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OPTIMIZATION OF ELUENT COMPOSITION AND COLUMN LENGTH IN LIQUID CHROMATOGRAPHY

SEPARATION OF NUCLEOTIDES BY ION-PAIR CHROMATOGRAPHY

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SUMMARY

Equations for optimum column length for two different, ideal, separation problems have been derived and the significance of simultaneous optimization of column length (or flow-rate) and eluent composition is demonstrated. To optimize the separation of a set of compounds, a simple iterative off-line computer program has been written. The data set necessary for this optimization comprises equations for the capacity factor and height of theoretical plate as a function of eluent composition. Practical applications are included for separation of various nucleotide mixtures by ion-pair chromatography with variable pH, methanol concentration and column length. In most cases, the adjustment of column length significantly decreased the time necessary for chromatography.

INTRODUCTION

The optimization methods in liquid chromatography may be divided into two broad groups: the first includes optimization of the separation of samples, the components of which are not known or identified; the second relates to the separation of samples where the number of components is known and single components are either available in pure form or may be identified in the chromatogram. Recently, the optimization procedure used in liquid chromatography has been reviewed[1] and the quality of criteria for optimization has been evaluated[2]. Whereas for the first group of methods the optimization procedure may never be clearly defined and the criteria of quality related to information theory are fully adequate, for the second group a single quality criterion of the separation process may be found. The usual assumption of constant column length leads to problems of relative weighting assigned to the time factor (elution time of the last peak) and separation factor (*e.g.*, resolution of two adjacent peaks)[3,4]. For a given separation problem of the second group, the optimum solution may be approached if eluent composition and column length (number of theoretical plates) are considered as variables, as has been proposed earlier by Svoboda[5]. Then, all chromatograms of the examined array may be

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compared, using the time factor as the single criterion, whereas the minimum resolution of two adjacent peaks is held constant and equal to a predetermined value.

Recently, a multiparametric optimization procedure was published, which included the column length among other parameters[6]. The significance of this parameter was demonstrated.

THEORY

It is difficult to treat the optimization of chromatographic separation as a general case. However, two idealized cases with emphasis on simplicity and the possibility of analytical solution can be described: firstly, the case of the separation of a certain number (n) of compounds, the elution of which may be arbitrarily regulated *e.g.* by eluent composition, choice of sorbents, etc.) so that the first one is eluted with the void volume and the others follow at regular intervals, secondly, the case of three analogous compounds, where two follow each other very closely in the chromatogram (critical pair, which determines the necessary efficiency of the separating column) and the third, eluted at a greater interval, determines the time necessary for completion of the whole chromatographic separation. The first case was introduced by Giddings[7] in his work related to the peak capacity of the chromatographic column and represents the case where the optimization of a chromatographic system is pushed to its limits; it can scarcely be reproduced in real systems. The opposite may be said about the second system; however, most practical situations (where the separation of more than three compounds need not be followed at all or is better than that of the critical pair) may be approximated by this case.

The case of n regularly eluted compounds

Obviously, the most compact arrangement of compounds in an isocratic chromatogram is that described by eqn. 1. The first compound is eluted with the void volume V_o; ($V_1 = V_o$) and the distance between all other adjacent peaks with elution volumes V_{i+1} and V_i is

$$V_{i+1} - V_i = R(\sigma_2 + \sigma_{i+1}) \tag{1}$$

Under this assumption, the nth compound is eluted at volume V_n

$$\ln(V_n/V_o) = (n - 1) \cdot 2R/\sqrt{N} \tag{2}$$

The total separation time (T) may be defined as

$$T = (V_n + R\sigma_n)/F \tag{3}$$

where F is the eluent flow-rate. We will tacitly assume that, for all compounds, the number of theoretical plates N has the same value:

$$N = (V/\sigma)^2 \tag{4}$$

The characteristics of the separation system are summarized by two linear equations:

$$N = l \cdot H \tag{5}$$

where l is the column length and H is the height of one theoretical plate, and

$$V = V_o/FN \tag{6}$$

Time τ may be interpreted as the time needed for the eluent to travel the distance equal to the height of one theoretical plate.

By substitution of variables from eqns. 6, 4 and 2 into eqn. 3, we obtain

$$T = \tau N (1 + R/\sqrt{N}) \exp[(N - 1) 2R/\sqrt{N}] = \tau p \tag{7}$$

Thus, for a given number of compounds to be separated, there exists a distinct minimum separation time with respect to the number of theoretical plates. Whereas the first multiplier of eqn. 7 increases linearly with N, the last one exponentially decreases with the square root of N.

To determine the minimum of T, we have to solve the simple differential equation (valid for $T \neq 0$)

$$(\mathrm{d}T/\mathrm{d}N)/T = 1/N - RN^{-3/2}/(1 + R/\sqrt{N}) - (n - 1)RN^{-3/2} \tag{8}$$

If the second term is omitted, we obtain a simple relationship for the optimal number of theoretical plates for a given set of compounds

$$N_{\mathrm{opt}} = R^2 (n - 1)^2 \tag{9}$$

The optimal time of separation (substitution of N_{opt} into eqn. 7) is then

$$T_{\mathrm{opt}} = 7.39\tau R^2(n - 1)^2 [1 + 2/(n - 1)] \tag{10}$$

The reduced volume of the last compound, eluted under optimal conditions, is always

$$V_n/V_{o_{\mathrm{opt}}} = e^2 = 7.39 \tag{11}$$

and the capacity factor ($k'_{n_{\mathrm{opt}}}$) is therefore 6.39.

Table I summarizes the computed values of coefficients p (eqn. 7). Values of p are omitted from this table when the capacity factor of the last compound is > 50. The optimum is rather shallow: both an excess of theoretical plates and a small number of them may have a deteriorating effect on the speed of chromatographic separation.

The case of three analogous compounds

The capacity factors of three analogous compounds (1, 2 and 3) are described by the following equations

$$\log k_1 = a + \mathrm{f}(c) \tag{12}$$
$$\log k_2 = a + \Delta a + \mathrm{f}(c) \tag{13}$$
$$\log k_3 = a_3 + \mathrm{f}(c) \tag{14}$$

where Δa is small compared with a, and where f(c) is a function of eluent composition

TABLE I

TIME NEEDED FOR SEPARATION OF REGULARLY ELUTED COMPOUNDS

Coefficient p from eqn. 7 × 10^{-3}. n = number of compounds.

n	*Theoretical plates*									
	200	*324*	*700*	*1444*	*4000*	*9604*	*20 000*	*39 204*	*80 000*	*160 000*
10	2.91	2.66	2.93	3.91	7.29	14.1	26.1	47.5	91.5	175.9
20			13.3	11.2	13.7	21.3	34.7	58.12	105.4	194.4
50					91.5	72.4	81.1	106.6	161.1	265.2
100						557	333.6	292.6	326.7	432.7

(concentration of one or more eluent components), *i.e.* a polynomial or logarithmic function. Further, values a, a_3 and f(c) are chosen so that the condition $k_1 < k_2 < k_3$ is valid for the whole described space.

Using eqn. 3, where $n = 3$, and two other obvious relations

$$N = 4V_1^2R^2/(V_2 - V_1) \quad (15)$$

$$k_3 = k_1 \exp(a_3 - a) \quad (16)$$

we arrive at the time necessary for our separation:

$$T = 4\tau R\,[(1 + k_1)^2/(\Delta a^2k_1^2)]\,[1 + k_1 \exp(a_3 - a)]\,(1 + R/\sqrt{N}) \quad (17)$$

Neglecting, as above, the third term and solving the equation for the first derivative of T according to k_1, we obtain the optimum capacity value

$$k_{1_{\rm opt}} = 0.5 + \sqrt{0.25 + 2\exp[-(a_3 - a)]} \quad (18)$$

$$N_{\rm opt} = 4R^2(1 + k_{1_{\rm opt}})/\Delta a^2k_{1_{\rm opt}}^2 \quad (19)$$

Under optimal conditions, the capacity factors of the compounds of the critical pair have to be adjusted so that they lie in the region between 1 and 2 (see eqn. 18).

The number of theoretical plates is determined predominantly by Δa. The optimum separation time T is influenced by both of these factors. The optimum is rather shallow, as in the preceding case. This type of optimization is found in many practical problems, as will be shown later.

Variation of the number of theoretical plates

The number of theoretical plates may be varied not only by varying the geometrical length of the column (l) (see eqn. 5), but also by changing the eluent flow-rate. Many equations have been proposed to relate the height of a theoretical plate with eluent velocity. However, due to the complexity of this phenomenon and, simultaneously, poor reproducibility of similar measurements, we must be satisfied with the simplified equation

$$h = 2B/v + A + Cv \quad (20)$$

Here, h is the reduced height of a theoretical plate, v is the reduced velocity of the eluent, and A, B and C are constants. In cases where v is rather high (as in most practical applications), the first term may be neglected and the only decisive factor is the constant C. If, as in ion-exchange chromatography with a polymer matrix or similar materials with slow transfer kinetics, constant $C = 1$, the height of a theoretical plate increases very rapidly with the flow-rate ($dh/dv = 1$), and time τ, in spite of increasing the eluent velocity, remains constant. Thus, the net effect of increasing the eluent speed is the increase in the necessary pressure at the column inlet and the decrease in the number of theoretical plates (at constant column length). Obviously, in this case it is easier to decrease the column length in order to decrease the number of theoretical plates. On the other hand, if $C \ll 1$ (as for most modern, silica gel-based sorbents), the decrease in the number of theoretical plates due to increased eluent velocity is accompanied by a decrease in τ. The whole separation then takes less time than that corresponding only to the shortening of the column length due to the decrease in the number of theoretical plates. Therefore, in this case, it is more advantageous to decrease N by increasing v, if increased pressure at the column inlet is permissible.

OPTIMIZATION

It is possible to optimize the separation of any combination of solutes for which data (coefficients) for eqns. 20 and 21 are available. We had at our disposal a set of coefficients for more than 80 nucleotides, measured under strictly controlled conditions in the pH range 2.7–4.7 at methanol concentrations of 0–40% (see below). The optimization procedure was as follows. After the combination of compounds is selected, the upper and lower limits of each variable are chosen, as well as the number of steps for every variable interval. Then, the whole subspace is searched, point by point, and the ten best conditions for constant column length (satisfying the condition of minimal resolution of adjacent peaks) and for variable column lengths are finally printed. For variable column lengths, the upper and lower limits may be set. For each point, the column length is adjusted in such a way that the minimal resolution of adjacent peaks equals the predetermined value of R (usually 2). Usually, *ca.* 0.02 units of pH or 0.1% (v/v) of methanol are used as step lengths.

EXPERIMENTAL

All measurements were made using an SP 8100 automatic liquid chromatograph (Spectra-Physics, San Jose, CA, U.S.A.) with programmable sample injector and SP 4200 integrator, programmed so that not only the elution time but also the number of theoretical plates of each isolated peak were measured and recorded. The columns were glass cartridges (150 × 3.2 mm I.D.) packed with 5-μm octadecyl silica (CGC Separon SIX C18). (Laboratorní přístroje, Praha, Czechoslovakia). Solutes were detected using an SP 8440 UV detector.

In most cases, the flow-rate was 0.5 ml/min. The columns were thermostated to 25°C with water jackets. The eluent (0.05 M sodium dihydrogen phosphate, 0.005 M tributylamine and 0–40% (v/v) methanol; pH 2.7–4.7) was prepared from analytical-grade reagents and doubly distilled water. Tributylamine (Aldrich, Milwaukee,

WI, U.S.A.) was purified by distillation over calcium hydride and calcium oxide; the pH was adjusted by addition of concentrated phosphoric acid. The nucleotides were purchased from Sigma (St. Louis, MO, U.S.A.).

Data were reduced by using the polynomial representation and least-squares approximation by the well-known Cholesky method[9] of matrix inversion. The capacity factor of every assayed compound was represented by the polynomial:

$$\log k' = a_0 + a_1 x + a_2 y + a_3 x^2 + a_4 y^2 + a_5 xy \quad (20)$$

and the reduced height of a theoretical plate h:

$$h = b_0 + b_1 x + b_2 y \quad (21)$$

where x is the pH of the eluent and y is the volume fraction of methanol. Both of these equations were found to represent the collected data with satisfactory precision. In cases where the data could not be measured with the needed precision (when a capacity factor was found to be higher than *ca.* 30), the data were linearly extrapolated with decreasing weight. The whole set of data for more than 80 nucleotides will be published elsewhere[8].

All programs were written in *FORTRAN* IV and run on a PDP 11/23 digital computer (Digital Equipment Corporation, Maynard, MA, U.S.A.); graphs were plotted on an HP 7225 A plotter (Hewlett-Packard, Palo Alto, CA, U.S.A.).

RESULTS AND DISCUSSION

A comparison of constant *vs.* variable column length optimization may be demonstrated with two sets of three-dimensional graphs. In the first case, four ribonucleotides: 5'-AMP, 5'-GMP, 5'-CMP and 5'-UMP are separated. The speed in

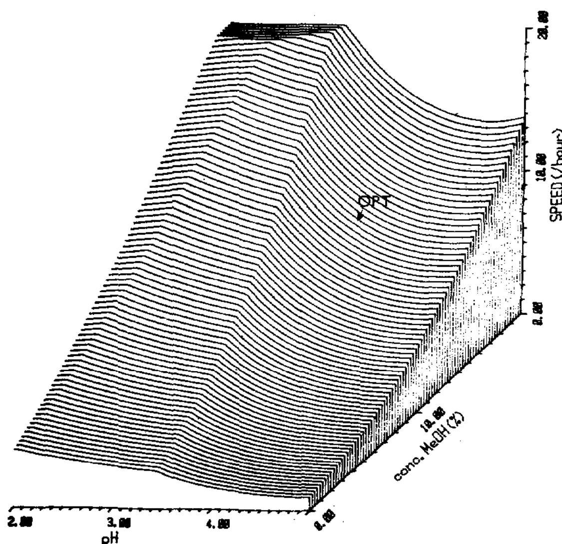

Fig. 1. Separation speed of four nucleotides (5'-AMP, 5'-CMP, 5'-UMP, 5'-GMP) at constant column length.

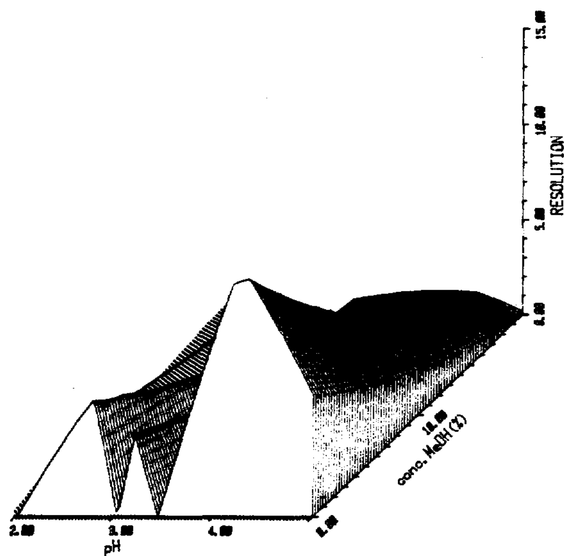

Fig. 2. Resolution of four nucleotides at constant column length.

these figures is defined as the number of separations that can be performed in one hour ($1/T$), it increases monotonously with increasing concentration of methanol (Fig. 1). However, the resolution (Fig. 2) not only decreases with increasing methanol concentration, but it drops to zero if the pH attains values where two solutes have identical elution volumes. If surfaces where the resolution drops below 2 are excluded from the graph in Fig. 1, we obtain three isolated areas (Fig. 3). The optimum is

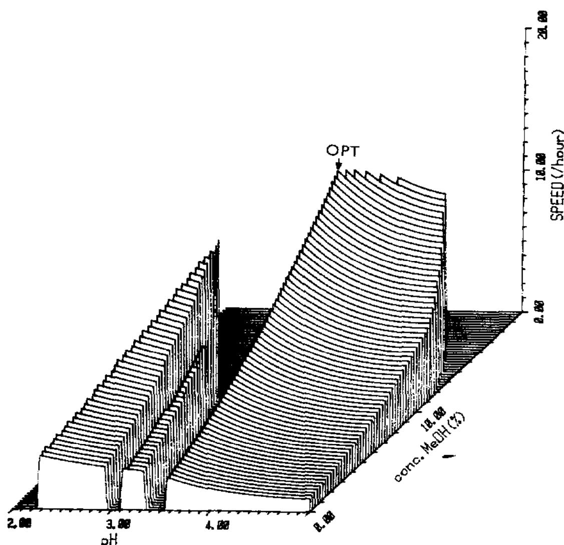

Fig. 3. Separation speed (as in Fig. 1) with those areas omitted where the resolution is less than 2.

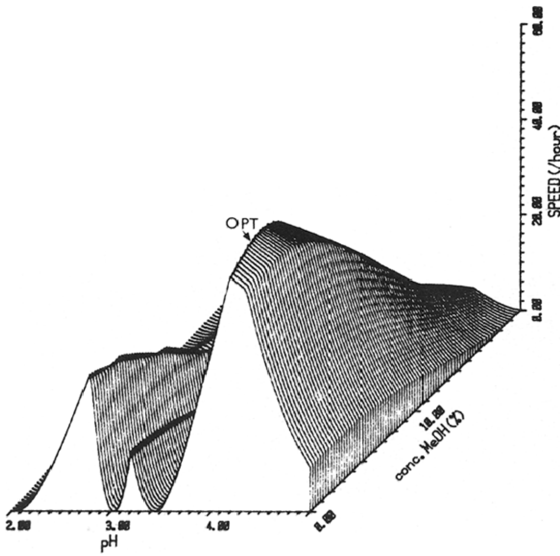

Fig. 4. Separation speed (four nucleotides, variable column length).

located on the border of one of them. The speed in the case of variable column lengths (Fig. 4) is influenced by the necessary variation in column length; its variations are recorded in Fig. 5. When areas with excessive column length are excluded (Fig. 6), the shape of the graph in Fig. 4 is changed only in the areas of lowest speed, but the most interesting areas are completely preserved. The optimum, in this case located on the apex, would not be disturbed, even if the permitted column length

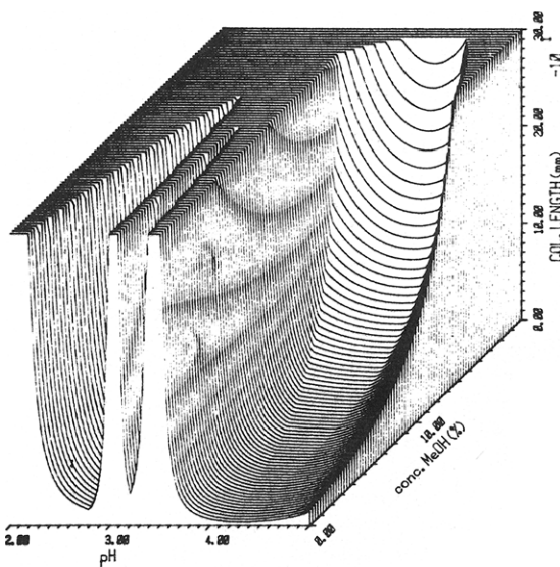

Fig. 5. Column length (four nucleotides, variable column length).

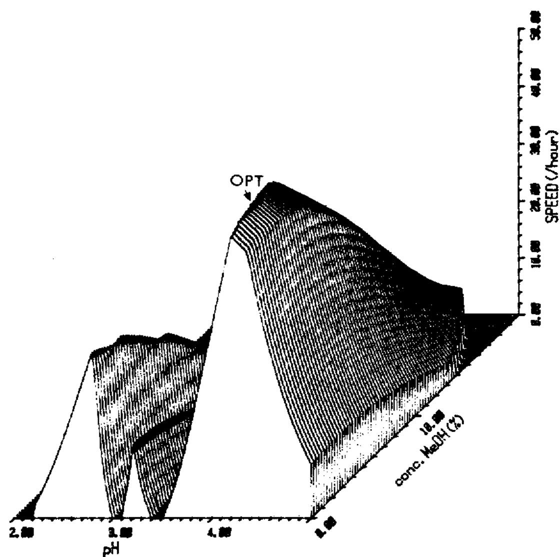

Fig. 6. Separation speed with those areas omitted where the column is longer than 300 mm (four nucleotides, variable column length).

were to be much shorter. The other series of similar graphs (Figs. 7–13) is more complicated: the separated mixture includes not only four ribonucleotides (5′-CMP, 5′-UMP, 5′-AMP, 5′-GMP), but also four deoxyribonucleotides (5′-dCMP, 5′-TMP, 5′-dUMP, 5′-dGMP). The shape of the speed surface at constant column length (Fig. 7) resembles that in Fig. 1, but the resolution surface is deeply separated into many ridges, running perpendicular to the pH axis (Figs. 8 and 9). The optimum, as in

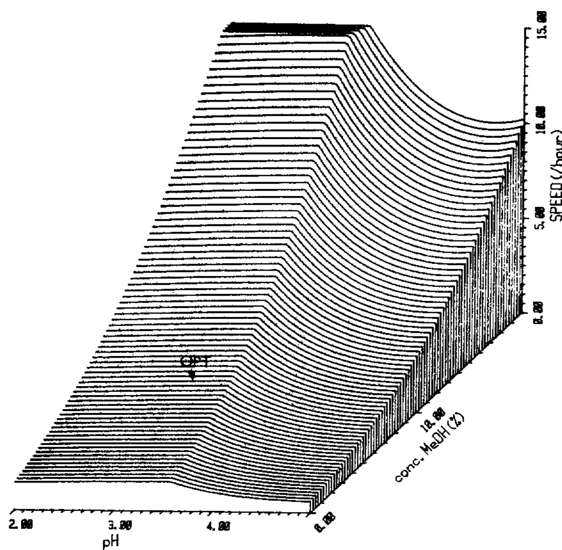

Fig. 7. Separation speed of eight 5′-nucleotides (AMP, GMP, CMP, UMP, TMP, dCMP, dUMP, dGMP).

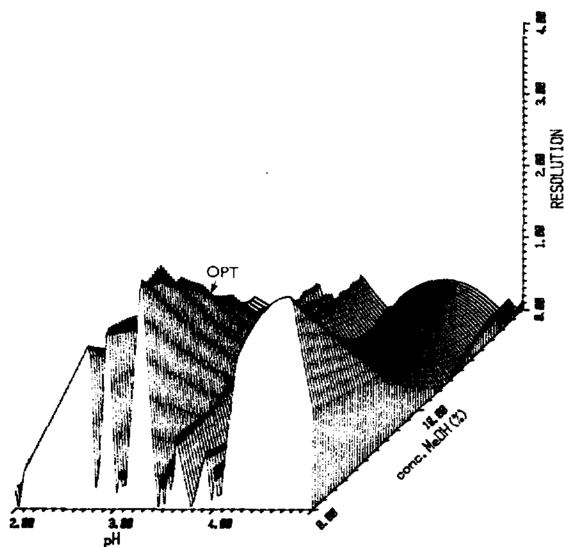

Fig. 8. Resolution of eight nucleotides at constant column length.

the preceding case, is again located just on the border of the isolated surface (Fig. 10), and every change in column length shifts the optimum to different coordinates. The optimum in the variable-column case (Figs. 11–13) is (as in the case of separation of four ribonucleotides) located on the peak of one of the "islands" and, again, no decrease in permissible column length would affect its location.

Some results of optimizations for several mixtures of nucleotides are summarized in Table II. With the exception of the last one, the optimum was always found

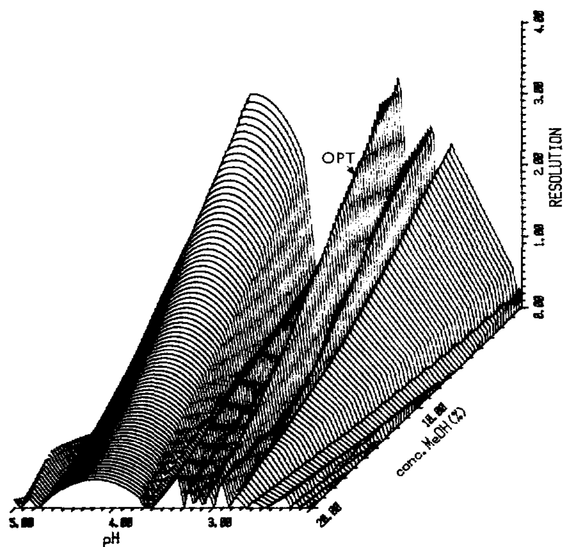

Fig. 9. As in Fig. 8, but the methanol concentration axis is reversed.

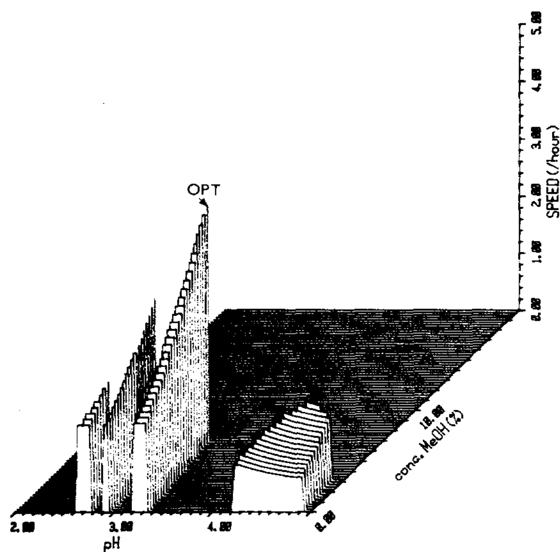

Fig. 10. As in Fig. 7, but the areas where resolution drops below 2 are omitted.

at a column length shorter than the original; the gain in separation speed was, in most cases, significant.

From the standpoint of the practical analyst, information on the stability of the optimal separation obtained is important. This is obvious from the form of the surface near the optimum. In our case, the ridge (Fig. 13) is rather sharp; small variations in pH may influence the resolution drastically.

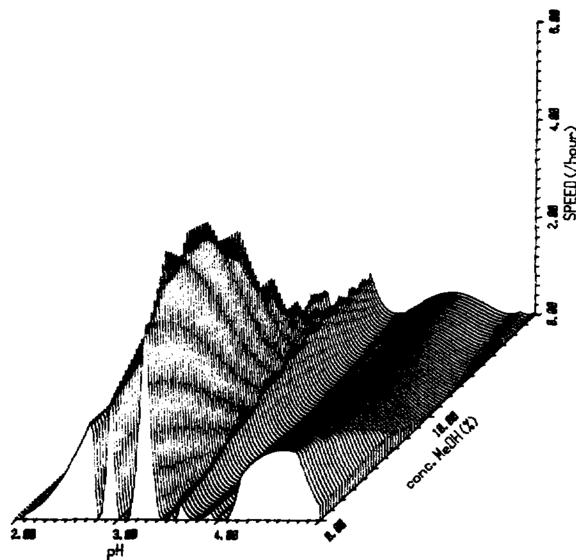

Fig. 11. Separation speed (eight nucleotides, variable column length).

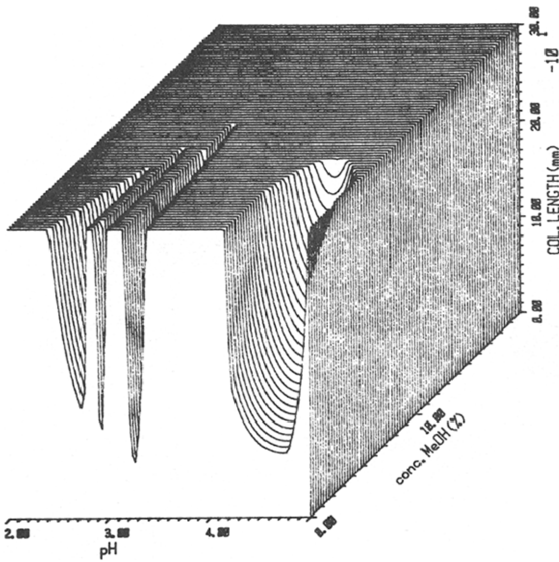

Fig. 12. Column length (eight nucleotides, variable column length).

A few words should be said about the searching methods. In our preceding paper, devoted to optimalization[8], the simplex search was successfully applied. This may be effective in less complicated systems. However, the best results are never guaranteed. If the systematic search is used, as in this case, then the attainment of optimal coordinates is always guaranteed at the price of longer computer time. With the advent of modern microcomputers, this disadvantage becomes less significant.

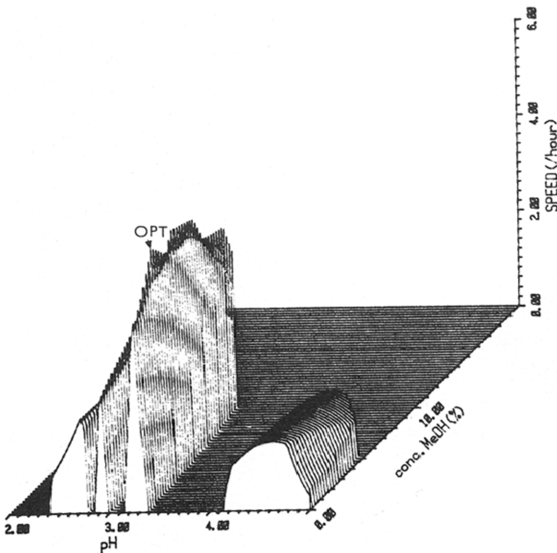

Fig. 13. As in Fig. 11, but the areas where columns are longer than 300 mm are omitted.

TABLE II

COMPARISON OF OPTIMAL SEPARATION AT CONSTANT OR VARIABLE COLUMN LENGTH

Compounds	*pH*	*Methanol (%)*	*Column length*	*Separation time*	*Mode*
5'-AMP, 5'-CMP, 5'-dCMP, 5'-UMP	3.20	7.00	150	14.48	Constant
5'-dAMP, 5'-TMP, 5'-GMP, 5'-dGMP	3.23	2.00	61	11.80	Variable
5'-AMP, 5'-UMP, 5'-GMP, 5'-CMP	3.56	15.75	150	9.16	Constant
	4.13	2.25	5.75	4.86	Variable
5'-AMP, 5'-dAMP, 5'-ADP, 5'-ATP,	3.55	20.00	150	8.62	Constant
5'-ADP, 5'-dATP	5.00	17.00	18.3	4.64	Variable
5'-CMP, 5'-CDP, 5'-AMP, 5'-CTP,	4.64	26.00	150	6.20	Constant
5'-ADP, 5'-ATP	4.65	18.50	45.5	3.80	Variable
5'-AMP, 5'-GMP, 5'-ADP, 5'-GDP,	2.75	16.50	150	9.44	Constant
5'-ATP, 5'-GTP	2.78	12.50	18.3	6.64	Variable
5'-AMP, 5'-UMP, 5'-ADP, 5'-UDP,	2.77	17.00	150	10.58	Constant
5'-ATP, 5'-UTP	3.53	16.50	63	6.26	Variable
7M5'-GMP, 5'-GMP, 3'-GMP, 2'-GMP,	3.75	12.50	150	16.72	Constant
2:3'-cGMP, 3':5'-cGMP	4.00	20.00	311.2	14.16	Variable

CONCLUSIONS

By optimizing the eluent composition simultaneously with varying the column length (*vs.* optimization at constant column length), a higher separation speed (at predetermined minimum resolution) can be achieved. The advantage of this may be very substantial. However, sometimes columns longer then the original may be optimal. A shorter column is equivalent to higher eluent speed; the transformation from column length to flow-rate may be limited at a maximum permitted pressure for a particular system. The optimization is performed by computer program, using a simple data set (*e.g.* six points for a second-degree polynomial (two variables) for one compound). Data may be gathered by using a pre-programmed automatic sampler. The method is suitable for all kinds of liquid chromatography (ion-pair, ion-exchange, reversed-phase or liquid–solid). No simple search algorithm is applicable (*e.g.* simplex, steepest gradient, etc.). Only net methods which need more computer time are really dependable (systematic search with small step-length). The stability of the optimum can be determined from the shape of the surface around the optimum.

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