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GRADIENT ELUTION IN LIQUID CHROMATOGRAPHY

IX. SELECTION OF OPTIMAL CONDITIONS IN STEPWISE-ELUTION LIQUID CHROMATOGRAPHY

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SUMMARY

A mathematical approach is described for stepwise-elution liquid chromatography that permits calculations of the optimum composition and volume of the mobile phase in each isocratic step in order to achieve the desired separation. The method is based on the theory published in the previous part of this series. Modifications of this approach are discussed and illustrated by practical examples.

INTRODUCTION

Gradient elution has become a widely accepted and efficient method for solution of the so-called general elution problem[1], *i.e.*, the separation of compounds having greatly differing retentions. The importance of this technique has increased with the growth in the application of liquid chromatography to the analysis of complex samples of naturally occurring substances, which have capacity ratios that may differ by several orders of magnitude in a given chromatographic system under isocratic conditions. Gradient-elution liquid chromatography also seems very promising as a method for the analysis of organic pollutants in environmental waters[2,3].

If gradient elution is to produce meaningful results, the assignment of peaks to the individual compounds becomes of major importance and the requirements for chromatographic reproducibility are rather high. A considerable effort has therefore been made to improve the performance of gradient-elution equipment in order to obtain satisfactory reproducibility[4,5].

On the other hand, for a more reliable identification of peaks it is highly useful to be able to calculate retention data in gradient-elution chromatography from the parameters determined in isocratic experiments. Further, a method of calculation of the "right" gradient conditions for a given practical separation problem can eliminate the need for tedious trial-and-error experiments.

In all calculations of retention data or conditions for elution in experiments using a programmed composition of mobile phase (stepwise or continuous gradient elution), the relationship between the capacity ratios, k', of the sample compounds and the concentration, c, of the efficient eluting component in the mobile phase must

be known. Three forms of this function describe most of the possible situations in adsorption, partition and ion-exchange chromatography[7–9]:

$$k' = k'_0 \cdot c^{-n} \tag{1}$$

and

$$k' = k'_0 \cdot 10^{-n \cdot c} \tag{2}$$

or

$$k' = (a + b \cdot c)^{-n} \tag{3}$$

Here, k'_0, n, a and b are experimental constants that depend on the character of the sample compound and the system used.

In the previous part of this series[7] we suggested an approach for calculations of the composition of the mobile phase that is necessary in order to achieve separation desired in isocratic elution liquid chromatography. In the present paper, a method is described that enables similar predictions to be made for stepwise elution liquid chromatography.

THEORETICAL

Retention volume in stepwise-elution chromatography

In Part II of this series[10] we presented a method for the calculation of retention volumes in stepwise-elution liquid chromatography. In this derivation, n steps were considered, with a constant composition of the mobile phase in each step i, which corresponds to the concentration c_i of the efficient eluting component.

Let us define the volume of mobile phase of constant composition delivered to the column in step i as V_{ei}. The capacity ratio of a sample compound eluted in step n is constant in each step i, *i.e.*, k'_i = constant. Thus, each step i ($1 \leqslant i \leqslant n$) contributes a partial volume V_i to the retention volume of the sample compound, V_R.

The migration of the band of a sample compound along a chromatographic column in the course of stepwise elution is shown schematically in Fig. 1 with respect to the different stages of elution (A–H) and the corresponding volumes of the eluate, $V_{e(A)}$–$V_{e(H)}$.

(A) Injection of the sample (IN), start of the chromatogram. The column is filled with mobile phase 1 (c_1) and the pump is delivering mobile phase 1; $V_{e(A)} = 0$.

(B) The pump starts to deliver mobile phase 2 (c_2); $V_{e(B)} = V_{e1}$. The sample band is still migrating in the mobile phase 1.

(C) The front of the mobile phase 2 has reached the centre of the sample band in the column at a position corresponding to the portion V_{m1} of the column void volume, V_m; $V_{e(C)} = V_{e1} + V_z + V_{m1}$; where V_z is the volume of the connecting tubing between the outlet of the gradient-generating device and the top of the column.

(D) The pump starts to deliver mobile phase 3 (c_3); $V_{e(D)} = V_{e1} + V_{e2}$. The sample band is still migrating in mobile phase 2.

(E) The front of the mobile phase 3 has reached the centre of the sample band

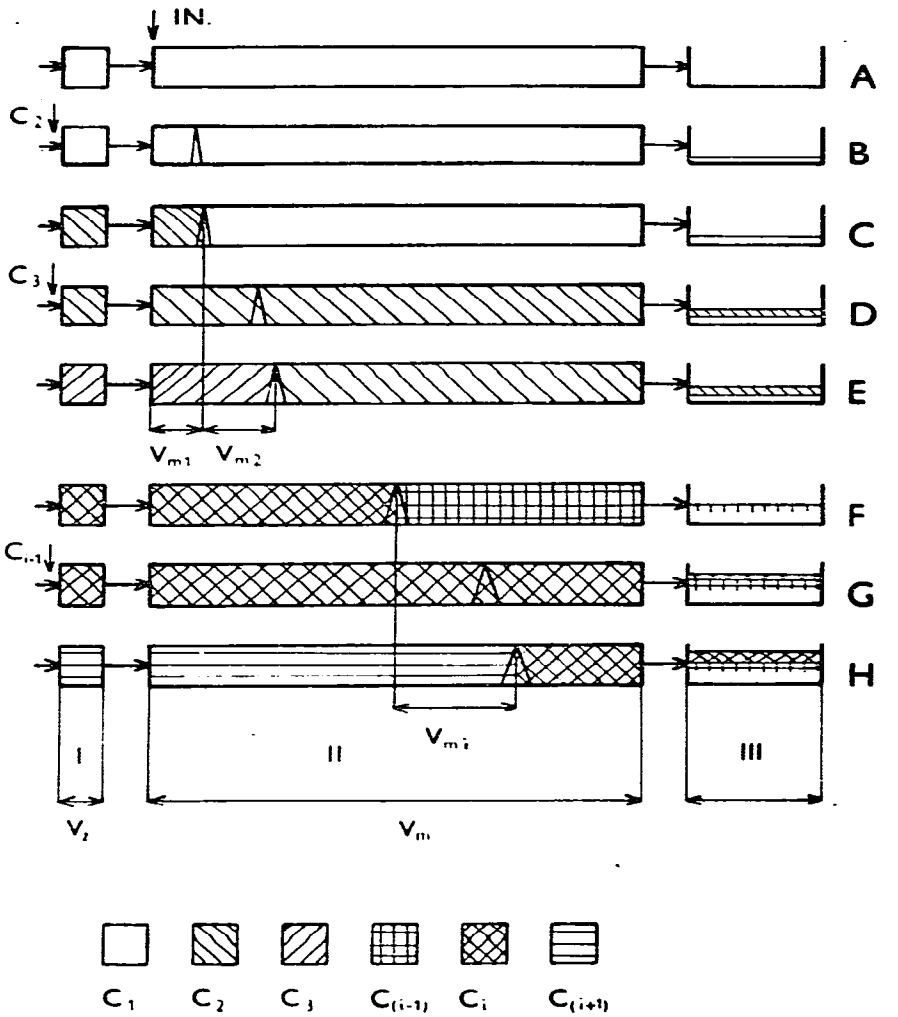


Fig. 1. Sample band migration in stepwise-elution liquid chromatography. The situation is drawn schematically for an arbitrary sample component band as the elution progresses from point A to H. I = Connection tubing from pump to the column, volume V_Z; II = separation column, V_m = volume of the mobile phase in the column; III = Eluate vessel, V_e = volume of the eluate; c_1, c_2, c_3, $c_{(i-1)}$, c_i and $c_{(i+1)}$ = concentrations of the efficient eluting component in the mobile phase in steps 1, 2, 3, $(i-1)$, i and $(i+1)$, respectively; V_{m1}, V_{m2}, V_{m3}, $V_{m(i-1)}$, V_{mi} and $V_{m(i+1)}$ = contribution of each step 1, 2, 3, $(i-1)$, i and $(i+1)$, respectively, to the total column void volume, V_m.

in the column at a position corresponding to the portion $V_{m1} + V_{m2}$ of the column void volume, V_m.

$$V_{e(E)} = V_{e1} + V_{e2} + V_z + V_{m1} + V_{m2} \quad (4)$$

(F) The front of the mobile phase i (c_i) has reached the centre of the sample

band in the column at a position corresponding to the portion $\sum_{i=1}^{i-1} V_{mi}$ of the column void volume, V_m.

$$V_{e(F)} = \sum_{i=1}^{i-1} V_{ei} + V_Z + \sum_{i=1}^{i-1} V_{mi} \tag{5}$$

(G) The pump starts to deliver mobile phase $(i + 1)$ (c_{i+1}). The sample band is migrating in mobile phase i.

$$V_{e(G)} = \sum_{i=1}^{i} V_{ei} \tag{6}$$

(H) The front of the mobile phase $(i + 1)$ has reached the centre of the sample band in the column at a position corresponding to the portion $\sum_{i=1}^{i} V_{mi}$ of the column void volume, V_m.

$$V_{e(H)} = \sum_{i=1}^{i} V_{ei} + V_Z + \sum_{i=1}^{i} V_{mi} \tag{7}$$

Consequently, we can consider the column to be composed of n parts with void volumes V_{mi}. Each sample compound is characterized by a different V_{mi} sequence. In each part of the column, isocratic elution is performed using a concentration c_i of the efficient eluting component in the mobile phase and with a corresponding capacity ratio, k'_i, of the sample compound. Thus, the following equations apply:

$$V_i = V_{mi}(1 + k'_i) \tag{8}$$

and

$$V_i - V_{mi} = V_i \cdot \frac{k'_i}{1 + k'_i} \tag{9}$$

The retention volume of the sample compound, V_R, is given by the sum of all the partial contributions V_i:

$$V_R = \sum_{i=1}^{n} V_i \tag{10}$$

Similarly:

$$V'_R = \sum_{i=1}^{n} (V_i - V_{mi}) = \sum_{i=1}^{n} V_i \cdot \frac{k'_i}{1 + k'_i} \tag{11}$$

With aid of Fig. 1, we can easily derive the relationship between the volume of the mobile phase delivered in step i, V_{ei}, and the contribution of step i, V_i, to the retention volume:

$$V_i = V_{e(H)} - V_{e(F)} = \sum_{i=1}^{i} V_{ei} + V_Z + \sum_{i=1}^{i} V_{mi} - \sum_{i=1}^{i-1} V_{ei} - \\ - V_Z - \sum_{i=1}^{i-1} V_{mi} = V_{ei} + V_{mi} \tag{12}$$

Thus, V_{ei} represents the contribution of the step i to the net retention volume, V'_{Ri}, of the sample compound:

$$V'_{Ri} = V_i - V_{mi} = V_{ei} \tag{13}$$

For simplicity V_z can be incorporated into V_{e1}.

The calculations of the eluting conditions in stepwise-elution chromatography (c_i, V_{mi}) should be performed sequentially, beginning with step 1. In this way, the values of c_i, k'_i, the contributions V_i, V_{mi} and the volumes V_{ei} are known for all steps from 1 to $(n-1)$ for a sample compound eluted in step n. V_n is related to the retention volume V_R by the equation:

$$V_n = V_R - \sum_{i=1}^{n-1} V_i \tag{14}$$

Thus, with the aid of eqns. 9 and 14, eqn. 11 can be rewritten in the form:

$$V'_R = \sum_{i=1}^{n-1} V_{ei} \cdot \frac{k'_i - k'_n}{k'_i} + V_m \cdot k'_n \tag{15}$$

Prediction of optimal conditions in stepwise-elution chromatography

The elution conditions in stepwise-elution chromatography have to be calculated separately for each individual step; beginning with step 1. The capacity ratio, k_n, of a compound to be eluted in a given step (n with respect to this compound) is calculated first, by considering the required resolution (or retention volume, V_{Rn}). Using the appropriate function $k' = f(c)$ (eqns. 1–3), the corresponding concentration c_n, of the efficient eluting component in the mobile phase in step n, can be determined. Lastly, the volume of the mobile phase V_{en} necessary in step n, is evaluated. This sequence of calculations should be repeated for each of the subsequent steps, until the conditions for elution of all the sample compounds have been estimated.

To simplify the calculations, the width of a peak (w) in stepwise-elution chromatography is assumed to depend mainly on the actual composition of the mobile phase in which the peak maximum is eluted[10]; for a compound eluted in step n it follows that

$$w = \frac{4V_m}{\sqrt{N}} \cdot (1 + k'_n) \tag{16}$$

where w is expressed in volume units.

The method of calculation of the capacity ratio, k'_n, is dependent on the requirements of optimalization. Generally, two different approaches seem reasonable.

(A) It is required that the elution of each sample compound is achieved in one, separate elution step, with a given resolution for two neighbouring peaks [one eluted in step $(n-1)$ and the other in step n]:

$$R_S = 2 \cdot \frac{V'_{Rn} - V'_{R(n-1)}}{w_n + w_{(n-1)}} \tag{17}$$

Thus, introducing eqn. 16, we obtain:

$$V'_{Rn} = V'_{R(n-1)} + R_S \cdot \frac{w_n + w_{(n-1)}}{2} = V'_{R(n-1)} + \frac{2R_S V_m}{\sqrt{N}} \cdot (k'_n + k'_{(n-1)} + 2) \quad (18)$$

After combination of this equation with eqn. 15, a relationship can be written which yields the capacity ratio k'_n necessary to achieve the resolution required:

$$k'_n = \frac{V'_{R(n-1)} - \sum_{i=1}^{n-1} V_{ei} + \frac{2V_m R_S}{\sqrt{N}} \cdot (k'_{(n-1)} + 2)}{V_m - \sum_{i=1}^{n-1} \frac{V_{ei}}{k'_i} - \frac{2V_m R_S}{\sqrt{N}}} \quad (19)$$

From k'_n, the value of c_n is estimated using the function $k' = f(c)$, which must be known. For convenience, the step n can be terminated immediately after the elution of the peak of the sample compound, and the volume of the mobile phase necessary in step n can be calculated from

$$V_{en} = V'_{R(n)} + \frac{2V_m}{\sqrt{N}} \cdot (1 + k'_n) - \sum_{i=1}^{n-1} V_{ei} \quad (20)$$

or:

$$V_{en} = V'_{R(n)} + \frac{2V_m R_S}{\sqrt{N}} \cdot (1 + k'_n) - \sum_{i=1}^{n-1} V_{ei} \quad (20a)$$

(B) Two compounds A and B are to be eluted in each step n with resolution

$$R_{SA,B} = 2 \cdot \frac{V'_{RB} - V'_{RA}}{w_B + w_A} \quad (21)$$

Using eqn. 15 for V'_{RA} and V'_{RB}, it follows that

$$V'_{RB} - V'_{RA} = \frac{2R_{SA,B} \cdot V_m}{\sqrt{N}} \cdot (k'_{nB} + k'_{nA} + 2) =$$

$$= k'_{nB} \cdot \left(V_m - \sum_{i=1}^{n-1} \frac{V_{ei}}{k'_{iB}}\right) - k'_{nA} \cdot \left(V_m - \sum_{i=1}^{n-1} \frac{V_{ei}}{k'_{iA}}\right) \quad (22)$$

where k'_{iA} and k'_{iB} denote the capacity ratios of compounds A and B in step i.

After introduction of the known relationships $k'_A = f_1(c)$ and $k'_B = f_2(c)$ into eqn. 22, the concentration of the efficient eluting component in the mobile phase, c_n, necessary for the required resolution of compounds A and B in step n, can be calculated. Generally, c_n cannot be expressed explicitly and its solution requires an approximation. Thus, V_{en} can be calculated as follows:

$$V_{en} = V'_{RB} + \frac{2V_m}{\sqrt{N}} \cdot (1 + k'_{nB}) - \sum_{i=1}^{n-1} V_{ei} \quad (23)$$

or

$$V_{en} = V'_{RB} + \frac{2V_m R_{SA,B}}{\sqrt{N}} \cdot (1 + k'_{nB}) - \sum_{i=1}^{n-1} V_{ei} \quad (23a)$$

Naturally, the approaches (A) and (B) can be combined for subsequent elution steps. It is convenient to calculate the conditions in step 1 for elution of two compounds by using the method for isocratic conditions[7].

If the value of c_n calculated from eqns. 19 and 1–3 or eqns. 22 and 1–3 is lower than $c_{(n-1)}$, the resolution required in step n can be achieved only in a mobile phase having a lower eluting strength than in step $(n-1)$. However, this difference must not be too large, otherwise serious difficulties can arise; thus very broad bands of irregular shape are observed for compounds eluted in step n. This difficulty can be surpassed if the conditions for elution in step $(n-1)$ are recalculated in order to accomplish the elution of the above compounds in step $(n-1)$.

Further, V'_{Rn} should be larger than $\sum_{i=1}^{n-1} V_{ei}$, otherwise the concentration in step $(n-1)$ is too high to permit the resolution required in step n. In this instance, the conditions for step $(n-1)$ should also be recalculated.

EXPERIMENTAL

Two practical examples were chosen to demonstrate this approach. A reversed-phase column (C_{18} chemically bonded on LiChrosorb Si-100, 10 μm) was used for the chromatographic separation, with a stepwise gradient of methanol concentration in a mobile phase composed of methanol and water. A low-pressure device was used to generate the gradient, and was equipped with a photoelectric curve follower, which made it possible to reproduce any preset mathematical form of concentration gradient. The mixed mobile phase was introduced into the inlet part of a Waters Assoc. M 6000 pump and delivered to the column (stainless steel, 300 × 4.2 mm I.D.) via a Waters Assoc. U6K injector.

The equipment described is able to reproduce the concentration gradient within the error limits of *ca.* 1 %, with no appreciable demixing of the gradient profile, and eliminates the errors caused by volume contractions connected with the mixing of the two liquid components comprising the concentration gradient. Further details will be provided elsewhere[11].

Two model sample mixtures were tested in this system. The first was composed of six barbiturates and the second contained a homologous series of four 3-alkyl-6-methyluracils. In isocratic experiments, the plots of log k' *versus* concentration of methanol (c) in the mobile phase were drawn for all sample compounds and the values of the parameters k'_0 and n of eqn. 2 were evaluated by linear regression analysis. While the semilogarithmic regression lines gave a good fit to the experimenal points for the barbiturates, the experimental data for the substituted uracils deviated significantly from a linear relationship and only the experimental points in the interval of $-0.4 < \log k' < 0.8$ could be used for regression analysis[11].

Using the parameters k'_0 and n evaluated for each compound, the conditions for stepwise elution were calculated with aid of eqns. 19, 2 and 20. The calculated values of the concentration of methanol in the mobile phase, c_i, in each step and the corresponding volumes of the mobile phase, V_i, necessary to achieve the resolution required were used for construction of the concentration gradient. In step 1, the elution of two compounds, and in each of the following steps the elution of one compound only was required, while the resolution is desired to remain constant for all compounds.

Practical separation experiments were performed under the calculated conditions and the retention characteristics from these experiments were compared with the values calculated using eqns. 18, 16 and 17. The results are shown in Tables I and II. The differences between the experimental and calculated retention volumes are within the limits of 0.25–0.3 ml, which represents a *ca.* 5% relative error for the barbiturates. The relative error for the V'_R values is higher for the substituted uracils due to their lower retention (and possibly also due to the poorer fit of the experimental data to eqn. 2). Most of the differences between the experimental and calculated peak widths do not exceed 10–15%, and the deviations in resolution are *ca.* 20%.

TABLE I

CALCULATION OF OPTIMUM CONDITIONS IN STEPWISE-ELUTION CHROMATOGRAPHY

Column: C_{18} Lichrosorb Si-100 (10 μm), 300 × 4.2 mm; V_m = 3.2 ml. Mobile phase: stepwise gradient, methanol–water; 0.97 ml/min. Compounds: A = barbital, n = 3.203, k'_0 = 21.81; B = heptobarbital, n = 3.711, k'_0 = 58.44; C = allobarbital, n = 3.547, k'_0 = 69.44; D = aprobarbital, n = 3.659, k'_0 = 106.96; E = butobarbital, n = 3.776, k'_0 = 187.41; F = hexobarbital, n = 3.766, k'_0 = 252.29, k'_0 and n are the parameters of eqn. 2 evaluated by linear regression analysis of the experimental $\log k' = f(c)$ plots (c = concentration of methanol in the mobile phase, % (v/v) × 10^{-2}). c_i (c in step i) and V_{ei} (volume of the mobile phase in step i) were calculated from eqns. 19, 2 and 20 for the separation of all compounds with R_s = 1.76. $N \approx 2330$ for all compounds (mean value from isocratic experiments). The expected values of V'_R, w and R_s were evaluated from eqns. 18, 16 and 17. Detection: UV, 254 nm, 0.32 a.u.f.s.

Step	c_i	V_{ei} *(ml)*	*Compound eluted*	V'_R *(ml)*		w *(ml)*		R_s	
				calc.	*exptl.*	*calc.*	*exptl.*	*calc.*	*exptl.*
1	0.52	2.37	A	1.48	1.54	0.39	0.39		
1	0.52	2 37	B	2.15	2.07	0.44	0.43	1.61	1.29
2	0.55	0.81	C	2.95	2.90	0.47	0.46	1.76	1.87
3	0.54	0.97	D	3.87	3.84	0.57	0.46	1.77	2.03
4	0.65	0.82	E	4.76	5.00	0.44	0.42	1.76	2.65
5	0.63	0.92	F	5.62	5.96	0.55	0.63	1.74	1.84

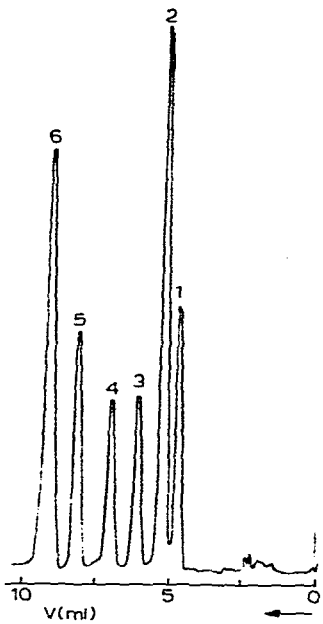
TABLE II

CALCULATION OF OPTIMUM CONDITIONS IN STEPWISE-ELUTION CHROMATOGRAPHY

Column: C_{18} Lichrosorb Si-100 (10 μm), 300 × 4.2 mm; V_m = 3.2 ml. Mobile phase: stepwise gradient, methanol–water; 0.93 ml/min. Compounds: A = 3,6-dimethyluracil, n = 4.399, k'_0 = 9.508; B = 3-ethyl-6-methyluracil, n = 3.690, k'_0 = 15.628; C = 3-*n*-propyl-6-methyluracil, n = 3.308, k'_0 = 29.978; D = 3-*n*-butyl-6-methyluracil, n = 3.246, k' = 67.205. Other operating conditions, and the meaning of symbols and methods of calculation, as in Table I. Resolution R_s = 1.94 required for all compounds; $N \approx 3350$ for all compounds (mean value from isocratic experiments).

Step	c_i	V_{ei} *(ml)*	*Compound*	V'_R *(ml)*		w *(ml)*		R_s	
				calc.	*exptl.*	*calc.*	*exptl.*	*calc.*	*exptl.*
1	0.51	0.79	A	0.18	0.43	0.23	0.24	1.94	1.68
1	0.51	0.79	B	0.66	0.85	0.27	0.26	1.94	2.23
2	0.66	0.52	C	1.18	1.43	0.27	0.26	1.94	2.53
3	0.75	0.53	D	1.70	2.15	0.27	0.31		

The experimental and calculated data seem to be in reasonable agreement, which supports the potential utility of the method. Figs. 2 and 3 show the chromatographic separation of sample mixtures under the calculated conditions of stepwise elution.



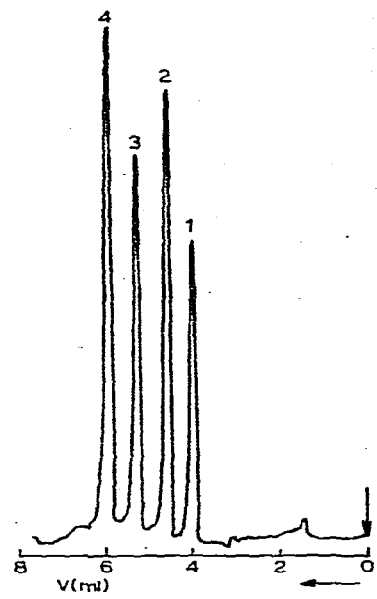


Fig. 2. Reversed-phase chromatographic separation of barbiturates by stepwise elution in water–methanol. Operating conditions as in Table I. Compounds: 1 = barbital; 2 = heptobarbital; 3 = allobarbital; 4 = aprobarbital; 5 = butobarbital; 6 = hexobarbital.

Fig. 3. Reversed-phase chromatographic separation of alkyluracils by stepwise elution in water–methanol. Operating conditions as in Table II. Compounds: 1 = 3,6-dimethyluracil; 2 = 3-ethyl-6-methyluracil; 3 = 3-*n*-propyl-6-methyluracil; 4 = 3-*n*-butyl-6-methyluracil.

In practical applications of this approach, the calculations should be performed with aid of a computer. Even small programmable desk and pocket calculators are suitable. All the calculations in this work were made using a TI-58 programmable pocket calculator (Texas Instruments) with a maximum program capacity of 480 steps. A program was written for this calculator, which makes possible calculations of elution conditions for stepwise elution in a maximum of 14 steps. The time required to perform a step-by-step calculation of the elution conditions for a sample mixture is only a few minutes.

APPENDIX

During the final preparation of this paper, two reports by Borówko *et al.*[12,13] appeared on the same topic. In many ways, their optimization approach is similar to ours. The principal difference between the two approaches consists in a different way of defining the respective portions of the column, V_{mi}, which leads to a considerably more complex solution for the model of Borówko *et al.*

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