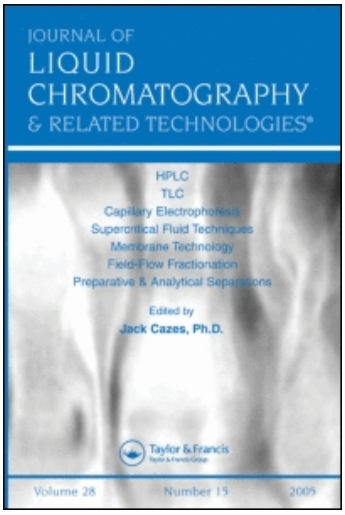



## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# PRACTICAL OPTIMIZATION OF SOLVENT SELECTIVITY USING GRADIENT ELUTION FOR RAPID SELECTION AND MIXTURE-DESIGN STATISTICAL TECHNIQUE FOR THE SEPARATION OF FAT-SOLUBLE VITAMINS

S. Wieliński[a]; A. Olszanowski[b]
[a] Glaxo Wellcome Poznań S.A, Poznań, Poland [b] Institute of Chemical Technology and Engineering, Poznań University of Technology, Poznań, Poland

Online publication date: 17 November 1999

J. LIQ. CHROM. & REL. TECHNOL., 22(20), 3115–3128 (1999)

# PRACTICAL OPTIMIZATION OF SOLVENT SELECTIVITY USING GRADIENT ELUTION FOR RAPID SELECTION AND MIXTURE-DESIGN STATISTICAL TECHNIQUE FOR THE SEPARATION OF FAT-SOLUBLE VITAMINS

S. Wieliński,[1] A. Olszanowski[2,]*

[1] GlaxoWellcome Poznań S.A,
ul. Grunwaldzka 18
60-322 Poznań, Poland

[2] Institute of Chemical Technology and Engineering
Poznań University of Technology
Pl. M. Skłodowskiej-Curie 2
60-965 Poznań, Poland



## ABSTRACT

It is shown that an initial scan of a mixture with a gradient elution programme offers a rapid and easy procedure for predicting the isocratic conditions. The experimental design methodology facilitates optimisation of the chromatographic separation of vitamin molecules dependent on the determination of mathematical equations. The specialised cubic equations have been elaborated. Examples are shown of chromatographic separations obtained using mobile phase combinations which were the best compromise of the two selected criteria.

## INTRODUCTION

Vitamins are substances essential for normal growth, development. and health. Their determination is of interest in biochemistry, pharmaceuticals, and the food sciences. High performance Liquid Chromatography (HPLC) has been applied successfully to the separation of fat-soluble vitamins in pure standard solution,[1] pharmaceutical formulations,[2] human serum,[3, 4] and food samples[5] by either gradient elution or isocratic separation techniques.

This paper describes practical optimisation using a gradient elution for rapid selection and mixture-design statistical technique for the separation of fat-soluble vitamins, such as retinol (acetate, palmitate), cholecalciferol, alphacalcidol, tocopherol (acetate), and phylloquinone, in capsules from a single sample extract, using HPLC reverse phase and column backflushing technique. A well known optimisation approach using multi-criterion decision-making (MCDM) was given by Nsengiyumva et al.[6, 7] They proposed using the Pareto-optimality concept. The Pareto-optimality concept has been discussed elsewhere.[8, 9]

Gradient elution can be used as a very powerful technique for unknown mixture samples. Gradient separation provides an immediate picture of the sample nature and from that separation, the necessary solvent strength for an isocratic separation can be predicted.[10]

One of the more widely used methods to optimise the mobile phase composition is ORM (overlapping resolution mapping). Glajch et al.'s procedure calculates retention time and resolution for each of seven chromatograms described by the simplex design and then fits the best second-order polynomial equation to four variables; that is, the tree solvent compositions and the dependent retention time or resolution.[11, 12]

This method permits the optimisation of a separation without requiring that the peaks be identified. It is also well suited to an automated system. With this method only seven experiments need to be carried out to locate the optimum conditions according to a mixture-design triangle.

Measured retention times and peak width at half height were fitted to a special cubic polynomial model with the vertices of the triangular solvent mixture as variables. The programme models the response surface for each pair of peaks related to the percentage of organic modifier.

Although software implementing overlapping resolution mapping has been reported in the literature,[13] a programme was designed by us for application to this approach. This software also presents the possibility of validation by making five more experiments.

## EXPERIMENTAL

### Chemical and Reagents

Vitamin K1 (phylloquinone) and A (all-trans-retinol acetate, all-trans-retinol palmitate) were obtained from Promochem (USP, BP-standard). Vitamin $D_3$ (cholecalciferol) and vitamin E (dl-α-tocoferol acetate) were purchased from Sigma. Alphacalcidol was purchased from Infarm, Poland. All other chemicals, such as n-hexane, acetonitrile, methanol, and tetrahydrofuran were commercially available (Merck, Baker). Water was obtained from a Maxima purification system (conductivity below 0,4μS/cm).

### Instrumentation and Equipment

The liquid chromatograph used was obtained from Hewlett Packard. The apparatus consisted of a pump module (HP1100), a photodiode-array detector (HP1100), an autosampler (HP1100), a degasser, and chromatography data station Chemstation rev. 5.01.



### Standard Preparation

Accurately weighed samples, estimated to contain 20 mg retinol palmitate (A palmitate) and 10 mg retinol acetate (A acetate), 7.5 mg cholecalciferol (D3) and alphacalcidol (Alfc), 60 mg phylloquinone (K1), and 30 mg tocopherol acetate (E acetate) were dissolved in 10 mL n-hexane and diluted to 100 mL with methanol. The standard was sonicated for 15 minutes at 30°C. Air should be avoided and excluded by working in a pure nitrogen-atmosphere, to avoid oxidative degradation.

### Chromatographic Condition

HPLC separation was performed on an 25 cm x 4.6 mm, i.d., stainless steel column packed with octyl stationary phase (5μm spherical particles, porosity 120A). The column was thermostatted at 30°C during all experiments. The flow rate of the mobile phase was 2.0 mL/min. Solvent was vacuum-degassed individually from reservoirs with an in-line degasser. Sample injections (20 μL) were made using a loop injection valve and autosampler. All data were obtained as Chromascan from which spectra and chromatograms could be taken using a Chemstation Data Station. Detection was performed at 280 nm with a photodiode array detector. The hold-up time ($t_0$) was estimated to be 1.33 min, by using replicate injections of $10^{-4}$M KI. Peak recognition was performed by injection of each individual solute.

**Software**

Generation of design points, and model building were supported by the Statistica, version for Windows (StatSoft, Inc.). Other calculations and graphical presentation were implemented with Excel software (Office 95 version).

## RESULTS AND DISCUSSION

An approach, used to predict isocratic separations from gradient elution may be found in a text by Schoenmakers.[14] The solvent strength parameter, S, is strongly correlated with $k_0$:

$$S = p + q \ln k_0 \quad (1)$$

where: $k_0$ is an imaginary (extrapolated) capacity factor in pure water; value expected for the solute using isocratic elution p and q are constant for a given binary mobile phase system. If p and q are known, then retention in an isocratic system requires only one parameter to be known (S or $\ln k_0$).

For a linear gradient, the retention behaviour of solute in a reversed-phase system can be expressed by the following linear relationship:

$$\ln k = \ln k_0 - S\,\varphi \quad (2)$$

where: k is the isocratic capacity factor as a function of composition; φ is the volume-fraction of the organic in the mobile phase. A mixture of vitamins was prepared in 10 mL n-hexane and diluted to 100 mL with methanol. To this mixture a linear gradient of 20 to 100% methanol – water was applied over a period of 18 min. The resulting chromatogram is shown in Figure 1. Hence, according to the procedure, proposed by Snyder, Glajch, and Kirkland,[15] binary mobile phase 99.5:0.5 MeOH: $H_2O$ was selected.

The next step in the procedure was to define the other corners of the triangle by calculating solvent compositions of the other two modifiers (acetonitrile and tetrahydrofuran) with water, which should have the same total solvent strength. With eqns. 3 and 4, as described by Snyder and Kirkland,[16] we were able to transfer from one organic modifier to another in order to change the selectivity, while keeping the retention approximately constant:

$$\varphi_{ACN} = 0.32\,\varphi^2_{MeOH} + 0.57\varphi_{MeOH} \quad (3)$$

$$\varphi_{THF} = 0.66\,\varphi_{MeOH} \quad (4)$$

where φ is the volume fraction of solvent.

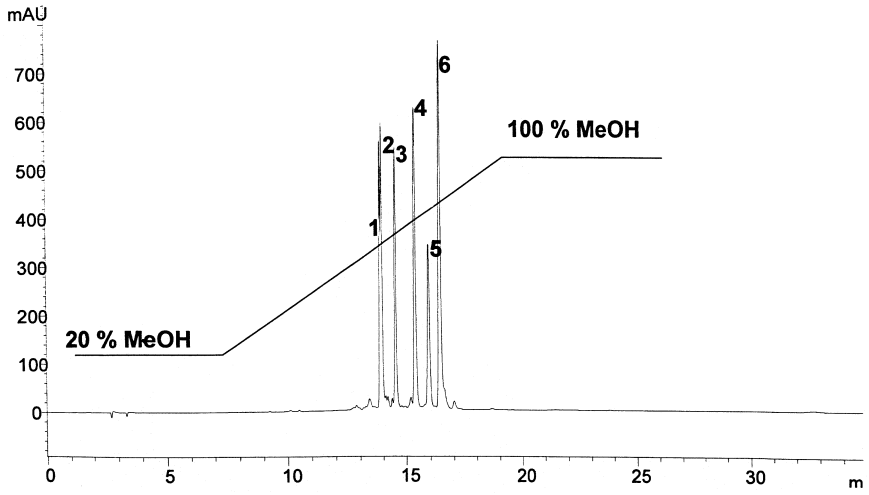



**Figure 1**. The chromatogram of mixture of vitamins when a linear gradient of 20 – 100% methanol-water was applied over a period of 18 min.; 250mm x 4.6mm I.D. column at 2 mL/min; UV detection (285 nm).



The three vertices of the Snyder selectivity triangle were identified as 99.5:0.5 MeOH:$H_2O$, 88:22 ACN:$H_2O$, and 66:34 THF:$H_2O$. After experimental adjustment we decided to change 88:22 ACN:$H_2O$ to 100% ACN (long retention time for K1 and A palmitate).

A mixture-design statistical technique was used to minimize the number of separate experiments needed to find the optimum solvent mixture. This concept was introduced in reversed-phase by Glajch et al.[17] This method permits the optimisation of a separation without peak identification and this is well suited to an automated system. By this method only seven experiments need to be carried out in order to locate the optimum conditions according to the mixture-design triangle.

Measured retention times and peak width at half height were fitted to a special cubic polynomial model with the vertices of the triangular solvent mixture as variables. The programme models the response surface for each pair of peaks related to the percentage of organic modifier. The response surface is calculated by adjusting the experimental values by non-linear multivariate regression to the equation:

$$Y = a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{123}X_1X_2X_3 \qquad (5)$$

**Table 1**

**Measured Retention Times (min) for the Fat-Soluble Vitamins**

| Run | φMeOH 99.5% | φACN 100% | φTHF 66% | A Acetate 1 | D3 2 | Alpha-Calcidol 3 | E Acetate 4 | K1 5 | A Palmitate 6 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 0 | 0 | 1.584 | 1.652 | 1.844 | 2.156 | 2.408 | 2.551 |
| 2 | 0 | 1 | 0 | 1.812 | 2.528 | 3.107 | 3.2 | 3.427 | 4.424 |
| 3 | 0 | 0 | 1 | 2.007 | 2.391 | 2.546 | 3.969 | 4.048 | 6.47 |
| 4 | 0.5 | 0.5 | 0 | 1.645 | 1.772 | 2.144 | 2.425 | 2.649 | 3.088 |
| 5 | 0.5 | 0 | 0.5 | 2.321 | 2.435 | 3.138 | 5.099 | 5.701 | 9.962 |
| 6 | 0 | 0.5 | 0.5 | 2.111 | 2.16 | 2.891 | 4.36 | 4.611 | 7.514 |
| 7 | 0.333 | 0.333 | 0.334 | 2.072 | 2.1 | 2.766 | 3.945 | 4.32 | 6.572 |
| 8 | 0.666 | 0.167 | 0.167 | 1.845 | 1.89 | 2.29 | 2.919 | 3.275 | 4.174 |
| 9 | 0.167 | 0.666 | 0.167 | 1.866 | 2.013 | 2.624 | 3.378 | 3.661 | 5.06 |
| 10 | 0.167 | 0.167 | 0.666 | 2.25 | 2.441 | 3.069 | 5.024 | 5.349 | 9.614 |
| 11 | 0.5 | 0.5 | 0 | 1.622 | 1.732 | 2.081 | 2.295 | 2.518 | 2.696 |
| 12 | 0.5 | 0 | 0.5 | 2.324 | 2.494 | 3.157 | 5.105 | 5.696 | 10.002 |
| 13 | 0 | 0.5 | 0.5 | 2.118 | 2.199 | 2.909 | 4.414 | 4.718 | 7.848 |
| 14 | 0.333 | 0.333 | 0.334 | 2.052 | 2.115 | 2.728 | 3.893 | 4.277 | 6.545 |
| 15 | 0.05 | 0.95 | 0 | 1.779 | 2.390 | 2.942 | 3.066 | 3.296 | 4.153 |



Where Y is the response to be modelled (retention time, resolution), $X_{1..3}$ are the volume fractions for each organic modifier, and $a_{1..3}$ are the coefficients of the model. Resolution $R_s$ between two peaks was calculated by use of the simple expression 6:

$$R_s = 1.18 (Rt_2 - Rt_1) / (Wh_1 + Wh_2) \quad (6)$$

where: $Rt_1$ is retention time of the first eluting peak, $Rt_2$ is the retention time of the next peak, $Wh_1$ and $Wh_2$ are the peak width at half height, respectively.

For our model, 10 experiments were undertaken. Experiments 11 to 15 were used to check the lack of fit between model and experimental results. (See Table 1).

The average relative deviation (ARD %) and the sum of squares of the absolute deviation (SSQ) between observed and calculated data, provides information on the accuracy of the model. They are summarised in Table 2. For retention, model building gave ARD values between 0.2% and 0.55%.

The squared multiple correlation coefficient ( $R^2$ ) can be used to measure the goodness of fit of a regression equation. Because the number of estimated parameters is less than the total number of design points, adjusted $R_a^2$ is preferred for measurement of the goodness of fit the model. The values of these two statistics are displayed in Table 3.

**Table 2**

**Average Relative Deviation and the Sum of Squares of the Absolute Deviations Between Observed and Calculated Data***

| | A Acetate 1 | D3 2 | Alpha-Calcidol 3 | E Acetate 4 | K1 5 | A Palmitate 6 |
|---|---|---|---|---|---|---|
| ARD% | 0.55 | 0.28 | 0.2 | 0.3 | 0.4 | 0.42 |
| SSQ | 0.018 | 0.009 | 0.012 | 0.016 | 0.017 | 0.019 |

* Retention time.

**Table 3**

**Evaluation of Special Cubic Models for Retention Times of Six Fat-Soluble Vitamins**

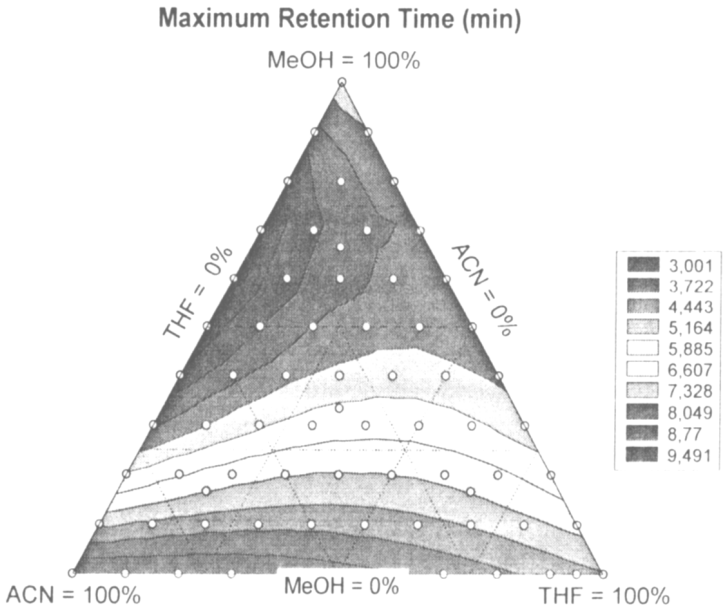
| | A Acetate 1 | D3 2 | Alpha-Calcidol 3 | E Acetate 4 | K1 5 | A Palmitate 6 |
|---|---|---|---|---|---|---|
| $R^2$ | 0.9951 | 0.9901 | 0.9997 | 0.9962 | 0.9956 | 0.9982 |
| Adjusted $R_a^2$ | 0.9962 | 0.9932 | 0.9998 | 0.9973 | 0.9963 | 0.9989 |

The retention times of the six fat-soluble vitamins resulted in an average $R^2$ (%) value of 99.58% for a special cubic model. The average Adjusted $R^2$ (%) values calculated were 99.7%. It was concluded that the best fit for retention data were obtained for the average Adjusted $R^2$.

The model coefficients for retention data are summarised in Table 4. Good agreement was obtained between observed and predicted retention times.

It was decided to optimise the chromatographic separation according to two main objective criteria: retention separation as a measure of the quality of separation and the analysis time as a measure of the cost of analysis. The requirements of separations were as follows:

$$Rt_{max} = 5\text{min};\ R_{smin} = 1.2 \quad (7)$$



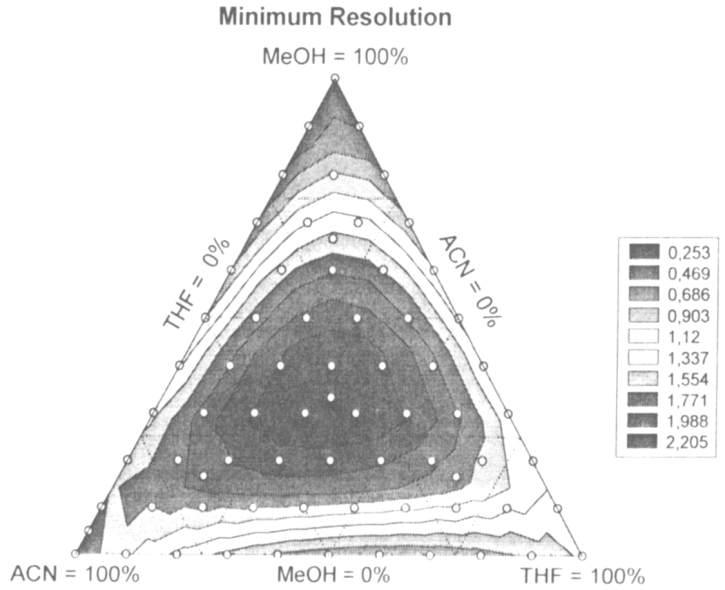




**Figure 2**. Mixture contour of minimum resolution and maximum retention time (min).

**Table 4**

**Special Cubic Model Coefficients and Standard Errors for Retention Time***

| Variable | A Acetate 1 | D3 2 | Alpha-Calcidol 3 | E Acetate 4 | K1 5 | A Palmitate 6 |
|---|---|---|---|---|---|---|
| 1 | **1.571** | **1.642** | **1.813** | **2.043** | **2.287** | **2.209** |
| $X_1$ | 0.013 | 0.011 | 0.031 | 0.113 | 0.121 | 0.342 |
| 2 | **1.803** | **2.498** | **3.075** | **3.184** | **3.417** | **4.375** |
| $X_2$ | 0.009 | 0.03 | 0.032 | 0.016 | 0.01 | 0.049 |
| 3 | **2.023** | **2.492** | **2.597** | **4.089** | **4.165** | **6.852** |
| $X_3$ | 0.016 | 0.485 | 0.05 | 0.12 | 0.117 | 0.382 |
| 4 | **-0.258** | **-1.353** | **-1.451** | **-1.273** | **-1.337** | **-2.384** |
| $X_1X_2$ | 0.46 | 0.081 | 0.125 | 0.261 | 0.263 | 0.768 |
| 5 | **2.108** | **1.711** | **3.809** | **8.159** | **9.883** | **21.886** |
| $X_1X_3$ | 0.006 | 0.057 | 0.039 | 0.013 | 0.009 | 0.08 |
| 6 | **0.82** | **-1.182** | **0.293** | **3.11** | **3.71** | **8.939** |
| $X_2X_3$ | 0.014 | 0.016 | 0.037 | 0.008 | 0.216 | 0.671 |
| 7 | **-1.175** | **-0.126** | **-1.664** | **-8.772** | **-10.17** | **-29.694** |
| $X_1X_2X_3$ | 0.404 | 0.135 | 0.764 | 0.654 | 0.927 | 0.705 |

* R. T., min.

Figure 2 shows the response surface of the minimum effective resolution and retention time obtained by means of special cubic model coefficients (10% increments of solvent volume fractions between each point). In this calculation, 66 solvent combinations were involved in the predictions.

Hence, the minimum effective resolution (resolution of the least well resolved pair of peaks) and the maximum retention time (for the last eluting peak) were selected for each of the 66 mobile phase compositions. A very large region along the acetonitrile volume fraction axis displayed conditions giving quality separation with minimum resolution, $1.3 \leq R_{smin} \leq 1.7$. There is a large zone in the centre, where the conditions lead to well resolved peaks with $1.7 \leq R_{smin} \leq 2.2$. We had to know how much analysis time is required in each selected zone. Examination of the contour plot of maximum retention time shows a zone with a short analysis time between $3\text{min} \leq Rt_{max} \leq 5.1$ min along the acetonitrile 90% volume fraction axis.

Different optimisation goals may conflict, which means that better resolution often requires an increase in analysis time. We needed to find a desirable or compromising solution to such multi-criteria problems.

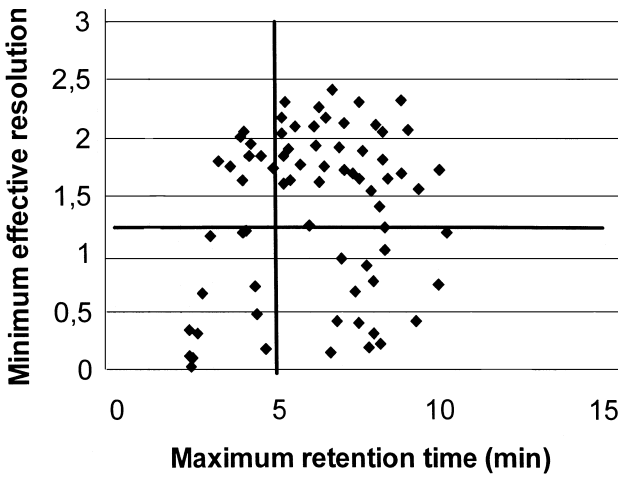



**Figure 3**. Plot of the feasible criteria space Pareto-optimality.



Simultaneous optimisation of both types of criteria (resolution and retention time) is achieved by using Pareto strategies. An experiment is called Pareto-optimal if there is no other experiment that has a better result on one criterion without having a worse result on another. The main advantages of this strategy are that no preliminary information is required and the compromise between the different criteria can be visualised. The Pareto-optimal plot is depicted in Figure 3.

The points (quadrant left-above) are called non-inferior solution or Pareto-optimal points. All other points in the feasible criteria space are inferior to these points (or solution). A points in the feasible criteria space are Pareto-optimal points if no other point exists in that space which yields an improvement in one criterion without causing a degradation in the other criterion.

Simplex coordinates of some Pareto-optimal points are displayed in Table 5. A compromise between time and separation quality is achieved.

The best condition is found at 95 % ACN, 5 % MeOH. Resolution and selectivity values between every pair of peaks are listed in Table 6 and the resultant chromatogram under these conditions is given in Figure 4.

When the chromatograms were recorded 3 months later under the same conditions, i.e. mobile phase, column, flow rate, the retention times showed a very good match (RSD 0.3%) with the previous results, thus confirming the stability of the chromatographic system.

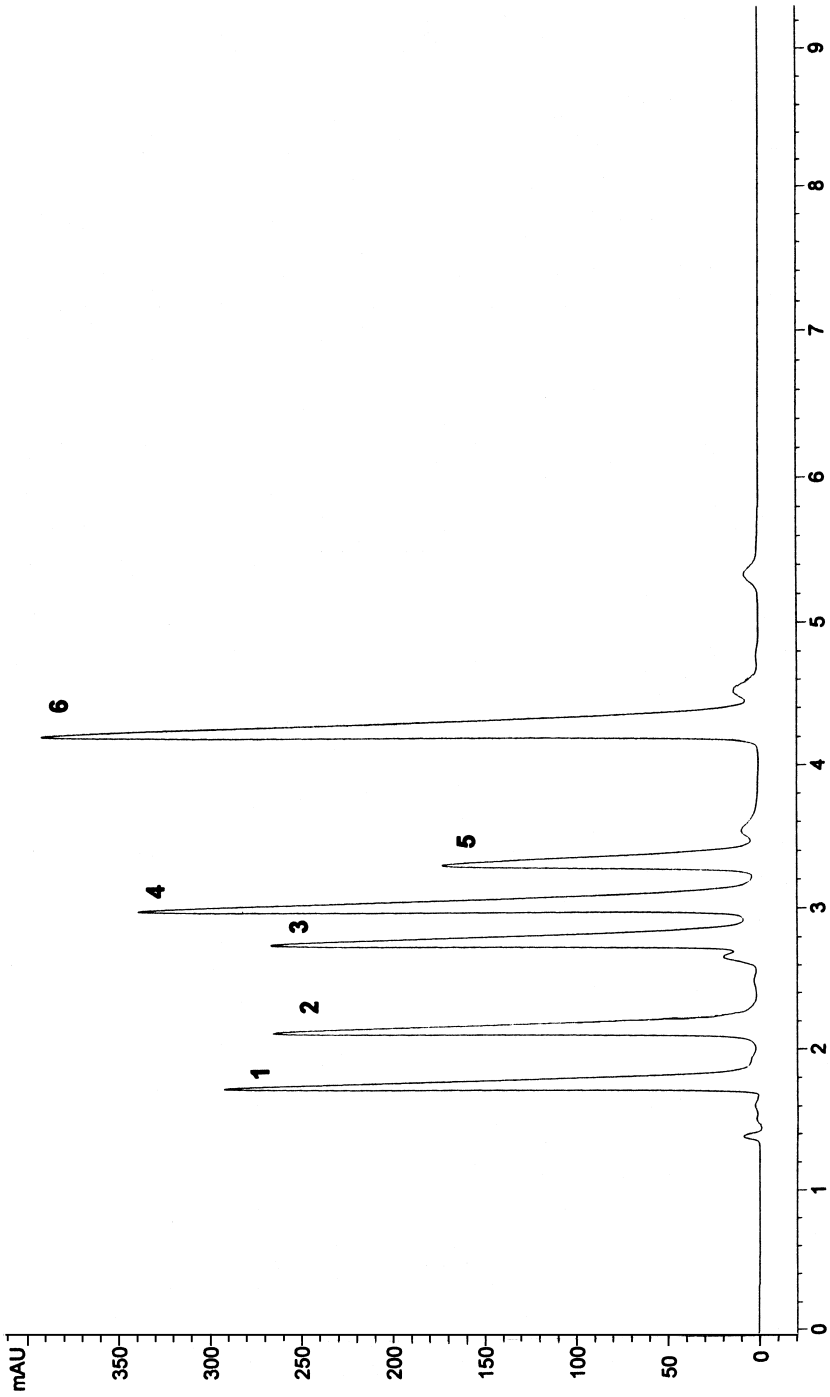



**Figure 4**. Optimized separation of the fat-soluble vitamins (1 - retinol acetate, 2 - cholecalciferol, 3 - alphacalcidol, 4 - alpha-tocopherol, 5-phylloquinone, 6 – retinol palmitate) with detection at 285nm.

**Table 5**

**Pareto-Optimal Points. Minimum Effective Resolution, $R_{smin}$ 1.2, Maximum Retention Time, $Rt_{max}$ 5 Min.**

| Maximum Retention Time $Rt_{max}$ | Minimum Effective Resolution $R_{smin}$ | ϕMeOH/ϕACN/ϕTHF |
|---|---|---|
| 3.94 | 1.63 | 0.6/0.3/0.1 |
| 3.56 | 1.75 | 0.9/0.8/0.0 |
| 3.22 | 1.79 | 0.0/0.1/0.9 |
| **4.29** | **1.84** | **0.5/0.95/0.0** |
| 4.50 | 1.85 | 0.2/0.7/0.1 |
| 3.92 | 2.01 | 0.5/0.4/0.1 |
| 4.00 | 2.05 | 0.4/0.5/0.1 |
| 4.91 | 1.73 | 0.1/0.8/0.1 |



**Table 6**

**Resolution and Selectivity Values Between Every Pair of Peaks for Optimum Separation**

| Peak Pair: | 1-2 | 2-3 | 3-4 | 4-5 | 5-6 |
|---|---|---|---|---|---|
| Resolution | 3.25 | 4.95 | 1.84 | 2.09 | 5.96 |
| Selectivity $\alpha=k_i/k_{i+1}$ | 1.84 | 1.74 | 1.17 | 1.17 | 1.47 |

## CONCLUSION

The results obtained in this study have successfully demonstrated the application of a systematic approach to the optimisation of mobile phase composition for HPLC. It was shown how gradient elution may be used as a scanning technique for quickly establishing proper isocratic conditions. The use of solvent-strength optimisation is facilitated by use of initial gradient elution experiments at the initial development of the method. The simplex centroid design applied enabled fitting of retention data to a special cubic model. The mixture design technique in combination with the systematic approach of optimisation were shown to be a convenient and powerful tool. Further work is underway to extend this systematic approach to optimize the effect of temperature on the separation process.

**ACKNOWLEDGMENT**

This work was supported by grant No. BW 32/287/98.

**REFERENCES**

1. W. O. Landen Jr., Chromatogr., **211**, 155 (1981).

2. R. van Haelen-Fastre, M. van Haelen, J. Chromatogr., **179**, 131 (1979).

3. W. J. Driskell, J. W. Neese, C. C. Bryant, M. M. Bashor, J. Chromatogr., **231**, 439 (1982).

4. C. A. Collins, C. K. Chow, J. Chromatogr., **317**, 349 (1984).

5. P. J. van Niekerk, S. C. C. Smit, J. A. O. C. S., **57**, 12, 417 (1980).

6. C. Nsengiyumva, J. O. De Beer, Chromatographia, **47**, 401 (1998).

7. C. Nsengiyumva, J. O. De Beer, Chromatographia, **44**, 634 (1997).

8. A. K. Smilde, A. Knevelman, P. M. J. Coenegracht, J. Chromatogr., **369**, 1 (1986).

9. P. F. Vanbel, B. L. Tilquin, P. J. Schoenmakers, J. Chromatogr., A, **697**, 3 (1995).

10. P. J. Schoenmakers, H. A. H. Billiet, J. Chromatogr., **205**, 13 (1981).

11. J. L. Glajch, J. J. Kirkland, L. R. Snyder, Chromatographia, **14**, 644 (1982).

12. J. L. Glajch, J. J. Kirkland, J. Chromatogr. Sci, **25**, 4 (1987).

13. J. C. Berridge, **Techniques for The Automated Optimization of HPLC Separations**, John Wiley & Sons, Inc., New York 1985.

14. P. J. Schoenmakers, **Optimization of Chromatographic Selectivity**, Elsevier, New York 1986.

15. L. R. Snyder, J. L. Glajch, J. J. Kirkland, **Practical HPLC Method Development,** John Wiley & Sons, Inc., New York 1988.

16. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York 1979.

17. J. L. Glajch, J. J. Kirkland, K. M. Squire, J. Chromatogr., **199**, 57 (1980).

Received January 1, 1999
Accepted February 8, 1999
Manuscript 4979

# Request Permission or Order Reprints Instantly!