CHROM. 23 144

Optimization of isotachophoretic analysis: use of the charge-based transient-state model

VLADISLAV DOLNÍK*, MIRKO DEML, PETR GEBAUER and PETR BOČEK

Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, Veveří 97, 611 42 Brno (Czechoslovakia)

ABSTRACT

A charged-based transient-state model of the isotachophoretic separation of a multi-component mixture is described. The model enables both the column hold-up required for the full separation of a multi-component sample and the analysis time to be calculated. The optimum pH of the leading electrolyte can be found by plotting the analysis time *vs.* the pH of leading electrolyte. The method was applied to the separation of a three-component mixture (acetate, lactate, butyrate) and the theoretical values of the required column hold-up were compared with those obtained experimentally. Good agreement between the theoretical and experimental values was found.

INTRODUCTION

In analytical isotachophoresis, there are several parameters (*e.g.*, analysis time, cost of analysis, sample volume) which are optimized during the elaboration of an analytical procedure. Because of its practical importance, the analysis time is the parameter that is optimized most frequently.

To achieve the complete separation of all sample components during isotachophoretic analysis, the separation capillary has to provide a sufficient separation capacity. This leads to the necessity to express the grade of the separation achieved quantitatively; until now, the separation of binary mixed zones was treated most frequently.

Capillary length [1], separation time [2–4] and electric charge passed through the column [5,6] have been used as quantitative parameters expressing the level of separation achieved. As far as a multi-component mixture is concerned, its separation should be solved as the separation of the binary mixture containing the pair of components that are the most difficult to separate [1,2]. Recently, Hirokawa *et al.* [7] described a transient-state model for a three-component system.

The optimum separation conditions, especially the pH of the leading electrolyte, have been determined typically by plotting the effective mobilities of the separated compounds in their pure isotachophoretic zones *vs.* the pH of the leading electrolyte. The values of the effective mobilities were obtained experimentally [8] or by calculation [9–11]. At the optimum pH of the leading electrolyte, the minimum mobility difference

between two adjacent zones for a given multi-component mixture reaches the maximum value in the tested pH interval.

This paper offers a possible solution of the optimization problem of the isotachophoretic separation of a multi-component sample. It is based on mathematical modelling of the separation process and the optimization is performed by using the pH of the leading electrolyte, pH_L as the variable parameter. We assume a constant detection sensitivity (which determines the given sample amount which is necessary to obtain sufficient quantification of all minor sample components). For each pH_L, the calculation provides the following output parameters: the column hold-up necessary for the separation of the sample, the effective mobility of the terminator (which should be as mobile as possible), the conductivity of its zone and the resulting maximum electric driving current (with constant cooling properties of the capillary) and the analysis time. The solution is then the pH_L at which the minimum analysis time is achieved.

THEORETICAL

Optimization of isotachophoretic analysis

The goal of the optimization of an isotachophoretic analysis can be stated as the establishment of conditions under which the necessary sample amount is separated (analysed) within a minimum time. The necessary sample amount is given by the requirement that all minor sample components must be determined with sufficient precision and accuracy.

Such time-based optimization is necessary especially if a routine analytical method is worked out, *i.e.*, if a large number of samples have to be analysed over a long time period. There are two ways to perform such an optimization: by experiment or by calculation. The former is very time consuming, which is why the latter is of special interest. The computational optimization must be based on knowledge of the sample as detailed as possible: one must know all ionogenic components of the sample, their ionic mobilities and pK_a values and the ranges of their possible concentrations. The determination of the necessary sample amount is then based on the minimum amount of the minor components that is necessary for good quantification. The estimated sample amount and the maximum expected concentration of the sample components serve as inputs for the mathematical modelling of the isotachophoretic separation.

The minimum analysis time of an n-component sample can be expressed as

$$t_{min} = \frac{Q_{L,req} + \sum_{i=1}^{n} Q_i}{I_{max}} \tag{1}$$

where I_{max} is the maximum electric driving current that can be used without danger of overheating, $\sum_{i=1}^{n} Q_i$ is the sum of zone passage charges of the individual sample components and $Q_{L,req}$ is the column hold-up required for the complete separation of the given sample. Note that both the sample amount and the separation capabilities of the system are expressed in terms of electric charge (the zone passage charge is the

electric charge required for the passage of an isotachophoretic zone through, *e.g.*, the detection cell; the column hold-up is the electric charge which transfers the rear boundary of the zone of leading electrolyte from the starting point to the detection cell [5,12]); this proved to be useful by providing a more general description than is possible with other quantities. Eqn. 1 may be rewritten in the form

$$
t_{min} = \frac{Q_{L,req} + \sum\limits_{i=1}^{n} Q_i}{\sqrt{\kappa_T}} \cdot const.
\tag{2}
$$

where κ_T is the conductivity of the terminating zone (of the zone with the lowest conductivity).

The optimization procedure may then be as follows. We assume we have an isotachophoretic column the hold-up of which can be varied in order to keep it always at the minimum necessary value, $Q_{L,req}$. If now, *e.g.*, the pH of the leading electrolyte, pH_L, is taken as the parameter, the values of all quantities in eqn. 2 may be calculated; by plotting the resulting t_{min} *vs.* pH_L, the optimum (minimum) value of t_{min} can be found.

Separation scheme for a multi-component mixture

When calculating the value of t_{min} from eqn. 2, the most difficult step is to obtain the necessary value of $Q_{L,req}$, especially for multi-component samples. In order to be able to calculate $Q_{L,req}$, it is useful to make some simplifications in the description of the system (which apply also to eqns. 1 and 2): (i) the sample is assumed to have the composition of an adjusted isotachophoretic mixed zone; (ii) the separation is assumed to proceed at constant temperature; (iii) we neglect diffusion, interactions between ions and other disturbing effects such as electroosmosis.

For the complete description of the separation process of an n-component sample, all partial Q_L values must be calculated which correspond to the extinction of all various mixed zones containing from 2 to n components. The symbol $Q_{L,i...j}$, *e.g.*, corresponds to the column hold-up necessary for the extinction of the transient mixed zone containing $j-i+1$ components, *viz.*, $i, i+1, ..., j-1, j$.

Fig. 1a, b and c show the schemes of the isotachophoretic separation of samples containing two, three and four components, respectively. The schemes are drawn as plots of the electric charge that passed through the column, Q, *vs.* the column hold-up, Q_L. The position of a point on the diagram may be understood as follows: its x-coordinate indicates the actual position in the isotachophoretic column (expressed in the term of Q_L) and its y-coordinate refers to a given point of the separation process (expressed in terms of Q). The scheme shows the composition of the stack of isotachophoretic (steady-state and transient) zones in each location in the column after electric charge Q has passed through the column. The lines in the scheme represent the boundaries between neighbouring zones, *i.e.*, for given Q and Q_L we obtain information on the location and size of the individual zones (including information on which zone is just in the detection cell). The slopes of these lines are equivalent to the reciprocal relative velocities of the respective boundaries (see eqns. 18 and 19 in the Appendix). The line parallel to the x-axis at a distance Q crosses the network
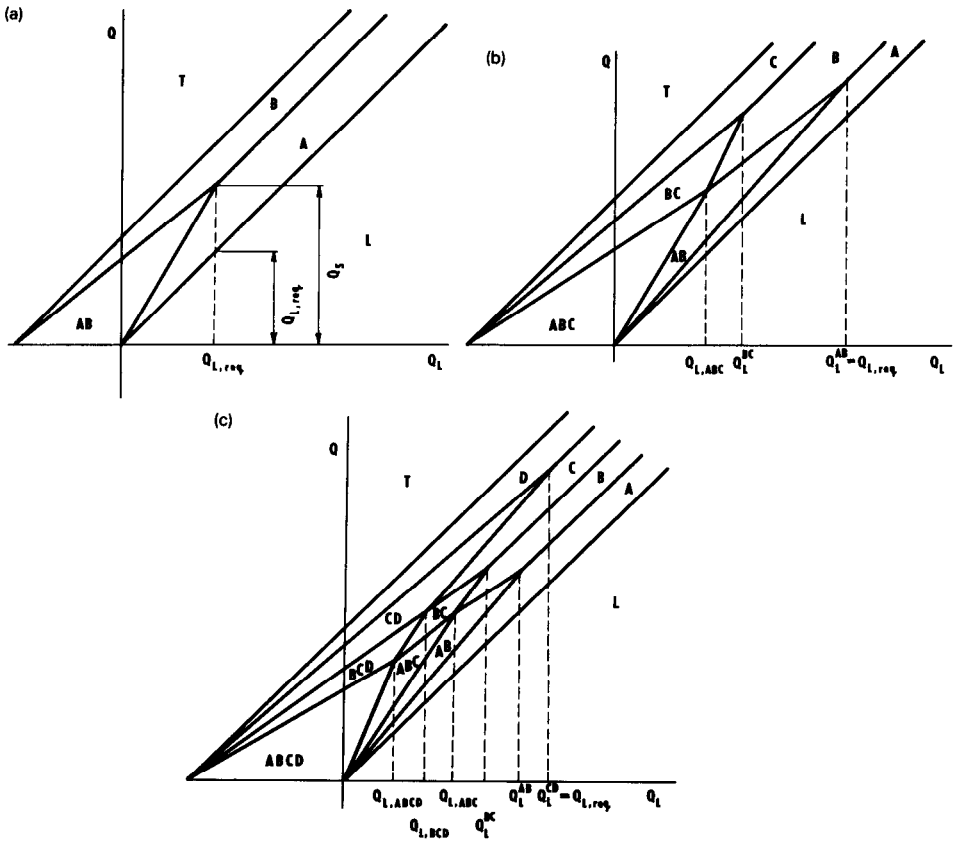
Fig. 1. Scheme of isotachophoretic separation of multi-component sample containing (a) two components A and B, (b) three components A, B and C and (c) four components A, B, C and D.

representing the zone boundaries showing the state of separation after charge Q has passed through the column. Similarly, the line parallel to the y-axis at a distance Q_L shows by its crossing the network how zones pass through the detection cell if the column hold-up is equal to Q_L.

From the viewpoint of the description of the separation process, the points of intersection of the lines corresponding to the zone boundaries are important: they are the points of extinction of mixed zones. The x-coordinate of such a point represents the column hold-up required for the disappearance of the respective mixed zone; the corresponding y-coordinate represents the appropriate separation charge. The largest of the Q_L^{XY} values (corresponding to the extinction of binary mixed zones) is then equal to the $Q_{L,req}$ value required for the complete separation of the sample.

It is obvious that in an optimum case the isotachophoretic column has a hold-up just equal to the value $Q_{L,req}$. If the actual Q_L is lower, then only incomplete separation is achieved. If the column has a higher Q_L value, complete separation is obtained but an additional electric charge is required to transfer the separated zones to the detection

cell. We can say that in an optimum case the binary mixture the separation of which is the most crucial is separated just when it reaches the detection cell.

The calculation of the $Q_{L,req}$ value depends on the number of the sample components. For a two-component sample, this value can be determined simply from (*cf.*, eqn. 25 in the Appendix)

$$Q_{L,req} = (Q_A + Q_B)\frac{\bar{u}_{B,AB}}{\bar{u}_{A,AB} - \bar{u}_{B,AB}} \tag{3}$$

where $\bar{u}_{i,j}$ is the effective mobility of substance i in zone j.

If the sample contains three or more components, it is necessary to calculate the Q_L^{XY} values for each pair of neighbouring components and to find the maximum value of them to obtain $Q_{L,req}$. For the simplest case of a three-component sample (containing substances A, B and C) we have to calculate Q_L^{AB} and Q_L^{BC}. According to eqns. 37 and 38 (see Appendix) their values can be obtained from

$$Q_L^{AB} = \frac{Q_A}{\dfrac{\bar{u}_{L,L}\kappa_{AB}}{\kappa_L \bar{u}_{B,AB}} - 1} \tag{4}$$

$$Q_L^{BC} = \frac{Q_C}{\dfrac{\bar{u}_{B,BC}\kappa_L}{\kappa_{BC}\bar{u}_{L,L}} - 1} - Q_A - Q_B \tag{5}$$

The column hold-up required for the complete separation of the sample is then given by the higher value of Q_L^{AB} and Q_L^{BC}.

The determination of $Q_{L,req}$ if the sample contains more than three components is analogous. A detailed mathematical description of the problem is given in the Appendix.

EXPERIMENTAL

All chemicals were of analytical-reagent grade from Lachema (Brno, Czechoslovakia).

The isotachophoretic experiments were carried out in an Agrofor instrument (JZD Odra, Krmelín, Czechoslovakia) at room temperature. For calculations, a PMD-85-2 computer (Tesla Piešťany, Czechoslovakia) was used.

Published ionic mobilities and dissociation constants [13] were used in the calculations.

RESULTS AND DISCUSSION

For the determination of the optimum pH of the leading electrolyte, a computer program was written that enables the separation of a three-component sample to be solved. The amount of separated components and their physico-chemical constants (ionic mobilities and dissociation constants have to be known for the use of the
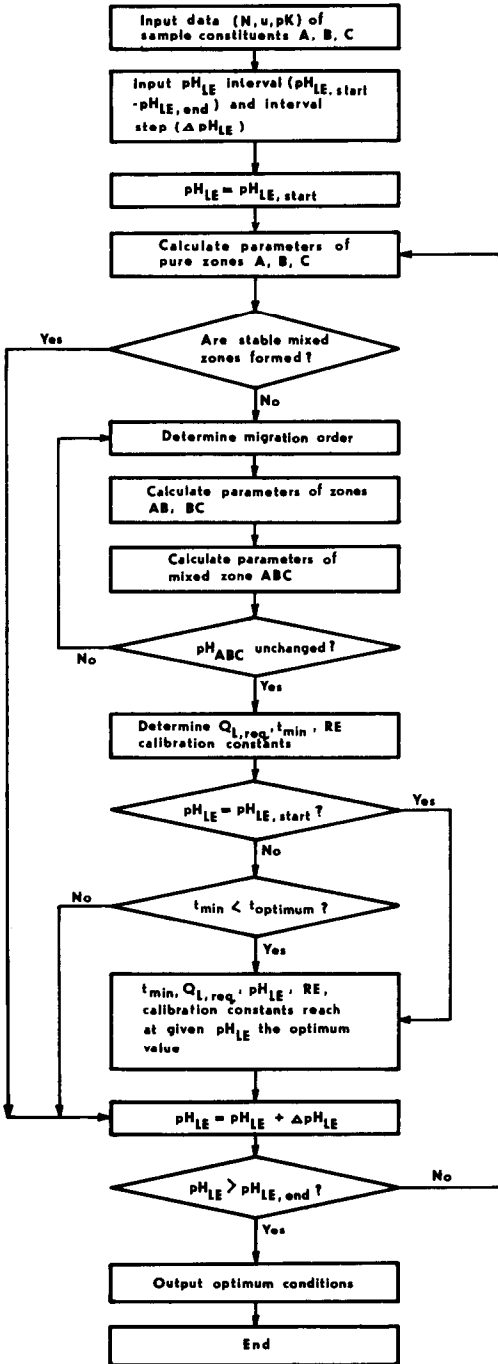
Fig. 2. Flow chart of optimization of isotachophoretic separation.

computer program). The program provides the composition of the optimum leading electrolyte, the required column hold-up, the recommended driving current and the time of analysis and further qualitative and quantitative parameters of the particular isotachophoretic zones (relative effective mobilities or *RE* values and calibration constants). A flow chart of the computer program is shown in Fig. 2.

To test the applicability of the computer program, the separation of lactate, acetate and butyrate in silages was selected [14]. To simulate a silage extract, a mixture of 37 mM acetate, 25 mM lactate and 22 mM butyrate was used [8]. The computer output is shown in Fig. 3. For the calculation of the time of analysis the parameters of the LKB Tachophor were used [15].

The minimum required column hold-up, 30.5 mC, was found at pH$_L$ = 3.4, but the optimum, *i.e.*, minimum, analysis time (2.74 min) was found at pH$_L$ = 4.5, when a column hold-up of 33.2 mC was required.

The plot of analysis time *vs.* pH$_L$ can be only partly verified experimentally.

```
SEPARATION OF ANIONS A,B,C


A: N= 3.7E-08 mol
    pK(1)= 4.756   pK(2)= 0      pK(3)= 0
    u(1)=-42.4     u(2)= 0       u(3)= 0

B: N= 2.5E-08 mol
    pK(1)= 3.86    pK(2)= 0      pK(3)= 0
    u(1)=-36.5     u(2)= 0       u(3)= 0

C: N= 2.2E-08 mol
    pK(1)= 4.82    pK(2)= 0      pK(3)= 0
    u(1)=-33.8     u(2)= 0       u(3)= 0


pH(LE)      Q(L)          TIME         pH(ABC)
            [C]           [MIN]

  3        .0357017      5.5999        3.86286
  3.5      .0304899      3.71887       4.24143
  4        .0318718      3.25428       4.50266
  4.5      .0332153      2.74921       4.8715
  5        .126952       6.97172       5.25818
  5.5      .245324       11.8057       5.67292
  6        .0981953      5.06607       6.13667
  6.5      .103402       5.2247        6.61783

RECOMMENDED LEADING ELECTROLYTE: 10 mmol/HCl
+ 6-AMINOCAPROIC ACID, pH(LE)= 4.5
REQUIRED COLUMN HOLD-UP Q(L)= .0332153 C
RECOMMENDED DRIVING CURRENT I= 375 /uA
ANALYSIS TIME t= 2.74921 min
RE(A)= 3.15652
RE(B)= 2.47397
RE(C)= 4.03037
1 mC= 6.09947 nmol A
1 mC= 5.7073 nmol B
1 mC= 5.52044 nmol C
```

Fig. 3. Computer output of optimization program.

However, the model can be tested by comparing theoretical and experimental values of the required column hold-up.

Calculated values of the required column hold-up and experimentally measured values are shown in Fig. 4. There is good agreement between the theoretical and experimental values. The differences are insignificant and are probably caused by the deviations from the introductory presumptions that the sample has the composition of an adjusted mixed isotachophoretic zone and that the ionic mobilities and dissociation constants do not depend on temperature.

The sufficient agreement between the theoretical and experimental values of the required column hold-up confirmed that the separation conditions of isotachophoretic analysis can be easily optimized by computer simulation using the charge-based transient-state model. If the sample contains only several identified compounds, the ionic mobilities, dissociation constants and concentration range of which are known, the labourious experimental determination of suitable separation conditions can be omitted and only optimization of the separation based on the theoretical model needs to be performed.

APPENDIX

Zone passage charge

The zone passage charge [6,8,12] is a general quantitative parameter of isotachophoretic analysis that is suitable for the quantitative description of an isotachophoretic zone. It is the electric charge that has to be passed through the isotachophoretic column to shift the rear boundary of an isotachophoretic zone to reach the position where the front boundary of that zone was prior to the passage of electric current. The expression of the zone passage charge can be derived by
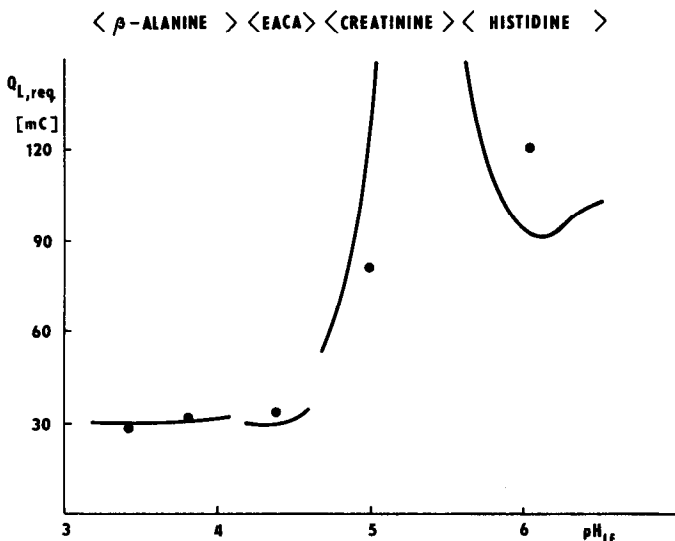

Fig. 4. Dependence of column hold-up required for separation of a sample containing 37 nmol acetic acid, 25 nmol lactic acid and 22 nmol butyric acid. Solid lines, calculated results; ●, experimental data. ⟨ ⟩ show the pH_{LE} regions of the used counterions. EACA = ε-aminocaproic acid.

combination of the modified Ohm's law (eqn. 6), the expression for the migration velocity of a substance (eqn. 7), electric charge (eqn. 8) and effective mobility (eqn. 9).

$$E_i = \frac{I}{S\kappa_i} \tag{6}$$

$$v_i = \frac{l_i}{t} = |\bar{u}_i| E_i \tag{7}$$

$$Q = It \tag{8}$$

$$\bar{u}_i = \frac{\sum\limits_{j=1}^{m} c_j u_j}{\bar{c}_i} \tag{9}$$

where I = electric current, S = cross-section of separation capillary, t = time, Q = electric charge, E_i = electric field strength in the ith zone, v_i = isotachophoretic velocity, l_i = migration path of substance i, $\bar{u}_i$ = effective mobility of substance i in its isotachophoretic zone, $\bar{c}_i$ = analytical concentration of substance i in its isotachophoretic zone, c_j = concentration of jth species of substance i dissociating into m ionic species and κ_i = conductivity of ith zone.

For the zone passage charge of substance i, we obtain by combination of eqns. 6–9

$$Q_i = N_i \cdot \frac{\kappa_i}{\sum\limits_{j=1}^{m} c_j |u_j|} \tag{10}$$

where $N_i = \bar{c}_i l_i S$ is the molar amount of substance i.

Concentration of counter ion in an n-component mixed zone

The counter-ion mass balance can be expressed for both front and rear boundaries of an isotachophoretic zone containing n components (see Fig. 5) by the equations

$$\bar{c}_{R,1...n-1}(\bar{u}_{R,1...n-1}E_{1...n-1} - v_{1...n\to1...n-1}) = \bar{c}_{R,1...n}(\bar{u}_{R,1...n}E_{1...n} - v_{1...n\to1...n-1}) \tag{11}$$

$$\bar{c}_{R,2...n}(\bar{u}_{R,2...n}E_{2...n} - v_{2...n\to1...n}) = \bar{c}_{R,1...n}(\bar{u}_{R,1...n}E_{1...n} - v_{2...n\to1...n}) \tag{12}$$

where the subscripts R, 1 and n refer to counter ion, component with the highest effective mobility and component with the lowest mobility in the mixed zone, respectively, v_1 and v_n are the migration velocities of the rear and front boundaries, for which

$$v_{2...n\to1...n} = |\bar{u}_{1,1...n}| E_{1...n} \tag{13}$$

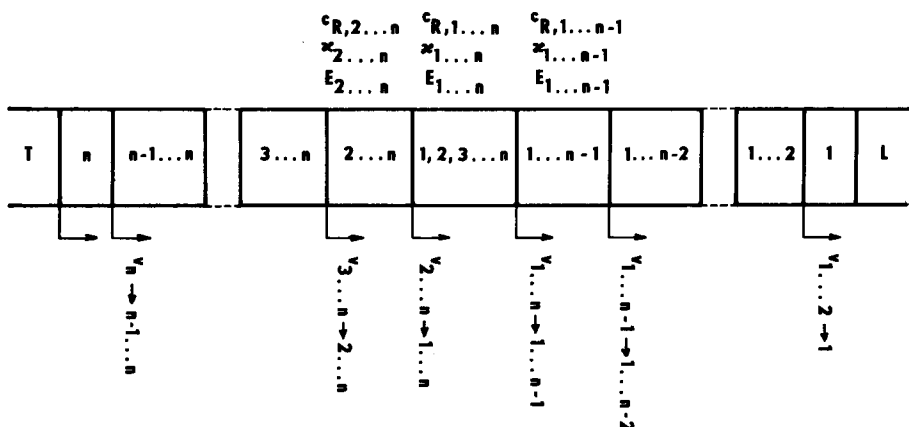$$v_{1...n\to1...n-1} = |\bar{u}_{n,1...n}| E_{1...n} \tag{14}$$

Fig. 5. Scheme of separation of an *n*-component mixed zone.

If eqns. 6 and 11–14 are combined, the following expressions for the concentration of the counter ion in the *n*-component mixed zone and the conductivity of this zone are obtained:

$$\bar{c}_{R,1\ldots n} = \frac{\bar{u}_{R,1\ldots n-1}\kappa_{2\ldots n}\bar{u}_{1,1\ldots n} - \bar{u}_{R,2\ldots n}\kappa_{1\ldots n-1}\bar{u}_{n,1\ldots n}}{\dfrac{\kappa_{2\ldots n}\bar{u}_{R,1\ldots n-1}}{\bar{c}_{R,2\ldots n}}(\bar{u}_{R,1\ldots n} - \bar{u}_{1,1\ldots n}) - \dfrac{\kappa_{1\ldots n-1}\bar{u}_{R,2\ldots n}}{\bar{c}_{R,1\ldots n-1}}(\bar{u}_{R,1\ldots n} - \bar{u}_{n,1\ldots n})} \tag{15}$$

$$\kappa_{1\ldots n} = \frac{\bar{c}_{R,1\ldots n-1}\bar{u}_{n,1\ldots n}(\bar{u}_{R,1\ldots n} - \bar{u}_{1,1\ldots n}) - \bar{c}_{R,2\ldots n}\bar{u}_{1,1\ldots n}(\bar{u}_{R,1\ldots n} - \bar{u}_{n,1\ldots n})}{\dfrac{\bar{c}_{R,1\ldots n-1}\bar{u}_{R,1\ldots n-1}}{\kappa_{1\ldots n-1}}(\bar{u}_{R,1\ldots n} - \bar{u}_{1,1\ldots n}) - \dfrac{\bar{c}_{R,2\ldots n}\bar{u}_{R,2\ldots n}}{\kappa_{2\ldots n}}(\bar{u}_{R,1\ldots n} - \bar{u}_{n,1\ldots n})} \tag{16}$$

General isotachophoretic condition for an n-component mixed zone

Let us consider the isotachophoretic system shown in Fig. 5. The general isotachophoretic condition for pure isotachophoretic zones can be expressed as [16]

$$W = \frac{|\bar{u}_{i,i}|}{\kappa_i} = \text{constant} \tag{17}$$

If we consider the migration of a mixed zone containing *n* components, the linear velocities of the front and rear boundaries are expressed by eqns. 13 and 14. If these are related to the linear velocity of the leading zone (eqn. 7), relative velocities of the boundaries, *R*, are obtained:

$$R_n = \frac{\bar{u}_{n,1\ldots n}\kappa_L}{\kappa_{1\ldots n}\bar{u}_{L,L}} \tag{18}$$

$$R_1 = \frac{\bar{u}_{1,1\ldots n}\kappa_L}{\kappa_{1\ldots n}\bar{u}_{L,L}} \tag{19}$$

The total zone passage charge of the components that passed through the front and rear boundaries of the n-component mixed zone until its extinction can be expressed as

$$Q_{\text{front},1...n} = \sum_{i=1}^{n} Q_{i,1...n} \frac{\bar{u}_{i,1...n} - \bar{u}_{n,1...n}}{\bar{u}_{1,1...n} - \bar{u}_{n,1...n}} \tag{20}$$

$$Q_{\text{rear},1...n} = \sum_{i=1}^{n} Q_{i,1...n} \frac{\bar{u}_{1,1...n} - \bar{u}_{i,1...n}}{\bar{u}_{1,1...n} - \bar{u}_{n,1...n}} \tag{21}$$

The separation charge that has to pass through the column to reach the extinction of zone $1...n$ can be expressed (see Fig. 1) as

$$Q_{\text{s},1...n} = \frac{\displaystyle\sum_{i=1}^{n} Q_{i,1...n}}{R_1 - R_n} = \frac{Q_{\text{front},1...n}}{1 - R_n} = \frac{Q_{\text{rear},1...n}}{R_1 - 1}. \tag{22}$$

By combining eqns. 18, 19 and 22, the isotachophoretic condition related to mixed zone $1...n$ is obtained:

$$W = \frac{\displaystyle\sum_{i=1}^{n} Q_{i,1...n} \bar{u}_{i,1...n}}{\kappa_{1...n} \displaystyle\sum_{i=1}^{n} Q_{i,1...n}} = \frac{\bar{u}_{i,i}}{\kappa_i} \tag{23}$$

Required column hold-up $Q_{\text{L},1...n}$

Let us consider the separation of the n-component mixed zone shown in Fig. 5. The front boundary migrates with relative velocity R_n and the rear boundary with relative velocity R_1. The zone passage charge of each component i is $Q_{i,1...n}$. Comparing the path (expressed as the electric charge) through which both front and rear boundaries migrate from the start of the analysis to the extinction of zone $1...n$, we obtain

$$R_1\left(Q_{\text{L},1...n} + \sum_{i=1}^{n} Q_{i,1...n}\right) = R_n Q_{\text{L},1...n} \tag{24}$$

By arrangement and combination with eqns. 18 and 19 we can write

$$Q_{\text{L},1...n} = \frac{\bar{u}_{n,1...n}}{\bar{u}_{1,1...n} - \bar{u}_{n,1...n}} \sum_{i=1}^{n} Q_{i,1...n} \tag{25}$$

Eqn. 25 is a general expression for the column hold-up required for the extinction of an n-component mixed zone. If $n = 2$ the expression also describes the column hold-up required for the full separation of the sample.

Separation charge

Separation charge is another important parameter of isotachophoretic separation [5]. It is the electric charge that has to pass across the isotachophoretic column to achieve extinction of the appropriate mixed zone. Let us consider the separation of the n-component mixed zone shown in Fig. 5. If the hold-up of the column between the n-component sample and the detection cell is $Q_{L,1...n}$, the n-component mixed zone disappears just in the detection cell. However, the electric charge that has to pass through the column to reach this situation is higher because not only leading zone but also all zones that are generated in front of the n-component mixed zone has to pass the detection cell, *i.e.*, it holds that

$$Q_{S,1...n} = Q_{L,1...n} + Q_{front,1...n} = \frac{\sum_{i=1}^{n} Q_{i,1...n}\bar{u}_{i,1...n}}{\bar{u}_{1,1...n} - \bar{u}_{n,1...n}} \tag{26}$$

Column hold-up required for the full separation of an n-component sample $Q_{L,req}$

There is no general equation describing the column hold-up required for the full separation of an n-component sample. Calculation of the required column hold-up, $Q_{L,req}$, depends on the number of sample components.

If the sample contains only two components (Fig. 1a), the required column hold-up can be determined in a simple way (see eqn. 3).

Solving the separation of a multi- component sample containing three and more components, the procedure for the calculation of the required column hold-up can be formulated as a parallel calculation of the column hold-up required for the extinction of all individual mixed zones and, as a result, binary-mixed-zone column hold-ups Q_L^{XY} are obtained. The Q_L^{XY} which reaches the largest value is then the final resulting $Q_{L,req}$.

If the sample contains three components, A, B and C, the column hold-ups required for the extinction of the mixed zone ABC and of the remaining binary mixed zones AB and BC have to be determined. According to eqns. 25 and 26, the column hold-up $Q_{L,ABC}$ and corresponding separation charge $Q_{S,ABC}$ can be calculated from the equations

$$Q_{L,ABC} = \frac{\bar{u}_{C,ABC}}{\bar{u}_{A,ABC} - \bar{u}_{C,ABC}}(Q_A + Q_B + Q_C) \tag{27}$$

$$Q_{S,ABC} = \frac{Q_A\bar{u}_{A,ABC} + Q_B\bar{u}_{B,ABC} + Q_C\bar{u}_{C,ABC}}{\bar{u}_{A,ABC} - \bar{u}_{C,ABC}} \tag{28}$$

If the column hold-up required for the separation of zone ABC is applied, parts of zone AB and BC remain unseparated. Their zone passage charges $Q'_{A,AB} + Q'_{B,AB}$ and $Q'_{B,BC} + Q'_{C,BC}$ can be calculated by the sumation of the appropriate contributions of individual constituents. For the content of component B in both zones it holds, according to eqns. 20 and 21, that

$$Q'_{B,AB} = \frac{\bar{u}_{B,ABC} - \bar{u}_{C,ABC}}{\bar{u}_{A,ABC} - \bar{u}_{C,ABC}} \cdot Q_B \tag{29}$$

$$Q'_{B,BC} = \frac{\bar{u}_{A,ABC} - \bar{u}_{B,ABC}}{\bar{u}_{A,ABC} - \bar{u}_{C,ABC}} \cdot Q_B \tag{30}$$

After passing electric charge $Q_{S,ABC}$ through the column, the zone passage charge of pure zone A is equal to the product of the difference in relative velocities of both boundaries of zone A and of charge passed:

$$Q'_{A,A} = Q_{S,ABC}\left(1 - \frac{\kappa_i \bar{u}_{B,AB}}{\kappa_{AB} \bar{u}_{i,i}}\right) \tag{31}$$

By rearrangement and combination, we obtain

$$Q'_{A,AB} = Q_A - Q_{S,ABC} \cdot \frac{Q_A(\bar{u}_{A,AB} - \bar{u}_{B,AB})}{Q_A \bar{u}_{A,AB} + Q_B \bar{u}_{B,AB}} \tag{32}$$

Analogously, it holds that

$$Q'_{C,BC} = Q_C - Q_{S,ABC} \cdot \frac{Q_C(\bar{u}_{B,BC} - \bar{u}_{C,BC})}{Q_B \bar{u}_{B,BC} + Q_C \bar{u}_{C,BC}} \tag{33}$$

The column hold-up Q_L^{AB} consists of three parts: (i) $Q_{L,ABC}$, (ii) zone passage charge $(Q'_{A,AB} + Q'_{B,AB})$ and (iii) $Q_{L,AB}$. By combining eqns. 25 and 27–30 we obtain

$$Q_L^{AB} = (Q_A + Q_B + Q_C) \cdot \frac{\bar{u}_{C,ABC}}{\bar{u}_{A,ABC} - \bar{u}_{C,ABC}} + \left[Q_A - \frac{Q_A \bar{u}_{A,ABC} + Q_B \bar{u}_{B,ABC} + Q_C \bar{u}_{C,ABC}}{\bar{u}_{A,ABC} - \bar{u}_{C,ABC}} \cdot \right.$$

$$\left. \frac{Q_A(\bar{u}_{A,AB} - \bar{u}_{B,AB})}{Q_A \bar{u}_{A,AB} + Q_B \bar{u}_{B,AB}} + Q_B \cdot \frac{\bar{u}_{B,ABC} - \bar{u}_{C,ABC}}{\bar{u}_{A,ABC} - \bar{u}_{C,ABC}} \right] \frac{\bar{u}_{A,AB}}{\bar{u}_{A,AB} - \bar{u}_{B,AB}} \tag{34}$$

The column hold-up Q_L^{BC} consists of two parts, (i) $Q_{L,ABC}$ and (ii) $Q_{L,BC}$, and can be expressed as

$$Q_L^{BC} = (Q_A + Q_B + Q_C) \cdot \frac{\bar{u}_{C,ABC}}{\bar{u}_{A,ABC} - \bar{u}_{C,ABC}} + \left[Q_B \cdot \frac{\bar{u}_{A,ABC} - \bar{u}_{B,ABC}}{\bar{u}_{A,ABC} - \bar{u}_{C,ABC}} + Q_C - \right.$$

$$\left. \frac{Q_A \bar{u}_{A,ABC} + Q_B \bar{u}_{B,ABC} + Q_C \bar{u}_{C,ABC}}{\bar{u}_{A,ABC} - \bar{u}_{C,ABC}} \cdot \frac{Q_C(\bar{u}_{B,BC} - \bar{u}_{C,BC})}{Q_B \bar{u}_{B,BC} + Q_C \bar{u}_{C,BC}} \right] \frac{\bar{u}_{C,BC}}{\bar{u}_{B,BC} - \bar{u}_{C,BC}} \tag{35}$$

If the separation of a multi-component sample is to be solved, another approach is suitable; however, the resulting Q_L^{XY} values are then expressed by more parameters than only zone passage charges and effective mobilities. In this approach general expressions for $Q_L^{1,2}$, $Q_L^{2,3} \ldots Q_L^{n-2,n-1}$, $Q_L^{n-1,n}$ can be derived. Let us consider the separation of the n-component sample shown in Fig. 5 (compare, e.g., Fig. 1c). The zone passage charge of the pure zone 1 can be expressed as the product of the charge

passed through the column until the complete separation of zones 1 and 2 and the difference in relative velocities of the front and rear boundaries of zone 1:

$$Q_1 = (Q_L^{1,2} + Q_1)\left(1 - \frac{\bar{u}_{2,1\dots2}\kappa_L}{\kappa_{1\dots2}\bar{u}_{L,L}}\right)$$ (36)

By rewriting eqn. 36 we obtain

$$Q_L^{1,2} = \frac{Q_1}{\dfrac{\bar{u}_{L,L}\kappa_{1\dots2}}{\kappa_L\bar{u}_{2,1\dots2}} - 1}$$ (37)

Analogously, the expression for $Q_L^{n-1,n}$ can be derived:

$$Q_L^{n-1,n} = \frac{Q_n}{\dfrac{\bar{u}_{n-1,n-1\dots n}\kappa_L}{\kappa_{n-1\dots n}\bar{u}_{L,L}} - 1} - \sum_{i=1}^{n-1} Q_i$$ (38)

The expression for $Q_L^{2,3}$, $Q_L^{n-1,n}$ and other Q_L^{XY} can be derived in a similar way, but the derivation and the resulting expressions are more complicated.

The required column hold-up is then obtained as the largest value of Q_L^{XY}.

Calculation of the composition of n-component mixed zone

The method of calculation of the composition of a mixed zone is very similar to that used for the calculation of individual isotachophoretic zones [17,18]. The isotachophoretic condition can be written in the form

$$Q_i^* = \frac{\kappa_i}{F|\bar{u}_{i,i}|} = \text{constant}$$ (39)

where F is the Faraday constant. By iteration, the pH of any mixed zone can be calculated by the method the algorithm of which is based on finding the roots of the function RFQ [17], defined as

$$RFQ = \frac{Q_L^*}{Q_i^*} - 1 = 0$$ (40)

The pH at which RFQ equals zero can be found by finding the pH range in which the function RFQ changes its sign and further by the method of interval bisection. A detailed description of the method can be found elsewhere [8].

For a mixed zone that contains n components we define the RFQ function as

$$RFQ = \frac{\kappa_L \sum\limits_{i=1}^{n} Q_{i,1\dots n}\bar{u}_{i,1\dots n}}{\bar{u}_{L,L}\kappa_{1\dots n} \sum\limits_{i=1}^{n} Q_{i,1\dots n}} - 1 = 0$$ (41)

To find the roots of the *RFQ* function, $\kappa_{1...n}$ has to be determined. For the use of eqn. 16 it is necessary to know the parameters of partially mixed zones which are calculated in the same way: first, binary mixed zones are calculated from the pure isotachophoretic zones, then ternary mixed zones from the binary mixed zones, etc. However, in partially mixed zones, the content of individual components expressed as $Q_{i,1...n}$ cannot be directly determined and has to be calculated by another iteration.

By using parameters of an *n*-component mixed zone, the binary zones have to be calculated and the composition of the *n*-component zone is recalculated using new values of the binary mixed zones. The procedure of both combined iteration steps is repeated as long as values of all mixed zones do not differ from those obtained in the previous step. For example, the composition of binary zones in the case of a three-component sample can be calculated from eqns. 29–33 and zone passage charges of individual components in the binary zones are obtained. Those are then substituted for $Q_{i,1...n}$ in eqn. 41 and used in the *RFQ* method. The procedure is repeated as described above.

Calculation of required column hold-up from experimental data

Let us consider the separation of a sample containing components A, B and C when complete separation is not achieved with column hold-up Q_L and the mixed zone BC can be observed in the isotachopherogram (see Fig. 6). The sampling ratio of components B and C expressed as the ratio of zone passage charges is *q*:

$$q = Q_C/Q_B \tag{42}$$

The separation charge of the partial separation of the mixed zone BC is, according to eqn. 26, equal to

$$Q'_S = Q_L + \bar{Q}_A + \bar{Q}_B = \frac{\bar{Q}_B \bar{u}_{B,BC} + \bar{Q}_B q \bar{u}_{C,BC}}{\bar{u}_{B,BC} - \bar{u}_{C,BC}} \tag{43}$$

when $\bar{Q}_i$ is the zone passage charge of the *i*th zone taken from the isotachopherogram. During passage of mixed zone BC through the detection cell, zone BC is further
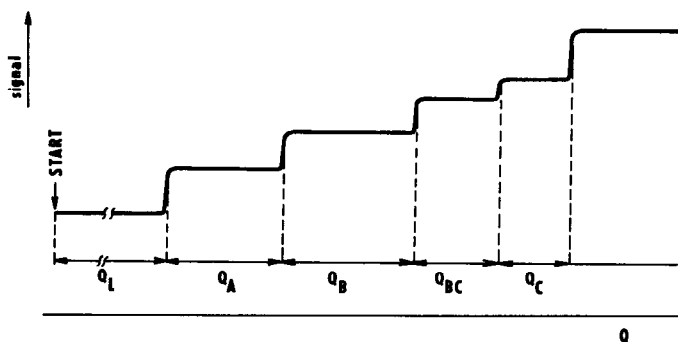
Fig. 6. Isotachopherogram of analysis of a sample with components A, B and C when only partial separation of components B and C is obtained.

separated so that zone C has at its passage through the detection cell zone passage charge Q_C. For the separation charge of this partial separation it holds that

$$Q_S'' = Q_L + \bar{Q}_A + \bar{Q}_B + \bar{Q}_{BC} = \frac{\bar{Q}_C \bar{u}_{C,BC} + \bar{Q}_C \bar{u}_{B,BC}/q}{\bar{u}_{B,BC} - \bar{u}_{C,BC}} \tag{44}$$

For the column hold-up Q_L and column hold-up $Q_{L,req}$ required for the complete separation, it holds according to eqn. 25 that

$$Q_L = (\bar{Q}_B + \bar{Q}_B q) \frac{\bar{u}_{C,BC}}{\bar{u}_{B,BC} - \bar{u}_{C,BC}} - \bar{Q}_A \tag{45}$$

$$Q_{L,req} = (\bar{Q}_B + \bar{Q}_{BC} + \bar{Q}_C) \frac{\bar{u}_{C,BC}}{\bar{u}_{B,BC} - \bar{u}_{C,BC}} - \bar{Q}_A \tag{46}$$

The zone passage charge of the zone A is subtracted from the column hold-up because also during migration of zone A through the detection cell the mixed zone BC is separated.

By combination of eqns. 43–46, an expression is obtained for the column hold-up required for the complete separation of sample ABC:

$$Q_{L,req} = \frac{(Q_L + \bar{Q}_A)(\bar{Q}_B + \bar{Q}_{BC} + \bar{Q}_C)(Q_L + \bar{Q}_A + \bar{Q}_B + \bar{Q}_{BC})}{(Q_L + \bar{Q}_A + \bar{Q}_B)(\bar{Q}_B + \bar{Q}_C) + \bar{Q}_B \bar{Q}_{BC}} - \bar{Q}_A \tag{47}$$

If the first substance forms a transient mixed zone, eqn. 47 can be rewritten by omitting $\bar{Q}_A$ and the equation already published [8] is obtained.

SYMBOLS AND ABBREVIATIONS

A	separated component of sample (separand)
B	separated component of sample (separand)
C	separated component of sample (separand)
$\tilde{c}_i$	analytical concentration of substance i
c_j	concentration of jth ion of substance i
$\tilde{c}_{R,1...n}$	total concentration of counter ion in mixed zone containing components 1,2,...n
$\tilde{c}_{1,1...n}$	total concentration of substance 1 in mixed zone containing components 1,2,...n
E_i	electric field strength in ith zone
$E_{1...n}$	electric field strength in mixed zone containing components 1,2,...n
F	Faraday constant
I	electric driving current
I_{max}	maximum electric driving current
l_i	migration path of substance i
N_i	amount of substance i
pH_L	pH of leading electrolyte

Q electric charge

Q_i zone passage charge of substance i

$Q'_{i,1...n}$ zone passage charge of substance i in partially separated zone $1...n$

$Q_{front,1...n}$ total zone passage charge of substances that passed the front boundary of mixed zone $1...n$

Q_L column hold-up

$Q_{L,req}$ column hold-up required for complete separation of sample

$Q_{L,1...n}$ column hold-up required for extinction of mixed zone containing $1,2...n$ components

Q_L^{AB} column hold-up required for the complete separation of sample when zone AB is separated last

$Q_{rear,1...n}$ total zone passage charge of substances that passed the rear boundary of mixed zone

Q_S separation charge

$Q_{S,1...n}$ separation charge corresponding to extinction of mixed zone containing components $1,2...n$

Q'_S quasi-separation charge if only incomplete separation is achieved

Q''_S quasi-separation charge if incomplete separation is obtained and mixed zone contributes to it

$\bar{Q}_i$ zone passage charge of ith zone taken from the isotachopherogram

Q_i^* $Q_i/V_i F$

$Q_{1...n}^*$ $\sum_{i=1}^{n} Q_i/V_i F$

$\sum_{i=1}^{n} Q_i$ zone passage charge of mixed zone partially separated

q sampling ratio

R_n relative velocity of boundary between zones $1...n$ and $1...n-1$

S cross-section of separation capillary

ΔT difference between mean temperature of isotachophoretic zone and thermostating temperature

t time

t_{min} minimum time of analysis

$\bar{u}_{i,1...n}$ effective mobility of substance i in mixed zone containing components $1,2...n$

u_j ionic mobility of jth subspecies of substance i

$\bar{u}_{R,1...n}$ effective mobility of counter ion in mixed zone containing components $1,2,...n$

v_i migration velocity of ith substance

$v_{1...n \to 1...n-1}$ velocity of boundary between zones $1...n$ and $1...n-1$

W charge-related volume velocity

κ_T conductivity of the zone with the lowest conductivity in the system

κ_i conductivity of zone of ith substance

$\kappa_{1...n}$ conductivity of mixed zone containing substances $1,2,...n$

REFERENCES

1 A. J. P. Martin and F. M. Everaerts, *Proc. R. Soc. London, Ser. A,* 316 (1970) 493.

2 G. Brouwer and G. A. Postema, *J. Electrochem. Soc.*, 117 (1970) 874.
3 F. E. P. Mikkers, F. M. Everaerts and J. A. F. Peek, *J. Chromatogr.*, 168 (1979) 293.
4 T. Hirokawa, K. Nakahara and Y. Kiso, *J. Chromatogr.*, 463 (1989) 51.
5 P. Boček, M. Deml, B. Kaplanová and J. Janák, *J. Chromatogr.*, 160 (1978) 1.
6 V. Dolník, M. Deml and P. Boček, *J. Chromatogr.*, 320 (1985) 89.
7 T. Hirokawa, K. Nakahara and Y. Kiso, *J. Chromatogr.*, 470 (1989) 21.
8 P. Boček, M. Deml, P. Gebauer and V. Dolník, *Analytical Isotachophoresis*, VCH, Weinheim, 1988.
9 T. Hirokawa and Y. Kiso, *J. Chromatogr.*, 257 (1983) 197.
10 T. Hirokawa, H. Takemi and Y. Kiso, *J. Chromatogr.*, 305 (1984) 429.
11 T. Hirokawa, T. Gojo and Y. Kiso, *J. Chromatogr.*, 390 (1987) 201.
12 P. Gebauer, V. Dolník, M. Deml and P. Boček, *Adv. Electrophoresis*, 1 (1987) 281.
13 T. Hirokawa, M. Nishino, N. Aoki, Y. Kiso, Y. Sawamoto, T. Yagi and J. Akiyama, *J. Chromatogr.*, 271 (1983) D1.
14 P. Boček, S. Pavelka, K. Grígelová, M. Deml and J. Janák, *J. Chromatogr.*, 154 (1978) 356.
15 Z. Ryšlavý, P. Boček, M. Deml and J. Janák, *J. Chromatogr.*, 144 (1977) 17.
16 P. Gebauer and P. Boček, *J. Chromatogr.*, 320 (1985) 49.
17 J. L. Beckers and F. M. Everaerts, *J. Chromatogr.*, 68 (1972) 207.
18 P. Boček and P. Gebauer, *Electrophoresis*, 5 (1984) 338.