Isotachophoresis and zone electrophoresis in narrow bore tubes

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ISOTACHOPHORESIS AND ZONE ELECTROPHORESIS IN NARROW BORE TUBES

PROEFSCHRIFT

ter verkrijging van de graad van doctor in de technische wetenschappen aan de Technische Hogeschool Eindhoven, op gezag van de rector magnificus, prof. ir. J. Erkelens, voor een commissie aangewezen door het college van dekanen in het openbaar te verdedigen op dinsdag 28 oktober 1980 te 16.00 uur

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Prof. dr. ir. C.A.M.G. Cramers
en
Prof. dr. ir. F.M. Everaerts

aan mijn ouders

Cover design

Frans E.P. Mikkers , 1970
Thyrsigeri multi, paucos adflavit Iacchus!

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GENERAL INTRODUCTION

For an adequate assessment of the structure or proper-
ties of a chemical substance it is often of decisive impor-
tance that the substance can be isolated from its native
environment. All branches of chemistry and biochemistry
therefore require separation methods for analytical and pre-
parative purposes. Most appropriate the Dutch word for the
entire science of chemistry, *Scheikunde*, means the art of
separation. During the last fifty years separation techni-
ques have evolved in a rather spectacular way and have be-
come a self-evident factor in analytical chemistry. Numerous
separation methods have been developed and fortunately for
the analytical chemist, with interest in separation, there
is no straightforward and simple answer to the question :
"which separation method is the best ?".

Electrophoresis can be used as a method for achieving
separation of ionic substances in solution using an electric
field. Furthermore the term electrophoresis is loosely ap-
plied to a number of industrial processes involving particle
precipitation onto a surface. Like all separation methods,
whether they involve dilution or concentration of the solu-
tes, electrophoresis aims to produce a relative increase of
concentration in a mixture of one solute with respect to
another, at least one solute being ionic.

The fundamental phenomenology of electrophoresis has
been described already in 1897 by Kohlrausch [1] and numerous
different techniques and procedures have been developed. As
a result electrophoresis proved to be a useful method for the
separation of many ionic solutes, especially for large bio-

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polymers. Whereas after World War II chromatography developed quite spectacular, electrophoresis remained at about the same level of utility as that of paper- and thin layer chromatography. In the early nineteen sixties, however, there has been an accumulation of new impulses in electrophoresis: *disc electrophoresis* [2], *isoelectric focusing* [3] and *isotachophoresis* [4]. Especially for the latter technique there has been a continuous effort in improving the instrumental conditions and the theoretical knowledge [5]. This thesis and the authors further work on electrophoresis aims to fit within this frame work as it was started with the second generation of isotachophoretic instruments [6] and ended with a third generation [7].

Nowadays isotachophoresis can compete with other analytical separation techniques. It has found its its applications in many fields, especially with low molecular weight substances, and has advantages due to its high resolving capabilities, accuracy and flexibility.

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PART 1

THEORETICAL PART

CHAPTER 1

Electrophoresis

Electrophoresis is an electric field induced transport process of electrically charged particles. It is a strongly regulated process, governed by the Kohlrausch regulating function concept. Basically four electrophoretic migration modes suffice to describe all electrophoretic separation techniques.

1.0. INTRODUCTION

As early as 1808 von Reuss[1] observed two important electrokinetic effects. He inserted two vertical glass tubes into a lump of moist clay, filled them with water and put an electrode in each. Applying a voltage over the electrodes, the water rose in one tube and sank in the other. Moreover, the water in the latter became turbid, since clay particles were moving in the opposite direction to the water. He thus discovered both electro-osmosis and electrophoresis. Since that time numerous investigations have shown that this electric field induced migration is a general electrokinetic phenomenon shown by colloidal particles. As a result electrophoresis is historically linked with colloid chemistry, where it proved to be a useful method for the measurement of electrophoretic mobilities of colloidal particles.

The concept of ionic migration was suggested by Hittorf[2]

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in 1853, and in the following years the measurement of the Hittorf transference numbers[3,4] obtained much attention.

At the end of the last century the early physico-chemical interest in electrophoresis diverged into a more analytical one: electrophoresis as a method for achieving separation. In this respect an important discovery was made by Hardy[5] in 1899, when he noticed that the net electrical charge of proteins could be changed in varying the pH of the protein solution. It was in fact Michaelis[6] who revealed the potential strength of electrophoresis on the separation and characterization of proteins. Substantial experimental improvements in electrophoretic techniques were introduced by Svedberg and Tiselius in 1926[7]. The real importance of electrophoresis for protein chemistry was stipulated by the work of Tiselius[8,9] who in 1937 described in detail his moving boundary equipment and for his work he obtained the Nobel Prize. Although Tiselius was convinced of the general applicability of electrophoresis, from that time on a close liaison between electrophoresis and proteins was established. In the early sixties this association was reinforced by the ingenious work of Ornstein[10] and Davies[11], with the development of discontinuous electrophoresis. About the same time the development of isoelectric focusing was initiated by Svensson[12], and particularly this technique proved to be extremely well applicable for proteins.

From the start, however, there was a problem which always worried research workers: stabilization in electrophoresis. Already in 1886 Lodge[13] observed that electrophoresis was very sensitive to convective disturbances and he appears to have been the first to attempt to use an anticonvective medium, gelatine in his case, in order to study the migration characteristics of inorganic ions in an electric field. Since that time numerous substances, such as glass wool, glass beads, cotton gauze, silk fibers, paper, gels of agarose and poly-acrylamide,etc, have been used, with more or less success. In fact the liaison electrophoresis-chromatography was born out of the need for

stabilization. This liaison not only increased the practical applicability of electrophoresis in protein chemistry but also allowed the use of electrophoresis as a practical separation technique for lower molecular weight substances.

A problem of comparible importance to stabilization has been detection. Even nowadays the most generally applied method of detection is based on a specific chemical reaction of the sample constituents with a chromogenic reagent. Numerous staining procedures, for post-run detection, have been developed for proteins as well as for low molecular weight substances. Many of the procedures are familiar with those used in paper- and thin-layer chromatography. Though generally rather elaborate some of these procedures have a high sensitivity and specificity. This is best illustrated by immuno-electrophoresis, in which the highly specific immunochemical precipitation of the antigen-antibody system is used. Various detection systems have been developed for in run detection, of which the Schlieren method proved to be useful for moving boundary electrophoresis with the Tiselius apparatus. This interference method, monitoring a change of refractive index, is useful for electrophoresis in free solution but not in gels and require rather elaborate optics.

In the early sixties both problems, stabilization and detection were again tackled by Konstantinov and Oshurkova[14] and Martin and Everaerts[15], in the development of isotachophoresis. The use of capillary systems (anticonvective carrier) and reliable detection systems (photometric, thermometric and conductimetric) in combination with a superb electrophoretic principle (isotachophoresis) proved to be successful. Whereas most of the earlier work did find its main field of application in proteins and proteinlike substances, this new approach was extremely well applicable to the lower molecular weight substances. The development of detection systems with a fast response and high sensitivity and the use of capillary configurations therefore can be a turning point in the development of electrophoresis.

1.1. ELECTROPHORESIS A REGULATED PROCESS

Electrophoresis can be considered as an electric field induced transport process of electric charge carrying species in a conductor, that consists of a solution of electrolytes with initially arbitrary local concentrations. As early as 1897 Kohlrausch[16] concluded that the change of local electrolyte concentrations cannot be an arbitrary process, but must be strongly regulated. This he formulated in his regulating function concept and its mathematical expression *"die beharrliche Function"*. Kohlrausch derived his regulating function not with respect to a specific electrophoretic principle but as a general concept. It comprised the transport phenomena, that occur during electrolysis of electrolyte solutions, without the analytical implications that nowadays are linked with electrophoresis. In his original work he applied his basic concept to several electrophoretic configurations. The experiments of Lodge[13], Wetham[17,18] and Nernst[19] were for him the basis to derive a specific function for the electrolyte configuration in which the constituents are electrophoretically displaced by a sharp moving boundary. These experiments can be considered as the onset of moving boundary electrophoresis, which proved to be a useful method for the measurement of mobilities and transference numbers.

When Tiselius[8] developed his moving boundary method into a quantitative analytical method, a new interpretation of the regulating function concept became desirable. The first attempt was made by Svensson[20], when he derived the moving boundary equation, in which the moving boundary velocity is related to local constituent concentrations and mobilities. Some years later the moving boundary equation was given in a more general form by Longsworth[21], Dole[22] and Alberty[23]. The regulating function concept as well as the moving boundary theory regained their importance with the development of isotachophoresis and discontinuous electrophoresis. These techniques clearly

showed that electrophoresis is a regulated process governed by four basic principles: Ohms law, electroneutrality, chemical equilibrium and balance of mass[26].

The Kohlrausch regulating functions

In electrophoresis the migration velocity, v, of a constituent i is given by the product of effective mobility $\bar{m}_i$ and the local electric field strength, E:

$$v_i = \bar{m}_i \, E \tag{1.1}$$

The electric field strength is vectorial so, the effective mobilities can be taken as signed quantities, positive for constituents that migrate in a cathodic direction and negative for those migrating anodically. As a constituent may consist of several forms of subspecies in rapid equilibrium, the effective mobility represents an average ensemble. Not considering constituents consisting of both positively and negatively charged subspecies in equilibrium, we can take concentrations with a sign corresponding to the charge of the subspecies. Thus the total constituent concentration, $\bar{c}_i$, is given by the summation of all subspecies concentrations:

$$\bar{c}_i = \sum_n c_n \tag{1.2}$$

where c_n is the subspecies concentration.
Following the mobility concept of Tiselius[24], the effective mobility is given by

$$\bar{m}_i = \sum_n \frac{c_n m_n}{\bar{c}_i} \tag{1.3}$$

where m_n is the ionic mobility of the sub-species. The effective mobility of the constituent can be influenced by several parameters, such as temperature, solvation, dissociation, complex-formation and permeability. Considering only dissociation equilibria the effective mobility can be evaluated using the degree of dissociation, α:

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$$\bar{m}_i = \sum_n \alpha_n m_n \tag{1.4}$$

The degree of dissociation can be calculated once the equilibrium constants, K_n, for the subspecies and the pH of the solution are known. Neglecting activity effects it follows:

$$\alpha_n = \left\{ \frac{\prod\limits_n K_n}{(c_H)^n} \right\}^{\pm n} \left\{ 1 + \sum\limits_n \frac{\prod\limits_n K_n}{(c_H)^n} \right\}^{\mp n} \tag{1.5}$$

where c_H is the proton concentration and K_n is the proto-lysis constant for the nth subspecies.

For monovalent weakly ionic constituents this equation can be transformed to the well known Henderson-Hasselbalch[25] relation

$$pH = pK \pm \log \left(\frac{1}{\alpha} - 1 \right) \tag{1.6}$$

where pK is the negative logarithm of the protolysis constant; the positive sign holds for cationic subspecies and the negative sign for anionic sub-species.

In electrophoresis we are dealing with the migration of electrically charged species in a solvent. This means that it is essentially a charge transport process and that Ohm's law is valid. In electrophoresis this law is most conveniently expressed in terms of electrical current density, J, specific conductance, κ, and electric field strength, E:

$$J = \kappa E \tag{1.7}$$

The advantage of this modified law of Ohm[26] is that the electric field strength, an electrophoretic important parameter, can easily be converted into a more chemical one, i.e. the specific conductance. In general, the electric conductance of a solution is the summation of contributions from all charged subspecies present. In spite of the fact that it is a non specific property, conductance gives useful information of the charged species present in a solution and the interactions with the solvent. The spe-

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cific conductance is given by the individual subspecies concentrations:

$$\kappa = F \sum_i \sum_n m_n c_n |z_n| \qquad (1.8)$$

where F is the Faraday constant and $|z_n|$ is the absolute value of the subspecies valency.

The equation of continuity states for the electrophoretic process that

$$\frac{\partial}{\partial t} c_n = - \frac{\partial}{\partial x} \left(\frac{\partial}{\partial x} D_n c_n - v_n c_n \right) \qquad (1.9)$$

where t and x are time and place coordinates, respectively and D is the diffusion coefficient. Assuming the presence of monovalent weakly ionic constituents eqn. 1.9 can be applied for each subspecies. Since the non-charged subspecies do not contribute in the migrational term and if the diffusion coefficient can be considered to be independent of the subspecies we obtain.

$$\frac{\partial}{\partial t} \bar{c}_i = - D_i \frac{\partial^2}{\partial x^2} \bar{c}_i + \frac{\partial}{\partial x} E m_i c_i \qquad (1.10)$$

where m_i and c_i are the mobility and the concentration of the charged species i. Neglecting diffusional dispersion we can apply eqn. 1.10 for each constituent and the over-all summation of the constituents gives

$$\frac{\partial}{\partial t} \sum_i \bar{c}_i = \frac{\partial}{\partial x} E \sum_i m_i c_i \qquad (1.11)$$

In combination with the specific conductance and the modi-fied Ohm's law it follows that

$$\frac{\partial}{\partial t} \sum_i \bar{c}_i = 0 \quad \text{or} \quad \sum_i \bar{c}_i = \text{constant} \qquad (1.12)$$

A function of the same constraint can be derived from eqn. 1.10. Division by m_i and application of the resulting relationship for each constituent and overall summation gives

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$$\frac{\partial}{\partial t} \sum_i \frac{\bar{c}_i}{m_i} = \frac{\partial}{\partial x} E \sum_i c_i \qquad (1.13)$$

Electroneutrality, however, demands $\sum_i c_i = 0$, so

$$\frac{\partial}{\partial t} \sum_i \frac{\bar{c}_i}{m_i} = 0 \quad \text{or} \quad \sum_i \frac{\bar{c}_i}{m_i} = \text{constant} \qquad (1.14)$$

Eqn. 1.14 is well known as the Kohlrausch regulating function.

Both regulating functions can be used for the calculation of local constituent concentrations in electrophoresis. The success of such an operation will largely depend on the complexity of the problem and the nature of the constituents involved. Only in one particular case the regulating functions will result in a direct solution: i.e. when an electrolyte solution of two constituents is displaced by a solution of two other constituents. In all other cases a further knowledge of the etiology of the electrophoretic process is required.

The moving boundary equation

In an electrophoretic system different zones can be present, in which a zone is defined as a homogeneous solution demarcated by moving and/or stationary boundaries[27]. We can apply the continuity principle to a boundary, Fig. 1.1, and derive the general form of the moving boundary equation[23].

$$\bar{m}_i \bar{c}_i E - \bar{m}_i^{K+1} \bar{c}_i^{K+1} E^{K+1} = v^{K/K+1}(\bar{c}_i^K - \bar{c}_i^{K+1}) \qquad (1.15)$$

where $v^{K/K+1}$ represents the drift velocity of the separating boundary between the zones K and $K+1$.
In the case of a stationary boundary, the boundary velocity is zero and eqn. 1.15 reduces to

$$\frac{\bar{m}_i^{K+1}}{\bar{m}_i^K} \cdot \frac{\bar{c}_i^{K+1}}{\bar{c}_i^K} = \frac{E^K}{E^{K+1}} = \text{constant} \qquad (1.16)$$
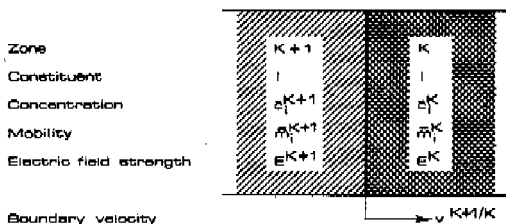
Fig. 1.1 A moving boundary.

From eqn. 1.16 it follows directly that for monovalent
weak and strong electrolytes all ionic species are diluted
or concentrated over the stationary boundary to the same
extent because

$$\frac{c_i^{K+1}}{c_i^K} = \text{constant} \tag{1.17}$$

It should be noted that this phenomenon holds only when
the constituent mobilities are relatively insensitive to
temperature and concentration effects, which generally
holds for dilute solutions over a limited temperature
range. The dilution or concentration over the stationary
boundary is directly related to the fact that the
Kohlrausch functions are locally invariable with time.
Hence a concentration boundary, with all constituents
present at each side of the boundary, will not be dis-
placed by electrophoresis.

A moving boundary will be present if at least one consti-
tuent disappears over the boundary. From Fig. 1.1 it fol-
lows that essentially two configurations are possible. If
the disappearing constituent is present in the zone K but
not in the zone $K+1$ the criterion for boundary stability
is given by

$$v_j^{K+1} > v_j^K \tag{1.18}$$

where v_j^K is the constituent velocity in the zone K and
v_j^{K+1} is the constituent velocity if the constituent j

might turn on in the *K+1* zone. A similar relation is found
if the disappearing constituent is present in the zone *K+1*
but not in the zone *K*.

It should be emphasized that the boundary velocity need
not to be constant, but may vary as a function of time.
The sharpness and structure of the transition boundary is
dependent on the local values of the electric field
strength, the mobility of the constituents and of the dis-
persive factors such as diffusion and uneven temperature
distributions. As long as an inhomogeneity in the electric
field exists any dispersion will be optimally levelled by
the selfsharpening effect, but always a finite transition
boundary will be present[34,39].

The criterion for separation

As in all differential migration methods, the crite-
rion for separation in electrophoresis depends simply on
the effect that two ionic constituents will separate when-
ever their migration rates in the mixed state are diffe-
rent. For two constituents i and j, this means that their
effective mobilities in the mixed state must be different:

$$\frac{\bar{m}_i}{\bar{m}_j} \neq 1 \tag{1.19}$$

When the effective mobility of i is higher than that of j
the latter constituent will lagg behind the former. Conse-
quently, two monovalent weakly anionic constituents will
fail to separate when the pH of the mixed state, pH^M, is
given by

$$pH^{MO} = pK_j + \log\left(\frac{1 - \dfrac{m_j K_j}{m_i K_i}}{\dfrac{m_j}{m_i} - 1}\right) \tag{1.20}$$

where K_i and K_j are the protolysis constants for the sub-
species of the constituents i and j and pK is the negative
logarithm of the protolysis constant.
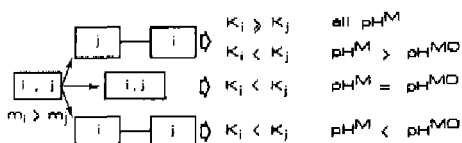
Fig. 1.2 Possible migration configurations for anionic constituents.

When the more mobile constituent has the larger protolysis constant the migration configuration will be independent of pH. If the more mobile constituent has the smaller protolysis constant the migration configuration is a function of pH, as can be seen from Fig. 1.2.
For two monovalent weakly cationic constituents the critical pH is given by

$$pH^{MO} = pK_j - \log\left(\frac{1 - \dfrac{K_i m_j}{K_j m_i}}{\dfrac{m_j}{m_i} - 1}\right) \tag{1.21}$$

It should be recognized that the criterion for separation gives only an academic answer to the question of whether constituents can be separated or not. Dealing with actual separability other parameters, such as time for resolution, resolution and load capacity, can be of decisive importance. Moreover, dealing with separability in its limiting case, i.e. $\bar{m}_i/\bar{m}_j \to 1$, dispersive factors become important and should be incorporated into the equation of continuity and its resulting relationships. Dispersion may have several causes, e.g. temperature distributions, hydrodynamic flow and density gradients, and they may exceed diffusional dispersion largely. This overall dispersion is closely related to the chosen operating conditions and the design of the equipment. Allowance can be made for such dispersive factors but the resulting uncertainty in the criterion for separation would cause this to remain academic[28]. The merit of the criterion for separation is that it gives the experimental conditions that should not be used if separation is pursued.

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1.2. ELECTROPHORETIC PRINCIPLES

In 1909 Michaelis[6] suggested that the migration of colloidal particles in an electric field should be called electrophoresis. The discrimination between the migration of colloidal particles and ions was reemphasized by Martin and Synge[29] designating the latter effect with the name ionophoresis. Though there may exist a difference, at least in the quantitative description of the two effects, they do not differ qualitatively. Noting that electric field induced migration in solution is applicable to ions, cells, biopolymers irrespective their size as well as colloids, its is obvious that the discrimination between electrophoresis and ionophoresis offers no real advantage. As a result the name electrophoresis nowadays is generally accepted.

TABLE 1.1

THE ELECTROPHORETIC CABOODLE

Agarophoresis	Ion focusing
Cataphoresis	Ionophoresis
Cons electrophoresis	Isoelectric focusing
Continuous flow electrophoresis	Isotachophoresis
Density gradient electrophoresis	Moving boundary electrophoresis
Deviation electrophoresis	Multi phasic zone electrophoresis
Dielectrophoresis	Omegaphoresis
Disc electrophoresis	Paper electrophoresis
Displacement electrophoresis	Paper ionography
Electrochromatography	Preparative electrophoresis
Electrorheophoresis	Pore gradient electrophoresis
Endless beld electrophoresis	Micro electrophoresis
Gel electrophoresis	Transphoresis
High voltage electrophoresis	Zone electrophoresis
Immuno electrophoresis	Slab gel electrophoresis

During its historical development numerous electrophoretic principles, methods and techniques have been developed. From Table 1.1, where a selection is given, it follows that this has resulted in a babylonian confusion. There are, however, several criteria that can be chosen to classify the various electrophoretic methods[28,30], but only one allows a systematic and simple classification. In his Nobel lecture Tiselius[31] pointed out that in chromatography basically three differential migration modes can be

distinguished: zonal, frontal and displacement. Surprisingly, however, Tiselius preferred to distinguish in electrophoresis only between boundary and zonal separations[32,33]. Martin and Everaerts[15,34], noting the analogy between electrophoresis and chromatography used again the differential migration criterion and distinguished three main principles.

Zone electrophoresis: wich can be compared with the elution principle in chromatography.
Moving boundary electrophoresis: as the analogon of chromatographic frontal analysis.
Isotachophoresis: the electrophoretic displacement principle.

These three main principles suffice to describe every migration configuration but need the addition of *Isoelectric focusing*.
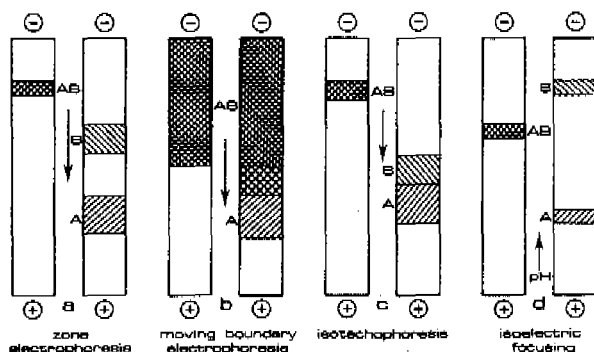
Fig. 1.3 The electrophoretic principles.

Combinations of these principles with one another can be made, e.g. disc electrophoresis[10] is a combination of isotachophoresis and zone electrophoresis. Moreover, additional force fields, multiple dimensions, additional separation mechanisms and methods of detection can be used.

Zone electrophoresis

In zone electrophoresis the separands are allowed to migrate in spatially separated zones. Such a separation

configuration, Fig. 1.3a, is most conveniently obtained by filling all the compartments of the equipment with one kind of electrolyte, the so-called carrier electrolyte. The sample containing the separands, is introduced as a small discrete band in the carrier electrolyte, Fig. 1.3a. Applying a voltage all constituents will migrate with their own velocity depending on the local experimental conditions, such as pH, conductance and driving current. After an appropriate time of analysis the various separands will migrate in different zones, spatially separated by the carrier electrolyte. Each separand zone migrates with its own velocity in a superimposed configuration with the carrier electrolyte. Zone electrophoresis can be performed on anticonvective media, such as paper, cellulose acetate, thin layers of silica or sephadex and in gels of starch, agar and polyacrylamide. Special chromatographic effects can be introduced through the use of ion exchange or pore gradient media. This principle of electrophoresis is comparable with the elution techniques in chomatography and probably the most popular technique in use[35,36].

Moving boundary electrophoresis

If moving boundary electrophoresis is considered as the analogon of frontal analysis the method should be depicted as shown in Fig. 1.3b. One part of the electrophoretic equipment is filled with the sample. In order to garantee a constant sample feed, the sample compartment should be large. The other part of the electrophoretic equipment is filled with a so-called leading electrolyte, consisting of a leading constituent, which has the same sign of charge as the separands, and a counter constituent to preserve electroneutrality. The effective mobility of the leading constituent should be higher than that of the separands and the polarity of the electric field should be chosen in such a way that the separands migrate towards the leading electrolyte. Applying a voltage the moving boundary separation process begins, characterized by the separation of the most mobile separand and mixed zones for

all other separands. Depending on the sample composition and the characteristics of the leading electrolyte, the zone boundaries can be very sharp[26,34,37]. As an analytical method moving boundary electrophoresis has only limited value, but it should be emphasized that the moving boundary principle is operative in almost every electrophoretic separation process in its initial phase. A classical example of this is given by the Tiselius boundary electrophoresis, which is in fact zone electrophoresis in its early stage, when the zones are not yet fully separated.

Isotachophoresis

Moving boundary electrophoresis fails as an analytical technique since no complete separation can be obtained, due to the fact that the amount of sample is unlimited. As a result the rationale for achievement of complete separation is straightforward: limitation of the amount of sample.
In isotachophoresis this is realized by displacement of a limited amount of the separands by a suitable constituent, the terminator, Fig. 1.3c. In order to obtain an isotachophoretic configuration some stringent requirements have to be met[38]. These comprise the application of a discrete amount of sample at the interface of two different electrolytes: the leading electrolyte and the terminating electrolyte. In its most simple configuration, both the leading electrolyte and the terminating electrolyte contain only one ionic constituent of the same charge sign as the separands and a counter constituent to preserve electroneutrality. The effective mobility of the leading constituent should be higher than that of any of the separands. The terminating constituent must have an effective mobility smaller than that of any of the separands. The electric field is applied in such a way that the direction of separand migration is towards the leading electrolyte. Applying the electric field a moving boundary separation process will begin and after sufficient time of migration

the separands will be completely separated. All separands will then be arranged in contiguous zones, generally in order of their mobilities. Provided that the current density is constant all zones will migrate at equal and constant velocity without further changes. The velocity of each separand zone is equal to the velocity of the leading electrolyte zone and as a result the electric field strength in each zone is inversely proportional to the effective separand mobility. Since mobilities are constituent dependent quantities, measurement of the electric field strength, or its related parameters, can be used for identification purposes. According to the Kohlrausch regulating function concept and the moving boundary phenomenon, the concentration within each zone is strictly regulated and the zone boundaries have self-sharpening capabilities against convective disturbances[34,39]. Within a zone the separand concentration is constant and measurement of zone length therefore provides quantitative information. Though the principle of isotachophoresis had been known for many years[40-46], it took until the late sixties before the basic instrumental requirements for isotachophoresis were developed by Everaerts[34].

Isoelectric focusing

In isoelectric focusing the migration behaviour of ampholytic molecules in a pH gradient is used to obtain their condensation in narrow zones that are stationary in an electric field. Depending on the pH of the solution ampholytic substances, such as proteins, can be positively and/or negatively charged. Hence an ampholyte in solution may electrophoretically migrate as a cationic or an anionic constituent or it may not migrate at all. In the latter configuration the ampholyte is called isoelectric. The pH value, at which this isoelectric state occurs is called the isoelectric point, *pI*. Generally the *pI* value coincides or is nearly equal to the isoprotic point, at which the molecule carries no net electric charge. Since many ampholytes have different *pI* values, isoelectric

focusing can be used as a separation principle. A basic
requirement for this is the availability of a stable pH-
gradient. By exposing a mixture of ampholytic substances
with different *pI* values to an electric gradient in a con-
vection free medium a natural pH gradient is formed by the
electric transport process itself. The most acidic ampho-
lyte, with the lowest *pI*, will condense in the most anodi-
cally position and the most basic ampholyte, with the
highest *pI*, will condense in the most cathodic position.
Ampholytes with intermediate *pI* values will condense in
intermediate positions. A mixture of proteins can be super-
imposed in the pH gradient and the various proteins will
migrate towards their respective *pI* values. During this
migration process their electrophoretic velocity gradually
decreases. As a result the time for reaching the condensed
state will be rather long. Moreover, the decrease in elec-
trophoretic velocity also implies a decrease in electric
conductance, and as a result local temperature regimes may
occur, that sometimes can be deleterious for proteins.
Since its introduction in biochemistry isoelectric focu-
sing has become very popular. The main field of applica-
tion is the separation of proteins. The resolved proteins
can be identified through their *pI* value. Detection by
means of a staining procedure is the most commonly used
method. UV-absorption, autoradiography and zymogram me-
thods are other possibilities for detection.

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CHAPTER 2

Zone electrophoresis

*Non symmetrical concentration
distributions in zone electro-
phoresis are inherent to the
separation principle. The mo-
bility of the separand rela-
tive to that of the carrier
constituent determines whether
the concentration distribution
is leading or tailing. Only at
very low sample loads an inde-
pendent retention behaviour
can exist.*

2.0. INTRODUCTION

Zone electrophoresis is the commonest and most widely
applied electrophoretic principle. Numerous zone electropho-
retic techniques and procedures have been developed, main-
ly on an empirical basis.
Being a zonal separation principle, zone electrophoresis
allows a separand to form a zone, separated from other
zones by separand free regions. When in zone electrophore-
sis longitudinal diffusion is the only mechanism of band
spreading and migration occurs at a constant velocity,
Gaussian concentration distributions are obtained[1,2].

The actual broadening, however, may exceed the diffusional broadening due to convection, electrodiffusion, electroosmosis and reversible adsorption. Such non-idealities have been discussed in detail by Wieme[3] and Boyack and Giddings[4] and are collectively responsible for what has been called "electrophoretic dispersion". They can be dealt with by using a pseudo-diffusion coefficient, that combines the adverse effects of this additional spreading[3].

In zone electrophoresis, however, frequently non-symmetrical concentration distributions are obtained. When adsorption processes can occur, non linear adsorption isotherms can explain the asymmetry[5-9]. The effect of an inhomogeneity of the electric field on the zone profile has been discussed by several workers[10-15]. This phenomenon is closely related to the fact that in electrophoresis one frequently encounters "boundary anomalies", i.e. stationary or moving boundaries, in which the migration velocity is a function of concentration[16-18]. It is generally assumed that in view of electrophoretic performance these "boundary anomalies" have to be avoided. This seems to be the result of the chromatographic tenet that any effect which improves the definition of the one boundary invariably causes deterioration of the other boundary. Thus, in chromatographic zonal separations generally the best resolution is obtained when these effects are absent and the zone boundaries are symmetrical.

Although there is a close analogy between chromatographic and electrophoretic separation principles, some important methodological differences exist. Probably the most profound difference is that in electrophoresis Ohm's law must hold and that the resulting Kohlrausch relations [16-21] govern the electrophoretic process. Any changing of concentrations during an electrophoretic process are ruled by these relationships. As a result, on the one hand the occurrence of "boundary anomalies" can be used in a favourable way, while on the other hand problems in retention behaviour arise.

2.1. GENERAL EQUATIONS

In all electrophoretic separation techniques changes
of electrolyte constituent concentrations will occur owing
to the action of an externally applied electric field. In
zone electrophoresis a discrete sample zone is eluted by
the so-called carrier electrolyte. Although gradient con-
figurations (dimensional, thermal or electrolytic) are
possible, we shall assume a separation compartment of
uniform dimensions, operated at a constant temperature and
filled with a homogeneous carrier electrolyte. This elec-
trolyte basically consists of a carrier constituent A,
which has the same sign of electrical charge as the sepa-
rand(s) J, and a counter constituent B, to preserve elec-
troneutrality.

A small-volume element of the separation compartment,
Fig. 2.1, that originally was filled with the carrier
electrolyte AB, will contain after an appropriate time of
analysis a mixture of the carrier constituent and one or
even more separands.
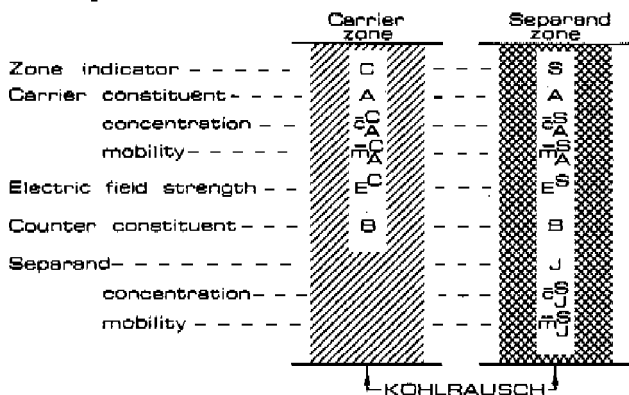
Fig. 2.1 A zone electrophoretic configuration.

After a even longer time, the sample will have left the
volume-element and the original situation will be resto-
red again. Assuming the presence of only monovalent weak
ionic constituents, two important *Kohlrausch* functions can
be derived, Part 1 chapter 1:

$$\sum_i \bar{c}_i = \omega_1 \quad \text{and} \quad \sum_i \frac{\bar{c}_i}{r_i} = \omega_2 \tag{2.1}$$

where $\bar{c}_i$ represents the molar concentration of the consti-
tuent I and r_i is its ionic mobility relative to an appro-
priate reference constituent. Obviously the carrier consti-
tuent offers the best reference mobility. It should be
noted that both the concentrations and the mobilities can
most conveniently be taken as signed quantities and that
the summation should be applied to all constituents, in-
cluding the common counter constituent. The use of rela-
tive mobilities will reduce the influence of temperature
and activity effects.

The numerical values of the *Kohlrausch* functions, ω_1
and ω_2, are locally invariable with time. Thus, taking the
carrier electrolyte as a frame of reference, it follows
for the configurations of Fig. 2.1

$$\bar{c}_A^C(x,t) = \bar{c}_A^S(x,t) + \sum_j k_j \bar{c}_j^S(x,t) \tag{2.2}$$

where

$$k_j = \left(\frac{r_j - r_B}{1 - r_B}\right) \cdot \frac{1}{r_B}$$

The summation indicates that within the volume-element se-
veral separands J can be present. If a constant electric
driving current and the presence of only strong ionic con-
stituents is assumed, it follows for the specific electri-
cal conductance κ, that

$$\kappa^S(x,t) = \kappa^C + \sum_j b_j c_j^S(x,t) \tag{2.3}$$

where

$$b_j = F\, m_A (r_j - r_B)(1 - \frac{1}{r_j})$$

F is the Faraday constant, m_A is the ionic mobility of the
carrier constituent and $c_j^S(x,t)$ is the total concentration
of the separand J.

Applying Ohm's law, we obtain for the electric field strength E:

$$E^S(x,t) = \frac{E^C}{1 - \sum_j a_j c_j^S(x,t)} \tag{2.4}$$

where

$$a_j = \frac{k_j}{c_A^C} (1 - r_j)$$

When only separand is present in the volume-element, an important conclusion can be drawn from eqns. 2.2 and 2.4. The electric field strength in the separand zone will allways be smaller than that in the carrier electrolyte, when the separand has a mobility higher than that of the carrier constituent, i.e. when $r_j > 1$. For other mobility configurations analogous conclusions can be drawn:

$$r_j > 1 \qquad E^S(x,t) < E^C$$
$$r_j = 1 \qquad E^S(x,t) = E^C$$
$$r_j < 1 \qquad E^S(x,t) > E^C$$

The inhomogeneity of the electric field strength will influence the concentration distributions of all ionic constituents. The equation of continuity states for the electrophoretic process

$$\frac{\partial}{\partial t} c_j(x,t) = \frac{\partial}{\partial x}(D_j \frac{\partial}{\partial x} c_j(x,t) - v_j(x,t)c_j(x,t)) \tag{2.6}$$

where D_j is the diffusion coefficient and v_j is the electrophoretic drift velocity of the constituent J. Assuming a constant velocity, Gaussian concentration distributions are obtained, in which a symmetrical broadening of the separand zone occurs due to diffusion. The distribution can be described by

$$c_j(x,t) = \frac{c_j(0,0)}{\sqrt{4\pi D_j t}} \cdot \exp(-\frac{(x - v_j t)^2}{4 D_j t}) \tag{2.7}$$

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In electrophoresis, however, one frequently encounters "boundary anomalies", in which the migration rate is a function of concentration. Virtanen[10] indicated that the electrophoretic velocity is not constant and gave an analytical solution of the equation of continuity, assuming that the drift velocity is linearly related to the separand concentration. According to eqn. 2.4 this can only be approximate. The equations describing this effect are non-linear and the description of non-linear migration in which diffusional dispersion occurs is laboursome. The effect of "boundary anomalies", however, can easily be deduced if one assumes that diffusional dispersion can be neglected. Eqn. 2.6 then reduces to

$$\frac{\partial}{\partial t} c_j(x,t) = - \frac{\partial}{\partial x} v_j(x,t) c_j(x,t) \tag{2.8}$$

If the presence of only one strong ionic separand J is assumed, combination of eqns. 2.4 and 2.6 gives

$$\frac{\partial}{\partial t} c_j^S(x,t) = - m_A r_j E^C \frac{\partial}{\partial x} \left\{ \frac{c_j^S(x,t)}{1 - a_j c_j^S(x,t)} \right\} \tag{2.9}$$

Introducing $\psi(x,t) = 1 - a_j c_j^S(x,t)$ this differential equation can easily be solved to give

$$\psi(x,t) = (\alpha x + \beta)^{-\frac{1}{2}} (t + \gamma)^{\frac{1}{2}} \tag{2.10}$$

The constants α, β and γ are determined by the actual boundary conditions.

2.2. CONCENTRATION DISTRIBUTIONS

During the migration process, several discontinuities can occur that are restricted both in place and time. A complete mathematical treatment of all possible configurations has been given elswhere[21]. After an appropriate time of migration the concentration distributions have a characteristic form. Fig. 2.2 gives the distributions for three possible cases of the separand mobilities.
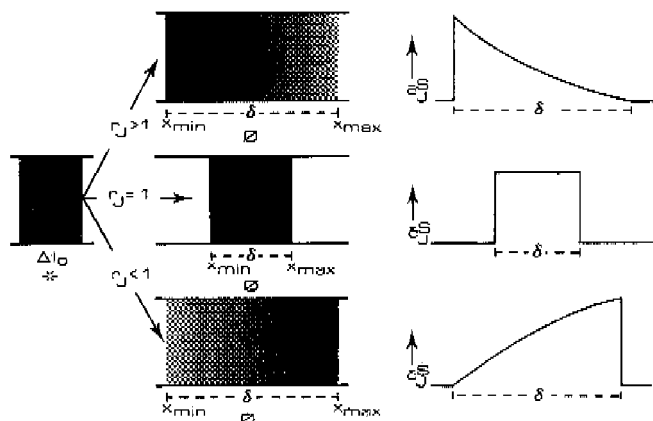
Fig. 2.2 Concentration distributions in zone electrophore-
sis as a function of the relative separand mobility.
*sampling compartment, ∅ separation compartment.

When the separand has a higher mobility than that of
the carrier constituent, $r_j > 1$, the leading side of the
separand zone will be diffuse, whereas the rear will be
sharp. This is caused by the fact that at the rear a sta-
ble moving boundary can be formed, whereas at the leading
side the criterion for stability cannot be met. Although
several time restrictions can occur during the initial
phase of the migration process[21], the final concentration
distribution will be given by

$$c_J^S(x,t) = \frac{1}{a_J} \left(1 - \sqrt{\frac{x_{max} - \Delta l_o}{x - \Delta l_o}}\right) \tag{2.11}$$

where Δl_o is the initial width of the sample pulse and
x_{max} is the maximal distance that the separand has migra-
ted in the given time interval. It follows that this maxi-
mal distance is given by

$$x_{max} = m_A r_J E^C t + \Delta l_o \tag{2.12}$$

It should be noted that for this mobility configuration
the maximal distance is linearly related with time, where-
as the minimal distance that the separand has migrated,

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x_{min}, is a rather complex function of all parameters involved. For the zone-width $\delta(t)$ it can be derived that

$$\delta(t) = x_{max} - x_{min} = -a_J\Delta l_o c_J^{S*} + 2 \sqrt{-m_A r_J E^C t a_J \Delta l_o c_J^{S*}}$$

(2.13)

where c_J^{S*} is the concentration of the separand in the original sample. Figure 2.3 shows the electrophoretic development as a function of time. From the concentration distribution after one sec it can be seen that the separand concentrates over the boundary between the sampling and the separation compartment. After five sec of migration the zone still contains a homogeneous part, but the
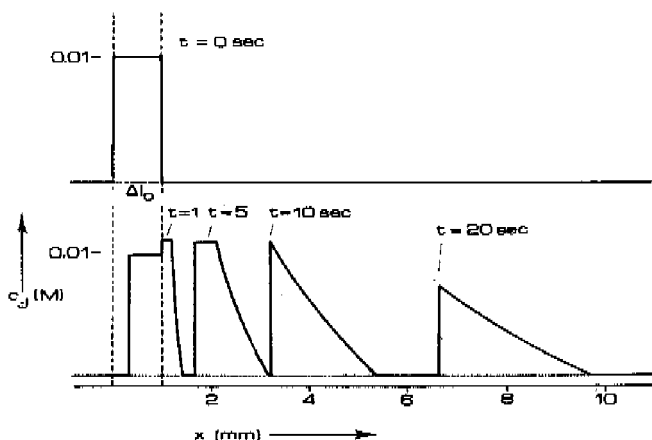
Fig. 2.3 Development of a zone electrophoretic process for a $r_J > 1$ configuration. c_J is the concentration of the separand, x is the migration coordinate (mm). t is the migration time (sec).

"diffuse" region is already clearly visible. After 10 sec the homogeneous part has just disappeared and complete elution starts. From this moment on the concentration distribution according to the eqn. 2.11 is present. It should be emphasized that the boundary velocity during the first ten sec is constant. The moment that true elution starts, the velocity of the moving boundary will gradually

increase untill it has reached the maximal migration velo-
city v_{max}. During this increase the sharpness of the boun-
dary decreases.

When the mobility of the separand is equal to the mo-
bility of the carrier constituent, $r_J = 1$, the sample con-
stituent is only diluted or concentrated over the statio-
nary boundary between the sampling and the separation com-
partment. If the sample again has been introduced as a
block pulse, the concentration distribution will be given
by

$$c_J^S = \frac{c_A^C}{c_A^{C*}} \ c_J^{S*}$$ (2.14)

where c_A^C/c_A^{C*} is the dilution factor over the stationary
boundary between the sampling and the separation compart-
ment. It follows that the concentration of the separand is
independent of time and that the maximal distance that the
separand has migrated is given by eqn. 2.12. Moreover, it
must be concluded that, after an initial elongation or
shortening, the zone width δ is independent of time

$$\delta = \frac{c_A^{C*}}{c_A^C} \ \Delta l_o$$ (2.15)

When the separand has a smaller mobility than that of
the carrier constituent, $r_J < 1$, the leading side of the
separand zone will be sharp, whereas the rear will be dif-
fuse. The final concentration distribution will be given
by

$$c_J^S(x,t) = \frac{1}{a_J} \left\{ 1 - \sqrt{\frac{x_{min} + \Delta l_o (c_A^{C*}/c_A^C - 1)}{x + \Delta l_o (c_A^{C*}/c_A^C - 1)}} \right\}$$ (2.16)

For the zone width $\delta(t)$ it follows that

$$\delta(t) = a_J c_J^{S*} \Delta l_o + 2 \sqrt{m_A r_J E^C t a_J \Delta l_o c_J^{S*}}$$ (2.17)

The minimal migrated distance, x_{min}, is given by

$$x_{min} = m_A r_J E^C t - \Delta l_o \left\{ \frac{c_A^{C*}}{c_A^C} - 1 \right\} \qquad (2.18)$$

2.3. ELECTRIC FIELD STRENGTH PROFILES

Electric field strength profiles and concentration distributions have different forms and there is a distinct difference between time-based and distance-based distributions. According to eqn. 2.4 the electric field strength readily can be calculated once the local constituent concentrations are known. Distance-based electric field strength profiles can be obtained by substitution of the appropriate concentration distribution in eqn. 2.4. When a fixed point electric gradient detection system is used, time-based distributions will be measured and it can easily be shown that for a $r_J \neq 1$ configuration it must hold that

$$\frac{E^S(x_{det}, t)}{E^C} = \sqrt{\frac{t_{det}}{t}} \qquad (2.19)$$

where E^S and E^C are the electric field strengths in the separand zone and in the carrier electrolyte respectively and x_{det} is the distance at which the electric gradient detector is located. For a $r_J > 1$ configuration t_{det} is given by the time interval at which the first separand ion reaches the point of detection. For $r_J < 1$ configurations, however, t_{det} is given by the time interval at which the last separand ion reaches the point of detection. The moment at which the last separand ion, for $r_J > 1$, or the first separand ion, $r_J < 1$, reaches the point of detection, i.e. t_{stop}, is related to the sampled amount, since

$$n_J = 0 \int_{t_{det}}^{t_{stop}} c_J^S(x_{det}, t) dt \qquad (2.20)$$

where n_J is the sampled amount of the separand J and 0 is the cross-sectional area of the separation compartment. So to describe the generated distribution function requi-

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res only the knowledge of t_{det} from either theoretical calculations or experimental observations. The sampled amount should be used to know the time interval during which the generated distribution function is applicable. Combining the eqns. 2.19 and 2.20 a very useful relation for quantitative determinations is found

$$n_J = \frac{0}{a_J} \left(t_{stop} - \frac{2}{3} \sqrt{\frac{t_{stop}^3}{t_{det}}} - \frac{1}{3} t_{det} \right) \qquad (2.21)$$

So for quantitative measurements it is not necessary to measure the exact form of the distribution, since the measurement of both t_{stop} and t_{det} will suffice.
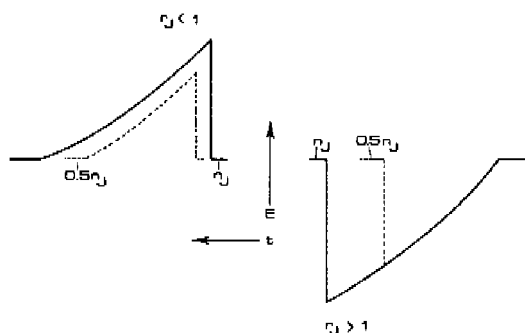
Fig. 2.4 Electric field strength profiles as a function of the sampled amount. E = electric field strength, t = migration time, n_J = sampled amount.

According to eqn. 2.12, t_{det} for a $r_J > 1$ configuration is independent of the sampled amount and is constant for a given separand and carrier electrolyte. For a $r_J < 1$ configuration however both t_{det} and t_{stop} are a function of the sampled amount. As can be seen from Fig. 2.4, not only the shape of the distribution but also its position is influenced by the sample load. Whereas it is possible to define a retention time for the $r_J > 1$ configuration, i.e. t_{det}, this is not possible for the $r_J < 1$ configuration. As mentioned already a $r_J = 1$ configuration will result in a constant electric field strength.

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2.4. RETENTION BEHAVIOUR

The separation of multicomponent samples will develop in a complicated manner, since the concentration distributions and the migration velocities of almost each separand is influenced by the physico-chemical characteristics and concentrations of all constituents present. The effect of mutual interactions in electrophoretic separation techniques is more pronounced than in chromatographic separation techniques. This adverse effect of Ohm's law can be suppressed only by the application of very small amounts of sample. The complexity is further increased as generally weak electrolytes will be applied.

It has been shown that, in isotachophoresis and moving boundary electrophoresis[22], the ratio of separand mobilities in the mixed state is important when separability and current efficiency are considered. The same holds for zone electrophoresis and generally the same optimization rationales can be followed. In anionic separations a low pH of the carrier electrolyte is preferable, whereas for cationic separations a high pH will give the better results. The current efficiency in zone electrophoresis, however, will be low in comparison with that in isotachophoresis owing to the continuous transport of carrier electrolyte.

In zone electrophoresis the zone characteristics will be determined by the carrier electrolyte and the separands. Using a fixed-point detection system, the time-interval that a separand needs to reach the detector, i.e. the retention behaviour, is strongly affected by the proper choice of operational conditions. Considering retention behaviour it can be concluded that the difference in the separand mobilities is important. In experimental practice, a compromise between current efficiency and retention behaviour has to be found. Obviously, pH and complex formation have a great influence on the retention behaviour. Assuming a well buffered electrolyte system and the application of a small amount of sample, pH deviations and inhomogeneities in the electric field can be neglected.

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For the retention time t_R it follows that

$$t_R = t_0/\bar{r}_J$$

where $\bar{r}_J$ is the effective mobility of the separand relative to that of the carrier constituent and t_0 is the retention time of the carrier constituent. Effective mobilities are strongly influenced by pH and the dissociation constants. Fig. 2.5 shows the relative retention as a function of the relative ionic mobility of the separand. The difference between the pK_a value of the separand and the pH of the carrier electrolyte has been used as a parameter.
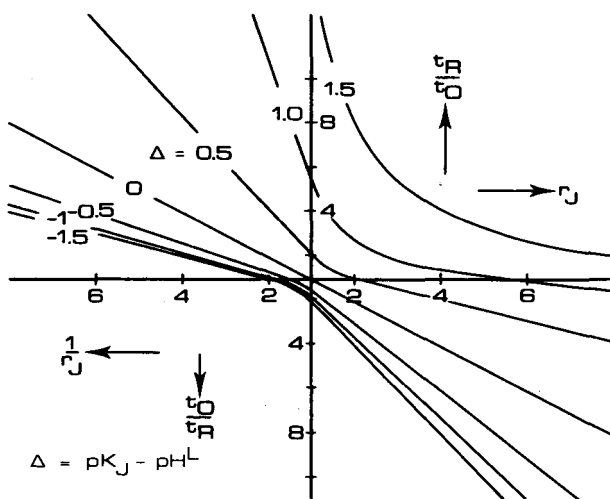
Fig. 2.5 The relative retention as a function of the relative ionic mobility of the separand. The difference between the pK_a of the separand and the pH of the carrier electrolyte has been used as a parameter.

The carrier constituent has been chosen for its optimal buffering capacity, i.e. $pH^C = pK_C$. A separand with a relative ionic mobility of 2 and a low pK value compared with the pH of the carrier electrolyte will have an inverse relative retention, t_0/t_R, of 4. This means that the sample constituent will migrate at a higher velocity than the carrier constituent. A separand with a relative ionic mobility of 0.5, i.e. $1/r_J = 2$, has an inverse relative retention of unity. Obviously this separand cannot be detected by

measurement of the electric gradient.

2.5. THE INFLUENCE OF DIFFUSION

In the given approach, diffusional effects were pur-
posely neglected in order to emphasize the important in-
fluence of the electrophoretic process on the concentra-
tion distributions. In this way the asymmetry, that fre-
quently occurs in zone electrophoresis can easily be ex-
plained as the result of the electrophoretic process. In
experimental practice, however, the diffusional effect
cannot be neglected and for the calculation of concentra-
tion distributions the diffusional effect should be incor-
porated in the equation of continuity. The importance of
diffusional and migrational dispersion, however, can be
evaluated rather easily. Using the appropriate relation-
ships, the eqns. 2.13 and 2.17 can be written in a somewhat
more practical form:

$$\delta(t) = \frac{c_J^{S*}}{c_A^C} \Delta l_o f(r) + 2 \sqrt{\frac{c_J^{S*}}{c_A^C} \Delta l_o f(r) v_J t} \qquad (2.22)$$

where $f(r)$ is a function of the ionic mobilities and v_J is
the migration velocity of the separand in the carrier elec-
trolyte. Both $f(r)$ and Δl_o will commonly show only a limi-
ted degree of freedom and both should be minimized. Neglec-
ting the initial discontinuities, band-spreading due to
diffusion and to electrophoretic migration is of the same
order of magnitude when

$$D_J \approx 0.1 \frac{c_J^{S*}}{c_A^C} f(r) \Delta l_o v_J \qquad (2.23)$$

Taking a diffusion coefficient, D_J, of 10^{-5} cm^2/sec at a
migration rate of 1 mm/sec and an initial band width of
1 mm, diffusion and electrophoretic migration will have a
comparable adverse effect at a concentration ratio c_J^{S*}/c_A^C
of 10^{-2}. Below this value band spreading is due mainly to
diffusion and above this value electrophoretic migration

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will mainly contribute. Assuming that the zone electro-
separations are carried out in a capillary system with an
inner diameter of 0.2 mm and using a carrier electrolyte
at a concentration of 10 mM, the migrational effect will
be appreciable when more then 3×10^{-12} mole of the sepa-
rand is injected. Obviously the detection of such a low
amount will put very high demands on the detection systems.
Generally a higher sample load will be used and consequent-
ly the migrational effect will dominate.

Other forms of dispersion, through which the effecti-
ve diffusion coefficient may exceed the thermal one, will
obscure the migrational dispersion and should be minimized.
It should be noted,however,that the occurence of "boundary
anomalies" counteracts the influence of non migrational
dispersion. This has been shown to be especially true for
isotachophoresis[24], but holds also for zone electrophore-
sis, although to a minor extent. The selfrestoring capabi-
lities, a direct result of the "boundary anomalies", are
decreasing when r_J approaches the critical value of unity.
This configuration will be particularly sensitive for non-
migrational dispersion.

The adverse effect of a relatively large sampling
width, Δl_ϕ, can be counteracted by the concentrating capa-
bilities of the electrolyte system. Choosing the condition
$c_J^{S*} \ll c_A^C$ and a high sampling ratio, i.e. $c_J^{S*} \gg c_A^{S*}$, the
separand will be concentrated over the stationary boundary
between the sampling and the separation compartment. This
concentrating effect is the result of the fact that in
electrophoresis the *Kohlrausch* regulating function concept
cannot be overruled by the electrophoretic process (Part 1
Chapter 1). It seems that this forms the most profound
difference between chromatographic an electrophoretic se-
paration principles. In experimental practice this means
that, in order to take full advantage of the concentrating
capabilities, the sample should not be equilibrated with
the carrier electrolyte.

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CHAPTER 3

Isotachophoresis

*A model for the separation
process of isotachophoresis
is given. The influence of
operational parameters on the
separation process is descri-
bed in terms of time for re-
solution, load capacity and
current efficiency. Optimiza-
tion favoured by a high dri-
ving current, a low mobility
of the counter constituent
and a low pH for anionic se-
parations, whereas for catio-
nic separations a high pH
will be favourable.*

3.0. INTRODUCTION

In isotachophoresis a steady state configuration is
obtained as the results of a separation process that pro-
ceeds according to the moving boundary principle[1]. Though
this has been recognized for many years most attention has
been paid to the development of theoretical models that
describe the steady state of isotachophoresis. These theo-
retical approaches have resulted in different computer
programs, that have shown to be in good agreement with one
another and with experimental data[2-5]. Though the steady

state, representing the pursued target, is important, additional features are decisive dealing with the practical applicability of a used isotachophoretic procedure. Most of these features are related to the separation process. Although the separation process is a transient state, it is governed by the same regulating function concept as the steady state. A quantitative description of the transient state provides information on the time needed for an isotachophoretic separation. Moreover, such a description requires the definition of resolution and separability in isotachophoresis and shows the results that can be expected from optimization procedures.

In the transient state model we will neglect several secondary effects, such as temperature distribution and activity effects. Although these effects are not always marginal, they will generally not have a drastic impact on the transient state model and its resulting guidances for optimization. With regard to uneven temperature distributions, either longitudinal or transversal[6], it should be emphasized that their effect will be deleterious only under extreme operating conditions. Working at moderate current densities, without excessive cooling, convective disturbances are negligible and temperature differences can be well controlled[7]. In special cases, temperature effects can have a favourable influence on separation, but so far temperature programming has not been studied in isotachophoresis.

The transient state model and its resulting implications will be developed for a sample containing two monovalent weakly ionic separands. The relative simplicity of such a model allows a fundamental understanding of the isotachophoretic separation process and provides a realistic view on what results can be expected from optimization procedures. It is obvious that the model can be extended to multivalent weak electrolytes. The efficiency of such considerations, however, will be poor as no other guidances will be found. Concerning multi-component samples it should be emphasized that the mathematical intricacy in-

creases very rapidly with increase in the number of sepa-
rands. For strong electrolytes extension of the model is
not difficult but is laborious and monastic for practical
purposes[3,8,14-16]. The major advantage of relatively sim-
ple model is that it clearly indicates the importance of
physico-chemical, operational and instrumental parameters.

3.1. THE SEPARATION PROCESS

Although essentially immaterial, we shall consider a
separation compartment of uniform dimensions operated at a
constant electric driving current and a constant tempera-
ture. We shall deal with the case where all constituents
involved are monovalent weak electrolytes. The development
of the separation process is shown in Fig. 3.1. In the ini-
tial situation, t_0, the sampling compartment has been
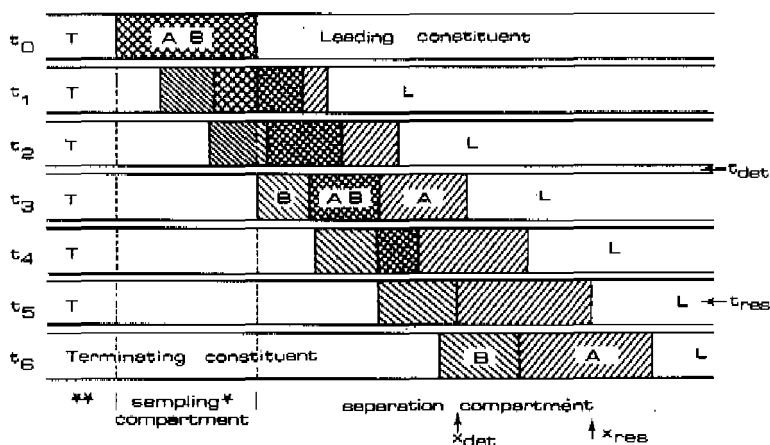
Fig. 3.1 The separation process.

filled with a homogeneous mixture of the two separands A
and B. The separation compartment contains the leading
constituent L and the terminating compartment is filled
with the terminating constituent T. A counter constituent
C, to preserve electroneutrality, is common to both the
sample constituents, the leading and the terminating con-
stituents. Electrolyte changes in the electrode compart-

ments, temperature effects and activity effects are ne-
glected. Each compartment has its own regulating function,
due to the initial composition of the electrolytes. Star-
ting from t_0 the separation of the sample will occur ac-
cording to the moving boundary principle. All zone charac-
teristics are, as long as they exist, constant with time.
At different times several moving boundaries can be pre-
sent: A/L, AB/A, B/AB, B/A, T/B, B^*/AB^*, T^*/B^*. Boundary
velocities are given by local conditions. The sampling
compartment causes the stationary boundaries: AB^*/AB, B^*/B,
T^*/T, T^{**}/T^*. At t_3 the sample is leaving the sampling
compartment and from this time on the total zone length of
the sample zone is constant. The properties of the mixed
zone in the separation compartment will be in agreement
with the local regulating function and the nature of the
sample. At t_5 resolution is obtained and from this moment
on the individual zone lengths will be constant. Resolu-
tion was obtained at t_{res} with a resolution length of x_{res}.
Using a fixed point detector, detection could have been
started at t_{det} with the detection system located at x_{det}.
It should be emphasized that within the separator three
different regions are present and each has its own regula-
ting behaviour. The regulating functions, eqns. 1.12 and
1.14, are the mathematical expression of this regulating
behaviour and locally they cannot be overruled by the elec-
trophoretic process[9]. All changes in electrophoretic para-
meters, e.g. concentration, pH and conductance, will be in
agreement with the local regulating function.
Applying eqn. 1.17 it follows that for monovalent weak and
strong separands all ionic subspecies are diluted or con-
centrated to the same extent over the stationary boundary
between the separation and the sampling compartment

$$\phi = \frac{c_B^{M^*}}{c_A^{M^*}} = \frac{c_B^M}{c_A^M} = \text{constant} \tag{3.1}$$

where the asterisk marks the mixed zone properties in the
sampling compartment. Hence the sampling ratio ϕ for the

charged sub-species is invariable. Taking the leading
electrolyte as a frame of reference, the regulating func-
tions will result in

$$\bar{c}_L^L + \bar{c}_C^L = \bar{c}_A^M + \bar{c}_B^M + \bar{c}_C^M \tag{3.2}$$

and

$$\frac{\bar{c}_L^L}{r_L} + \frac{\bar{c}_C^L}{r_C} = \frac{\bar{c}_A^M}{r_A} + \frac{\bar{c}_B^M}{r_B} + \frac{\bar{c}_C^M}{r_C} \tag{3.3}$$

where C is the counter constituent common to all separands
and r_I is the mobility of the constituent I relative to
that of an appropriate reference constituent, in this case
the leading constituent. Chemical equilibrium and electro-
neutrality imply that

$$\bar{c}_A^M = c_A^M(1 + 10^{\pm(pK_A - pH^M)})$$

$$\bar{c}_B^M = \phi c_A^M(1 + 10^{\pm(pK_B - pH^M)}) \tag{3.4}$$

$$\bar{c}_C^M = - (1 + \phi) c_A^M(1 + 10^{\pm(pH^M - pK_C)})$$

Combining the eqns. 3.2, 3.3 and 3.4 we obtain

$$\bar{c}_L^L + \bar{c}_C^L = c_A^M \left\{ \frac{1}{\alpha_A^M} + \frac{\phi}{\alpha_B^M} - \frac{(1 + \phi)}{\alpha_C^M} \right\} \tag{3.5}$$

and

$$\bar{c}_L^L (1 - r_C) = c_A^M \left\{ \frac{(r_A - r_C)}{r_A \, \alpha_A^M} + \frac{(r_B - r_C)}{r_B \, \alpha_B^M} \right\} \tag{3.6}$$

We now introduce the relative leading concentration ρ and
the reduced mobility k_I

$$\rho = \frac{\bar{c}_C^L}{\bar{c}_L^L} \quad \text{and} \quad k_I = \frac{1 - r_C}{r_I - r_C} \tag{3.7}$$

Elimination of c_A^M gives a quadratic equation for the pro-
ton concentration in the mixed zone. Only one root will
have physical significance.

$$a \ 10^{2\text{pH}^M} + b \ 10^{\text{pH}^M} + c = 0 \tag{3.8}$$

The constants a, b and c for the equation are given in Table 3.1. Once the pH in the mixed zone has been calculated, all dynamic process variables can be calculated by using the appropriate equations. Moreover, steady-state configurations are obtained by using a zero sampling ratio.

TABLE 3.1

CONSTANTS FOR THE pH OF THE MIXED ZONE (EQN. 3.8)

Anionic constituents	Cationic constituents
$a = (1+\phi)10^{-pK_C}$	$a = (\frac{1+\rho}{r_A k_A} - 1)10^{-pK_A} + \phi(\frac{1+\rho}{r_B k_B} - 1)10^{-pK_B}$
$b = (1+\rho)(\frac{1}{r_A k_A} + \frac{\phi}{r_B k_B})$	$b = (1+\rho)(\frac{1}{r_A k_A} + \frac{\phi}{r_B k_B})$
$c = (\frac{1+\rho}{r_A k_A} - 1)10^{pK_A} + \phi(\frac{1+\rho}{r_B k_B} - 1)10^{pK_B}$	$c = (1+\phi)10^{pK_C}$

Moving boundary experiments can be simulated by the introcution of a high sample load. Computerization allows multiple calculations of all dynamic process variables[10]. As the criterion for separation, Part 1 Chapter 1, has to be satisfied, the pH of the mixed zone is of decisive importance. According to eqn. 3.8, this pH is influenced by the physico-chemical characteristics of the separands and of the common counter constituent, by the sampling ratio, ϕ, and the relative leading concentration, ρ. The last parameter is closely related to the pH of the leading electrolyte and the former one to the pH of the sample. We shall consider anionic separations, but equivalent relationships and conclusions can be made for cationic separations.

In isotachophoresis the leading constituent generally must have a high effective mobility, so strong ionic constituents like chloride are commonly used[11]. In this instance it follows that

$$-\infty < \rho = -\frac{1}{\alpha_C^L} < -1 \tag{3.9}$$

At $\rho \to -1$ the counter constituent is used far below its pK
value and it behaves like a strongly ionic species. In
this case the leading electrolyte has no buffering capaci-
ty. At $\rho = -2$ the counter constituent is used at its pK
value, $pH^L = pK_C$, and therefore exhibits its full buffe-
ring capabilities. High negative values for the relative
leading concentration again imply a low buffering capacity.
Moreover, in this case the concentration of the counter
constituent will be high in comparison with that of the
leading constituent, which can be favourable in complex
formation. It is easily shown that for increasing $pK_J - pH^L$,
i.e. the separands are only partially dissociated at
the pH of the leading electrolyte, the difference in pH
between the mixed zone and the leading electrolyte will in-
crease. Separands that are already completely ionized at
the pH of the leading electrolyte will induce only a
slight elevation of pH^M and therefore will be separated as
strong electrolytes. Counter constituents with a low pK
value in comparison with the pH of the leading electrolyte
show a tendency to diminish this increase in pM^M. When the
leading constituent is a strongly ionic species the pH of
any following zone will be higher than the pH of the lea-
ding zone. If however, a weak constituent is chosen as the
leading constituent negative pH steps can occur under the
appropriate conditions[11].

a: pK_C = 2.0
b: pK_C = 3.0
c: pK_C = 4.0
d: pK_C = 5.0
e: pK_C = 6.0
f: pK_C = 7.0
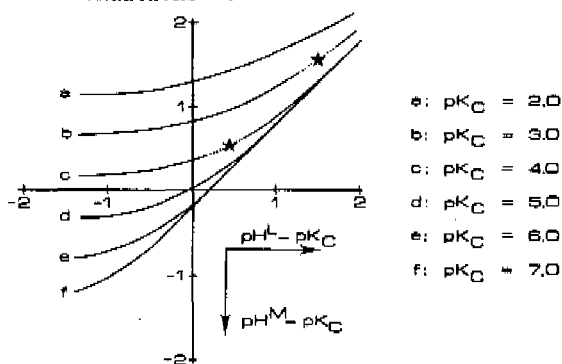
*Fig. 3.2 The influence of the pH of the leading electrolyte
on the pH of the mixed zone. Constituent data: Table 3.2.*

Obviously, problems in separation generally occur when both the pK values and the mobilities of the separands show only little differences. An example of such a pair is given in Fig. 3.2. When the more mobile constituent has the larger dissociation constant, the criterion for separation will always be satisfied. Optimization in this instance is very straightforward: low pH^L and low pK_C. However, when the more mobile constituent has the smaller equilibrium constant, the criterion for separation need not always be satisfied. It will depend on the proper choice of pH^L and pK_C whether the critical pH, as indicated in Fig. 3.2 with an asterisk, will be obtained.

TABLE 3.2

CONSTITUENT DATA FOR FIG. 3.2 AND FIG. 3.3

constituent	mobility cm^2/Vsec	pK_a value
Leading, L	-77×10^{-5}	$pK_L = -2$
Counter, C	$+30 \times 10^{-5}$	pK_C variable
Separand, A	-45×10^{-5}	$pK_A = 4.5$
B	-30×10^{-5}	$pK_B = 4.0$

Sampling ratio: Fig. 3.2 $\phi = 1$, Fig. 3.3 variable.

From eqn. 1.20, it follows that the given pair will not separate at $pH^M = 4.52$. If a counter constituent is chosen with $pK_C = 4$, there will be no separation at $pH^L = 4.40$. Above this pH^L, constituent B will migrate behind A, whereas the order will be reversed at low pH^L. It is easily shown that the lower pH of the leading electrolyte will give a more favourable ratio of effective mobilities. It should be emphasized that the mobilities of the separands have only a marginal influence owing to their limited numerical extension.

The influence of the sampling ratio is shown in Fig. 3.3, where limiting values of ϕ are given. At zero sampling ratio the pH of the "mixed" zone will be that of the isotachophoretic A zone, whereas at very large sampling ratio the pH^M will be governed by the separand B.
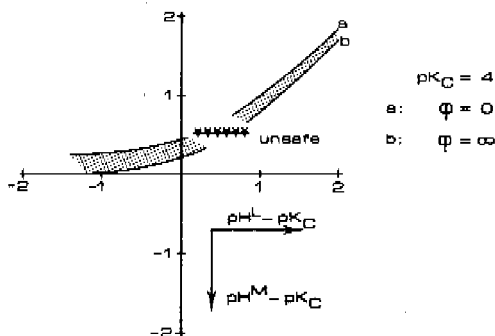
Fig. 3.3 The influence of the sampling ratio on the pH of the mixed zone. Constituent data: Table 3.2.

Hence, whatever the pH of the sample or its molar concentration ratio, the pH of the mixed zone will always lie between the pH values of the completely resolved zones. In common practice sampling ratios can show appreciable fluctuations due to the sample pH or the molar concentrations. Fig. 3.3 therefore gives an indication of the "unsafe" margin, which in this particular case extends over 0.4 pH unit. It is obvious that the pH of the leading electrolyte must be chosen well out of this "unsafe" region. Sampling ratios can show an even larger influence, when the pK values of the separands show more distinct differences.

3.2. RESOLUTION

Once the criterion for separation, $m_i/m_j \neq 1$, has been satisfied, the time needed for resolution becomes important. When a separand zone contains all of the sampled amount, resolution has been obtained for that constituent. We therefore define the resolution, R, as the separated fractional amount of the constituent:

$$R_i = \frac{\text{separated amount of I}}{\text{sampled amount of I}} \tag{3.10}$$

From this definition, it follows that during the separation process the resolution increases from zero to its maximal value, unity. Constituents that fail to separate,

remain at zero resolution and can be termed ideally mixed zones[12].

Complete separation of a sample requires the resolution values of all separands to be unity. As expected the resolution and its time derivative are complex functions of the constituents involved and the driving forces applied. Moreover, the mathematical intricacy involved in calculating optimal process variables increases rapidly with increasing number and complexity of the separands. For strong electrolytes relevant mathematical formulations have been published[8] but most separations nowadays concern weak electrolytes. In this case dissociation equilibria, and therefore a proper choice of pH, are tools in the control and optimization of the separation process. When dealing with complex formation, association equilibria should be involved. It has been suggested[13] that the difference in migration rates, i.e. $v_i - v_j$, is of decisive importance in separation. However, in isotachophoresis and moving boundary electrophoresis this does not apply, and in these instances it is more beneficial to optimize the ratio of the migration rates, i.e. v_i/v_j. Whereas the velocity difference will reach a maximal value as a function of pH, the ratio shows no such optimum. As the local electric field strength for both separands in the mixed zone will be the same, it follows directly that the mobility ratio must be maximized or minimized, depending on the migration configuration, Part 1 Chapter 1. Introducing equilibrium constants and ionic mobilities it follows that in anionic separations the lowest pH will give the better mobility ratio, and mutatis mutandis holds for cationic separations. It should be emphasized however, that pH extremes have only limited experimental applicability and that practical considerations often govern the proper choice of pH. Moreover, a low numerical value of the effective mobility will require a high electric field strength in order to obtain an appreciable migration rate and other electrokinetic effects may then prevail.

From Fig. 3.1 it can be concluded that the time

60

needed to reach the maximal resolution for the constituent A can be expressed as a function of the boundary veloci- ties:

$$t_{res} = \frac{\ell_A}{v_{L/A} - v_{A/A+B}} \qquad (3.11)$$

where ℓ_A is the steady state zone length of the separand zone A and $v_{I/J}$ is the boundary velocity.
Using the appropriate relationships, we obtain

$$t_{res} = \frac{n_A \, F}{I} \left\{ \frac{1 + \phi \, \dfrac{k_A}{k_B}}{1 - \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}} \right\} \left\{ 1 - \frac{r_C}{r_A} \right\} \qquad (3.12)$$

Hence it follows that the time of resolution is a complex function of the concentration and the pH of both the lea- ding electrolyte and the sample, of the sampled amount, the sampling ratio, the electric driving current and all ionic mobilities and dissociation constants. It should be noted that in eqn. 3.12 it is the ratio of the effective separand mobilites and not their difference that is impor- tant. Further this equation emphasizes the importance of the pH of the mixed zone.

For the length of the separation compartment, needed to contain the completely resolved state, x_{res}, it follows that

$$x_{res} = \frac{n_A}{Oc_L^{-L}} \left\{ \frac{1 + \phi \, \dfrac{k_A}{k_B}}{1 - \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}} \right\} \frac{1}{k_A r_A} \qquad (3.13)$$

where O is the cross sectional area of the separation com- partment.

For a given sample and electrolyte system, the resolu- tion length, x_{res}, is independent of the applied electri- cal driving current, whereas the time of resolution, t_{res}, is inversely related to the driving current. From the eqns.

3.12 and 3.13 it follows that the separation of a
given sample load requires a definite amount of coulombs
and column volume. Taking limiting values for eqn. 3.12,
it follows that

$$\frac{n_A F}{I} < t_{res} < \sim \qquad (3.14)$$

The relationship between the sampled amount and the time
of resolution is obviously a linear one. Moreover, for a
two-separand sample, resolution for both separands will be
obtained simultaneously.

The influence of the counter constituent

From eqns. 3.12 and 3.8 it can be concluded that both
the time of resolution and the pH of the mixed zone are
affected by the mobility of the common counter constituent.
Fig. 3.4 shows the variation of the time of resolution as
a function of the mobility of the counter constituent rela-
tive to that of the leading constituent. It follows that a
low mobility favours the time of resolution, partly becau-
se of its influence on the pH of the mixed zone and partly
because it increases the efficiency of the current trans-
port.
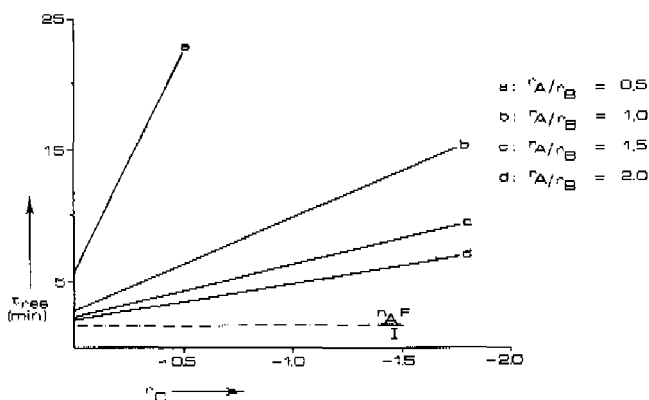
*Fig. 3.4 The time of resolution as a function of the rela-
tive counter constituent mobility. Constituent data: Table
3.3.*

TABLE 3.3
CONSTITUENT DATA FOR FIG. 3.4

Constituent	mobility cm^2/Vsec	pK_a value
Leading, L	-70×10^{-5}	$pK_L = 0$
Counter, C	variable	$pK_C = 4.0$
Separand, A	variable	$pK_A = 4.0$
B	-30×10^{-5}	$pK_B = 5.0$

Leading electrolyte: $pH^L = 4.0$, $\bar{c}^L = -0.01$ M
Sample: $pH^{M*} = 4.0$, $n_A = n_B = 10^{-7}$ mole, $\bar{c}_A^{M*} = \bar{c}_B^{M*} = -0.05$ M
Driving current: $I = 100$ μA,O = 0.002 cm^2.

The influence of the mobility of the counter constituent
on the pH of the mixed zone is, however, marginal. For the
lower limiting value of zero it follows that the pH of the
mixed zone becomes independent of the constituent mobili-
ties.

As eqn. 3.12 is a function of the effective separand
mobilities in the mixed zone, the pH of this zone is of de-
cisive importance. Recognizing that all mixed zone characte-
ristics are determined by the leading electrolyte as well
as by the sample, it is obvious that the relative leading
concentration, ρ, and the sampling ratio, φ, can be used
in optimization procedures. Both ρ and φ are functions of
pH and can be chosen arbitrarily within practical limita-
tions.

The influence of the pH of the leading electrolyte

From the practical point of view the choice of the
pH of the leading electrolyte offers the best possibili-
ties in optimization procedures. In Fig. 3.5 the influence
of the pH of the leading electrolyte, for different mobili-
ties of the separands, on the time of resolution is shown.
The counter constituent has been chosen for its maximal
buffering capacity at the pH of the leading electrolyte.
Dealing with monovalent anionic constituents it follows
that, whenever the more mobile separand has the larger
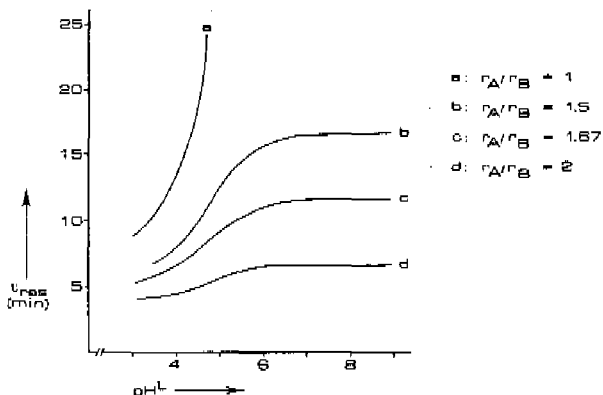
Fig. 3.5 Time of resolution as a function of the pH of the leading electrolyte. Constituent data: Table 3.5.

dissociation constant, resolution is favoured by a low pH of the leading electrolyte. Mutatis mutandis holds for cationic separands, for which a high pH will be favourable. It should be noted that the effect on the time of resolution can be quite appreciable. When the ionic mobilities are equal only a low pH^L will result in an acceptable time of resolution. For separands that have already large differences in their ionic mobilities, the effect of decreasing the pH of the leading electrolyte is much less pronounced. The flattening of the sigmoidal curves at high pH indicates that the sample constituents are being separated as monovalent strong ions, in which event there is, of course, no influence of pH^L.

Though the rationale for optimization of the separation process is rather straightforward, care should be taken in choosing extreme pH values for the electrolyte systems. Electrolyte systems that induce a low effective mobility of the constituents, e.g. smaller than 10^{-4} cm^2/Vsec, will require the use of high electric gradients, e.g. larger than 200 V/cm. In analytical practice such electrolyte systems have only limited value[11]. At low high pH the contribution of protons or hydroxylions, being rather mobile species, to the conductance can not be neglected and their deleterious effects will be dealt with in Chapter 3.4.

TABLE 3.4

CONSTITUENT DATA FOR FIGS. 3.5 AND 3.6

Constituent	mobility $cm^2/Vsec$	pK$_a$ value Fig. 3.5	pK$_a$ value Fig. 3.6
Leading, L	-77×10^{-5}	$pK_L = -2$	$pK_L = -2$
Counter, C	$+30 \times 10^{-5}$	$pH^L = pK_C$	$pH^L = pK_C$
Separand, A	variable	$pK_A = 4.0$	$pK_A = 4.5$
B	-20×10^{-5}	$pK_B = 4.5$	$pK_B = 4.0$

Leading electrolyte: pH^L variable, $\bar{c}_L^L = -0.01$ M
Sample: $pH^{M*} = 4.0$, $n_A = n_B = 10^{-7}$ mole, $\bar{c}_A^{M*} = \bar{c}_B^{M*} = -0.05$ M
Driving current: $I = 100$ μA, $O = 0.002$ cm^2.
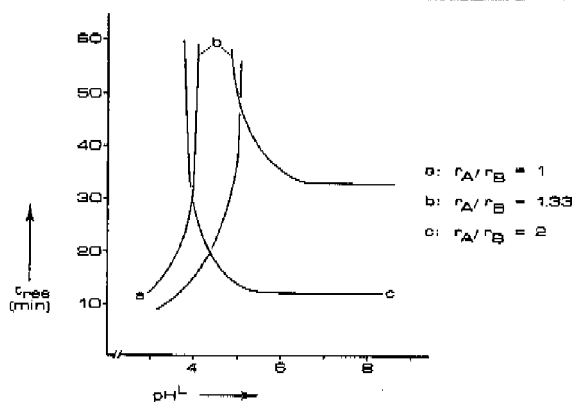
Fig. 3.6 *Time of resolution as a function of the pH of the leading electrolyte. Constituent data: Table 3.4.*

If the more mobile separand has the lower dissocia-
tion constant, the situation becomes more complex. The pH
of the mixed zone, at which, in this case, no separation
occurs and its relation to the pH of the leading electro-
lyte,have already been discussed. From Fig. 3.6 it follows
that the pH of the leading electrolyte must be at least
one pH unit different from the critical pH^L in order to
obtain an acceptable time of resolution. The question of
whether a high or a low pH must be chosen depends on the

physico-chemical characteristics of the separands. Never-
theless, the tendency that a low pH^L is favourable still
holds. For example, with the mobility ratio of 1.33 reso-
lution will be obtained at $pH^L = 7$, but a higher resolu-
tion rate will be obtained at $pH^L = 3$. At high pH the se-
parands will migrate in order of ionic mobilities, whereas
at low pH they will migrate in order of dissociation con-
stants.

The influence of the pH of the sample

Although in practice the pH of the sample can not be
varied arbitrarily, its influence can nevertheless be sub-
stantial, as can be seen from Fig. 3.7.
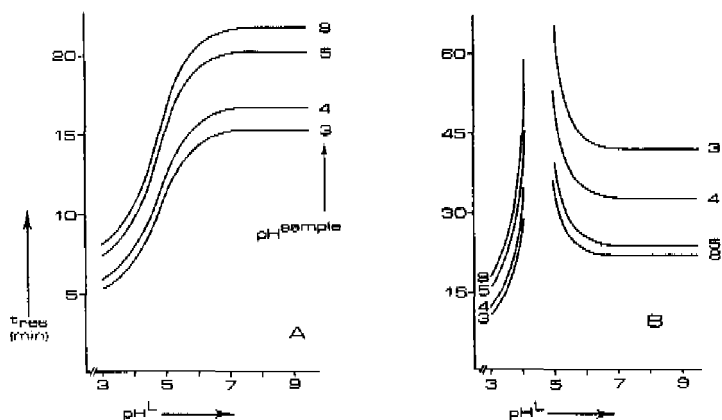
*Fig. 3.7 The influence of the pH of the sample on the time
of resolution. Constituent data: Table 3.5.*

Again, resolution is favoured by a low pH of the sample.
When the more mobile constituent has the larger dissocia-
tion constant, Fig. 3.7A, the effect is straightforward.
Resolution is favoured by a low pH of both the sample and
the leading electrolyte. From Fig. 3.7B where $r_A > r_B$ and
$K_A < K_B$, the guidance for this "reversed pair" of sepa-
rands can be deduced. When running such a sample at a high
pH of the leading electrolyte it is also preferable to use
a sample with a high pH.

66

TABLE 3.5

CONSTITUENT DATA FOR FIG. 3.7

| Constituent | mobility $cm^2/Vsec$ | pK$_a$ value | |
		Fig. 3.7A	Fig. 3.7B
Leading, L	-70×10^{-5}	$pK_L = -2$	$pK_L = -2$
Counter, C	$+30 \times 10^{-5}$	$pH^L = pK_C$	$pH^L = pK_C$
Separands, A	-40×10^{-5}	$pK_A = 4.0$	$pK_A = 4.5$
B	-30×10^{-5}	$pK_B = 4.5$	$pK_B = 4.0$

Leading electrolyte: pH^L variable, $\bar{c}_L^L = -0.01$ M

Sample: pH^{M*} variable, $n_A = n_B = 10^{-7}$ mole, $\bar{c}_A^{M*} = \bar{c}_B^{M*} = -0.05$ M

Driving current: $I = 100$ μA, $O = 0.002$ cm^2.

Optimal conditions are obtained, however, at a low pH of both the sample and the leading electrolyte. In both cases the migration configuration of the separands in the sampling compartment and the separation compartment will be the same. If, however, a low pH of the leading electrolyte is combined with a high pH of the sample, *B* will separate as well in the sampling compartment as in the separation compartment.
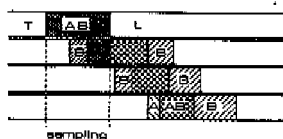
Fig. 3.8 The dual separation phenomenon.

As soon as the sample train has left the sampling compartment, Fig. 3.8, a zone-electrophoretic elution of the mixed zone in the separation compartment will start. As soon as the terminating constituent has reached the rear side of the zone-electrophoretic concentration distribution, a new discontinuity, the separand zone *A*, will start to grow. This phenomenon of zone-electrophoretic rearrangement, has no other influence on the separation process, as

has been discussed already.

3.3. TIME OF DETECTION AND LOAD CAPACITY

Eqn. 3.13 suggests that a fixed-point detector must be located at x_{res} from the sampling compartment and that detection can be started at t_{res}. From Fig. 3.1, however, it follows that this is not always the case, as detection can already have commenced before the sample has been resolved completely. The criterion for detection states, that only resolved separands must be detected, i.e. the mixed zone should resolve the moment reaches the point of detection. Hence, for the minimal length at which the detector must be located, x_{det}, it follows that

$$x_{det} = t_{res} \; v_{A/A+B} \tag{3.15}$$

and, for the moment at which detection must be started, t_{det},

$$t_{det} = x_{det}/v_L \tag{3.16}$$

It follows directly that the time of resolution will be larger than or equal to the time of detection, as holds for the resolution distance and the detection distance. Using the appropriate relationships we obtain

$$\frac{x_{det}}{x_{res}} = \frac{t_{det}}{t_{res}} = \frac{\phi \; \dfrac{k_A}{k_B} + \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}}{1 + \phi \; \dfrac{k_A}{k_B}} \tag{3.17}$$

For a fixed point detector it is important to minimize both x_{det} and t_{det}, and optimization procedures are analogous to the minimization of the time of resolution. Fig. 3.9 shows the influence of the sampling ratio on the ratio of detection time and resolution time. Depending on the actual parameters, the effect can be considerable. For a low sampling ratio and a low mobility ratio, the time of detection will be very small compared with the time of resolution. In practical terms this means that,
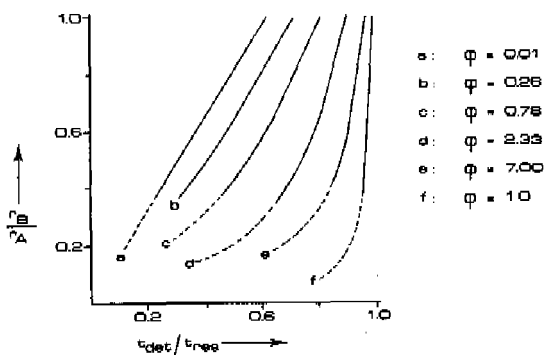
Fig. 3.9 Relationship between the time of detection and the time of resolution. Constituent data Table 3.6.

TABLE 3.6

CONSTITUENT DATA FOR FIG. 3.9 AND FIG. 3.10

Constituent	mobility cm^2/vsec		pK_a value	
	Fig. 3.9	Fig. 3.10	Fig. 3.9	Fig. 3.10
Leading, L	-77×10^{-5}	-77×10^{-5}	$pK_L = -2$	$pK_L = -2$
Counter, C	30×10^{-5}	30×10^{-5}	$pK_C = 4$	$pK_C = 4$
Separand, A	variable	variable	$pK_A = 4$	$pK_A = 4$
B	-30×10^{-5}	-30×10^{-5}	$pK_B = 4.5$	$pK_B = 4.5$

Leading electrolyte: $pH^L = 4$, $\bar{c}_L^L = -0.01$ M
Sample: Fig. 3.9 $pH^{M*} = 4$, ϕ variable, $n_A = 10^{-7}$ mole
         Fig. 3.10 pH^{M*} variable, $n_A = n_B$
Driving current: Fig. 3.9 $I = 100$ µA, $O = 0.002$ cm^2.

whenever the more mobile separand has a high concentration compared with that of the less mobile separand, detection can be started early and only a short separation compartment is needed. At high sampling ratios, the time of detection will be almost equal to the time of resolution.

In common practice, however, the detector will be located at a fixed position in the separation compartment,

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x_{detfix}, so it is impossible to choose the actual length of the separation compartment. For the maximal sample load, n^{max}, for the column we obtain

$$n_A^{max} = n_L^{load} r_A k_A \left\{ \frac{1 - \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}}{\phi \dfrac{k_A}{k_B} + \dfrac{\alpha_B^M r_B}{\alpha_A^M r_A}} \right\} \tag{3.18}$$

where n_L^{load} is the amount of the leading constituent filling the separation compartment, from the sampling compartment to the detector.
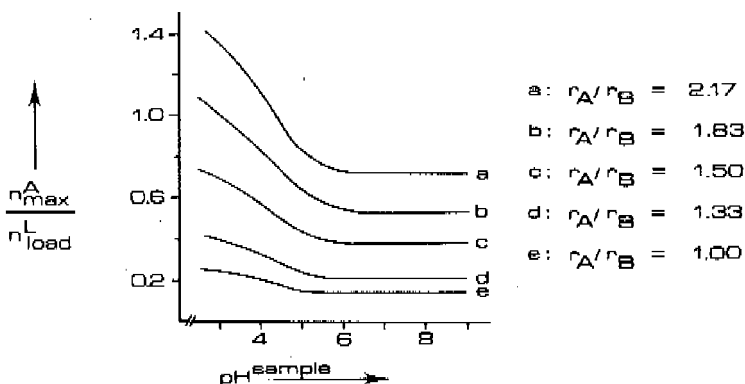
Fig. 3.10 Influence of the sample pH on the load capacity. Constituent data: Table 3.6.

The maximal sample load for the second separand follows directly from the given definitions. Moreover, eqn. 3.18 can be transformed directly into a time-based or distance-based form, using

$$n_L^{load} = x_{detfix} \, \bar{c}_L^L = t_{detfix} \, \frac{I}{F(1 - r_C)} \tag{3.19}$$

Eqn. 3.19 is very useful, since it gives directly the amount of the leading electrolyte, n_L^{load}, once the product of time and driving current, i.e. the amount of Coulombs used, is measured and the transference number of the leading constituent is known. Since the amount of leading constituent can be arbitrarily chosen, we define for the load capacity C

$$C_{load} = \frac{n_A^{max}}{n_L^{load}} \tag{3.20}$$

A maximal load capacity is obtained by optimization of the separation process. For anionic separations a low pH of both the sample and the leading electrolyte will favour the load capacity, as can be seen from Fig. 3.10. For cationic separations a high pH of the leading electrolyte will be preferable.
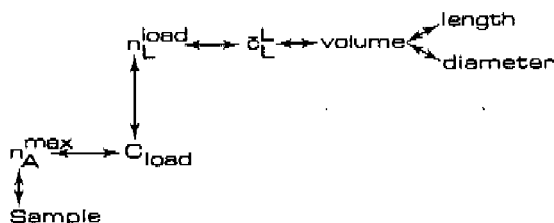
Fig. 3.11 Column evaluation.

The load capacity can be used for defining the required column dimensions or vice versa. The evaluation procedure is schematically given in Fig. 3.11.

3.4. CURRENT EFFICIENCY

In the previous sections it was shown how important parameters, such as time of resolution and load capacity, are related with operational and instrumental conditions. In addition,however, it is important to know how efficient the applied electric driving current is used for separation. Eqn. 3.12 offers a unique possibility to derive the dimensionless separation number S. The advantage of this

separation number is that it is essentially independent of the amount of sample, column geometry and the electric driving current. Differentiation of the separated amount of the constituent A with respect to time and multiplication by F/I gives

$$S_A = \frac{F}{I} \frac{\partial}{\partial t} n_A = \left\{ \frac{1 - \frac{\alpha_B^M r_B}{\alpha_A^M r_A}}{1 + \phi\frac{k_A}{k_B}} \right\} \left\{ \frac{r_A}{r_A - r_C} \right\} \tag{3.21}$$

The physical significance of the dimensionless separation number is that it gives the relative part of the driving current, that is actually used for the separation of the separand A. It should be noted that the separation number is not equivalent with the transference number T. For the transference number of the constituents A and B in the mixed zone it follows that:

$$T_A^M = \frac{1}{1 + \phi\frac{k_A}{k_B}} \left\{ \frac{r_A}{r_A - r_C} \right\} \quad \text{and} \quad T_B^M = \frac{\phi\frac{k_A}{k_B}}{1 + \phi\frac{k_A}{k_B}} \left\{ \frac{r_B}{r_B - r_C} \right\} \tag{3.22}$$

In the limiting cases, where $\phi = 0$ or $\phi = \infty$, the transference numbers are equal to those in the separated zones. Combining the eqns. 3.21 and 3.22 we obtain for the separation number

$$S_A = \left\{ 1 - \frac{\alpha_B^M r_B}{\alpha_A^M r_A} \right\} T_A^M \tag{3.23}$$

Taking limiting conditions for the pH of the mixed zone and the sampling ratio into consideration it follows that:

$$0 < S_A < T_A \tag{3.24}$$

The separation number of the second separand, B, is closely related to that of A, as

$$S_B = \left\{ \frac{\alpha_A^M r_A}{\alpha_B^M r_B} - 1 \right\} \cdot T_B^M = \frac{S_A T_B^M}{T_A^M - S_A} = \chi S_A \qquad (3.25)$$

where χ is the molar concentration ratio $\bar{c}_B / \bar{c}_A$ in the sample. The relationship between χ, ϕ and the pH of the sample is straightforward:

$$\phi = \chi \cdot \frac{(1 + 10^{+(pK_A - pH^{M*})})}{(1 + 10^{+(pK_B - pH^{M*})})} \qquad (3.26)$$

In Fig. 3.12 the influence of the sampling ratio on the separation number is shown for various separands. The counter constituent again has been chosen for its optimal buffering capacity in the leading electrolyte.
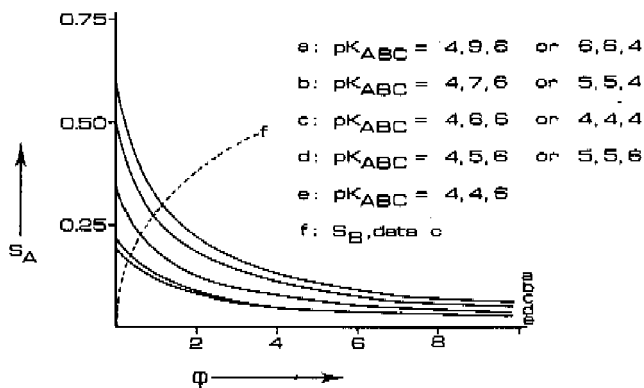
Fig. 3.12 *The influence of the sampling ratio on the dimensionless separation number.*
$pH^L = pK_C$, $r_A = 0.6$, $r_B = 0.4$, $r_C = -0.4$.

It follows that the separation number decreases rapidly with increasing sampling ratio. Numerical calculations have shown[10] that many of the curves, that are obtained when the dissociation constants and mobilities are varied, show congruent behaviour. It should be recognized that generally exact data for the separands are not known and therefore a variation in the input data has to be taken into consideration. Reasons for these variations are

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obvious: lack of data, unreliable data, temperature and
activity effects. The influence of parameter fluctuations
is however marginal[10], as many of them are counter active.
From the curve Γ in Fig. 3.12 it follows that the separa-
tion number for the second separand increases with increa-
sing sampling ratio.

Eqn. 3.22 again gives the optimization rationale for
anionic separations. As long as the transference number in
the mixed zone is independent of pH, a low pH will have a
favourable influence on separation. At a low pH, however,
the contribution of protons in the transport process can-
not be neglected and the transference number for the separ-
and A can be written as:

$$T_A^M = \frac{r_A}{r_A - r_C} \cdot \left\{ \frac{1}{1 + \phi\dfrac{k_A}{k_B} + \dfrac{c_H^M}{c_A^M} \cdot \dfrac{k_A}{k_H}} \right\} \qquad (3.27)$$

where c_H^M and c_A^M are the proton concentration and the sepa-
rand concentration in the mixed zone. From eqn. 3.27 it
follows that the ratio of the proton and the separand con-
centration may have a large influence on the transference
number. At a relatively high proton concentration the fa-
vourable influence of the low pH on the ratio of effective
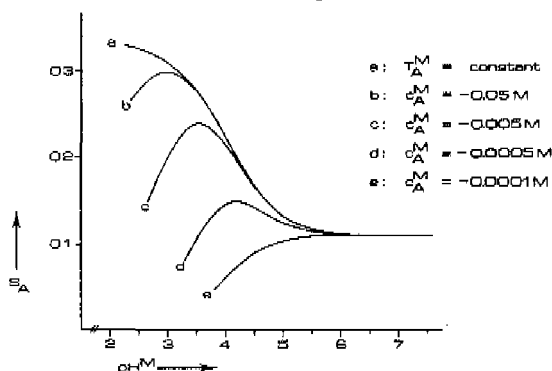
*Fig. 3.13 The influence of a relatively high proton con-
centration on the separation number.*
$r_A = 0.6, \ r_B = 0.4, \ r_C = 0.4, \ r_H = 6, \ \phi = 1, \ pK_A = -2, \ pK_B = 4.$

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separand mobilities is counteracted by the deleterious effect of pH on the transference number. Depending on the actual conditions the separation number can show an optimum. In Fig. 3.13 an example is shown. The sampling ratio was chosen to be unity and the concentration of the separand A was chosen as a parameter. It follows that, at a low separand concentration and a low pH of the mixed zone, the effect can be appreciable.

The optimal pH of the mixed zone is influenced by all the physico-chemical constants of the constituents in the mixed zone. A small difference in dissociation constants and/or mobilities will make the separation number rather insensitive for pH, but it should be remembered that such separands, per se, will have a low separation number. Separand pairs that have a large difference in pK values will be more sensitive in this respect and the optimum in pH increases with increasing pK values. It should be recognized, however, that a low separand concentration and low pH of the mixed zone, at this sampling ratio, cannot be generated by an arbitrarily chosen leading electrolyte.

Curve e of Fig. 3.13 can only be generated by using a rather low concentration of the constituent. Such electrolyte systems are known to have only a rather small range of pH applicability[11]. Depending on the concentration of the leading constituent the transference number of the leading constituent is influenced by the pH of the leading
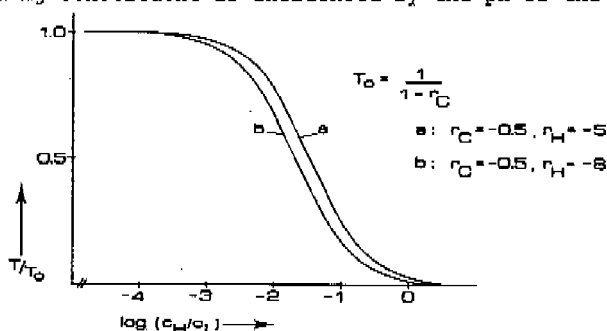
Fig. 3.14 The transference number of leading constituent as a function of pH.

electrolyte, as can be seen from Fig. 3.14. If the ratio of the proton concentration and leading constituent concentration is small, the transference number is constant. With increasing ratio, however, it decreases rapidly, and almost the total electric driving current is carried by the rather mobile protons. In practice, the concentration ratio will be chosen smaller than 0.1. Therefore, working at a leading constituent concentration of 10 mM, the pH of the leading electrolyte should be chosen higher than pH = 3. Working at a concentration of 1 mM the lower limit of pH is already 4, which emphasizes the limited applicability of very dilute electrolyte systems. Though we did consider only the deleterious influence of protons at low pH, similar considerations can be done for the influence of hydroxyl ions at a high pH.

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PART 2

EXPERIMENTAL PART

CHAPTER 1

High performance zone electro-phoresis

An experimental approach is given to high performance zone electrophoresis. The asymmetric concentration distributions are the result of migration dispersion and asymmetry can be only suppressed by the application of very low amounts of sample. Non migrational dispersion can be well controlled by the use of narrow bore tubes. Plate heights smaller than 10 μm can easily be obtained. The practical applicability of HPZE is discussed.

1.0. INTRODUCTION

Many of the problems in the development of electrophoresis are caused by convection and detection. In experimental practice there are three alternative approaches to alleviate the problem of convection. One approach emphasizes the use of additional force fields, e.g. gravitational or electromagnetic, to eliminate the disturbing influence of convection[1,2]. In a second, somewhat more practical approach, stabilizing media such as paper,

cellulose acetate or gels are used[3]. Although in this way convection can be effectively suppressed, it inherently introduces an interaction between the solutes of interest and the anticonvective medium. Such interactions may well be beneficial in many applications, but are generally not desirable. The third approach, which has proven to give a satisfactory solution to obtain a stable electrophoretic performance, is the use of the anticonvective "wall-effect"[4]. Hence stability can be obtained by decreasing the diameter of circular columns or decreasing the gap distance for flat columns. In moving boundary electrophoresis and isotachophoresis such configurations have been applied successfully, mainly by using narrow-bore tubes of chemically and electrically non conducting materials[5-12], such as glass and PTFE.

In conventional zone electrophoresis generally "off-line" detection methods, such as colour reactions and subsequent optical scanning, are used. A prerequisite for high performance zone electrophoresis in narrow-bore tubes is however the availability of a reliable, fast responding and sensitive detection system. Untill now only the conductimetric and UV detection systems[12] seems to have some compatibility with the required demands.

Zone electrophoresis in narrow bore tubes has attracted less attention, although several suggestions about its feasibility have been made[13-15]. Hjertén[13] performed zone electrophoresis in tubes of quartz glass coated with methyl cellulose and used UV detection. The adverse effect of the relatively large inner diameter of the separation compartment was reduced by rotating it about its longitudinal axis. Although the operating conditions seem somewhat complex, he clearly showed the feasibility of the technique.

Everaerts and Hoving Keulemans[14] used an isotachophoretic instrument equipped with a thermometric detector. The separation compartment was of PTFE tubing and with instrument they were able to perform zone electrophoretic separations and they detected highly asymmetric zones.

A detailed study was made by Virtanen[15], who used a glass capillary with an inner diameter of 0.21 mm and potentiometric detection. The performance of the equipment however was poor as the experiments were extremely sensitive for disturbances.

Giddings[16] evaluated theoretically the ultimate capabilities of zone electrophoresis by introducing the HETP concept. Provided that a low dispersive performance can be achieved, he suggested that plate heights down to 10 μm should be possible. Untill recently this limit of performance had never been reached, but the use of capillary configurations seemed to be promising in this respect. In analogy with modern chromatographic methods, the low load capacity of capillary systems will put high demands on detection. For moving boundary electrophoresis and isotachophoresis a satisfactory solution has been found. In zone-electrophoresis however, a higher selectivity is needed. Moreover, there are some methodological problems, that may hamper the development of high performance zone electrophoresis.

1.1. EXPERIMENTAL

All chemicals used were of analytical reagent grade or additionally purified by conventional methods. The operational systems and conditions are given in Table 1.1. Counter constituents were chosen for their optimal buffering capacity at the operative pH, whereas the carrier constituent was chosed according the specific requirements of the analytical problem.
In all cases a viscous additive was used to increase the electrophoretic performance. Driving currents were chosen in such a way that an acceptable compromise was found between the time for analysis, heat production and required voltage.

The major part of the experiments were performed in the electrophoretic equipment developed by Everaerts et al[12]. The separation compartment was formed by PTFE narrow
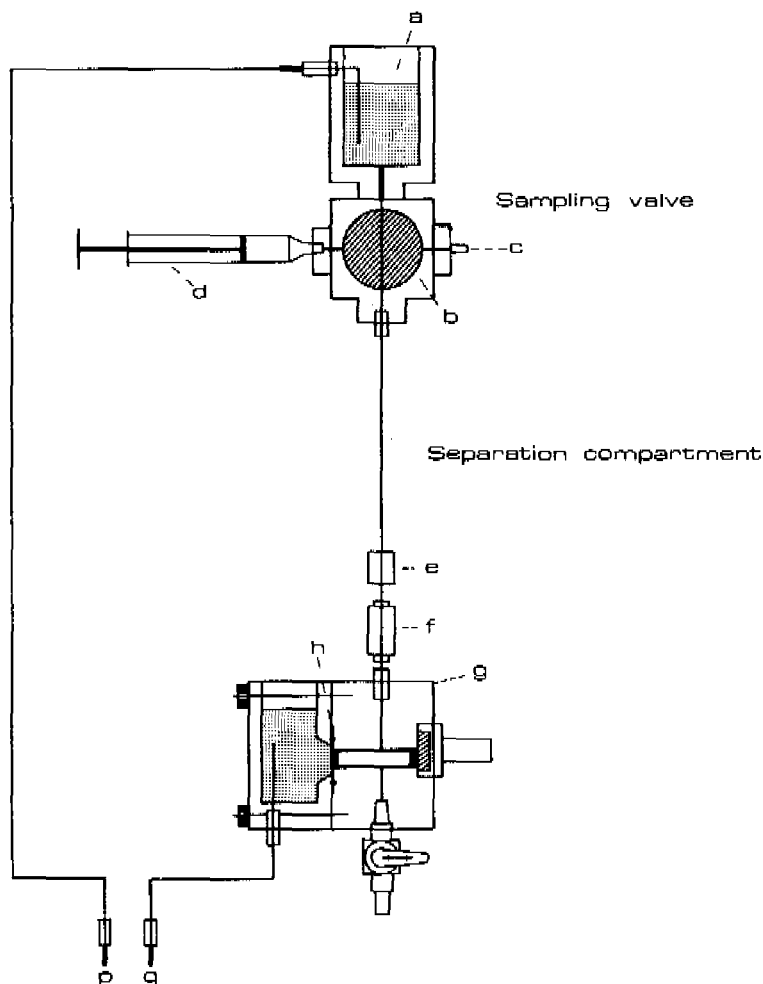
Fig. 1.1 Zone electrophoretic equipment.
a: electrode compartment, b: sampling valve, c: drain,
d: sampling syringe, e: UV detector, f: conductimeter,
g: electrode compartment, h: membrane, p and q: leads to
power supply.

TABLE 1.1

OPERATIONAL SYSTEMS

Parameter	System No.			
	1	2	3	4
Carrier constituent	MES	Acetic acid	Acetic acid	HEPES
Concentration (M)	0.01	0.005	0.1	0.1
Counter constituent	HIST	HIST	GABA	TRIS
pH	6.05	6.02	4.00	6.00
Additive	0.1% HEC	0.1% HEC	0.1% HEC	0.1% HEC
Inner diameter (mm)	0.2	0.2	0.2	0.45
Driving current (µA)	20	30	100	150
Temperature (°C)				20

bore tube with an inner diameter of 0.2 mm and an outer
diameter of 0.35 mm. Samples were introduced by means of
a microliter syringe or a specially constructed four-way
injection valve with a volume of 0.7 µl and an inner dia-
meter of 0.3 mm. Electric gradient detectors were used in
the conductance mode[12] and UV absorption signals (254 nm)
were converted electronically into absorbance. The direct
and constant driving current was taken from a modified
Brandenburg (Thornton Heath, Great Britain) high voltage
power supply.
A schematic representation of the experimental set up is
given in Fig. 1.1. Additional experiments were performed
with the LKB Tachophor (LKB Bromma, Sweden), equipped with
a 0.45 I.D. narrow bore tube. Only the experiments with
the LKB Tachophor were performed at a constant temperature
of 20°C, in all other experiments the temperature was am-
bient.

1.2. RESULTS AND DISCUSSION

According to our theoretical considerations, Part 1
Chapter 2, the effective mobility of the separand, relati-
ve to that of the carrier constituent, determines the con-
contration distribution of the separand. A separand with
an effective mobility smaller than that of the carrier

constituent must migrate with a sharp leading side, where-
as the back side of the zone must be "diffuse". From Fig.
1.2b it can be seen that the electric gradient profile
of propionate conforms with theory when acetate is used as
the carrier constituent. In this case a stable moving boun-
dary is formed at the leading side of the zone, whereas at
the back side the criterion for stability can not be met.
The steepness of the voltage gradient, of course, determines
the actual sharpness of the moving boundary.
On the other hand the electric gradient profile of a se-
parand with an effective mobility larger than that of the
carrier constituent must migrate with a sharp back side
and a "diffuse" leading side. In Fig. 1.2a the distribu-
tion of formate detected under the same operational condi-
tions, (system 2, Table 1.1) is given.
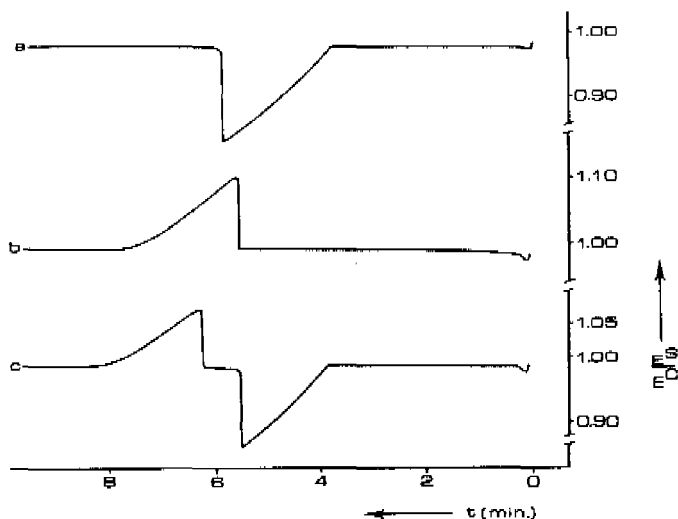
Fig. 1.2 Zone electrophoretic separation of formate and
propionate in the operational system 2 (Table 1.1).
a) formate, 3.5 x 10^{-9} mole; b) propionate, 3.5 x 10^{-9}
mole; c) formate + propionate, each 2.33 x 10^{-9} mole.
E^S/E^C = electrical field strength in the separand zone re-
lative to the carrier electrolyte; t(min) = time of analy-
sis.

Again the actual gradient profile conforms with theory.
The result of the analysis of a mixture of the two sepa-
rands is shown in Fig. 1.2c. It can be seen that neither
the retention behaviour nor the distribution function of
the more mobile separand, i.e. formate, is affected by the
presence of the second separand, whereas the opposite is
clearly not true. This is caused by the fact that for a
$r_J > 1$ configuration the time needed for the first sepa-
rand ion to reach the point of detection, t_{det}, is inde-
pendent of the sampled amount and the presence of other
separands, whereas for a $r_J < 1$ configuration no indepen-
dence exists. Therefore Fig. 1.2 not only proves theory
but also emphasizes the complex nature of retention in
zone electrophoresis. This complexity may hamper the hand-
ling of multicomponent samples in which large concentra-
tion differences occur.

When an fixed point detection system is used, the
electric gradient profile for a $r_J > 1$ is given by:
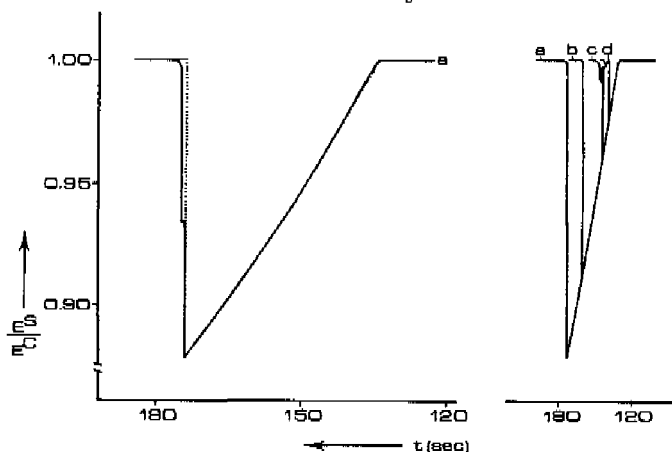
Fig. 1.3 Electric gradient profile of a chloride zone, mi-
grating in the operational system 1 of Table 1.1.
a) 707 x 10^{-12} mole Cl^-; b) 354 x 10^{-12} mole Cl^-; c) 70.7
x 10^{-12} mole Cl^-; d) 21.1 x 10^{-12} mole Cl^-; E^S/E^C relative
electric field strength; t(sec) = time of analysis.

$$E^S(x_{det}, t)/E^C = \sqrt{t_{det}/t} \qquad (1.1)$$

In Fig. 1.3 an electric gradient profile of a chloride zone migrating in the operational system 1 of Table 1.1 is shown. From the theoretical profile, given in Fig. 1.3 by the dotted line, and the experimental profile it must be concluded that the theoretical profile is in close agreement with experimental practice. It should be emphasized that the theoretical model, Chapter 2 Part 1, essentially was developed for monovalent strong electrolytes. The operational system 1 of Table 1.1, however, is well buffered, as both the carrier constituent and the counter constituent have pK values that are close to the pH of the carrier electrolyte. Any pH shift in this electrolyte system is unlikely and therefore the theoretical considerations will apply. From Fig. 1.3 we conclude that dispersive factors, other than electrophoretic migration, are well controlled and have a negligible influence on the concentration distributions. Owing to the high relative mobility of the separand and its local concentration, an appreciable inhomogeneity in the electric field occurs, resulting in stable boundaries. It can be calculated that under the given operational conditions diffusional dispersion becomes important at the picomole level for the sampled amount.

TABLE 1.2

RETENTION DATA

c.v. coefficient of variation								
No. of determinations	Sample load (mole x 10^{-12})	Peak width		Peak height		t_{stop}	t_{det}	t_{gr}
		sec	C.V.(%)	mm	C.V.(%)	sec	sec	sec
3	707	39.1	1.5	1230	0	172.4	133.3	159.2
4	500	32.4	2	1094	0.4	166.1	133.7	155.0
4	354	27.4	2	908	0.2	162.2	134.8	153.0
4	225	21.8	2	732	0.5	154.9	133.1	147.6
4	70.7	11.8	6	427	1.0	146.2	134.4	142.3
4	21.2	7.53	12	243.5	1.0	140.7	133.2	138.2
3	7.07	6.14	5	165.2	3.0	138.7	132.6	136.7

Theoretically, it has been shown, Part 1, Chapter 1, that t_{det} is independent of the sampled amount, as is experimentally confirmed by the right hand side of Fig. 1.3 and the data of Table 1.2. Decreasing the sample load has no effect on the shape of the concentration distribution and only the moment the last sample ions are reaching the point of detection, t_{stop}, is determined by the sampled amount and the generated distribution function.

From the data in Table 1.2, it follows that neither the peak height nor the peak width is linearly related to the amount sampled. For quantitative determinations we derived:

$$n_s = k(t_{stop} - \frac{2}{3} \sqrt{\frac{t_{stop}^3}{t_{det}}} - \frac{1}{3} t_{det}) \qquad (1.2)$$

where k is a calibration constant depending on the cross-sectional area, the current density and the mobilities. The data of Table 1.2 fit this eqn. with a correlation coefficient of 0.9997. Again it must be concluded that the experiments performed were essentially non-diffusional.
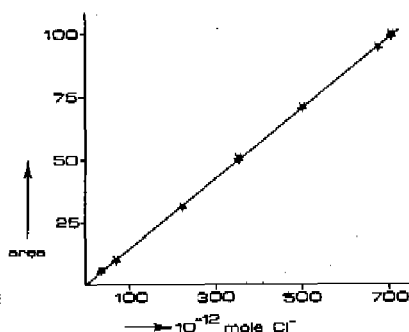
Fig. 1.4 Calibration graph for chloride determinations.

In those instances in which somewhat blurred concentration distributions are obtained, e.g. when injection is made with a microliter syringe, it is necessary to use the peak area. As the difference in specific conductance between a sample zone and the carrier electrolyte is directly related to the concentration of the separand concentra-

tion, it is obvious that the conductance-based peak area must be used. Though the applied detection system is of the electric gradient or resistance type[12], the electric gradient peak-area, in a triangle approximation, proved to be linearly related with the sampled amount. Figure 1.4 shows the calibration graph for the separand chloride.

For identification purposes a retention time has to be defined. Both the time-based centra of gravity, t_{gr}, and the time of the peak maximum, t_{stop}, are affected by the sample load as can be seen from Table 1.2. For practical reasons, generally the time of the peak maximum will be preferred.

At high sample loads probably an appropriate empirical correction function can be used. The use of a constant voltage instead of a constant driving current may well be favourable in this respect. From the Fig. 1.3 it can be seen that the sample did not only contain chloride, but also an impurity, which was identified as sulphate. At a high sample load it is difficult to decide whether separation is complete or not. Although asymmetric concentration distributions are frequently encountered in free zone electrophoresis, they also occur when anticonvective media such as gels or cellulose acetate are used[18]. Using capillary systems, however, they seem to be more pronounced because most forms of non migrational dispersion can be well controlled. In fact the high asymmetry of the separations shown in Figs. 1.2 and 1.3 gives an indication of the low dispersive performance of the equipment. Working with syringe injection also very reproducible and low dispersive separations can be done, as can be seen from Fig. 1.5. Manual injection must be done with great care in order to ensure that the sample is introduced in the ideal plug-form. The use of a sampling valve obviously is superior in this respect but has the disadvantage of a relatively large and invariable volume. The adverse effect of a relatively large sampling volume can often be decreased by the concentrating capabilities of the electrolyte system. The left hand side of Fig. 1.6 shows a separation of three
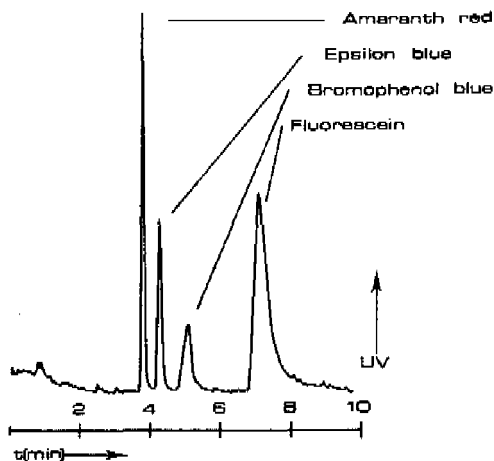
Fig. 1.5 Separation of some anionic dyes on the LKB Tacho-phor, t = time of analysis, UV = UV absorption at 254 nm Operational system 4 of Table 1.1.

separands, analysed in the operational system 3 of Table 1.1, when the four-way sample valve is used. The relative-ly high concentration of the carrier constituent and the fact that the separands were not dissolved in the carrier electrolyte guarantees a concentration of the sample over the stationary boundary between the sampling and the sepa-ration compartment, Part 1 Chapter 2.

Irrespective the long sample width, i.e. 10 mm, a good separation is obtained. A comparable result can be obtai-ned by disc electrophoresis[19,20], by choosing a suitable stacking electrolyte or by using a two dimensional coupled column system[21]. The right hand side of Fig. 1.6 shows the same separation, but now the separands are dissolved in the carrier electrolyte. In this instance the electrolyte system has no concentrating effect and the adverse effect of the relatively long sample width is clearly visible. Therefore, in experimental practice the sample should not be equilibrated with the carrier electrolyte. Selective removal of any separand, in which one is not interested,
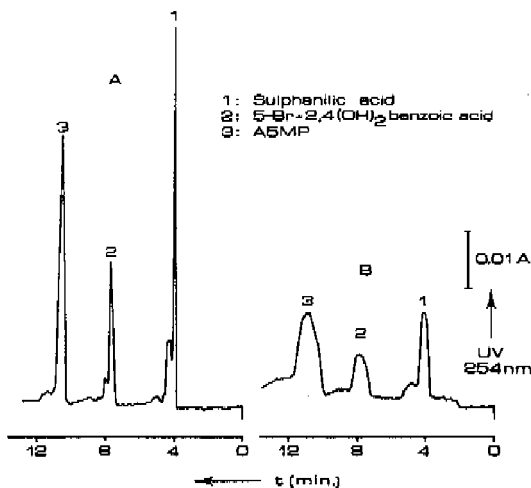
Fig. 1.6 The influence of the sampling width.
Operational system 3, Table 1.1; t (min) = time of analy-
sis; UV = UV absorbance at 254 nm; A) Separands dissolved
in water; B) Separands dissolved in carrier electrolyte.

or replacement of it by a less mobile constituent, will
enhance resolution. Theoretically it is possible to obtain
peak widths that are smaller than the sample width. An-
other important consequence of the concentrating capabili-
ties should be mentioned. Since local separand concentra-
tions depend on the concentration of the carrier constitu-
ent, Part 1, Chapter 2, a high concentration of the latter
one involves a relatively high concentration of the sepa-
rand. Working with concentration dependent detection sys-
tems, such as UV, this may have a favourable effect on the
signal to noise ratio. For non specific detection systems,
such as the conductimeter, no direct advantage is obtained
from highly concentrated electrolyte systems. In order to
obtain a comparable time of analysis the driving current
should be taken proportional to the concentration of the
carrier constituent. The resulting increase in heat
production may well have a deleterious effect on separa-
tion.

    As mentioned already quantification in zone electro-

phoresis is not straightforward. Especially with UV detection, being a concentration dependent detector, some care should be taken. At a large peak width, e.g. the separations of Fig. 1.2, appreciable velocity differences exist within the concentration distribution, that is passing the detection system. As a result the integrated peak area can not be linearly related to the sampled amount. At small peak widths however, deviations will be small.

The applicability of a non specific detection system follows from Fig. 1.7, where a separation of a 16-component sample is shown in the operational system 1 of Table 1.1. Because only 17.5 pmole of each constituent were injected, reasonably symmetric distributions are obtained. From the separation shown in Fig. 1.7, it follows that the respons of the conductimetric detector[17] decreases with increasing retention time. Moreover, divalent separands have a considerable higher respons than monovalent separands. For a quantitative evaluation at this low concentration level, the integrated peak area can be used directly.
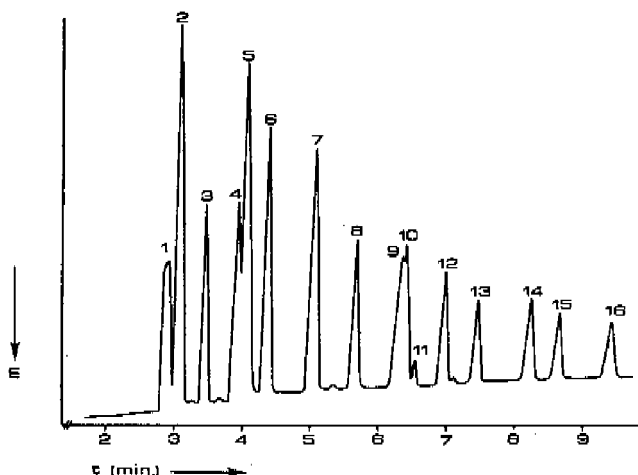
Fig. 1.7 Zone-electrophoretic separation of a 16 component sample. t(min) = time of analysis; E = increasing electrical field strength, sample load: each separand 17.5 x 10⁻¹² mole. Operational system: system 1 Table 1.1 separands: Table 1.3.

The peak maxima can be used most conveniently for reten-
tion purposes, because at this low sample load they are
fairly independent of the amount sampled. As their repea-
tability is very high, as can be seen from Table 1.3,
they can be used for identification.

Column efficiency is generally expressed as the theo-
retical plate number N or the related quantity H, the
height equivalent to a theoretical plate:

$$N = \left(\frac{t_R}{\sigma_t}\right)^2 \quad \text{and} \quad H = \frac{L}{N}$$

where t_R is the retention time, σ_t is the standard devia-
tion of the concentration distribution and L is the column
length. Though there is still some asymmetry left we used
the well known chromatographic relation, $N = 5.55 \ (t_R/w_{\frac{1}{2}})^2$,
where $w_{\frac{1}{2}}$ is the peak width at half height.
Giddings[16] predicted that the minimal plate height that
can be obtained in zone electrophoresis is about 10 μm.
According to Fig. 1.7 and the data of Table 1.3, this mini-
mal value is easily obtained for several sample constitu-
ents.

As there is still some asymmetry left, it must be concluded
that at a lower sample load even better HETPs can be ob-
tained. This, however, will place even higher demands on
the detection system, which essentially was developed for
isotachophoresis and moving boundary electrophoresis[12].
Using the appropriate equations and the data of Table 1.2,
it follows that 1 pmole of chloride, analysed in the ope-
rational system 1 of Table 1.1, will cause a maximal de-
viation of the baseline signal of 0.5%. As most separands
will have a lower response than chloride, an even higher
stability is required. At a very high sensitivity, e.g.
0.01% of the conductance of the carrier electrolyte, the
conductivity signal often shows a poor reproducibility and
time dependent instabilities, that are not of electronic
origin. As a result an irreproducible drift of the base-
line, signal wander and ghost peaking occur. These effects
are mainly due to electrochemical effects at the micro

TABLE 1.3

PERFORMANCE CHARACTERISTICS

Separand	No	Retention time		HETP	N
		sec	cv%	μm	
Chloride	1				
Sulphate	2	185.3	0.8	38	5300
Chlorate	3	208.2	0.8	20	10000
Malonate	4	236.3	0.2	--	-
Chromate	5	244.1	0.6	22	9200
Pyrazole-3,5-dicar-					
boxylate	6	262.6	0.8	18	11000
Adipate	7	304.2	0.9	18	11000
Acetate	8	339.4	0.9	8.7	23000
Propionate	9	376.9	0.9	-	-
β-Chloropropionate	10	382.7	0.6	-	-
Benzoate	12	416.4	0.6	7.1	28000
Naphtalene-2-mono					
sulphonate	13	443.2	0.9	5.6	36000
Glutamate	14	486.9	1.3	6.3	32000
Enanthate	15	514.0	1.0	5.6	36000
Benzylaspartate	16	558.0	1.0	5.9	34000

Length of the separation compartment: L = 20 cm

HETP = Height equivalent to a theoretical plate

N = theoretical plate number

cv. = coefficient of variation n = 6.

sensing electrodes, adsorption and the presence of impurities in the electrolytes. Temperature effects seem to have only a minor influence[18]. Signal stability causes less problems when UV detection is used. Noting that in the conventional equipment[12] and optical path-length of only 0.2 mm is present, a proportional increase in sensitivity can be achieved by increasing this length. Other advantages of UV detection are the selectivity and its compatibility with electrophoretic gradient elution techniques by pH programming.

1.3. CONCLUSIONS

Concentration distributions in free zone electrophoresis are strongly influenced by the effective mobility of the separand relative to that of the carrier constituent. Leading distributions will be obtained whenever the effective mobility of the separand is larger than that of the carrier constituent. Tailing distributions will result when the relative mobility of the separand is smaller than unity.

High performance zone electrophoresis at a low concentration level is possible and gives reproducible results. Adequate design of the equipment[12] and the use of narrow bore tubes guarantees a low dispersive performance and allows a simple and easy operation.

The retention mechanism in zone electrophoresis is complex, especially when large concentration differences in the separands occur. The retention behaviour is strongly affected by the nature of the sample.

A high stability and of the detection systems is required. UV detection seems to be most promising in this respect.

A fundamental question is whether zone electrophoresis is superior to isotachophoresis or not. It should be noticed that the separation processes of both electrophoretic principles mainly proceed according to the moving boundary principle. In isotachophoresis a high current efficiency is coupled with the need for a relatively high sample load. In zone electrophoresis the current efficiency is rather low, due to the continuous transport of carrier electrolyte, but fortunately only a low sample load is required. The overall result is that both principles differ not appreciable in their time of analysis. Problems considering separability with the two principles are not very likely to be different. Since the retention behaviour in zone electrophoresis is dependent on the presence of other sample constituents, isotachophoresis has a small advantage considering qualitative aspects.

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CHAPTER 2

The transient-state characteristics of isotachophoresis

Resolution, load capacity and current efficiency are experimentally evaluated. The pH of the leading electrolyte gives the best rationale for optimization, whereas the pH of the sample has only restricted possibilities. The dimensionless separation number and the loadcapacity can be used for determination and standardization of experimental performance. Steady state configurations in which the separands are not migrating in order of decreasing effective mobilities are shown and discussed.

2.0. INTRODUCTION

Resolution in isotachophoresis has been defined as the fractional separated amount of the separands under investigation. According to this definition, its numerical value may vary between the limiting values of unity and

zero. At zero resolution no separation has occurred and the constituent forms an ideally mixed zone with at least one other constituent. Obviously the maximal resolution value of unity should be reached in the shortest time possible and with the most convenient experimental conditions.

Basically the three optimization rationales are given by the electric driving current, the mobility of the counter constituent and the chemical equilibria of the electrolytes[1].

The electric driving current acts directly on the time needed to resolve a sample. It should be recognized, however, that for the separation of a given sample a definite amount of coulombs is necessary, but that the time interval in which this amount must be delivered is immaterial[2]. The time needed for resolution and the electric driving current are inversely related, so minimization of the former involves maximization of the latter. High driving currents result in an appreciable temperature effect, which can be deleterious for the separation. The use of flat columns[3,4] works favourable in this respect, since they allow a better removal of the heat produced. Neglecting differential temperature effects, the driving current has no influence on the current efficiency of the separation process. It follows that for a given sample the volume of the separation compartment, required for the separation is independent of the driving current.

The mobility of the counter constituent acts directly on the transference number and should be as low as possible. The mobility of the counter constituent has only a small influence on the pH of the mixed zone.

For an optimal current efficiency both the ratio of effective separands mobilities and their transference numbers should be optimized. A well known mechanism influencing effective mobilities selectively is given by dissociation and complex formation[5-7]. In the former instance the pH can be used in optimization procedures, and until now it has been the most frequently used parameter[5].

The direct result of optimal current efficiency will

be a favourable time of resolution and an optimal load ca-
pacity. The current efficiency is best expressed by the
dimensionless separation number, Part 1 Chapter 3, which
has its maximal value at unity. This separation number is
neglecting temperature effects, independent of various
operational conditions, such as the electric driving cur-
rent and the column geometry. It is, however, strongly af-
fected by the nature of the electrolytes and therefore can
be used to compare the efficiency of different analyses.
The load capacity gives direct information on the amount
of separand that can be separated in a given operational
system. The load capacity can be optimized by following
the same rationales as for the current efficiency. Both
parameters can be used to evaluate the experimental perfor-
mance of an isotachophoretic separation.

2.1. EXPERIMENTAL

All experiments were performed using the isotachopho-
retic equipment developed by Everaerts et al[5]. The separa-
tion compartment, Fig. 2.1, consisted of PTFE narrow bore
tubing with inner diameters of 0.45, 0.2 or 0.15 mm and
corresponding outer diameters of 0.75, 0.4 and 0.3 mm. The
direct constant electric driving current was obtained from
a modified Brandenburg (Thornton Heath, Great Britain)
high voltage power supply. Electric gradient detectors,
applied in either the electric gradient or resistance mode,
were used for the measurement transient- state and steady
state characteristics. UV detection was performed in the
absorption mode at 254 nm.

2.2. RESULTS AND DISCUSSION

Transient-state characteristics can be easily obtai-
ned experimentally and several important parameters can be
evaluated directly as most are interrelated. For isotacho-
phoreic analyses it is most convenient to use a separation
compartment of well defined and constant volume and to ap-
ply a constant electric driving current. Using a fixed

TABLE 2.1

OPERATIONAL SYSTEMS

Parameter	System No.				
	1	2	3	4	5
pH of leading electrolyte	3.60	4.03	4.60	5.04	6.02
Leading constituent	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-
Concentration (M)	0.01	0.01	0.01	0.01	0.01
Counter constituent	BALA	GABA	EACA	CREAT	HIST
Terminating constituent	C_2H_5COOH	C_2H_5COOH	C_2H_5COOH	C_2H_5COOH	MES**
Concentration (M)	0.005	0.005	0.005	0.005	0.005
Additive to leading electrolyte	0.05%	0.05%	0.05%	0.05%	0.05%
	MOWIOL	MOWIOL	MOWIOL	MOWIOL	MOWIOL
Temperature	Ambient	Ambient	Ambient	Ambient	Ambient
Current density (A/cm^2)					
d_i = 0.45 mm	0.0503	0.0503	0.0503	0.0503	0.0503
d_i = 0.20 mm	0.0796	0.0796	0.0796	0.0796	0.0796
d_i = 0.14 mm	0.1415	0.1415	0.1415	0.1415	0.1415
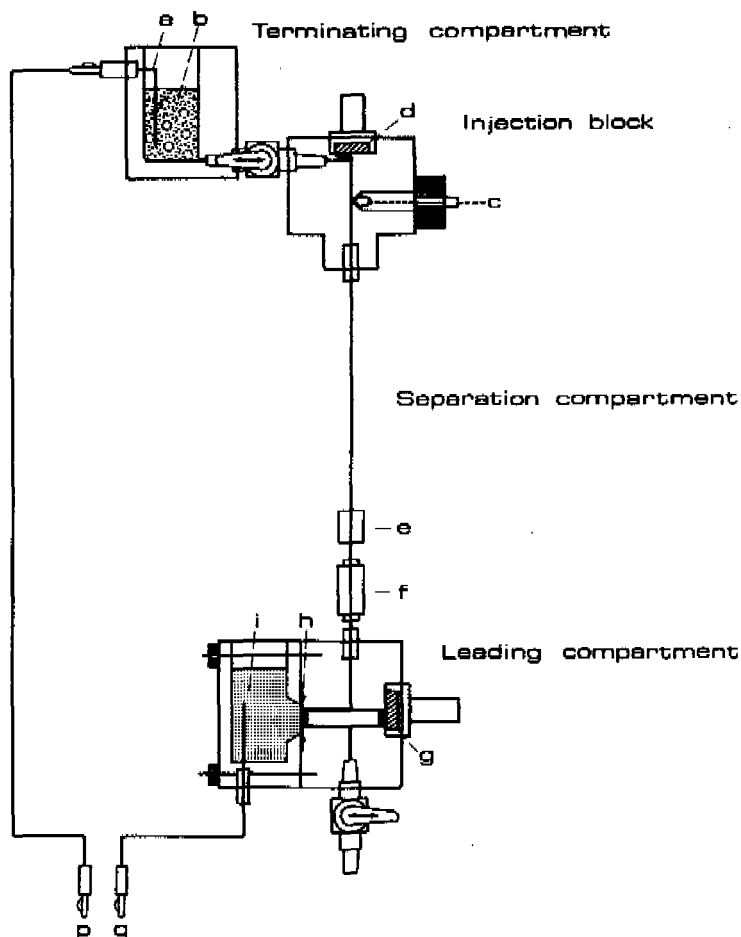
Fig. 8.1 Isotachophoretic equipment.
a: Pt-electrode, b: terminating electrolyte, c: drain,
d: septum, e: UV detector, f: conductimeter, g: septum,
h: membrane, i: Pt-electrode, p and q leads to power supply.

point detector and a given electrolyte system, all charac-
teristics can be evaluated by injection of known amounts of
sample and accurate measurement of all electric gradient
and time events.

TABLE 2.2
CHARACTERISTICS OF THE LEADING ELECTROLYTE, SYSTEM 2 TABLE
2.1.

Parameter		value
Leading Constituent, chloride	m_{Cl^-}	$= -77 \times 10^{-5}$ cm^2/Vsec
concentration	$\bar{c}_L^L$	$= -0.01$ M
Counter constituent, GABA	m_{GABA}	$= 30 \times 10^{-5}$ cm^2/Vsec
	pK_{GABA}	$= 4.03$
Electric driving current	I	$= 80$ µA
Diameter of the sep. compartment	d_i	$= 0.45$ mm
Appearance of 1st boundary	t_{detfix}	$= 1112$ sec
Response leading constituent	$\Delta n_L / \Delta t$	$= 0.592$ nmole/sec
Leading load	n_L^{load}	$= 658$ nmole
Transference number: experimental	T_{exp}	$= 0.714$
theoretical	T_{theor}	$= 0.720$

In this case, the amount of the leading constituent fil-
ling the separation compartment, n_L^{load}, is constant. Provi-
ded the sample did not contain the leading constituent,
the first boundary, that reaches the detector, will always
be registered after the same time interval, t_{detfix};

$$t_{detfix} = n_L^{load} \frac{F}{I} (1 - r_C) \qquad (2.1)$$

where F is the Faraday constant, I the applied current and
r_C the ionic mobility of the counter constituent, relative
to the mobility of the leading constituent. Experimentally
the amount of the leading constituent can be determined by
the injection of a known amount of the leading constituent
Δn_L, and the measurement of the resulting time delay, Δt,

with respect to t_{detfix}:

$$n_L^{load} = t_{detfix} \frac{\Delta n_L}{\Delta t} \qquad (2.2)$$

Moreover, because for a one-constituent monovalent weakly ionic constituent zone the dimensionless separation number S, is identical with the transference number, T, the experimental and theoretical transport efficiency can be compared:

$$T_L = \frac{F}{I} \cdot \frac{n_L^{load}}{t_{detfix}} = \frac{m_L}{\sum\limits_i m_i} \qquad (2.3)$$

where m_i si the ionic mobility of the constituent i. It should be noted that the transference number of monovalent weakly ionic constituents does not contain effective mobilities, but rather ionic mobilities, because, owing to electroneutrality, the degree of dissociation cancels out. Some experimental results are given in Table 2.2.

Eqn. 2.3 is useful, as it provides a practical procedure for the determination of the amount of the leading constituent, once the ionic mobilities and t_{detfix}, are known. It also provides a method for the determination of counter constituent mobilities from experimental results, as

$$r_C = 1 - \frac{1}{T_{exp}} \qquad (2.4)$$

It should be emphasized, however, that at extreme values of pH the contribution of hydroxyl-ions and protons should be taken into consideration.
Obviously, a high transport efficiency due to a low ionic mobility of the counter constituent, is always favourable as it garantees an efficient use of the current applied. For the operational systems given in Table 2.1 we can expect transport efficiencies between 70 and 75%.

As already mentioned, the characteristics of the separation process can be evaluated, by the injection of known amounts of sample. An example of such a procedure is
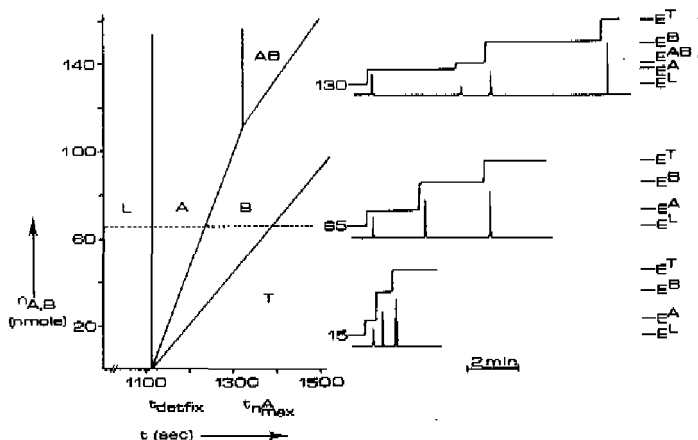
Fig. 2.2 Resolution lines for a two separand sample.
Operational system: Table 2.1, system no 2 and Table 2.2
L = chloride, A = formate, B = glycolate, T = propionate;
$E^{L,A,B,T}$ = electric field strength; n = sampled amount.
Sample: $c_{formate}$ = -0.05 M, $c_{glycolate}$ = -0.05 M, pH = 5.0.

given in Fig. 2.2 and relevant parameters are summarized
in Table 2.3. The fact that, at a constant load of the
leading constituent, the first boundary will always be
detected at the same time interval, t_{detfix}, is illustra-
ted in Fig. 2.2 by the resolution line L/A. The low coef-
ficient of variation, Table 2.3, confirms the excellent
performance of the equipment. Injection of a small amount
of sample will cause two zones, stacked between the lea-
ding constituent L and the terminating constituent T. A
sample load of 1.3 μl of the separand sample, see Fig. 2.2,
where n_A = 65, will give a time based zone length of 124.4
sec for constituent A and detection must be started 1112
sec after injection. The zone length of the second sepa-
rand, B, will be 148.1 sec. Other sample loads will give
proportional zone lengths. The characteristics of these
steady state zones will not be discussed here but the close
agreement of the calculated and experimental resolution
lines, L/A, A/B, and B/T, indicates the reliability of the
calculations.

TABLE 2.3

RESOLUTION DATA

For operational systeem see Fig. 2.2 and Table 2.2

Resolution line: $n = at - b$ (nmole).

Boundary	No. of determinations	a		b		C.v. or C.c.
		Exp.	Theor.	Exp.	Theor.	
L/A	53	0	0	-1112	-1112	0.8%
A/B	13	0.525	0.530	584	590	1.000
B/T	45	0.242	0.251	270	279	1.000
A/AB	6	0	0	-1328	-1317	0.4%
AB/B	6	0.321	0.316	314	312	0.998

Parameter	Experimental	Theoretical
Load of leading constituent (n_L^{load})	658 nmole	647 nmole
Maximal sample load (n_A^{max})	113 nmole	108 nmole
Separation number (S_A)	0.103	0.099
(S_B)	0.103	0.099
Load capacity (C_{load})	0.172	0.167

C.v. = coefficient of variation

C.c. = correlation coefficient

As the separation compartment has a limited load ca-
pacitiy, at a high sample load a mixed zone will be detec-
ted. The characteristics of these mixed zones are deter-
mined by both the leading electrolyte and the sample and
are constant with time, as long as they exist. The time
interval $t_{n_A^{max}}$, at which the mixed zone will be detected,
is again constant, as illustrated in Fig. 2.2 by the reso-
lution line A/AB:

$$t_{n_A^{max}} = t_{detfix} \cdot \frac{\overline{m}_L^L \ E^L}{\overline{m}_B^M \ E^M} \tag{2.5}$$

The maximal zone length for the resolved separand A on a
time base, is given by

$$\ell_A^{max} = t_{n_A^{max}} - t_{detfix} \tag{2.6}$$

The maximal sample load, n_A^{max}, is given by the intercepts of the resolution lines A/B A/AB and AB/B. For the given pair of separands, formate and glycolate, the maximal sample load was 113 nmole, which was close to the theoretical value, Table 2.3. From the maximal sample load the load capacity, C_{load}, and the dimensionless separation number, S, can be calculated directly. Optimal column dimensions can be derived from the load capacity, whereas the dimensionless separation number gives the relationship between the amount sampled and the electric driving current or time of resolution. The appropriate procedure is given in Fig. 2.3

Dimensions	$\longrightarrow$	diameter, length
Volume	$\uparrow$	29.1 μl
Concentration	$\bar{c}_L^L$	-0.01 M
Leading load	n_L	29.1 nmole
Load capacity	C_{load}	0.172
Sample	n_J	5 nmole
Separation number	S_J	0.103
Charge	$\longrightarrow$	4.68 mC time current
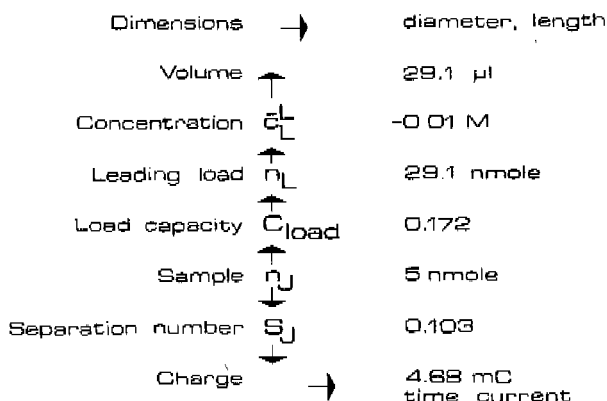
Fig. 2.3 Column evaluation.

From the load capacity, C_{load}, of 0.172, it follows that for a sample that contains 5 nmole of both separands, 29.1 nmole of the leading constituent chloride are necessary. The required volume can now be calculated once the concentration of the leading constituent has been chosen, *e.g.* $\bar{c}_L^L = -0.01\ M$. If the inside diameter of the separation compartment is 0.2 mm the length must be 9.25 cm. Obviously, if we had chosen a higher concentration of the leading constituent with the same dimensions of the separation compartment, the maximal sample load would have

been proportionally higher. From the dimensionless separa-
tion number it follows that for a resolution time of 100
sec, an electric driving current of 46.8 μA must be ap-
plied. At a driving current of 25 μA we can expect a reso-
lution time of 187.4 sec, since the sample requires 4.68
mC of electric charge. Although all resolution lines were
determined experimentally, the load capacity and the sepa-
ration number can be obtained with sufficient accuracy
from only a few experiments, in which a mixed zone is pre-
sent. The number of necessary determinations depends lar-
gely on the performance of the equipment. A high perfor-
mance implies that the coefficients of variations in both
t_{detfix} and $t_{n_4^{max}}$ are low. It should be emphasized that
the column evaluation of Fig. 2.3 applies only to one spe-
cific sample. Generally, a sample will show fluctuations
in composition and an appropriate safety margin should be
used. In analytical practice the fluctuations in composi-
tion may be due to concentration and/or to pH. Once the
extreme values of these fluctuations are known, the safety
margin can easily be calculated.

In the experimental determinations, not only time
events are being registered, but also electric gradients.
As eqn. 2.5 contains only one unknown quantity, $\bar{m}_B^M$, the ef-
fective mobility of the trailing constituent in the mixed
zone can be obtained from the experimental results. For
the glycolate constituent in the mixed zone of Fig. 2.2 it
follows that

$$\frac{\bar{m}_{glycolate}^{-Mixed}}{\bar{m}_{chloride}^{-Leading}} = \frac{1112}{1328} \cdot 0.481 = 0.403$$

Provided that the ionic mobility of glycolate is known,
the pH of the mixed zone can be derived. Using the appro-
priate relationships it follows that $pH^M = 4.28$, which is
very close to the theoretically expected value of 4.30,
that can be calculated with the transient state model.
From this we can evaluate how much the ratio of effective
mobilities in the mixed state differs from the critical

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value of unity at which no separation occurs.

$$\frac{\overline{m}_{glycolate}^{Mixed}}{\overline{m}_{formate}^{Mixed}} = 0.73$$

In combination with the separation number, $S = 0.103$, we find for the transference number of formate in the mixed zone: $T_{formate} = 0.38$.

From Fig. 2.2 and the data of Table 2.3 we conclude that even in the presence of a mixed zone, the relationship between the total zonelength of the sample and the sample load is perfectly linear. Deviations[12,13,14] from this rule are the result of experimental inaccuracies, such as hydrodynamic flow, improper injection and mixing of leading electrolyte with sample and/or terminating electrolyte.
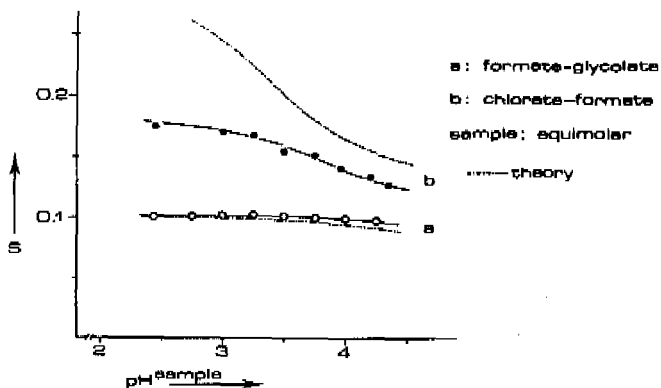
Fig. 2.4 The influence of the pH of the sample on the dimensionless separation number.
Operational system: Table 2.1, system no 2.

As the separation process is strongly dependent on the pH of both the sample and the leading electrolyte, optimization by choosing suitable electrolyte conditions should be possible. The effect of the pH of the sample has been given in Fig. 2.4. Owing to the small difference in the dissociations constants of the separands glycolic acid

and formic acid, $\Delta pK = 0.08$, the effect of the pH of the sample on the current efficiency is minimal. For the given pair of separands the theoretical and experimental results show good agreement. When the difference in the dissociation constants is larger, the effect of the sample pH is much more pronounced, as is confirmed by the constituent pair chlorate-formate. In the instance a low pH of the sample clearly favours resolution. Although the theoretical considerations suggest a rapid, continuous increase in efficiency, decreasing the pH of the sample, the experimental curve indicates only a moderate increase. In the transient state model, Part 1 Chapter 3, we made no allowance for the influence of a relatively high proton concentration at low pH. Functioning as a mobile counter constituent, protons, at a relatively high concentration will decrease the efficiency of the transport process, and as such the current efficiency of the separation process. When the separands have only a small difference in their dissociation constants, such as formic acid and glycolic acid, the theoretical and experimental results will show only small differences. When the pK values of the separands differ substantially, higher pH shifts can be expected and larger deviations will result. Irrespective the numerical discrepancy for anionic separations a low pH of the sample favours resolution. Appropriate incorporation of the hydrogen and/or hydroxyl constituent into the mathematical formulations will improve the theoretical results.

In common practice the pH of the sample shows only a small degree of freedom and the most useful optimization parameter is the pH of the leading electrolyte. As long as the pH has no influence on the transference number the lower pH of the leading electrolyte will increase the current efficiency for anionic separations. Table 2.4 gives some experimental results for the separands of Fig. 2.4. From both the theoretical and the experimental results it follows that, for constituents that have only a small difference in their pK values, the pH of the leading electro-

lyte is not very useful for optimization. According to
theory the ratio of effective separand mobilities in the
mixed state should be optimized. In case of only small pK
differences, the pH of the leading electrolyte will have
only a minor influence, as can be seen from the Fig. 2.5.

TABLE 2.4

INFLUENCE OF THE pH OF THE LEADING ELECTROLYTE ON THE
DIMENSIONLESS SEPARATION NUMBER

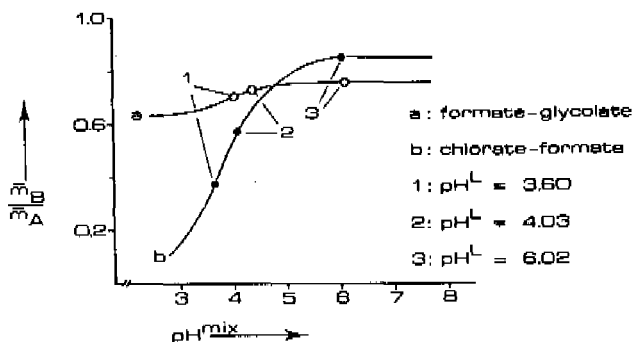| Parameter	Separands			
	Chlorate-Formate		Formate-Glycolate	
Concentration (M)	-0.05	-0.05	-0.05	-0.05
pH^{sample}	2.41		2.41	
pH^L Counter Constit.	S$_{exp}$	S$_{theor}$	S$_{exp}$	S$_{theor}$
3.60 BALA	0.259	0.368	0.095	0.099
4.03 GABA	0.1799	0.275	0.098	0.100
6.02 HIST	0.075	0.105	0.101	0.092

Fig. 2.5 The influence of the pH of the mixed zone on the
ratio of effective separand mobilities.
Operational system: Table 2.1.

It can easily be shown that for the limiting values of the
mobility ratio it must hold that:

$$10^{(pK_A - pK_b)} \cdot \frac{m_B}{m_A} < \frac{\bar{m}_B}{\bar{m}_A} < \frac{m_B}{m_A} \qquad (2.7)$$

As a result, for the separand pair chlorate-formate the ratio of effective mobilities can vary between its maximal value of 0.764 at high pH and its minimal value of 0.365 at low pH. Hence any pH shift, due to either the leading electrolyte or the sample, has hardly any influence on the mobility ratio. Owing to the relatively low mobility of the counter constituent histidine, the separation at pH^L 6.02 has the higher efficiency, although differences are only marginal. When the differences in pK values are more substantial the rationale for optimization is more clear, as can be seen from the second separand pair, chlorate-formate, in Table 2.4 and Fig. 2.5. In this instance the mobility ratio can vary between almost zero at low pH and 0.846 at high pH. A high pH of the leading electrolyte will cause a high pH of the mixed zone, resulting in an unfavourable ratio of the separand mobilities. A low pH of both the leading electrolyte and the sample will result in an optimal ratio and therefore optimal current efficiency.

From the Fig. 2.5 it can also be seen that for small differences in pK values, the difference in the pH values of the mixed zone and the leading zone is relatively high. When the sample contains stronger acids, this difference is considerably smaller, resulting in a relatively low pH in the mixed zone. Whenever the pH of the leading electrolyte is substantially higher than the pK values of the

TABLE 2.5

CONSTITUENT DATA FOR FIG. 2.5

Parameter	Separand	
	Acetate	Naphtalene-2-sulphonate
Mobility (cm^2/Vsec)	-41×10^{-5}	-30×10^{-5}
Concentration (M)	-0.005	-0.005
pK value	4.75	0
pH^{sample}	4.75	4.75
No separation at $pH^M = 5.19$		
No separation at $pH^L = 4.98$	($\rho = -2$, $m_C = 30 \times 10^{-5}$ cm^2/Vsec)	
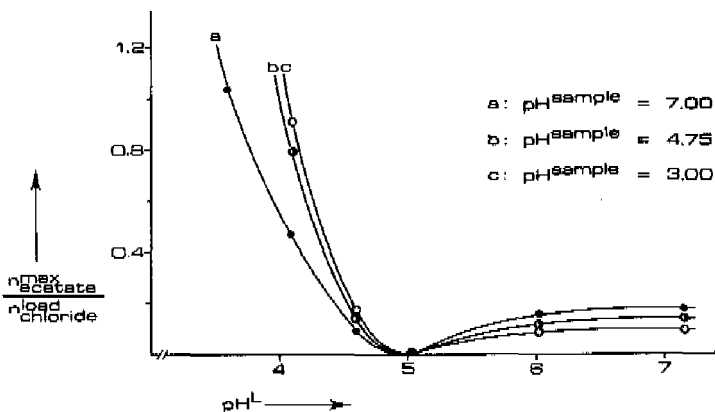
Fig. 2.6 Influence of the pH of the loading electrolyte on the load capacity.

Operational systems: Table 2.1, Constituent Data: Table 2.5.

separands, the difference will be small and the separands will be separated as strongly ionic species. From the example given it follows directly that a separation based on pK values is generally more effective than one based on ionic mobilities.

For pairs of separands, where $m_B < m_A$ and $pK_A < pK_B$, the rationale for optimization is straightforward: low pH. For anionic pairs, where $m_B > m_A$ and $pK_A > pK_B$, the rationale is more complex. It has been shown, Part 1 Chapter 1, that for such pairs a pH will exist, at which no separation occurs. Of course, this pH will cause an infinite time of resolution, zero separation number and zero load capacity. Moreover, at this pH the order in which the separands migrate will be reversed. Experimental results concerning the load capacity of such a pair are given in Fig. 2.6 as a function of the pH of the leading electrolyte and the pH of the sample. The experimental curves confirm the theoretically predicted behaviour. Using the appropriate data, Table 2.5, and relevant mathematical formulations, it follows that the criterion for separation, Part 1 Chapter 1, will not be satisfied at a mixed zone pH of 5.19. Obviously, this pH can be generated by numerous combina-

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tions of leading electrolytes and sample compositions. Working at the maximal buffering capacity of the counter constituent, i.e. $\rho = -2$ or $pH^L = pK_C$, and introducing an acceptable ionic mobility for the counter constituent, $m_C = 30 \times 10^{-5}$ $cm^2/Vsec$, the critical pH of the leading electrolyte is 4.98. This was confirmed experimentally by the separation at $pH^L = 5.04$, at which hardly any load capacity was present. At a pH^L higher than the critical value, separands migrate in order of ionic mobilities, and separations can be performed with only moderate efficiency. At low pH^L, however, separands are migrating in order of their pK values and a much higher efficiency can be obtained, resulting in a high load capacity. For example, the resolution of a 1.5 nmole sample, an absolute amount that can be detected without difficulty, would take about 23 sec, $S = 0.26$, at $pH^L = 4.10$ and $pH^S = 3.0$, whereas the same sample can be resolved in 133 sec, $S = 0.045$, at $pH^L = 7.10$. The required length of the separation compartment in the former instance is 5.8 times shorter than in the latter. Obviously, for specific samples rigid optimization procedures can be followed, resulting in very short analysis time, small dimensions of the separation compartment and efficient use of the power applied. It must be emphasized, however, that the success of optimization procedures depends largely on the physico-chemical characteristics of the species to be separated and the performance of the equipment. When there are only small differences in ionic mobility and dissociation constants, optimization procedures are elaborate and result in a rather small increase in efficiency.

Another important conclusion can be drawn from the results shown in Fig. 2.6. It follows that efficient separations can be achieved whenever the sampling ratio ϕ, i.e. the concentration of the charged trailing separand divided by the concentration of the charged leading separand, is small. Biochemical samples often contain substantial amounts of very mobile species such as sodium, chloride or perchlorate. Such samples represent typical low

"φ *cases*", that can be separated in a relatively short co-
lumn. The time of analysis, however, will increase sub-
stantially in the presence of these mobile separands. In
very special cases lowering of the sample pH by the addi-
tion of, for example, hydrochloric acid will increase the
load capacity of the column by the proposed mechanism.

TABLE 2.6

INFLUENCE OF THE INSIDE DIAMETER OF THE SEPARATION COMPART
MENT ON THE DIMENSIONLESS SEPARATION NUMBER

Parameter	Inside diameter (d_i, mm)		
	0.45	0.20	0.15
Driving current (I, μA)	80	25	25
Sample load (n_A^{max}, nmole)	87.5	23.5	16.6
(n_B^{max}, nmole)	87.5	23.5	16.6
Resolution time (t_{res}, sec)	1081	1030	703.7
Separation number (S_{theor})	0.100	0.100	0.100
($S_{A'exp}$)	0.098	0.088	0.091

Sample: A = formate (∼0.05 M); B = glycolate (∼0.05 M)
pH^{sample} = 2.41. Operational system: Table 2.1, system 2.

The dimensionless separation number seems to be a re-
liable quantity for describing the current efficiency, as
it is independent of operating conditions such as the elec-
tric driving current and column geometry. Table 2.6
gives the observed experimental efficiencies for the same
sample under different operating conditions. From these re-
sults it follows that, although differences in the separa-
tion numbers occur, the efficiency is not significantly
different. In our theoretical approach, Part 1 Chapter 2,
we introduced relative mobilities to suppress the influen-
ce of temperature effects. Comparing literature data on
ionic mobilities[9,10], it must be concluded that these tem-
perature effects are only partially eliminated in this way,
as many non-linear effects occur. Moreover, when conside-
ring temperature effects, the dissociation constants

should also be corrected. Mathematical iteration procedures to involve temperature corrections for various physico-chemical parameters can be introduced into the transient state model with a probable consequent, but small, increase in accuracy. There is however, a lack of reliable data on physico-chemical parameters and the present model seems sufficient reliable, the predicted parameters being confirmed experimentally.
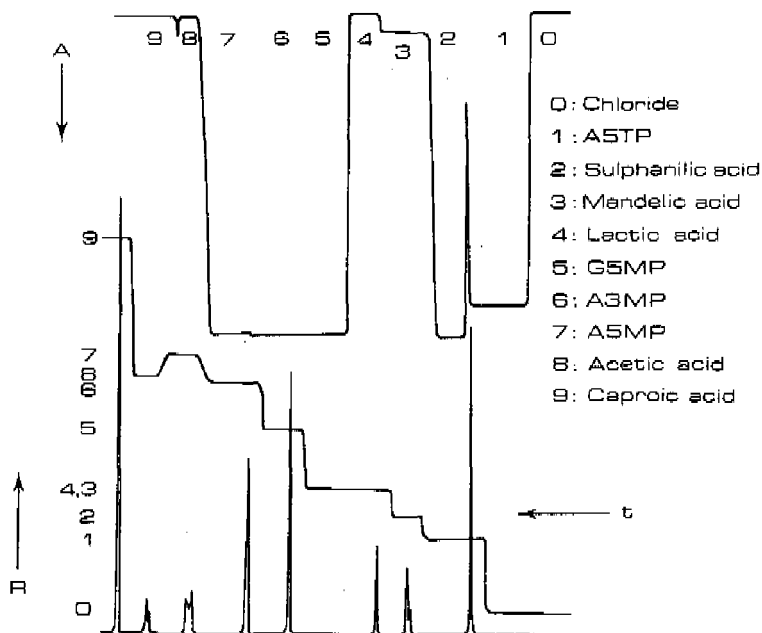
Fig. 2.7 Isotachophoretic state configurations.
Operational system: Table 2.1 system no 2, $pH^L = 3.95$
R = increasing resistance. A = UV absorption 254 nm.

From the theoretical formulations, it follows that the steady state effective separand mobilities can be only used as an indication for experimental separability. Constituents that have almost equal steady state mobilities can sometimes be separated quite efficiently. Moreover, it should be emphasized that the effective steady state

mobilities are only indicative for the steady state migra-
tion configuration; enforced isotachophoretic configura-
tions[14], in which a more mobile separand migrates behind
a less mobile separand, can be perfectly stable with res-
pects to time. An example of the possible configurations
is shown in Fig. 2.7. Several separands confirm that gene-
rally constituents in isotachophoresis are migrating in
order of decreasing effective separand mobilities.

TABLE 2.7

COMPARISON OF ZONE CHARACTERISTICS

Trans = computerized transient-state model; X3 = computerized steady-state model[14]							
Parameter	Chloride zone, experimental	Mandelate zone			Lactate zone		
		Trans	X3	Exp.	Trans	X3	Exp.
pK	-2	3.37			3.86		
$m.10^{-5}(cm^2/Vsec)$	-77	-28.1			-33.9		
pH	3.95	4.21	4.22	4.25	4.27	4.28	4.29
E^L/E^X	1.00	0.319	0.320	0.322	0.318	0.319	0.322
$\bar{c}_X^X(mM)$	-10.00	-6.47	-6.34		-7.16	-7.06	
$\frac{m_{mandelate\ zone}^{lactate}}{m_{mandelate\ zone}^{mandelate}}$		0.956	0.960	0.974			
$\frac{m_{lactate\ zone}^{lactate}}{m_{lactate\ zone}^{lactate}}$					1.027	1.017	1.010
$\frac{m_{mixed\ zone}^{mandelate}}{m_{mixed\ zone}^{lactate}}$	1.027						

The constituents lactic and mandelic acid, Fig. 2.7 no 3
and 4, however show virtually no difference in effective
mobilities, as for their isotachophoretic migration the
same electric gradient seems to be necessary (Fig. 2.6
and Table 2.7). From the linear conductance trace it ap-
pears that this pair has not resolved during the separa-
tion process. The UV trace, however, clearly indicates
that mandelic acid has been resolved from lactid acid and
that the former migrates in front of the latter. The tran-
sient state model reveals that the pH of the mixed zone,
from which the pure zones are formed, is just below the
critical pH of 4.32 at which no separation occurs. At the
pH of the leading electrolyte mandelic acid will be

resolved in front of lactic acid.

From the data in Table 2.7 it follows that the experimental and theoretical zone characteristics are in good agreement. The minor differences between the transient state and the steady state results, have already been mentioned. The occurrence of a lactate ion in the resolved mandelic acid zone will cause a 2.6% deviation from the critical value of unity for the ratio of effective constituent mobilities. Mandelate ions, in the resolved lactic acid zone, would lead to a difference of 1%. From the UV trace it follows that these deviations are large enough to garantee a satisfactory separation. The theoretical calculations show a larger difference and for the ratio of effective mobilities in the mixed zone a 2.6% deviation from unity was calculated. The experimental separation confirms that this deviation is sufficient to obtain resolution. It must be emphasized, however, that these small deviations result in a low efficiency and column overloading can easily occur. Fig. 2.7 nevertheless indicates clearly that isotachopherograms in which only one detection system is used must be interpreted with great care. The same, of course, applies when only UV detection is used. From the UV trace in Fig. 2.7 it would be concluded that the nucleotides G5MP and A5MP have not been resolved. The conductance trace however, clearly confirms the separation of the two separands. On most occasions small amounts of impurities, with either UV-absorbing or non UV-absorbing properties, will indicate the separation boundary.

The separand A5MP and acetate, constituents 7 and 8, are migrating in an enforced isotachophoretic configuration. The effective mobility of the acetate constituent in its proper zone is higher than that of the nucleotide A5MP in its proper zone, as indicated in Fig. 2.7 by the lower resistance of zone 8 in comparison with zone 7. For the relative effective mobilities it follows

$$\frac{\overline{m}^{Acetate}_{Acetate}}{\overline{m}^{Chloride}_{Chloride}} = 0.212 \quad \text{and} \quad \frac{\overline{m}^{A5MP}_{A5MP}}{\overline{m}^{Chloride}_{Chloride}} = 0.198$$

The 7% deviation from unity of the mobility ratio allows
a satisfactory separation between the two separands. The
reason for the stability can be found in the difference in
pH values of the two resolved zones. Using the appropriate
relationships it follows that the pH of the acetate zone
is 4.57. A nucleotide ion, lost owing to the convection or
diffusion from its proper zone into the acetate zone, will
migrate there with a higher effective mobility than the
acetate constituent. In the nucleotide zone the pH is 4.32,
so any acetate ion in the nucleotide zone will migrate
with a considerably lower velocity than the nucleotide.
Hence the self-sharpening capabilities of the separation
boundary allow the enforced isotachophoretic configuration
to be stable with respect to time. The transient state mo-
del predicts even in this case, the separation configura-
tion correctly. It should be noticed however, that enfor-
ced isotachophoretic configurations will not be encountered
very frequently in common practice.

2.3. CONCLUSIONS

From both our previous theoretical considerations,
Part 1 Chapter 3, and the experimental evaluation presen-
ted here, it follows that through optimization a conside-
rable increase in current efficiency and load capacity and
a decrease in time for resolution can be obtained. We res-
tricted our theoretical and experimental considerations
mainly to two separand samples but the same optimization
rationales hold, be it to a lesser extent, for multicompo-
nent samples[8].
In optimization procedures three rationales should be fol-
lowed:
i   The electrical driving current should be maximized
     during the separation process.
ii   The mobility of the counter constituent should be mi-
     nimized.
iii A low pH of both sample and leading electrolyte
     should be used for anionic separations. In case of
     cationic separations a high pH will be preferable.

Extreme pH's where the proton or hydroxyl ionconcen-
trations have a deleterious influence on the transfe-
rence number should be avoided. The pH at which no
separation occurs can be calculated and hence can be
avoided.

It must be emphasized that the result of any optimization
procedure depends on the nature of the sample and calcula-
tions of isotachophoretic steady state characteristics are
only indicative for separability. For very complex mixtu-
res in which multicomponent information must be obtained,
optimization can sometimes be elaborate and difficult. As
a result analyses in more than one operational system are
inevitable.

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CHAPTER 3

Column coupling in isotachophoresis

*A two dimensional electropho-
retic instrument is described
that allow the application
of large sample loads, with-
out appreciable increase in
time of analysis. The instru-
ment is fully automised and
can be used for micro-prepara-
tive and two dimensional sepa-
rations.*

3.0. INTRODUCTION

Separands are often present within a sample together
with numerous other substances in which there is no inte-
rest. As long as the separand concentration predominates
that of the interfering solutes qualitative and quantita-
tive determination of the separand will cause only minor
problems. Unfortunately this is generally not the case and
according to our theoretical considerations, Part 1 Chap-
ter 3, such samples have a deleterious impact on both the
time for resolution and the maximal allowable sample load.

There are several approaches to alleviate this pro-
blem. First of all, sample pretreatment procedures, such
as solvent extraction with or without extract concentra-
tion, can be used for either removal of interfering sub-
stances or selective enrichment of separands. Being gene-
ral procedures they can be used successfully in isotacho-

phoresis[1]. Any sample pretreatment procedure, however, is sensitive for quantitative inaccuracy and as a result recovery studies have to be applied.

A somewhat more elegant method is the use of selective detection systems of high sensitivity, as they allow quantitative determinations at a low sample load. In this respect the UV-absorbance detector can be applied quite successfully, using the "UV-spike" method[2-6]. In this method the separand migrates in a very small zone, stacked between two non UV-absorbing spacers and the integrated peak area or the peak-height is used for quantitation.

Not considering selective detection systems, the quantitative aspects in isotachophoresis are directly related with the zone volume of the separand. With decreasing zone volume the quantitative reliability decreases. Moreover, for a fast responding detection system, such as the conductimeter, the volume of the detector is important. Due to the presence of a zone profile, a finite thickness of the transition boundaries and the presence of convective instabilities, theoretical calculations on the minimal detectable amount or the minimal required zone volume are rather academic[4]. For accurate quantitative determinations the zone volume should be maximized whereas the detector volume should be minimized. It should be noted that neither dilution of the leading electrolyte nor reduction of the diameter of the separation compartment, carried out as a uniform operation, will work advantageous since the maximal sample load is proportional with the concentration of leading electrolyte as well as the column hold-up volume. Moreover, a low concentration of the leading electrolyte, lower than 1 mM, will have a restricted pH applicability and detection systems of very small inner diameters, smaller than 0.1 mm, are difficult to realize.

According to theory the time-based zone length is linearly related with the sample load and inversely proportional to the driving current and the transference number. The latter quantity is invariable for a given separand in an optimized electrolyte system, whereas the

driving current has a limited degree of freedom. For a large time-based zone length a low driving current is favourable, whereas the separation process requires a maximized driving current. The maximal value during separation is governed by power dissipation and thermostating and the minimal value during detection is governed by the required sharpness of the transition boundaries. As a result time current programming can be useful as long as the resulting transient temperature regimes can be avoided.

The most straightforward method for increasing the actual or time-based zone length is by increasing the sample load. As mixed zones have to be avoided a larger column volume will be necessary and there are several possibilities to realize this.

Generally the length of the separation compartment can be elongated without much difficulty. This method has only limited practical applicability since both the time of analysis and the voltage drop over a column of uniform dimensions are proportional with its length. It will suffice for those analytical problems that require only a doubling of the sample load. When the sample load must be increased by at least one order of magnitude elongation of the separation compartment will fail due to the long time of analysis and the resulting unworkably high endvoltage. These disadvantages can be solved to some extent by using a separation compartment of non-uniform dimensions, e.g. conical compartments[7], non uniform concentration distributions[8] and time-current programming.

The second method that has been proven to be useful is counterflow of electrolyte[9-14]. An inherent drawback of counter flow of electrolyte is that its flow profile not only has a disturbing effect on the zone boundaries[9] but also reduces the current efficiency of the separation process, introducing antiseparative velocity distributions in the mixed zones. As a result relatively large differences in the effective mobilities of the separands are necessary. The major benefit of counterflow of electrolyte compared with elongation of the separation compartment is the low

end-voltage and its versatility, especially when it is ful-
ly automized[9]. If counterflow is not applied during detec-
tion the reproducibility of the analysis in comparable
with that of analysis without it. Counterflow of electro-
lyte during detection is possible but its reliability has
not been investigated yet and probably requires an accu-
rate control system.

The use of continuous sampling systems[15,16], with or with-
out counterflow of electrolyte does not provide a direct
way of increasing the maximal sample load, but rather a
large sample volume. The major drawback of counterflow of
electrolyte is its decreased efficiency and the resulting
long time of analysis.

3.1. COLUMN COUPLING

A more general solution to the problem of maximal al-
lowable sample load is given by the use of two separation
compartments with different inner diameters as shown sche-
matically in Fig. 3.1. The first separation compartment
has a relatively large volume, necessary for the resolu-
tion of a high sample load. Its large inner diameter al-
lows the use of a high driving current. Only the separand
zones of interest are allowed to migrate into the second
separation compartment, where they can be analyzed further
at a lower driving current. Hence by selective switching
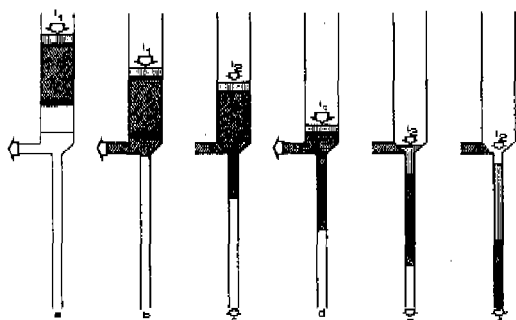
Fig. 3.1. Column Coupling
a,b,d: preseparation mode, c,e,f: separation mode.

interfering substances can be removed from the isotacho-
phoretic sample train.

The ultimate capabilities of the coupled column system can
be evaluated by comparing its time of resolution, for a
given sample, with that of a conventional instrument. As
for the separation of a sample only the time-current inte-
gral is important, the required amount of coulombs Q is
the same for both instruments.

For the coupled column system it follows that,

$$Q = I_1 t_1 + I_1 dt + I_2 t_2 \qquad (3.1)$$

where I_1 is the driving current in the preseparation com-
partment during the time t_1 and dt is the time an inter-
fering zone needs to pass the bifurcation. I_2 and t_2 are
respectively the driving current and time in the final se-
paration compartment. For a conventional instrument it
follows that,

$$Q = t_3 I_2 \qquad (3.2)$$

where t_3 is the resolution time in the conventional instru-
ment. It should be emphasized that for a correct compari-
son the inner diameter of the final separation capillary
in the coupled column system is chosen to be equal of the
conventional equipment.
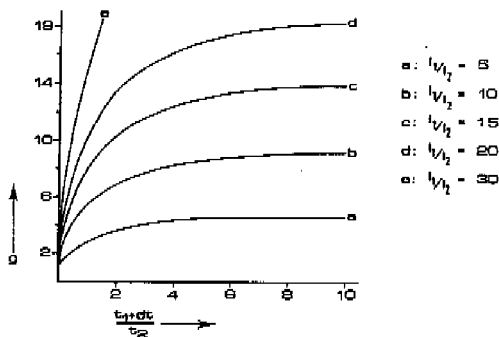
Fig. 3.2 The relative gain in time for resolution.

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For the absolute profit in time for resolution t_g we find:

$$t_g = t_3 - (t_1 + dt + t_2) \qquad (3.3)$$

or

$$t_g = (t_1 + dt) \left\{ \frac{I_1}{I_2} - 1 \right\} \qquad (3.4)$$

The relative gain, g, is given by the ratio of the resolution time in the conventional equipment and that of the coupled column system. It follows that

$$g = \frac{\dfrac{I_1}{I_2} \left\{ \dfrac{t_1 + dt}{t_2} \right\} + 1}{\left\{ \dfrac{t_1 + dt}{t_2} \right\} + 1} \qquad (3.5)$$

In Fig. 3.2 the relative gain has been plotted as a function of the ratio fo the preseparation time and the separation time. It follows that whenever $t_1 + dt$ is large in comparison with t_2 a constant relative gain is obtained and that a large benefit can be found in maximizing the ratio of the driving currents. The maximal value of I_1 is governed by power dissipation and the effectivity of thermostatting, whereas the value of I_2 can not be chosen arbitrarely low since at very low driving currents considerable remixing of the separands during trapping can occur.

Instrumentation

The coupled column system essentially consists of three sections, Fig. 3.3:
   - the preseparation compartment
   - the bifurcation block
   - the separation compartment
The preseparation compartment has the larger inner diameter resulting in a large column volume and allows the use of a high sample load and a high electric driving current. In order to alleviate the deleterious effects of relatively high power dissipations, this PTFE capillary can be thermostatted. A vertical orientation with downward

INJECTION BLOCK

PRESEPARATION
COMPARTMENT

BIFURCATION BLOCK
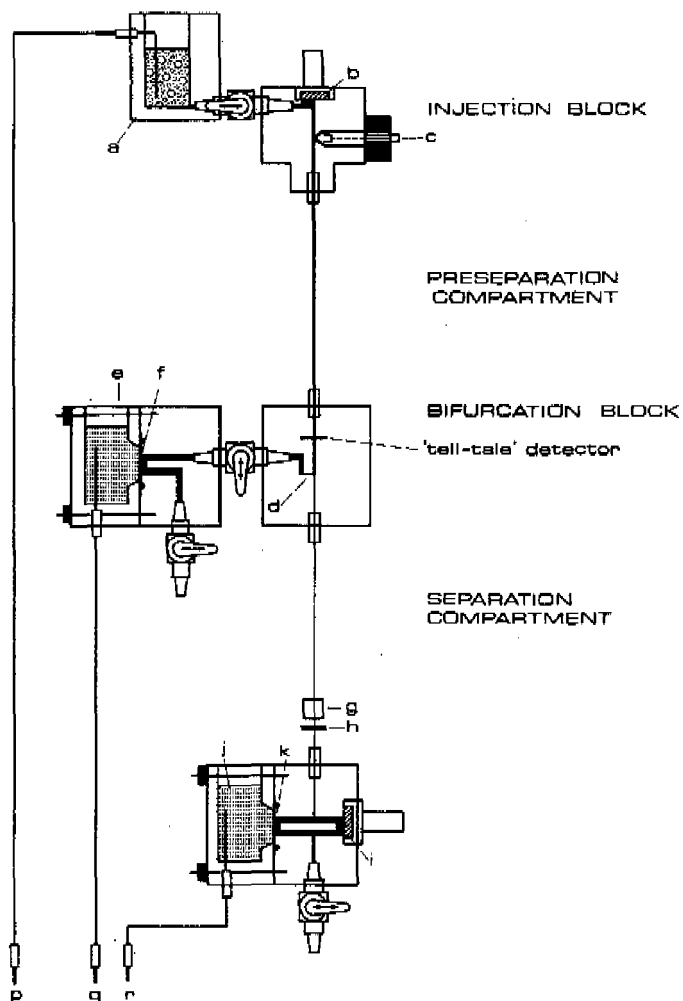
'tell-tale' detector

SEPARATION
COMPARTMENT

Fig. 3.3 The coupled column system.
a: terminating electrode compartment, b: septum, c: drain,
d: flat channel, e: counter electrode compartment presepara-
tion mode, f: membrane, g: UV detector, h: conductimeter,
i: septum, j: counter electrode separation mode, k: membrane,
p,q,r: leads to power supply.

migration of the separands has proven to give the most gravitationally stable configuration. The length and/or its inner diameter can be varied, though inner diameters larger than 1.2 mm are not generally applicable, since they require the use of relatively low current densities and the use of viscous electrolytes to prevent gravitional settling.

The separation compartment has a smaller inner diameter and due to its favourable thermal behaviour[17] no thermostatting is necessary. Moreover, its anticonvective wall-effect[17] garantees an orientation independent stable performance. All electrode compartments and detection systems are of the conventional type and have been described in detail elswhere[9].

Fig. 3.3 shows the bifurcation block in which the two separation compartments are coupled in an "in-line" configuration. The bifurcation block, made of acrylic, contains three different channels. The wide bore with an inner diameter of 0.8 mm, contains a conductimetric detection system, the "tell-tale" detector, and can be connected with the preseparation compartment. A second boring with a small inner diameter, e.g. 0.2 mm, lies in line with the wide boring and forms the connection to the separation compartment. The connection with the counter electrode of the preseparation mode, Fig. 3.3-e, is formed by a flat channel, width 1 mm and height 0.05 mm, and is perpendicular to the "in-line" separation compartment borings.

In the preseparation mode the electrode compartments a and e are used and the sample is separated at a high driving current. The sample zones migrate toward the branching of in the bifurcation block. Using the electrode compartment e not relevant zones are allowed to vent, whereas separand zones can be trapped into the separation compartment using the counter electrode j.

The dimensions of the flat channel are chosen as to minimize diffusional and disturbing effects and allow optimal trapping. Though the flat channel has only a gap width of 0.05 mm still high driving currents in the
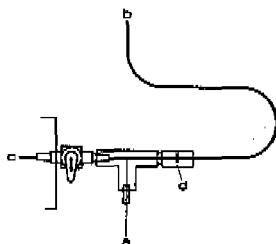
Fig. 3.4 The out of line bifurcation.
a: separation capillary, b: preseparation capillary,
c: vent, d: tell-tale detector.

preseparation mode can be used due to the effective heat
removal of the flat channel. In the preseparation boring
of the bifurcation block a conductimetric detection system,
the "tell-tale" detector, is mounted. The separand zones,
passing this detector, will need a definite time to cover
the distance between the point of detection and the bran-
ching off. For a given electrolyte system and driving cur-
rent this time the delay time t_d is invariable and can
easily be measured. As a result this detector can be used
to decide at which moment the instrument should be swit-
ched from the preseparation mode to the separation mode.

Another construction for coupling the two separation
compartments is shown in Fig. 3.4. In this case the two
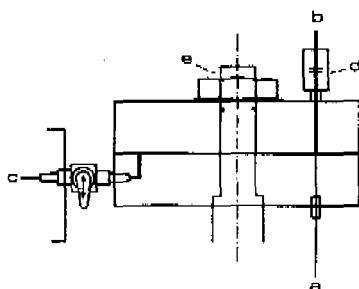separation compartments are not in-line. The bifurcation

Fig. 3.5 The trapping valve.
a: separation capillary, b: preseparation capillary,
c: vent, d: tell-tale detector, e: adjusting-nut.

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block consists of an acrylic T-piece with a large boring of 0.8 mm and perpendicular to this the small boring of 0.2 mm. The "tell-tale" detector is mounted separately. Though this type of coupling has proven to work satisfactorely the in line configuration has to be preferred, since the latter prevents gravitional settling.

The third possibility for coupling is the use of a disk-type valve instead of the bifurcation block, Fig. 3.5. Again a conductimeter is used to control the switching of the valve. Since the trapping volume can be varied using a disk with multiple borings, this configuration seems particular applicable for micropreparative work and off-column techniques.

Essential for optimal functioning of the coupled column system is that the two counter electrode compartments can be connected with the power supply separately. In the preseparation mode the electrode compartment of the separation capillary must be detached from the power supply and no leak current through the separation compartment should occur. In the trapping and separation mode the counter electrode of the preseparation compartment must be detached from the power supply, whereas the electrode of the separation compartment must be connected. During trapping and the final separation no leak current should occur through the preseparation electrode compartment and since this compartment can be at relatively high voltage it requires a perfect electric insulation. It should be emphasized that even a small current leak during trapping will result in incomplete and irreproducible trapping. Any current leak during detection will have a deleterious effect on the time-based zonelength. In the coupled column system this galvanic separation of the two electrode compartments is realized by the use of vacuum high voltage relays (Kilovac Corp. St. Barabara, Cal. USA). To prevent any current leakage through the "tell-tale" detector this detection system is mechanically disconnected in the final separation mode.

Automation of the coupled column system

Though the coupled column system can be operated manually its automated operation is more convenient. The basic operations for automatic handling are shown in Fig. 3.6
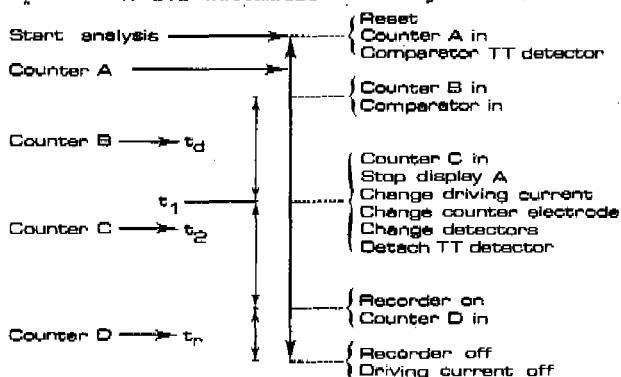
Fig. 3.6 *Automation procedure.*
t_1 *is the preseparation time,* t_d *is the delay time,* t_2 *is the final separation time,* t_r *is the recording time.*

Before the analysis is started the driving current for the preseparation compartment, I_1, and the final separation, I_2 should be chosen. Various times, the delay time t_d, the separation time t_2 and the recording time can be stored in the memory of three counters. The output level of the "tell-tale" conductimeter is fed to a level comparator. The conductivity level of the selected zone must be chosen for the level comparator.
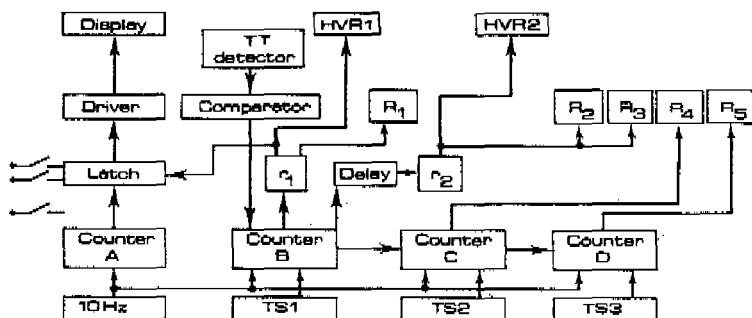
Fig. 3.7 *Scheme of the electronic process control.*

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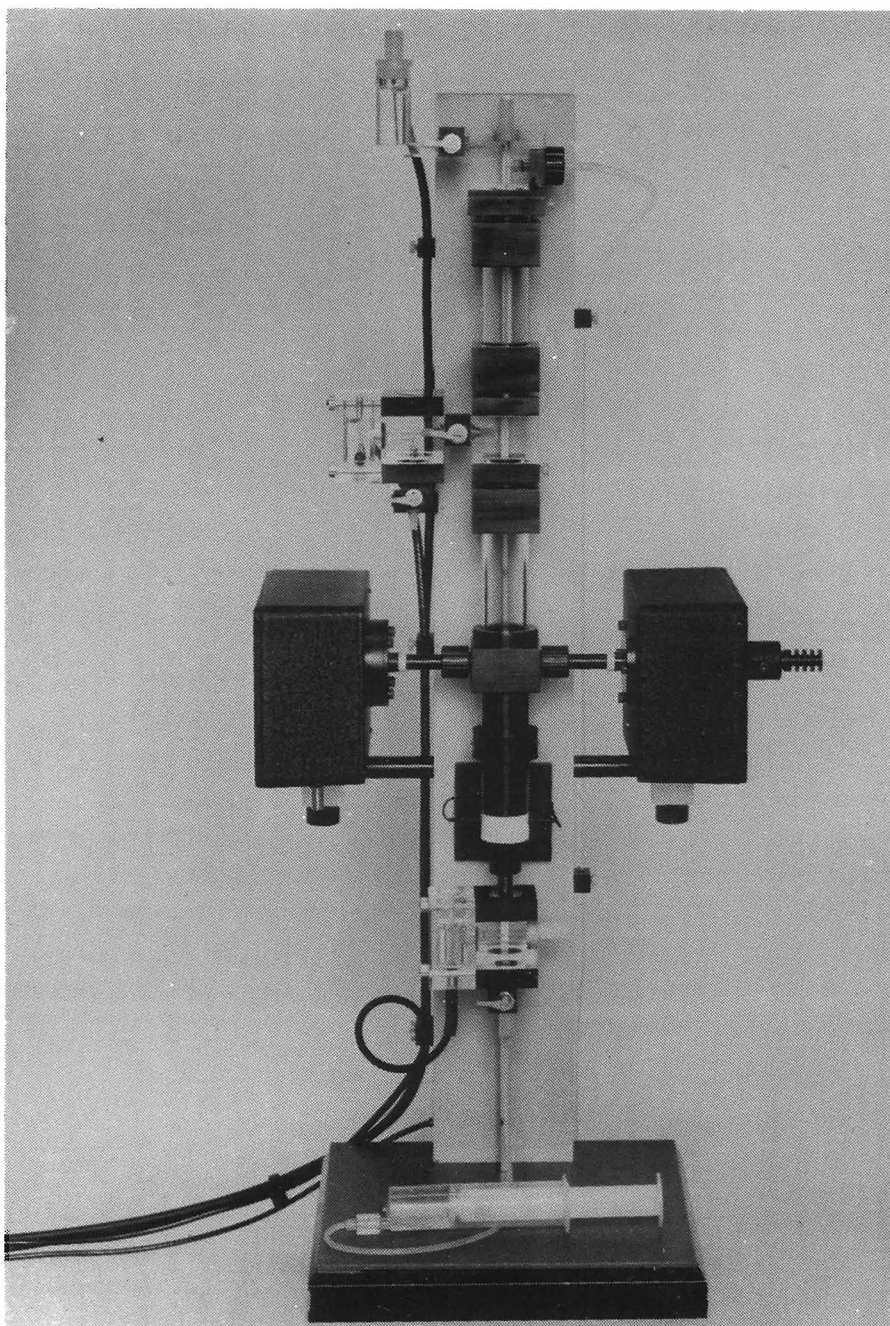
Fig. 3.8 Photograph of the coupled column instrument.

As soon as the analysis is started the counter A is
activated in combination with the display. The various
times can be measured with this counter. As soon as the
signal derived from the "tell-tale" detector matches the
chosen value in the level comparator, counter B is activa-
ted. In the memory of this counter the delay time t_d is
stored. After the delay time the following operations are
started simultaneously; the latch is activated and the dis-
play of counter A is stopped, the driving current is chan-
ged from its value I_1 to I_2 via the relays $R1$, counter C
is activated and the separation compartment is attached to
the current-stabilized power supply via the high voltage
relay $HVR1$.
After 100 msec the counter electrode of the preseparation
compartment is detached via the second high voltage relay
$HVR2$. Simultaneously, the "tell-tale" detector is mechani-
cally, i.e. motor driven, disconnected by means of the re-
lay $R2$. Moreover the conductimeter is switched from the
"tell-tale" detector to the detector of the separation com-
partment, via relay $R3$. The display of counter A can be
reactivated manually through the latch. After the final
separation time, stored in counter C, has passed the paper
transport of the recorder is started via relay $R4$. Simul-
taneously the counter D is activated. After t_r seconds,
the recording time, the recorder is stopped and the dri-
ving current I_2 is switched off. The equipment is now
ready for a new run and resetting.

3.2. EXPERIMENTAL PERFORMANCE

One of the most important features of the coupled co-
lumn system is that it allows the analysis of samples in
which an interfering constituent is present at a relative-
ly high concentration. In Fig. 3.9 an example is shown of
the analysis of ascorbic acid in a natural orange juice
performed with the conventional instrument. The apparatus
was equipped with a PTFE separation capillary with an in-
ner diameter of 0.2 mm and the electrolyte systems and ope-
rational conditions are summarized in Table 3.1.
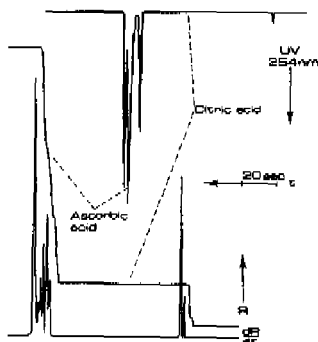
Fig. 3.9 Isotachophoretic separation of orange juice.
Operational conditions Table 3.1, Conventional equipment.
Sample load; 0.75 µl, 1/10 diluted.

TABLE 3.1

ELECTROLYTE SYSTEMS AND OPERATIONAL CONDITIONS

	preseparation compartment	separation compartment	terminating compartment
Anion	Chloride	Chloride	MES
Concentration	0.01 M	0.01 M	0.005 M
Counter const.	HIST	HIST	TRIS
pH	6.02	6.02	6.4
Additive	0.2% HEC	0.2% HEC	--
Driving current	250 µA	25 µA	
UV		254 nm	

In the conventional equipment, the separation compartment conditions of the coupled column system were used.

Even when the conventional equipment was operated at its full load capacity, only the citrate zone, the interfering constituent, could be measured with sufficient accuracy. The separand of interest, ascorbic acid, is present as a small zone and the sample load should be at least one order of magnitude higher for an accurate determination and this is far beyond the capabilities of the conventional equipment. The same orange juice in its undiluted form was analyzed with the coupled column system at a much
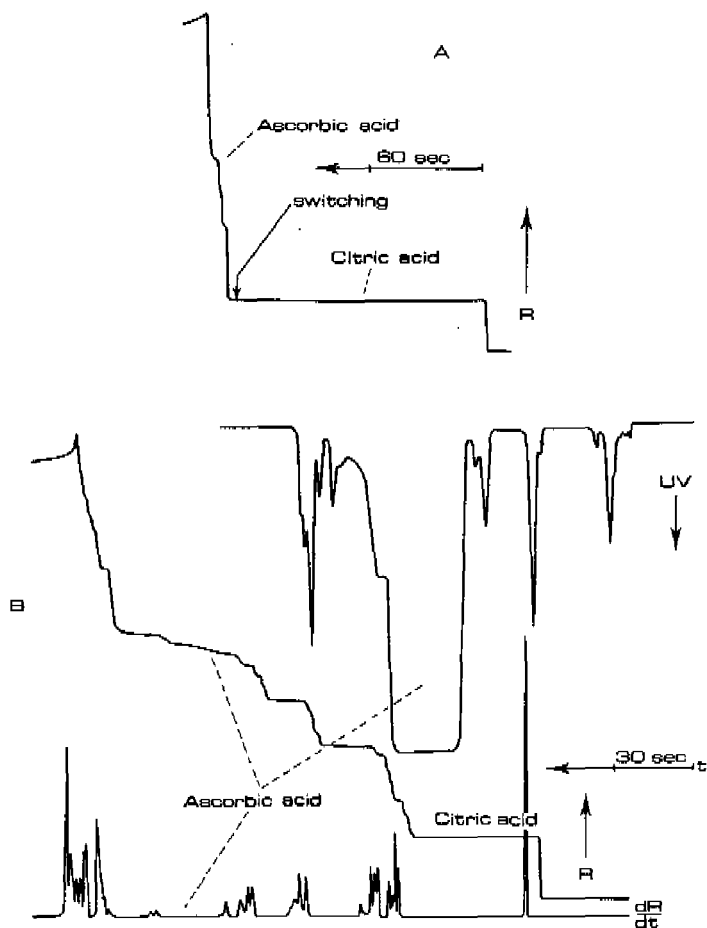
Fig. 3.10 Isotachophoretic analysis of orange juice with the coupled column system.

A. Detection trace from the tell-tale detector in the pre-separation mode.

B. Detection traces in the separation mode.

Operational conditions: Table 3.1, Sample load: 1 µl.

higher sample load. The preseparation compartment with an
inner diameter of 0.8 mm was coupled with a separation ca-
pillary i.d. 0.2 mm, using the off-line bifurcation. Again
the operational conditions of Table 3.1 were used. The re-
sult, as obtained from the conductimetric tell-tale detec-
tor, mounted in the preseparation compartment, is shown in
Fig. 3.10 A. The leading boundary of the citrate zone pas-
sed the tell tale detector after 8 minutes. The swamping
amount of citrate was allowed to pass the bifurcation and
approximately 4.5 sec before the next zone boundary reach-
ed the bifurcation the system was switched in its separa-
tion mode. Only a small amount of the citrate was allowed
to migrate into the separation capillary together with all
the following separands. The result is shown in Fig. 3.10
B. The total time of analysis was about 15 min. Full quali-
tative and quantitative information could be obtained from
both the conductivity and the UV detector. Using the cali-
bration data the ascorbate content proved to be 586 mg/l.
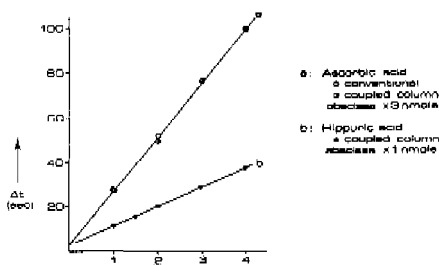
Fig. 3.11 Calibration data.

An essential requirement for the coupled column sys-
tem is that the separand zones must be trapped quantitati-
vely into the final separation capillary and that its re-
sults should be consistent with those obtained with the
conventional equipment. In a series of experiments, the
calibration characteristics of the coupled column system
were evaluated using ascorbic acid and hippuric acid. The
latter sample consisted of 5×10^{-4} M hippuric acid in 0.2
M NaCl solution, whereas the former contained only ascor-
bic acid. Again the operational conditions of Table 3.1

were used. Due to the swamping amount of chloride the hippuric acid sample could not be analyzed with the conventional equipment. From the calibration graphs of Fig. 3.11 it follows that the quantitative characteristics of the coupled column system are comparable with that of the conventional equipment. It should be noted that the calibration lines do not pass the origin. This is the result of the fact that some impurities were present in the electrolyte systems and is not related with trapping. The use of the coupled column system requires the application of ultrapure electrolyte systems. In fact the purity of the electrolyte systems can be limiting factor for realizing even higher relative gains.

A second benefit of the coupled column system is that it allows the use of different electrolyte systems in the preseparation compartment and in the separation compartment.

TABLE 3.2

ELECTROLYTE SYSTEMS AND OPERATIONAL CONDITIONS

	preseparation compartment	separation compartment	terminating compartment
Anion	Chloride	Chloride	Glutamate
Concentration	0.01 M	0.01 M	0.007 M
Counter const.	HIST	BALA	HIST
pH	6.02	3.00	6
Additive	0.2% HEC	0.2% HEC	--
Driving current	250 µA	25 µA	

The analysis in the conventional equipment was performed at $pH^L = 6.02$, at a driving current of 25 µA.

In Fig. 3.12 A the separation of some acids is shown in the conventional instrument. At $pH^L = 6.02$ the sequence of the separand zones is; sulphate, formate, citrate, acetate, phosphate + lactate. The separands lactate and phosphate can not be separated at this pH. Using the coupled column system the preseparation compartment was filled with the leading electrolyte at pH 6, whereas the final separation
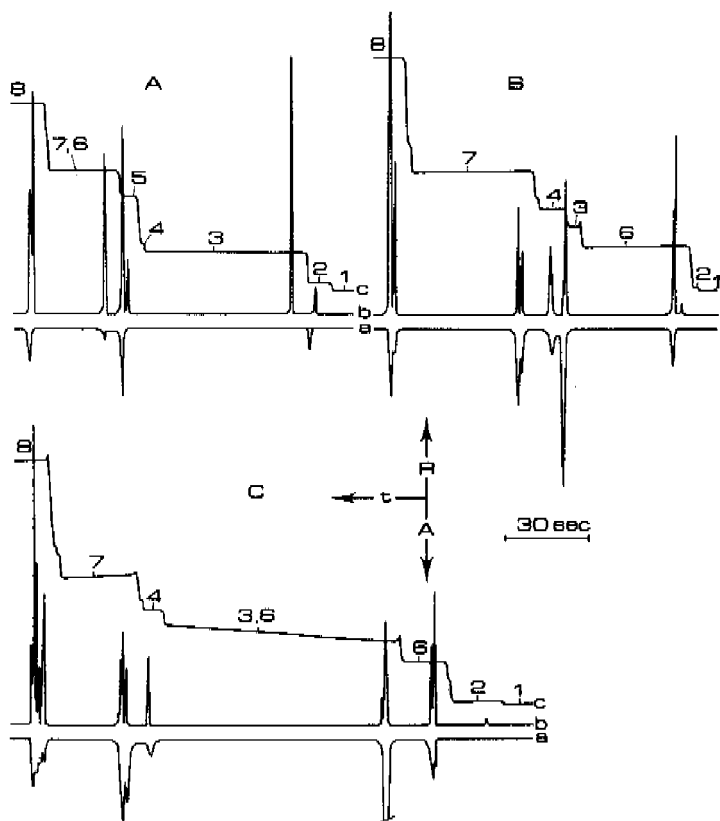
Fig. 3.12 The use of different electrolyte systems in the coupled column system.

1 = chloride, 2 = sulphate, 3 = formate, 4 = citrate, 5 = acetate, 6 = phosphate, 7 = lactate, 8 = glutamate.
R = increasing resistance, A = UV at 254 nm, t = time

A: Separation at pH^L = 6 in the conventional equipment, samle load 1 µl.

B: Separation with the coupled column system. Preseparation compartment pH^L = 6, separation compartment pH^L = 3. Sample load 3 µl., selective trapping.

C: As B, complete trapping. Sample load 1.5 ul.

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compartment was filled with the leading electrolyte at pH 6, whereas the final separation compartment was filled with the leading electrolyte at pH 3, Table 3.2. At pH 3 the sequence of the separand zones is: sulphate, phosphate, formate, citrate and lactate. In Fig. 3.12B the result is shown when two different electrolyte sysyems are used in the coupled column system. Formate was allowed to pass the bifurcation and therefore only a small formate zone is present in the final separation. The separands phosphate and lactate are now completely resolved. Acetate, constituent no 5, is not visible anymore as it has a smaller effective mobility than the terminating constituent at this pH. It should be emphasized that in using these two different electrolytes a total rearrangement of the separation configuration has occurred. This, of course, will have not always a favourable effect on the maximal allowable sample load as can be seen from the separation in Fig. 3.12C. In this case a lower sample load was applied and all the separand zones were trapped into the final separation compartment. From the separation it follows that the mixed zone between the relatively large amount of formate and phosphate hase not been resolved.
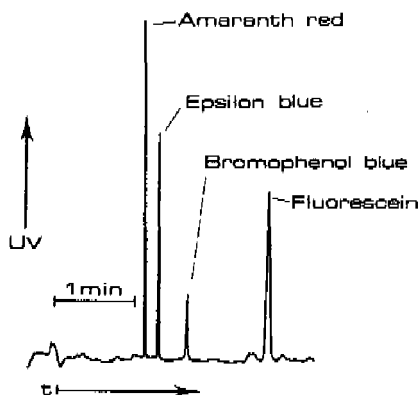
Fig. 3.13 Zone electrophoretic separation with the coupled column system.

Therefore the choice of electrolyte systems and the moment of column switching is extremely important when different electrolyte systems are used in the coupled column instrument.

Another field of application of the coupled column system is that it can be used for the combination of different electrophoretic principles.

TABLE 3.3

ELECTROLYTE SYSTEMS FOR DISCONTINUOUS ELECTROPHORESIS

	preseparation compartment	separation compartment	terminating compartment
Anion	Chloride	MES	MES
Concentration	0.01 M	0.005 M	0.005 M
Counter const.	HIST	HIST	HIST
pH	6.02	6.5	6.5
Additive	0.2 % HEC	0.2 % HEC	
Driving current	250 μA	50 μA	

In discontinuous electrophoresis isotachophoretically stacked zones are zone electrophoretically eluted by choosing the appropriate electrolyte and experimental conditions [19]. With the coupled column system the electrolyte conditions can be found rather easily: in the preseparation capillary a isotachophoretic leading-terminating electrolyte system has to be applied, whereas in the final separation capillary a zone electrophoretic electrolyte system must be used. Fig. 3.13 shows the zone electrophoretic separation of some anionic dyes, analyzed with the operational system of Table 3.3. The isotachophoretically migrating separands were trapped into the zone-electrophoretic electrolyte system, filling the final separation compartment. As can be seen from Fig. 3.13 a good separation is obtained and the result is at least comparable with the zone electrophoretic separations shown in Part 2 chapter 1.

Due to the fact that the migration configuration has not changed the isotachophoretic separation functioned as a almost ideal injection for the zone electrophoretic analysis.

3.3. CONCLUSIONS

Column coupling in isotachophoresis alleviates the problems that are encountered analyzing samples that contain ionic solutes at large concentration differences. Using a two dimensional system interfering ionic solutes can be removed from the isotachophoretically migrating sample by selective column switching. In comparison with conventional equipment the coupled column instrument allows a high sample load without a considerable increase in the analysis time. As the instrument comprises two separation compartments different electrolyte systems can be applied and combinations of different electrophoretic principles are allowed.

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CHAPTER 4

Separation of uremic metabolites

*Uremic blood sera contain, in
comparison with normal sera a
large excess of low molecular
weight anionic solutes, that
are only partially removed by
hemodialysis. Investigations
on Middle Molecule fractions
with gas chromatography, iso-
tachophoresis and liquid chro-
matography could not substan-
tiate the Middle Molecule hypo-
thesis.*

4.0. INTRODUCTION

One of the main functions of the kidneys in man is
the excretion of metabolic waste products. They are one of
the most important operating units in maintaining the fa-
vourable chemical environment of the body fluid system.
The kidneys can be affected selectively by diseases that
cause either gradual or sudden impairment of function. In
terminal chronic renal failure life cannot be sustained
without some substitution for renal function. Though trans-
plantation is probably the most optimal solution, patients
with chronic renal failure generally have to be submitted
to intermittent hemodialysis or other dialysis and ultra-

filtration procedures. The patients show a complex of symptoms, usually called "the uremic syndrome" or "uremia". Many of these symptoms are related to a disturbance in the homeostatic or regenerative function of the kidneys, which results in retention of metabolic products and in disorders of hormonal and metabolic function.

Until the early 1960's, dialysis activities were limited to the treatment of acute renal failure. But when the problem of repeated access to the blood circulation was solved by Scribner[1], regular dialysis treatment of terminal renal failure became possible. By removing, be it partially, the accumulating waste products, the clinical manifestations of uremia can be alleviated and many of the patients can persevere life in an "acceptable" way. Adequate dialysis implies the removal, or even retention, of the right substances in the right way and to the right extent. Though this may seem to be a medical-technical problem, it is obvious that analytical chemistry can provide important "diagnostic" tools.

With the increasing refinement in analytical chemistry the number of chemical substances, that have been proven to accumulate in chronic renal failure, has grown vastly[2-5]. Nevertheless it has not been possible to attribute the broad spectrum of uremic symptoms to the accumulation of known chemical substances. This can be due to the fact that the criteria for a uremic toxin are not uniform, whereas in addition antagonistic, additive and synergistic effects may play an important role. The search for uremic toxins has been highly specific, assessing the possible toxicity of single known substances. The importance of various inorganic substances, such as sodium, potassium, magnesium, trace elements, phosphate and even water is well documented[7-13]. But even in the absence of gross abnormalities in water and electrolyte metabolism many uremic symptoms occur in patients with terminal renal failure.

Numerous organic substances are known to accumulate in chronic renal failure, varying from very simple mole-

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cules, e.g. urea, to complex molecules like β_2-microglobulin. There are indications that many of the retained components can act as cell toxins and enzyme inhibitors or may cause abnormal membrane transport in tissue and cells[5]. Many authors have investigated the role of lower molecular weight substances, such as guanidines, guanidino acids, amino acids, amines, phenolic acids, polyols etc.[2,4,14-18]. Though in many cases appreciably increased concentrations in the body fluids were found, the relationship between the uremic symptoms and the increased concentration levels generally failed. In this respect it should be emphasized that most of these studies aimed at a specific accumulant or a specific class of accumulants. It seems that for an adequate assessment of their significance a multifactorial approach is necessary. In 1965 Scribner[19] suggested that with more permeable dialysis membranes certain accumulating substances could be more efficiently removed. This suggestion stemms from the clinical finding that patients on long term peritoneal dialysis were doing well, in spite of the relatively high serum concentrations of creatinine, uric acid and urea. It proved that not only the permeability was important for the efficiency of dialysis, but also the membrane surface area and the number of hours of dialysis per week. In the resulting *Square-Meter-Hour* hypothesis these parameters were related[20]. The original suggestion however postulated the presence of unknown, but pathophysiologically important molecules with a molecular weight intermediate to that of rather small accumulating substances, i.e. molw. < 500, and large solutes such as lipoproteins, polypeptides and proteins. In 1972 the name m^2-hr hypothesis was changed to *Middle Molecule* hypothesis[21]. Since the proposal of the middle molecule hypothesis, large surface-area dialyzers have been increasingly used to reduce dialysis time. Babb and Scribner[22] developed the concept of the dialysis index to facilitate quantitation and prescription of dialysis therapy.

Though there was some clinical evidence for the

existence of middle molecules, their role in uremic toxi-
city remains controversial. Nowadays the middle molecule
hypothesis comprises serum solutes with a molecular weight
range of 500 to 2000. In dialysis patients such molecules
may accumulate to a greater extent than small molecules,
due to their relative low permeability across the dialysis
membrane.

Obviously many investigators have tried to analyze, iden-
tify and isolate middle molecules. Most groups have used
gelfiltration for separation and identification of com-
pounds in the middle molecular weight range and used UV-
detection[22-36]. Only few investigators claimed to have iso-
lated or identified a specific solute of the required mo-
lecular weight[37-40]. Generally the results of gelfiltra-
tion analysis are taken as a quantitative measure for ac-
cumulation and elimination of middle molecules. Moreover
middle molecular weight fractions of gelfiltration showed
in vitro toxicity effects, such as inhibition of glucose
utilization[41], inhibition of phagocytic activity and inhi-
bition of activity of a number of enzymes[43-44,14,37].
Though many investigators emphasize the importance of mid-
dle molecules it should be noted that no convincing ana-
lytical demonstration of their presence has been given. As
such the middle molecule hypothesis is still a hypothesis
that remains to be proven.

In recent years however some research groups have
followed a screening approach to the problem, in which che-
mical multicomponent analyses of biological fluids, deri-
ved from uremic patients, play an important role. The ob-
vious drawback of such an approach is the extraordinary
complexity of such fluids. Many methodological choices
have to be made as the accumulating solutes can be very
different; volatile-non volatile, low molecular weight-
high molecular weight, ionic-non ionic. As a result va-
rious analytical techniques have to be used. Analytical
chemistry has been successfull in the development of tech-
niques that permit separation, identification, quantifica-
tion and isolation of many metabolites in biological

fluids . The choice which techniques have to be applied is
in practice further limited by financial and manpower facili-
ties. In our screening approach four different techniques
are used: *Isotachophoresis, gas chromatography, liquid chro-
matography and mass-spectrometry*. For identification of
solutes and to increase the differentiating capabilities
the various techniques can be combined. The results of
such a multifactorial approach will have its spin off in
several ways. They should decrease our ignorance[2] on which
accumulating solutes are important and will increase the
knowledge on metabolism and homeostasis.

For the analysis of ionic solutes only few analytical
techniques are available. Isotachophoresis is compatible
with the basic requirements for screening approach proce-
dures; multicomponent information, rapid completion, re-
liability and low cost. Moreover, the flexibility of the
technique offers a vast spectrum of possibilities for de-
tailed studies. Therefore isotachophoresis will provide
useful information on the occurrence of ionic solutes in
uremic biological fluids.

4.1. EXPERIMENTAL

Isotachophoresis was performed using the equipment
developed by Everaerts et al.[45]. The separation compart-
ment consisted of a polytetrafluoro-ethylene capillary
with an inner diameter of 0.2 mm and an outer diameter of
0.4 mm. The direct and constant electric driving current
was taken from a Brandenburg (Thornton Heath, UK) high
voltage power supply. Separated zones were detected by
measuring the electric conductance as well as the UV-ab-
sorbance at 254 nm. Electrolyte systems and other opera-
tional conditions are summarized in Table 4.1.
To increase the experimental performance 0.2% hydroxyethyl-
cellulose was used as a viscous additive. The electrolyte
system 3 of Table 4.1 is of a non-conventional
type as two counter constituents are used. The leading
electrolyte was prepared by dissolving TRISHCl buffer in

TABLE 4.1

ELECTROLYTE SYSTEMS AND OPERATIONAL CONDITIONS

Parameter	System no.		
	1	2	3
pH	3.50	6.02	8.10
Leading constituent	Cl^-	Cl^-	Cl^-
Concentration (M)	0.01	0.01	0.01
Counter constituent	BALA	HIST	$TRIS/NH_4^+$
Terminating constituent	CAPROIC	HEPES	OH^-
Concentration (M)	0.01	0.01	~
Additive	0.2% HEC	0.2% HEC	0.2% HEC
Driving current	25 µA	20 µA	30 µA

water at a concentration of 0.01 M. The desired pH was ob-
tained by the addition of ammonia. The use of two counter
constituents allows a large pH difference between the lea-
ding and the terminating electrolyte. As a result hydroxyl-
ions can be used as the terminator. A solution of barium-
hydroxide, pH 10.5, was used as the terminating electrolyte
and the precipitate of barium-carbonate, due to the car-
bon-dioxide content of the water, was removed by filtra-
tion. The performance characteristics of this electrolyte
system are better than those of conventional electrolyte
systems, in which generally an amino acid of doubtful pu-
rity is used. Separation times were generally less than
twenty minutes and samples were introduced using a micro-
liter syringe.

In the screening approach also high resolution gas chromato-
graphic separations were performed following the procedure
of Schoots[46]. In the GC-procedure sample pretreatment com-
prised ultrafiltration, evaporation and derivatization.
For identification mass-spectrometry was used[46,47]. High
performance liquid chromatographic separations were done in
the reversed phase mode using a buffered water-methanol
gradient[48]. Serum samples were ultrafiltered before appli-
cation. Gelfiltration was performed on Sephadex G-15 in an
ammonium acetate buffer[49]. The eluate was fractionated and

lyophilized. Relevant fractions were reanalyzed by isota-
chophoresis, gas chromatography and liquid chromatography.

If the removal of high molecular weight substances
was required an ultrafiltration procedure was used. Pres-
sure ultrafiltration was carried out in a micro-cell[50]
using membranes of the required molecular weight cut-off.
All membranes were carefully soaked in distilled water
before use. In additional experiments cone-type ultrafil-
tration filters, that can be used in a centrifuge, were
applied. All membranes, including the cone type, were pur-
chased from Amicon (Oosterhout, The Netherlands). For re-
moval of proteins the filters XM50, PM30 and CF25, with
nominal mol. wt. cut-off of respectively 50.000, 30.000 and
25.000 were used.

Phagocytosis was studied by a modified method of
Baehner at the analytical laboratory of the Nephrological
division of the University Hospital of Ghent (Belgium)[42].
The burst of the metabolic activity, that is normally as-
sociated with phagocytosis was measured directly by $^{14}CO_2$
generated from glucose-1-^{14}C by the hexose monophosphate
shunt. Whole blood, collected from normal persons, was
incubated with glucose-1-^{14}C. Phagocytosis was initiated
by the addition of polystyrene latex beads or zymosan at
$37^{\circ}C$ for 1 hour. $^{14}CO_2$ was trapped on a filter and coun-
ted with liquid scintillation. Inhibition of phagocytosis
by uremic ultrafiltrate, gelfiltration fractions, normal
urine and appropriate reference samples was studied.

Blood samples of uremic patients, before and after
hemodialysis, ultrafiltrates and urines were obtained from
the Nephrological Division of the University Hospital of
Ghent (Belgium). Patients had different etiologies and
were subjected to several dialysis strategies. All samples
were stored at $-20^{\circ}C$ until use.

4.2. RESULTS AND DISCUSSION

Applying isotachophoresis as a screening approach
much information must be obtained in a relatively short

time of analysis. We therefore limited the time of analy-
sis to 20 minutes at moderate current densities. Concer-
ning the multicomponent information a methodological choi-
ce has to be made between anionic and cationic separations.
Preliminary experiments showed that cationic profiling of
uremic sera gave only minor differences in comparison with
normal sera. On the other hand, anionic separations showed
more deviating results. So we confined our investigations
mainly to the anionic separation mode. Anionic separands
will have sufficient electrophoretic mobility, provided
the pH of the leading electrolyte is sufficiently high.
For the anionic profiling of uremic sera, Fig. 4.1, an
electrolyte system at a high pH was used. The operational
conditions for this electrolyte system are given in Table
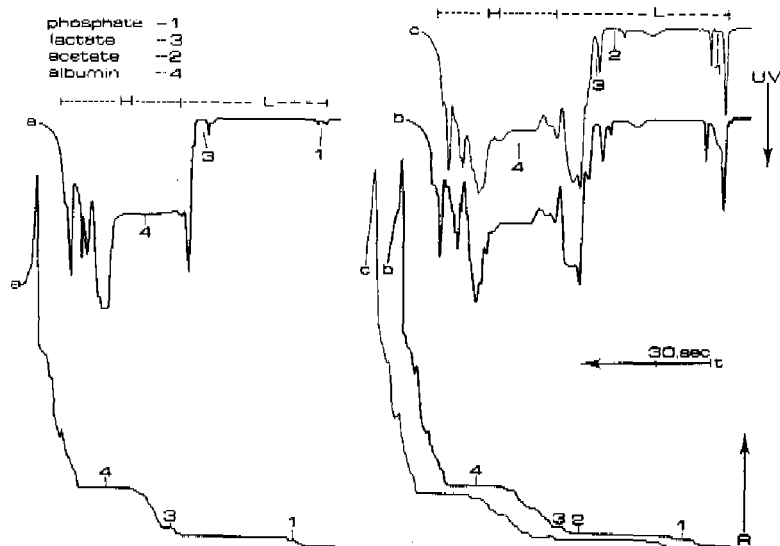4.1, system 3.

Fig. 4.1 Isotachophoretic serum analyses at high pH
a: normal serum, b: uremic serum pre dialysis, c: uremic
serum post dialysis. Injected volume: 0.2 µl.
UV: UV absorption at 254 nm, R = increasing resistance,
t: time, H: High molecular weight region, L: Low molecular
weight region.

Fig. 4.1 shows the analytical results when 0.2 μl of normal, uremic pre and uremic postdialysis. Sera were injected. The uremic samples were obtained from a male patient, age 63 with chronic glomerulonephritis, on intermittent hemodialysis from January 1977. Dialysis was performed with a poly-acrylonitrile membrane three times per week for 4 h. in a RP6 (Rhône Poulenc, France) open system[51]. Due to the swamping amount of chloride and the relatively high protein content only a low sample load can be applied. The differences between the uremic and the normal sera are evident. The protein region in Fig. 4.1 indicated with H, in which albumin is the most abundant solute, shows only minor differences. They can be due to the proteins or to low concentrations of some amino acids, peptides, purines and pyrimidines, with fairly high pK_a values[52]. The major differences however occur in the lower molecular weight region and many of the accumulating solutes have UV absorption at 254 nm. The somewhat poor differentiation in this region is the analytical price for the succesful attempt to stack as much anionic solutes as possible. The large zone between lactate and phosphate is mainly carbonate, originating from the electrolyte system and the sample. Comparing the analyses before and after hemodialysis, it follows that the accumulating solutes have been removed only partially. Moreover acetate, originating from the dialysate, is clearly visible. Though this electrolyte system seems very attractive for studying proteins in uremia it should be recognized that only moderate differentiation is obtained. This differentiation can be increased by the use of spacers[53], but probably more interesting separations will be obtained with isoelectric focusing.

More differentiation can be obtained when the pH of the leading electrolyte is lowered. The electrolyte system at pH = 6, which covers a large spectrum of anionic solutes, is given in Table 4.1. It should be noted that many of the proteins, peptides, amino-acids, purines and pyrimidines, will not migrate isotachophoretically in this

electrolyte system. Generally, anionic solutes with pK_a values higher than that of the terminator, pK_a (HEPES) = 7.5, will not be stacked. As some solutes are immobilized at this pH, a higher sample load can be applied. Uremic sera, however, can show appreciable fluctuations and therefore it is wise to work well below the maximal allowable sample load. In Fig. 4.2 the separations of uremic sera are compared with the result of a normal serum. Obviously the normal serum differs significantly from the uremic sera before and after hemodialysis. Several accumulating solutes have been identified[54]. From the UV-detec-
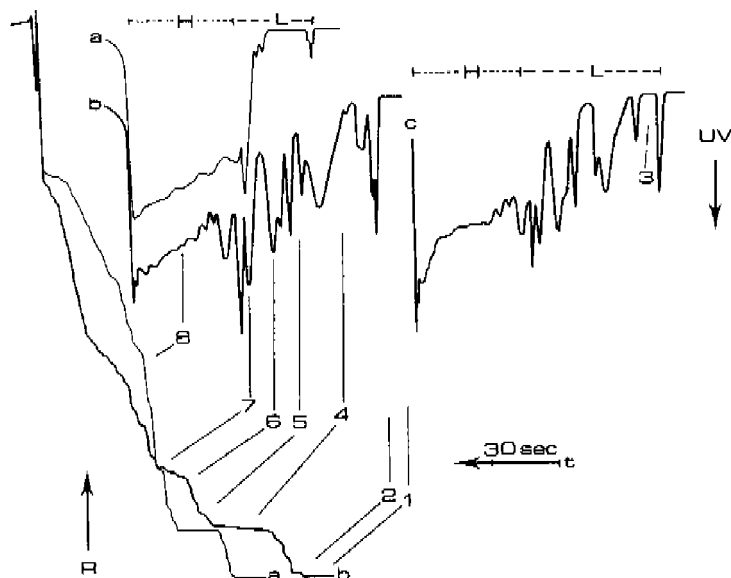

Fig. 4.2 Anionic profiling of uremic and normal sera at pH 6.

a: normal serum, 0.3 µl, b: uremic pre dialysis, 0.3 µl, c: uremic post dialysis, 0.3 µl.

1 = chloride, 2 = sulphate, 3 = acetate, 4 = phosphate + lactate, 5 = β-OH-butyrate, 6 = hippurate, 7 = urate, 8 = albumin.

R: increasing resistance. UV: UV-absorption 254 nm, t: time.

tion traces it follows that the major differences between the uremic and the normal sera occur in the more mobile part of the isotachopherogram, indicated in Fig. 4.2 by L. In this part the anionic low molecular weight substances with relatively low pK_a values, i.e. pK_a < 5.5, will migrate. Comparing the UV-traces before and after hemodialysis it follows that the accumulating solutes have been removed only partially. Moreover, acetate, originating from the dialysate, is clearly visible in the uremic serum after hemodialysis. In the second part of the isotachopherogram, in Fig. 4.2 indicated by H, anionic solutes with a rather low effective mobility will migrate. This part of the isotachopherogram is mainly occupied by albumin, as only few low molecular weight substances have pK_a values between 5.5 and 7.5. Using an ultrafiltration step the H region can be easily removed from the isotachopherogram[55]. From the separation profiles it follows that the differences between the normal and the uremic sera are much less pronounced in this part. The minor differences can be caused by the occurence of albumin-heparin complex in the post-treatment samples. It has been shown that by the formation of such complexes not only the mobility of albumin, or other proteins, can be increased, but also that in this way protein binding of low molecular weight solutes, can be reduced[56]. In fact the increased mobility of the albumin zone is characteristic for the post-treatment samples.

As the excess of low molecular weight substances is a characteristic difference between normal and uremic samples, it is advantageous to describe this difference in a single parameter. This is most conveniently done by taking the quotient of the two zone-lengths, H and L. The transition point between the albumin-zone and the lower molecular weight region is characteristic for all separations, done until now, and therefore this point is used for determining the two zone-lengths. It should be emphasized that the HL ratio does not provide direct quantitative information. The HL-ratio for normal sera always is signifi-

cantly higher than unity, indicating a relatively small
content of the anionic low molecular weight solutes. For
uremic sera the pre dialysis values are generally smaller
than unity, due to the excess of the accumulating solutes.
As acetate is metabolized rather quickly after hemodialy-
sis[57,58], the HL-ratio should be corrected for this zone-
length[54]. In order to study the relevance of this ratio a
patient was screened during several weeks of hemodialysis.
The patient (female, age 38, chronic glomerulonephritis),
was since August 1975 on intermittent hemodialysis. Dialy-
sis was performed with a RP6 (Rhône Poulenc, France) open
system three times per week for 4 h. The strong similari-
ty of the separation profiles indicated that the patient
was well stabilized and gave a fairly constant respons to
intermittent hemodialysis[54]. In Table 4.2 the measured HL-
ratios have been summarized.

TABLE 4.2

HL-RATIOS: NORMAL-, PRE- AND POST-DIALYSIS VALUES

Serum	HL-ratio								mean	cv%
Normal	1.10	1.25	1.22	1.17	1.18				1.18	5
Uremic pré	0.86	0.91	0.91	0.79	0.86	0.84	0.77	0.72	0.83	8
Uremic post	1.19	1.15	0.94	0.90	1.10	1.03	0.98	1.00	1.06	10
c.v. coefficient of variation										

Post dialysis values, corrected for the acetate contents,
were slightly larger than unity, suggesting a correction
towards the normal value.
As can be expected patients with chronic renal failure do
not form a homogeneous group. Figure 4.3 gives some sepa-
ration profiles of different patients before hemodialysis.
Patients with a very large excess of anionic lower molecu-
lar weight solutes will have a low ratio, e.g. HL = 0.68.
As can be seen the separation profiles can be very diffe-
rent. The example with HL = 1.19 is hardly deviating from
normal. The samples show as well differences in the lower
molecular weight part, L, as in the higher molecular
weight part, H. The differences in the latter part, how-
ever, are much less pronounced. In Fig. 4.3 also the
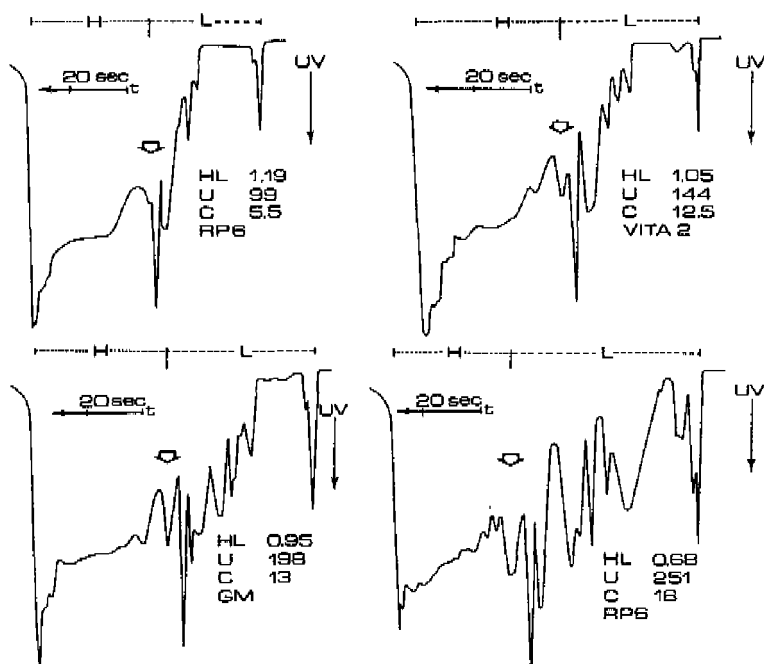
Fig. 4.3 UV-separation profiles of different patients.
Operational system 2, Table 4.1, injected volume 0.3 μl
serum. HL: HL-ratio, U: urea (mg/100 ml), C: creatinine
(mg/100 ml), RP6: Polyacrylonytrile, 1.0 m^2, Rhône Poulenc
(France), VITA 2: Cuprophan, 1.2 m^2, Bellco Spa (Italy),
G.M.: Cuprophan, 1.5 m^2, Ab. Gambro (Sweden).

urea and creatinine concentrations and membranes of the
dialyzer are given.

A HL ratio, which is close to normal, hardly improves by dia-
lysis whereas the effect on low HL ratios can be apprecia-
ble. From Fig. 4.2 it follows that not only the concentra-
tion levels of the various acumulating solutes can differ
but also the acumulating solutes can be different. The se-
paration profiles of Fig. 4.2 are some representative
examples, taken from a group of 27 different patients.
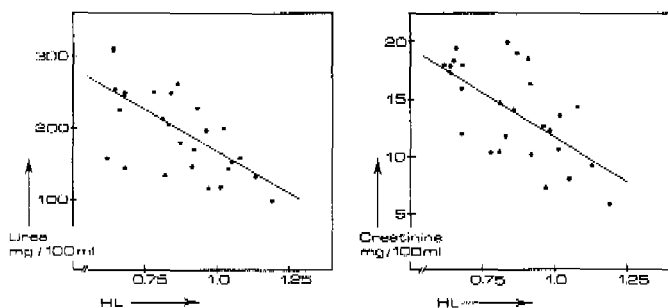
Fig. 4.4 HL-ratios versus urea and creatinine concentrations, pre dialysis values.

From the sera of these patients the HL-ratios, creatinine and urea concentrations were determined. In Fig. 4.3 the pre dialysis values are compared. As well the urea concentration as the creatinine concentration proved to be significantly correlated to the HL-ratio on the 95% probability level. From both the Figs. 4.3 and 4.4 it can be concluded that uremic patients form a rather heterogenic group. This heterogeneity can be the result of the impairment of renal function, secondary complications, differences in biological parameters, such as age and body weight, differences in dialysis treatment, in diet and way of living, renal restfunction etc. An adequate assessment of all these factors will require a large and more selected group of patients. All HL-ratios improved after hemodialysis but nog significant correlation was found any more for the urea and creatinine concentrations with the HL ratio.

Whereas the electrolyte system at pH 6 can be used for a fast screening approach, more differentiation is obtained at low pH. The operational system for anionic separations at pH 3.5 is given in Table 4.1. Fig. 4.5 gives the separation profiles of a normal and a pre dialysis sample.
Whereas in the normal serum profile, lactate and phosphate are the most abundant solutes (constituents 5 and 17), many other anionic solutes are present in the uremic serum.

Fig. 4.5 Anionic separation at low pH of a normal and a uremic serum pre dialysis.
Operational system. Table 4.1 system 1. Injected volume
0.8 μl. R: increasing resistance, UV: UV-absorption 254 nm,
t: time, Constituents: Table 4.2

Caproate was used as the terminating ion and therefore generally only anionic solutes with a pK_a values smaller than that of caproic acid, $pK_a = 4.8$, will migrate isota-chophoretically. As many of the solutes do not migrate at this low pH, the sample load can be increased in compari-son with the analyses at pH 6. Several solutes have been identified[54,57] and are given in Table 4.2.

TABLE 4.2
ANIONIC UREMIC SOLUTES, FIGS. 4.5 AND 4.6

t: tentatively

no	constituent	no	constituent
0	chloride	17	lactate
1	sulphate	19	β-OH-butyrate
2	ATP	20	hippurate
3	pyruvate	21	nicotinate (t)
4	indoxylsulphate	24	ascorbate
5	phosphate	25	succinate
8	ADP (t)	28	glutathion (t)
9	citrate	30	acetate
10	orotate	32	caproate

The concentration levels of the various accumulating solu-
tes again can show appreciable differences, but many of
them are present just below the mM. range. For a quantita-
tive determination the UV-spike method or the coupled co-
lumn system, Part 2 Chapter 3, has to be used. In Fig. 4.6
the separation profile of the uremic serum after hemodia-
lysis is given. In comparison with the predialysis sample
the excess of solutes, as analyzed by isotachophoresis,
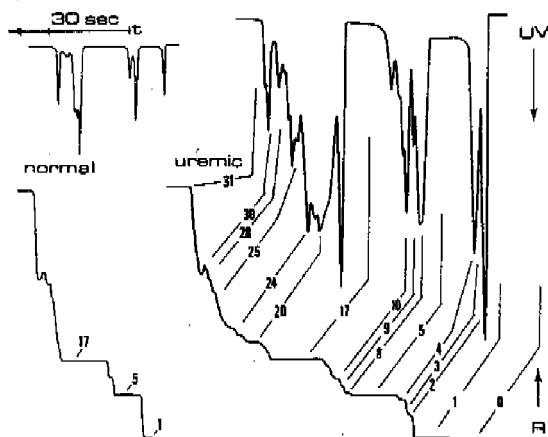has been reduced for approximately 60%.
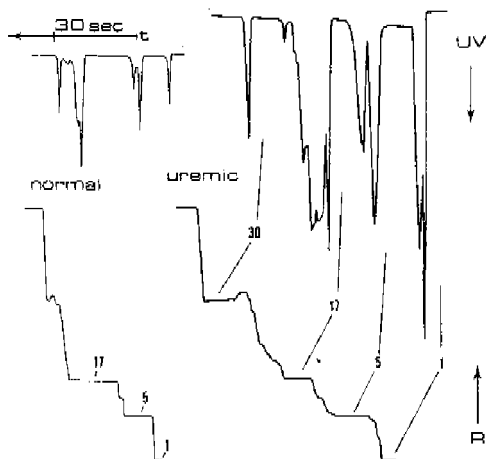
*Fig. 4.6 Anionic separation at pH 3.5 of a normal and a
uremic serum after hemodialysis.*
*Operational system, Table 4.1 system 1, Injected volume
0.8 µl. R: increasing resistance, UV: UV-absorption 254 nm,
t: time, constituents: Table 4.2.*

Nevertheless the uremic serum after hemodialysis still dif-
fers considerably from the normal one. Moreover, acetate,
originating the dialysate, is clearly visible and can be de-
termined without problem[54]. This operational electrolyte sys-
tem additionally has been used for the separation of normal
and uremic urines. Normal urine and serum give completely
different separation profiles[55] as a result of the selective
and concentrating capabilities of the normally functioning
kidneys. In case of renal restfunction, the separation

profiles of uremic urine and uremic serum show a large
similarity. Almost each solute, which is present in the
uremic sera, proved also to be present in the uremic uri-
nes, though individual concentration differences do exist.
Uremic urine, that was produced during hemodialysis or
shortly after it can contain an appreciable acetate con-
centration.

From all the isotachophoretic separations shown it
follows that the excess of relatively low molecular weight
solutes is characteristic for uremic serum samples. Pre-
dialysis and post dialysis serum samples have also been
investigated with gas-chromatography[46] and liquid chroma-
tography[48]. Representative chromatograms are shown in the
Figs. 4.7 and 4.8.
In the gaschromatographic procedure high molecular weight
solutes were removed by pressure ultrafiltration through
membrane filters with a mol. wt. cut-off at 50.000. Ultrafil-
trates were evaporated to dryness and derivatized to silyl
derivatives to enable gaschromatographic analysis. Separa-
tions were performed on glass capillary columns, coated
with SE-30[46]. The method proved to be reliable, reproduci-
ble and relatively fast. Solutes that were separated and
detected by this method are related to carbohydrate meta-
bolism, such as aldoses, aldonic acids and polyols. Also
other organic acids and some nitrogen containing compounds
were detected. Some thirty solutes have been identified
in combination with mass-spectrometry[47]. From the chromato-
grams of Fig. 4.7 it follows that the differences in con-
centration of various solutes are large. A group of ten
patients showed similar profiles, though individual dif-
ferences did occur. Profiles from pre and post dialysis
serum show that hemodialysis results in a significant de-
crease of the concentration of many solutes. Comparison
of the separation profiles of different patients re-empha-
sized the heterogeneity of the uremic patient group.
In the liquid chromatographic procedure[48] proteins were
removed from serum using Amicon CF 25 ultrafiltration fil-
ters with a mol. wt. cut-off at 25.000. Analyses

Fig. 4.7 Gaschromatographic profiles of ultrafiltered serum from an uremic patient before and after hemodialysis and from a pool of non-uremic sera.

9: urea, 10: phosphoric acid, 11: glycerol, 18: tartronic acid, 22: threonine, 26b: homoserine, 30: Δ-pyrrolidone-5-carboxylic acid, 32: threitol, 33: erythritol, 35: erythronic acid, 40a: tartaric acid, 49: arabinitol, 55: arabinonic acid, 56: citric acid, 58: fructose 61: galactose, 66: glucono-1,4-lactone, 67: α-D-glucose, 72: mannitol, 75: β-D-glucose, 76a: gluconic acid, 78: myo-inositol.

were done in the reversed phase mode, C_{18} chemically modified silica, using a buffered water methanol gradient. Elution was achieved with a 60-minute linear gradient programmed form 0.05 M aqueous ammonium formate, pH 4, to methanol. The column effluent was monitored with UV detection at 254 nm, and several peaks were collected and identified with off-line mass-spectrometry. From Fig. 4.8 the differences between the normal and the uremic sera can be seen. In the normal serum uric acid, hippuric acid and hypoxanthine are the most abundant solutes. The uremic sera, how-

Fig. 4.8 High performance liquid chromatography profiles
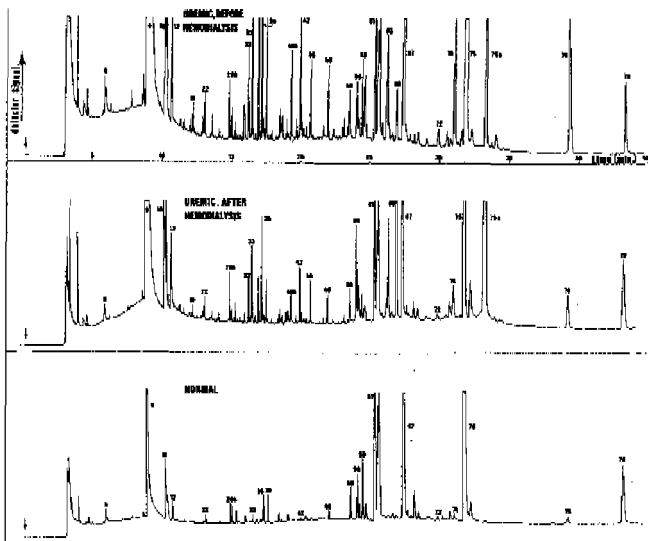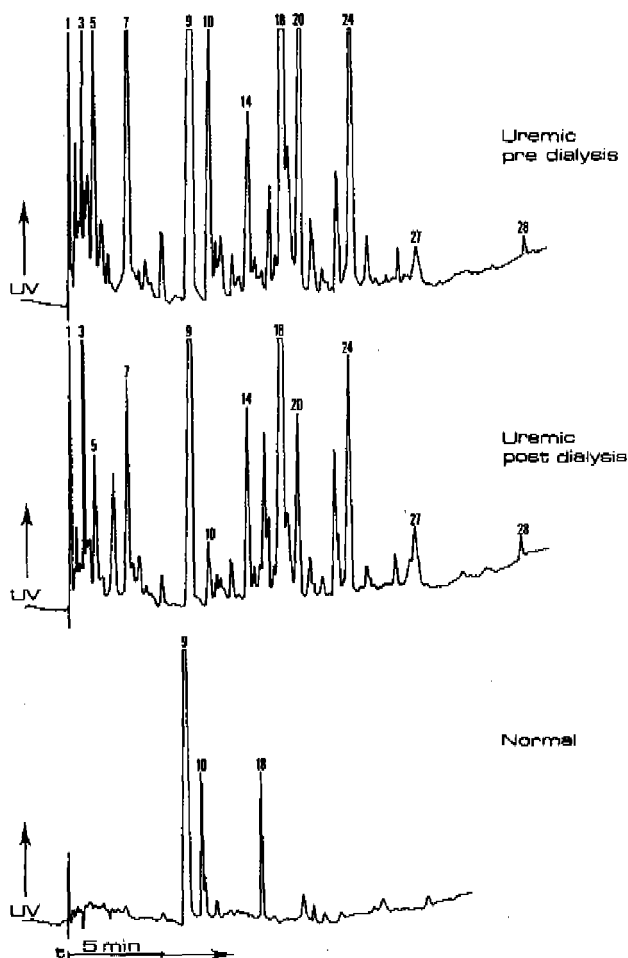of ultrafiltered serum from an uremic patient before and
after hemodialysis and from a pool of non uremic sera.

UV: UV-absorbance 254 nm, t: time

1 = Ascorbic acid

9 = uric acid, 10 = hypoxanthine, 18 = hippuric acid.

however, contain numerous other solutes with UV-absorbing properties at 254 nm. Some of them are given in the legend of Fig. 4.8. Other UV-absorbing solutes probably belong to the class of purines, pyrimidines, phenolic and indolic acids[59,60].

From all the separations shown, it follows that the excess of relatively low molecular weight substances is characteristic for the uremic state. According to the middle molecule hypothesis[20,21] solutes in the molecular weight range of 500-2000 should accumulate in uremic serum. The allocation of this molecular weight range is mainly based on gelchromatographic results. The relation between retention volume and molecular weight for these substances is however rather poor[32], as many non size related retention mechanisms dominate[61]. Zimmerman reanalyzed middle molecular weight gelfiltration fractions and ion-exchange reprocessed subfractions with isotachophoresis[40]. Both the gelfiltration fractions and the subfractions proved to contain several UV-absorbing solutes of rather high effective mobilities. The applied electrolyte system is almost equal to the system 1 of Table 4.1 at pH 3.5. The authors suggest that the separated solutes are peptides with a molecular weight of approximately 1000. This is however rather unlikely as such peptides only can be analyzed as anionic constituents by isotachophoresis at a high pH of the leading electrolyte. At low pH they will migrate as cations or will not migrate at all. The mobilities and UV-absorbing properties indicate that the analysed solutes belong to the class of low molecular weight organic acids, with fairly low pK_a values. Possible candidates probably can be found amongst the substitued hippuric- and benzoic acids or nucleotides.

In order to obtain more detailed information on middle molecules gelchromatographic fractions were analyzed with isotachophoresis, high performance liquid chromatography and high resolution gas chromatography[62]. Lyophilized gelchromatographic fractions from ultrafiltered mal serum, normal urine and several uremic ultrafiltrates

were investigated. The operational conditions for gelchromatography are given in Table 4.3

TABLE 4.3
OPERATIONAL CONDITIONS FOR GELCHROMATOGRAPHY

Column	length 60 cm, i.d. 1.6 cm
Gel	Sephadex G-15
Eluent	0.3 M NH$_4$Acetate
Flow	15 ml/h
Detection	UV: 206 nm, 280 nm
Fractions	5 ml
Sample load	uremic ultrafiltrate: 5 ml
	normal serum: 2 ml
	normal urine: 2 ml

The ammonium acetate eluent was chosen as it is easily removed during lyophilization. Each fraction was divided in three parts which were, after lyophilization, used for analysis and the measurement of phagocytosis inhibition. In Fig. 4.9 the gelchromatogram and the fractions are given. Middle molecules are supposed to eluate in fractions 11-15. The fractions of the ultrafiltered normal serum showed no signigicant inhibition of phagocytosis, whereas the middle molecule fractions gave considerable inhibition. All fractions of the first part of the gelchromatogram, i.e. fraction no. 3-18 in Fig. 4.9, were analyzed with isotachophoresis, gas chromatography and liquid chromatography. It proved that almost the entire spectrum of accumulating low molecular weight solutes, that normally are found in uremic ultrafiltrate, eluate in the middle molecular weight region. In Fig. 4.9 the distribution of some indentified solutes is given. Moreover numerous solutes, that have not yet been identified were present. Some representative results are shown in Fig. 4.10. All the identified solutes have rather low molecular weight, but nevertheless eluate in the middle molecule region. Though some separation has occurred Figs. 4.9 and 4.10 emphasize the fact that gelchromatography cannot be used as a size discriminating separation technique for solutes of intermediate molecular weight

Fig. 4.9 Gelchromatographic separation of uremic ultrafil-
trate. The fractions were analyzed by gas chromatography,
liquid chromatography and isotachophoresis and tested for
inhibition of phagocytosis. The distribution of various
solutes is shown: ● high concentration, ○ low concentra-
tion. The analyses of fractions 13 and 14 are shown in
Fig. 4.10.
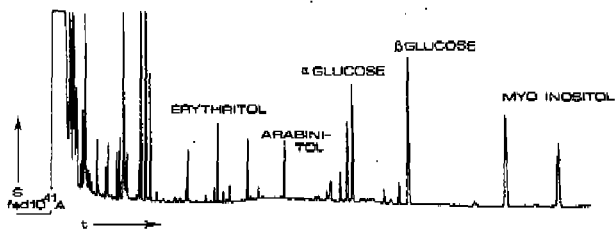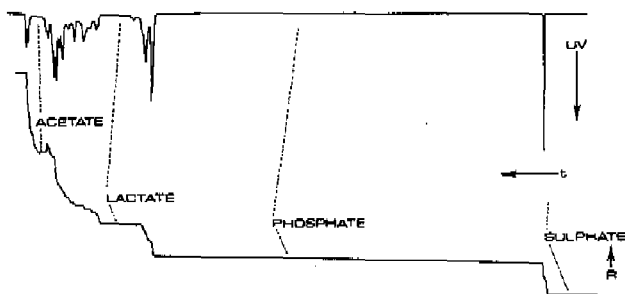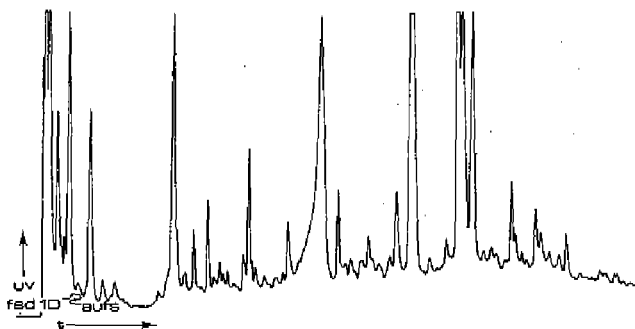
Fig. 4.10 Analysis of a middle molecule fraction with gas chromatography, liquid chromatography and isotacho-phoresis.

4.3. CONCLUSIONS

Isotachophoresis provides multicomponent information of uremic serum samples in a relatively short time of analysis. Due to the minimal sample pretreatment and standardized experimental conditions the results have good reproducibility (and a high reliability).
In anionic separations appreciable differences are found between uremic and normal sera, whereas in cationic separations only minor differences occur. Differentiation can be increased by choosing appropriate electrolyte systems. Anionic separations at a high pH of the leading electrolyte will include the analysis of proteins. Due to the relatively high protein contents of serum only a low sample load can be applied. Anionic profiling at pH = 6 will involve the analysis of albumin and the low molecular weight anionic solutes. Uremic sera show a large excess of low molecular weight solutes in comparison with normal sera. For isotachophoresis this can be expressed in the ratio of high molecular weight solutes to low molecular weight anionic solutes: the HL-ratio. Uremic sera show HL-ratios that are smaller than normal sera, dialysis increases the HL-ratio. Uremic patients form a heterogenic group, when the accumulating solutes are measured with isotachophoresis, gas chromatography and high performance liquid chromatography. Several solutes have been identified and proved to have a rather low molecular weight.

Investigations on middle molecule fractions, obtained with gel chromatography, showed that these fractions contain numerous solutes of low molecular weight. As a result the significance of the determination of "middle molecules", having molecular weights between 500 and 2000, by gelchromatography is highly questionable.

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CHAPTER 5

Determination of valproic acid in human serum

*An isotachophoretic procedure
for the determination of the
anticonvulsant valproic acid
in serum is given. The proce-
dure requires only micro li-
ter amounts of sample and no
sample pretreatment is neces-
sary. Quantitative determina-
tions can be done at the the-
rapeutic level within fifteen
minutes. Results are compared
with a routine gaschromatogra-
phic procedure.*

5.0. INTRODUCTION

Sodium valproate is known as a useful anticonvulsant
drug in primary generalized epilepsy[1]. The concentration
of the anticonvulsant in serum is of importance for the
correct treatment of epileptic patients, especially in es-
tablishing the pharmacotherapy. Optimal therapeutic serum
concentrations are known to be approximately 60 µg/ml.

Several gaschromatographic procedures have been des-
cribed[2-9], each with its own advantages and limitations.

A disadvantage, common to all gaschromatographic procedures, is the treatment of the sample prior to chromatography. Dependent on the specific procedure, solvent extraction, derivatisation and evaporation have to be used.

Isotachophoresis[10] requires no sample pretreatment and only minute amounts of sample are necessary for an accurate determination. Since valproate is an ionic solute and its therapeutic concentration level is just below the millimolar range, it is possible to determine it directly by analytical isotachophoresis.

5.1. EXPERIMENTAL

All chemicals were of analytical grade or additionally purified by conventional methods. Sodium valproate was obtained from Labaz (Maassluis, The Netherlands). Test- and reference sera were obtained from a hospital dispensory (Apotheek Haagse Ziekenhuizen, Den Haag, The Netherlands). In addition to valproic acid the testsera contained phenobarbital, phenytoin, ethosuximide, primidone, clonazepam, carbamazepine and 10,11-epoxycarbamazepine.
Serum samples were taken from venous blood; after clotting and centrifugation the sera were stored at -20°C until use.

Gaschromatography

For the gaschromatographic determinations a Packard Becker 421 chromatograph was used. Separations were performed in a glass column 1.5 m x 4 mm I.D., packed with 5% FFAP on Chromosorb WHP, 80-100 mesh (Free Fatty Acid Phase, Chrompack, Middelburg, The Netherlands). The injection temperature was 160°C, whereas the oven temperature was kept isothermal at 150°C. Nitrogen was the carrier gas and FID detection at 175°C was used. Cyclohexanecarbonic acid was applied as the internal standard.

Isotachophoresis

For the isotachophoretic separations the coupled column system, Part 2 Chapter 3, was used. The inner diameter

of the preseparation compartment was 0.8 mm. At a leading
ion concentration of 0.01 M an electrical driving current
of 377 µA was used. The valproate zone was trapped into
the analytical column, which had an inner diameter of 0.2
mm. The electric driving current in the analytical co-
lumn was 10 µA. The electrolyte systems and other opera-
tional conditons are given in Table 5.1.

TABLE 5.1
ELECTROLYTE SYSTEMS AND OPERATIONAL CONDITIONS

	preseparation compartment	separation compartment	terminating compartment
Anion	Chloride	Chloride	MES
Concentration	0.01 M	0.005 M	0.005 M
Counter Const.	EACA	EACA	TRIS
pH	5.00	5.00	6.50
Additive	0.3% HEC	0.3% HEC	--
Current Density	0.075 A/cm^2	0.0318 A/cm^2	
Temperature	22°C	ambient.	
UV	--	254 nm	

The constant electric driving current was taken from a
modified Brandenburg power supply (Thornton Heath, UK).
The voltage varied between 1 and 15 kV. Serum samples were
injected directly, using a microliter syringe. Separated
zones were detected by measuring the electric conductance
as well as the UV absorption at 254 nm.

5.2. RESULTS AND DISCUSSION

One of the major advantages of isotachophoresis is
that ionic solutes often can be analyzed without sample
pretreatment. Therapeutic levels of valproate in serum,
however, differ at least two orders of magnitude from the
physiological chloride concentration. Due to this unfavou-
rable sampling ratio, the electrolyte system will have a
rather low current efficiency[12]. Hence, for a reliable de-
termination, a large column volume must be available, re-
sulting in a large time of analysis. Most of these pro-

blems can be solved using a coupled system. This system not only allows the use of high sample load, but also the use of different electrolyte concentrations, e.g. Table 5.1. For the determination of valproate the concentration of the leading ion in the preseparation compartment was 0.01 M. At a high driving current serum samples were separated in approximately 6 minutes. The swamping amount of chloride was allowed to pass the bifurcation with the analytical column. The valproate zone was trapped into the analytical column, which contained the leading ion at a concentration of 0.005 M. Figure 1 shows a representative result when 3 µl of a patient serum was injected. The total time of analysis was less than 15 minutes. Since trapping was started two seconds before the valproate zone
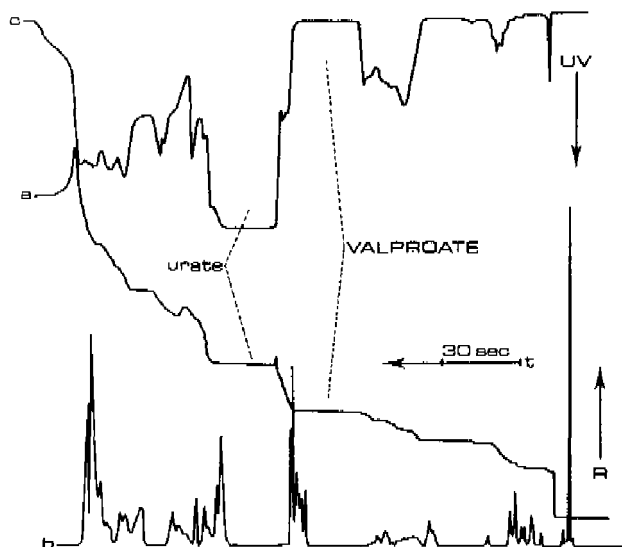
Fig. 5.1 Isotachophoretic separation of a patient serum. UV = UV absorption at 254 nm; R = increasing resistance; t = increasing time. Injected volume: 3.0 µl of serum. a. Conductimetric trace; b. differentiated conductimetric trace; c. UV trace, 254 nm.

reached the bifurcation point, some other solutes have
been analyzed additionally. The valproate zone, however,
easily can be localized in both the UV trace, Fig. 5.1c,
and the conductimetric trace, Fig. 5.1a. From the separa-
tion in Fig. 5.1 it follows that uric acid could have been
used as the terminating ion, instead of morpholine-ethane-
sulfonic acid. As a result a lower end-voltage would have
been obtained allowing a further optimization of the time
for analysis.

For the quantitative analyses, the characteristics of
the calibration line, i.e. zone-length versus amount of
valproic acid, were determined. The calibration points
were measured with standard valproate solutions in water
and in serum. Additionally several testsera, containing
various other drugs were analyzed. A good linear relation
was found with a correlation coefficient of 0.99914 (n =
26). The response was found to be 6.12 ng/sec and the mean
error per point was 3.8 ng in the detection range of 50-
500 ng. The additional other drugs did not interfere. The
gaschromatographic procedure yielded also a good calibra-
tion line, with a correlation coefficient of 0.99969. In
the detection range of 2-20 ng the mean error per point
was 0.1 ng. Using these calibration data, valproic acid
concentration levels were determined in the sera of twenty
patients. The isotachophoretic results were compared with
the gaschromatographic results. As can be seen from Fig.
5.3 there is a good relation. The experimental slope de-
viates only one percent from the ideal value of unity. A
positive intercept of 4.9 ng valproic acid, however, is
present. The group mean was 62.1 µg/ml for the isotacho-
phoretic determinations and 57.8 µg/ml for the gaschroma-
tographic determinations. The origine of the systematic
deviation is still under investigation. The results of
four different testsera, bovine sera, were for both me-
thods in good agreement with the expected values.
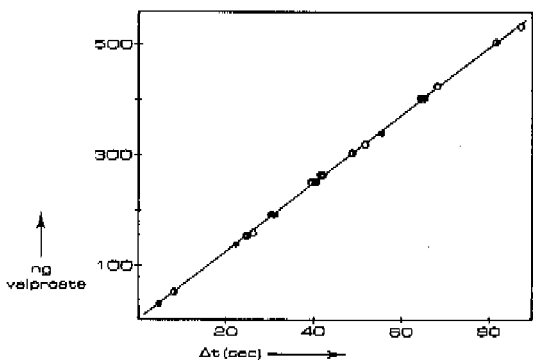
Fig. 5.2 *The calibration graph for isotachophoretic val-*
proate determinations. ○ *standard solution in water*
◑ *standard solution in serum*
● *test sera.*
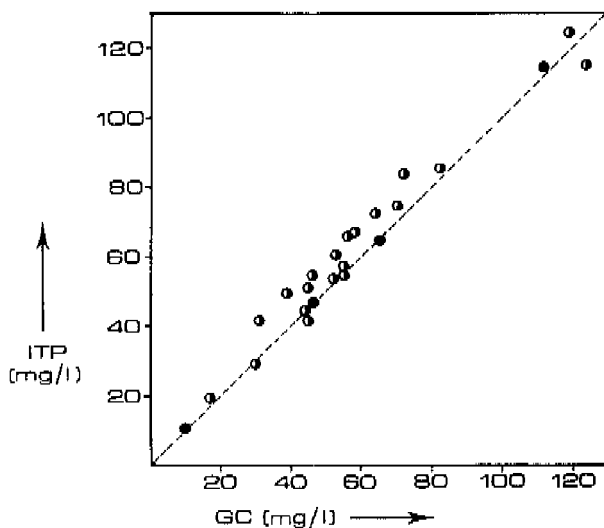
Fig. 5.3 *Comparison of gaschromatographic and isotacho-*
phoretic results. ◑ *patient sera*
● *test sera.*

5.3. CONCLUSIONS

Valproate in serum can be determined quantitatively by isotachphoresis. The procedure requires no sample pretreatment and only 3 µl of serum is necessary for the analysis. Results agree reasonably well with gaschromatographic results, though a systematic deviation exists.

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CHAPTER 6

Determination of uric acid in human serum

*An operational system is des-
cribed for the isotachophore-
tic determination of urate in
serum. Results show a good
agreement with a standard en-
zymatic procedure. For the
isotachophoretic determina-
tion only 3 µl of serum is re-
quired and the sample can be
analysed without any pretreat-
ment.*

6.0. INTRODUCTION

Uric acid is the end-product of purine catabolism in
man. An abnormal concentration of uric acid in the body
fluids may be indicative for a number of disturbances. An
increased serum concentration, hyperuricemia, is seen in
primary gout: although there is a normal excretion rate,
uric acid is overproduced or it is underexcreted[1]. In se-
condary gout, hyperuricemia might result either from in-
creased nucleic acid turn-over e.g. hematologic disorders,
or from decreased renal excretion of uric acid induced by
drugs or dietary factors. Also in a number of genetic dis-
orders, such as glycogen storage disease, Down's syndrome

and psoriasis, hyperuricemia is a common feature[2,1].

The methods currently in use to assay uric acid in serum are based on either chemical or enzymatic oxidation to allantoin. The enzymatic procedure seems to be the method of choice because of its sensitivity, accuracy and specificity[3,4]. Analytical techniques such as high performance liquid chromatography and isotachophoresis can be applied for the determination of various metabolites in biological fluids. Methods using HPLC have been reported for urate in serum[5] and urine[6]. Isotachophoresis has been employed in the analysis of nucleotides in muscle extracts[7] and urinary purines and pyrimidines, including uric acid[8].

Unlike other available methods, such as the colorimetric[9] and the enzymatic[3,4] methods, the determination of uric acid by HPLC and isotachophoresis is much less hampered by interfering substances, such as drugs and biological metabolites. In contrast to HPLC, where generally the proteins have to be removed by an appropriate sample pretreatment, isotachophoresis requires no sample pretreatment. Moreover the ratio of free to protein urate can be determined conveniently using a simple ultrafiltration procedure.

6.1. EXPERIMENTAL

In this study the isotachophoretic coupled column system, Part 2 Chapter 3, was used. This system is of special interest for the analysis of biological samples. In such samples separands are often present in a relatively low concentration and the concentration of other solutes can differ by an order of magnitude. This is the case in serum, for example, where the chloride concentration can exceed the concentration of uric acid by a factor of up to 500. In such mixtures the analysis time, required to obtain sufficient information, is rather large because a high sample load must be applied. The coupled column system alleviates this problem. The system comprised a preseparation capillary with an inner diameter of 0.8 mm and a final separation capillary with an inner diameter of 0.2 mm. The

driving current was taken from a Brandenburg, (Thornton Heath, UK) power supply and separated zones were detected using conductimetric and UV-detection systems[10]. Electrolyte systems and additional operational conditions are summarized in Table 6.1.

The enzymatic determinations of serum uric acid were performed in the laboratory of the Department of Neurology (University Hospital, Nijmegen) with an ABA 100 bichromatic analyzer (Abbot, UK). The determination of uric acid is based on the successive action of three purified enzymes which are added to the reaction mixture: uricase, catalase and aldehyde dehydrogenase[4]. The formation of NADPH from $NADP^+$ in the latter reaction, measured at both 340 and 380 nm) is used for the quantification of uric acid.

TABLE 6.1

ELECTROLYTE SYSTEMS AND OPERATIONAL CONDITIONS

	preseparation compartment	separation compartment	terminating compartment
Anion	Chloride	Chloride	MES
Concentration	0.01 M	0.01 M	0.005 M
Counter Const.	EACA	EACA	TRIS
pH	5.00	5.00	6.5
Additive	0.25% HEC	0.25% HEC	--
Driving current	250 µA	20 µA	

Serum was prepared from venous blood after clotting, 2h at room temperature, and centrifugation for 10 min at 1000 g at 4 ^{O}C. The samples were stored at - 20 ^{O}C until use. All chemicals used were of analytical grade or purified by conventional methods. Ultrafiltration CF 25 centriflow filters, nominal mol. wt. cut-off 25.000, were purchased from Amicon (Oosterhout, The Netherlands).

6.2. RESULTS AND DISCUSSION

Physiological uric acid serum concentrations normally range between 0.15 and 0.45 mM. The calibration line for uric acid in the given operational system proved to be

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linear and had a response of 0.0474 nmole urate per sec.
The identity of uric acid was confirmed in several ways.
In Fig. 6.1 an experiment is shown which demonstrates that
the uric acid zone is completely abolished by pre-incuba-
tion of the sample with purified uricase[11]. Moreover in-
jection of an additional amount of uric acid increased the
zone-length of the urate zone, whereas its isotachophore-
tic mobility, as measured by the conductimetric step
height, remained constant.
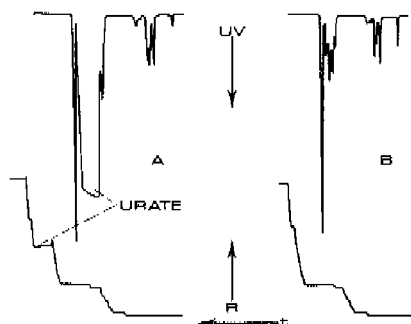
*Fig. 6.1 Effect of incubation of serum with purified uri-
case.*
A. Serum ultrafiltrate before incubation,
B. Serum ultrafiltrate after enzymatic incubation.

When standard solutions of uric acid were added to
serum, extensively dialysed against 0.9% NaCl solution,
the isotachophoretic analyses yielded recoveries of 99.0 –
100.5%. From this it can be concluded that the uric acid
protein binding is broken during the isotachophoretic ana-
lysis. Some serum samples showed turbidity, as judged from
visual inspection. Those samples were rapidly passed
through a Millipore filter, Millex®, 0.22 μm. The recovery
of uric acid was not affected by this procedure.

Serum samples from six healthy controls were assayed
for uric acid using the isotachophoretic and the enzymatic
procedure. The samples were used either directly or after
ultrafiltration. The serum uric acid values obtained with
the enzymatic and the isotachophoretic procedure showed

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an acceptable correlation with a correlation coefficient
of 0.980. The uric acid determinations in the ultrafilte-
red samples showed also an acceptable correlation, corre-
lation coefficient 0.976, but the group mean of the enzyma-
tic method was somewhat lower than that of the isotacho-
phoretic method. The precision of both methods for a re-
peatedly tested sample was better than 2%. The isotacho-
phoretic and the enzymatic procedures showed day-to-day
variations of less than 2% and 6% respectively.

TABLE 6.2

ISOTACHOPHORETIC AND ENZYMATIC RESULTS FROM SIX CONTROLS

No	Isotachophoresis			Enzymatic		
	NF	UF	Bound	NF	UF	Bound
	μM	μM	%	μM	μM	%
1	374	329	12	390	303	22
2	392	282	28	383	283	26
3	294	224	24	292	233	20
4	483	415	14	483	400	17
5	361	298	17	375	317	15
6	463	385	17	498	365	27
mean	395	332	19	403	317	21

NF is non ultrafiltered, UF is ultrafiltered.

The recoveries were not affected by the use of the CF-25
ultra filter membranes; recoveries of standard solution
using the ultrafiltration membrane were 99.4%.
From the table 6.2 it follows that protein binding varies
between twelve and twenty-eight percent. The physiological
significance of urate binding to plasma protein binding is
still disputed. Reversible interactions between urate and
serum albumin, low-density-lipoprotein, β_2-macroglubulins
and α_1, α_2 globulins have been reported[12,13].
Percentages of 20-40% of bound urate have been described
under different conditions of temperature, ionic strength,
buffers, etc.[12,13,14]. Our values agree with these data.
Reduced binding capacity of plasma proteins might lead to
higher levels of free uric acid; in patients with gout
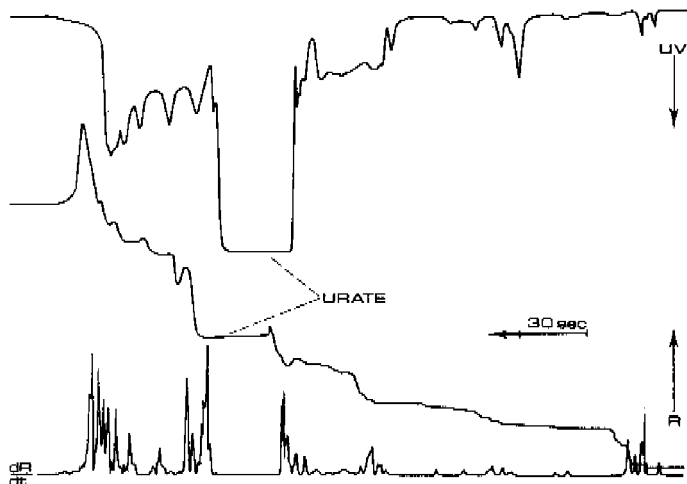
Fig. 6.2 Isotachophoretic separation of a hyperuricemic serum. R = increasing resistance, UV = UV transmission at 280 nm, t = time, $\frac{dR}{dt}$ = differential of the linear conductimetric trace.

such a decreased binding capacity has been reported[15]. It has also been shown that several drugs, such as salicylates, phenylbutazone and probenecid, reduce urate binding in vitro[16]. It should be stressed however, that all these studies, including our own were done under non-physiological conditions. Therefore, no conclusion can be drawn regarding the physiological significance, especially because some in vivo measurements have shown that at 37 $^{\circ}$C the percentage of urate bound is small[17].

TABLE 6.3

THE EFFECT OF HOMOGENTISIC ACID ON THE URIC ACID DETERMINATION

Addition	Isotachophoresis	Enzymatic
None	348 µM	348 µM
0.5 g/l homogentisic	348 µM	364 µM
5.0 g/l homogentisic	346 µM	676 µM

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Several metabolites can interfere with the enzymatic method for the determination of uric acid in serum. An example is homogentisic acid, a compound which occurs in increased quantities in urine of patients with alkaptonuria, in inborn error of amino acid metabolism[18,19]. It interferes with the enzymatic uric acid determination at 340 nm by giving lower values than those actually present. Even when unphysiologically high amounts of homogentisic acid were added to serum samples, no effect on the isotachophoretic determinations was seen. Increased levels however were found with the enzymatic procedure carried out with the bichromatic (340 and 380 nm) analyzed ABA-100. No attempts were done to elucidate this experimentally, but the differences might be attributed to the use of the bichromatic analyzer, in contrast to a monochromatic determination at 340 nm only[4].

TABLE 6.4
SERUM URIC ACID CONCENTRATIONS IN PATIENTS RECEIVING VARIOUS DRUGS

Diagnosis	Medication	Uric Acid µM	
		Enzymatic	Isotachophoresis
Gout (male, age 63)	Zyloric	286	299
Rheumatoid arthritis with hyperuricemia (female, age 68)	Hygroton, Selokene Penicillinamide, Indocid Seresta	620	647
Rheumatoid arthritis with hyperuricemia (female, age 44)	Baktrimel, Primtison, Torecon	356	366

Serum uric acid values in three initially hyperuricemic rheumatologic patients, who were treated with a number of drugs, were in close agreement when determined with both procedures. None of the drugs seemed to interfere with the uric acid determination both procedures.

6.3. CONCLUSIONS

The isotachophoretic procedure for the determination of uric acid in serum is quantitative, reliable and reproducible. In contrast to the general practice in HPLC there is no need for deproteinisation of the serum samples. Once the electrolyte system has been optimized for the isotachophoretic determination, analyses can be done with a low day-to-day variation. At present the isotachophoretic method is more accurate than the enzymatic method, although the latter is faster when automated.

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Abbreviations of chemical substances

A3MP	adenosine 3'-monophosphate
A5MP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BALA	β-alanine
CREAT	creatinine
EACA	ε-aminocaproic acid
HEC	hydroxyethyl cellulose (Polysciences Inc, Warrington Pa, USA, Cat. no 5568)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIST	L-histidine
GABA	γ-amino-n-butyric acid
G5MP	guanosine 5'-monophosphate
MES	2(N-morpholino)ethane sulfonic acid
MM	middle molecule
MOWIOL	polyvinylalcohol (N 8-88 Hoechst A.G., Frankfurt, GFR)
TRIS	tris(hydroxymethyl)aminomethane
U	Urea

Symbols

	linescript	superscript	subscript	designation
A	o			ionic species, constituent
A	o			UV absorption
A		o		zone indicator
A			o	constituent indicator
a	o			constant
α	o			degree of dissociation
α	o			constant
B	o			ionic species, constituent

```
        linescript
        ↓ superscript
          ↓ subscript
            ↓
```

	linescript	superscript	subscript	
B	o			zone indicator
B		o		constituent indicator
b	o			constant
β	o			constant
C	o			load capacity
C	o			ionic species, constituent
C		o		zone indicator
C			o	constituent indicator
c	o			constant
c	o			subspecies concentration ($geq.cm^{-3}$)
$\bar{c}$	o			constituent concentration ($mole.cm^{-3}$)
γ	o			constant
D	o			diffusion coefficient ($cm^2.sec^{-1}$)
d	o			diameter (cm)
$\Delta\ell_o$	o			sampling width (cm)
Δt	o			time-based zone length (sec)
δ	o			zone width (cm)
E	o			electric field strength ($V.cm^{-1}$)
F	o			constant of Faraday ($C.geq^{-1}$)
φ	o			sampling ratio
g	o			relative gain in time
H	o			high molecular weigth region
H	o			heigth equivalent to a theoretical plate (cm)
HL	o			HL ratio
I	o			electric driving current (A)
I	o			ionic species, constituent
I		o		zone indicator
I			o	constituent indicator
i	o			constituent
i		o		constituent indicator
J	o			electric current density ($A.cm^{-2}$)

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Symbol				Description
J	o			ionic species, constituent
J		o		zone indicator
J			o	constituent indicator
j	o			constituent
j			o	constituent indicator
K	o			equilibrium constant
		o		zone indicator
k	o			constant
κ	o			specific conductance $(\Omega^{-1}.cm^{-1})$
L	o			column length (cm)
L	o			low molecular weight region
L	o			leading constituent
L		o		zone indicator
L			o	constituent indicator
ℓ	o			zone length
M		o		mixed zone indicator
MO		o		mixed zone indicator
m	o			ionic mobility $(cm^2.v^{-1}.sec^{-1})$
$\bar{m}$	o			effective mobility $(cm^2.v^{-1}.sec^{-1})$
N	o			number of theoretical plates
n	o			amount
n	o			sample load (mole)
n	o			subspecies
O	o			cross-sectional area (cm^2)
pH	o			pH
pI	o			isoelectric point
pK	o			negative logarithm of the protolysis constant
Π	o			product function
Q	o			amount of electric charge (C)
R	o			Resolution
R	o			resistance (Ω)
r	o			relative mobility
$\bar{r}$	o			relative effective mobility

	linescript	superscript	subscript	
ρ	o			relative leading concentration
S	o			separation number
S	o			separand
S		o		zone indicator
S			o	constituent indicator
Σ	o			summation
σ	o			standard deviation
T	o			transference number
T	o			terminating constituent
T		o		zone indicator
T			o	constituent indicator
t	o			time (sec)
t_{det}	o			time of detection (sec)
t_g	o			gain in time (sec)
t_{gr}	o			time-based centre of gravity
t_r	o			retention time (sec)
t_{res}	o			time of resolution (sec)
t_{stop}	o			time of detection (sec)
UV	o			Uv absorption
v	o			migration velocity (cm.sec^{-1})
x	o			place coordinate
x_{det}	o			length of detection (cm)
x_{max}	o			maximal migrated distance (cm)
x_{min}	o			minimal migrated distance (cm)
x_{res}	o			length of resolution (cm)
z	o			valency
ω	o			Kohlrausch function
*		o		indicator sampling compartment
**		o		indicator terminator compartment

Summary

Electrophoresis can be used as a method for achieving separation of ionic solutes in solution and is based on the differential migration of the separands in an electric field. This thesis describes the theoretical, experimental and instrumental developments of zone electrophoresis and isotachophoresis in narrow bore tubes.

Electrophoresis inherently involves the local change of electrolyte constituent concentrations. This changing of concentrations is not an arbitrary process, but is a strongly regulated one,governed by the Kohlrausch regulating functions. This Kohlrausch concept has been applied to zone electrophoresis and isotachophoresis and allows a fundamental understanding of the capabilities of both analytical techniques.

In zone electrophoresis the effect of electrophoretic migration on the concentration distributions is evaluated, using a non-diffusional model. It follows that the sample constituents, that have an effective mobility larger than that of the carrier constituent, migrate with concentration distributions that are diffuse at the front and sharp at the rear of the zone. The reversed holds for sample constituents that have an effective mobility lower than that of the carrier constituent. As a result of the regulating functions no independent retention behaviour can exist in zone electrophoresis. In an experimental approach to high performance zone electrophoresis it is shown that non migrational dispersion can be well controlled by the use of narrow bore tubes. The measured concentration distributions conform with theory and their asymmetry can only be suppressed by the application of very small amounts of sample. High performance zone electrophoretic separations, using UV absorbance and conductimetric detection, are shown. The theoretical and practical limitations of zone electrophoresis are indicated.

The initial stage of zone electrophoresis is essential-
ly not different from moving boundary electrophoresis or the
separation process in isotachophoresis. The latter is eluci-
dated using a transient-state model for monovalent weakly
ionic constituents. The influence of operational parameters,
such as pH, electric driving current, sample load and coun-
ter constituent, is described in terms of time of resoluti-
on, load capacity and current efficiency. Theoretically and
experimentally it is shown that optimization procedures in
isotachophoresis are governed by three rationales: the pH,
the counter constituent and the electric driving current.
For anionic separations a low pH of both the leading electro-
lyte and the sample will favour resolution, whereas for cat-
ionic separations a high pH will be preferable. The delete-
rious effects of extreme values of pH are discussed. The
counter constituents should have a low mobility and the elec-
tric driving current should be maximized. Steady-state con-
figurations, in which the sample constituents are not migra-
ting in order of mobility, are shown and discussed.

The time-based zone length is in isotachophoresis di-
rectly related to the amount of sample. To avoid column-
overloading only a limited sample load can be applied in
conventional instruments. A new two-dimensional, fully auto-
mized, electrophoretic instrument is described, which allows
the application of high sample loads, two dimensional sepa-
rations and feasibility for micro-preparative applications.

Both the theoretical concepts and instrumental develop-
ments were applied to several biomedical research topics.
In the search for uremic toxins, isotachophoresis was used
for the determination of ionic solutes in the body fluids of
patients with terminal chronic renal failure. It is shown
that uremic patients form a rather heterogenic group and that
hemodialysis removes only partially the accumulating meta-
bolic products from the body fluids of patients with chronic
renal failure. The existence of toxic molecules with inter-
mediate molecular weight, the so-called middle molecules,
which are supposed to accumulate in chronic renal failure,
could not be substantiated with isotachophoresis, gas chro-

matography or high performance liquid chromatography. The
middle molecular weight fractions, as obtained with gel chro-
matography, proved to contain numerous solutes of rather low
molecular weight.
Isotachophoresis was successfully applied for the direct de-
termination of the anticonvulsant drug valproate in serum
and for the determination of uric acid in serum.

Samenvatting

Electroforese wordt veelal toegepast als analytische scheidingsmethodiek voor ionogene stoffen. Dit proefschrift beschrijft zowel de theoretische achtergronden als de instrumentele ontwikkelingen van zone electroforese en isotachoforese in capillaire kolommen.

Gedurende een electroforetisch proces treden, plaatselijk, veranderingen in de electroliet concentratie(s) op. Dit proces verloopt niet willekeurig, maar zeer stringent gereguleerd, hetgeen beschreven kan worden met behulp van de zgn. Kohlrausch functies. Hierdoor kan een inzicht verkregen worden in de analytische mogelijkheden van zowel zone electroforese als isotachoforese.

Uitgaande van een zone electroforetisch model, waarin de invloed van diffusie verwaarloosd wordt, blijken asymmetrische concentratieverdelingen op te kunnen treden. Ionogene componenten, met een effectieve mobiliteit groter dan die van de drager, blijken een asymmetrische concentraverdeling te hebben, die aan de voorkant diffuus is terwijl de achterkant scherp blijft. Mutatis mutandis geldt voor ionogene componenten met een kleinere effectieve mobiliteit dan die van de drager. Doordat het zone electroforetische proces zeer stringent gereguleerd verloopt, wordt het retentiegedrag van vrijwel iedere ionogene component beinvloed door de aanwezigheid van andere. Dispersie kan in zone electroforese geminimaliseerd worden door deze techniek uit te voeren in capillaire kolommen. De experimenteel gemeten concentratieverdelingen bevestigen de juistheid van de theoretische berekeningen. De asymmetrie van de verdelingen kan slechts onderdrukt worden door het inbrengen van zeer geringe hoeveelheden monster. Zone electroforetische scheidingen, waarbij gebruik gemaakt wordt van zowel conductometrische als UV-absorptie detectie, tonen de praktische en theoretische beperkingen van deze techniek.

Het scheidingsproces van zone electroforese en isota-
choforese verlopen beide volgens het bewegende grensvlak
principe. Het scheidingsproces van isotachoforese wordt be-
schreven in een mathematisch model, dat toepasbaar is bij
de analyse van eenwaardige zwakke electrolieten. Uit de mo-
delbeschouwingen volgt dat de optimalisering van een isota-
choforetisch scheidingsproces beheerst wordt door de pH van
de electrolieten, de aard van het tegenion en de toegepaste
stroomsterkte. De invloed van deze, experimenteel belang-
rijke, parameters komt tot uitdrukking in de resolutietijd,
de beladingscapaciteit en het stroomrendement. De resolutie-
tijd is omgekeerd evenredig met de electrische stroomsterkte,
zodat deze laatste gemaximaliseerd dient te worden.
Bij scheidingen van anionen worden de optimale condities
verkregen bij een lage pH van zowel loopelectroliet als mon-
ster. In het geval van de scheiding van cationen verdient
een hoge pH de voorkeur. De tegenionen dienen altijd een
kleine mobiliteit te bezitten. Zowel theoretisch als expe-
rimenteel wordt aangetoond, dat de ionogene componenten van
een monster zich bij isotachoforese niet altijd rangschikken
in volgorde van effectieve mobiliteit.

De hoeveelheid ingebrachte stof is bij isotachoforese
direct gerelateerd aan de zone-lengte. Teneinde grote hoe-
veelheden van een monster te kunnen analyseren werd een nieuw
tweedimensionaal instrument ontwikkeld. De voordelen van dit
volledig geautomatiseerde instrument ten opzichte van conven-
tionele apparatuur worden beschreven.

Zowel de theoretische inzichten als de instrumentele
ontwikkelingen werden toegepast op diverse onderwerpen uit
het biomedisch geliëerd onderzoek.
Isotachoforese blijkt geschikt te zijn voor de analyse van
ionogene stoffen in de lichaamsvloeistoffen van chronische
nierpatienten. Uit de isotachoforetische analyses blijkt
dat nierpatienten, wat betreft de accumulerende stoffen,
een inhomogene groep vormen. De stoffen, die in de lichaams-
vloeistoffen van chronische nierpatienten accumuleren, wor-
den slechts ten dele door hemodialyse verwijderd.
De " middle molecule " hypothese kon niet bevestigd worden

met behulp van isotachoforese, gaschromatografie of vloei-
stofchromatografie.

Isotachoforese werd met succes gebruikt voor de directe be-
paling van het geneesmiddel valproinezuur in het serum van
patienten met epilepsie. De resultaten werden vergeleken met
die van een standaard gaschromatografische analysemethode.
De isotachoforetische bepaling van urinezuur in serum bleek
goed in overeenstemming te zijn met de resultaten van een
enzymatische bepalingsmethodiek.

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From all I learned something, from a few a learned a lot,
only few may have the opportunity to teach me something in
future.

Curriculum vitae

The author was born on november 16th 1947 in Valkenswaard, the Netherlands. After graduating from the " Gymnasium Augustinianum " he started his studies in chemical engineering at the Eindhoven University of Technology (Eindhoven, the Netherlands) in 1967. The graduation work was carried out at the Laboratory of Instrumental Analysis and he obtained his "ir" degree, cum laude, in 1974.

After a short but sharp carreer in military service, he rejoined the research group on electrophoresis at the Eindhoven University of Technology. In november 1976 the author started the research described in this thesis.

Mane de Tabula

STELLINGEN

1. De veronderstelling dat bij zone electroforese de beste
    scheidingscondities verkregen worden wanneer het verschil
    van de beweeglijkheden van de te scheiden monstercompo-
    nenten maximaal is *, lijkt aantrekkelijk maar is onjuist.

    * R. Consden, A.H. Gordon en A.J.P. Martin, Biochem. J.,
      40 (1946) 33.

2. Het scheidingsproces bij isotachoforese wordt bepaald
    door de condities in de mengzone(s). Getabelleerde iso-
    tachoforetische beweeglijkheden * geven slechts een in-
    dicatie of monstercomponenten al dan niet met behulp
    van isotachoforese gescheiden kunnen worden.

    * F.M. Everaerts, J.L. Beckers en Th.P.E.M. Verheggen,
      Isotachophoresis, J. Chromatogr. Libr., Vol. 6,
      Elsevier, Amsterdam-Oxford-New York, 1976.

3. Wanneer bij de verklaring van de kathodische drift bij
    iso-electrische focussering geen rekening gehouden wordt
    met het feit dat de absolute waarden van de electrofo-
    retische beweeglijkheden van protonen en hydroxylionen
    niet gelijk zijn *, dient deze verklaring in twijfel te
    worden getrokken.

    * N.Y. Nguyen en A. Crambach, Anal. Biochem., 82 (1977) 226.
      B.A. der Lan en A. Crambach, Electrophoresis, 1 (1980) 23.

4. Chromatofocussering, als scheidingsmethodiek voor eiwit-
    ten, verdient meer aandacht.

    L.A.AE. Sluyterman en O. Elgersma, J.Chromatogr., 150
    (1978) 17.
    L.A.AE. Sluyterman en J. Wijdenes, J.Chromatogr., 150
    (1978) 31.

5. Het piekoppervlak uit een chromatogram, verkregen bij
    de gelchromatografische scheiding van uremisch serum,
    kan niet als kwantitatieve maat * dienen voor het voor-

komen van de zogenaamde "middle molecules" in uremisch
serum.

* L. Migone, P. Dall'Aglio en C. Buzo, Clin. Nephrol.,
  3 (1975) 82.
  J. Bergström en P. Fürst, Clin. Nephrol., 5 (1976) 144.
  J.L. Funck Brentano, G.F. Cueille en N.K. Man, Kidney
  Internat., 13 (1978) 31.

6. De uitspraak van Bergström * dat de zogenaamde "middle
   molecules" bestaan en dat deze biologisch actief zijn,
   moet als triviaal beschouwd worden.

   * J. Bergström, P. Fürst en L. Zimmerman, Clin. Nephrol.,
     11 (1979) 229.

7. Bij het gebruik van electro-osmotische vloeistofpompen
   dient men er rekening mee te houden, dat electro-osmose
   beinvloed wordt door de aanwezigheid van oppervlakte-
   actieve stoffen.

   * V. Pretorius, B.J. Hopkins en J.D. Schieke, J. Chro-
     matogr., 99 (1974) 23.
     Z. Ryslavy, P. Bocek, M. Deml en J. Janak, J. Chroma-
     togr., 147 (1978) 446.

8. De toename van het gebruik van mini- en microcomputers
   in het analytische laboratorium en de daaraan blijkbaar
   inherente verslaving, zal leiden tot een vermindering
   van de inzet op het eigen, d.w.z. analytische, vakgebied.

9. De opmerking van Schumacher * dat het proefschrift van
   Everaerts ** uitmunt door het niet refereren naar Schu-
   macher *** is niet juist.
   *   E. Schumacher en P. Ryser, Chimia, 30 (1976) 105.
   **   F.M. Everaerts, Proefschrift, Technische Hogeschool
         Eindhoven, 1968.
   ***   E. Schumacher en T. Studer, Helv. Chim. Acta, 18
         (1964) 957.

Eindhoven   28 october 1980   F.E.P. Mikkers