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OPTIMIZATION IN ISOTACHOPHORESIS

THE CONCEPT OF SELECTIVITY AND SEPARATION SPEED

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SUMMARY

A general approach to the optimization of isotachophoretic separations of two-component samples is given. It is shown that this optimization may be performed in a correct way by simple experiments and should be based on two quantities: selectivity and separation speed. The former is defined as the relative difference of the effective mobilities of the separands in the mixed zone and expresses the maximum relative separation volume; the latter expresses the volume velocity of separation. The optimum conditions for a separation may be found from the experimental dependences of selectivity and separation speed on pH and concentration of the leading electrolyte.

INTRODUCTION

The first step in an analytical isotachophoretic problem is usually aimed at finding an electrolyte system with which the substances in question are separable. This selection is based on approximation rules, experience, the nature of the substances to be separated and tabulated data, if available.

The experimental investigation of the separability of substances in a selected electrolyte system is usually performed by calibration with standard model mixtures and by following the qualitative character of the isotachopherogram[1]. The theoretical approach is based on an understanding[2] of separability as the existence of an unambiguous migration order of all zones which may be expressed in the terms[3] of the parameters of the $\bar{u}_{i,j}$ type (effective mobility of a substance i in zone j).

When the separability of substances is achieved in principle, another analytical problem arises concerning quantitation. Here, the absolute amount of the sample that we are able to separate (as expressed by the concepts of the separation[4] and/or load[5] capacity) is not of importance, but rather the amount that provides sufficiently long zones even for minor components in order to ensure satisfactory quantitative analysis.

For this amount of sample, in the selected system complete resolution of all zones must be obtained. Usually this is not the case and the system must be optimized

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either by changing its pH or concentration or in another, more elaborate, way. When acid–base equilibria, for example, are involved, then maximum resolution is provided[5] by a high pH for cations and a low pH for anions.

For optimization, in practice the effective mobilities (or proportional detection signals) of the separands in their zones are commonly measured[6] in several systems and the maximum difference in their values is taken as the optimum. In the same way, computer simulation[7,8] of isotachopherograms has been used instead of experimental measurement. Although in most instances the above approach leads to the required separation, it may fail in some instances[3] because, as shown by Mikkers *et al.*[5], it is not the difference but the ratio of the effective mobilities that controls the separation and, moreover, the effective mobilities must relate to the transient-state mixed zone. Unfortunately, no direct method is available for measuring the effective mobilities in a mixed zone at present, and laborious experiments are frequently necessary in order to find the optimum.

When the separability and the quantitation are ensured, an effort is made to minimize the analysis time, especially if the investigated separation is scheduled to be used as a routine analytical procedure. No universal method for achieving the minimum analysis time is available at present. Usually, a higher driving current and the use of a less mobile counter ion can help, but the level of separation and quantitation already attained may then deteriorate.

The aim of this paper is to give objective factors that can be used for optimization and are available from simple experiments. The proposed procedures deal with the optimization of the analysis of two-component samples. However, we believe that by selecting suitable pairs of components that are difficult to separate, analyses of multi-component samples may also be optimized.

THEORETICAL

For an isotachophoretic system, the basic condition $E_i \bar{u}_{i,i}$ = constant holds[9], where E_i is the electric potential gradient in zone i. Assuming that the column cross-section and driving current (I) vary with the longitudinal coordinate and time, respectively, its more general form derived from the moving boundary equation may be stated:[10]

$$\frac{\bar{u}_{i,i}}{\kappa_i} = \text{constant} = W = \frac{w}{I} = \frac{V_L}{Q_L} \tag{1}$$

where κ_i is the conductivity of zone i, V_L is the volume of the separation column filled with the leading electrolyte and Q_L is the amount of electricity needed to pass through the column in order to bring the rear boundary of the leading zone into the detector (the column hold-up[4]); w is the volume velocity of migration of the isotachophoretic boundaries. The constant W expresses the volume covered by the movement of the isotachophoretic zone boundary per coulomb of electricity passed. It is equal to the ratio V_L/Q_L and hence is independent of both the column geometry and the electrical operating conditions.

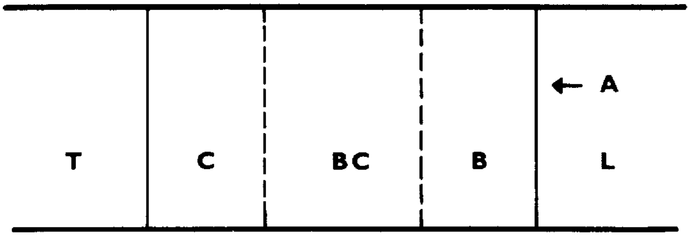


Fig. 1. Scheme of the separation system.

The concept of selectivity

Let us consider the isotachophoretic system shown in Fig. 1, where between the leading electrolyte (L and A) and the terminator (T) a sample of two substances B and C is placed and the composition of the mixed zone is already adjusted to the leading electrolyte.

If the substances B and C have different effective mobilities in this transient-state mixed zone, then the separation of B and C proceeds in the already well described way[9]. Fig. 2a shows the scheme of the initial state where the fixed detection point and the volumes occupied by the leading electrolyte and the mixed zone of B and C are demarcated.

Let us assume that substance B is the more mobile one in the mixed zone, *i.e.*,

$$\bar{u}_{B,BC} > \bar{u}_{C,BC} \tag{2}$$

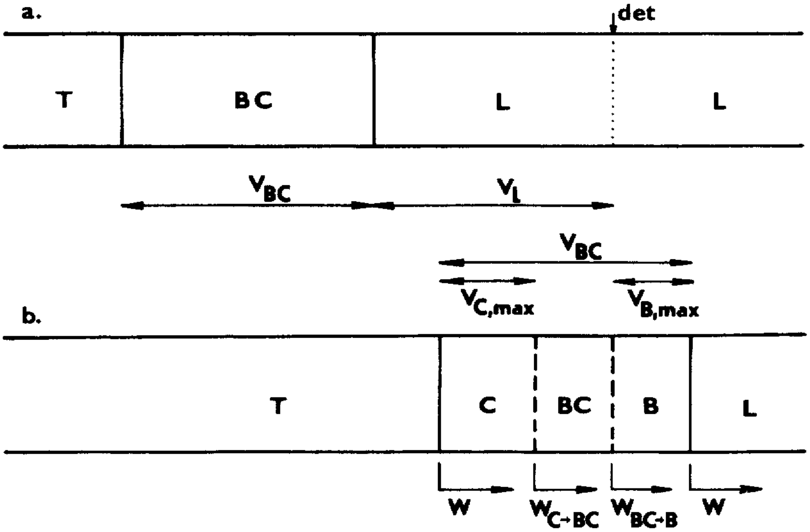


Fig. 2. Scheme of the separation process. (a) In the initial state the mixed zone BC of volume V_{BC} is placed between the leading zone L and terminating zone T. The volume of the leading electrolyte between the detection point "det" and zone BC is V_L. (b) At the end of the analysis the boundary BC → B is in the detector. The (maximum) separated volumes of pure zones of B and C are $V_{B,max}$ and $V_{C,max}$, respectively. The charge-based volume velocities of migration of the isotachophoretic (W) and transient-state separation boundaries ($W_{C \to BC}$, $W_{BC \to B}$) are also shown.

where the mobility values are taken as positive and the subscript BC relates to the transient-state mixed zone of B and C. Under these conditions, the zone of pure B is formed in front of the mixed zone and the zone of pure C is formed behind the mixed zone. Fig. 2b shows the situation at the maximum useful resolution of B and C, *i.e.*, when the boundary BC → B reaches the detector. The maximum resolved volumes of pure B and C are $V_{\mathrm{B,max}}$ and $V_{\mathrm{C,max}}$, respectively.

The charge-related volume velocities of the boundaries C → BC and BC → B are obviously different from the value $W = V_{\mathrm{L}}/Q_{\mathrm{L}}$ (see eqn. 1 and Fig. 2b) and it can be shown that

$$W_{\mathrm{BC}\rightarrow\mathrm{B}} = \frac{\bar{u}_{\mathrm{C,BC}}}{\kappa_{\mathrm{BC}}}; \qquad W_{\mathrm{C}\rightarrow\mathrm{BC}} = \frac{\bar{u}_{\mathrm{B,BC}}}{\kappa_{\mathrm{BC}}} \tag{3}$$

For the total amount of electricity needed to perform the analysis, *i.e.*, to bring the boundary BC → B into the detector, $Q_{\mathrm{L}} + Q_{\mathrm{B,max}}$, the following relationship may be written (see eqn. 1):

$$\begin{aligned} Q_{\mathrm{L}} + Q_{\mathrm{B,max}} &= \frac{V_{\mathrm{L}} + V_{\mathrm{B,max}}}{W} = \frac{V_{\mathrm{B,max}} + V_{\mathrm{C,max}}}{W_{\mathrm{C}\rightarrow\mathrm{BC}} - W_{\mathrm{BC}\rightarrow\mathrm{B}}} \\ &= \frac{V_{\mathrm{B,max}}}{W - W_{\mathrm{BC}\rightarrow\mathrm{B}}} \end{aligned} \tag{4}$$

After substitution from eqn. 3, elimination of W and κ_{BC} and rearrangement, we obtain

$$\frac{V_{\mathrm{B,max}} + V_{\mathrm{C,max}}}{V_{\mathrm{L}}} = \frac{\bar{u}_{\mathrm{B,BC}}}{\bar{u}_{\mathrm{C,BC}}} - 1 = p \tag{5}$$

This relationship shows that the maximum separable amount of a binary mixture expressed as the sum of the volumes of the zones of pure substances (equal to the volume of the adjusted transient-state mixed zone corresponding to this amount) related to the volume of the leading electrolyte used is equal to the relative difference in the effective mobilities of the two substances. We can therefore express the value of the maximum relative separation volume as a separation parameter, p, representing the selectivity between the separands ($\Delta\bar{u}/\bar{u}$), as defined by Giddings[11] for zone electrophoresis.

When considering a column of constant cross-section, the volumes in eqn. 5 may be replaced with zone lengths. The relationship so obtained confirms the equation $\Delta l/l = \Delta u/u$ postulated by Martin and Everaerts[12] and shows that the effective mobilities in the mixed zone should be applied in such a way that p is always positive.

The concept of selectivity introduced in the above way has two unique features that make it very suitable for use in optimization procedures:

(i) in an ideal case it is independent of the working conditions (particularly of the driving current) and equipment (column and power supply) used and it involves the appropriate influence of the leading electrolyte on the effective mobilities only;

(ii) it is directly accessible by experiment: for a fixed-point detector of constant cross-section we can write

$$p = \frac{Q_{\mathrm{B,max}} + Q_{\mathrm{C,max}}}{Q_{\mathrm{L}}} \tag{6}$$

and assuming also a constant driving current then

$$p = \frac{t_{\mathrm{B,max}} + t_{\mathrm{C,max}}}{t_{\mathrm{L}}} = \frac{d_{\mathrm{B,max}} + d_{\mathrm{C,max}}}{d_{\mathrm{L}}} \tag{7}$$

where t and d are the time-based zone lengths and steplengths on the record, respectively. Obviously, eqns. 6 and 7 relate the selectivity to quantities commonly used[1] for quantitation in isotachophoretic measurements. It should be pointed out that in isotachophoresis the zone length related to the zone volume and to the cross-section of the detection cell is of much more interest than the absolute amount of sample as the parameter that should be maximized.

When, however, the separable number of moles of a substance is needed, the following relationship can be used:

$$N_{\mathrm{B,max}} = p\, c_{\mathrm{B,BC}}\, V_{\mathrm{L}} = p_{\mathrm{B}}\, c_{\mathrm{B,B}}\, V_{\mathrm{L}} \tag{8}$$

where the value of V_{L} may be substituted by $Q_{\mathrm{L}}\, \bar{u}_{\mathrm{L,L}}/\kappa_{\mathrm{L}}$ from eqn. 1. In eqn. 8, $c_{i,i}$ is the analytical concentration of substance i in zone i and p_{B} represents a partial value of the maximum relative separation volume:

$$\begin{aligned} p_{\mathrm{B}} &= \frac{V_{\mathrm{B,max}}}{V_{\mathrm{L}}} = p\left(1 + \frac{V_{\mathrm{C,max}}}{V_{\mathrm{B,max}}}\right)^{-1} \\ &= p\left(1 + q \cdot \frac{c_{\mathrm{B,B}}}{c_{\mathrm{C,C}}}\right)^{-1} \end{aligned} \tag{9}$$

where $q = c_{\mathrm{C,BC}}/c_{\mathrm{B,BC}}$ is the molar sampling ratio. The partial quantities p_{B} and p_{C} relate to selectivity only in the way that $p_{\mathrm{B}} + p_{\mathrm{C}} = p$.

The separation speed

As already pointed out above, when the separability and quantitation are ensured, the minimum analysis time represents a further requirement for the optimization procedure. To cover both maximum separation and minimum analysis time, let us define the separation speed $\bar{w}$ as the volume of the mixed zone separated per unit time:

$$\bar{w} = V_{\mathrm{BC}}/(t_{\mathrm{L}} + t_{\mathrm{B}}) \tag{10}$$

By using eqns. 1, 5, 9 and 10, the separation speed (as the volume velocity of separation) may be expressed by using the volume velocity of migration as

$$\bar{w} = \frac{p}{1 + p_{\mathrm{B}}} \cdot I\, W = \frac{p}{1 + p_{\mathrm{B}}} \cdot w \tag{11}$$

It is obvious that a maximum separation speed is obtained by both a maximized separation volume, $V_{BC} = V_{B,max} + V_{C,max}$, and a minimized analysis time, $t = t_L + t_{B,max}$. The maximum separation volume relates predominantly to the selectivity and for a given column it may therefore be optimized only by appropriate changes in the leading electrolyte. The minimum attainable analysis time depends on many more factors that include not only the nature of the electrolytes and separands but also the parameters of the column and power supply used. The latter factors represent a suitable basis for treating the limits of maximum separation speed, where three basic instrumental parameters may be discussed: the maximum working voltage, the maximum final dissipated electric power and the maximum driving current.

Operation at maximum working voltage. Here a maximum working voltage, U_{max}, is applied across the separation column of length l_L. When considering constant-current operation, the maximum working voltage is reached at the end of the analysis when the whole separation column is filled with the least conductive terminating electrolyte. Ohm's law for the terminating zone can be written as

$$I = \frac{U_{max}}{R_T} = \frac{U_{max}}{r} \cdot \kappa_T \tag{12}$$

where r is a geometrical factor relating the electric resistance of the column with the conductivity of the electrolyte, $R = r/\kappa$. For a given column, r is a constant, with $r = l_L/S$ for a uniform cross-section S.

By combining eqns. 1, 11 and 12, we obtain

$$\bar{w}_U = \frac{p}{1 + p_B} \cdot \bar{u}_{T,T} \cdot \frac{U_{max}}{r} = \frac{p}{1 + p_B} \cdot \bar{u}_{T,T}\, a \tag{13}$$

showing the dependence of the separation speed on the parameters of the device used (given by constant a) and on the effective mobility in the terminating zone. Obviously, this effective mobility in the terminating zone should be as high as possible with regard to the given substance C in order to obtain the maximum separation speed.

Operation at maximum final dissipated electric power. In this instance the applied driving voltage and/or current are limited by the cooling capability of the column and they must be controlled in order to prevent overheating of the electrolyte. For the difference of the thermostatted temperature and the maximum permitted temperature in the column we have[13]

$$\Delta T = kEI \tag{14}$$

where EI is the electric power dissipated in a unit length of the column and k is an appropriate quotient characterizing a given column of constant longitudinal characteristics.

In a column of non-constant cross-section, the narrowest location is critical from the viewpoint of overheating, especially when operating with constant current. Let the cross-section of this location be S_c; by combining eqns. 12 and 14 with the well known relationship $I = E\kappa S$ we obtain

$$\frac{\Delta T\, S_c}{k} = \frac{UI}{r} = \frac{P_{max}}{r} \tag{15}$$

relating the maximum final electric power dissipated in the column, $P_{\max}$, to parameters characteristic of this column (k relates here to the critical location). It should be emphasized that the value of $P_{\max}$ corresponds to the situation at the end of the analysis when the whole column is filled with the adjusted terminator. By using eqns. 1 and 11, we can now write for the separation speed

$$\bar{w}_P = \frac{p}{1 + p_B} \sqrt{\bar{u}_{T,T}\, W \cdot \frac{P_{\max}}{r}} \tag{16}$$

Assuming a linear dependence of conductivity on concentration of the leading electrolyte, we can re-write eqn. 16 as

$$\bar{w}_P = \frac{p}{1 + p_B} \sqrt{\frac{\bar{u}_{T,T}}{c_L}} \cdot b \tag{17}$$

where b is a constant.

Operation at maximum driving current. This case may occur when performing separations in more concentrated electrolytes with higher conductivity and the driving current that can be provided by the power supply used is limited in its maximum value. Then the separation speed may be expressed, according to eqn. 11, as

$$\bar{w}_I = \frac{p}{1 + p_B} \cdot W\, I_{\max} = \frac{p}{1 + p_B} \cdot \frac{d}{c_L} \tag{18}$$

where d is a constant introduced under the same assumption as made for eqn. 17.

The utilization of p and $\bar{w}$: model examples

From the instrumental point of view, the limitations of driving current and voltage can be outlined by a working diagram as shown in Fig. 3. Here, the possible actual working regimes are depicted as points inside the area contoured by the maximum values corresponding to the three operating regimes.

When calculating the maximum separation speed according to eqns. 13, 16 and 18, three different values may usually be obtained. Obviously, as the actual separation speed the minimum of the three values should be taken:

$$\bar{w} = \min\,(\bar{w}_U, \bar{w}_P, \bar{w}_I) \tag{19}$$

which indicates also the actual operating regime.

The given theory is applicable to both anionic and cationic isotachophoresis. In order to show in an explicit way the factors that influence the separation quantities introduced (especially the concentration and pH of the leading electrolyte), the following model examples of cationic systems are discussed.

Strong electrolytes. Here a pair of cations B and C is assumed, with effective mobilities equal to their ionic mobilities irrespective of the composition of the electrolytes used. Thus the selectivity p is constant and so is p_B, if it is assumed also that $c_{B,B}/c_{C,C}$ and q are constant. If, further, termination by a strong electrolyte with $u_T \approx u_C$ is assumed, then the separation speed is independent of pH.

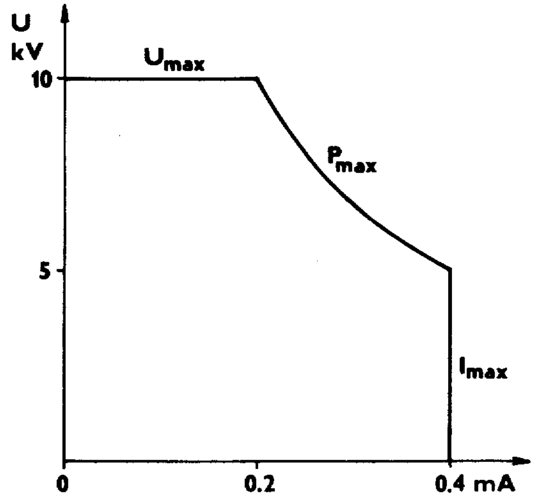


Fig. 3. Working diagram (voltage U *vs.* current I) of a model device with maximum parameters from Table I.

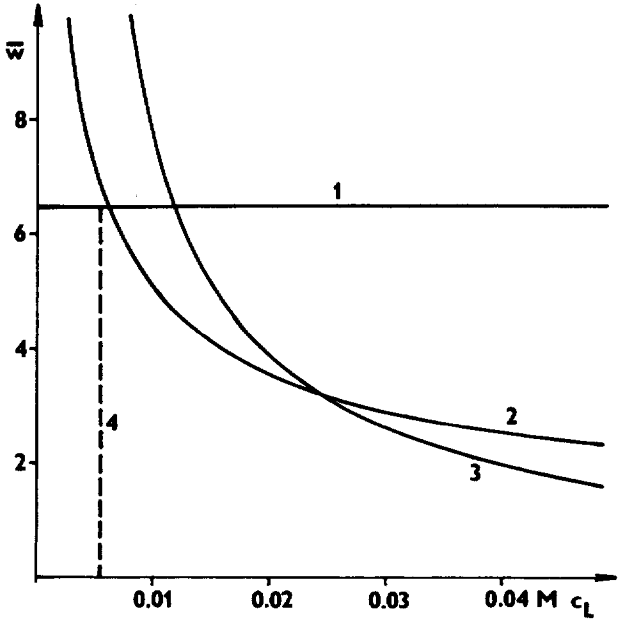


Fig. 4. Calculated dependence of the separation speed $\bar{w}$ (10^{-5} cm^3/sec) on the concentration of the leading electrolyte, c_L. The mobilities of the pair of strong cations in question are u_B = 30 and u_C = 22.5 · 10^{-5} cm^2/V · sec and it was assumed that $c_{B,B}/c_{C,C}$ = constant = 1.21 and q = 1. For other parameters of the calculation, see Table I. The individual curves correspond to (1) $\bar{w}_U$, (2) $\bar{w}_P$ and (3) $\bar{w}_I$; line 4 indicates the critical concentration when buffered acetate is assumed as the leading electrolyte, $[K]_{crit}$ = 0.0054 M.

TABLE I

PARAMETERS OF THE MODEL DEVICE USED FOR CALCULATIONS

Parameter	*Value*
U_{max}	10^4 V
P_{max}	2 W
I_{max}	$4 \cdot 10^{-4}$ A
r	10^4 cm^{-1}
a	1 V cm
b	$1.35 \cdot 10^{-6}$ V cm mol sec^{-1}
d	$2.70 \cdot 10^{-6}$ mol sec^{-1}

The dependence of $\bar{w}$ on the concentration of the leading electrolyte is shown in Fig. 4 for a model calculation (the parameters of the model device in question are given in Table I). It can be seen that the value of $\bar{w}_U$ is independent of concentration and represents the maximum attainable separation speed in the given system. The values of $\bar{w}_P$ and $\bar{w}_I$ show concentration dependence (in accordance with eqns. 17 and 18) and are functions of $c_L^{-1/2}$ and c_L^{-1}, respectively.

In general, we can say that $\bar{w}$ decreases with increasing concentration and a low concentration of the leading electrolyte should thus be optimum for the separation. The lowering of the concentration has limitations, however. In cationic systems, the concentration of the leading electrolyte cannot be lower than its critical value[14] for the given terminator in order to keep the isotachophoretic system correct[15] with regard to H^+ migration. In Fig. 4, this condition is represented by the dashed line (4).

Weak bases (straight pair). Here we consider a pair of weak bases where that with higher ionic mobility of the protonated form has also the higher pK_a value. With weak bases in general, the selectivity depends on the pH of the transient-state mixed zone:

$$p = \frac{u_{BH}}{u_{CH}} \frac{K_{CH} + [H]_{BC}}{K_{BH} + [H]_{BC}} - 1 \tag{20}$$

As has already been shown earlier[5], optimum selectivity is found at a high pH for the system in question. Fig. 5 shows the result of the calculation for a model system. It can be seen that at low pH, a low selectivity corresponding to the limiting case $p = (u_{BH}/u_{CH}) - 1$ exists. At high pH, a high selectivity is reached, limiting to the value $p = (u_{BH}K_{CH}/u_{CH}K_{BH}) - 1$.

Fig. 6 shows the calculated dependence of the separation speed on the pH of the transient state mixed zone for the system in Fig. 5. It can be seen that although a high pH means high selectivity, too high a pH value results in a very low separation speed. From the phenomenological point of view, this can be explained in such a way that the relative difference in effective mobilities is increasing with increasing pH, but simultaneously the effective mobilities themselves decrease considerably. This results in a very low conductivity of the terminating zone and thus (owing to the above-mentioned limitations of the electric driving parameters) in a low separation speed. Bearing in mind that the actual separation speed is the minimum of the values

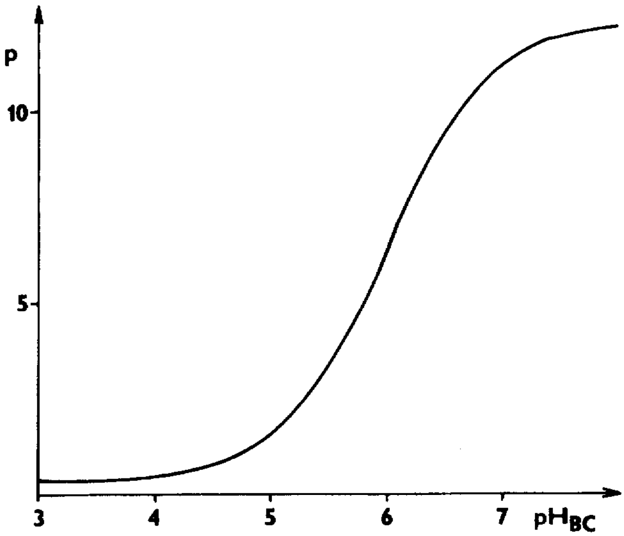


Fig. 5. Calculated dependence of selectivity p for the pair of weak bases B and C (u_{BH} = 30, u_{CH} = 22.5 $\cdot 10^{-5}$ cm^2/V $\cdot$ sec, pK_{BH} = 6, pK_{CH} = 5) on the pH of the transient-state mixed zone.

of $\bar{w}_U$ (curve 1), $\bar{w}_P$ (curve 2) and $\bar{w}_I$ (curve 3) (*cf.*, eqn. 19), it can be seen from Fig. 6 that $\bar{w}$ shows an optimum in the pH dependence (see arrow).

Weak bases (reverse pair). The last example is represented by a pair of weak bases where that with higher ionic mobility has the lower pK_a value. In this instance, inversion of the migration order[3] will occur.

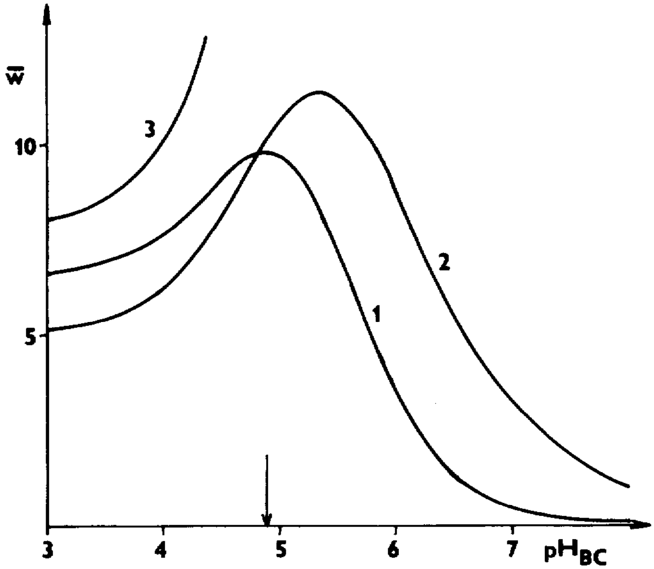


Fig. 6. Calculated dependence of the separation speed $\bar{w}$ (10^{-5} cm^3/sec) on the pH of the mixed zone for the pair of bases in Fig. 5. The parameters for the calculation were $c_{B,B}/c_{C,C}$ = constant = 1.21, q = 1, c_L = 0.01 M and $\bar{u}_{T,T} = \bar{u}_{C,BC}$. The curves correspond to (1) $\bar{w}_U$, (2) $\bar{w}_P$ and (3) $\bar{w}_I$.

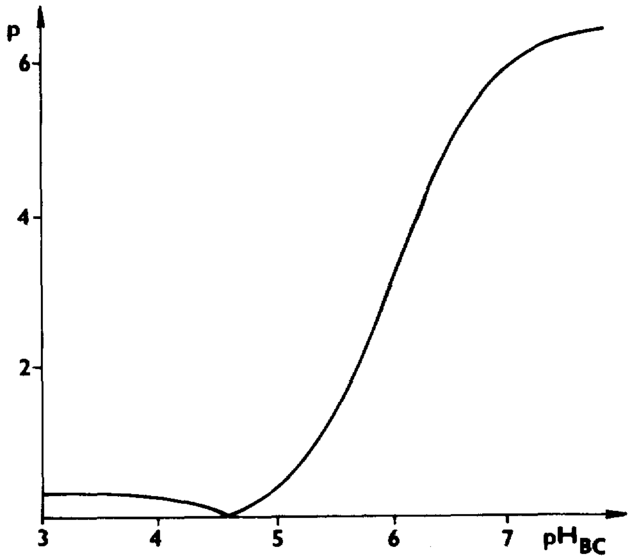


Fig. 7. Calculated dependence of selectivity p for the pair of weak bases B and C (u_{BH} = 30, u_{CH} = 22.5 $\cdot$ 10^{-5} cm^2/V $\cdot$ sec, pK_{BH} = 5, pK_{CH} = 6) on the pH of the transient-state mixed zone.

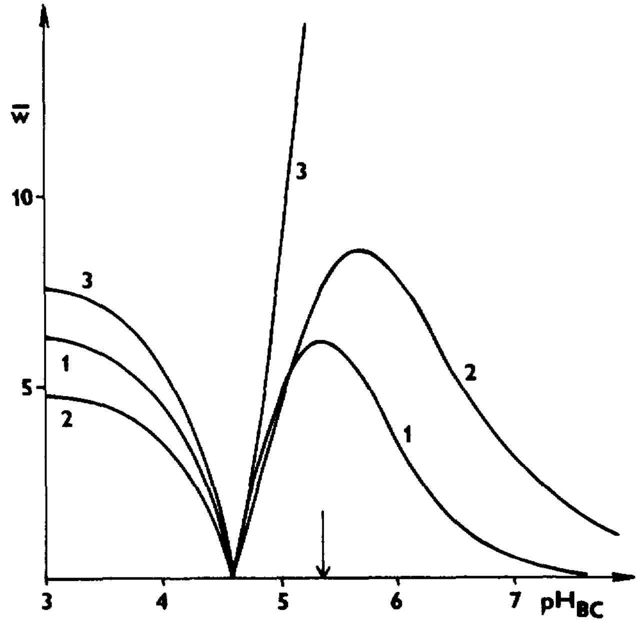


Fig. 8. Calculated dependence of the separation speed $\bar{w}$ (10^{-5} cm^3/sec) on the pH of the mixed zone for the pair of bases in Fig. 7. For the other parameters used for calculation, see Fig. 6. The curves correspond to (1) $\bar{w}_U$, (2) $\bar{w}_P$ and (3) $\bar{w}_I$.

Fig. 7 shows the calculated dependence of selectivity on pH for a model pair of weak bases. It can be seen that the selectivity becomes zero at a pH corresponding to the steady-state mixed zone of B and C[3,5]. For calculation of the selectivity, the migration order must be respected, and the base migrating first must always be taken as B in eqn. 20 in order for p to be positive. In general, also in this instance we can say that a high selectivity is reached at high pH.

Fig. 8 shows, by analogy with Fig. 6, the dependence of the separation speed on the pH of the transient-state mixed zone for the model pair in Fig. 7. A decrease in the separation speed to zero at the pH of the steady-state mixed zone can be seen. Further, depending on the sampling ratio, the formation of this steady state mixed zone must be expected in the region of *ca.* 0.1 pH unit around the pH value mentioned. Also in this instance, an optimum in the separation speed can be found in the high pH region, as indicated by the arrow.

EXPERIMENTAL

The experiments were carried out in an isotachophoretic column made of Perspex with a separation capillary of rectangular cross-section[16]. The potential gradient detector and the high-voltage supply of stabilized current have been described earlier[17]. The isotachopherograms were recorded with a Servogor RE 571 line recorder (Goertz, Vienna, Austria).

For the determination of the separation speed, both voltage and current were measured at the end of the analysis and their magnitudes were regulated in such a way that one of the following conditions was fulfilled: U_{max} = 7.0 kV, P_{max} = 1.5 W, I_{max} = 300 μA. The electric current was measured with a 2.5% moving coil ammeter. The voltage on the column was measured by a voltmeter equipped with an HV probe.

The pH of the solutions was measured with an OP 208 digital pH meter (Radelkis, Budapest, Hungary) using a glass and a saturated calomel electrode.

4-Aminobutyric acid (Serva, Heidelberg, F.R.G.), β-alanine (Austranal, Fischamend, Austria) and the other chemicals used (Lachema, Brno, Czechoslovakia) were of analytical-reagent grade.

RESULTS AND DISCUSSION

The method of measuring the selectivity between the separands in a two-component sample follows directly from its definition (see eqns. 5 and 7) and can be performed (at constant-current operation) from the time-based zone lengths of one experiment with an incompletely resolved sample. A problem is that instead of $t_{C,max}$, a greater value, $t_{C,det}$, is obtained as the separation proceeds also during the time t_{BC} of passage of the rest of the mixed zone through the detector. This effect can be eliminated by replacing $t_{C,max}$ in eqn. 7 with $t_{C,det}$ $(t_L + t_{B,max})/(t_L + t_{B,max} + t_{BC})$. Another problem arises from the fact that usually the separation boundary C → BC is not sharp enough to allow a sufficiently accurate determination of t_{BC} and $t_{C,det}$.

We therefore used another approach, based on two measurements, one of them with a completely resolved sample and one with a non-resolved sample. This enabled us to obtain the correlation $t_{C,max}/t_{B,max} = t_C/t_B$ and to evaluate the selectivity from

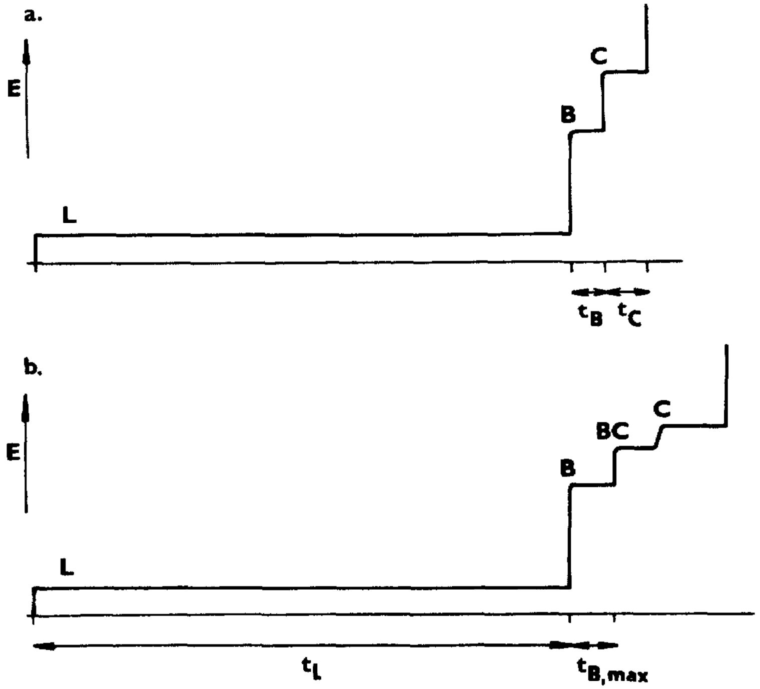


Fig. 9. Experimental isotachopherograms of the separation of lithium and tetraethylammonium (TEA) in the system 0.01 *M* potassium acetate (leading electrolyte), 0.02 *M* acetic acid (terminator). Sample: (a) 3 μl and (b) 6 μl of a 0.01 *M* equimolar solution of LiCl and TEAI. $I = 120\ \mu A$. L = K^+; B = Li^+; C = TEA^+.

$$p = \frac{t_{B,max}}{t_L}\left(1 + \frac{t_C}{t_B}\right)$$

Fig. 9 shows application of the described method to the example of the separation of lithium and tetraethylammonium (TEA) in a common acetate system. Here, the method of obtaining the required values of t_C and t_B (Fig. 9a) and t_L and $t_{B,max}$ (Fig. 9b) is seen.

From the same experiment, the separation speed can be simultaneously evaluated (*cf.* eqn. 10):

$$\bar{w} = p(t_L + t_{B,max})^{-1} V_L$$

If the volume of the separation compartment is not known, the relative value of $\bar{w}/V_L$ can be used directly for optimization without loss of information.

In the Theoretical section it was proposed that for strong electrolytes the selectivity is, to a first approximation, independent of the experimental conditions, including the electrolyte system used. Table II shows some experimental values obtained for the above-mentioned pair Li–TEA in various electrolytes. It can be seen that the selectivity values coincide well (with regard to possible experimental errors) and are independent of pH, the concentration of the leading electrolyte and the

TABLE II

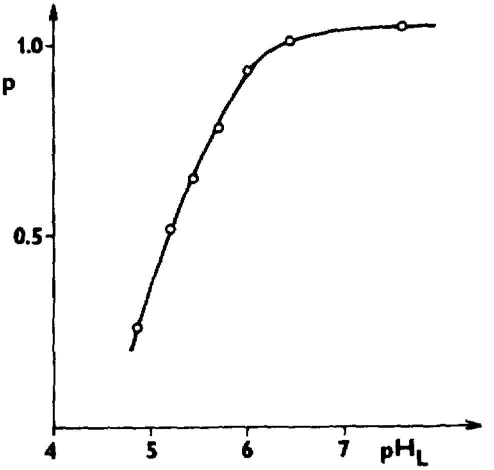
EXPERIMENTAL VALUES OF SELECTIVITY (p) BETWEEN THE PAIR LITHIUM-TETRAETHYLAMMONIUM UNDER VARIOUS CONDITIONS

The given values of p are averages from four measurements. All experiments were performed on a flat column except the last one, which was carried out on a commercial device (URVJT, Spišská Nová Ves, Czechoslovakia). The terminator was 0.02 M acetic acid in all instances. The values of p marked with asterisks relate to non-ideal sampling of the mixture (into the leading electrolyte[16]). The sample in question was an equimolar (0.01 M) solution of LiCl and TEAI.

Leading electrolyte	*Driving current (μA)*	*p*
0.01 M potassium acetate + 0.01 M acetic acid	150	0.199
		0.150*
	60	0.204
		0.163*
0.01 M potassium acetate	120	0.197
0.05 M potassium formate	420	0.193
0.005 M potassium butyrate + 0.005 M butyric acid	150	0.181

driving current applied. Further, it can be seen that ideal sampling (exactly between the leading and terminating solutions) is an important prerequisite for obtaining correct selectivity values; non-ideal sampling results in their decrease.

The theoretical predictions made for weak bases are illustrated by Figs. 10 and 11, where the experimental dependences of selectivity and separation speed on the pH of the leading acetate are shown for the analysis of a mixture of histidine and creatinine terminated by β-alanine. From Fig. 10 it can be seen that the selectivity,



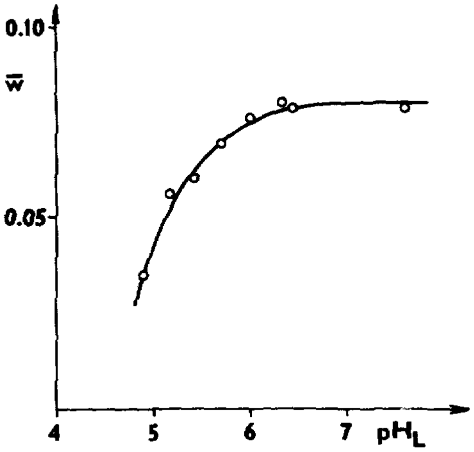
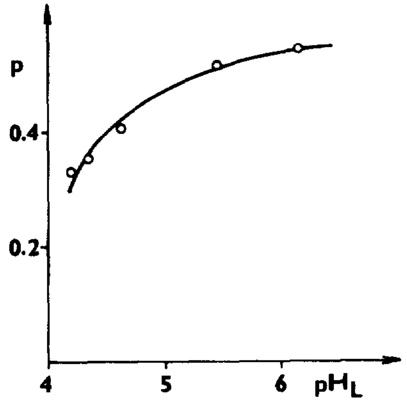


Fig. 10. Experimental dependence of the selectivity p between histidine and creatinine on the pH of the leading electrolyte, pH_L (0.01 M potassium acetate). For the sample composition, see Fig. 11.

Fig. 11. Experimental dependence of the separation speed $\bar{w}$ (μl/sec) on the pH of the leading electrolyte, pH_L (0.01 M potassium acetate). The sample in question was an equimolar 0.05 M solution of histidine and creatinine (pH = 5.3) and β-alanine was the terminator. The operating regime was U_{max} = 7 kV.



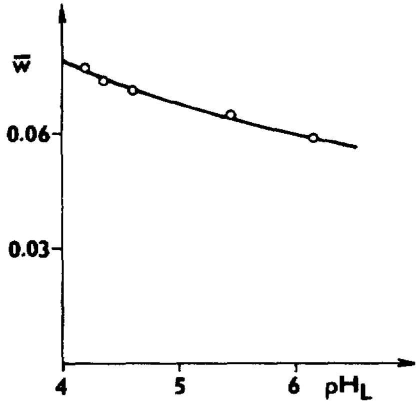


Fig. 12. Experimental dependence of the selectivity p between aniline and ε-aminocaproic acid on the pH of the leading electrolyte, pH$_L$ (0.01 M potassium acetate). For the sample composition, see Fig. 13.

Fig. 13. Experimental dependence of the separation speed $\bar{w}$ (μl/sec) on the pH of the leading electrolyte, pH$_L$ (0.01 M potassium acetate). The sample in question was an equimolar 0.02 M solution of aniline and ε-aminocaproic acid and γ-aminobutyric acid was the terminator. The operating regime was $U_{max} = 7$ kV.

in accordance with theory, increases with increasing pH. The optimum separation speed in this instance lies at the end of the working range of the acetate system and $\bar{w}$ also increases with increasing pH (see Fig. 11).

Figs. 12 and 13 show the results for another system. Here, the sample was formed by aniline and ε-aminocaproic acid, with γ-aminobutyric acid as the termi-

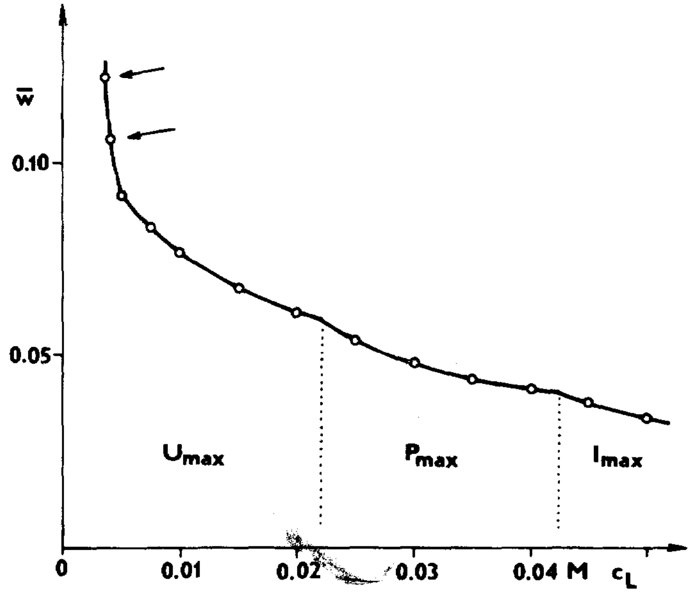


Fig. 14. Experimental dependence of the separation speed $\bar{w}$ (μl/sec) on the concentration of the leading electrolyte, c_L (0.01 M potassium acetate, pH 6.3). For the sample and the terminator used, see Fig. 11; for further explanation, see text.

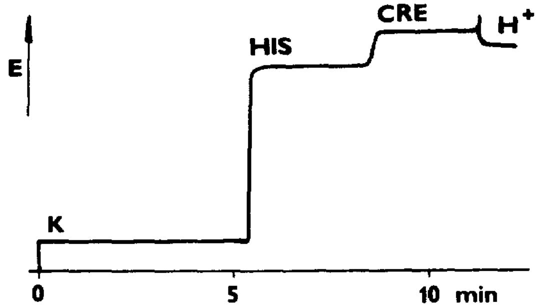


Fig. 15. Experimental isotachopherogram of a separation of histidine (HIS) and creatinine (CRE) using 0.0035 *M* potassium acetate of pH 6.3 as the leading electrolyte. The sample was 75 nmol each of HIS and CRE (1.5 μl of an equimolar 0.05 *M* solution of pH 5.3). The terminator was 0.02 *M* acetic acid. $I = 63$ μA; $U_{max} = 7$ kV.

nator. From Fig. 12 it can be seen that also in this instance the selectivity increases with increasing pH. Fig. 13 shows that the maximum separation speed is found at low pH, however, and this fact must be taken into consideration for optimization.

Fig. 14 shows the dependence of the separation speed on the concentration of the leading electrolyte for histidine–creatinine as the sample with β-alanine as the terminator. It can be seen that in general $\bar{w}$ decreases with increasing concentration. The breaks in the curve are caused by changes in the operating regime as indicated in the figure (with increasing concentration from U_{max} to P_{max} and I_{max}). The separation speed for U_{max} is not independent of concentration (as predicted for strong electrolytes; see Fig. 4), as the effective mobility of the terminator used (β-alanine as a weak base) shows a considerable dependence on c_L. At low concentrations, termination with hydrogen ions occurs[14] as β-alanine becomes slower than H^+ (c_L is lower than the critical value[14] for β-alanine). This results in a further increase in the separation speed (see the experimental points marked by arrows).

Fig. 15 shows the example of an optimum separation based on the above results. Here, the separation of histidine and creatinine in low-concentration acetate of high pH resulted in both a high selectivity ($p = 1.10$) and a high separation speed ($\bar{w} = 0.122$ μl/sec).

CONCLUSIONS

Optimization in isotachophoresis can be briefly characterized as a search for good separation and correct quantitation in a short time. It should be always carried out when a routine method of the analysis is developed. Optimization should be always based on effective mobilities of the separands in the transient-state mixed zone. For a two-component sample, the concept of selectivity expressing the relative difference in the effective mobilities can be introduced as a factor suitable for optimization. It is directly accessible by experiment, being equal to the maximum relative separation volume (eqn. 5). For strong electrolytes, selectivity is independent of pH and concentration of the leading electrolyte. For weak bases, for example, selectivity increases with increasing pH.

From the quantitative point of view, an isotachophoretic separation should be optimized not according to absolute amounts of substances but according to the zone volumes that can be separated whilst still keeping the resolution at unity. These zone volumes relate directly to zone lengths (when assuming passage through a fixed-point detector of constant cross-section) and thus to selectivity.

In order to involve the requirement of minimum analysis time in the optimization process, the separation speed is defined as the volume of the mixed zone separated per unit time (eqn. 12). For a given system, the actual value of the maximum separation speed is, depending on concentration, limited either by the maximum working voltage, the maximum final dissipated electric power or the maximum driving current. In general, the maximum separation speed for strong electrolytes and for weak bases, for example, decreases with increasing concentration. For weak bases, an optimum separation speed can be found depending on pH of the leading electrolyte.

REFERENCES

1 P. Boček, *Top. Curr. Chem.*, 95 (1981) 131.
2 P. Boček and P. Gebauer, *Electrophoresis*, 5 (1984) in press.
3 P. Gebauer and P. Boček, *J. Chromatogr.*, 267 (1983) 49.
4 P. Boček, M. Deml, B. Kaplanová and J. Janák, *J. Chromatogr.*, 160 (1978) 1.
5 F. E. P. Mikkers, F. M. Everaerts and J. A. F. Peek, *J. Chromatogr.*, 168 (1979) 293.
6 V. Dolník and P. Boček, *J. Chromatogr.*, 225 (1981) 455.
7 T. Hirokawa and Y. Kiso, *J. Chromatogr.*, 257 (1983) 197.
8 T. Hirokawa, H. Takemi, Y. Kiso, R. Takiyama, M. Morio, K. Fujii and H. Kikuchi, *J. Chromatogr.*, 305 (1984) 429.
9 F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis —Theory, Instrumentation and Applications* (*Journal of Chromatography Library*, Vol. 6), Elsevier, Amsterdam, Oxford, New York, 1976.
10 P. Boček, M. Deml and J. Janák, *J. Chromatogr.*, 91 (1974) 829.
11 J. C. Giddings, *Separ. Sci.*, 4 (1969) 181.
12 A. J. P. Martin and F. M. Everaerts, *Proc. R. Soc. London, Ser. A*, 316 (1970) 493.
13 Z. Ryšlavý, P. Boček, M. Deml and J. Janák, *J. Chromatogr.*, 144 (1977) 17.
14 P. Boček, P. Gebauer and M. Deml, *J. Chromatogr.*, 217 (1981) 209.
15 P. Boček, P. Gebauer and M. Deml, *J. Chromatogr.*, 219 (1981) 21.
16 P. Boček, M. Deml and J. Janák, *J. Chromatogr.*, 106 (1975) 283.
17 M. Deml, P. Boček and J. Janák, *J. Chromatogr.*, 109 (1975) 49.