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# MULTIFACTOR MODEL FOR THE OPTIMIZATION OF SELECTIVITY IN REVERSED-PHASE CHROMATOGRAPHY

MATTHIAS OTTO* and WOLFHARD WEGSCHEIDER*
*Institute for Analytical Chemistry, Micro- and Radiochemistry, Technical University of Graz, Technikerstrasse 4, 8010 Graz (Austria)*

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## SUMMARY

Three variables characterizing the mobile phase composition, pH, elution strength and ionic strength, have been studied in order to construct a three-dimensional semiempirical model for predicting retention times of dibasic substances. The solutes treated quantitatively include dibasic acids and bases, an amino acid and two dipeptides. Experimental effort was minimized by arranging them as 6 × 3 × 2 factorial design and deriving the coefficients of the model with a variable projection algorithm that separates linear from non-linear parameters. The coefficients are then used to predict capacity factors, $k'$, and relative retentions, $\alpha$, for all solute pairs in a computerized grid search. Within the limits of the model, it is an easy task to reduce the grid size to calculate all combinations of 25 pH, 20 elution strength and 10 ionic strength values. The predicted optimal selectivity was verified experimentally and the experimental retention data found to be in good agreement with the computed retention times.

## INTRODUCTION

The difficulties in optimizing chromatographic separations frequently arise from the existence of multiple optima over the domain of factor space. This is particularly relevant in high-performance liquid chromatography (HPLC) where the retention order is affected by a large number of mobile phase variables such as pH, elution strength, concentration of surface-active ions, ionic strength or temperature.

Lacking a full understanding of specific solvation effects one has to consider the widespread interactions between the various factors, so that the attainment of the "global" optimum is not guaranteed by varying one factor at a time. Systematic studies covering the entire domain of experimental variables are not only hampered by the need to select the correct increment grid size for each variable, but lead in general also to an unmanageable workload prior to the analytical routine.

* On leave from: Department of Chemistry, Analytical Centre, Karl Marx University, Liebigstrasse 18, 7010 Leipzig, G.D.R.

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Conceptually, all optimizations can be regarded as two-stage processes: in the first stage a quantitative definition of what is to be regarded as optimal, of how the optimum is quantified, is made, while in the second stage an attempt is made to locate exactly the coordinates of the optimum in the space of the experimental variables. The problem of a quantitative definition that also encompasses aspects of total analysis time was discussed previously[1]. In the present paper emphasis is placed on the estimation of elution times as this variable can be used to predict selectivity factors as well as the resolution[2] and total analysis time. So far, optimization in reversed-phase HPLC of ionogenic substances based on semi-empirical models has been restricted to the simultaneous dependence on two factors at the most, these being either solvent strength and the concentration of an ion interacting reagent (IIR)[3,4] or the pH of the mobile phase and the concentration of an IIR[5,6].

Numerous mechanistic and semiempirical models for description of the elution of ionogenic substances have been described, linking retention to pH[7–9], solvent strength[10,11], ionic strength[12], silanophilic interactions[13–14], concentration of IIRs[15,16] and solvent properties like proton acceptor or donor strength and dipole moment[17–19]. It is, however, very rare indeed that any of these models can replace optimization of a particular separation problem. This is not to say that one should overlook the wealth of experimental variables that may aid in finding a solution to a separation problem, but a semiempirical or even mechanism-based approach including many variables in one set of experiments designed to lead to a satisfactory separation would be impractical.

We present a semiempirical model for description of the retention behaviour of diprotic species in three-variable space, pH, methanol content and ionic strength. The solutes have been chosen to include an amino acid with a low second dissociation constant relative to the pH limit imposed by the column support, two dipeptides as an example of molecules forming zwitterions, a weak diprotic acid and three isomeric aminobenzoic acids with an uncharged intermediate. This choice, although somewhat arbitrary by necessity, nevertheless includes one species of several ionogenic types of molecules that are routinely separated by reversed-phase HPLC, exhibiting retention characteristics quite opposite to each other in the variables under study. Thus it is possible to show how the general approach is used to derive, from generally accepted principles of acid–base equilibria, species-specific modifications that lead to consistent estimates of retention times. These are then employed to predict numerically, with suitably small resolution in the experimental variables, all selectivity factors, the smallest of which can be used to define a hypersurface whose maximum is indicative of the chromatographic conditions giving the highest selectivity.

## EXPERIMENTAL

### *Chromatographic system*

For the HPLC experiments a Waters 6000 M pump, an automatic injection system Waters WISP 710 A and a Waters UV detector (M 440) were applied. Digitizing of retention data was achieved with the Waters data module and the Waters system controller 720. For automated runs, a home-made device for selecting among eight mobile phases was controlled by the system controller unit. LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.), particle size 7 $\mu$m, was packed into a stainless-steel

column (150 × 3.2 mm I.D.) by a slurry technique. The column was operated at 22°C. Flow-rates were maintained at 1.0 ml $min^{-1}$. From injections of KBr solutions, the time equivalent to the void volume was found to be $t_0 = 0.75$ min.

*Mobile phases*

Mobile phases were prepared from reagent grade methanol (Merck) and aqueous potassium hydrogen phosphate solutions giving a final buffer concentration of 15 m*M*. The ionic strength was kept constant by addition of potassium chloride, taking into account the ionic contributions from the buffer species at different pH values. The pH of the aqueous methanol solutions was adjusted pH-metrically and corrected for by use of the $\gamma$ values according to Bates *et al.*[20].

All mobile phases were aspirated through a 0.45-$\mu$m Sartorius 11306 filter and degassed in an ultrasonic bath before use.

*Samples*

The chemicals used were analytical reagent grade from Fluka (Buchs, Switzerland) or Merck. The dipeptides L-leucyl-L-tyrosine, D-leucyl-L-tyrosine and L-tyrosine were purchased from Sigma (St. Louis, MO, U.S.A.).

*Experimental design and computations*

A three-factor 6 × 3 × 2 level factorial design was used for adjusting the 36 different mobile phases to all combinations of six pH values (2.0, 3.0, 4.0, 5.0, 6.0, 7.0), three methanol contents (10, 20, 30%, v/v) and two ionic strengths (0.1 and 0.2 *M*). The run order was randomized with respect to cost (minimizing the risk to ruin the column by excessively frequent large changes of parameters) and time according to Joiner and Campbell[21].

The one-dimensional fits of pH dependences were based on measurements of six additional mobile phases, the results of which have been included in the final three-factor model computations.

For fitting the experimental data, two computer programs for multiple non-linear least squares estimates were applied: a standard routine based on the Marquardt algorithm[22] and a program for solving problems whose variables can be separated utilizing a variable projection algorithm[23]. The programs were written in FORTRAN IV and run on an UNIVAC 1100 computer.

The generation of asymmetric peaks were carried out with the help of an HP 97 calculator (Hewlett-Packard, Loveland, CO, U.S.A.). Pseudo-three-dimensional response surfaces were drawn from digitized data on a Model 9862 A calculator-plotter connected to a Model 9830 digital computer (both from Hewlett-Packard).

## RESULTS AND DISCUSSION

*Dependence on single factors*

The influence of pH on the retention of each of the eight solutes is shown in Fig. 1. Some of the dependences exhibit two inflection points in the studied pH range, arising from consecutive protolysis equilibria. In order to predict this behaviour by a mathematical model all acid–base equilibria as well as partition of all ionized forms of the solutes have to be considered. A general expression for the description of the

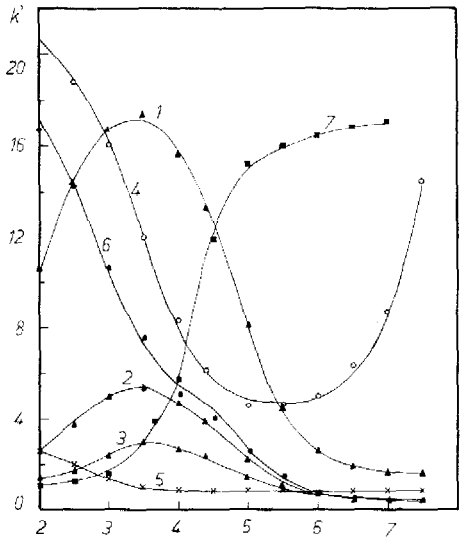


Fig. 1. Influence of pH on solute retention at 10% (v/v) methanol and ionic strength 0.1 *M*. Solutes: 1 = anthranilic acid; 2 = *p*-aminobenzoic acid; 3 = *m*-aminobenzoic acid; 4 = L-leucyl-L-tyrosine; 5 = L-tyrosine; 6 = phthalic acid; 7 = dimethylaminoantipyrine.

capacity factor, $k'$, for zwitterionic solutes (L-leucyl-L-tyrosine, D-leucyl-L-tyrosine and L-tyrosine) or aminobenzoic acids (anthranilic acid, *m*-aminobenzoic acid and *p*-aminobenzoic acid) as a function of pH is given by[7,8]

$$k' = \frac{k_0 + k_1 \cdot \frac{[H^+]}{K_{a_1}} + k_{-1} \cdot \frac{K_{a_2}}{[H^+]}}{1 + \frac{[H^+]}{K_{a_1}} + \frac{K_{a_2}}{[H^+]}} \quad (1)$$

where $k_0$, $k_1$, $k_{-1}$ represent the (modified) distribution coefficients for the species HS (undissociated), $H_2S$ (protonated) and S (deprotonated), respectively, and $K_{a_1}$, $K_{a_2}$ are the consecutive acid dissociation constants. (The dissociation of the hydroxy group of tyrosine can be neglected in the studied pH range since $pK_{OH} > 9.0$.) This model (eqn. 1) holds irrespective of the charge on any of the three species, and thus also for the diprotic phthalic acid (species $H_2S$, $HS^-$, $S^{2-}$). For fitting the base 4-dimethylaminoantipyrine only one dissociation constant has to be considered, the two forms being $HS^+$ and S.

The validity of these models was checked by computing the linear and non-linear parameters with two programs, a Marquardt algorithm-based routine[22] and a variable projection algorithm[23]. Initially, deviations from theoretical behaviour were indicated at pH values lower than 3.0 for dipeptides and at pH > 6.0 for dimethylaminoantipyrine. Inspection of the recorded chromatograms revealed the existence of asymmetric peaks at these pH values explained by the low methanol content (10%, v/v). In order to obtain a more correct measure of retention time than the retention of the maximum, the first statistical moment (the mean) was evaluated graphically from

generated normalized asymmetric peaks using a Gram–Charlier polynomial[26], the procedure being similar to that recently proposed by Barber and Carr[27]. The best parameter estimates are compiled in Table I. No remarkable differences were found in parameters evaluated with the different programs; the data are therefore reported only for computations with Golub's program[23].

The good agreement between the fitted model and the experimental data can be deduced from the residuals, $s$, in Table I and from Fig. 1 where the curves shown were calculated using the parameters in Table I. The residuals, which include random effects (errors in the determination of $k'$) as well as inadequacies in the model, are computed as the square root of the mean square deviations between measured and estimated $k'$ values.

At present, the effect of organic modifiers in the mobile phase on retention behaviour is frequently described by linear plots of log $k'$ *vs.* [%M], where [%M] is volume per cent methanol[28,29]. Deviations from linearity have been explained in terms of silanophilic interactions[13,14], conformational changes of the solute[30] and changes in secondary equilibria[31].

In our case linear dependences could be observed only at selected pH values where one protolytic form is dominant. In the absence of other effects in the studied range of elution strength and molecular size, it was thought appropriate to attribute the non-linearity primarily to a shift of the secondary equilibria: different ionic species are present whose relative abundance is dependent on the value of the protolysis constants which in turn are affected by the methanol content of the mobile phases. For this reason the effect of organic modifier on the value of the protolysis constants had to be taken into account in the retention model (see below). Linear interpolations between experimental data in two-factor space (pH and methanol content) are presented in Fig. 2 for phthalic acid, L-leucyl-L-tyrosine and anthranilic acid.

In order to correct the retention data for the effect of ionic strength, $I$, different equations have been tested for calculating activities in the mobile phases. As previously shown by Van de Venne *et al.*[12], good agreement between experimental and calculated data was achieved by use of the Davies equation[32]

$$\log k' = \log k'_0 - \frac{Az^2 I^{1/2}}{1 + I^{1/2}} + 0.04\, z^2 I \qquad (2)$$

where $z$ is the charge on the solute, $k'_0$ the capacity factor at zero ionic strength and $A$ is a constant known to be 0.512 at 25°C[12,31].

Further corrections, *e.g.*, for the influence of methanol on activities[18], made by use of more sophisticated expressions such as those of Horváth *et al.*[15], have been omitted as they would fall within experimental error.

*Three-factor numerical model*

The problem of combining the influences of different factors lies in the fact that the model must account for the partition and protolysis equilibria of all forms of the solute and their dependence on methanol content and ionic strength.

As the base for a three-dimensional model the pH dependence in eqn. 1 was

TABLE I

ESTIMATED PARAMETERS FOR PREDICTION OF CAPACITY FACTOR DEPENDENCE ON pH

Methanol content: 10%. Ionic strength: 0.1 *M*.

| *Solute* | $k_0$ | $k_1$ | $k_{-1}$ | $K_{a_1}$ (*M*) | $K_{a_2}$ (*M*) | $s$ |
|---|---|---|---|---|---|---|
| Anthranilic acid | 18.53 ± 0.27 | 4.76 ± 1.74 | 1.60 ± 0.13<br>lit. 24* | $(7.34 \pm 2.07) \cdot 10^{-3}$<br>$1.00 \cdot 10^{-2}$ | $(1.67 \pm 0.11) \cdot 10^{-5}$<br>$1.62 \cdot 10^{-4}$ | 0.242 |
| *m*-Aminobenzoic acid | 3.55 ± 0.17 | 1.18 ± 0.12 | 0.55 ± 0.04 | $(1.15 \pm 0.32) \cdot 10^{-3}$ | $(2.38 \pm 0.40) \cdot 10^{-5}$ | 0.138 |
| *p*-Aminobenzoic acid | 6.05 ± 0.07 | 1.17 ± 0.18 | 0.35 ± 0.03 | $(3.93 \pm 0.45) \cdot 10^{-3}$ | $(2.20 \pm 0.10) \cdot 10^{-5}$ | 0.111 |
| L-Leucyl-L-tyrosine | 4.31 ± 0.10 | 20.16 ± 0.30 | 27.02 ± 3.06<br>lit. 25* | $(3.32 \pm 0.35) \cdot 10^{-4}$<br>$6.31 \cdot 10^{-4}$ | $(2.51 \pm 0.11) \cdot 10^{-8}$<br>$1.86 \cdot 10^{-8}$ | 0.252 |
| D-Leucyl-L-tyrosine | 12.17 ± 0.30 | 93.68 ± 29.05 | 23.99 ± 15.4<br>lit. 25 | $(1.45 \pm 1.01) \cdot 10^{-3}$<br>$1.45 \cdot 10^{-3}$ | $(4.79 \pm 0.97) \cdot 10^{-9}$<br>$4.79 \cdot 10^{-9}$ | 0.393 |
| Phthalic acid | 5.16 ± 0.22 | 18.99 ± 0.50 | 0.30 ± 0.16<br>lit. 24 | $(1.67 \pm 0.87) \cdot 10^{-3}$<br>$1.78 \cdot 10^{-3}$ | $(1.17 \pm 0.22) \cdot 10^{-5}$<br>$1.17 \cdot 10^{-5}$ | 0.182 |
| L-Tyrosine | 0.97 ± 0.13 | 3.24 ± 0.57 | 0.72 ± 0.11<br>lit. 24 | $(4.10 \pm 2.80) \cdot 10^{-3}$<br>$6.79 \cdot 10^{-3}$ | $(9.12 \cdot 10^{-10})$<br>$9.12 \cdot 10^{-10}$ | 0.200 |
| 4-Dimethylamino-antipyrine** | 17.09 ± 0.43 | 0.84 ± 0.48 | – | $(5.46 \pm 1.05) \cdot 10^{-5}$ | – | 0.254 |

* $I$ = 0–1 *M*; 25°C.

** At 30% methanol.

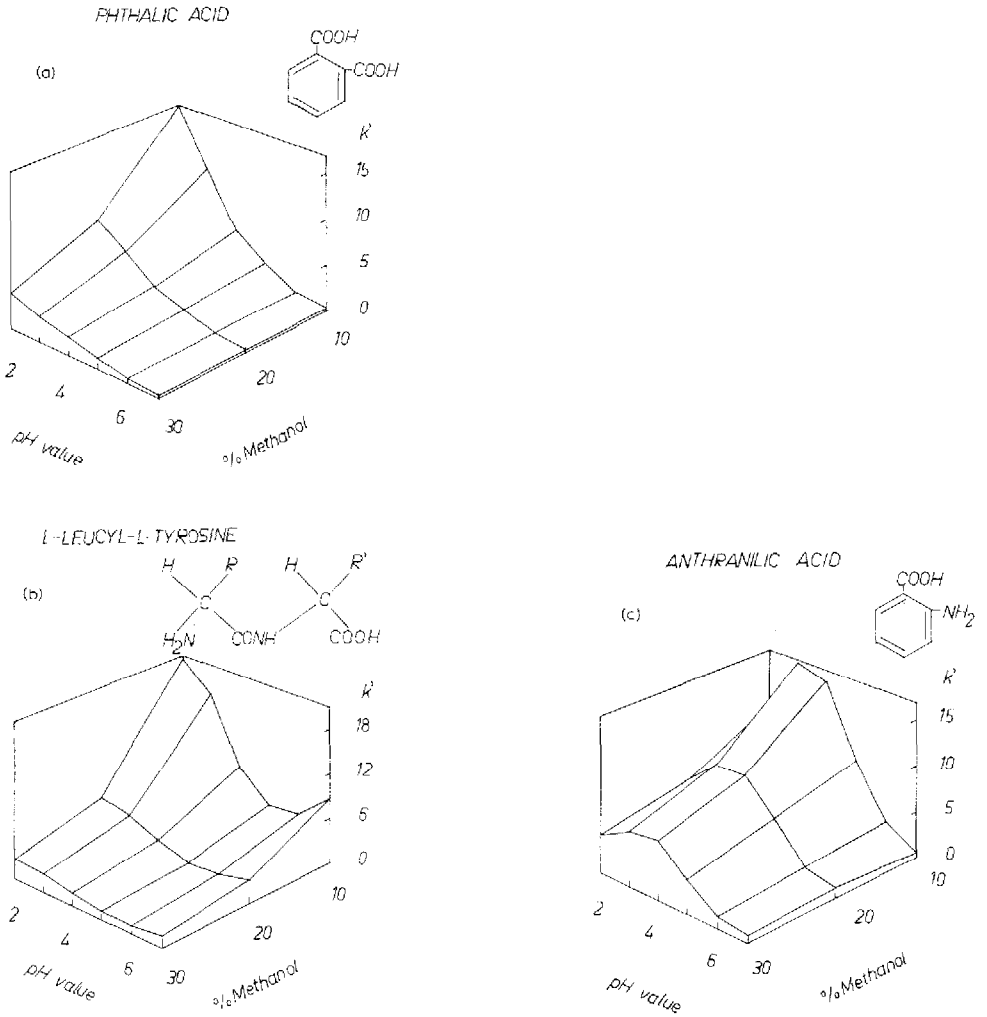


Fig. 2. Combined effect of pH and methanol content on retention of dibasic compounds existing in different ionized forms. R = $-CH_2-CH(CH_3)_2$; R' = $-CH_2-C_6H_4-OH$.

used. The effect of methanol on the retention of the different ionized forms of the solutes may be expressed as follows (*cf.*, ref. 3)

$$k_0 = C_0(F_1 + F_2 \cdot e^{-K_3[\%M]}) \tag{3}$$

$$k_1 = C_1(F_3 + F_4 \cdot e^{-K_4[\%M]}) \tag{4}$$

$$k_{-1} = C_{-1}(F_5 + F_6 \cdot e^{-K_5[\%M]}) \tag{5}$$

where, $F_1$, $F_3$, $F_5$ describe the translation along the $k'$ axis, the linear constants $F_2$, $F_4$ and $F_6$ represent the slope of the exponential function for HS, $H_2S$ and S, respective-

ly, and the non-linear constants $K_3$, $K_4$ and $K_5$ reflect the curvature in the $k'$ *vs.* [%M] plot. The constants $C_0$, $C_1$ and $C_{-1}$ are ionic strength corrections with respect to all species participating in the partition equilibrium, and are calculated separately for each solute; *e.g.*, for a solute in the ionic form $H_2S^+$ the constant $C_1$ is:

$$C_1 = 10^{\left[\frac{1.024\, I^{1/2}}{1 + I^{1/2}} - 0.08\, I\right]}$$

Also the protolysis constants, $K_{a_1}$ and $K_{a_2}$ (eqn. 1), are dependent on the ionic strength and methanol content and are fitted by a factor $P$

$$K_{a_{1,2}} = K^0_{a_{1,2}} P_{1,2} \tag{6}$$

where $K^0_{a_{1,2}}$ are the protolysis constants at zero ionic strength and 100% water as the mobile phase; $P_{1,2}$ accounts for ionic strength according to the Davies equation (*cf.*, eqn. 2) and for the influence of the organic modifier as follows

$$P_1 = 10^{\left\{\frac{-0.512\,(\Sigma z_i^2)\, I^{1/2}}{1 + I^{1/2}} + 0.04\,(\Sigma z_i^2) I + K_6\,[\%\mathrm{M}]\right\}} \tag{7}$$

$$P_2 = 10^{\left\{\frac{-0.512\,(\Sigma z_i^2)\, I^{1/2}}{1 + I^{1/2}} + 0.04\,(\Sigma z_i^2) I + K_7\,[\%\mathrm{M}]\right\}} \tag{8}$$

where $K_6$ and $K_7$ describe the dependence of the protolysis constants on the content of organic modifier.

Finally, the dependence of the overall capacity factor, $k'$, on pH, [%M] and ionic strength is described by six linear parameters ($F_1$ to $F_6$) and seven non-linear parameters ($K_1$ to $K_7$). Fits to the complete model for seven of the studied solutes (4-dimethylaminoantipyrine cannot be investigated at methanol contents lower than 30% because of strong asymmetric retention behaviour) with Golub's program[23] gave good agreement between model and experimental data; however, some of the parameters were estimated only with very low precision or their values were physically meaningless. This was due either to high correlations between parameters or to the fact that some ionic forms of the solutes contribute too little to the overall $k'$ value to be modelled by the whole set of constants. Thus, different reduced models have been tested and the fitted parameters are given in Table II.

It is important to realize that clues to the redundance of some of the factors are offered by the software itself and that physical understanding leads to a decision as to which of two highly correlated parameters is to be omitted. From Table II it is evident that for the same type of solute, *e.g.*, anthranilic acid, *m*-aminobenzoic acid or *p*-aminobenzoic acid, the same model is valid. In the case of aminobenzoic acids the neutral HS-form is the most strongly retained species (*cf.*, Fig. 1) so that the retention behaviour of the forms $H_2S^+$ and $S^-$ can be modelled with the reduced set of parameters (Table II). The same is true for the zwitterionic dipeptides and, in general, for tyrosine and phthalic acid. Apart from variables omitted because they describe ionic forms whose presence in the pH range studied can be neglected, the variables most

TABLE II

PARAMETER ESTIMATES FOR THE THREE-FACTOR MODEL

| *Parameter* | *Anthranilic acid* | *m-Aminobenzoic acid* | *p-Aminobenzoic acid* | *L-Leucyl tyrosine* | *D-Leucyl tyrosine* | *Tyrosine* | *Phthalic acid* |
|---|---|---|---|---|---|---|---|
| $F_1$ | 3.16 ± 0.51 | 1.25 ± 0.27 | 1.15 ± 0.22 | – | – | 0.28 ± 0.18 | – |
| $F_2$ | 32.26 ± 0.81 | 5.18 ± 0.91 | 13.78 ± 1.00 | 9.47 ± 0.66 | 37.22 ± 3.02 | 0.95 ± 0.08 | 4.72 ± 0.95 |
| $F_3$ | – | – | – | 1.37 ± 0.18 | 1.61 ± 0.45 | 0.50 ± 0.13 | 2.87 ± 1.98 |
| $F_4$ | 3.84 ± 0.98 | 0.24 ± 0.26 | 0.48 ± 0.38 | 52.23 ± 5.51 | 72.08 ± 9.08 | 2.97 ± 0.69 | 52.34 ± 5.88 |
| $F_5$ | – | – | – | – | – | – | 0.045 ± 0.021 |
| $F_6$ | 1.46 ± 0.18 | 0.42 ± 0.09 | 0.28 ± 0.06 | 71.13 ± 14.20 | 34.77 ± 15.96 | – | – |
| $pK_{a_1}^0$ | 2.02 ± 0.06 | 2.08 ± 0.10 | 2.26 ± 0.11 | 3.21 ± 0.05 | 3.17 ± 0.07 | 2.46 ± 0.26 | 2.49 ± 0.11 |
| $pK_{a_2}^0$ | 4.42 ± 0.05 | 4.49 ± 0.15 | 4.27 ± 0.10 | – | – | – | 4.26 ± 0.05 |
| $K_3$ | 7.39 ± 0.41 | 10.34 ± 2.56 | 10.13 ± 0.91 | 7.27 ± 0.57 | 11.13 ± 0.75 | 5.34 ± 2.79 | 5.52 ± 1.27 |
| $K_4$ | 2.32 ± 0.78 | −0.82 ± 2.74 | −0.69 ± 2.38 | 16.40 ± 1.02 | 13.75 ± 1.09 | 12.59 ± 3.16 | 11.21 ± 1.74 |
| $K_5$ | 4.81 ± 0.90 | 2.65 ± 1.28 | 1.59 ± 1.27 | 13.45 ± 1.95 | 4.32 ± 4.03 | – | – |
| $K_6$ | −1.48 ± 0.21 | −2.00 ± 0.39 | −1.28 ± 0.58 | – | – | 1.62 ± 1.61 | −0.06 ± 0.32 |
| $K_7$ | −1.35 ± 0.29 | −0.63 ± 1.15 | −1.40 ± 0.68 | – | – | −5.77 ± 2.56 | −1.54 ± 5.47 |
| $s$ | 0.171 | 0.141 | 0.130 | 0.225 | 0.333 | 0.090 | 0.245 |

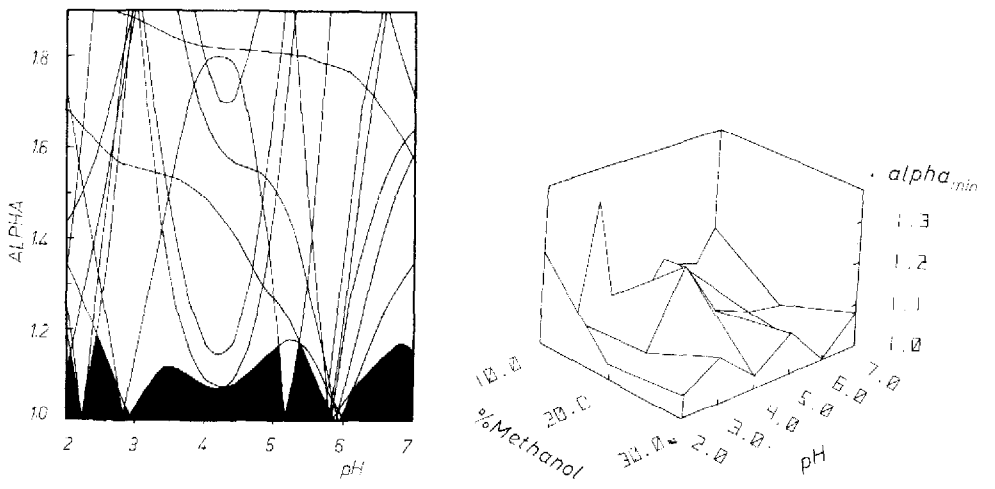


Fig. 3. Window diagram as a function of pH for the seven-component mixture at 20% methanol and ionic strength 0.1 *M*.

Fig. 4. Two-factor window diagram for the seven-component mixture at fixed ionic strength (0.1 *M*).

often deleted are the offset terms of the $k'$ *vs.* elution strength relationship, *i.e.*, $F_1$, $F_3$, $F_5$.

The parameters in Table II enable one to calculate the $k'$ values of each solute at pH values from 2.0 to 7.0, at 10–30% methanol content and at ionic strengths between 0.1 and 0.2 *M*.

*Optimization: the search for the global optimum*

As an initial attempt, the relative retention values, $\alpha$, for pairs of all seven compounds calculated with the one-dimensional model (eqn. 1) were plotted against pH giving so-called window diagrams[33]. Fig. 3 shows such a diagram at 20% (v/v) methanol and an ionic strength of 0.1 *M*. The aforementioned existence of several

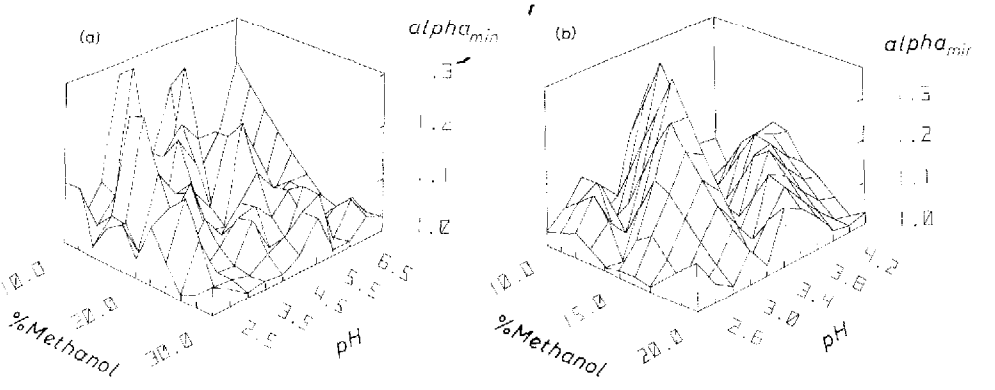


Fig. 5. Minimum alpha plots for the seven-component mixture at optimal ionic strength. Step-widths: a, $\Delta$pH = 0.5, $\Delta$[%M] = 2%; b, $\Delta$pH = 0.2, $\Delta$[%M] = 1%.

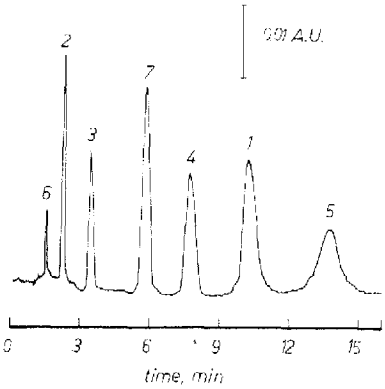


Fig. 6. Chromatogram of the seven-component mixture under optimal conditions. Peak numbers refer to the solutes in Table III.

local optima is relevant also in the present separation problem and no superior window can be selected from the figure.

Even two-dimensional minimum alpha plots[3] constructed from experimental data did not reveal a superior global optimum as shown in Fig. 4 at fixed ionic strength. Thus, an exhaustive search for the optimum in all three dimensions was undertaken by computerized grid search[22] with the following step-widths: $\Delta$pH = 0.1; $\Delta$[%M] = 2%; $\Delta I$ = 0.01 $M$. The optimum chromatographic performance as determined by the $\alpha$ value of the least resolved pair ($\alpha$ = 1.375) was found at pH = 3.20, 14% (v/v) methanol and ionic strength 0.18 $M$. Minimum alpha plots at optimal ionic strength (Fig. 5) demonstrate that the global optimum could be estimated only with step-widths as small as 0.2 pH units and 1% methanol content.

A chromatogram under optimum conditions is shown in Fig. 6 and the measured retention data are compared to the theoretically expected values in Table III. The agreement between experimental and theoretical retention data is within experimental error (standard deviations calculated from six parallel chromatograms). Thus the presented three-factor model is quite useful for computer location of the global optimum.

TABLE III

COMPARISON OF EXPERIMENTAL AND COMPUTED RETENTION DATA AT OPTIMUM CHROMATOGRAPHIC PERFORMANCE

| *No.* | *Solute* | $k'$ | |
|---|---|---|---|
| | | *Predicted* | *Experimental* |
| 1 | Anthranilic acid | 13.50 | 13.44 ± 0.12* |
| 2 | *m*-Aminobenzoic acid | 2.19 | 1.92 ± 0.05 |
| 3 | *p*-Aminobenzoic acid | 3.90 | 3.56 ± 0.62 |
| 4 | L-Leucyl-L-tyrosine | 9.78 | 9.55 ± 0.24 |
| 5 | D-Leucyl-L-tyrosine | 18.60 | 18.30 ± 0.36 |
| 6 | L-Tyrosine | 0.94 | 0.92 ± 0.04 |
| 7 | Phthalic acid | 6.84 | 6.68 ± 0.11 |

* Standard deviation from six determinations.

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