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Chiral separations in supercritical fluid chromatography: a multivariate optimization method*

Patrik Petersson, Niklas Lundell and Karin E Markides

Department of Analytical Chemistry, Uppsala University, P O Box 531, S-751 21 Uppsala (Sweden)

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ABSTRACT

A multivariate optimization method, based on the method of steepest ascent, is proposed for the separation of two enantiomers on a chiral stationary phase in open-tubular column supercritical fluid chromatography The objective of the method is to optimize start density, density gradient and temperature in order to locate a desired resolution in a first step and, if desired, minimize the retention time in a second step without decrease in resolution

INTRODUCTION

Open-tubular column supercritical fluid chromatography (SFC) is a promising technique for chiral separations [1,2] It possesses important advantages over gas chromatography (GC) and liquid chromatography (LC) such as combined low-temperature elution and access to a wide range of detection methods, including "universal" and sensitive flame ionization detection (FID) Low temperature is important when working with chiral separations as it will increase the chiral selectivity [3] of the chromatographic system and reduce the probability of racemization or thermal decomposition of the enantiomers

There are several factors that simplify the optimization of a chiral separation in comparison with the separation of more complex mixtures In most instances, the purpose of the separation is to determine the ratio of the amounts of the two enantiomers of a chiral compound and therefore only the resolution and retention times of the two enantiomers have to be monitored This eliminates the need for the definition of more or less imaginative quality measures, often refered to as response functions [4,5] In addition, there have been no reports, to our knowledge, of a reversal of elution order of enantiomers in SFC due to different elution temperature or density Therefore, there should be no need for peak tracking [6] during the optimization The number of variables that can be used to optimize a chiral separation using SFC–FID with a certain column, restrictor and mobile phase are limited to density/pressure, temprature and gradients of these, as organic modifiers (possibly with the exception for formic acid) are not suitable in combination with FID In the method described in this paper, the start density, density gradient and temperature are the variables chosen for optimization, a compromise between the number of variables/experiments and possibilities available to achieve the desired separation

Multivariate optimization of separations in SFC is a relatively new area where, until now, only two papers have been published, neither of which treated chiral separations Crow and Foley [7] described the use of a modified simplex algorithm whereas Ong *et*

Correspondence to Dr K E Markides, Department of Analytical Chemistry, Uppsala University, P O Box 531, S-751 21 Uppsala, Sweden

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al [8] chose to use overlapping resolution mapping In the former study it was concluded that the parameters that control the separation are highly synergistic and therefore it would not be appropriate to use an univariate optimization strategy

For the separation of two enantiomers by open-tubular column SFC–FID, a multivariate optimization method consisting of two steps is suggested the first step locates and verifies the conditions of the desired resolution after approximately six experiments and the second step provides the possibility of continuing with a minimization of the retention time without a decrease in resolution In this step sequential experiments are carried out until little progress is made A minimization of retention time should result in an increase in peak height and therefore a more favourable signal-to-noise ratio for the determination of optical purity This optimization method has been designed in a way that does not require any advanced software or skills in programming

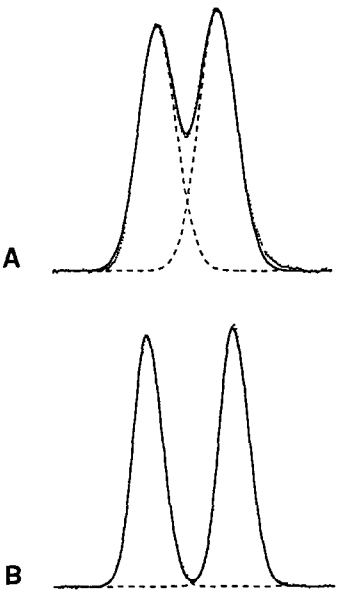

Fig 1 Overlapping (CR = −0 316) and baseline-resolved (CR = 0 274) peaks of the enantiomers of diethyl tartrate illustrating the advantage of fitting Gaussian functions for the estimation of peak widths and retention times Conditions (A) CO_2, 78°C, start density 0 325 g ml^{-1}, density gradient 0 037 g ml^{-1} min^{-1}, (B) CO_2, 91°C, start density 0 175 g ml^{-1}, density gradient 0 054 g ml^{-1} min^{-1}

THEORY

Measure of resolution

The chiral resolution (CR) was calculated as a measure of the separation of two enantiomers This measure was defined by Aichholz *et al* [9] as a chiral analogy to the trennzahl (TZ) or separation number (SN)

$$CR = \frac{(t_R)_2 - (t_R)_1}{(w_h)_2 + (w_h)_1} - 1 \qquad (1)$$

where t_R is retention time and w_h is peak width at half-height

Determination of resolution

Chiral resolution was calculated from retention times and peak widths obtained by the fitting of two Gaussian functions to the recorded data As long as the peaks have a shape that is approximately Gaussian, as in most instances in open-tubular column SFC, these variables can easily be estimated even for overlapping peaks (Fig 1) The peak fitting of overlapping peaks is simplified by the assumption of equal widths for both peaks

Steepest ascent

Box and Wilson first described the method of steepest ascent in 1951 [10] In this method, a first-order polynomial is fitted from initial experiments in a subregion of the experimental domain The response is maximized by sequential experiments in the direction that results in the largest gain in response This is done by moving along the vector described by the coefficients of the polynomial Subsequent experiments along the vector are continued until the curvature is considerable or little progress is made If the former applies the optimization is ended or repeated around the experiment which resulted in the largest response

Step one location of the desired resolution

Five experiments are performed in a half-fractional factorial design [11] shaped as a cube with experiments located in the centre and in four of the corners (Fig 2A) (the experimental domain is chosen from the result of previous "scouting", always necessary to judge if the stationary phase possess chiral selectivity for the analyte in question) From these five experiments a first-order polynomi-

al (eqn 2) is fitted as a model of chiral resolution The fitting is performed by the general least-squares matrix solution for linear models [11] (eqn 3) If the variables (x_1–x_3) are coded in three levels, −1, 0 and +1, as illustrated in Table I, the fitting is simplified, and only the matrix of the response has to be changed in order to fit different models

$$CR = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 \quad (2)$$

$$\mathbf{A} = \begin{bmatrix} x_0 \\ \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{bmatrix} \quad \mathbf{X} = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 1 & -1 & 1 & 1 \\ 1 & 1 & -1 & 1 \\ 1 & 1 & 1 & -1 \\ 1 & -1 & -1 & -1 \end{bmatrix} \quad \mathbf{CR} = \begin{bmatrix} CR_1 \\ CR_2 \\ CR_3 \\ CR_4 \\ CR_5 \end{bmatrix}$$

$$\mathbf{A} = (\mathbf{X}'\mathbf{X})^{-1}\mathbf{X}'\,\mathbf{CR} \quad (3)$$

The empirical coefficients of the polynomial (α_1–α_3) provide the direction which results in the largest gain in resolution, the vector of steepest ascent (Fig 2B) The point on this vector where the desired resolution (CR^*) is obtained is given by eqns 4–6 in coded values The scale factor (Δ), which is related to the distance from the centre of the design, is calculated according to eqn 7 If there is an unacceptable deviation between model and reality, the scale factor is adjusted towards higher/lower resolution

$$x_i = \alpha_i \Delta \quad (4)$$

$$x_j = \alpha_j \Delta \quad (5)$$

$$x_k = \alpha_k \Delta \quad (6)$$

$$\Delta = \frac{CR^* - \alpha_0}{\alpha_i^2 + \alpha_j^2 + \alpha_k^2} \quad (7)$$

Step two minimization of retention time

If the retention time is critical for the analysis, it is possible to minimize it without reducing the resolution This is done by a modified method of steepest ascent as described below

From five experiments, a half-fractional factorial design around the experiment which gave the desired resolution (Fig 2C), first-order polynomials are fitted as models for the chiral resolution and the retention time of the last-eluting enantiomer (eqns 8 and 9)

$$CR = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 \quad (8)$$

$$(t_R)_2 = \gamma_0 + \gamma_1 x_1 + \gamma_2 x_2 + \gamma_3 x_3 \quad (9)$$

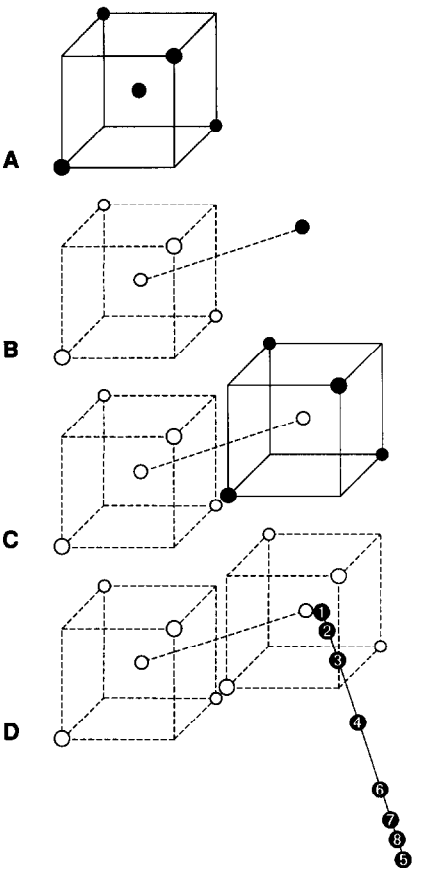

Fig 2 Schematic illustration of the multivariate optimization method (A) Five experiments to estimate a linear model for chiral resolution, (B) calculation of the location of the desired resolution along the vector of steepest ascent, (C) four experiments to estimate linear models for the chiral resolution and the retention time of the last-eluting enantiomer, (D) minimization of the retention time by a line search along the vector of steepest ascent for the retention time of the last eluting enantiomer The chiral resolution is kept above a specified value

The coefficients of eqn 9 give the vector of steepest ascent for the retention time However, in order to keep the resolution equal to or higher than the desired resolution during the minimization, eqn 9 has to be modified This is done by isolating one of the variables of eqn 8, as a dependent variable (x_k), and including the desired resolution in this expression (eqn 10) The dependent variable is chosen so that the starting point of the line search (see below) is located as close as possible to the centre of the experimental design (*i e*, $x_i = x_j = 0$) The com-

bination of eqns 9 and 10 provides an expression (eqn 11) that allows the minimization of the retention time without a decrease in resolution

$$x_k = \frac{CR^* - \beta_0 - \beta_i x_i - \beta_j x_j}{\beta_k} \quad (10)$$

$$(t_R)_2 = \gamma_0 + \gamma_i x_i + \gamma_j x_j + \gamma_k \left(\frac{CR^* - \beta_0 - \beta_i x_i - \beta_j x_j}{\beta_k} \right) \quad (11)$$

To minimize the retention time, an initial step size for one of the independent variables (x_i) is chosen by the analyst while the other independent variable (x_j) is calculated by this initial step size and the coefficients of eqn 9 (eqns 12 and 13)

$$\delta = x_i/\gamma_i \quad (12)$$

$$x_j = \gamma_j \delta \quad (13)$$

In order to reduce the number of experiments along the vector, a method for line search is applied (Fig 2D), *i e*, the step size is doubled for every step until the direction of the search is changed for the first time From this moment the step size is halved for every step no matter what the direction is (Fig 3) The direction of the line search is changed when the resolution decreases or the retention time increases relative to the best experiment in the line search (when a specified limit is passed, *i e*, the lower/upper limit of a confidence interval for the variable) The line search is ended at the point where the step size is limited by the chromatographic instrumentation Four experiments in a half-fractional factorial design centred around the last experiment in the line search will give new linear models and another vector of steepest ascent to follow This procedure is repeated until little progress is made

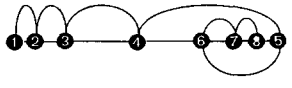

Fig 3 Illustration of the line search along the vector of steepest ascent the step size is doubled for every step until the direction of the search is changed (5) From this moment the step size is halved for every step The direction of the line search is changed when the resolution decrease or the retention time increase relative to the best experiment in the line search (5 and 6) The line search is ended at the point where the step size is limited by the chromatographic instrumentation (8)

EXPERIMENTAL

Instrumentation

The chromatograms were obtained with a Series 600-D SFC system (Dionex, Salt Lake City, UT, USA) equipped with a flame ionization detector (350°C) The injector consisted of a Model CI4W 2 high-pressure four-port valve injector with a 0 2-μl sample loop (Valco Instruments, Houston, TX, USA) and a splitter (300 μm I D) (SGE, Austin, TX, USA) SFC-grade CO_2 (Scott Specialty Gases, Plumsteadville, PA, USA) was used as the mobile phase at an average linear velocity of *ca* 1 5 cm s^{-1} (60°C, 0 30 g ml^{-1}) controlled by a deactivated frit restrictor (50 μm I D) obtained from Dionex The chromatograms were registered with a Model SP4290 integrator (Spectra-Physics, San Jose, CA, USA) and transferred to a Macintosh IIfx computer (Apple Computer, Cupertino, CA, USA) for further treatment

Column

An open-tubular column (5 m × 50 μm I D) coated with a chiral copolymeric stationary phase, poly[(1*R*)-*trans*-N,N′-1,2-cyclohexylenebisbenzamide]oligoalkylsiloxane [12] (film thickness ≈ 0 25 μm), was used

Samples

(−)- and (+)-diethyl tartrate were purchased from Aldrich (Steinheim am Albuch, Germany) Ethyl-(2*R*,3*S*)-dihydroxyoctanoate and ethyl-(2*S*, 3*R*)-dihydroxyoctanoate were provided by K B Sharples (Massachusetts Institute of Technology) and (±)-mephenytoin by Sandoz (Basle, Switzerland) Ethanol was used as the solvent except for mephenytoin, which was dissolved in toluene

Software

The chromatograms were transferred from the integrator and subsequently decoded with an in-house written routine in Microsoft QuickBasic (Microsoft, Redmond, WA, USA) A program for general graphing and data analysis, Igor (Wave Metrics, Lake Oswego, OR, USA), was used for peak fitting and Microsoft Excel was used for all other calculations

RESULTS AND DISCUSSION

The optimization method is exemplified in Tables I–VI with the separation of ethyl-(2*R*,3*S*)-dihydroxyoctanoate and ethyl-(2*S*,3*R*)-dihydroxyoctanoate According to the two-step theoretical discussion, a two-step optimization was performed, as follows

Step one conditions for the desired resolution were located as follows The levels of the variables were coded (Table I) and five experiments performed according to a half-fractional factorial design An experiment in the centre of the design was included to give the possibility of detecting large deviations from the linear model (Table II) Using

TABLE I

Step one coding of variables

Coded variable[a]	−1	0	+1
D_0 Start density (g ml^{-1})	0 270	0 300	0 330
t Temperature (°C)	47	50	53
D_g Density gradient (g ml^{-1} min^{-1})	0 007	0 010	0 013

[a] $x_1 = (D_0 - 0\ 300)/0\ 030$, $x_2 = (t - 50)/3$, $x_3 = (D_g - 0\ 010)/0\ 003$

TABLE II

Step one half-fractional factorial design for the location of the desired resolution

Experiment No	Coded variables[a]			D_0 (g ml^{-1})	t (°C)	D_g (g ml^{-1} min^{-1})	CR	$(t_R)_2$ (min)
	x_1	x_2	x_3					
1	0	0	0	0 300	50	0 010	0 825	17 336
2	−1	1	1	0 270	53	0 013	0 756	16 270
3	1	−1	1	0 330	47	0 013	0 516	15 077
4	1	1	−1	0 330	53	0 007	0 659	15 943
5	−1	−1	−1	0 270	47	0 007	1 219	25 980

[a] See Table I

TABLE III

Step two first half-fractional factorial design for the minimization of the retention time

Experiment No	Coded variables[a]			D_0 (g ml^{-1})	t (°C)	D_g (g ml^{-1} min^{-1})	CR	$(t_R)_2$ (min)
	x_1	x_2	x_3					
6	0	0	0	0 344	52	0 013	0 281	12 251
7	−1	1	1	0 314	55	0 016	0 365	11 882
8	1	−1	1	0 374	49	0 016	0 050	11 180
9	1	1	−1	0 374	55	0 010	−0 009	10 964
10	−1	−1	−1	0 314	49	0 010	0 632	16 416

[a] In analogy with Table I, $x_1 = (D_0 - 0\ 344)/0\ 030$, $x_2 = (t - 52)/3$, $x_3 = (D_g - 0\ 013)/0\ 003$

TABLE IV

Step two line search along the first vector of steepest ascent for the retention time

Experiment No	D_0 (g ml^{-1})	t (°C)	D_g (g ml^{-1} min^{-1})	δ	CR	$(t_R)_2$ (min)
11	0 343	52	0 013	0 000	0 327	12 303
12	0 338	53	0 014	−0 281	0 306	11 998
13	0 327	55	0 016	−0 842	0 283	11 423
14	0 306	59	0 020	−1 965	0 231	10 454
15	0 316	57	0 018	−1 404	0 264	10 944
16	0 311	58	0 019	−1 684	0 255	10 704

TABLE V

Step two second half-fractional factorial design for the minimization of the retention time

Experiment No	Coded variables[a]			D_0 (g ml^{-1})	t (°C)	D_g (g ml^{-1} min^{-1})	CR	$(t_R)_2$ (min)
	x_1	x_2	x_3					
17	0	0	0	0 311	58	0 019	0 255	10 704
18	−1	1	1	0 281	61	0 022	0 278	10 413
19	1	−1	1	0 341	55	0 022	0 086	9 734
20	1	1	−1	0 341	61	0 016	0 041	9 491
21	−1	−1	−1	0 281	55	0 016	0 547	13 629

[a] In analogy with Table I, $x_1 = (D_0 - 0\ 311)/0\ 030$, $x_2 = (t - 58)/3$, $x_3 = (D_g - 0\ 019)/0\ 003$

TABLE VI

Step two line search along the second vector of steepest ascent for the retention time

Experiment No	D_0 (g ml^{-1})	t (°C)	D_g (g ml^{-1} min^{-1})	δ	CR	$(t_R)_2$ (min)
22	0 305	58	0 019	0 000	0 303	10 911
23	0 298	59	0 020	−0 385	0 284	10 738
24	0 283	61	0 022	−1 156	0 280	10 410
25	0 254	65	0 025	−2 698	0 221	9 969
26	0 269	63	0 023	−1 927	0 245	10 177
27	0 276	62	0 022	−1 542	0 265	10 293

the coefficients of the polynomial fitted for the chiral resolution (eqn 14), the location of the desired resolution, $CR^* = 0\ 274$, along the vector of steepest ascent was calculated

$$CR = 0\ 7949 - 0\ 2001x_1 - 0\ 0799x_2 - 0\ 1512x_3 \quad (14)$$

The suggested conditions (Table III, experiment 6) gave a CR value (0 281) that is within an acceptable resolution range To estimate this standard error of resolution, a large number of experiments were carried out and it was found that $CR^* \pm 0\ 025$ is a reasonable range (90% confidence interval for base-

line resolution, $n = 18$) This estimate is not required in order to perform an optimization, it was merely a necessary step in the development of the method

Step two the retention time was minimized according to the modified method of steepest ascent A half-fractional factorial design was located around the point of the desired resolution and the levels of the variables were coded in analogy with Table I From the results of these five experiments (Table III), polynomials were fitted for the chiral resolution and the retention time of the last eluting enantiomer, respectively (eqns 15 and 16)

$$CR = 0\ 2637 - 0\ 2394x_1 - 0\ 0815x_2 - 0\ 0520x_3 \quad (15)$$

$$(t_R)_2 = 12\ 5387 - 1\ 5388x_1 - 1\ 1875x_2 - 1\ 0794x_3 \quad (16)$$

The coefficients of these polynomials were used to follow the vector of steepest ascent for the retention time of the last-eluting enantiomer (Table IV) In this line search, the coded temperature (x_2) was chosen as the independent variable which determined the initial step size The coded start density (x_1) was chosen as the dependent variable [As a result of the definition of the dependent variable (eqn 10), the first experiment in a line search does not necessarily result in a retention time that is shorter than in the previous experiment, as seen in Table IV] After six experiments along the vector, the smallest possible change in temperature was reached and four experiments in a half-fractional factorial design (Table V) gave two new polynomials (eqns 17 and 18) and a third vector to follow (Table VI)

$$CR = 0\ 2413 - 0\ 1746x_1 - 0\ 0786x_2 - 0\ 0558x_3 \quad (17)$$

$$(t_R)_2 = 10\ 7943 - 1\ 2043x_1 - 0\ 8648x_2 - 0\ 7430x_3 \quad (18)$$

After this second line search, the optimization was ended (Fig 4) At this point the retention time had been reduced by 16% compared with the point where the desired resolution was achieved

A more detailed investigation of the response surface around the last experiment was performed by a central composite design [11] Second-order polynomials fitted to these results as models of resolution and retention time revealed that the area of optimum conditions had been reached

Before deciding to use the method of steepest ascent, one alternative approach using a central composite design was evaluated Unfortunately, the desired resolution often covered a large experimental domain and a central composite design could therefore not provide models which were good enough to locate optimum conditions However, these experiments clearly indicated the need to optimize the start density, density gradient and temperature Further, it showed that a first-order polynomial should be a good approximation of models for both resolution and retention time in a subregion of the experimental domain (the response surfaces obtained from the central composite designs were smooth and slightly parabolic)

Figs 5 and 6 show two other applications of the optimization method where the enantiomers of (±)-mephenytoin, an anticonvulsant drug, and (±)-diethyltartrate were separated at retention times comparable to those of a rapid GC analysis In the three examples the decreases in retention time, from the point of location of the desired resolution to the point of location of the area of optimum conditions, are in the range 3–19%

One problem that often is associated with sequential methods, such as the method of steepest ascent or the simplex algorithm, is the risk of finding a local

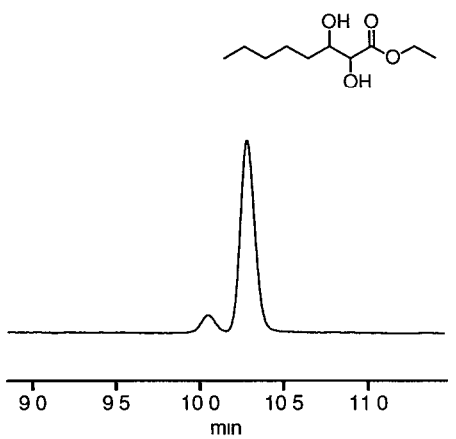

Fig 4 Optimized separation of ethyl-(2*R*,3*S*)-dihydroxyoctanoate and ethyl-(2*S*,3*R*)-dihydroxyoctanoate The desired resolution was located after six experiments An additional 20 experiments resulted in a 16% decrease in the retention time of the last-eluting enantiomer Conditions CO_2, 62°C, start density 0 276 g ml^{-1}, density gradient 0 022 g ml^{-1} min^{-1}

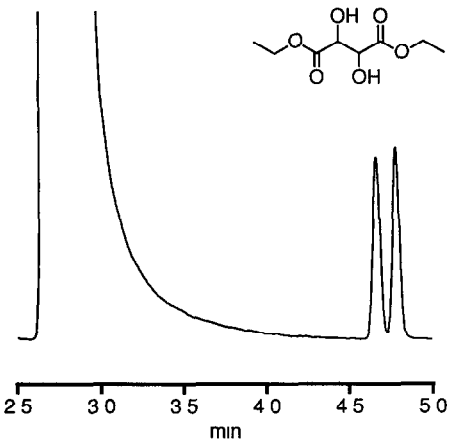

Fig 5 Optimized separation of the enantiomers of the anticonvulsant drug mephenytoin The desired resolution was located after seven experiments An additional 18 experiments resulted in a 3% reduction of the retention time of the last eluting enantiomer Conditions CO_2, 92°C, start density 0 555 g ml^{-1}, density gradient 0 024 g ml^{-1} min^{-1}

optimum instead of the true optimum As only the two enantiomers are to be separated and, as mentioned above, the response surfaces for both resolution and retention time are smooth and slightly parabolic, there should be no risk of hitting a local optimum with the described optimization procedure Therefore, the minimization of retention time

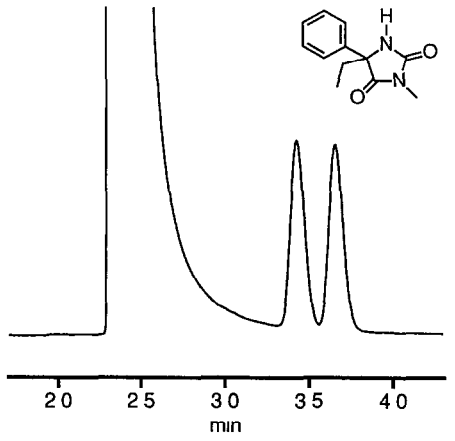

Fig 6 Optimized separation of the enantiomers of diethyl tartrate The desired resolution was located after seven experiments An additional 21 experiments resulted in a 19% decrease in the retention time of the last-eluting enantiomer Conditions CO_2, 91°C, start density 0 175 g ml^{-1}, density gradient 0 054 g ml^{-1} min^{-1}

in step two should also reach the optimum area sooner or later by a successive application of the method The reliability of this location of optimum conditions is dependent on the number of experiments and therefore at least two vectors of steepest ascent ought to be followed An assumption made in this second step, probably of no practical importance, is that the shortest possible retention time at a certain resolution increases with increasing resolution

The method outlined is designed to optimize the separation of two enantiomers without any interfering compounds present in the sample If interfering compounds are present a modified approach should be used Step one provides conditions that give the desired resolution of a racemic standard solution A central composite design, or alternatively a modified simplex algorithm, located in this limited experimental domain should then have good possibilities of finding optimum conditions

The deterministic gradient theories that have been developed for GC [13–15] and LC [16,17] with good results may also be useful for the optimization of separations performed by SFC There are, however, a number of complications such as changes in efficiency/mass transfer, linear velocity, etc , during a density program

CONCLUSIONS

It has been shown that the suggested method can be used to optimize chiral separations in open-tubular column SFC It has also been shown that it is sufficient to use three parameters, start density, density gradient and temperature, in order to reach a desired resolution within a specified range (if possible with the chromatographic system) and, if the retention time or signal-to-noise ratio is critical, minimize the retention time without a reduction in the resolution The method was demonstrated using open-tubular column SFC, but it is also applicable to chiral separations in GC It should be noted that all calculations are possible with commercial software and without skills in programming

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