Analytical isotachophoresis

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7. Analytical isotachophoresis

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1. Introduction
   Electrophoretic separation techniques nowadays seem to have an inevitable connection with protein chemistry and chromatography. The early develop-
ments on electrophoresis, however, were in the field of low molecular weight substances and colloids. Moreover chromatography was at that time, i.e. the turn of the century, still in its infancy.

A very important discovery was made by Hardy [11] in 1899. He noticed that proteins may be either negatively or positively charged, depending on the acidity of the solution. It was, in fact, Michaelis [2] who revealed the potential strength of electrophoresis for the separation and characterization of proteins. Substantial experimental improvements in electrophoretic techniques were introduced by Svedberg and Tiselius [3] in 1926. The real importance of electrophoresis for protein chemistry was stimulated by the wonderful work of Tiselius [4], who in 1937 described in detail his moving boundary equipment, which won him the Nobel Prize. In spite of the fact that Tiselius was convinced of the general analytical applications of electrophoresis, the close connection between electrophoresis and proteins was established.

From the start however there was a problem which always worried research workers: stabilization in electrophoresis. In fact from this the connection between electrophoresis and chromatography emerged. It must be emphasized that the connection in many respects was very beneficial for electrophoresis.

After World War II chromatography developed in a rather spectacular way. Although Tswett [5] introduced liquid chromatography, it was Martin and Syng [6] who understood the requirements of the new analytical method for large-scale purposes. As a result gas chromatography underwent an explosive development; principally because many industrial processes could make direct use of it. The potential capabilities of liquid chromatography nevertheless were well understood, a reason why this technique has also contributed to the development of isotachophoresis as can be found in a book devoted to this subject [20].

As a result isotachophoresis has developed as a technique, which competes with chromatographic techniques.

Isotachophoresis is applicable in many fields, especially with low molecular weight ionic substances, due to its high resolution, short time of analysis, high accuracy and tremendous flexibility. It now seems that the general application of electrophoresis, as a separation technique, may be shown to its full advantage, and that electrophoresis deserves more general attention, than it has been given until now.

1.1. List of symbols

\[ a \] degree of dissociation
\[ A \] constituent to be separated
\[ B \] constituent to be separated
\[ \dot{c} \] constituent concentration (mol cm\(^{-3}\))
\[ c \] sub-species concentration (equiv cm\(^{-3}\))
\[ C \] counter constituent
\[ D \] diffusional coefficient (cm\(^2\) sec\(^{-1}\))
\[ E \] electrical field strength (V cm\(^{-1}\))
\[ F \] Faraday constant (C equiv\(^{-1}\))
\[ i, j \] constituent, sub-species
\[ J \] electrical current density (A cm\(^{-2}\))
\[ k \] electrical specific conductance (\(\Omega^{-1}\) cm\(^{-1}\))
\[ k \] reduced mobility
\[ K \] dissociation constant
\[ L \] leading constituent
\[ l \] zone length (cm)
\[ m \] mobility (cm\(^2\) V\(^{-1}\) sec\(^{-1}\))
\[ n \] effective mobility (cm\(^2\) V\(^{-1}\) sec\(^{-1}\))
\[ n \] sub-species, A, B, C, L, T
\[ n \] amount of constituent (mol)
\[ O \] area (cm\(^{2}\))
\[ pK \] negative logarithmic transform of \(K\)
\[ \phi \] sampling ratio
\[ \gamma \] molar sampling ratio
\[ r \] relative mobility
\[ R \] resolution
\[ \rho \] relative leading concentration
\[ S \] separation number
\[ T \] terminating constituent
\[ t \] time coordinate (sec)
\[ t_{res} \] time of detection (sec)
\[ t_{det} \] time of resolution (sec)
\[ t_{def} \] running time (sec)
\[ v \] linear velocity (cm sec\(^{-1}\))
\[ \lambda \] place coordinate (cm)
\[ \lambda_{le} \] length of resolution (cm)
\[ \lambda_{de} \] length of detection (cm)
\[ \lambda_{def} \] running length (cm)

Subscripts
\[ i, j, n \] A, B, L, T, C constituent, species indicator

Superscripts
\[ K \] A, B, L, T, M (mixed) zone indicators

* The development of isoelectric focusing, immunoelectrophoresis and disc-electrophoresis is not discussed in this chapter, although to a large extent these techniques contributed to the separation of proteins.
In isotachophoresis, a steady-state configuration is obtained as the result of a separation process that proceeds according to the moving boundary principle (cf. Chapter 1). Although this separation process is a transient state, it is governed by the same regulating function concept as the steady state. Quantitative and qualitative descriptions of the transient state provide information on the time needed for an isotachophoretic separation [20, 21]. Moreover, such a description requires the definition of resolution in isotachophoresis and shows the results that can be expected from optimization procedures.

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

\[
v_i = \bar{m}_i E
\]  

(1)

The electrical field strength is vectorial so the effective mobilities can be taken as charged 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 sub-species in rapid equilibrium, the effective mobility represents an average ensemble. Ignoring 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, \( \bar{c}_n \), is given by the summation of all of the subspecies concentrations, \( c_n \):

\[
\bar{c}_i = \sum_n c_n
\]  

(2)

Following the mobility concept of Tiselius [4], the effective mobility is given by

\[
\bar{m}_i = \sum_n c_n m_n \bar{c}_i
\]  

(3)

where \( m_n \) is the ionic mobility of the subspecies. In dissociation equilibria the effective mobility can be evaluated using the degree of dissociation, \( a \)

\[
\bar{m}_i = \sum a_i m_n
\]  

(4)

The degree of dissociation can be calculated once the equilibrium constant, \( K \), for the subspecies and the pH of the solution are known. For a restricted pH range a very useful relationship has been given by the Henderson Hasselbalch equation [22, 23]

\[
pH = pK_a \pm \log \left( \frac{1}{a} - 1 \right)
\]  

(5)

where \( pK_a \) is the negative logarithm of the protolysis constant; the positive sign holds for cationic subspecies.

All electrophoretic processes are essentially charge-transport processes that obey Ohm's law. In electrophoresis this law is most conveniently expressed in terms of electrical current density, \( J \), specific conductance, \( k \), and electrical field strength, \( E \):

\[
J = k E
\]  

(6)

The specific conductance is given by the individual constituent contributions

\[
k = F \sum_i \bar{c}_i \bar{m}_i
\]  

(7)

where \( F \) is the Faraday constant.

The equation of continuity states for any electrophoretic process that

\[
\frac{\partial}{\partial t} \bar{c}_i = -\frac{\partial}{\partial x} \left( \frac{\bar{c}_i}{\bar{c}_i} D \bar{c}_i - v_i \bar{c}_i \right)
\]  

(8)

where \( t \) and \( x \) are time and place coordinates, respectively, and \( D \) is the diffusion coefficient. Neglecting diffusional dispersion we can apply equation (8) for each constituent and the overall summation of the constituents gives

\[
\frac{\partial}{\partial t} \sum \bar{c}_i = \frac{E}{\bar{c}_i} E \sum \bar{m}_i \bar{c}_i
\]  

(9)

In combination with the specific conductance (equation 7) and modified Ohm's law (equation 6), it follows that
For monovalent weakly ionic constituents, equation (8) can be written as

\[ \frac{\partial}{\partial t} \sum_i c_i = 0 \quad \text{or} \quad \sum_i \frac{\partial c_i}{\partial t} = \text{constant} \]  

(10)

where \( m_i \) and \( c_i \) are the mobility and the concentration of the charged species \( i \). Division by \( m_i \) and application of the resulting relationship for each constituent, gives for overall summation

\[ \frac{\partial}{\partial t} \sum_i \frac{c_i}{m_i} = \frac{\partial}{\partial x} E \sum_i c_i \]  

(11)

Electroneutrality, however, demands \( \sum_i c_i = 0 \), so

\[ \frac{\partial}{\partial t} \sum_i \frac{c_i}{m_i} = 0 \quad \text{or} \quad \sum_i \frac{\partial c_i}{\partial t} = \text{constant} \]  

(12)

Equation (13) is well known as the Kohlrausch regulating function [4]. In an electrophoretic system different zones can be present, in which a zone is defined [23, 24] as a homogeneous solution demarcated by moving and/or stationary boundaries. We can apply the continuity principle (equation 8) to a boundary and derive the general form of the moving boundary equation [13].

\[ \tilde{m}_i \tilde{c}_i \tilde{E}_i - \nu^{K+1} \tilde{c}_i \tilde{E}^{K+1} = \nu^{K+1} \tilde{E}_i \]  

(14)

where \( \nu^{K+1} \) 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 equation (14) reduces to

\[ \frac{\tilde{m}_i^{K+1} \tilde{c}_i^{K+1}}{\tilde{m}_i^{K+1} \tilde{E}_i^{K+1}} = \frac{\tilde{E}_i^{K}}{\tilde{E}_i^{K+1}} = \text{constant} \]  

(15)

From equation (15) it follows directly that for monovalent weak and strong electrolytes all ionic subspecies are diluted or concentrated over a stationary boundary to the same extent, because

\[ \frac{\tilde{m}_i}{\tilde{m}_j} = \frac{\tilde{E}_i^{K}}{\tilde{E}_j^{K+1}} = \text{constant} \]  

(16)

In isotachophoresis, sample constituents migrate in a stacked configuration under a steady-state condition, between a leading ionic constituent of high effective mobility and a terminating constituent of low effective mobility.

From the moving boundary equation (equation 9) it follows directly that, in a separation compartment of uniform dimensions at constant electrical driving current, all boundary velocities within the isotachophoretic framework are equal and constant. According to Joule's law, heat generation will occur, resulting in different regions that are moving or stationary. In order to reduce the effects of temperature, relative mobilities, \( r \), can be introduced. Obviously the leading ion constituent, \( L \), provides the best reference mobility

\[ r_i = \frac{m_i}{m_L} \]  

(17)

Moreover, as in most isotachophoretic separations, for simplicity, only one counter-ion constituent, \( C \), will be present, the reduced mobility, \( k \), can be introduced

\[ k_i = \frac{1 - r_i}{r_i - r_c} \]  

(18)

Using the derived equations it is possible to calculate all dynamic parameters of analytical importance [21]. Moreover, the model considerations can be extended to moving boundary electrophoresis as well as zone electrophoresis.

2.2. The criterion for separation

As in all differential migration methods, the criterion for separation in isotachophoresis depends simply on the fact that two ionogenic constituents will separate whenever their migration rates in the mixed state are different. For two constituents \( i \) and \( j \), this means that according to equation (1) their effective mobilities in the mixed state must be different

\[ \frac{\tilde{m}_i}{\tilde{m}_j} = \frac{\tilde{E}_i^{K}}{\tilde{E}_j^{K+1}} = \text{constant} \]  

(19)

When the effective mobility of \( i \) is higher than that of \( j \) the latter constituent will migrate behind the former. Consequently, two monovalent weakly
anionic constituents will fail to separate [21] when the pH of the mixed state, 
$pH^{m}$, is given by

$$pH^{m} = pH^{m} = pK_j + \log \left( \frac{l - \frac{r_j K_i}{r_i K_j}}{\frac{r_i}{r_j} - l} \right)$$

(20)

where $K_i$ and $K_j$ are the protolysis constants for the subspecies of the 
constituents $i$ and $j$. When the more mobile constituent has a higher protolysis 
constant, we have a ‘straight’ pair of constituents; when the more mobile 
constituent has a lower protolysis constant, we have a ‘reversed’ pair of 
constituents, for which the separation configuration is a function of the pH.

2.3. Resolution

Once the criterion for separation has been satisfied, the time needed for 
resolution becomes important. When a constituent 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

$$R_i = \frac{\text{separated amount of } i}{\text{sampled amount of } i}$$

(21)

From the definition, it follows that during the separation process the 
resolution increases from zero to its value, unity. Constituents that fail to 
separate remain at zero resolution and can be termed ideally mixed zones [25].

Complete separation of a sample requires the resolution values of all 
constituents of interest to be unity. Maximum speed of separation is obtained 
whenever the resolution rate, $\partial R_i/\partial t$, is optimized during the separation 
process. As expected, the resolution and its time derivatives 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 
sample constituents. For strong electrolytes relevant mathematical 
formulations have been published [26, 27], but most separations nowadays 
[26, 27] are concerned with weak electrolytes. In this case dissociation 
equilibria, and therefore a proper choice of pH, are tools in the control and 
optimization of separation process. When dealing with complex formation, 
association equilibria should be involved. Others [15] have suggested that the 
difference in migration rates, e.g., $v_i - v_j$, 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, e.g., $v_i/v_j$. Whereas the velocity difference will reach a 
maximum value as a function of pH [28], the ratio shows no such optimum 
[21, 22]. As the local electrical field strength for both constituents will be the 
same, it follows directly that equation (19) must be maximized or minimized, 
depending on the migration configuration. By introducing equilibrium 
constants and ionic mobilities it follows that in anionic separations the lowest 
 pH will give the better mobility ratio, and vice versa for cationic separations. 
It should be emphasized, however, that pH extremes have only limited 
experimental application and that practical considerations often govern the 
proper choice of pH. Moreover, a low numerical value of the effective mobility 
will induce a high electrical field strength in order to obtain an appreciable migration rate and then other electrokinetic effects may prevail.

2.4. Steady state

A unique feature of isotachophoresis is that, once the separation process has been 
completed, all electrophoretic parameters remain constant with time. Assuming 
a uniform current density, all sample constituents within the leading-
terminating electrolyte framework will migrate at identical speeds. Moreover, 
at constant current density local migration rates will be constant. In this 
steady state, resolution values of stacked constituents will be either unity or zero. The basic features of steady-state configurations have been discussed 
[19, 20].

2.5. The separation process

The application of the above equations and definitions and the resulting 
implications are best illustrated by using a relatively simple two-component 
sample. We shall deal with the case where all constituents involved are 
monovalent weak electrolytes. Although essentially immaterial, we shall 
consider a separation compartment of uniform dimensions at a constant 
electrical driving current and a constant temperature. The separation process 
and some relevant information are given in Fig. 1. Full information about this 
separation will be given later.

It should be emphasized that within the separator three different regions are 
present and each has its own regulating behaviour. The regulating functions 
(equations 10 and 13) are the mathematical expression of this regulating 
behaviour and locally they cannot be over-rulled by the electrophoretic 
process [10]. All changes in electrophoretic parameters, e.g. concentration,
At $\rho = -1$ the counter ion constituent is used far below its $pK$ value and it behaves like a strongly ionic species. In this event the leading electrolyte has no buffering capacity. At $\rho = -2$ the counter ion constituent is used at its $pK$ value, $pH^L = pK_n$, and therefore it exhibits its full buffering capabilities. High negative values for the relative leading ion concentration again implies low buffering. Moreover, the concentration of the counter ion constituent will be high in comparison with that of the leading ion constituent, which can be favourable in complex formation. It is easily shown that for increasing $pH^L - pK_{i,j}$, i.e. the constituents to be separated are only partially dissociated at the pH of the leading electrolyte, $pH^L - pH^L$ will increase. Constituents that are 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 ion constituents with a low $pK$ value in comparison with the pH of the leading electrolyte show a tendency to diminish this increase in $pH^M$. When the leading electrolyte is a strongly ionic species the pH of any following zone will be higher than the pH of the leading zone. If, however, a weak electrolyte is chosen as the leading ion constituent, negative pH steps can occur under appropriate conditions [20].

2.7. Time of resolution and length of resolution

Resolution has been defined as the separated fractional amount of the constituent under investigation. Maximum resolution, $R = 1$, is obtained whenever the constituent zone contains all of the sampled amount $n$. From Fig. 1 it can be concluded that the time for resolution of the boundary velocities $v_{L/A}$ and $v_{A+(A+B)}$

$$t_{res} = \frac{I_A}{v_{L/A} - v_{A+(A+B)}}$$  (23)

The time of resolution is a complex function [21, 22, 29] of the concentration and the pH of both the leading electrolyte and the sample, of the sampled amount, the sampling ratio, the electric driving current and all ionic mobilities and dissociation constants involved. No attention will be paid here to the mathematical formulae, because it is out of the scope of this book [20].

For a given sample and electrolyte system, the resolution length is independent of the applied current density or electrical field strength, whereas the time of resolution is inversely related to the electrical driving current. From the resolution length the load capacity of the column can be deduced [21, 22]. Obviously, a high load capacity is always favoured by a low resolution time.
More information about, e.g. resolution, resolution rate, ease of separation, fixed-point detectors, etc. can be found in [21, 22, 29].

2.8. Discussion

In a steady state, constituent ions will generally migrate in order of their effective mobilities, i.e. the effective mobilities decrease from leading to terminating electrolyte. In special cases, however, a constituent ion with low effective mobility can migrate isotachophoretically in front of a constituent ion with a relatively high effective mobility. Such separation configurations have been called 'enforced isotachophoresis' [20] and are stable with respect to time. It follows that measurement of the step heights of single constituent ions gives only an indication of the separation configuration for a mixture of constituent ions. Moreover, it has been shown that, depending on pH, constituent ions can migrate in a different steady-state configuration. The importance of the pH of the leading electrolyte in this respect has been extensively discussed [20–22, 29]. In Fig. 2 a mixture of anions isotachophoretically separated in an apparatus equipped with both a conductivity detector and a uv detector, is shown. Some 'abnormalities' that can be found during isotachophoretic analyses are shown.

It is generally assumed that the nature of the sample, especially its pH and concentration, has no influence on the steady state. Dealing with a reversed pair of sample constituent ions near the critical pH of the leading electrolyte, at which a reversal of order can occur, the pH of the sample may, theoretically, be the deciding factor. Hence it must be concluded that the steady-state characteristics of the zone are not influenced by the pH of the sample, but this can affect the separation configuration. However, in practice this will not generally occur. It is obvious that, for constituent ions separating very slowly, it will be difficult to conclude whether the steady state has been reached or not. This holds especially for complex mixtures, such as natural protein mixtures in which the numerous constituent ions, each with possible microheterogeneity, may give rise to a continuous mobility spectrum. Such complex mixtures require a relatively long separation time. Obviously the use of spacers for such samples, whether ampholytes or discrete substances, will decrease the efficiency of the separation process, but can increase the interpretability. Whenever possible the use of discrete spacers at low concentration is to be preferred.

In optimization procedures three rationales can be recognized which, of course, are not completely independent:

a. The electrical driving current acts directly on the time of analysis. As the time for resolution is inversely related to the electrical driving current, it is obvious that this operational parameter must be maximized. In

![Fig. 2. Isotachophoretic steady-state configurations. pH leading electrolyte, 3.95; chloride (0.01 M)-GABA; terminating electrolyte: C,H,COOH (ca. 0.05 M); additive: 0.05% PVA (N-88, Hoechst, Frankfurt, GFR). R, increasing resistance; A, uv-absorption at 254 nm; T, increasing time; I, 5-ATP; 2, sulphamic acid; 3, DL-mandelic acid; 4, DL-lactic acid; 5, 5-GMP; 6, 3-AMP; 7, 5-AMP; 8, acetic acid. * A pair of constituents for which conductimetric detection indicates no resolution, whereas uv detection does. ** A pair of constituents for which uv detection indicates no resolution whereas conductimetric detection does; *** A pair of constituents in an enforced [20] isotachophoretic configuration.](image)
practice this will mean that a compromise must be found between the quantitative and qualitative accuracy required and the possible driving current. The electrical driving current, if temperature effects are neglected, has no influence on the efficiency of the separation process, so the length of resolution, the location of the detection system and the load capacity are all independent of it. This is consistent with the fact that the current-time integral is important. In order to separate a given sample a definite number of coulombs are necessary but the time interval in which this amount must be delivered is immaterial. When performing isotachophoretic analyses, it is therefore not necessary to work at a constant electrical driving current. Using a fixed point detector, however, a constant electrical driving current greatly facilitates the interpretation of the isotachopherograms obtained. Further, the operating conditions are more easily standardized and better controlled.

b. The efficiency of the current transport is directly influenced by the mobility of the common counter ion constituent. The favourable effect of a counter ion constituent with a low ionic mobility is directly reflected in the time for resolution, time for detection, separation number and load capacity. In practice, however, only few substances will satisfy all the requirements of low mobility, low buffering capacity and no uv absorption.

c. The efficiency of the separation process is determined by the properties of the mixed zone. The transient-state model shows that these properties are also governed by the nature of the leading electrolyte as well as the nature of the sample. Considering the ratio of effective constituent mobilities in the mixed state, it follows that, owing to the limited numerical extension of ionic mobilities, pH or complex formation provides the best optimization parameter. In anionic separations a low pH of both the leading electrolyte and the sample will favour a high resolution rate and a high separation number. For cationic separations a high pH will be preferable. The presence of reversed pairs of constituent ions may complicate the optimization procedure. In general, it can be taken that the pH values of the leading electrolyte and the sample should not differ too much. For known species the critical pH values at which separation will not occur can easily be calculated and hence can be avoided.

3. Practical evaluation

All experiments discussed here were performed using the isotachophoretic equipment developed by Everaerts et al. [20]. The separation compartment consisted of PTFE narrow-bore tubing with ID 0.45, 0.2 or 0.15 mm and corresponding OD 0.75, 0.4 and 0.3 mm. The direct constant electrical driving current was obtained from a modified Brandenburg (Thornton Heath, Great Britain) high-voltage power supply. Potential gradient detectors, used in either the potential gradient or the conductance mode, were used for the determination of transient-state and steady-state characteristics. All chemicals used were of pro analisi grade and additionally purified by conventional methods (operational conditions Table 6). Theoretical calculations were performed with the computerized transient-state model [21, 22] and physicochemical data were taken from references [30-32].

For isotachophoretic analyses it is most convenient to use a separation compartment of well defined and constant volume and to apply a constant electrical driving current. Using a fixed point detector and a given operational electrolyte system, the characteristics of all electrical gradients and time events are constant because, under these conditions, the amount of the leading ion constituent filling the separation compartment, \( n_{0 \text{load}} \), is constant, so that the first boundary that reaches the detector will always be registered after the same time interval, \( t_{\text{det}} \). Some experimental results are given in Table 1. Obviously, a high transport efficiency, due to a low ionic mobility of the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leading ion constituent, chloride</td>
<td>( m_{\text{Cl}} ) = (-77 \times 10^{-5}) cm(^2) v(^{-1}) sec(^{-1})</td>
</tr>
<tr>
<td>Concentration</td>
<td>( C^+ ) (-0.01) M</td>
</tr>
<tr>
<td>Counter ion constituent, ( \beta )-aminobutyric acid</td>
<td>( m_{\text{GABA}} ) = (30 \times 10^{-5}) cm(^2) v(^{-1}) sec(^{-1}), ( \text{pK}_{\text{GABA}} ) = 4.03</td>
</tr>
<tr>
<td>Electrical driving current</td>
<td>( I ) = 80 ( \mu)A</td>
</tr>
<tr>
<td>Diameter of separation compartment</td>
<td>( d_s ) = 0.45 mm</td>
</tr>
<tr>
<td>Appearance of the first boundary</td>
<td>( t_{\text{det}} ) = 1112 sec</td>
</tr>
<tr>
<td>Amount of leading ion constituent samples</td>
<td>( \Delta n_L ) = 100 nmol</td>
</tr>
<tr>
<td>Time delay to amount sampled</td>
<td>( \Delta t ) = 59.2 sec</td>
</tr>
<tr>
<td>Response</td>
<td>( \frac{\Delta n_L}{\Delta t} ) = 0.592 nmol sec(^{-1})</td>
</tr>
<tr>
<td>Load of leading ion constituent</td>
<td>( n_{0 \text{load}} ) = 658 nmol</td>
</tr>
<tr>
<td>Transport number: experimental</td>
<td>( T_{\text{exp}} ) = 0.714</td>
</tr>
<tr>
<td>Transport number: theoretical</td>
<td>( T_{\text{theo}} ) = 0.720</td>
</tr>
<tr>
<td>Transport efficiency: experimental</td>
<td>( t_{\text{exp}} ) = 71%</td>
</tr>
<tr>
<td>Transport efficiency: theoretical</td>
<td>( t_{\text{theo}} ) = 72%</td>
</tr>
</tbody>
</table>
counter ion constituent, is always favourable as it guarantees efficient use of the power applied. The characteristics of a separation process can be evaluated by the injection of known amounts of sample.

Relevant parameters are summarized in Table 2. The fact that, at a constant load of leading ion constituent, the first boundary will always be detected at the same time interval, \( t_{\text{def,fix}} \), is illustrated in Fig. 1 by the resolution line \( L/A \). The low coefficient of variation confirms the excellent performance of the equipment. Injection of a small amount of sample will cause two zones, stacked between the leading ion constituent \( L \) and the terminating ion constituent \( T \). A sample load of 1.3 \( \mu l \) of the constituent mixture (Fig. 1) where \( n_A = 65 \) will give a time-based zone length of 124.5 sec for constituent ion \( A \) and detection must be started 1112 sec after injection. The zone length of the second constituent ion, \( B \), will be 148.1 sec. Other sample loads give proportional zone lengths. The characteristics of these steady-state zones have already been discussed extensively and the close agreement of the calculated and experimental resolution lines, \( L/A \), \( A/B \) and \( B/T \), indicates the reliability of the calculations. As the separation compartment has a limited load capacity, at a high load a mixed zone will be detected. The characteristics of these mixed zones are determined by both the leading electrolyte and the sample and are constant with time, as long as they exist. The time interval, \( t_{n_A^{\text{max}}} \), at which the mixed zone will be detected, is again constant, as illustrated in Fig. 1 by the resolution line \( A/AB \) [21, 22].

\[
I_{n_A^{\text{max}}} = t_{\text{def,fix}} \frac{m_A^L E^L}{m_B^M E^M} \tag{24}
\]

The maximum zone length for the resolved constituent ion \( A \), on a time base, is given by \( t_{n_A^{\text{max}}} - t_{\text{def,fix}} \). The maximum sample load, \( n_A^{\text{max}} \), is given by the intercept of the resolution lines \( A/B \), \( A/AB \) and \( AB/B \). For the given pair of constituent ions, formate and glycollate, the maximal sample load was 113 nmol, which was close to the theoretical value (Table 2). From the maximum sample load the load capacity [21], \( C_{\text{load}} \), can be calculated directly. Optimum column dimensions can be obtained from the load capacity. For anionic straight pairs of constituent ions, where \( m_B < m_A \) and \( pK_A < pK_B \), the rationale for optimization is straightforward: low pH of the leading electrolyte and the sample. With anionic reversed pairs, where \( m_B > m_A \) and \( pK_A < pK_B \), this rationale is more complicated. It has been shown that for such pairs a pH will exist, \( pH^{\text{cr}} \), at which no separation occurs. Of course, this pH will cause an infinite time for resolution, zero separation number and zero load capacity.

Moreover, at this critical pH the order in which the constituent ions migrate will be reversed. Experimental results concerning the load capacity for a
Table 2. Resolution data. For operational system see Fig. 1 and Table 1. Resolution line: n = at (nmol).

<table>
<thead>
<tr>
<th>Boundary</th>
<th>No. of determinations</th>
<th>a</th>
<th>b</th>
<th>Coefficient of variation or Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>L:A</td>
<td>53</td>
<td>0</td>
<td>0</td>
<td>-1112 - 1112</td>
</tr>
<tr>
<td>A, B</td>
<td>13</td>
<td>0.525</td>
<td>0.530</td>
<td>584</td>
</tr>
<tr>
<td>B, T</td>
<td>45</td>
<td>0.242</td>
<td>0.251</td>
<td>270</td>
</tr>
<tr>
<td>A/AB</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>-1328 - 1317</td>
</tr>
<tr>
<td>AB, B</td>
<td>6</td>
<td>0.321</td>
<td>0.316</td>
<td>314</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experimental</th>
<th>Theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Load of leading ion constituent (n_Load)</td>
<td>658</td>
<td>647</td>
</tr>
<tr>
<td>Maximal sample load (n_Mass)</td>
<td>113</td>
<td>108</td>
</tr>
<tr>
<td>Separation number (S_A)</td>
<td>0.103</td>
<td>0.099</td>
</tr>
<tr>
<td>(S_B)</td>
<td>0.103</td>
<td>0.099</td>
</tr>
<tr>
<td>Load capacity (C_Load)</td>
<td>0.172</td>
<td>0.167</td>
</tr>
<tr>
<td>Separation efficiency (r. &quot;a&quot;)</td>
<td>21</td>
<td>20</td>
</tr>
</tbody>
</table>
reversed pair arc given in Table 3, it follows that the criterion for separation will not be satisfied at a mixed zone pH of 5.19. Obviously, this pH can be generated by numerous combinations of leading electrolytes and sample compositions. Working at the maximum buffering capacity of the common counter ion constituent, i.e. \( \rho = -2 \) or \( \text{pH}^{\text{L}} = \text{pK}^{'}, \) and introducing an acceptable ionic mobility for the counter ion constituent, \( m_c = 30 \times 10^{-5} \text{ cm}^2 \text{V}^{-1} \text{sec}^{-1} \), the critical pH of the leading electrolyte, at which no separation occurs, is 4.98. This was confirmed experimentally by the separation at \( \text{pH}^{\text{L}} = 5.04, \) at which hardly any load capacity was present. At a pH higher than the critical value, sample constituent ions migrate in order of ionic mobilities, and separations can be performed with only moderate efficiency. At low pH, however, constituent ions are migrating in order of their pK values and a much greater efficiency can be obtained, resulting in a high load capacity. For example, the resolution for a 1.5 nmol sample (an absolute amount that can be detected without difficulty) would take about 18 sec, at \( \text{pH}^{\text{L}} = 4.10 \) and \( \text{pH}^{\text{S}} = 3.00, \) whereas the same sample can be resolved in 105 sec, at \( \text{pH}^{\text{L}} = 7.10. \) 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 times, 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 mobilities and dissociation constant, optimization procedures are elaborate and result in only a small increase in efficiency.

The interpretation of Fig. 2 must be simple now. Several sample constituent ions confirm the general principle that constituent ions in isotachophoresis are migrating at equal velocity in order of decreasing effective mobilities. The constituent ions lactate and mandelate (constituents 3 and 4), however, show virtually no difference in effective mobilities, as for their isotachophoretic migration the same electrical gradient seems to be necessary. From the linear trace it appears that this pair has not resolved during the separation process. The uv trace, however, indicates clearly that the mandelate (3) has been resolved from the lactate (4) and that the former migrates in front of the latter. The transient-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. As this is a reversed pair, the mandelate will be resolved in front of the lactate.

From the date in Table 4 it follows that the experimental and theoretical zone characteristics are in good agreement. The minor difference between the transient-state and the steady-state results has already been mentioned. The
Table 3. Data for a reversed pair of sample constituent ions

<table>
<thead>
<tr>
<th>Constituent ion</th>
<th>Mobility (cm$^2$ V$^{-1}$ sec$^{-1}$)</th>
<th>pK</th>
<th>Concentration (M)</th>
<th>pH$^{sample}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>$-41 \times 10^{-5}$</td>
<td>4.75</td>
<td>$-0.005$</td>
<td>4.75</td>
</tr>
<tr>
<td>Naphthalene-2-sulphonate</td>
<td>$-30 \times 10^{-5}$</td>
<td>0</td>
<td>$-0.005$</td>
<td>4.75</td>
</tr>
</tbody>
</table>

No separation at pH$^{H^+}=5.19$

No separation at pH$^{L}=4.98$

$(\mu=-2, m^0=30 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ sec}^{-1})$
Table 4. Comparison of zone characteristics. Trans = computerized transient-state model; \times 3 = computerized steady-state model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chloride zone, experimental</th>
<th>Trans</th>
<th>Mandelate zone</th>
<th>Experimental</th>
<th>Trans</th>
<th>Lactate zone</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>( pK_m ) (cm(^2) V(^{-1}) sec(^{-1}))</td>
<td>-2</td>
<td>3.37</td>
<td>( \times 3 )</td>
<td>Experimental</td>
<td>3.86</td>
<td>( \times 3 )</td>
<td>Experimental</td>
</tr>
<tr>
<td>pH</td>
<td>(-77 \times 10^{-5})</td>
<td>(-28 \times 10^{-5})</td>
<td>4.21</td>
<td>4.22</td>
<td>4.25</td>
<td>4.27</td>
<td>4.28</td>
</tr>
<tr>
<td>( E^c/E^x )</td>
<td>1.00</td>
<td>0.319</td>
<td>0.320</td>
<td>0.322</td>
<td>0.318</td>
<td>3.319</td>
<td>0.322</td>
</tr>
<tr>
<td>( c^x ) (mM)</td>
<td>-10.00</td>
<td>-6.47</td>
<td>-6.34</td>
<td>-7.16</td>
<td>-7.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mandelate zone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>mandelate</td>
<td></td>
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<tr>
<td>mandelate zone</td>
<td></td>
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<td>mandelate</td>
<td></td>
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<td></td>
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<tr>
<td>lactate zone</td>
<td></td>
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<tr>
<td>mandelate</td>
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</tr>
<tr>
<td>lactate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mixed zone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mandelate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mixed zone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
deviation from the critical value of unity for the ratio of the effective constituent mobilities is small. Mandelate ions, in the resolved lactate zone, would lead to a 1\(^n\) deviation. From the uv trace it follows that these deviations are large enough to guarantee a sharp separation boundary. The theoretical calculations show a greater difference and for the ratio of effective mobilities, in the mixed zone, a 2.6\(^n\) deviation from unity was calculated. The experimental separation confirms that this deviation is sufficient to obtain resolution. It must be emphasized, however, that the small deviation results in a low separation efficiency and column overloading can easily occur. Fig. 2 nevertheless indicates clearly that isotachopherograms in which only one universal detection system is used must be interpreted with great care. The same applies, of course, when only uv detection is used. From the uv trace in Fig. 2 it would be concluded that the nucleotides 5-GMP and 5-AMP have not been resolved. The conductance trace, however, clearly confirms the separation of these two constituents. On most occasions small amounts of impurities, with either uv absorbing or uv non-absorbing properties, will indicate the separation boundary. Moreover, in this particular instance, a difference is visible when the uv results are being traced in the absorbance mode.

The sample constituent ions 5-AMP and acetate (constituent ions 7 and 8) are migrating in an enforced isotachophoretic configuration. The effective mobility of the acetate ion constituent in its proper zone is higher than that of the nucleotide 5-AMP in its proper zone, as indicated in Fig. 2 by the lower conductance of zone 7 in comparison with zone 8. For the relative effective mobilities it follows that

\[
\frac{\bar{m}_{\text{Acetate}}}{\bar{m}_{\text{Chloride}}} = 0.212 \quad \text{and} \quad \frac{\bar{m}_{5-\text{AMP}}}{\bar{m}_{\text{Chloride}}} = 0.198
\]

The 7\(^o\) deviation from unity of the mobility ratio \((\bar{m}_{5-\text{AMP}}/\bar{m}_{\text{Acetate}} = 0.93)\) allows a satisfactory sharpness of the separation boundary between the two constituent ions. The reason for the stability can be found in the difference in the pH value in the two resolved zones. Using the appropriate relationship it follows that the pH of the acetate zone is 4.57. A nucleotide ion, lost owing to convection or diffusion from its proper zone (7) into the acetate zone, will migrate with a higher effective mobility than that of the acetate ion 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, i.e. \(\bar{m}_{5-\text{AMP}}/\bar{m}_{\text{Acetate}} = 1.37\).

Hence the self-restoring capabilities of the separation boundary allow the enforced isotachophoretic configuration to be stable with respect to time. It should be noted, however, that enforced isotachophoretic configurations will
F. EVERAERTS AND T. VERHEGEN

4. Qualitative and quantitative aspects

4.1. Introduction

As is shown in Fig. 3 the signals derived from a conductivity detector (potential gradient, thermometric) gives general characteristics. From these signals qualitative and quantitative information can be deduced. Various ways can be given to list the qualitative information, so that various laboratories working with other equipment can make use of it. For a correct qualitative evaluation it is always important to know whether the temperature difference between leading electrolyte and terminating electrolyte is large or small. The final temperature not only influences the effective mobility (2%, per °C), but also the $pK_a$ values, especially of cationic species. For quantitative evaluation the temperature is of less influence, as long as calibration curves are used. If ionic species are present that are migrating faster than the leading ion this can be seen in the linear signal of the conductivity detector, which indicates a conductivity lower than that of the leading ion. The qualitative information of all other ions, migrating in between the leading zone and the terminating zone is not lost. If desired, a leading ion can be chosen with a higher effective mobility, in order to have all constituent ions between the leading and terminating zone. If an ion is present in the sample with an effective mobility equal to that of the leading ion the conductivity of the leading electrolyte is not changed. Quantitative information can still be obtained by measuring the retardation of the appearance of the first separation boundary. Of course in this case the conductivity and zone lengths of all other zones are not influenced by this ionic species.

4.2. Qualitative aspects

In Fig. 4 an isotachopherogram is given as obtained with a potentiometric detector. A comparable result can be obtained from a thermometric detector, or a conductimetric detector. For that reason not only the potential gradient (E) is indicated, but also the values for the conductimetric detector (R) and the thermometric detector (T). Figure 4 shows the linear trace of the potential gradient detector, the differential of this and the linear trace of the uv absorption detector. Quantitative information is derived from the linear trace of the potential gradient detector. The quantitative information is obtained by measuring the steplengths (distances between peaks of the differential trace). The uv absorption detector gives additional qualitative (specific) information. For listing the qualitative data in operational systems, four different methods are given:

a. the stepheight $h_i$

b. the reduced stepheight $h_i - h_L$

c. the stepheight-unit value or SU-value

$$SU = 100 \frac{h_i}{h_L}$$
d. the reference-unit value or RU-value

\[ \text{RU} = 100 \times \frac{h_x - h_k}{h_k - h_l} \]

From these possibilities \( h_x \) gives obscure information, especially if qualitative information, obtained from the various detectors, is compared. The conductometer indicates \( E_L \), which is constant at various current densities, assuming the temperature differences are small enough. The potential gradient detector and the thermometric detector do not give any signal at \( I = 0 \) mA. Both \( T_L \) and \( E_L \) are determined by the current density applied. \( T_L \) has a square relationship and \( E_L \) has a linear relationship with the current density. The stepheights and the reduced stepheights vary, moreover, if the amplification is changed. The SU-values, the potential unit values, the temperature unit values and the conductivity unit values, are applicable, as they give a ratio in \( \mu_{\text{effective}} \). In practice, we prefer to work with SU-values. Moreover, a correction is made for the amplification of the electric circuitry applied, although this holds also for the RU-value. The RU-value is less dependent on the current density than the SU-value, because two reference species are considered. Therefore the accuracy of a RU-value can be greater than that of a SU-value.

For listing qualitative data it is always important that the conditions of the operational electrolyte systems applied are well defined, the current density is fixed, the output signals of the detectors are linearized and, for a high accuracy, the diameter of the narrow bore tube is carefully chosen. We found an optimum for this diameter at 0.2 mm inside diameter.

4.3. Quantitative aspects

As Fig. 4 shows, the quantitative information in isotachophoretic analyses can be obtained by measuring the zone lengths. There is a linear relationship between the zone length of an ionic species and the amount of that ionic species introduced as a sample, assuming the electric current is stabilized. The calibration curves of these mgic species are given in the Fig. 5.

If mobility and \( pK_a \) values are known a calibration constant can be determined [20]. This is a constant in each operational system, if the operational conditions are fixed, for all ionic species. The use of an internal standard for quantitative evaluation is described in reference [20].

It is preferable to work with calibration curves. It has been found that this
method is more accurate, because it is practically determined and corrects for, e.g. dissociation, complexation, temperature, solvation and activity.

5. Commercially available equipment

Analytical isochromatographic experiments are commonly performed in equipment consisting of a narrow bore tube of Teflon® with a diameter of 0.2 - 0.4 mm. An extensive discussion of commercial equipment can be found in reference [20]. The equipment with coupled columns is described in references [33, 34].

For simultaneous detection both the conductivity and the uv-absorption detectors are applied. Commercially available is the Tachophor (LKB produkter AB, Bromma, Sweden) equipped with a conductometric and a uv-absorption detector. This equipment is used for micro-preparative experiments, using the Tachofrac (see Chapter 8). The isochromatographic analyser (Shimadzu, Oakridge Industrial Center, 9147-H Red Branch Road, Columbia, Maryland 21045, USA) is equipped with a potential gradient and uv-absorption detector.

The experiments shown in this chapter were performed in home-made equipment (Eindhoven University of Technology, Department of Instrumental Analysis).

Nearly all values, especially the minimum detectable amounts, refer to the equipment using a narrow bore tube of Teflon® having an inside diameter of 0.2 mm and an outside diameter of 0.35 - 0.40 mm.

6. Choice of electrolyte systems

Reference [20] gives a discussion on the method of finding the optimum electrolyte system for an isochromatographic separation.

In the near future operational systems suitable for isochromatographic analysis will be commercially available.

7. Instrumentation

7.1. The conventional equipment

An extensive description of the equipment, suitable for isochromatographic analyses, is given in references [20, 29]. It will suffice therefore to give only a brief description here (Fig. 6).

The equipment consists of two electrode compartments (Fig. 6: 1, 7) which are directly connected with the current stabilized, power supply, an injection block (Fig. 6: 3) and a narrow bore tube (Fig. 6: 4). To prevent a hydrodynamic flow between the two electrode compartments, a semi-permeable membrane (Fig. 6: 7) is mounted. The separation compartment (Fig. 6: 4) is a narrow bore tube of Teflon® (PTFE) with an inside diameter of 0.2 mm and an outside diameter of 0.4 mm. This diameter is found to be optimal, amongst others because the temperature difference between the various zones is small. Moreover the convective disturbances are small and the zone profile is minimal. The sample can be introduced with a microlitre syringe in the injection block (Fig. 6: 3).

As in isochromatographic analyses the sample zones are separated in consecutive zones according to their effective mobilities, all zones have their characteristic features: temperature, conductance, pH and potential gradient. Moreover a zone may have uv absorption or optical rotation; alternatively fluorescence or radioactive compounds may be present. A thermometric detector [10] (e.g. a thermocouple made of Cu-Constantan wires with a diameter of approximately 25 μm) was developed initially as the detection system. This detector is mounted on the outside of the narrow tube. The response of the thermometric detector is rather low, but its sensitivity however is still comparable with that of the high resolution detectors; conductometric, potential gradient and uv-absorption detector. From thermometric detection universal information can be derived. From the potential gradient detector (Fig. 6: 6), with micro-sensing electrodes (10 μm Pt-Ir 10%), in direct contact with the electrolytes, universal information can also be derived, as is explained in Section 4. Generally the conductivity probe (housing) is made of acrylic resin, although for nonaqueous solutions Delrin (PTFE) can be used. The electrodes are mounted so that the electrolyte remains surrounded by an uninterrupted cylindrical wall. A contact cement has been used for the construction of the probe. The cell volume is approximately a few nl. In our equipment [20] a uv-absorption detector is also mounted. The uv source is a microwave mercury electrode-less lamp. The uv light is guided by a quartz rod of optical quality into a slit with a diameter of 0.1 mm. The uv light passes the narrow bore tube and is guided by another quartz rod towards a uv-sensitive photodiode. The wavelength is selected by an interference filter. Teflon®-lined valves are used at various places in the equipment, for the connection with the electrolyte reservoirs and the drain. Figure 6 shows clearly distinguishable parts in the electrophoretic equipment: the reservoir for the terminating electrolyte (Fig. 6: 1); the places where the sample can be introduced (Fig. 6: 3); the separation compartment (Fig. 6: 4); the places where the detectors are mounted (Fig. 6: 5, 6).
7.2. The coupled-column system

7.2.1. Introduction

Separands of interest are often present in solution together with numerous substances at higher concentrations and thus sample pre-treatment procedures, such as extraction, column techniques or salting-out procedures have to be used.

Using the column-coupling system a large amount of sample can be introduced, without sample pre-treatment. The equipment described below has a greater potential than simply increasing the maximum load capacity.

Combination with other separation techniques, such as liquid chromatography or zone electrophoresis, can be considered.

7.2.2. Instrumentation

In Fig. 7 a schematic drawing of the coupled-column system is given. The apparatus (Fig. 7) consists essentially of three sections:

a. the pre-separation compartment;
b. the bifurcation block with the 'tell-tale' detector;
c. the final separation compartment.

Besides the bifurcation block, all other compartments and the way of mounting the detectors and the capillaries are extensively discussed in reference [20].

The bifurcation block consists of three different channels:

a. A wide boring, e.g. 0.8 mm, in which the 'tell-tale' detector is mounted. This boring is connected with a wide bore PTFE pre-separation tube.
b. A narrow boring, e.g. 0.2 mm, in line with the wide boring. Onto this boring the narrow bore PTFE separation tube is mounted.
c. A flat channel (1 mm width and 0.05 mm depth) perpendicular on the borings of 0.8 mm and 0.2 mm. This channel forms the connection with the counter-electrode compartment, applied during the pre-separation.

The dimensions of the flat channel are chosen in such a way that diffusional effects are minimized and an optimal trapping is allowed. Since the separation compartments are in line with each other, there is only a minimal mixing of separated zones during trapping. It should be emphasized that the distance between the pre-separation and separation compartment in the bifurcation block is only 0.05 mm. Because the heat-transfer in this channel is excellent, high pre-separation currents are permitted. The 'tell-tale' detector identifies the pre-separation. Because the zones, under carefully chosen operational...
conditions, all migrate with equal velocity, the time needed for a zone boundary to cover the distance between the ‘tell-tale’ detector and the bifurcation is constant. The delay time, $t_1$, is readily determined using dyes or by measuring the $dV/ dt$ of the current stabilized power supply in a leading electrolyte/terminating electrolyte experiment. Once the zone(s) have been identified by the ‘tell-tale’ detector, the moment at which they reach the branching-off point of the pre-separation column into the analytical column is established. Hence the zone(s) of interest can be easily selected, even if they are not migrating consecutively. This zone(s) is further analysed and detected in the analytical column using both the uv and conductivity detectors. The maximum load capacity can be increased by an order of magnitude, without increasing the total analysis time. The automation of the column-coupling system is schematically given in the Figs 8 and 9. Before the analysis is started the driving currents for the pre-separation ($I_1$) and final separation ($I_2$), the delay-time $t_1$ (via TS1), the separation time $t_2$ (via TS2), the recording time $t_3$ (via TS3) and the conductivity level of the selected zone for the level comparator must be chosen. As soon as the analysis is started the counter A is activated in combination with the display. With this counter the various times can be measured.

As soon as the signal derived from the ‘tell-tale’ detector matches the pre-set value in the level comparator, the counter B is activated. In the memory of this counter the delay-time $t_1$ is ‘stored’ via the thumbwheel-switch TS1.

After $t_1$ sec the following procedures are started simultaneously:

a. the counter C is activated;

b. the latch is activated, which stops the display of the counter A;

c. the driving current is lowered from its value $I_1$ to $I_2$ via the relay R1;

d. the analytical column is connected to the current stabilized power supply via the relay HVRI (make contact 15 kV).

After 100 msec the first counter electrode (used in the pre-separation mode) is disconnected via the high-voltage relay HVR2 (break contact 30 kV).

Simultaneously the ‘tell-tale’ detector is mechanically (motor-driven) disconnected via the relay R2 from the coupling transformer 2, whose galvanic insulation is ca 6 kV*. Moreover, the electronics of the conductivity detector is switched from the ‘tell-tale’ detector towards the conductivity detector in the analytical column, using the relay R3. The display of the counter A can be activated again manually by deactivating the latch. The pre-

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* At the end of the analysis the ‘tell-tale’ detector may reach a potential of ca 15 kV towards ground level. The galvanic insulation of the low-voltage measuring electronics and the ‘isotachophoretic’ high voltage withstands approximately 6 kV.
Fig. 8. Automatic handling of the isotachophoretic equipment with coupled columns. For more information see text and Fig. 9. The analysis is started with resetting the electronics (Fig. 9). Counter A is started for measuring the pre-separation and final separation time. The comparator is pre-set at a level equal to that of the zone of interest. As soon as the zone of interest reaches the 'tell-tale' detector, counter B is started. The time needed for this zone to reach the bifurcation point is 'stored' in a thumbwheel-switch. As soon as the zone of interest reaches the bifurcation point, counter C is started. The display is stopped and the pre-separation time can be measured. By hand the display is started again. Simultaneously with stopping the display, the electric driving current is lowered, the counter-electrode compartment is switched and the 'tell-tale' detector is mechanically detached. As soon as the zone of interest is registered, the recorder is switched off and the current stabilized power supply is switched off. A new analysis can be performed.

separation time \( t_0 \), at which the display stops, can provide valuable information about the amount of ionic material that passed the bifurcation in the pre-separation mode.

The final separation time in the analytical column is 'stored' in the memory of the counter C, using the thumbwheel switch TS2. After this time has passed, the paper transport of the recorder is started via the relay R4. Simultaneously the counter D is activated. In the memory of the counter the recording time \( t_3 \) is 'stored' using the thumbwheel switch TS3. After \( t_3 \) see the recorder is stopped and the driving current (\( I_2 \)) is switched off. The equipment is now ready for resetting.

The equipment with the coupled column was tested and compared with the conventional equipment \([20, 33]\). Straight calibration curves, injected amount versus zone length were obtained and no loss of material due to the construction of the bifurcation block could be measured. The equipment proved to be very useful when ionic material in urine or serum needed to be analysed. Reproduction, even day to day variation, was better than 98%.

8. Fields of application

It is difficult to describe all possible fields of application of analytical isotachophoresis. To predict feasibility for isotachophoresis as an analytical method, the ratio of molecular weight to effective charge can be used. Generally this ratio should not exceed 3000. Needless to say the compound must have a sufficient solubility in the solvent chosen. A brief survey of possible fields will be given, further information can be found in the references \([20, 33]\).

8.1. The purity of drugs

In the system listed in Table 5, many components such as metal ions and organic bases can be analysed. In Figs 10 and 11 the isotachophoretic analyses
Fig. 10. The isotachophoretic separation of atropine, badly sterilized. 1. K⁺; 2. Na⁺; 3. atropine; 4. epsilon-aminocaproate; 5. chloride; 6. sulphate; 7. MES. R. increasing resistance; A. increasing UV absorption and t. increasing time. Time for analysis is approximately 12 min.

Fig. 11. The isotachophoretic analysis of atropine, properly sterilized. For further information see text and Fig. 10.

Table 5. Conditions for the separation of cations at pH 5.

<table>
<thead>
<tr>
<th>Cation</th>
<th>Leading electrolyte</th>
<th>Concentration</th>
<th>Counter ion</th>
<th>Terminating electrolyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cation</td>
<td>K⁺</td>
<td>0.01 M</td>
<td>CH₃COO⁻</td>
<td>H⁺</td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
<td>0.04 M</td>
<td>CH₃COO⁻</td>
<td>4.0</td>
</tr>
<tr>
<td>Additive</td>
<td>0.25% HEC</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
</tbody>
</table>

of atropine are shown. In Fig. 10 atropine is injected (2.98 nmol); the drug had been badly sterilized (30 min, 130 °C). In the left hand side isotachopherogram the degradation products tropane and methylamine are clearly shown. The time for analysis was 12 min. In order to show that tropanic acid also was present the right hand side isotachopherogram is given. The analysis is carried out in the system listed in Table 6. From a calibration curve it can be calculated that the amount of atropine is reduced by bad sterilization to 60%. In Fig. 11 the analysis of atropine, properly sterilized at 115 °C for about 15 min, is shown. Again the analysis is carried out in the system listed in Table 5. No degradation products are present. In the right hand side isotachopherogram a double amount (see Fig. 10) has been injected. From such isotachopherograms (Figs 10 and 11) a mass balance can be made to study the degradation (in other cases, kinetics as a function of time, but also to check the accuracy of an analysis). Isotachophoresis can be applied for controlling the production steps of ionic solutes after synthesis, extraction or recrystallization.
Table 6. Conditions for the separation of anions at pH 6.

<table>
<thead>
<tr>
<th>Anion</th>
<th>Leading electrolyte</th>
<th>Terminating electrolyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>MES</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>0.01 M</td>
<td>0.005 M</td>
</tr>
<tr>
<td>Counter ion</td>
<td>histidine⁺</td>
<td>Tris⁺</td>
</tr>
<tr>
<td>pH</td>
<td>6.0</td>
<td>ca 6</td>
</tr>
<tr>
<td>Additive</td>
<td>0.25% HEC</td>
<td>none</td>
</tr>
</tbody>
</table>

8.2. The composition of Raney-nickel

Figure 12 shows a separation of Ni and Al present in a Raney-nickel catalyst. These cations were analysed in the system, listed in Table 7, migrating as EDTA complexes*. The time for analysis is approximately 12 min.

Table 7. Conditions for the separation of anions at pH 7.5.

<table>
<thead>
<tr>
<th>Anion</th>
<th>Leading electrolyte</th>
<th>Terminating electrolyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>MES</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>0.01 M</td>
<td>ca 0.01 M</td>
</tr>
<tr>
<td>Counter ion</td>
<td>Tris⁺</td>
<td>Tris⁺</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
<td>ca 6</td>
</tr>
<tr>
<td>Additive</td>
<td>0.25% HEC</td>
<td>none</td>
</tr>
</tbody>
</table>

8.3. The determination of uric acid in serum

To serum, extensively dialysed against 0.9% NaCl, a standard amount of uric acid was added, giving a final concentration of 474 μM (see also Section 8.5). The isotachophoretic analysis yielded recoveries of 99.0–100.5%. To estimate the amount of uric acid bound to serum proteins under our experimental conditions the recovery from ultra-filtered and non-filtered samples was compared. When undialysed serum was passed through an Amicon CF 25 filter (MW cut off: 25 000) 85.1% of the total serum uric acid was recovered in the ultra-filtrate, indicating that approximately 15% was bound to protein with a MW exceeding 25 000. The lower amounts of uric acid in the ultrafiltrate as compared to non-filtered samples was not due to the CF 25 filter; when a standard solution of uric acid (474 μM in water) was passed through it, the recovery was 99.4%.

* The cationic species mentioned cannot be analysed in the operational systems, developed for cationic analyses [20].

In addition, the effect of high pH on the binding of urate to serum protein was studied. The pH of normal serum samples (pH 7.2–7.4) was adjusted to pH 10.0 and after ultra-filtration still approximately 7% (instead of 15–20%) of total urate was bound. A small part of the sera showed some turbidity, as judged from visual inspection. Those samples were rapidly passed through a Millipore filter (Millex: 0.22 μm). This did not affect the recovery of uric acid.

The identity of uric acid was confirmed in several ways. In Fig. 13 an experiment is shown which demonstrates that the uric acid zone is abolished by pre-incubation of the sample with uricase. Injection of an extra small amount of uric acid gave an increased length of the uric acid zone. Furthermore, the conductivity signal (step height) was specific for uric acid. The analyses were performed under conditions listed in Table 8 and with the equipment described in reference [33].
Fig. 13. Effect of incubation of serum with purified uricase. (a) before incubation with uricase; the arrows indicate the uric acid zone, as determined by the uv detector in an isotachophoretic analysis (uv) and by the conductivity detector (c.s.). (b) after incubation with uricase.

Table 9 shows that there exists a good correlation between the data obtained with sera (not filtered and ultra-filtered over CF 25) from 4 controls analysed with the enzymic and the isotachophoretic method. The day-to-day variance was about 2% with the isotachophoretic and approximately 10% with the enzymic method. The assays were performed at the laboratory of the Department of Neurology (Faculty of Medicine, Nijmegen) with an ABA I 00 bichromatic analyser (Abbott). 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. The formation of NADPH from NADP⁺ in the latter reaction (measured both at 380 and 340 nm) is used for the quantification of uric acid. As standards, sera with known concentrations of uric acid were used.

8.4. The analysis of purine and pyrimidines in serum

With the low-pH system (pH 3.9) a rapid (10 min analysis time) and reproducible separation was obtained of a standard solution containing 11 purine and pyrimidine nucleotides (Fig. 14A; uv trace). The uv trace of a standard solution consisting of 9 bases (nucleosides) and some other metabolites shows that with the high-pH system (pH 7.75) these metabolites can also be separated (Fig. 14B). Both systems have been applied to the analysis of serum (Table 10).

As could be anticipated not many nucleotides were detected with the pH 3.9 system (data not shown). Bases and nucleosides were present at high concentrations. A number of them were detected in the serum of a hypouricemic individual (Fig. 15A). A preliminary identification of several uv absorbing compounds was attempted with standard solutions. An example of this is shown in Fig. 15B, where the analysis of a mixture of the same hypouricemic serum and a standard solution is given. Further identification of this sample was not attempted. The same holds for the uv trace of normal serum (pooled from several controls) and of serum from a Lesch-Nyhan patient (not under allopurinol treatment), shown in Fig. 15C, 15D, respectively.

A uv trace of the electrolyte system showed some minor impurities. These peaks will also feature in the metabolic profiles. A possibility to differentiate between the electrolyte impurities and the separands might be to increase the volume of the sample injected: the metabolite zones will increase whereas the
Table 10. Conditions for the separation of purine and pyrimidine nucleotides and purine and pyrimidine bases and nucleosides.

<table>
<thead>
<tr>
<th>System I (pH = 3.9) for separation of purine and pyrimidine nucleotides</th>
<th>Leading electrolyte</th>
<th>Terminating electrolyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anion</td>
<td>Cl⁻</td>
<td>Caproate</td>
</tr>
<tr>
<td>Concentration</td>
<td>0.01 M</td>
<td>0.005 M</td>
</tr>
<tr>
<td>Counter ion</td>
<td>GABA⁺</td>
<td>Tris⁺</td>
</tr>
<tr>
<td>pH</td>
<td>3.9</td>
<td>6.5</td>
</tr>
<tr>
<td>Additive</td>
<td>0.25ⁿ</td>
<td>none</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>System II (pH = 7.75) for separation of purine and pyrimidine bases and nucleosides</th>
<th>Leading electrolyte</th>
<th>Terminating electrolyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anion</td>
<td>Cl⁻</td>
<td>OH</td>
</tr>
<tr>
<td>Concentration</td>
<td>0.01 M</td>
<td>saturated Ba(OH)₂</td>
</tr>
<tr>
<td>Counter ion</td>
<td>0.01 M Tris⁺/Li⁺</td>
<td>Ba⁺⁺</td>
</tr>
<tr>
<td>pH</td>
<td>7.75</td>
<td>11.5</td>
</tr>
<tr>
<td>Additive</td>
<td>0.25ⁿ, HEC</td>
<td>none</td>
</tr>
</tbody>
</table>

Fig. 14. (A) Analyses of standard solution at high and low pH. A, uv trace obtained at low pH (pH = 3.9) of nucleotides. 1. UTP; 2. GTP; 3. ATP; 4. UDP; 5. CTP; 6. GDP; 7. ADP; 8. UMP; 9. GMP; 10. cAMP; 11. AMP. (B) uv trace obtained with the high-pH system (pH = 7.75) of bases, nucleosides and some other metabolites. 1. orotic acid; 2. uric acid; 3. hippuric acid; 4. xanthine; 5. hypoxanthine; 6. inosine; 7. allopurinol; 8. guanosine; 9. adenine.

Fig. 15. Analyses of serum at pH = 7.75. (A) uv trace of a serum from a hypouricemic individual; (B) same serum, but 'spiked' with a standard solution (see Fig. 14B); (C) uv trace of a serum from a healthy control individual; (D) uv trace of a serum from a patient with Lesch-Nyhan syndrome (not under allopurinol treatment).

Interfering electrolyte zones will increase much less. It should be noted that the uv absorption of the hippuric acid zone is not constant (Fig. 15). This is due to the fact that a steady-state mixed-zone [20] is formed with a constituent which has an effective mobility equal to that of hippurate. Figure 15B shows that several metabolites can be identified directly. However, to be absolutely sure about the identity of a certain metabolite, 'spiking' is not sufficient. Further possibilities to identify compounds include:

a. The step height of the conductivity signal (universal detector [20]) which gives qualitative information regarding the constituents involved.

b. The enzymic conversion of metabolite by purified enzymes which is a sensitive and specific way to identify the metabolite.
c. Information regarding extinction, e.g. the $E_{280}/E_{254}$ ratio.
d. Changes in the operational electrolyte systems (such as complexing agents, solvents, mixtures of solvents, pH; see also reference [20]).

8.5. The determination of valproic acid in serum of epileptic patients

8.5.1. Introduction

It was mentioned in Section 7.3 that uric acid in serum can be analysed directly isotachopheretically in the operational system listed in Table 8.

Another isotachopherogram is shown in Fig. 16 of an analysis of uric acid in serum of a gouty patient. In this operational system (Table 8) the valproate concentration in sera of epileptic patients was also determined [35]. Sodium valproate is known as a useful anticonvulsant drug in primary generalized epilepsy [36]. The determination of the anticonvulsant in serum is of importance for the correct treatment of epileptic patients, especially in establishing the pharmacotherapy. Optimal therapeutic serum concentrations are known to be about 60 μg cm$^{-3}$. Several gas-chromatographic procedures for the analysis of valproate have been described [37–44], each with its own advantages and limitations. A disadvantage, common to all gas-chromatographic procedures, is the treatment of the sample prior to chromatography. Dependent on the specific procedure, solvent extraction, derivatization and evaporation have to be used.

Isotachophoresis is an electrophoretic method that can provide both qualitative and quantitative results on ionic solutes in a relatively short time of analysis. The method 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.

8.5.2. Materials and methods

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 dispensary (Apotheek Haagse Ziekenhuizen, Den Haag, The Netherlands). In addition to valproic acid the test sera contained fenobarbital, fenitoine, ethosuximide, primidon, clorazepate, carbamazepine and 10,11-epoxy carbamazepine.

Serum samples were taken from venous blood; after clotting and centrifugation the sera were stored at $-20^\circ$C until required.

8.5.2.1. Gas chromatography. For the gas chromatographic determinations a Packard Becker 421 chromatograph was used. Separations were performed in a glass column, packed with 5% FFAP on Chromosorb WHP. 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. Cyclohexanecarboxylic acid was applied as the internal standard.

8.5.2.2. Isotachophoresis. For the isotachophoretic separations the coupled column system, developed by Verheggen et al. [34] was used (Section 7.2). The inner diameter of the pre-separation 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 electrical driving current in the analytical column was 10 μA. The electrolyte systems and other operational conditions are given in Table 8.

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

8.5.3 Results and discussion

One of the major advantages of isotachophoresis is that ionic solutes often can be analysed without sample pretreatment. Therapeutic levels of valproate in serum, however, differ by at least two orders of magnitude to the physiological chloride concentration. Due to this unfavourable sampling ratio, the electrolyte system will have a rather low separation efficiency [21]. Hence, for a reliable determination, a large column volume must be available resulting in a long time of analysis. Most of these problems can be solved using a coupled-column system [33, 34]. This system not only allows the use of a high sample load, but also the use of different electrolytes, e.g. Table 8. For the determination of valproate the concentration of the leading ion in the pre-

![Fig. 17. Isotachophoretic separation of epileptic 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.](image1)

![Fig. 18. Calibration graph for isotachophoretic valproic acid determinations. (●) Standard solutions in water; (○) standard solutions in serum; (△) test sera, commercially available.](image2)

separation compartment was 0.01 M. At a high driving current serum samples were separated in approximately 6 min. 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 17 shows a representative result when 3 μl of a patient’s serum was injected. The total time of analysis was less than 15 min. Since trapping was started 2 sec before the valproate zone reached the bifurcation point, some other solutes have been analysed additionally. The valproate zone, however, easily can be localized in both the uv trace, Fig. 17c, and the conductimetric trace, Fig. 17a. From the separation in Fig. 17 it follows that uric acid could have been used as the terminating ion, instead of morpholine-ethanesulfonic acid.

As a result of lower end voltage which would have been obtained, a further optimization of the time for analysis would have been allowed. 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 serum.
Additionally several test sera, containing various other drugs were analysed. A good linear relationship was found with a correlation coefficient of 0.99914 (n = 26). The response was found to be 6.12 ng sec⁻¹ and the mean error per point was 3.8 ng in the detection range of 50-500 ng. Reproducibility was better than 98% and day-to-day variations were small. The additional other drugs did not interfere. The gas chromatographic procedure also yielded a good calibration 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. Reproducibility and day-to-day variations were better than 95% (Fig. 18).

Using these calibration data, valproic acid concentration levels were determined in the sera of twenty patients. The isotachophoretic results were compared with the gas chromatographic results. As can be seen from Fig. 19 there is a good relationship. The experimental slope deviates only 1% 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 cm⁻³ for the isotachophoretic determinations and 57.8 µg cm⁻³ for the gas chromatographic determinations. The origin of the systematic deviation is still under investigation. The results of four different test sera and calf serum, were in good agreement with the expected values for both methods.

9. Acknowledgements

The work described in this chapter was prepared at the subject group 'Instrumental Analysis' of the Eindhoven University of Technology.

10. References


Fig. 19. Comparison of gas chromatographic and isotachophoretic results. Abscissa: µg/ml valproic acid by gas chromatography. Ordinate: µg/ml valproic acid by isotachophoresis. (★) Patient sera; (▲) test sera.