relative viscosity of the three organic solvents in water is MeOH > THF > ACN. Increasing the temperature will compensate for the increase in methanol content by decreasing the viscosity of the methanol and thereby increasing the diffusion coefficient. It is quite reasonable to expect that in future optimizations of other mixture separations via HPLC, that other three-way interactions such as gradient and initial and final organic composition will be significant.

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