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Separation Science and Technology

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Thormann, Wolfgang(1984) 'Principles of Isotachophoresis and Dynamics of the Isotachophoretic Separation of Two Components', Separation Science and Technology, 19: 8, 455 — 467
To link to this Article: DOI: 10.1080/01496398408060328
URL: http://dx.doi.org/10.1080/01496398408060328

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SEPARATION SCIENCE AND TECHNOLOGY, 19(8 & 9), pp. 455-467, 1984

REVIEW

Principles of Isotachophoresis and Dynamics of the Isotachophoretic Separation of Two Components

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Abstract

This paper reviews the principles of isotachophoresis, a separation method in which a migrating steady-state zone structure is established through electrical transport. Prior to attaining steady state, a moving transient system of homogeneous zones is gradually reduced to consecutive sample zones of constant length, where all boundaries migrate with equal velocity. The separation process of a pair of sample components is discussed to illustrate the dynamics of this unique electrophoretic method. Solutions in terms of separation time, separation rate, and separation distance are given. Furthermore, techniques for the enhancement of the maximum sample load of a column are discussed. The features of isotachophoresis are presented to illustrate the advantages of multichannel zone detection in an isotachophoretic instrument.

INTRODUCTION

Isotachophoresis (ITP) is a rapid, high resolution electrophoretic method for the quantitation and identification of metal ions, organic acids, amino acids, peptides, proteins, and other charged compounds (*1–7*). Sample components are introduced in small quantities at the interface of a discontinuous buffer system, the so-called leading and terminating electrolytes. The availability of a virtually infinite supply of these solutions prevents the interference of the electrolysis products with the components under observation. Under the influence of an applied electric dc current, sample components separate according to their net mobilities by forming discrete,

0149-6395/85/1908-0455$3.50/0

consecutive zones with homogeneous concentrations. This system reaches a steady state in which each sample zone moves with constant velocity, the zone length being proportional to the amount of a sample constituent. The zones are characterized by having a distinct, constant voltage gradient, resulting in a stepwise increase of the electric field from the leading to the terminating electrolyte. Each boundary between a pair of sample components is also marked by a sudden change in a number of other physical properties: conductivity, refractive index, temperature, pH value, absorbance, etc.

The purpose of this paper is to discuss the principles of ITP. No attempt will be made to give a comprehensive review of its fields of application in instrumental analysis. A simple 5-component system will be analyzed to provide insight into the features of ITP. Our considerations are particularly focused on the dynamics of the isotachophoretic separation of two sample components (*6*, *8–11*). Solutions in terms of separation time, separation rate, and separation distance are given. In addition, enhancement of the sample load is discussed by means of a leading electrolyte cascade (*3*, *12*) and/or by changes in the volume of a sensorless precolumn (*13*). The presentation of the features of this unique separation method is prompted by the design of a new analytical ITP apparatus with multichannel zone detection (*5*, *6*). The introduction of an array of sensors along the separation trough permits monitoring of the attainment of the steady isotachophoretic state. This is not possible with just one detector at the end of the separation space (*2–4*). Thus, an array detector is necessary for automation of analytical ITP.

CHARACTERISTICS OF THE STEADY ISOTACHOPHORETIC STATE

The whole ITP zone structure is normalized under the moving boundary conditions. This fundamental feature of ITP was first described by Kohlrausch (*14*) with the introduction of his regulatory function. It comprises the characterization of component displacement under the influence of an applied electric field and is restricted to strong electrolytes. The appropriate relation, extended for strong and monovalent weak electrolytes (*1–3*, *6*, *15*, *16*), is given by

$$\omega = \sum_j (C_j/u_{0j}) = \text{constant} \tag{1}$$

where $C_j = c_{0j} + c_{1j}$ equals the total concentration of component j (mol/cm^3) and u_{0j} is the mobility of the charged species of this component (cm$^2\cdot$mol/V$\cdot$A$\cdot$s^2). The mobility u_{ij} is defined as the average velocity of the species ij in the solution when acted upon by the force of 1 N/mol, independent of the origin of the force (*17*). Thus an uncharged species does not need to have a mobility of zero. The electrophoretic mobility equals $Fz_{ij}u_{ij}$ (cm^2/V·s). The mobility u_{ij} and the diffusion coefficient D_{ij} are related by the Einstein expression $D_{ij} = RTu_{ij}$, where R is the gas constant (8.314 V·A·s/K·mol) and T is the absolute temperature (K). The regulating function ω can be derived from the continuity equation if diffusion, convection, electroosmosis, and temperature gradients are neglected (*16*). Thus it describes the influence of electromigration only. The value of the ω function is simply the sum of the concentrations of the components divided by the mobility of the charged species. With the initial electrolyte

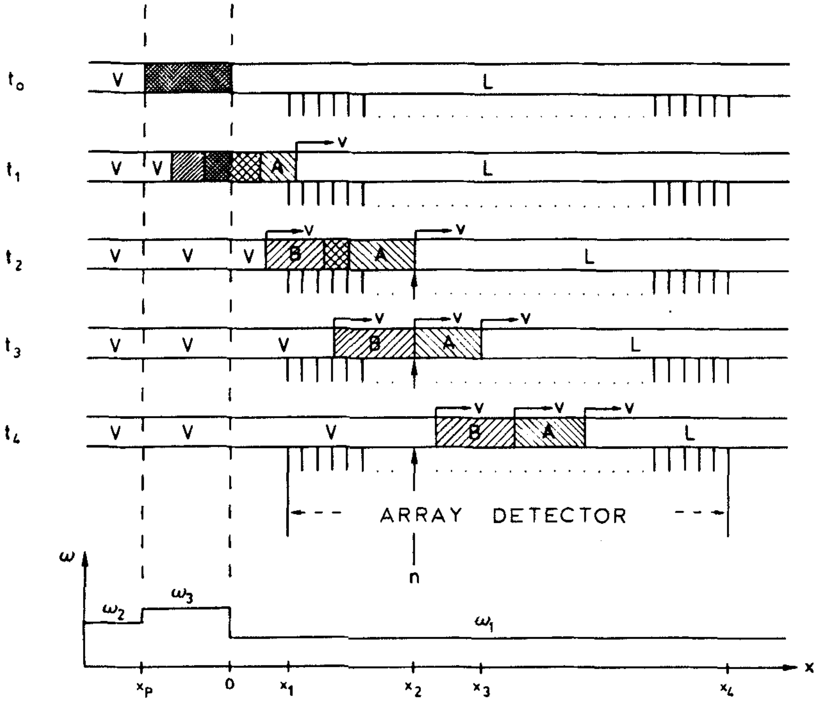

FIG. 1. Schematic of an isotachophoretic separation of two components (A, B) between leading (L) and terminating electrolyte (V) in a column with multiple detection channels. The array detector is located between positions x_1 and x_4 of the trough. The separation of the two components is assumed to be finished at time t_3, the so-called separation time. The separation distance, denoted by x_3, is defined as the location of the first boundary at this time. The steady-state boundaries between zones are represented by straight lines. Bottom: Regulating function ω, which is the sum of the concentrations of the components divided by the mobilities of their charged species. ω_1 is the value for the leading electrolyte, ω_2 for the terminating electrolyte, and ω_3 for the sample solution, which is initially located as block pulse between $x_p < x < 0$. Note that the value of the ω function does not change due to the current flow (*1–7, 16, 22*).

arrangement, given in the top line of Fig. 1, the value is predetermined over the whole column. Electromigration will not change the value.

All concentrations in a zone pattern are adjusted for a fixed concentration of the leader (C_L). This results in a precisely constant ratio of any sample component concentration C_S to C_L,

$$C_S = a_S \cdot C_L \qquad (\mathrm{mol/cm^3}) \tag{2}$$

where a_S is a function of the mobility coefficients of the charged species involved (*1–7*). The a_S value is a criterion for component identification. Considering monovalent components only, a_S equals T_S/T_L, the ratio between the transference numbers of components S and L. For these electrolytes the transference number T_j is given by $u_{0j}/(u_{0j} + u_{0X})$ assuming only one counter-constituent, component X, is present (*1, 16*). C_S is known from Eq. (2) and the quantity n_S (mol) of a sample component is given by the volume which the separated component occupies between its two moving boundaries. For a given operational system with a fixed concentration C_L, a constant driving current i (A) and a constant cross-sectional area q (cm^2) of the separation column, the quantitative result is obtained by measuring the zone length (distance between the two moving boundaries).

The leading electrolyte also determines the velocity of all boundaries in the steady-state zone structure. This value is given by the Moving Boundary Equation (*2, 3, 6, 15, 16*). For monovalent components ($z_{ij} = +-1$) the velocity is simply expressed by

$$v = (T_L/F) \cdot (1/C_L) \cdot I \qquad (\mathrm{cm/s}) \tag{3}$$

where T_L is the transference number of the leading component L in the leading electrolyte. F is the Faraday constant (96,487 A·s/mol) and I equals i/q is the current density applied (A/cm^2).

REQUIREMENTS FOR ISOTACHOPHORESIS

Establishment of ITP zones requires that the net mobilities of samples, M_S, be intermediate to the mobilities of leader L and terminator V:

$$|M_V| < |M_S| < |M_L| \tag{4}$$

Mobilities M_j can be expressed by

$$M_j = F \sum_i z_{ij} u_{ij} \alpha_{ij} \qquad (\mathrm{cm^2/V \cdot s}) \tag{5}$$

where F is the Faraday constant, z_{ij} is the charge number, and u_{ij} is the mobility of species ij of component j. α_{ij} is the dissociation coefficient of a chemical equilibrium process. If more than one such process is involved, a coefficient for each has to be introduced. The velocity of a component is then given by

$$v_j = E \cdot M_j \qquad \text{(cm/s)} \tag{6}$$

where E is the local electric field strength (V/cm). As in all differential migration methods, separation requires that the constituents have different migration rates in the mixed state. Several approaches can be used to increase mobility differences between sample components: (a) changing the pH value of the leading electrolyte (separation due to different pK_a values of proton transfer reactions (*1, 5, 6, 18–20*), (b) introducing specific counter-components to the leading electrolyte which form complexes with sample components (separation due to different complex stability (*3, 6, 21*)), (c) combinations of both effects mentioned above, and (d) mobilities of species, u_{ij}, are dependent upon the composition of the solvent, the temperature, and the ionic strength. The effect of these physical parameters is usually much less pronounced than the result of the chemically modified mobilities.

All sample components which do not fulfill Eq. (4) migrate zone electrophoretically within the leading electrolyte ($|M_S| > |M_L|$) or displacing electrolyte ($|M_S| < |M_V|$). Here the entire shape of a moving sample zone never reaches a steady state (*22, 23*).

DESCRIPTION OF THE SEPARATION OF TWO COMPONENTS

The development of the separation of two components whose mobilities satisfy Eq. (4) is depicted schematically in Fig. 1. The initial distribution consists of a homogeneous mixture of the two separands introduced between leading (L) and terminating (V) electrolytes. Each compartment of the column has its own regulating value ω, established by the initial composition of the electrolytes. With the application of the electric current the separation starts according to the moving boundary principle, forming a set of sequential steady-state moving boundaries. Their velocities are dependent upon the local conditions. The sample constituent with the higher mobility, A, forms its own zone on the front whereas Component B forms a zone on the rear side of the moving zone structure. Note that at the location of the original boundary between sample and leading electrolyte ($x = 0$), all zones are adapted from ω_3 to ω_1 (stationary boundary). This is shown at time t_1 for the mixed zone. At t_2 all sample components have left the sample compartment for a while but the separation is not yet complete. Both the first and the last boundary migrate at the rate determined by the leader and the applied current density (see Eq. 3), whereas the intermediate boundaries have different velocities. Notice that the whole zone structure has already reached its final length. The middle zone of the present three zone structure is still a transient, mixed zone composed of the two sample components. This zone is resolved by t_3, the separation time, where the expected two-zone structure is first established. The mixed zone gradually vanished between the two stages. The position of the first boundary at this time is the separation distance. Now the steady-state structure continues to move in the separation column as long as the current is maintained. The three boundaries represent steady-state concentration and field profiles which all migrate with the same velocity.

The following mathematical treatment, unless stated otherwise, is limited to monovalent electrolytes and to the assumption of constant mobilities u_{ij} throughout the column. The mobilities of the species are assumed to be independent of the composition and ionic strength of the electrolytes. Therefore this description is restricted to dilute solutions. The presence of a total of five components is considered. Components L, A, B, and V have the same migration direction whereas the common counter-constituent, X, moves in the opposite direction, thereby fulfilling electroneutrality throughout the column. Furthermore, the separation space is assumed to have a uniform cross-sectional area. The influence of diffusion on zone boundaries is neglected and the boundaries are considered to be a plane perpendicular to the direction of migration, having a surface area equal to the column cross section.

Components A and B must have different migration rates in both mixed zones to achieve their separation. The adaptation over the stationary boundary at $x = 0$ is characterized by a constant ratio of all charged species on either side of the boundary:

$$c_{0j}(\omega_3)/c_{0j}(\omega_1) = \text{constant} \tag{7a}$$

It follows from this relationship that there is equality of the ratio of the charged species concentrations in both mixed zones:

$$c_{0B}(\omega_3)/c_{0A}(\omega_3) = c_{0B}(\omega_1)/c_{0A}(\omega_1) = \phi \tag{7b}$$

Note that the ratio ϕ is equal to C_B/C_A for monovalent strong electrolytes only.

Let us consider an initial mixed zone, μ, where $1^{\mu}_{t=0}$ is the length the zone would occupy if adjusted to the omega value of the leading electrolyte, ω_1. The origin of the separation capillary is assumed to be at the interface between this mixed zone and the leading electrolyte. Under these conditions the separation time, t_{sep}, is given by

$$t_{sep} = l^{\mu}_{t=0}/(v^{\mu}_{A} - v^{\mu}_{B}) \tag{8}$$

The velocities v_j are expressed by Eq. (6). Considering that $E = I/\kappa$, with $\kappa = F^2 \sum_j u_{0j}c_{0j}$ as the specific conductivity (S/cm), that there is electroneutrality within the mixed zone, and that the concentration of A in the mixed zone is $n_A/l^{\mu}_{t=0} \cdot q$, the following expression is obtained:

$$t_{sep} = Fn_A\rho/(iT_A) \qquad \text{(sec)} \tag{9}$$

with

$$\rho = \frac{\alpha_{0A}u_{0A}(1 + \phi((u_{0B} + u_{0X})/(u_{0A} + u_{0X}))}{\alpha_{0A}u_{0A} - \alpha_{0B}u_{0B}} \tag{10}$$

where T_A is the transference number of Component A in its separated zone. ρ is the separation parameter which is a dimensionless function of both the mobility difference and the ratio ϕ, and is thus dependent upon the composition of the mixed zone. Its value is >1. The above expression for the separation time is identical with that derived by Mikkers et al. (*10*). For monovalent strong electrolytes it passes into the relation obtained by Boček et al. (*9*). It follows from Eq. (9) that the two-zone pattern develops in a time proportional to the amount of one of the sample constituents and to the separation parameter ρ. Furthermore the separation time is inversely proportional to the current applied. Note that the time necessary for full resolution is independent of the column geometry. The amount of electricity needed for the complete separation of the two components is given by the product of the constant driving current and the separation time. Thus the factor $S_A = T_A/\rho$ represents the efficiency of the separation. Its value is proportional to the mobility u_{0A} (*6*), is always positive, and $<T_A$. As ρ approaches 1, an optimization of this process is accomplished through the establishment of favorable conditions. The separation rate, $v_{sep} = \partial n_A/\partial t$, is given by

$$v_{sep} = iT_A/(\rho F) \qquad \text{(mol/s)} \tag{11}$$

The location of the first boundary when the separation is completed is the separation distance. It can be expressed as $t_{sep} \cdot v$, where v is the velocity of the boundary according to Eq. (3). It follows that the separation distance is given by

$$x_{sep} = l_A \cdot \rho \qquad \text{(cm)} \qquad (12)$$

where $l_A = n_A/qC_A$ is the steady-state zone length of Component A (cm). For the example discussed, a sensor at position $x_2 = l_A(\rho - 1)$ would be the first at which the steady-state structure could be observed. This is illustrated by detector channel n in Fig. 1. By monitoring at locations $x < x_2$, the mixed zone is detected, and at $x > x_2$ the two-zone structure is observed. The separation distance is dependent upon the amount of sample and the separation conditions, but independent of the electric current. The multichannel detector provides a method for determining where the mixed zones vanish, which is an important requirement for automation of ITP.

THE CONCEPT OF SEPARATION CAPACITY

The maximum amount of a sample component which can be separated in a given column, the separation capacity, is given by

$$n_A(\max) = n_L \cdot (1/(\rho - 1)) \cdot (T_A/T_L) \qquad \text{(mol)} \qquad (13)$$

where n_L is the amount of the leading component initially introduced between the sample compartment and the last detection position (end of separation space). This relation is obtained through combining Eqs. (2) and (12) for that position. The ratio n_A (max)/n_L is called the load capacity. This quantity is a constant in a given operational system. It is uniquely defined by the separation parameter ρ and the ion mobilities of the components involved. Thus its maximization is reached through optimizing the separation conditions. Note that the load capacity is independent of volume and geometry of the separation space.

Several approaches can be used to enhance the maximum sample load. For that purpose n_L has to be increased, which follows from the linear relationship between the two quantities (see Eq. 13). An elegant method represents the introduction of a higher leading electrolyte concentration in a section of the separation capillary, e.g., in a sensorless pre-separation column. This procedure was first reported to be useful by Boček et al. (*12*). The main advantage of this leading electrolyte cascade, which is illustrated in Fig. 2(a), is to increase n_L without changing the geometry of the separation trough. Under such conditions the whole zone structure is first adjusted to ω_4, the ω value of the higher leading electrolyte concentration. Then at $x = x_1$ all zones are diluted across the ω gradient (stationary boundary) at this position.

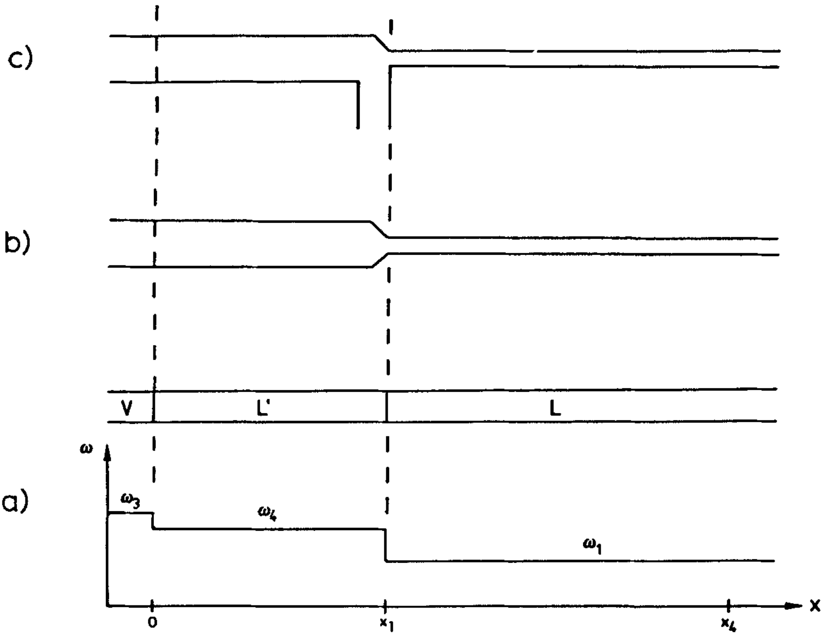

FIG. 2. Schematic of a leading electrolyte cascade and various forms of the separation column. The separation space is divided in a sensorless precolumn ($0 < x < x_1$) and the separation/detection trough ($x_1 < x < x_4$) with the array detector. Figure 2(a) presents a schematic of the profile of the ω function under the condition of a leading electrolyte cascade. The initial concentration of the leader (L') in the precolumn is assumed to be higher than that at locations $x > x_1$. In Fig. 2(b) the cross section of a two-stage separation column is given. Figure 2(c) represents a schematic of two coupled columns where both are connected to an electrode compartment (*23, 24*).

Another approach is a volume change of the separation space. This is preferably achieved by the use of variable column diameters of sensorless precolumns which can simply be exchanged (volume coupling (*13*)). With such a two-stage migration tube system, shown in Fig. 2(b), the high resolution obtained in the main capillary remains unchanged. The coupling of two columns, each with its own electrode compartment (Fig. 2c), permits the further use of various leading electrolytes as well as the selective input of specific sample zones into the main capillary (*23, 24*).

An increase of the separation trough length with uniform inner diameter is less useful because it often results in very high end voltages. The use of a pulse-free counterflow of the leading electrolyte is an alternate way to increase the separation capacity without changing the physical column length

(*25*). However, if not carefully applied, this method causes disturbances of zone boundaries. Combinations of the methods mentioned above are also conceivable.

THE BOUNDARY WIDTH

Another basic feature of ITP is the shape of the steady-state moving boundary. The width of the front between two homogeneous zones, e.g., between Zones L and A shown in Fig. 1, is influenced by diffusion, is a function of the boundary velocity, and is dependent upon the mobility difference of the two components (*6, 26, 27*):

$$W = \delta D_L m / v \qquad (\mathrm{cm}) \qquad (14)$$

where δ is a factor representing the mole fraction change of the two components across the boundary from $n\%$ to $100 - n\%$. D_L is the diffusion coefficient of the leading component L (cm^2/s) and v is the boundary velocity according to Eq. (3). The factor m includes the mobility difference of the two components.

For monovalent strong electrolytes m equals $u_A/(u_L - u_A)$ and the width factor δ is given by $2 \cdot \ln((100 - n)/n)$ (*6, 27*). With monovalent weak protolytes, m is more complex due to the involvement of the protolysis equilibria. In particular, m is dependent upon the separation parameter ρ. In the limit in which mobilities of the species of Components A and B are assumed to be equal, m becomes $K_a(A)/(K_a(L) - K_a(A))$, where $K_a(j)$ is the protolysis constant of component j. The width parameter δ for this case is given by $((1/m) + 2) \cdot \ln((100 - n)/n)$ (*27*).

It follows from Eq. (14) that the mobility of the leading component must exceed that of the displacing constituent for the maintenance of the front. The same conclusion can be drawn for the dissociation constants in the case in which separation occurs due to pK_a differences only. Furthermore, the larger the difference, the sharper the transition. Larger currents also enhance the sharpness of the boundary whereas an increase of the leading concentration spreads the boundary (cf. Eq. 3). The widths have to be much smaller than the zone lengths within an ITP pattern for quantitation by zone length measurements. A sufficiently large mobility difference is not only the fundamental requirement for sharp boundaries but for fast separations as well. Therefore, enhancements of mobility differences through the techniques mentioned above are most important for an isotachophoretic separation.

DISCUSSION

The isotachophoretic separation process can be described by a model based on electromigration only (*6, 8–10*) or by computer simulations (*11*). Both methodologies predict that prior to attaining the steady state, the sample comprises a moving transient system of homogeneous zones which is ultimately reduced to zones with constant length. The beginning and end of the transient mixed zones are marked by moving boundaries which are characterized by changes in the same physical properties as the boundaries in the steady-state zone structures. Their velocities, however, are unequal. It is important to realize that a precise description of the moving boundaries must consider the effects of diffusion. Analytical solutions can be found for the boundary width (*26, 27*); however, boundary shape can only be predicted by computer simulations (*11, 20, 27, 28*).

The separation process for two components has been verified experimentally by means of an array detector along the separation trough (*5, 6, 29*) as well as with instruments comprising one sensor at the end of the separation column (*9, 10, 23*). The quantitative results compare well with the theoretical description. The time required for the development of a steady-state ITP pattern is proportional to the amount of sample injected and to the mobility difference of the least separable pair in the sample. This time is inversely proportional to the current applied. The location where steady state is attained is independent of the current, but a function of the sampled amount and operational system. The separation of components in a given electrolyte system is associated with the properties of the leading electrolyte which determine the separand pattern. It can be fully predicted if mobilities and equilibrium constants are known.

This behavior of electrical transport systems is unique and not realizable with any heterogeneous type of chromatography or with any type of field-flow fractionation (*30*). The ability to predict the order of components, the separation scheme, and the shape of zone boundaries in an ITP spectrum is a prerequisite for automation. By having many equidistant detectors along the column (array detector (*5, 6, 29*)), or by scanning the separation trough repeatedly with a moving detector (*31*), transient and steady-state zone distributions can be observed. When measuring a general physical property, the temporal development of each zone boundary can be monitored, including the vanishing of mixed zones. This gives a computer interpretable criterion of the steady state, which is a mandatory condition for automation. If single channel detection (*2–4*) at the end of the separation space is used (as in all commercial ITP instruments), much available information of analytical relevance is lost: now knowing whether the steady state has been attained is a particularly severe limitation of this mode of operation.

Automated ITP is an alternative to automatic titration (*32*). In fact, this new method is far more sophisticated because it allows, in a single run, the monitoring of as many as 20 components in nano to picomol quantities each (1 ppm level). This is difficult or impossible to perform in conventional titrimetry. Sample preparation is minimal or unnecessary and experimental results are available in only a few minutes with a precision of +/−2% (*33*). The leading electrolyte plays the role of a standard solution in titration. The detection of moving boundaries is similar to the end point detection in titrimetry.

Acknowledgments

The author would like to express his gratitude to Drs M. Bier and R. A. Mosher for helpful discussions. This work was partly sponsored by NASA grant NSG-7333 and by a grant from the Swiss National Science Foundation.

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Received by editor December 5, 1983
Revised February 22, 1984