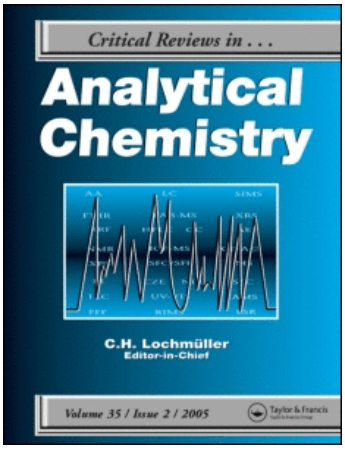


Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

Capillary Isotachophoresis

Sven-Göran Hjalmarsson; F. M. Everaerts

To cite this Article Hjalmarsson, Sven-Göran and Everaerts, F. M.(1981) 'Capillary Isotachophoresis', Critical Reviews in Analytical Chemistry, 11: 4, 261 — 352
To link to this Article: DOI: 10.1080/10408348108542743
URL: http://dx.doi.org/10.1080/10408348108542743

CAPILLARY ISOTACHOPHORESIS*

Authors: **Sven-Göran Hjalmarsson**
Astor Baldesten
Research Department
LKB Produkter AB
Bromma, Sweden

Referee: F.M. Everaerts
Department of Instrumental Analysis
Eindhoven University of Technology
Eindhoven, The Netherlands

TABLE OF CONTENTS

Mr. Hjalmarsson's present address is Tecator AB, Box 70, S-26301, Höganäs, Sweden.

I. INTRODUCTION

The use of isotachophoresis, the latest of the electrophoretic separation techniques, has grown very rapidly during the last few years in various application areas. The reason for the increased acceptance of analytical isotachophoresis is the appreciation of its attributes, such as small sample requirements in the picomole range, short analysis time of about 10 to 30 min, and ease of quantification. The present status of capillary isotachophoresis is described in this review article. A historical review is given in the first section followed by a discussion of the basic theory. Further on the selection of electrolyte systems and spacer ions is discussed, as well as aspects of quantification and different detection systems. The emphasis has, however, been laid on the description of various application areas, most of them illustrated by several characteristic practical examples of separations.

II. HISTORICAL BACKGROUND

Isotachophoresis as a working method is a young technique, although the laws which govern isotachophoretic separation were laid down in 1897. The German chemist, Kohlrausch,[1] published a theoretical treatment of the conditions at a migrating boundary between two salt solutions and showed that the concentrations of ions at the boundary

were related to their effective mobilities (Kohlrausch's regulating function). Many investigators very soon applied Kohlrausch's theory on the moving boundary for the determination of transference numbers. However, it was not until 1923 that anyone made use of the basic principle formulated by Kohlrausch for the purpose of separating ions. In 1923 and later years, Kendall and co-workers described the separation of some ions by an "ionic migration technique"[2-5] which, in fact, was isotachophoresis. The experiments of Kendall et al. were aimed at the separation of isotopes. However, these attempts failed and Kendall's pioneering work did not receive much attention until 1953 when a paper was published by Longsworth,[6] who realized its importance. In a Tiselius moving-boundary apparatus, he separated a mixture of metal ions (Ca, Ba, and Mg) between two ions which he called the leading solution (CsCl) and the trailing solution (LiCl). Schlieren scanning patterns clearly showed the sharpness of the boundaries between the zones. Longsworth also showed that when all the components were separated a steady state was reached on passage of a constant current.

Even though several research groups had worked over a long period with systems which were regulated by the Kohlrausch function, it was not until the 1960s that Kendall's so-called ionic migration technique received full attention. In 1962, Konstantinov et al.[7] observed the orderly succession of ionic boundaries as well as the relationship between concentration and mobility of the ions on the two sides of a boundary, in accordance with the Kohlrausch equation. This led Konstantinov and Oshurkova[8] to develop a "moving boundary method" for the microanalysis of, mainly, metal ions. Independently, in 1963, Everaerts[9] and Martin started to work systematically with the technique of isotachophoresis from both theoretical and practical points of view. In 1964, Ornstein[10] and Davis[11] introduced disk electrophoresis. In fact, they were the first to apply the Kohlrausch function to the separation of proteins. They placed a protein mixture between a terminating ion (glycine) and a leading ion (chloride). Because of the concentration phenomenon of isotachophoresis, the proteins became stacked in narrow zones between the two electrolytes ("steady state stacking"). The zones were very narrow, however, and in immediate contact with each other, so that even a high-resolution detector could not distinguish between them. Therefore, in the second step of the analysis, zone electrophoresis was used, which allowed the different proteins to move with different velocities. The concentrating step was, however, nothing but isotachophoresis. An important step forward in the applicability of isotachophoresis was the introduction of the so-called spacer technique by Vestermark.[12]

In 1968, Verheggen and Everaerts built a capillary tube apparatus for analytical isotachophoresis, based on the work of Everaerts[13] and Martin and Everaerts.[14] This became the basis for the commercial development and production of isotachophoretic equipment by LKB-Produkter AB of Bromma, Sweden.

Up to 1970 several names had been used for what Kendall initially called the ionic migration technique; these included moving boundary method, displacement electrophoresis, steady state stacking, cons electrophoresis, and ionophoresis.

In 1970 Haglund,[15] together with a group of researchers in this field, introduced a name based upon an important phenomenon of the electrophoretic technique, namely the identical velocities of the sample zones at equilibrium, isotachoelectrophoresis (Greek: *iso,* equal; *tacho,* speed) or isotachophoresis for short. The name isotachophoresis was readily accepted and has since been the acknowledged denomination.

III. PRINCIPLES

Isotachophoresis means a migration in an electric field of ion species of the same sign, all having a common counter-ion. Isotachophoresis will take place when an electric field is applied to a system of electrolytes consisting of:

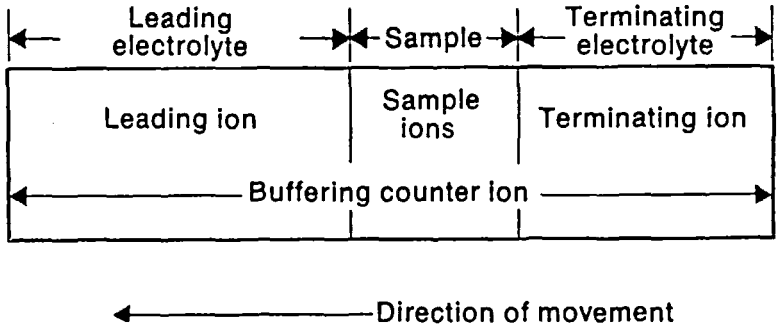


FIGURE 1. See text.

1. A sample solution containing the sample ions (of the same sign) to be separated, introduced as a zone between
2. One electrolyte, the leading electrolyte, which must contain only one ion species, the leading ion, L^-, having the same sign as the sample ions to be separated, and an effective mobility higher than that of any of the sample ions
3. A second electrolyte, the terminating electrolyte, which contains one ion species, the terminating ion, T^-, having the same sign as the sample ions to be separated, and an effective mobility lower than that of any of the sample ions

The polarity of the electric field must be such that the leading ion migrates to the electrode that is placed on the same side of the sample as the leading electrolyte (see Figure 1). When the system has reached equilibrium, all the ions move with the same speed, individually separated into a number of consecutive zones in immediate contact with each other, and arranged in order of effective mobility.

IV. BASIC THEORY

The ionic concentration in a separated sample zone will adapt itself to the concentration of the preceding zone. This was theoretically described by Kohlrausch[1] in the so-called regulating function which is the basis of the theory of isotachophoresis. Consider, for example, two negatively charged ions, A^- and B^-, with a common positively charged ion, R^+. The ratio between the concentrations C_A and C_B of ions A^- and B^- is given by

$$\frac{C_A}{C_B} = \frac{m_A}{m_A + m_R} \cdot \frac{m_B + m_R}{m_B} \qquad (1)$$

where m = ionic mobility (cm^2/volt·sec), and the subscripts A, B and R refer to the ions A^-, B^-, and R^+, respectively. The Kohlrausch equation thus gives the conditions at a boundary between two ions (A^-, B^-) with one ion in common (R^+) when the boundary migrates in an electric field.

Suppose the migration takes place in a narrow tube (Figure 2). The figure illustrates the starting conditions for the separation of ions A^- and B^- which are introduced between the leading and terminating electrolytes (containing ions L^- and T^-). The effective mobilities of the participating ions are selected so that $m_{L^-} > m_{A^-} > m_{B^-} > m_{T^-}$ (for simplicity, the counter-ion is not included in Figure 2).

When the electric current is applied between the electrodes, the negatively charged ions

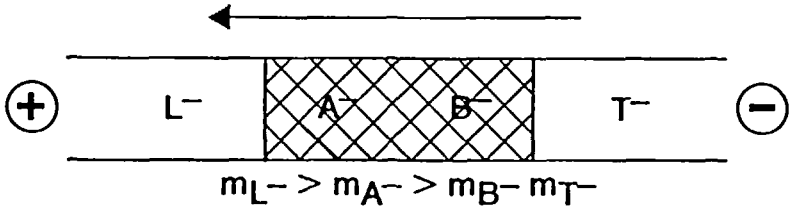


FIGURE 2. See text.

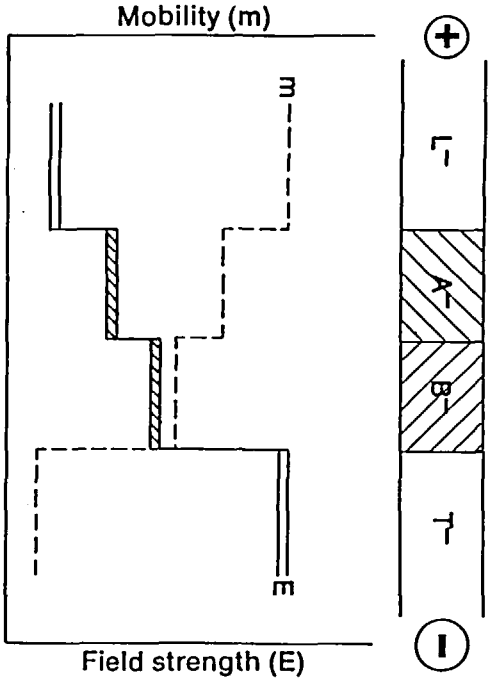


FIGURE 3. See text.

will start migrating towards the anode with a velocity depending upon the effective mobility of the leading ion, according to the formula

$$v = m \cdot E \qquad (2)$$

The ions with the highest effective mobility will move first and those with lower effective mobilities will follow in decreasing order of effective mobility. The current density in such a system must be homogeneous and the zones are therefore forced to follow in direct contact with each other with equal velocity. To fulfill Equation 2, the electrical field strength, E, is therefore increased when the mobility decreases, and at equilibrium the situation shown in Figure 3 is reached. The fall in effective mobility at a zone boundary is accompanied by a proportionally large rise in field strength (E is inversely proportional to m). In general, isotachophoresis is performed with a constant current. This maintains a constant field strength over each individual sample zone since the resistance in the zone is homogeneous. The velocity of zone movement will thus not change during the experiment.

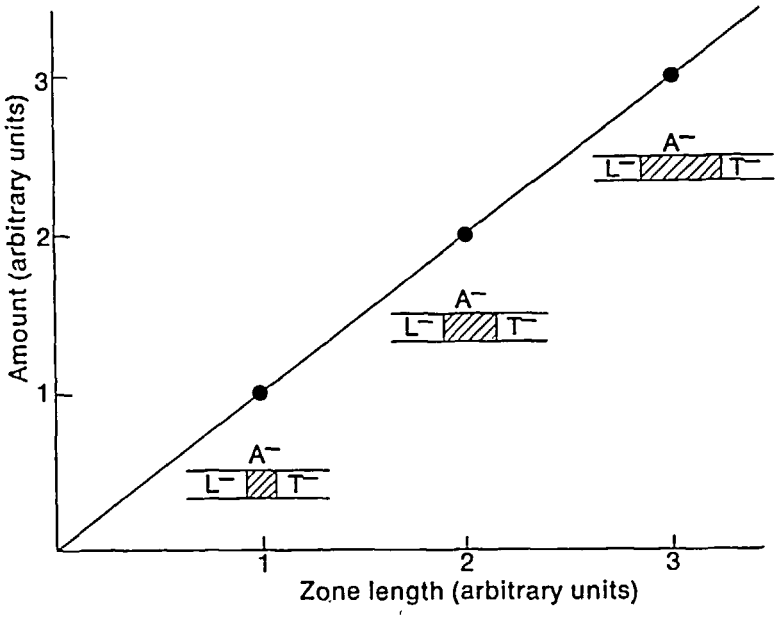


FIGURE 4. The zone length is directly proportional to the amount of ion in it.

Since the effective mobility of each of the participating ions is constant under defined conditions, we can re-write the Kohlrausch formula (1) as follows:

$$C_{A^-} = C_{L^-} \cdot \text{constant}$$

This indicates that, at equilibrium, the sample ion concentration (C_{A^-}) is directly proportional to the leading ion concentration (C_{L^-}). The ion concentration in each zone is, therefore, constant. The amount of ions in each zone can thus only be changed by proportionally changing the length of the zone. This gives us the unique feature of isotachophoresis: zone length is directly proportional to amount of ions in it. This is illustrated in Figure 4.

A. Concentrating Effect

An important consequence of the concentration properties of isotachophoresis is the so-called concentrating effect. If a component A^- (Figure 5a) is introduced at very low concentration, the Kohlrausch equation will also apply to A^-. Since the mobility of A^- lies in between the mobilities of the leading and terminating ions, the concentration of A^- at equilibrium will be intermediate to the concentrations of L^- and T^-. This will result in A^- being concentrated until it reaches the theoretically defined concentration (Figure 5b). If a concentrated sample is introduced into the isotachophoretic system, the reverse discussion applies and a dilution takes place until the correct equilibrium concentration is reached (Figure 5c).

B. Zone Sharpening Effect

As a result of, for example, diffusion, a sample ion may move out of its own zone into another. If an A^- ion diffuses in the L^- zone, it will experience a lower field strength there than in the A^- zone (see Figure 6). Since the mobility of A^- can be considered constant, the A^- ion in the L^- zone will have a reduced velocity compared with the L^- ions ($v = m \cdot E$);

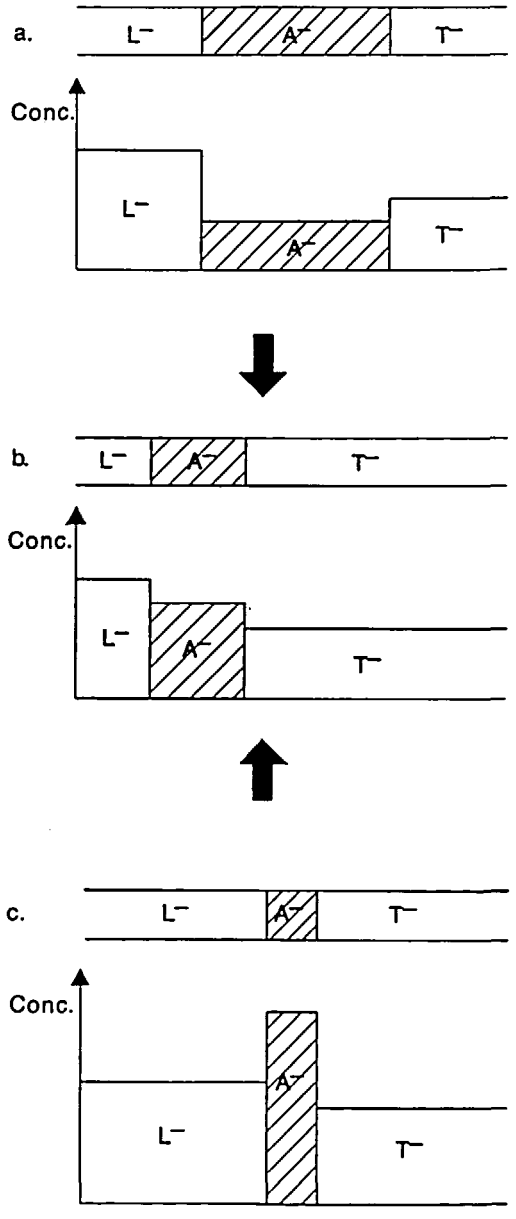


FIGURE 5. The concentrating effect (see text).

the A^- ion will, therefore, be readily caught up by its own zone. Similarly, if an L^- ion diffuses into the A^- zone, with its higher field strength, its velocity will be increased. It will immediately reenter the L^- zone and its velocity will return to that of the other L^- ions. As a consequence of these effects, there is in isotachophoresis an efficient counteraction of diffusion, resulting in sharp zone boundaries and, thus, high resolution.

The basic theory of isotachophoresis and an explanation of factors affecting an isotachophoretic separation have been treated by several authors—for instance, Everaerts et al.,[16-18] Routs,[19] Beckers,[20] Haglund,[15,21] Strongin et al.,[22] Neumann,[23] Ryser,[24] Miedziak and Waksmundski,[25] Ball and Hjalmarsson,[26] Van Der Moosdijk,[27] Nagayanagi,[28] and Arlinger.[29,30]

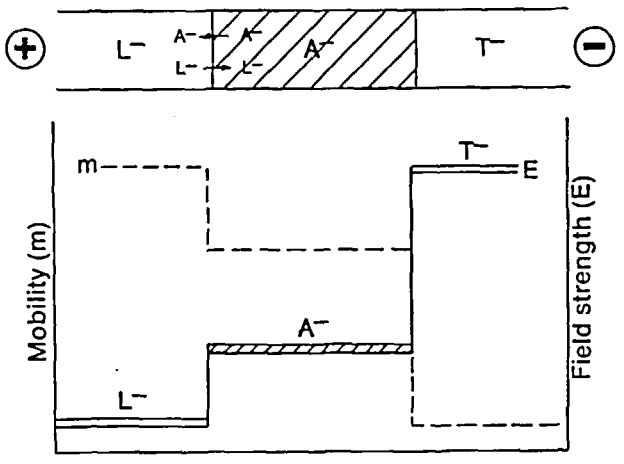


FIGURE 6. The zone sharpening effect (see text).

V. CHOICE OF ELECTROLYTE SYSTEMS

A. Basic Principles

When selecting a system for the isotachophoretic separation of ionic species, it is necessary to obtain differences in effective mobilities of the participating ions. The effective mobility is defined by

$$m_{eff} = \Sigma_i \alpha_i \gamma_i m_i$$

where α_i is the degree of dissociation, γ_i is a correction factor for the influence of relaxation and retardation effects and m_i is the absolute ionic mobility. These factors are described in detail by Everaerts et al.[16]

The degree of dissociation, α_i, is the parameter which mainly influences the effective mobility. It is also the factor which in practical work is most liable to be affected experimentally. It is, therefore, of vital importance to control the degree of dissociation when optimizing the electrolyte solution for the separation. The degree of dissociation is directly related to the pH and for a weak electrolyte, HA, this can be expressed by the well-known equation

$$pH = pK + \log \frac{[A^-]}{[HA]}$$

where K is the dissociation constant for the weak acid HA. When the pH is increased, the electrolyte HA is further dissociated and thereby its effective mobility is increased.

According to the Kohlrausch equation, the electrolyte conditions in the leading zone determine all the parameters in the succeeding zones. The pH in the leading zone thus determines the degree of dissociation and, thereby, the effective mobility of the following zones. The selection of a leading electrolyte system for a sample consists, therefore, of the choice of a leading ion with an effective mobility higher than any of the sample ions and the choice of a pH in the leading ion zone which will give optimal differences in effective mobility of the sample zones.

The pH of the leading zone is determined by the nature and the pK value of the counter-ion. The pK of the counter-ion should be such that it ensures a good buffering capacity in the pH interval within which the separation takes place. The zones will then

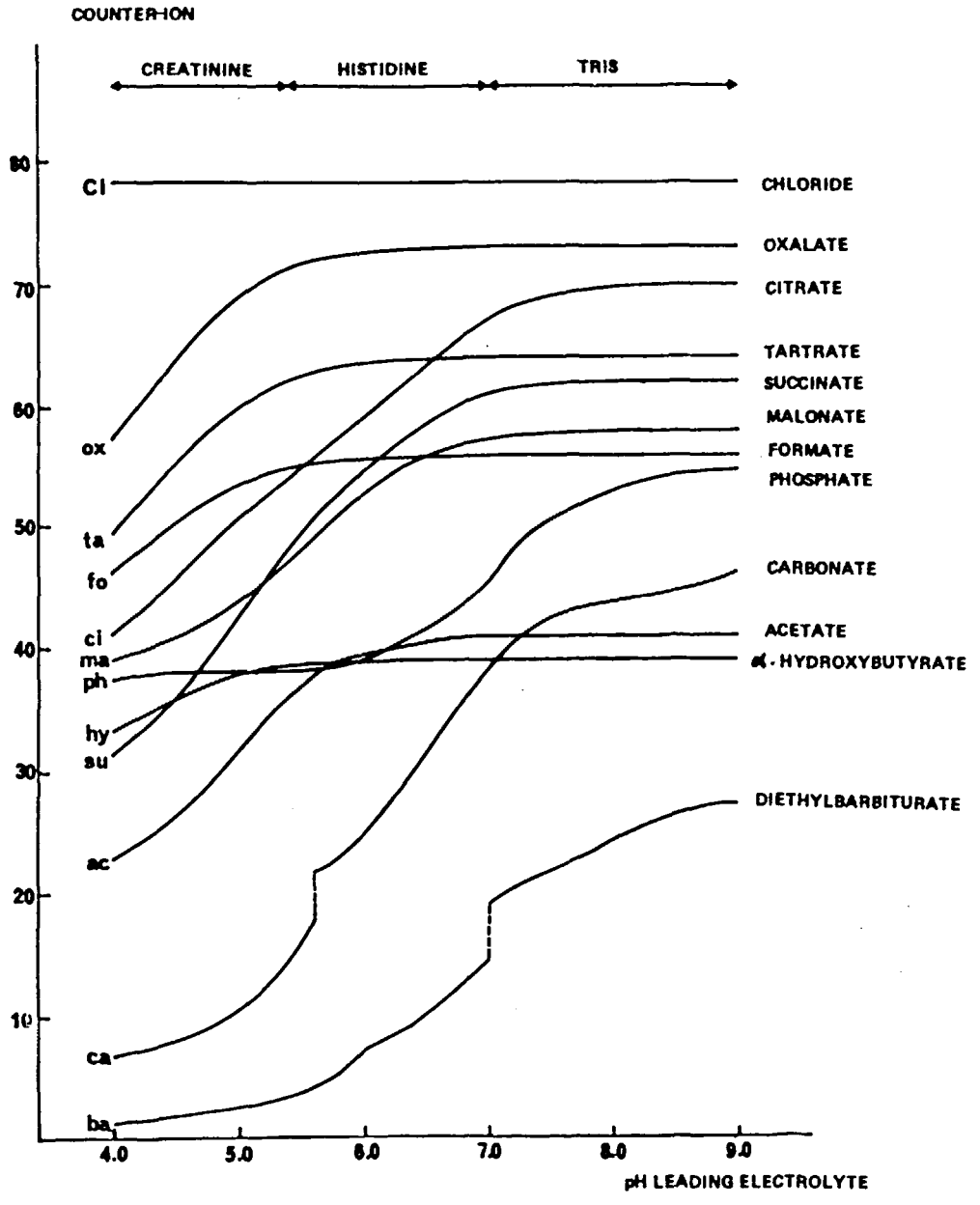


FIGURE 7. The net mobility of 11 weak acids as function of the pH in the leading electrolyte. The leading ion is 0.01 *M* HCl. The choice of the counter-ion species and concentration depends on the pH in the leading electrolyte: creatinine for the pH interval 4.0 to 5.4, histidine for 5.4 to 7.0, tris for 7.0 to 9.0.

possess good stability against pH disturbances. Mikkers et al.[31] have pointed out that a low mobility of the counter-ion is always favorable, since it gives the most efficient use of applied power for the separation of the sample ions, and Kaniansky et al.[32] have, in a recent article, studied the role of the charge number of counterionic constituents. Figure 7 shows examples of how the pH of the leading electrolyte will affect the effective mobility of a number of compounds. Literature data on mobilities or conductances,[33] pK values,[34-37] and pI values[38] will usually supply the basic information of possible pH ranges to select for the leading electrolyte.

The terminating electrolyte must always be chosen to have an effective mobility lower than that of the sample ions of interest. It is advisable to adjust the pH and concentration to fulfill the equilibrium conditions, as described, for instance, by Baldesten.[39] Routs,[19] Everaerts et al.,[16] and Baldesten[39] have discussed the principle of how to select electrolyte solutions.

B. Aqueous Electrolyte Systems

In most cases, water is the most suitable solvent for an electrolyte system. By the addition of an acid or a base, thereby changing the pH, it is a simple matter to obtain the desired degree of dissociation of the participating ions. Water is also an excellent medium for the additives needed to protect a sample against oxidation or reduction or to prevent, for instance, interactions between sample components. It is possible to add urea (up to at least 6 *M*), mercaptoethanol, dithiothreitol, nonpolar detergents, and all sorts of water-miscible organic solvents to an isotachophoretic electrolyte system, as long as the additives are not themselves dissociated at the chosen pH.

When an electric field is applied to an electrolyte system, an electroendosmotic flow, defined as the movement of the liquid with respect to the wall(s), is obtained.[40] In isotachophoresis the situation is complicated since the electroendosmotic flow differs from zone to zone. To overcome disturbances in the flow profile, different additives with high viscosity have been proposed. Martin and Everaerts[41] suggested the use of long-chain soluble polymers to increase the viscosity and thereby overcome unwanted electroendosmotic movement of the electrolytes. Arlinger[42] used hydroxypropylmethyl-cellulose (HPMC) and Everaerts[16] showed that Triton® X-100 and polyvinylalcohol are suitable. The polymers can be purified on mixed-bed ion-exchangers.[16] According to Delmotte,[43] it is preferable to dialyze HPMC before use. Woledge et al.[44] reported that 2% (w/v) HPMC in the leading electrolyte gave sharper zones compared with the previously used concentration of 0.2 to 0.5%. Several reports indicate that the effect of counteracting electroendosmosis by the above-mentioned substances lasts for several runs. The most commonly used additives are summarized in Table 1.

In general, it is very important to prepare the electrolyte solutions with the utmost care. Impurities in the chemicals used show up as unwanted peaks or zones and thereby often influence the separation and interpretation in a negative fashion. It is, therefore, advisable to recrystalize compounds intended for use in electrolyte solutions. Fredriksson[45] recently showed that bleeding from the septums in the injection valves can also give rise to undesired zones. Complete or patchwise adherence of compounds to the wall of the capillary can also disturb the separation, due to the slow release from the patches of previously adsorbed sample or electrolyte components. Such disturbances can take the form of a drastic alteration in the duration of an experiment, a loss in boundary sharpness, a change in temperature profile or drift of the UV baseline. Most of the electrolytes should be made up fresh in order to avoid growth of microorganisms, since excretion products may spoil the resolution. A summary of aqueous electrolyte systems is given in Table 2 and 3.

C. Nonaqueous Electrolyte Systems

Solvents other than water may sometimes be needed in order to achieve a complete separation. They can be used if the sample species have about the same effective mobilities and pK values and/or are only slightly soluble in water. Experimental data for isotachophoretic separations in nonaqueous media have been mainly presented for methanolic and ethanolic systems.[16,20,46-50] The use of acetone as solvent has recently been described.[51] Beckers[20] has described in detail the concept of determination of pK values in methanolic systems. The pK values for several organic acids in 95% methanol were calculated.

Table 1
SOME ADDITIVES USED IN ISOTACHOPHORESIS

Compound	Recommended concentration (% w/v or molarity)	Prepurification	Commercial source
Hydroxypropylmethylcellulose (HPMC)	0.05—0.5%	Dialysis[43]	Dow Chemicals, Co., Midland, Mich., U.S.
Hydroxyethylcellulose (HEC)	0.1—0.2%	Dialysis or ionexchange[130]	Polysciences, Warrington, Pa., U.S.
Methylcellulose (MC)	0.1—0.5%		Eastman Kodak Company, Rochester, N.Y., U.S.
Polyvinylalcohol (PVA)	0.05—0.2%	Mixed-bed ionexchanger[16]	Hoechst, Frankfurt, FRG
Triton® X-100	0.05—0.2%	Mixed-bed ionexchanger	Rohm & Haas, Philadelphia, Pa., U.S.
Urea	~6 *M*	[a]	Merck, Darmstadt, FRG

[a] Ultrapure quality must be used.

Table 2
AQUEOUS ELECTROLYTE SYSTEMS

Separation of Anions

	Leading electrolyte[a,b]			Terminating electrolyte	
No.	Leading ion	Counter-ion[c]	pH_L (pH in the leading electrolyte)	Terminating ion[b]	Ref.
1	0.005 *M* HCl	0.001 *M* NaCl	2.25	0.005 *M* succinic acid or 0.01 *M* formic acid	110, 122, 123
2	0.005 *M* HCl	Glycine	2.50	0.005 *M* caproic acid	
3	0.012 *M* HCl	α-naphthyl amine	2.85	0.01 *M* pivalic acid	107
4	0.007 *M* HCl	Glycyl-glycine	2.9	0.005 *M* caproic acid or 0.005 *M* benzoic acid	112, 113
5	0.005—0.010 *M* HCl	β-alanine	3.0—4.2	0.005—0.02 *M* caproic acid	55, 68, 110, 145
6	0.010 *M* HCl	Adenosine	3.4	0.01 *M* caproic acid	144
7	0.006 *M* Cd $(NO_3)_2$	—	—	0.01 *M* tartaric acid	105
8	0.010 *M* HCl	α-naphthyl amine	3.7	0.01 *M* caproic acid	144
9	0.010 *M* HCl	β-alanine	3.8	0.004 *M* acetic acid	116
10	0.010 *M* HCl	β-alanine	3.8	0.01 *M* p-amino-benzoic acid, pH = 4	16
11	0.010 *M* HCl	ε-aminocaproic acid	4.0—5.0	0.005 *M* Glutamic acid	16, 67
12	0.010 *M* HCl	Creatinine	4.2—5.2	0.01 *M* pivalic acid or 0.01 *M* cacodylic acid	55
13	0.010 *M* HCl	Aniline	4.2—4.6	0.01 *M* pivalic acid	144
14	0.010 *M* HCl	Pyridine	4.2	0.01 *M* pivalic acid with TRIS added to pH 4.0	16
15	0.010 *M* HCl	Histidine and ε-amino-caproic acid (1:1)	4.5	0.01 *M* ACES[d]	17
16	0.010 *M* HCl	ε-aminocaproic acid or histidine	4.5—4.7	0.010 M MES[e] with TRIS added to pH 7	16
17	0.010 *M* HCl	Aniline	4.6	0.005 *M* glutamic acid	116
18	0.010 *M* HCl	ε-aminocaproic acid and histidine	4.7	0.01 *M* MES[e]	211
19	0.010 *M* HCl	Histidine	5.0—7.0	0.01 *M* of any of the following ions: phenylacetic acid cacodylic acid caproic acid glycine MES[e] ACES[d]	
20	0.010 *M* HCl	Pyridine	5.0	0.01 *M* cacodylic acid	144
21	0.010 *M* HCl	Urotropine (hexamethylene tetra-amine)	5.1	0.005 *M* glutamic acid	116
22	0.010 *M* HCl	Histidine	6.0—6.9	0.01 *M* TES[f] with histidine to pH 6 or 0.01 *M* MES[e], with TRIS to pH 6 or 10 m*M* Hepes[g] with TRIS to pH 8.50	17, 223
23	0.040 *M* H_3PO_4	0.06 *M* TRIS	6.7	0.23 *M* ε-aminocaproic acid with TRIS added to pH 9.1	216

Table 2 (continued)
AQUEOUS ELECTROLYTE SYSTEMS

Separation of Anions

	Leading electrolyte[a,b]			Terminating electrolyte	
No.	Leading ion	Counter-ion[c]	pH_L (pH in the leading electrolyte)	Terminating ion[b]	Ref.
24	0.005—0.010 *M* HCl	TRIS	6.9—9.0	0.01 *M* of any of the following ions: glutamic acid glycine phenol β-alanine ε-aminocaproic acid (add Ba [OH]$_2$ until pH = 8—10)	55
25	0.010 *M* HCl	Imidazole	7.0	0.01 *M* benzyl-dl-asparagine	144
26	0.005 *M* HCl	Imidazole	7.0	0.005 *M* glutamic acid or 0.005 *M* caproic acid	140 215
27	0.013 *M* cacodylic acid	0.012 *M* TRIS	7.0	0.013 *M* β-alanine	143
28	0.010 *M* HCl	Imidazole	7.05	0.01 *M* MES[e] with TRIS added to pH 6	16
29	0.005 *M* glutamic acid	TRIS	7.2	0.005 *M* glycine with TRIS added to pH = 8.5 and thereafter Ba(OH)$_2$ to pH = 9.2	
30	HCl 5% (w/v) dextrose	TRIS	7.2	Glycine with TRIS added to pH = 8.3 5% (w/v) dextrose	217
31	0.010 *M* HCl	Imidazole	7.4	0.005 *M* ascorbic acid	116
32	0.005 *M* cacodylic acid	TRIS	7.4—7.5	0.005 *M* β-alanine (Ba[OH]$_2$ added to pH = 9.2)	185
33	0.011 *M* TES[f]	0.010 *M* TRIS	7.65	0.01 *M* β-alanine (Ba[OH]$_2$ added to pH 9)	224
34	0.005 *M* glycyl-glycine	TRIS	8.4	0.01 *M* glycine (Ba[OH]$_2$ added to pH 9) or 0.005 *M* serine (Ba[OH]$_2$ added to pH 9.5)	62
35	0.010 *M* HCl	TRIS	8.50	0.01 *M* EPPS[h] with TRIS added to pH 8.5	67
36	0.005—0.010 *M* HCl	Ammediol[i]	8.4—9.6	0.005—0.010 *M* of any of the following ions: glycine phenol β-alanine ε-aminocaproic acid (add Ba[OH]$_2$ until pH = 8.5—10)	55

Table 2 (continued)
AQUEOUS ELECTROLYTE SYSTEMS

Separation of Anions

	Leading electrolyte[a,b]				
			pH_L (pH in the leading	Terminating electrolyte	
No.	Leading ion	Counter-ion[c]	electrolyte)	Terminating ion[b]	Ref.
37	0.005—0.010 *M* MES[e]	Ammediol[i]	8.8—9.6	See system 36	55

[a] To all the aqueous leading electrolytes various additives should be used (in most cases 0.2—0.5% HPMC), see Table 1.
[b] The concentration of the electrolytes can be lowered, but preferably not below 0.002 *M*.
[c] When no concentration value is given it is assumed that the leading ion is buffered by the counter-ion to the desired pH.
[d] *N*-(2-acetoamido)-2-amino-ethane sulfonic acid.
[e] 2-*N*-(morpholino)-ethane sulfonic acid.
[f] *N*.-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid.
[g] *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid.
[h] 4-(2-hydroxyethyl)-1-piperazine-propane sulfonic acid.
[i] 2-amino-2-methyl-1,3 propanediol.

The measurement of pH in organic solvents is difficult. Because of the different liquid junction potential for a solution of a buffer in water and a solution of a buffer in an organic solvent, it is advisable to use the same kind of organic solvent for both the standard and the unknown solution. By utilizing this technique, the two liquid junction potentials will cancel each other out and the measured pH can be interpreted in terms of hydrogen activity. Tables 4 and 5 provide a summary of nonaqueous electrolyte systems.

VI. COUNTERFLOW OF ELECTROLYTES

A counterflow is a hydrodynamic flow of electrolyte in the opposite direction to the migration of the sample zones to be analyzed. The technique is used to improve a separation. Everaerts et al.[52] showed that the counterflow improves the separation substantially if the mobility differences are large and, particularly, if the aim is to analyze small amounts of an ion in the presence of large amounts of another sample constituent. They also clearly showed that if the mobility difference between two ions is small, a counterflow has no significant importance for a better separation. A counterflow greater than 30% of the electrical migration causes unduly disturbed borders between adjacent zones and is of no practical interest. The use of counterflow of electrolyte has also been shown[16] to have other drawbacks, such as:

1. A relatively long analysis time is needed and the effective elongation of the narrow bore tube is only about 60%.
2. In a long run the ionic impurities present in the solutes and solvents used often disturb or obscure the final result.

A major benefit, however, from using a counterflow of electrolyte compared with, elongation of the capillary, for example, is the low end-voltage at detection. Akiyama and Mizuno[53] and Shiogai and Akiyama[54] have, in two extensive articles, shown that

Table 3
AQUEOUS ELECTROLYTE SYSTEMS

Separation of Cations

	Leading electrolyte			Terminating electrolyte	
No.	Leading ion	Counter-ion	pH_L	Terminating ion	Ref.
38	0.010 *M* HNO_3	—	1.9	0.01 *M* TRIS	197
39	0.010 *M* HCl	—	2.0	0.01 *M* LiCl or 0.01 *M* TRIS	55
40	0.010 *M* sulfanilic acid	HCl	2.4	0.01 *M* LiCl	55
41	0.010 *M* KOH	Ascorbic acid	4.1	0.01 *M* LiCl	55
42	0.010 *M* KOH	Fumaric acid	4.3	0.01 *M* Li_2SO_4	55
43	0.005—0.010 *M* KAc	Acetic acid	4.0—5.5	0.01 *M* of any of the following ions: glycyl-glycine, β-alanine, α-alanine, ε-aminocaproic acid, TRIS	55
44	0.005—0.01 *M* KOH	Cacodylic acid	6.3—7.0	0.01 *M* creatinine with 0.005 *M* HCl added to pH ~ 5 or 0.01 *M* β-alanine with 0.005 *M* HCl added to pH 4.	55
45	0.005 *M* TRIS	Cacodylic acid	5.5—7.0	See system 44	184
46	0.01 *M* KOH	Diiodotyrosine	7.4	0.01 *M* TRIS	197
47	0.005 *M* $Ba(OH)_2$	Valine	9.3	0.02 *M* TRIS with HCl added to pH 8.4	164
48	0.005 *M* $Ba(OH)_2$	Glutamine	9.25	0.02 *M* triethylene-diamine	143
49	0.005 *M* $Ba(OH)_2$	0.015 *M* valine	9.9	0.02 *M* TRIS with HCl added to pH 8.3	55

Table 4
NONAQUEOUS ELECTROLYTE SYSTEMS

Separation of Anions

	Leading electrolyte			Terminating electrolyte	
No.	Leading ion, solvent	Counter-ion	pH_L	Terminating ion	Ref.
50	0.01 *M* HCl in 90% ethanol or 96% methanol	0.0085—0.02 *M* TRIS	—	0.01 *M* of any of the following ions: cacodylic acid, lithocholic acid, caproic acid	16, 202
51	0.005 *M* HCl ethanol: acetone: H_2O (4:1:5)	Histidine	—	0.01 *M* caproic acid in H_2O	106
52	0.01 *M* NaI in methanol	—	—	0.01 *M* H_3PO_4 in methanol	46

Table 5
NONAQUEOUS ELECTROLYTE SYSTEMS

Separation of Cations

	Leading electrolyte			Terminating electrolyte	
No.	Leading ion, solvent	Counter-ion	pH_L	Terminating ion	Ref.
53	0.01 *M* HCl in methanol	—	—	0.01 *M* $CdCl_2$ in methanol	16, 197
54	0.01 *M* KAc in methanol	Acetic acid	6.3—7.4	0.01 *M* $CdCl_2$ in methanol	16, 197
55	0.0089 *M* NaCl and 0.0007 *M* sodium-acetate in methanol	Saturated sulfonic acid	5.0	0.02 *M* $Zn(Ac)_2$ in methanol	65
56	0.01 *M* $(CH_3)_4$ NCl in methanol with sulfanilic acid and 10% $(CH_3)_4$ NOH	—	4.4	0.002 *M* $Zn(Ac)_2$ in methanol	55
57	0.01 *M* $(CH_3)_4$ N^+ in methanol	Acetic acid	6.9	0.005 *M* $CdCl_2$ in methanol	16, 197

with large mobility differences it is possible with counterflow to improve the separation of certain acids. Counterflow can be performed with a microsyringe dosage pump,[55] a gas-membrane pump as applied by Everaerts,[16] or with an osmotic pressure system as described by Rýslavý et al.[56]

VII. THE SPACER TECHNIQUE

In isotachophoresis the resolved sample zones are forced to run in immediate contact with each other. In many cases this can lead to practical difficulties in detecting the separation which has been achieved. As early as 1965, Vestermark[12] drew attention to this problem and suggested adding intermediate-mobility compounds to the sample solution. In such a way it would be possible to force two consecutive sample zones apart by an intermediate-mobility compound and, thereby, make possible analytical detection as well as the preparation of small amount of adjacent compounds. Vestermark named such intermediate-mobility compounds *spacers*. The spacer technique has since been further developed and several authors have described the use of this technique.

Spacing can be done either with a continuous mobility gradient[57-60] or by using discrete spacers.[61-63] A discrete spacer is, in principle, a single compound chosen to have an intermediate mobility to two sample components of interest. The discrete spacer can either be UV-absorbing or non-UV-absorbing. In the former case, two non-UV-absorbing sample components can, for example, be spaced apart by the UV-absorbing spacer ion and in this way an improved UV detectability is obtained. The same applies when adding non-UV-absorbing spacer ions to UV-absorbing sample ions.

A spacing mobility gradient contains a large number of ions which possess mobilities very close to each other. So far only Ampholine® carrier ampholytes have been used. The Ampholine® fraction used is nothing but a complex mixture of polyaminopolycarboxylic acids with a great number of pK-values distributed over a certain pH range. When this mixture is added to the sample in an isotachophoretic system its components arrange themselves in order of their effective mobility. The result is an isotachophoretically moving mobility and pH gradient. The mobility gradient spacers are mainly used in

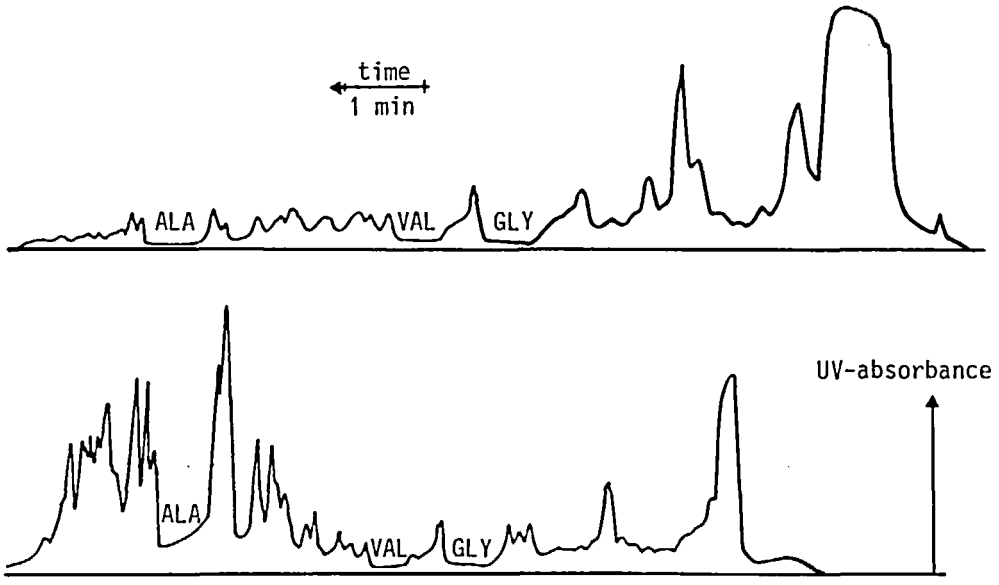


FIGURE 8. Comparison of cerebrospinal fluid patterns from a normal case and a multiple sclerosis patient. Upper tracing: separation pattern of normal cerebrospinal fluid. Lower tracing: cerebrospinal fluid from a multiple sclerosis patient. Fractions slower than amino acid valine are immunoglobulin G. Electrolyte system No. 37, $pH_L = 9.1$, Table 2. (From Delmotte, P., *Electrophoresis '78*, Catsimpoolas, N., Ed., Elsevier North-Holland, Amsterdam, 1978. With permission.)

protein separations, where the sample solutions are often very heterogeneous and where difficulties therefore arise in detection. When a protein mixture is run together with Ampholine® carrier ampholyte spacers, some of the spacer ions will have very similar effective mobilities to the protein components. Due to these very small differences in effective mobility, there is often some mixing of sample and spacer at the ends of each zone, leading to a rounded-off appearance (see, for example, Figure 8).

The optimal selection of spacer ions often has to be determined experimentally. The Ampholine® carrier ampholytes are commercially available (LKB-Produkter AB, Sweden) in pH-fractions selected according to the corresponding pI values of the ampholyte species. The proper choice of the ampholyte pH range is important in order to obtain the correct mobility gradient and, thereby, optimal separation. This has been treated by Hjalmarsson[64] in connection with the preparative separation of serum proteins. In capillary isotachophoresis, a practical recommendation is to start with a small amount of Ampholine® (0.1 to 1 $\mu\ell$ of a 1% solution) of a narrow pH range and then, if need be, select another pH range, increase the amount and/or add suitable amounts of Ampholine® of another range. Informative practical examples have recently been demonstrated by Delmotte,[43] where both discrete spacers and mobility gradient are used in protein analysis. The technique with discrete spacers has been optimized to perfection by Lange[63] who "framed" the protein of interest with the discrete spacers glycylglycine and aspartic acid (see Figure 9).

VIII. DETECTION SYSTEMS

At present, four different types of detection systems are used for measurements in isotachophoretic analyses: ultraviolet (UV)-absorbance detector, conductometric detector, potential gradient detector, and thermometric detector. The properties of the detectors are summarized in Table 6.

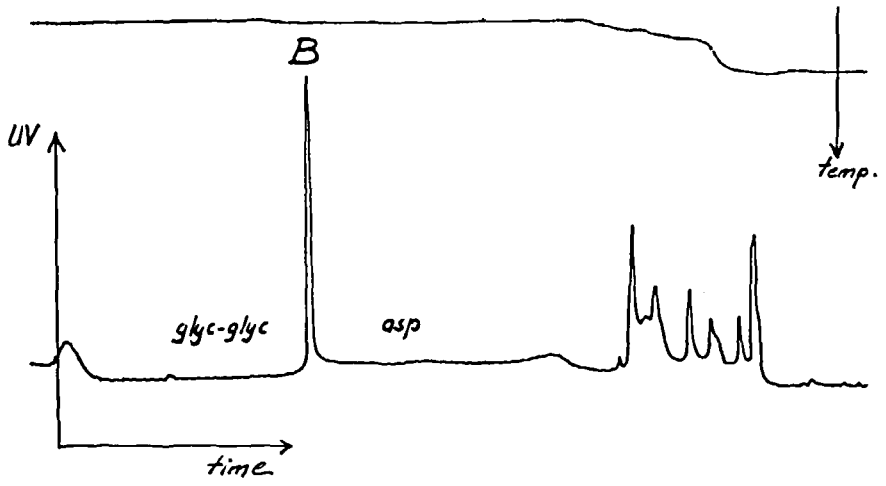


FIGURE 9. Analysis of 10 μg soluble protein from hippocampus of rat (B). As discrete spacers the amino acids glycyl-glycine and asparagine were used. Electrolyte system No. 36, $pH_L = 9.2$, Table 2. (From Lange, P. W., *Proc. 1st. Int. Symp. Isotachophoresis*, Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980. With permission.)

A. UV Detection

In the dynamic equilibrium stage of an isotachophoretic experiment all the ions move with the same speed in individual zones and only the counterion is homogeneously mixed with them. Hence, the registration of the UV absorption can give valuable information about some of the ions (semiqualitative) and at the same time serve to detect zone boundaries.

In practice, UV absorption is measured directly through the capillary (often made of Teflon®). The total internal volume of the complete capillary is so small (on the order of 40 to 100 $\mu\ell$) that only an extremely small cell volume is available for the actual detection. Enlargement of the cell volume by measuring absorption in an axial direction, as is commonly done in equipment for liquid chromatography, is impossible due to the very narrow separated zones in capillary isotachophoresis. Therefore, stringent conditions must be placed on the light source, the detector, and the signal amplifier. In most cases only the wavelengths 254 and 280 nm have been utilized for detection. Since the capillaries used in isotachophoresis are mostly made of Teflon®, this excludes the possibility of detecting below about 230 nm due to UV dispersion and absorption by the Teflon®. In some instances it is possible to detect boundaries between two consecutive non-UV-absorbing zones because of the trace amount of UV-absorbing impurities which are present in most electrolytes and which show up as "markers" in between the separated non-UV-absorbing zones (see Section VII.).

Arlinger[65] has demonstrated the use of UV-absorbing counter-ions in order to detect non-UV-absorbing compounds. Each zone has its own defined pH and concentration. By choosing a counter-ion which has a large difference in molar absorptivity between acidic and basic forms, the pH and concentration difference will give rise to an absorbance difference sufficiently large to be detectable.

B. Conductometric Detection

Each individual separated zone has its defined resistance and, thereby, a defined conductivity. The conductivity of the various zones can be determined in different ways; with microsensing electrodes not in direct contact with the electrolytes or with microsensing electrodes in direct contact with the electrolytes inside the capillary. A

Table 6
SURVEY OF THE DETECTORS USED IN CAPILLARY ISOTACHOPHORESIS

Type	Mode of Registration	Minimum detectable amount (in moles)	Minimum amounts for quantification (in moles)	Dependence on driving current	Comments	Ref.
UV (zone length measurement[a])	Microcell, fixed wavelength 256, 280, 340, 365nm	$0.5 \cdot 10^{-11}$	$2.0—5.0 \cdot 10^{-11}$	No	Quantitative, specific detector	16
UV (peak height measurement[a])	Microcell, fixed wavelength 256, 280, 340 365nm	$<10^{-12}$	$1.0—2.5 \cdot 10^{-11}$	No	Quantitative, specific detector	30, 62, 66
UV (steady state mixed zone[a])	Microcell, fixed wavelength 256, 280, 340, 365nm	$<10^{-12}$	$<10^{-11}$	No	Quantitative, specific detector	67, 69
Conductivity	Microsensing electrodes in contact with the electrolytes	$0.5 \cdot 10^{-11}$	$1.6 \cdot 10^{-11}$	No	Quantitative, qualitative, general detector	16, 67
Potentiometric	Microsensing electrodes in contact with the electrolytes	$0.5 \cdot 10^{-11}$	$1.6 \cdot 10^{-11}$	I	Quantitative, qualitative, general detector	16, 67
Thermometric	Cupper-constantan thermocouple 25μm	$2.0—5.0 \cdot 10^{-10}$	$8.0 \cdot 10^{-10}$	I^2	Quantitative, qualitative, general detector	16, 18, 67

Note: The minimum values given (for detection and quantification) are approximate and are valid for a capillary I.D. of 0.45 mm and a UV slit width of 0.25 mm. With a 0.2 mm I.D. capillary the sensitivity is increased by a factor of about 5 (see Refs. 18, 67)

[a] For details, see Section IX.

detailed description of the various methods for conductivity measurement is found in the book by Everaerts et al.[16]

The most commonly used method for conductivity detection is that of measuring the resistance by using an external measuring alternating current which is passed through the capillary. The microsensing electrodes are mounted equiplanar. Various noble metals can be used for the electrodes. However, an alloy of Pt-Ir (10 to 30%) with a thickness of 10 μm was found to be the best.[16] The absolute value of the conductivity signal provides a qualitative measurement characteristic of each component under defined operational conditions, and the length of the zones provides quantitative information.

C. Potentiometric Detection

The potentiometric type of measurement of the resistance of the various zones makes use of the potential gradient that characterizes each zone. The potential gradient detector consists of two electrodes inserted through the capillary wall and situated after each other longitudinally.[16] In potential gradient detection, the driving DC current is itself applied as the current source for the measurement (in conductivity detection an external AC current source is applied for measuring the resistance). Since the potential gradient in a given zone is correlated with the effective mobility of the ion in that zone, the voltage drop between the electrodes provides qualitative information.

D. Thermometric Detection

Another often used detection method is thermometric detection, the monitoring of the relative temperature differences between the separated zones. As mentioned before, isotachophoresis is performed at a constant current. Since power (P) is the product of field strength (E) and current (I), $P = E \cdot I$, the joule heat produced will change stepwise at each zone boundary. The position of each zone boundary is thus indicated by a temperature rise which can be detected by means of a sensitive thermocouple.[16] Since the temperature of each zone is related to the net mobility of the ion in it, the height of the thermal record (the "thermal step height") can be used to identify each sample ion qualitatively.

IX. QUANTIFICATION

A. Zone Width Determination

In capillary isotachophoresis the separated components form homogeneous zones. The zone lengths are directly proportional to the amount of ions in them (see Figure 4), and quantification is in general simplified to measuring the lengths of the individual zones as they appear on the recorder chart. This is illustrated in Figure 10 where three different amounts of ATP have been analyzed and quantified by measuring the zone length (in this case, the UV-absorbance signal). Note:

1. The sharp zone boundaries which are due to the zone sharpening effect. The resolved zones appear as rectangular peaks, not as the common Gaussian curves one is used to from chromatography.
2. The constant height of each peak due to the uniform sample concentration in each zone. (The peak height reflects the molar absorption at the given concentration.)

In order to measure the UV peak width, the component of interest must, at detection, fill the UV slit completely: this is shown in Figure 10 where the peak height is constant during the period of detection. It is also possible to determine the length of a non-UV-absorbing zone if it is bracketed by compounds which are UV-absorbing. The zone

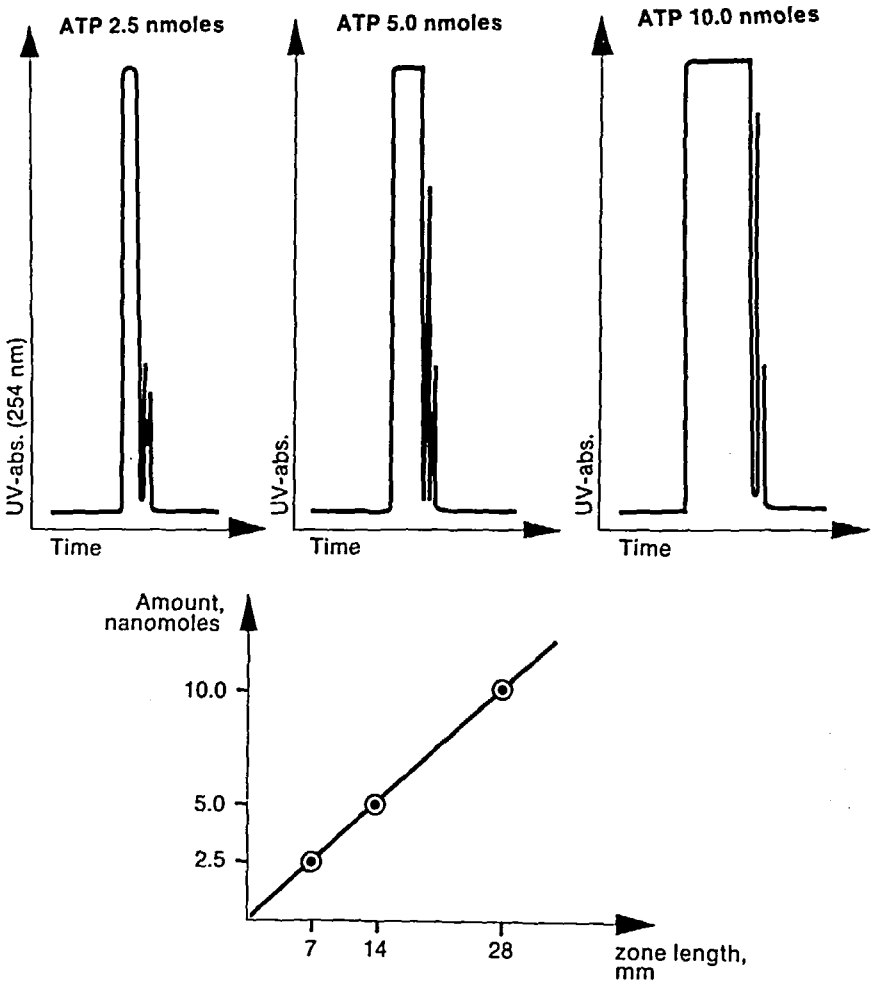


FIGURE 10. Quantification by measuring the zone length.

widths certainly can also be measured from conductometric, potentiometric, and thermometric signals. To facilitate the calculations in these cases, it is usual to differentiate the signals and to measure the distance between the inflection points.

B. UV Peak Height Determination

It is possible to quantitate tiny amounts of compounds which do not fill the UV slit completely by measuring the peak height. Arlinger[30] showed that there is a connection between peak height and amount when the zone length drops beneath the length of the UV slit. Svoboda et al.[66] deduced that for light-absorbing compounds there must be a linear relationship, assuming that the zones move in the capillary as ideal disks and that the registered absorption is a simple addition of the absorptions of the trace component and of the ions in the adjacent zones. Wielders[67] gives a more thorough explanation and shows that the theoretical assumptions are only valid if the zones on either side are long enough to fill the UV slit completely during the time the compound of interest is passing the slit. If, however, an adjacent zone is of comparable minute length to that of the trace component, quantification becomes difficult. Svoboda and Vacik[66] quantified different naphthalenesulphonic acids (Figure 11) down to a few picomoles and Moberg et al.[62]

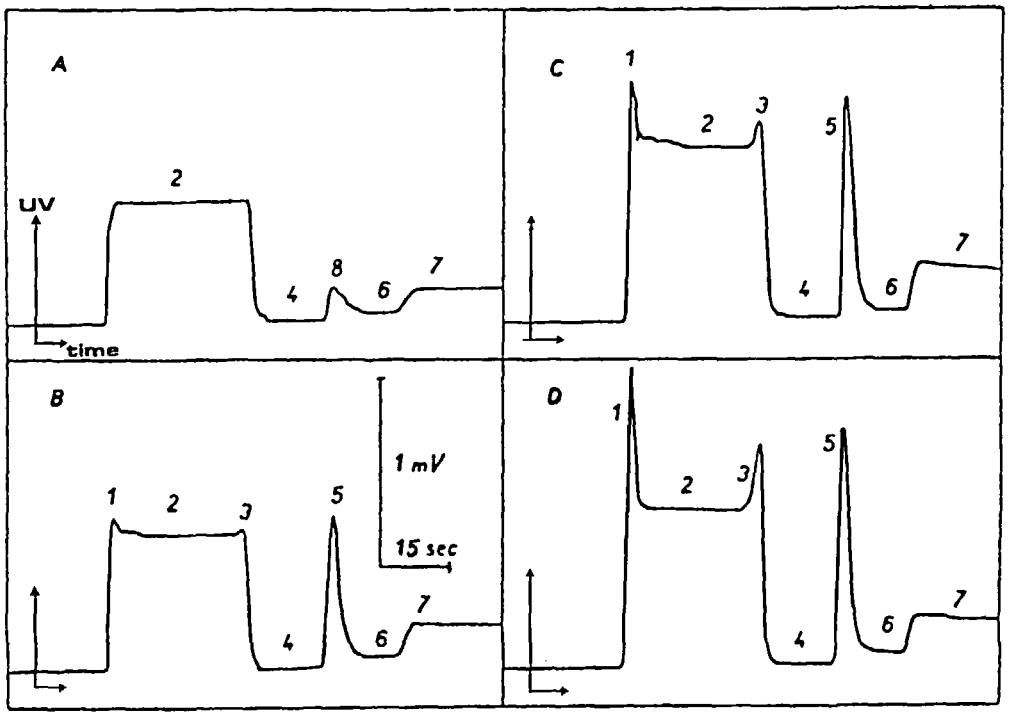


FIGURE 11. Trace analysis of naphthalene sulfonic acids (NSA). Zones: 1 = 1,3,5-naphthalene trisulfonic acid; 3 = 1,5-naphthalene disulfonic acid; 5 = 1-naphthalene sulfonic acid; 2 = sulfoisophthalic acid; 4 = malonic acid; 6 = β-chloropropionic acid; 7 = phenylacetate; 8 = unidentified impurity. Concentration of individual NSA compounds in the sample: A, no sample; B, 4μ*M*; C, 8 μ*M*; D, 16 μ*M*. Total amount: B, 16 pmol; C, 32 pmol; D, 64 pmol. (From Svoboda, M. and Vacik, J., *J. Chromatogr.*, 119, 539 [1976]. With permission.)

theophylline (Figure 12) down to 25 pmol by measuring peak heights. For most UV-absorbing components there is obviously a range over which peak height is proportional to amount. For low peaks and peaks which are close to the optimal height the reproducibility is poor, however. Gower and Woledge[68] discussed the integration of the total area under the curve. This will make it possible to obtain a linear relationship over the whole range for which separation is possible by applying a mathematical expression. Delmotte[43] also integrated the area under the peak and obtained a straight line relationship for serum protein fractions.

C. Steady-State Mixed Zones and Trace Analysis

In isotachophoretic analysis the concentration in a pure zone at equilibrium is much higher than that which is needed for registering it. Therefore, if a trace component could be forced to move at a much lower concentration than the other ions of the system (but still homogeneous) the threshold for quantitative measurements would be lowered substantially. Wielders et al.[69] and Wielders[67] show elegantly the possibility of quantifying UV-absorbing compounds by establishing what they call steady-state mixed zones. With this technique, the authors quantify ADP by mixing it with acetate. This is done in the presence of large amounts of interfering substances (Figure 13). However, it should be noted that finding the correct operational conditions is difficult and time-consuming, especially if several substances require analysis simultaneously. In practice, large differences in concentrations of the substance to be analyzed and other ionic species

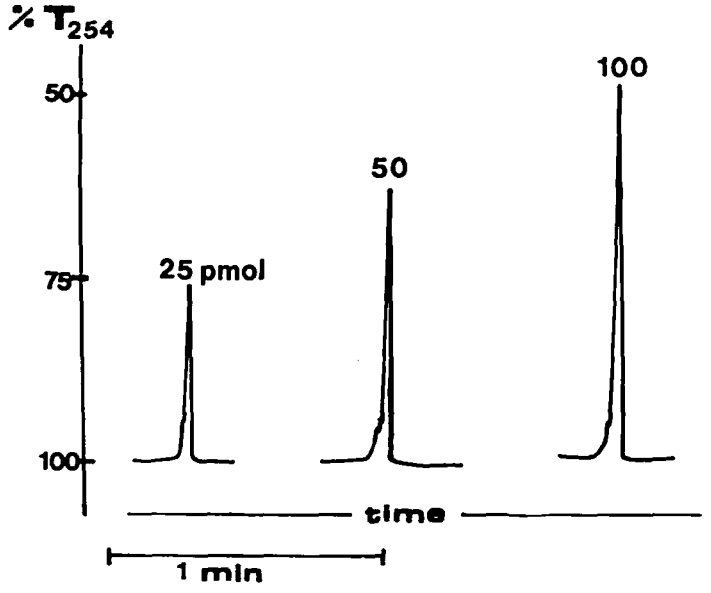


FIGURE 12. Trace analyses of theophylline. (From Moberg, U., Hjalmarsson, S-G., and Mellstrand, T., *J. Chromatogr.*, 181, 147 [1980]. With permission.)

in the sample are not permitted because the steady-state mixed zone condition is readily disturbed and two consecutive zones of carrier and substance are formed.

Holloway et al.[70] have quantified the β-glucuronides of 1- and 2-naphthol by measuring both the height and the width of the single peak. The width gives the total amount and, since the two isomers have a large difference in light absorption under the isotachophoretic conditions, the peak height gives the ratio of the two substances.

A frequent problem in analytical chemistry is to quantify impurities present in small amounts in a certain sample together with a major component. If the mobility difference between the two components is large, it is always possible to inject great amounts of sample and still achieve the desired and needed equilibrium. When an ion is present in small amounts in a dilute solution Ryšlavý et al.[71] suggest the use of a continuous sampling technique; with the use of a precolumn system, Everaerts et al.[72] have elegantly extended the injection of large volumes to include complex samples such as plasma or wine for the quantification of trace components. Both of these techniques are described later on, in Section XIV. If the mobility difference is sufficient and it is only the impurities that are to be quantitated, it is sometimes possible to use the main component as leading or terminating ion in an isotachophoretic system. This is illustrated in Figure 14, in the quantification of aconitic acid in large amounts of citric acid where the citric acid has been used as terminating ion.

X. DETECTION LIMITS

The detection limits in capillary isotachophoresis have so far not been studied in great detail. Everaerts et al.,[16] Wielders,[67] and Svoboda and Vacik[66] give the most extensive discussions on detection limits, and Arlinger[30] hinted early on at the possibility of detecting trace amounts isotachophoretically.

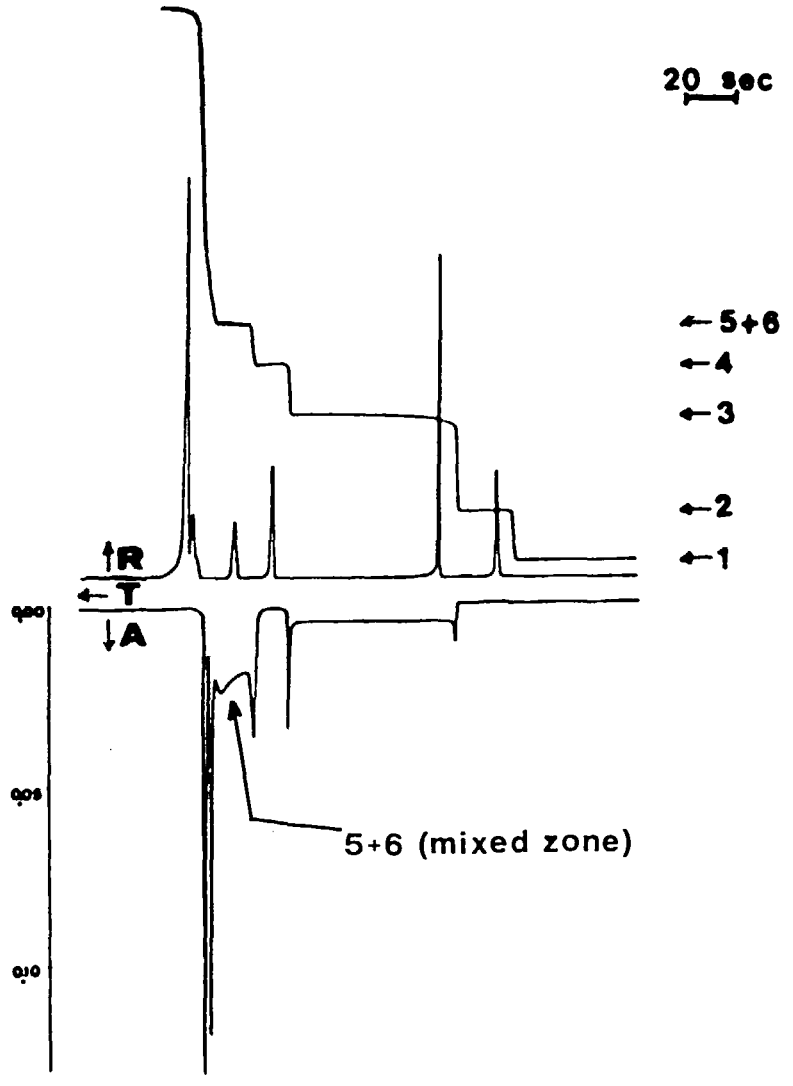


FIGURE 13. Isotachophoretic analysis of a sample that contained 6.23 μM ADP, 1 mM acetate, 2 mM EDTA, 2 mM chlorate, 7.5 mM chloride and 15 mM tris. R = increasing resistance; T = increasing time; A = increasing absorbance ($^{10}\log I_o/I$). 1 = chloride; 2 = chlorate; 3 = EDTA; 4 = bicarbonate; 5 = acetate; 6 = ADP; 7 = glucuronate. (From Wielders, J. P. M. and Everaerts, F. M., in *Electrofocusing and Isotachophoresis,* Radola, B. J. and Graesslin, D., Eds., Walter de Gruyter, Berlin, 1977, 527. With permission.)

The detection limit must always be related to the component to be detected. It is important, therefore, to consider the following when discussing the detection limits:

1. Are the chemicals pure enough?
2. Is the operational system well chosen, i.e., such that the proportion of the electric current carried by the buffering ion is small and the buffering capacity enough, and is the solvent well chosen?
3. Is the detector sensitive enough?
4. Is the time of analysis well chosen, i.e., is the capillary long enough and the diameter well chosen and is the driving current chosen in an optimal way?

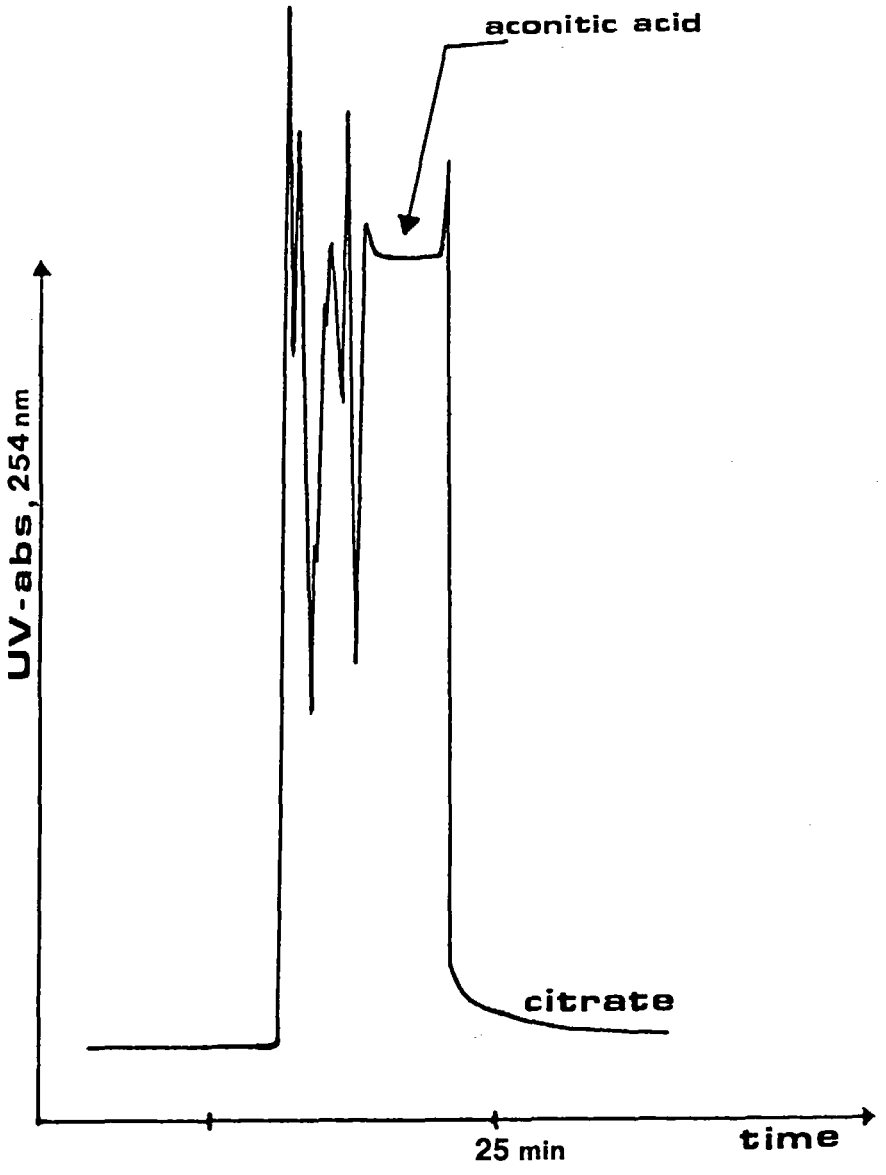


FIGURE 14. Isotachophoretic separation of aconitic acid in a sample mainly containing citric acid. Citric acid is used as terminating ion.

From this it is obvious that there are several different factors which may influence the lower limit of detection. Convincing evidence that demonstrates the variations in detection limits that can be obtained is provided by the difference in resolution attained by thermometric, conductometric, potentiometric, and UV-absorbance detection. A low-resolution detector gives no information about the real detection limits of the isotachophoretic separation process.

The use of electrolytes at low concentration limits the choice of pH and hence the operational systems to be used because of increasing influence of OH^- and H^+ ions in the electrophoretic separation procedure. However, other solvents which alter these limits may be examined. In most aqueous electrolyte systems a 0.002 to 0.005 *M* concentration

of the leading ion is the lower limit in practical work. The driving current balances the velocity of the zones when they pass the detector. It is always possible to elongate the recorded curve by decreasing the detection current. It is possible to do this until the zone-sharpening effect becomes smaller than the diffusion over the zone boundaries. For many common electrolyte systems it seems as if the lower limit of driving current is around 20 μA (with a capillary inner diameter of 0.5 mm).

Arlinger[30] showed in a series of experiments the minimal detectable amount of ATP. When the leading ion concentration was 0.01 *M* the minimal amount needed for detection by the UV detector (UV slit width 0.2 mm) was found to be about 25 p*M*. By diluting the leading electrolyte 20-fold (i.e., leading ion concentration was 0.0005 *M*) the results showed that a few picomoles of ATP could be detected. Arlinger[30] pointed out, however, that a further dilution of the leading ion is of doubtful value since the boundary sharpness is considerably decreased at a lowered concentration.

Another effect of dilution is that impurities from the solvent, glassware, etc., can easily contaminate the sample and can give rise to nonreproducible results. The study of detection limits and resolution in Arlinger's work was restricted to the UV detector. The same investigation could be made for the thermal detector, but this is known from experience to have about 1/50 of the resolution of the UV detector.

Everaerts et al.[16] have investigated the minimal amount needed for detection by the method of conductivity determination. It was found to be the same as that of the UV-detection method. Everaerts et al. further have thoroughly investigated the changes in detection limit produced by diluting the leading ion. It was found that a dilution by a factor of ten decreased the limit of detection only by a factor of about two. This is ascribed to the poor development of the boundary profile at the low electrolyte concentration (because the electroendosmotic flow cannot be sufficiently suppressed), and to the increase in electrophoretic transport by the more mobile ions (impurities, H^+ and OH^-). Changes in the temperature of the thermostated capillary tube were shown to have only a slight effect on the detection limit.

Wielders[67] has theoretically calculated the minimal detectable amounts of sample where he has taken into account the inner diameter of the capillary, the detection method used, the leading ion concentration and the injection volume. However, it must be mentioned that the theoretical considerations do not take into account the parabolic appearance of the boundary.[16] The curvature of the boundary is related to the temperatures of the neighboring zones. Hence, a UV-absorbing zone of very short length will be bent in a way that is dependent on the constituents of the zones ahead of and behind it and this will influence the detection limit and resolution.

It is somewhat premature at the present stage of isotachophoretic development to determine the ultimate detection limit of this technique. More research aimed at optimizing detector equipment and operational systems will improve the detection limits in the future. Particularly by using microscale preparation equipment (see Section XIII), it is possible to combine various specific detection techniques and, thereby, decrease the detection limits considerably.

XI. MATHEMATICAL MODELS OF ISOTACHOPHORESIS

It has already been pointed out that the mobilities of the sample ions must be intermediate to those of the leading and terminating ions in order to obtain an isotachophoretic separation. For the separation of metals and strong acids and bases, the literature[33] contains values of the mobilities which will give direct information for the choice of the leading and terminating systems. When dealing with partially ionized material, the problem becomes more complex. It is the effective mobility, rather than the

mobility of the totally ionized molecules, which determines the isotachophoretic behavior of weak ions. As described earlier, the effective mobility of an ion species and its place in the isotachophoretic separation is dependent on the electrolyte conditions in the leading zone. Several authors[1,16,19,24,73-77] have derived equations to describe isotachophoretically moving zones. In practice, all the theoretical models which have been described are essentially based on Kohlrausch's theory.[1] However, it was first through the work of Everaerts,[16,73] Routs,[19] and Beckers[20] that the theoretical models of isotachophoresis were experimentally verified. For the derivation of the formula needed for computation in buffered isotachophoretic systems, some assumptions must be made: the electric current is constant; diffusion, hydrostatic flow, and electroendosmosis are negligible; the cross-section of the capillary tube is constant; the activity coefficients are equal to one; and the influence of the temperature on electrophoretic effects is negligible. For the computation, the following must be considered: equilibrium equations; isotachophoretic conditions; mass balance of the buffer; electroneutrality principle; and the balance of electric current.

Computer programs with the assumptions mentioned above have been developed by Everaerts,[73] Routs,[19] Beckers,[20] and Ryser[24] for the computation of quantities such as:

1. Concentrations of sample and buffer ionic species
2. Electrical conductivities of the zones
3. pH values of the zones
4. Net mobilities of the ionic species in the zones during the steady state

For the calculations, the composition of the leading electrolyte zone and the ionic mobilities and pK values of all the ionic forms must be known.

Mikkers et al.[31] have very extensively described the fundamental concepts of isotachophoretic resolution, load capacity, and separation efficiency. The isotachophoretic separation process is elucidated using a mathematical model for monovalent weakly ionic constituents. The influence on the separation process of operational parameters, (pH, electric driving current, sample load, and counter constituents) is described in terms of resolution time, detection time, and load capacity. The theoretical results are verified by real experiments. It was clearly shown that the pH of the leading electrolyte is the best parameter for optimization, whereas, for example, the pH of the sample has only a slight influence on the optimization of the separation system.

XII. INSTRUMENTATION

A. Early Achievements

In 1963 the first article on isotachophoresis in a capillary column with a detector arrangement was published by Konstantinov and Oshurkova.[8] The separation took place in very primitive equipment, consisting essentially of a quartz capillary with an inner diameter of 0.1 mm. An electric field of 20 to 50 V/cm was applied over the capillary. In this way it was possible to separate a large number of metal ions such as $K,^{+}$ $Na,^{+}$ $Ca,^{2+}$ $Mg,^{2+}$ $Th,^{4+}$ $Al,^{3+}$ $Fe,^{3+}$ $Zn,^{2+}$ and $In.^{3+}$ H^{+} was used as leading ion and Cd^{2+} as terminating ion. The interfaces between the separated sample zones were detected by measuring the differences in refractive indices and the lowest detectable quantity for most of the compounds was about 10^{-7} to 10^{-8} g. Konstantinov and Oshurkova also indicated the possibility of registering the sample zones by measuring specific resistances and temperatures.

In 1967 Martin and Everaerts[14] published an article on displacement electrophoresis (an early name for isotachophoresis) in which they described an apparatus consisting of a

thin glass capillary (length 1 m, inner diameter 0.4 mm). Each end of the capillary was connected with its own four-way tap to a separate electrolyte reservoir. The reservoirs, of infinite volume compared with the tube, contained an anode and a cathode, respectively. The sample was introduced into the capillary and a constant current was applied between the electrodes. The relative temperature differences were measured by a thermocouple (copper-constantan wire) fixed to the capillary wall. A recorder connected to the thermocouple registered the series of increased temperatures in the consecutive sample zones migrating past the thermocouple. The registered thermal steps were simultaneously differentiated in order to obtain a more convenient measurement of the position of the zone boundaries.

In 1969 Fredriksson[78] described an apparatus for isotachophoresis, basically consisting of a narrow U-tube of glass surrounded by a cooling mantle of glass. The separated sample zones passed a pair of conductance electrodes. The conductance electrodes were coupled to an AC circuit and measured the conductivity in each zone migrating past the detector. However, at the end of the 1960s and the beginning of the 1970s, thermal detection was the most often used detection principle in capillary isotachophoresis. In 1970 Everaerts and Verheggen[79] described a greatly improved thermal detector fixed onto the outside of a Teflon® capillary tube (I.D. 0.45 mm). The capillary was embedded in a groove in an aluminium block and the block was efficiently thermostated by circulating cooling water through it. By thermostating the entire system more accurate values, quantitatively as well as qualitatively, were obtained. A secondary but very important effect was the shorter analysis times, since the increased heat transfer meant that higher currents could be used. Due to the diffusion of heat in the capillary wall along the axis of the tube, the infinitely sharp zone boundaries cannot all be registered by a thermal detector. In order to achieve a more highly resolved record of the true separation of the zones, a UV-absorption detector was developed by Arlinger and Routs.[58] In their paper, separation experiments with hemoglobin and ceruloplasmin were discussed. The UV detector was a modified commercial UV photometer which was mounted after a thermal detector. A comparison of the signals from the UV and thermal detectors clarified the necessity of using UV detection, e.g., protein analyses by isotachophoresis. The thermocouple does not fully reproduce the sharpness of the zone boundary. Because of its working principle, however, the thermal detector is a universal detector. A highly resolving universal detection method was therefore an obvious need.

In 1972 Everaerts and Verheggen[80] presented their excellent work on conductivity detection in a newly constructed apparatus. The capillary tube was made of Teflon® (I.D. 0.45 mm) and was cooled directly with kerosene. The direct cooling with a nonconductive organic solvent proved to be much more efficient than the previous use of a thermostated aluminium block and, thus, higher current densities could be applied. This resulted in both a shorter time for analysis and a higher resolving power. The conductivity electrodes were mounted at one end of the capillary tube. The electrodes were made of thin platinum foil (0.1 mm). For the measurement of the resistance between the electrodes, both a DC method and an AC method are described in detail with several application examples in reference.[16] The development of a highly sensitive conductivity detector by Everaerts et al.[80] and the previously developed UV detector by Arlinger and Routs[58] were very great steps forward in the applicability of capillary isotachophoresis to analytical chemistry.

The development of a potential gradient detection system by Haruki and Akiyama[81] in 1973 was also an important contribution to the previously developed detection methods. Haruki and Akiyama[81] also made an important contribution in the development of what they called potential gradient detection (same in the DC method). Intensive work has been carried out by several research groups in order to refine the basic concepts of

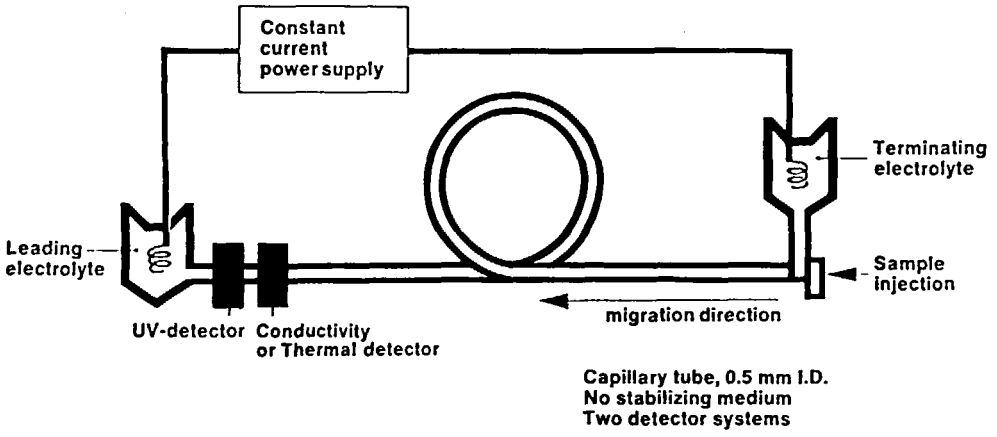


FIGURE 15. Schematic diagram of the LKB 2127 Tachophor.

thermal, conductivity, UV, and potential gradient detection used in capillary isotachophoresis. Several articles along these lines have been published.[16,82-85]

B. Commercial Instrumentation

In 1974, the first commercially produced apparatus for capillary isotachophoresis was introduced,[30,86] the LKB 2127 Tachophor (LKB-Produkter AB, Bromma, Sweden). The equipment (see schematic diagram in Figure 15) contains two detectors, one thermal and one for UV absorbance. The separation takes place in a Teflon® capillary (I.D. 0.5 mm). The capillary and the detectors are surrounded by kerosene. The liquid is thermostated by Peltier elements, so that separation can be performed at any temperature from 3 to 29°C. Four different lengths of capillary are available (230, 430, 610, and 800 mm). The power supply can supply up to 30,000 V with a constant current ranging from 0 to 500 μA. The very high outputs give high resolution and short separation times (often 5 to 30 min). Very recently, LKB-Produkter introduced a conductivity detector built into the instrument. The detector has all the advantages of conductivity detection and also the possibility of working at high voltages (up to 3.0 kV to ground), which is often necessary when analyzing low-mobility compounds.

The second company presently offering an instrument for isotachophoretic analyses is Shimadzu Seisakusho Ltd., Kyoto, Japan. In 1974 Shimadzu introduced an apparatus called IP-1. It has later been slightly modified to the presently available instrument IP-2A. The apparatus is equipped with two detectors (UV and potential gradient detectors). The capillary, made of Teflon®, can be selected in various lengths and is air-cooled. The capillary system is filled with electrolytes by the help of gas pressure.

XIII. PREPARATIVE CAPILLARY ISOTACHOPHORESIS

A. Introduction

One restriction in the use of capillary isotachophoresis was, for a long time, the impossibility of investigating the highly resolved sample zones with other than the "ordinary" detectors, i.e., UV absorbance, thermal, conductivity, or potentiometric detectors. Nor was it possible to use capillary separation as an integral step in preparation procedures. It was, therefore, considered of great value to use capillary isotachophoresis as a preparative procedure without losing the rapidity and the resolving power inherent in the method. The first successful experiments were made as early as 1970, but they were

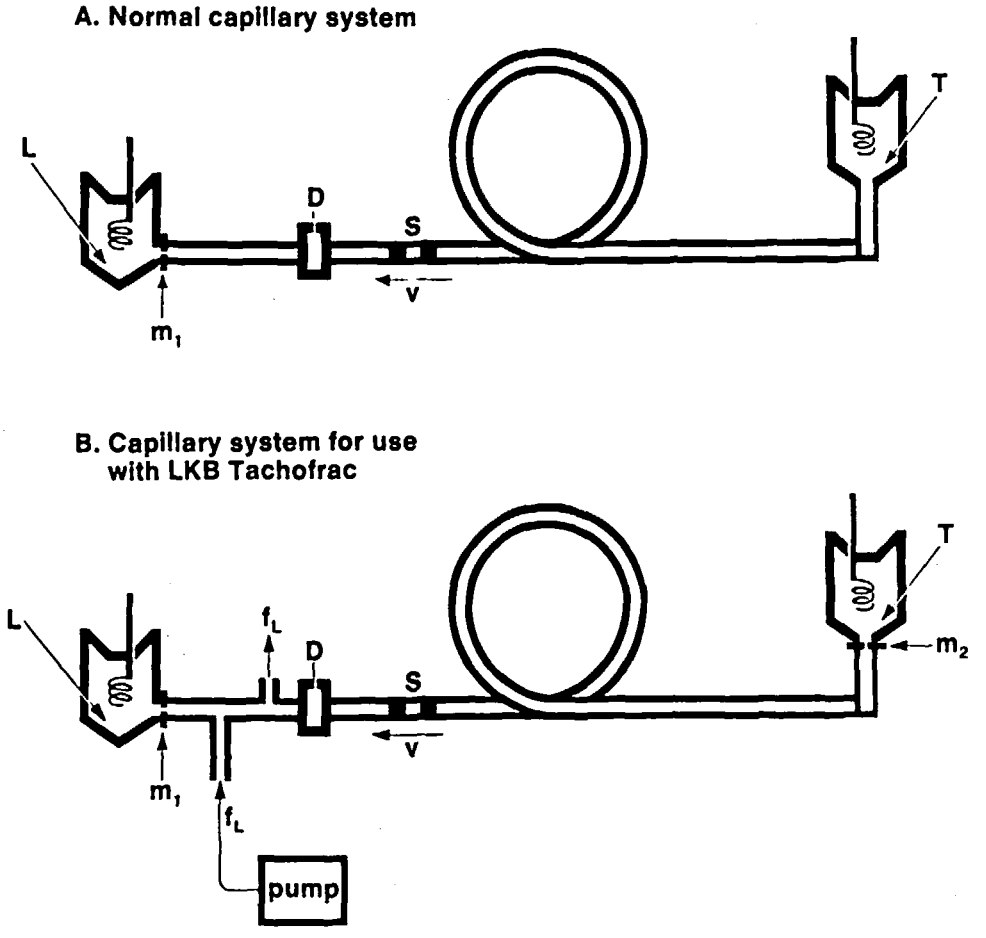


FIGURE 16. Principle of preparative capillary isotachophoresis. (A) Schematical diagram of basic analytical equipment; (B) preparative version with an additional flow of leading electrolyte. L = leading electrolyte; T = terminating electrolyte; D = UV detector site; S = sample ions; v = migration velocity; m_1, m_2 = semipermeable membranes; f_L = flow of L to elute the sample zones.

only made use of several years later when Arlinger[87] developed a fraction collector device for capillary isotachophoresis.

Figure 16A shows a schematical diagram of a basic analytical instrument (as described earlier), where m_1 is a semipermeable membrane separating the leading electrolyte reservoir from the capillary compartment in order to prevent hydrodynamic flow. The technique chosen for sample collection was to use a T-tube through which the sample is swept by a flow of leading electrolyte. This is shown schematically in Figure 16b, where m_2 is a membrane inserted in the terminator reservoir to prevent a counterflow through the whole capillary. After passing the UV detector, the zones will be swept out through the T-tube, provided that the liquid flow rate from the pump is greater than the corresponding migration rates of the ions. As the liquid flow will dilute the sample, the flow rate is set only a few per cent higher than the migration rate. The transport situation for the leading sample and counter-ion at the site of the T-tube has been thoroughly investigated and described in the article by Arlinger.[87]

B. The Fraction Collector Device

The sample zones may have volumes as low as 10 to 20 nℓ, and traditional fraction collection is not easily used. In the apparatus designed by Arlinger, the sample zones were collected on a cellulose acetate strip passing by the outlet of the T-tube. The cellulose acetate strip was chosen because it can be obtained in thin sheets with a smooth surface. It is also easy to handle and is a generally well-known and acceptable material. The identification of sample positions on the strip and recorder chart is a vital part of the procedures. Arlinger[87] solved this problem by a simultaneous and automatic marking of the strip and chart. Identification of the position of the sample is, therefore, reduced to noting the starting positions of the marks on the strip and chart, respectively. Since the T-joint is placed after the UV slit, there is a constant time difference between measurement of the UV absorbance of an ion and the moment the ion reaches the strip. This constant need be established only once for a given leading electrolyte, by simply running a colored compound, and is valid for a specific current and the corresponding liquid flow rate.

The initial development of a fraction collector device has been further refined in the present commercially available LKB 2127 Tachofrac, which is a preparative accessory to the previously described LKB 2127 Tachophor.

The article by Arlinger[87] gave several examples where the collected zones were detected and identified by methods new to capillary isotachophoresis, e.g., immunological and zymogram techniques, as well as radioactivity methods. Moberg et al.[88] and Kjellin[89] have shown the use of immunological detection and identification of isotachophoretically separated serum and cerebrospinal fluid proteins (see Section XV.G.).

XIV. RECENT DEVELOPMENTS IN INSTRUMENTATION

A. Continuous Sampling

An essential characteristic of quantitation in isotachophoresis is that the length of a zone is proportional to the amount of a component injected. It is, therefore, obvious that the zone must be long enough to be measured, i.e., the component in question must be present in an amount exceeding the minimum detectable amount. A very dilute sample must be injected into the column in a large volume. However, when injecting a large volume of sample solution, the sample will occupy a large part of the capillary column and the separation path is therefore reduced. Moreover, a long column containing a low-concentration sample suffers from considerable electric resistance, which prevents the use of higher electric currents in order to keep the separation times short. The construction of the capillary (small total volume) also limits the volume of the sample solution injected.

Ryšlavý et al.[71] have recently developed a method for sampling that permits an increase in the total volume of the sample solution in the isotachophoretic system so that initially dilute sample components can be detected and evaluated. The method is based on the simultaneous pumping of the leading electrolyte and the sample into the isotachophoretic column. A schematic diagram of the arrangement is shown in Figure 17. A Teflon® capillary tube was inserted via a septum into the injection port of the LKB 2127 Tachophor. The capillary was connected by means of a 200 μℓ micropipette to a homemade electroosmotic pump.[90] Similarly, another capillary connected the counterflow input to a second electroosmotic pump. After the electric current had been switched on, the rate of movement of the boundary between the leading and terminating electrolytes was decelerated to 5% and the sample was pumped into the injection port

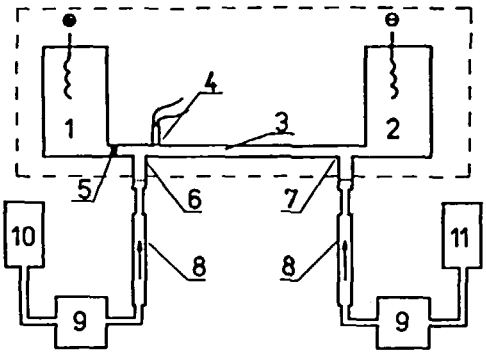


FIGURE 17. System for the continuous sampling technique (anionic separations). 1 = anode; 2 = cathode compartments; 3 = capillary; 4 = detectors; 5 = membrane; 6 = counter-flow input; 7 = injection port; 8 = micropipette; 9 = electroosmotic pump; 10 = leading electrolyte; 11 = sample reservoirs. (From Ryšlavý, Z., Boček, P., Deml, M., and Janák, J., *J. Chromatogr.*, 147, 369 [1978]. With permission.)

with a pumping rate experimentally adjusted so that the sample was not flushed out of the capillary into the terminating electrolyte reservoir. By using the continuous-sample technique, Ryšlavý et al. showed experimentally an increased operational capability towards lower concentrations by one to two orders of magnitude.[71]

B. Preseparation Column and Column-Coupling

Substances to be analyzed are very often present in a solution together with numerous other substances at higher concentration, and sample pretreatment procedures such as extraction and derivatization are, therefore, often necessary. In isotachophoretic analyses other methods can also be used to obtain qualitative and quantitative information about sample components present at low concentrations. Examples of such methods are the use of counterflow, steady-state mixed zones, and continuous sampling, which have all been described previously. However, these methods all show various drawbacks and therefore the preseparation column and column-coupling system by Everaerts[72] is probably the most efficient technique for isotachophoretic determination of substances at low concentrations in complex mixtures.

The equipment designed by Everaerts et al.[72] is shown schematically in Figure 18. Two Teflon® narrow-bore capillaries with different internal diameters are used. In the so-called preseparation column (5) (inner diameter 0.8 mm), a high preseparation current is permitted, c. 300 to 400 μA. At a well-defined distance from a conductivity detector (the so-called "tell-tale" detector) (6) mounted in the preseparation column, the final separation column (12) is coupled to the preseparation column via a T-connection (7) bifurcation. The narrower internal diameter (0.2 mm) of the separation column permits work at a higher current density during the final separation. Detection is by means of both UV-absorbance (13) and conductivity (14) detectors. The first (tell-tale) detector (6) registers the preseparation, and as soon as the zones of interest pass this detector, the electric current is switched over onto the analytical column (the time for a zone to migrate from the tell-tale detector [6] to the bifurcation [7] is determined in advance). The trapped zones can be further separated in the analytical column in an electrolyte system, if necessary, different from that applied during preseparation. In this way a zone or zones of interest can easily be isolated even if they are not migrating consecutively, and high

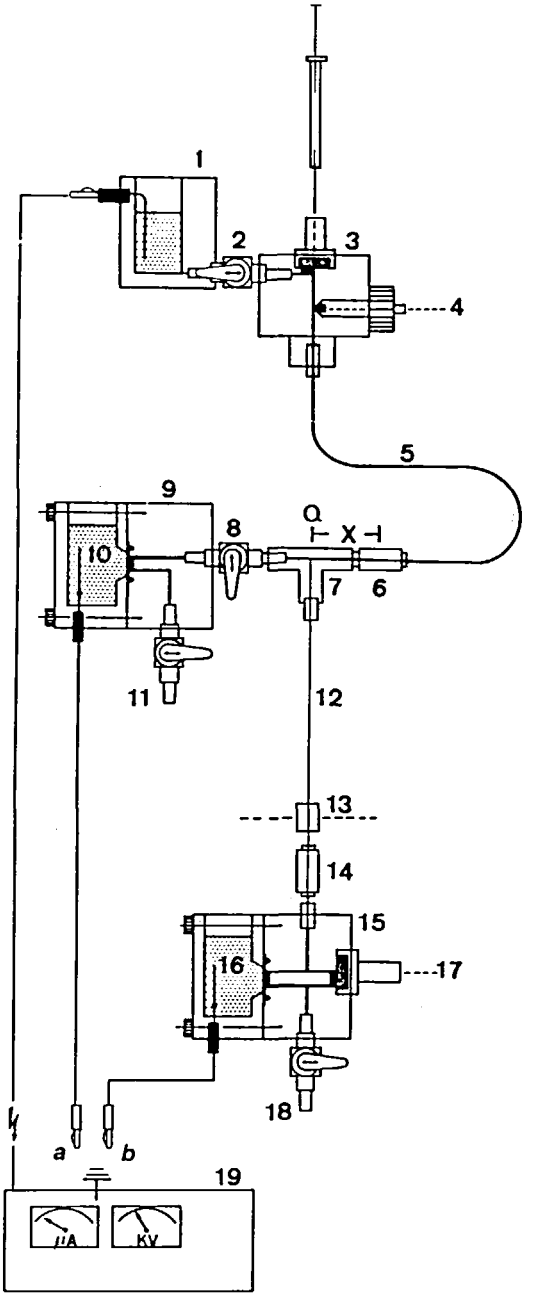


FIGURE 18. Device for column coupling in isotachophoresis. 1 = compartment filled with terminating electrolyte; 2 = PTFE-lined valve (1MM1; Hamilton, Bonaduz, Switzerland); 3 = injection block with septum; 4 = connection towards drain; 5 = preseparation narrow-bore tube (0.8 mm I.D.); 6 = conductivity detector; 7 = T-piece with a bore of 0.8 mm and a bore of 0.2 mm; 8 = PTFE-lined valve; 9 = counter-electrode compartment with semipermeable membrane; 10 = counter electrode, immersed in doubly distilled water to be used in mode a (preseparation); 11 = PTFE-lined valve; 12 = separation narrow-bore tube (0.2 mm I.D.); 13 = photometric detector (256 nm); 14 = conductivity detector; 15 = counter-electrode compartment with semipermeable membrane; 16 = counter electrode, immersed in doubly distilled water to be used in mode b (final separation and detection); 17 = septum through which a counter-flow of electrolyte can be applied; 18 = PTFE-lined valve; 19 = current stabilized power supply. The time needed by all the zones to migrate over a distance x is well defined under chosen conditions (i.e., isotachophoresis). (From Everaerts, F. M., Verheggen, Th. P. E. M., and Mikkers, F. E. P., *J. Chromatogr.*, 169, 21 [1979]. With permission.)

concentrations of, e.g., salt ions can be allowed to quickly pass by the bifurcation and migrate towards the counter-electrode compartment (10).

A major benefit of using the device with coupled columns is its flexibility in the use of different operational systems within an individual run. In this way a considerable gain in selectivity can be obtained. Everaerts gives an example[72] in which two totally different operational systems in the preseparation and final-separation columns are used. In this way it was possible to separate a mixture of phosphate and lactate in a solution in which formate was present in high concentration. In summary, the column-coupling system offers several advantages:

1. High sample load is permitted.
2. The total analysis time is kept low even if a large amount of sample is loaded.
3. High ratios of concentrations between the sample species are permitted.
4. Different operational systems can be applied in the separation and preseparation compartments.

XV. APPLICATION AREAS

A. Inorganic Anions

Inorganic anions mostly have high mobilities in an isotachophoretic system and they have frequently been used as model substances. Even with their primitive equipment, Kendall and White[5] succeeded in separating iodide and thiocyanate as early as 1924. In several articles Everaerts and co-workers[13,14,17,18,46,48,52,75,91-95] have described the separation of a large number of inorganic anions. Their work has been compiled in a book.[16] The mobility values are tabulated over the pH range 3.0 to 7.5 and various suitable electrolyte systems are given. On the whole the work has little connection with practical problems, but is an outstanding survey of the possibility of separating common inorganic anions quantitatively.

The halides have almost identical mobilities in water and cannot be separated in any known aqueous electrolyte system. The literature indicates that hexacyanoferrate, $Fe(CN_6)^{4-}$ has a mobility in water higher than that of chloride and, therefore, would be suited as leading ion. Experiments by Everaerts et al.[16] show, however, that Cl^- moves isotachophoretically faster than the hexacyanoferrate ion. It is, however, possible to separate the halides quantitatively by using methanol as solvent for the electrolytes. The differences in mobilities for these ions in water systems and in methanol systems are summarized[16] and a separation is illustrated in Figure 19.

The hydroxyl ion, OH^-, has the highest mobility of all anions in aqueous solution and it is, thus, not possible to determine this ion isotachophoretically in water. In an early paper by Beckers and Everaerts[46] it is shown, however, that OH^- has a substantially lower mobility when methanol is used as solvent and hydroxyl ions can, therefore, be quantified in a methanolic system.

Because of its importance in biological reactions, orthophosphate, PO_4^{3-}, has been determined by several workers.[96-99] Van der Hoeven[100] quantified PO_4^{3-} in dental plaques together with various organic acids. Gower and Woledge[68] have studied the possibility of measuring orthophosphate in muscle extracts, but found that the orthophosphate zone also contained other unidentified compounds originating from the extract. Boček et al.[101,102] have used isotachophoresis to quantify ortho- and pyrophosphate in artificial fertilizers after a 1000-fold dilution. The analysis was done in less than 4 min without any sample pretreatment. The addition of $Na_5P_3O_{10}$ and $Na_4P_4O_{12}$ to the sample did not interfere with the analytical result. In a recent publication, Boček et al.[103] have extended the work to include also quantitative

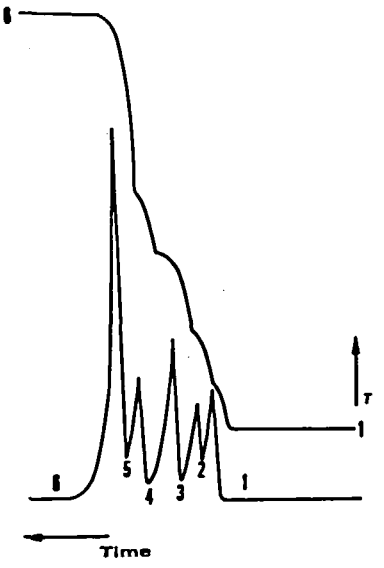


FIGURE 19. Isotachopherogram of the separation of halides in the electrolyte system 50, Table 4 (solvent: methanol, terminating ion: cacodylic acid). Formic acid is included for comparison. 1 = I^-; 2 = Br^-; 3 = Cl^-; 4 = $CHOO^-$; 5 = F^-; 6 = $H_2PO_4^-$.

measurements of nitrate and sulfate. Everaerts et al.[17] showed the quantification of pyrophosphate and orthophosphate together with dexamethazone sodium phosphate. The same paper also shows the improvement of the resolution between nitrate and sulfate by using D_2O as solvent instead of water.

In quality control during the manufacture of alpha olefin monsulfonates and disulfonates, these ions have been determined quantitatively together with inorganic sulfates.[103] The separation was done in a water/acetone system and the analysis time was about 30 min.

Boček et al.[104] have quantified nitrate together with hypophosphite, phosphite, orthophosphate, and lactate in baths from nickel-plating processes. The content of nitrate in the different batches varied from 0 to 14.8% of the total amount of soluble substance in the sample. The analysis time was about 6 min and the relative coefficient of variation about 2%.

Boček et al.[105] have separated the halides quantitatively together with sulfate and carbonate. By using Cd(II) as counter-ion and NO_3^- as leading ion, it was possible to obtain a complete separation of the halides (Figure 20). The technique has also been applied to the quantification of salt anions in mineral water. In the same type of sample Ryšlavý et al.[71] have determined chloride and sulfate, but have included the continuous sampling technique (see Section XIV.A.) to make possible the measurement of the tiny amounts of salts which are available. The authors report standard deviations of 1.4% and 1.0% for chloride and sulfate, respectively.

The analyses of thiosulfate, sulfate and sulfite in factory wastewater, with chloride as leading ion, have recently been described by Akiyama:[106] 50% acetone in water was used as solvent for the electrolytes. Chloride and sulfate in wastewater from a cardboard factory were also determined using hyposulfite as leading ion.

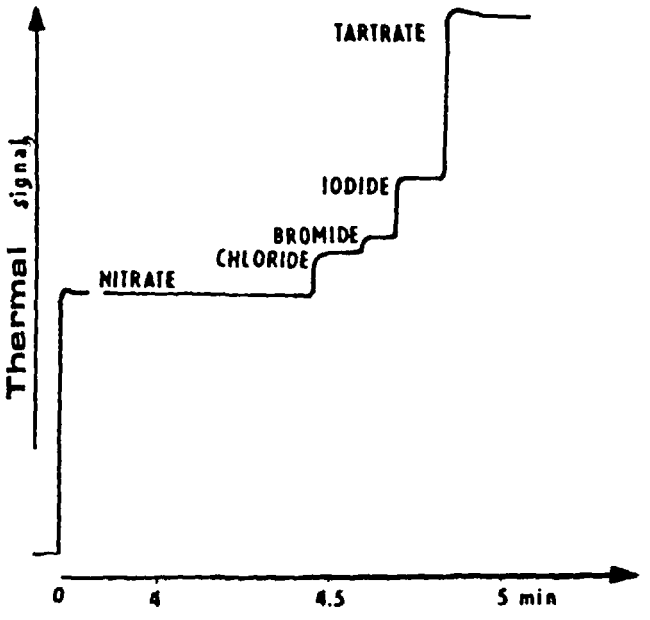


FIGURE 20. Analysis of a mixture of chloride, bromide and iodide. The volume injected was about 2 μℓ and the concentration of each component was about 0.01 *M*. Electrolyte system No. 7, Table 2. (From Boček, P., Miedziak, I., Deml, M., and Janák, J., *J. Chromatogr.*, 137, 83 [1977]. With permission.)

B. Organic Acids

Organic acids are very well adapted for isotachophoretic separations. They can often be separated in a highly dissociated form, but if this fails, the degree of dissociation can be diminished by lowering the pH, and accordingly the effective mobility is reduced to obtain the desired separation. The organic acids are also well adapted for analyses in which organic additives or solvents are added to the leading electrolyte in order to obtain an improved separation.[16] Everaerts et al.[18] have furthermore shown the possibility of altering separation of some acids by using a counter-ion which interacts with the sample ions. The list of aliphatic and aromatic acids which have been analysed isotachophoretically as model substances is very long. Most of them are listed in Reference 16.

An early practical application was published by Everaerts and Konz,[107] describing the quantitative determination of the products formed by the catalytic oxidation of sorbose and fructose. The analyses were performed directly on the reaction mixture and the main component of interest, 2-keto-l-gluconic acid, was determined.

Juices, jams, and other kinds of food contain a great number of organic acids in varying amounts. Such acids are easily quantified isotachophoretically with no sample treatment other than water extraction and/or centrifugation. Everaerts et al.[48,108] compared the acid composition of cherry juice prepared from the pulp with that of the juice obtained by cooking the stones with the remaining pulp. The first juce contained mainly citric acid, whereas the cooked juice contained large amounts of lactate and less citrate. In a later work Everaerts et al.[109] tabulated the content of acids in some citrus fruits and berries. Baldesten et al.[110] showed the separation of ascorbic acid in juice and yogurt as well as the seperation of citrate/isocitrate and cyclamate (Figure 21). Lactate and citrate have been quantified by Yagi et al.[111] in samples of lactic acid beverages. A more extensive survey of the possibility of quantitatively analyzing organic acids in different kinds of food has recently been published by Kaiser and Hupf.[112,113] The authors

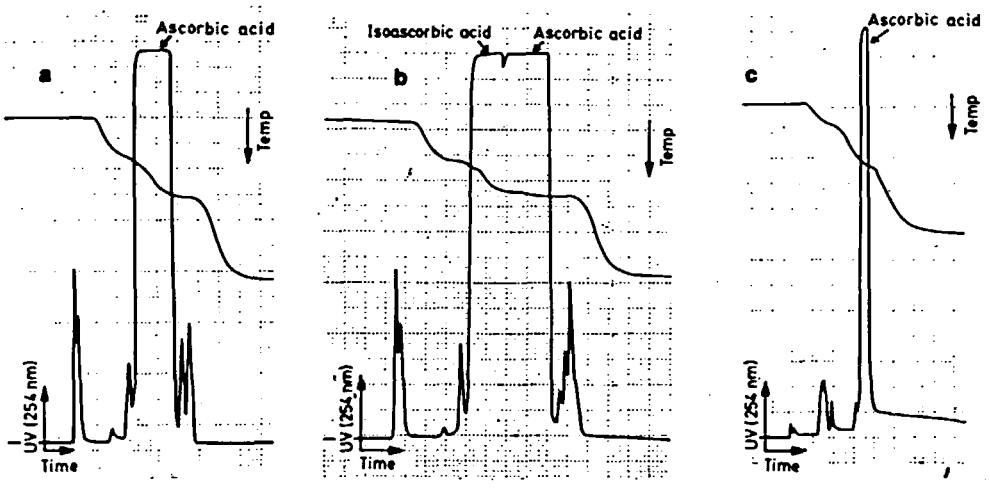


FIGURE 21. Determination of ascorbic acid in orange juice (a,b) and fruit yogurt (c). Injected volume of untreated juice was 1.6 μℓ (a), 2.0 μℓ with isoascorbic acid added (b). The yogurt was centrifuged and 2.0 μℓ of the supernatant was injected (c). Electrolyte system No. 5 (pH_L = 3.97), Table 2. (From Baldesten, A., Hjalmarsson, S-G., and Neumann, G., *Fresenius Z. Anal. Chem.*, 290, 148 [1978]. With permission.)

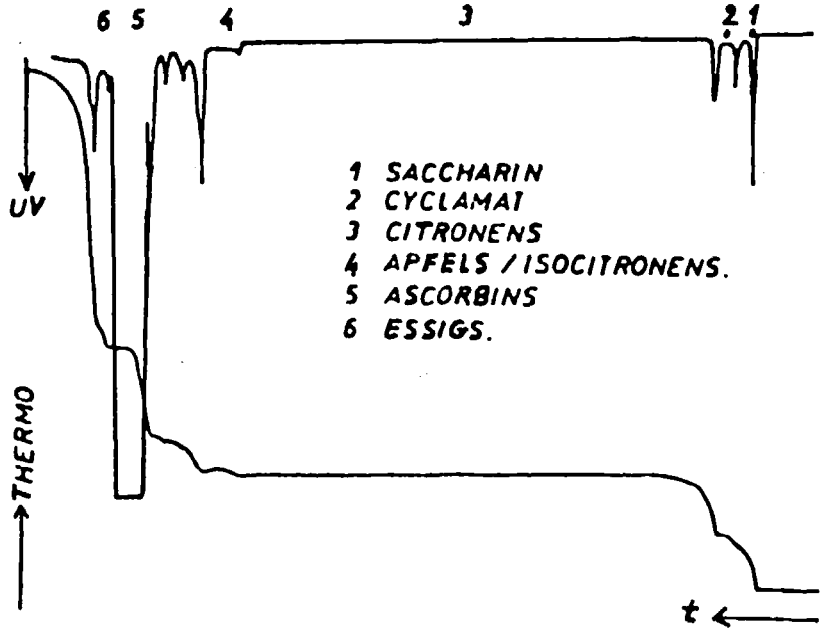


FIGURE 22. Analysis of diet beverage containing saccharin and cyclamate. 3 μℓ injected: 1. saccharin, 2. cyclamate, 3. citric acid, 4. malate and isocitric acid, 5. ascorbic acid, 6. acetic acid. Electrolyte system No. 4, Table 2. (From Kaiser, K-P. and Hupf, H., *Dtsch. Lebensm. Rundsch.*, 75(11), 346, [1979]. With permission.)

found a standard deviation of 2 to 3% for a number of common fruit acids. They also measured the content of acids in different wines and pointed out the possibility of using succinic acid as a marker in following the degree of fermentation. Their articles contain several examples, showing the separation of acids from various kinds of food, some of which are shown in Figures 22 to 24.

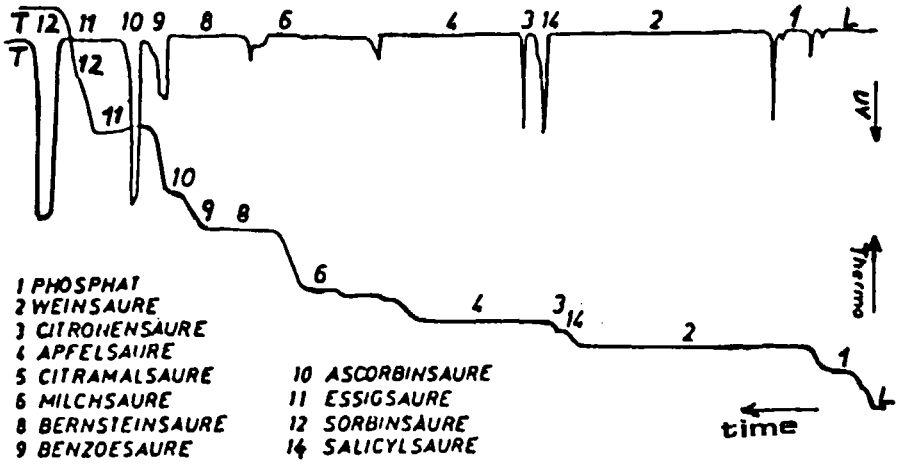


FIGURE 23. Analysis of French white wine (3 $\mu\ell$) to which salicylic acid, benzoic acid, ascorbic acid, and sorbic acid have been added: 1. phosphate, 2. tartaric acid, 3. citric acid, 4. malic acid, 5. citramalic acid, 6. lactic acid, 8. succinic acid, 9. benzoic acid, 10. ascorbic acid, 11. acetic acid, 12. sorbic acid, 14. salicylic acid. Electrolyte system No. 4, Table 2. (From Kaiser, K-P. and Hupf, H., *Dtsch. Lebensm. Rundsch.*, 75(11), 346 [1979]. With permission.)

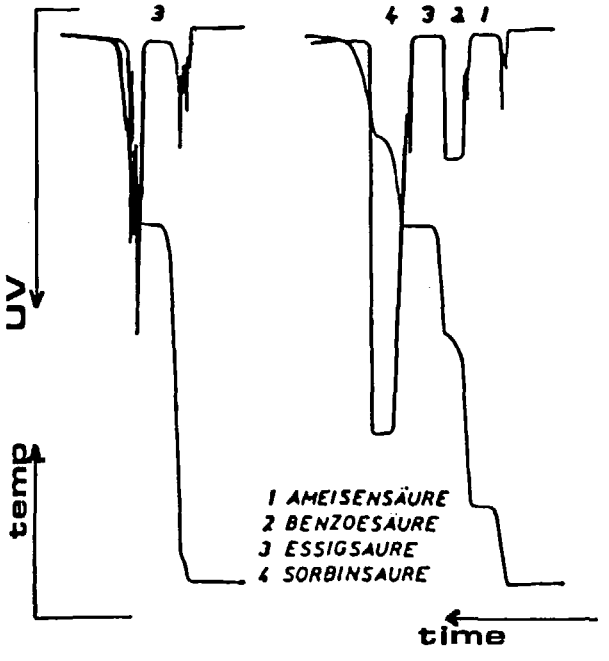


FIGURE 24. Mayonnaise without (left) and with perservatives (right) after water extraction. 3 $\mu\ell$ injected: 1. formic acid, 2. benzoic acid, 3. acetic acid, and 4. sorbic acid. Electrolyte system No. 4, Table 2. (From Kaiser, K-P. and Hupf, H., *Dtsch. Lebensm. Rundsch.*, 75(11), 346 [1979]. With permission.)

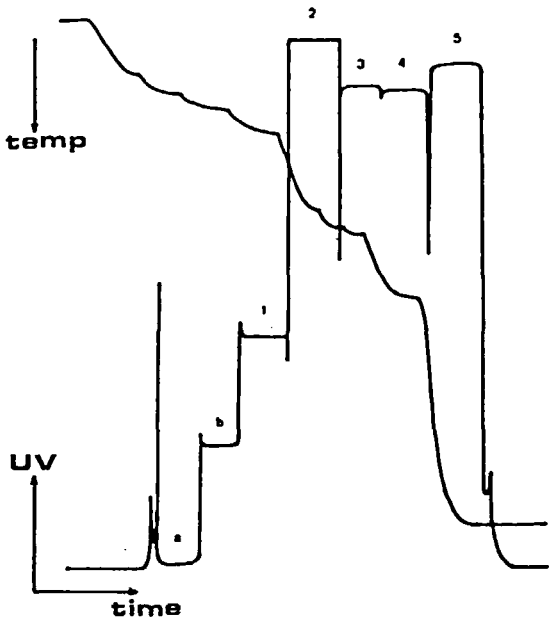


FIGURE 25. Isotachophoretic analysis of preservatives: a. propionic acid, b. salicylic acid, 1. benzoic acid, 2. sorbic acid, 3. p-hydroxybenzoic acid ethylester, 4. p-hydroxy-benzoic acid propylester, 5. p-hydroxybenzoic acid methylester. Electrolyte system No. 36, Table 2. (From Rubach, K., Breyer, C., and Kirkhoff, E., *Z. Lebensm. Unters. Forsch.*, 1, 4 [1979]. With permission.)

Kaiser and Hupf[113] also describe the isotachophoretic separation of several preservatives. Rubach et al.[114] have further studied a number of different acids and esters commonly used as bacteriostatics in food. The isotachophoretic analysis was carried out after removing lipophilic compounds on an Extralut® column ("Extralut", Neues verfahren zur Extraktion lipophiler Stoffe, E. Merck, Darmstadt). The standard deviation was found to be 2% or less. The content of preservatives found in some kinds of food was tabulated. Figure 25 shows a typical pattern from a mixture of preservatives.

The possibility of isotachophoretically analyzing compounds from enzymatic reactions in different organisms was considered quite early on. Dunn and Kemp[97] obtained glucose-6-phosphate and fructose-6-phosphate as a mixed zone in the extract from perfused mouse liver cells. Sjödin et al.[98,99] further analyzed several sugar derivatives. Boček et al.[115,116] studied the Krebs cycle acids and gave mobility curves in the pH interval 3.0 to 8.0. They selected pH 3.8 in the leading electrolyte as the most suitable for this group of acids. Lactate and creatinine phosphate are readily quantified isotachophoretically in extracts from tissues[68,99] without special sample treatment and the reproducibility has been found to be very good.

Microorganisms living on the teeth produce various organic acids as fermentation products. The analysis of these acids is an important tool in the identification of anaerobic bacteria. Van der Hoeven et al.[117,118] have quantified low molecular weight carboxylic acids in dental plaque from gnotobiotic rats inoculated with *Streptococcus mutans* and have also estimated the acid fermentation products from cultures of *S. mutans* grown in various media. An isotachophoretic analysis of rat dental plaque is shown in Figure 26.

The quality of silage extracts has been determined isotachophoretically by Boček et al.,[119] who quantified butyrate, acetate, and lactate without pretreatment of the sample.

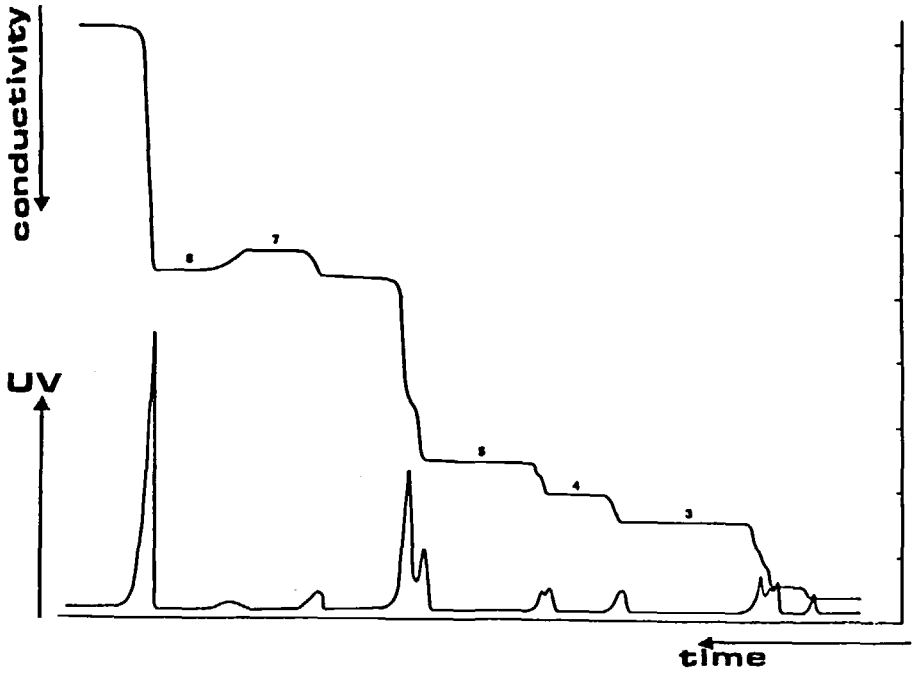


FIGURE 26. Analysis of rat dental plaque: 3. phosphate, 4. acetoacetate, 5. lactate, 7. 3-hydroxybyturate, 8. acetate. (From Van der Hoeven, J. S. and Franken, H.C.M., in *Proc. Ist Int. Symp. Isotachophoresis*, Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980. With permission.)

Urine contains many organic acids which can often give information about biotransformation processes in the body. An excellent example of this has been shown by Sollenberg and Baldesten,[120] who quantified the aromatic acids excreted in urine after exposure to styrene, toluene, and xylene. The acids determined were phenylglyoxylic acid (PGA), mandelic acid (MA), hippuric acid (HA), and methylhippuric acid (MHA). The concentrations of these compounds in urine are drastically increased after occupational exposure to certain organic solvents and, therefore, the use of these biotransformation products as biological indicators after exposure to styrene (PGA and MA), toluene (MA), and xylene (MHA) is of great importance. The acids were recovered by a simple ether extraction and a volume of 1 to 10 $\mu\ell$ was analyzed in the LKB Tachophor. The minimum measurable amount was 0.5 nM and the analysis time was about 20 min. Figure 27 illustrates the analysis of urine from an unexposed person and from a person exposed to styrene. Vesterberg an Sollenberg[121] showed by using isotachophoresis that the excretion of mandelic acid and methylhippuric acid is mainly completed 5 to 8 hr after exposure to the corresponding organic solvent.

The determination of urinary oxalate has been described in a recent series of papers.[122-125,126-128] Accurate determination of urinary oxalate seems to be pertinent to the understanding of renal stone disease and problems related to oxalate metabolism. Isotachophoresis for the determination of urinary oxalate offers the combined advantages of simplicity and avoidance of loss of oxalate with pretreatment procedures since isotachophoresis can be performed with untreated urine.

To verify that the separate zone of oxalate was homogeneous, Tschöpe et al.[122-124] treated the sample with oxalate decarboxylase. The result showed that oxalate was found in two separate zones, one containing the pure oxalate and the other a complex of oxalate

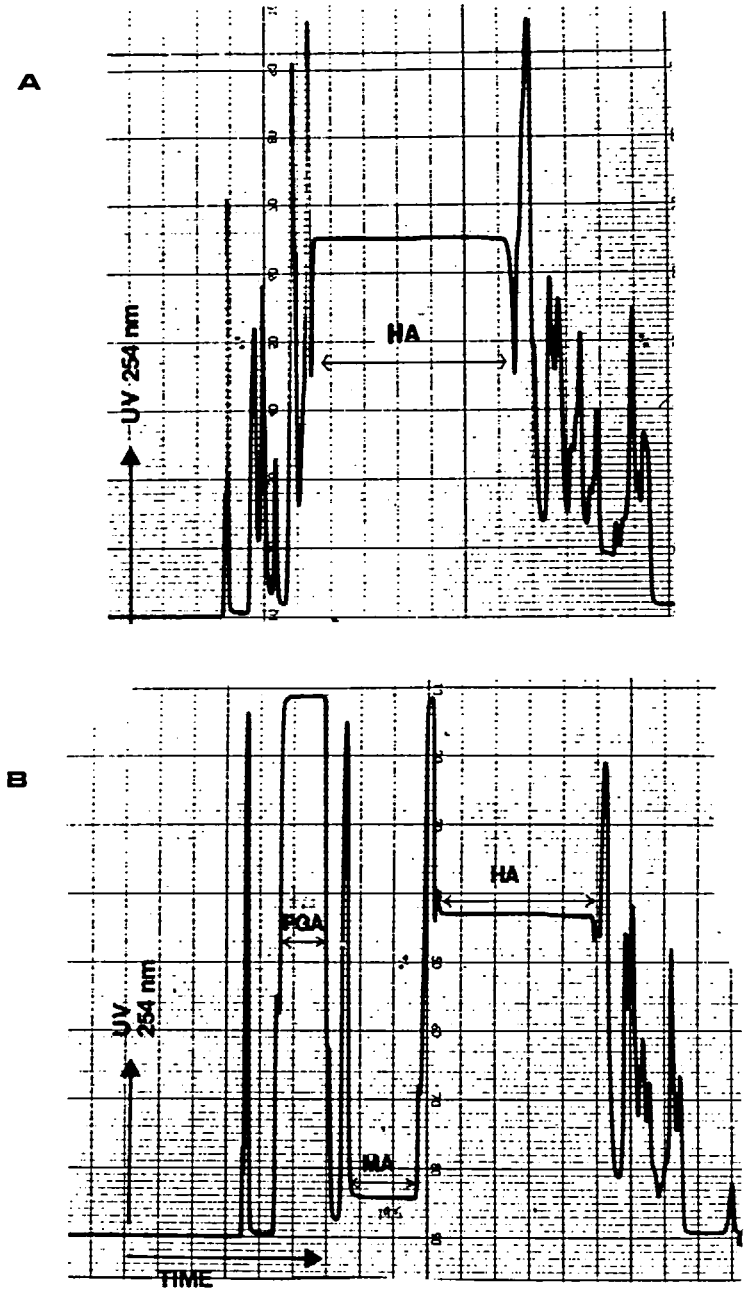


FIGURE 27. A. 2 $\mu\ell$ of urine from an unexposed person. B. 2 $\mu\ell$ of a urine sample from a person exposed to styrene. Phenylglyoxylic acid = 2.4 nmol/$\mu\ell$. Mandelic acid (MA) = 3.6 nmol/$\mu\ell$. Hippuric acid (HA) = 8.0 nmol/$\mu\ell$. Electrolyte system No. 5, Table 2. (From LKB Application Note 301, LKB Produkter AB, Bromma, Sweden. With permission.)

and ferric ions. This finding has been supported by Fredriksson,[129] who studied the formation of oxalate-ferric ion complexes. He has shown that even a very low amount of ferric ions originating, for instance, from the injection needle was sufficient to be detected as an oxalate-ferric ion complex. In order to avoid formation of a complex between the

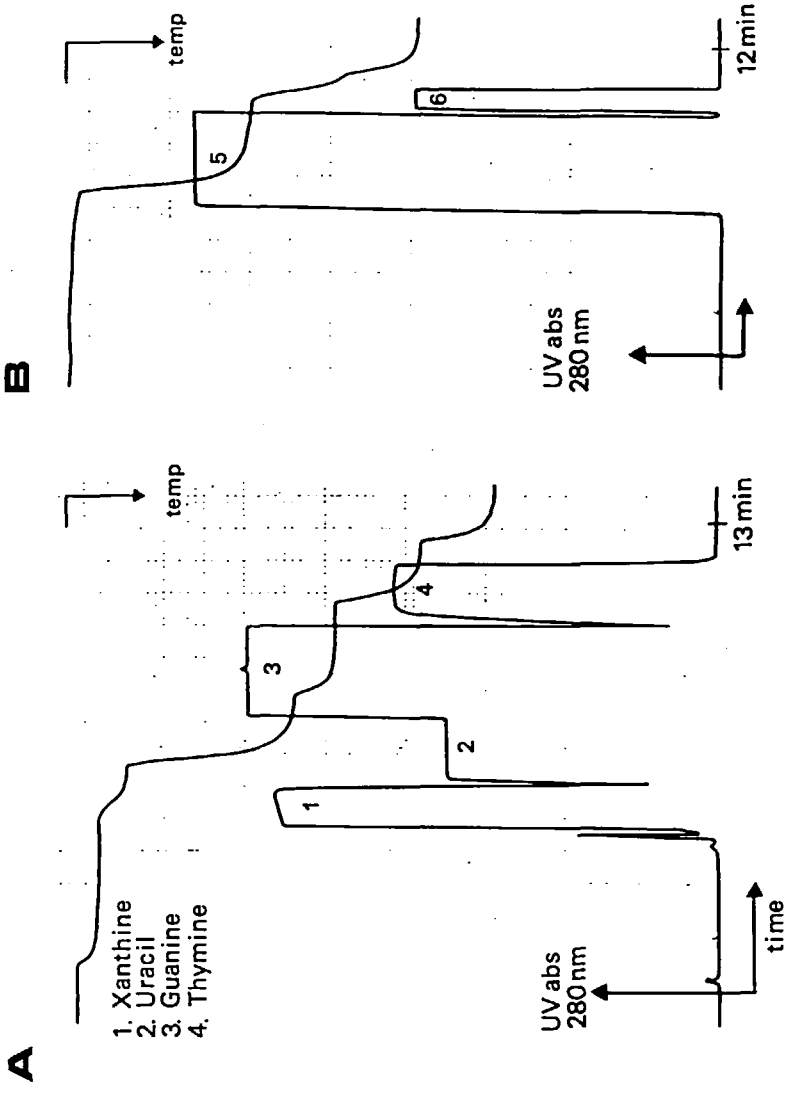


FIGURE 28. A. Separation of xanthine (5 nmol), uracil (7 nmol), guanine (10 nmol) and thymine (6 nmol). Electrolyte system No. 24, Table 2. (pH_L = 7.8, terminating ion: β-alanine). B. Separation of cytosine (15 nmol) and adenine (3 nmol). Electrolyte system No. 43, Table 3. (pH_L = 5.2, terminating ion: α-alanine). (From LKB Application Laboratory, Bromma, Sweden.)

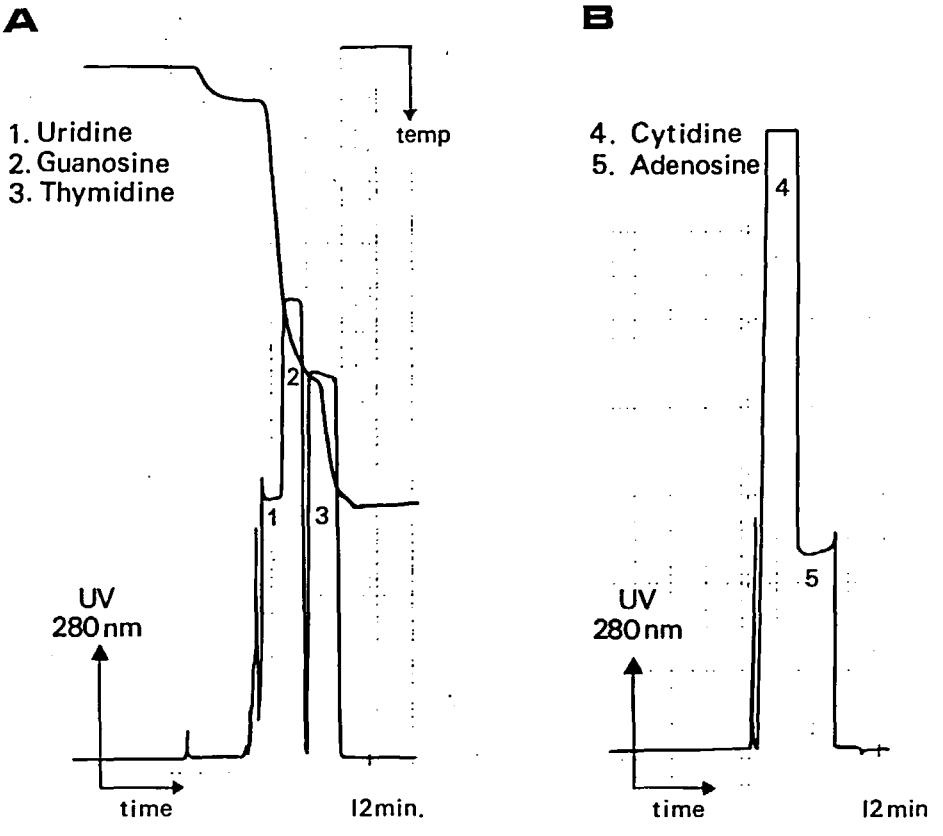


FIGURE 29. A. Separation of the ribonucleosides uridine (3 nmol), guanosine (3 nmol), thymidine 4 (nmol). Electrolyte system No. 36, Table 2 (pH_L = 8.6, terminating ion: ϵ-aminocaproic acid). B. Separation of cytidine (5 nmol) and adenosine (5 nmol). Electrolyte system No. 43, Table 3. (pH_L = 5.2, terminating ion: α-alanine). (From LKB Application Laboratory, Bromma, Sweden.)

oxalate and ferric ions, Tschöpe et al.[122-124] added complexing agents (EDTA or EGTA) to the urine.

Schmidt et al.[126-128] used another approach in the determination of urinary oxalate. The urine was first precipitated with calcium chloride. The calcium oxalate precipitate was dissolved and the oxalate could then be quantified by isotachophoresis down to a few milligrams per liter. The possibility of also quantifying orotic acid in urine has been demonstrated by Tschöpe et al.[124]

Mikkers[130] measured the serum levels of lactate and acetate before and after hemodialysis. The blood was analyzed after centrifugation and dilution with water.

The release of acetate masking the *N*-terminus of peptides has been studied isotachophoretically by Manabe et al.[131] They identified *N*-acetylaspartate and *N*-acetylaspartylglutamate on ninhydrin-negative peptides purified from bovine brain.

Different naphthalene sulfonic acids (see Figure 11) have been quantified down to 16 pmol[66] in a ratio of 1:500, and thymolphthaleinphosphate and tetrasulfonated indigo are other examples of isotachophoretically analyzed complex organic acids.[132]

C. Organic Bases

During the development of the isotachophoretic technique, Everaerts and co-workers used different organic bases (aliphatic and aromatic amines, purines, and pyrimidines)

Table 7
COMPOUNDS SCANNED IN ENZYME DEFICIENCIES

Type of enzyme deficiency		Compound in excess (determined isotachophoretically)
Adenine phosphoribosyl transferase (APRT)		Adenine
Hypoxanthine guanine phosphoribosyl transferase (HGPRT)		Uric acid
Xanthinuria		Xanthine
Purine nucleoside phosphorylase		Deoxyinosine, deoxyguanosine
Adenosine deaminase		Deoxyadenosine as adenine after acid hydrolysis
Orotidine-5'-phosphate pyrophosphorylase	OPRT/ODC	Orotic acid
Orotidine-5'-phosphate decarboxylase	OPRT/ODC	Orotic acid

in model experiments. About 40 different organic bases have been analyzed and are listed in the book by Everaerts et al.[16]

The quantitative isotachophoretic separation of hypoxanthine, xanthine, and uric acid in water solution has been described by Arlinger.[29] Several similar purine and pyrimidine bases have been separated at the LKB Application Laboratory and some experimental results are shown in Figures 28 and 29.

The usefulness of isotachophoresis for screening of inborn errors of purine and pyrimidine metabolism has been shown by Simmonds et al.[133] and Sahota et al.[134] By using a few milliliters of appropriately diluted urine, the pattern of purine and pyrimidine metabolites and related products has been scanned in six purine and pyrimidine enzyme deficiencies (see Table 7). The isotachophoretic technique permits screening to be performed on readily available urine, allows the monitoring of allopurinol therapy, and can confirm adequate drug dosage. Because of its simplicity, the isotachophoretic technique is considered superior to HPLC. Examples of screenings are shown in Figures 30 and 31.

Oerleman et al.[135,136] have recently analyzed serum levels of purines and pyrimidines after removing proteins by ultrafiltration. Uric acid in serum was quantified with a recovery of 100% ± 2%. The protein-bound urate could be estimated if the serum sample was ultrafiltered and the filtrate analyzed. The results were not influenced by natural metabolites or drugs present in the sample.

Theophylline[62] and 5-fluorouracil,[137,138] two commonly used drugs, have been quantified in serum as described in Section XV.J. In a recent work Gustavsson et al.[139] have determined hypoxanthine in serum and tissues.

Kiso[140] studied the mobilities of various monoammonium alkanes (C_1 to C_6 and C_8) and obtained differences which permitted a simultaneous separation of these compounds. Akiyama[141] analyzed monoethanolamine in turning oil to follow its breakdown and, indirectly, the formation of the *n*-nitriso-compounds. The di- and triethanolamines were also separated in the same electrolyte system.

No simple method for quantification of 1,2-diaminomethane has previously been available. Sollenberg and Lundberg,[142] however, recently showed the possibility of using isotachophoresis to analyze the content of 1,2-diaminomethane in air after enrichment in water. They also separated 1,3-diaminopropane, 1,4-diaminobutane (putrescin), and 1,5-diaminopentane (cadaverine) and showed that they did not interfere. The separation of a standard solution of putrescine, spermidine, and spermine in water solution is described in Reference 143.

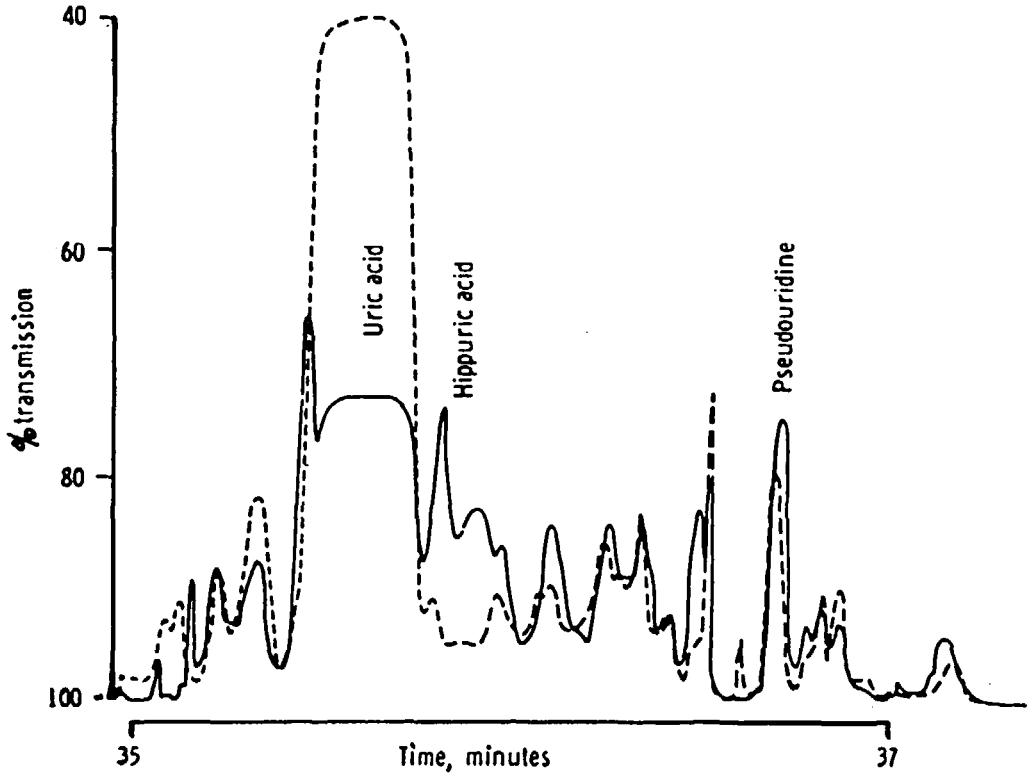


FIGURE 30. Isotachophoretic scan of a urine of a control child. 5 μℓ (1 in 5 dilution) were injected. (——) 254 nm; (----) 280 nm. Electrolyte system No. 24, (pH_L 6.9) Table 2. (From Sahota, A., Simmonds, A., and Payne, R. H., *J. Pharmacol. Meth.*, 2, 263 [1979]. With permission.)

D. Nucleotides and Related Compounds

The introduction of capillary isotachophoresis in the separation of nucleotides has proven to be a great improvement in the resolving power and sensitivity, especially when analyzing nucleotides in complex sample solutions. Beckers and Everaerts[144] investigated the separability of the 5'-substituted mono-, di- and triphosphates of adenosine, cytidine, guanosine, and uridine. The nucleotides were thermally detected and the thermal step-heights were measured for the nucleotides in different electrolyte systems where the pH of the leading electrolyte was varied from 3.4 to 7.0. The curve of thermal step-height vs. pH in the leading electrolyte thus showed (Figure 32) which pH in the leading electrolyte should be chosen when a certain set of nucleotides was to be separated. From this initial work it was obvious that the effective mobility differences between the different nucleotides decreases with increasing pH.

If a complicated mixture is to be analyzed, a low pH for the leading electrolyte is, thus, preferable. This is illustrated in Figure 33, where 13 nucleotides were separated in one run using a 63-cm capillary and a pH of 3.89 in the leading electrolyte.[145] The pH was found to be very crucial for achieving a good separation of all the nucleotides and should be pH 3.9.

Several papers have appeared in which capillary isotachophoresis has been used in the separation and quantification of nucleotides and related compounds in complex sample mixtures. Kopwillem[146,147] described the analysis of ATP, ADP, $NADP^+$, NADPH, glucose-6-phosphate, and 6-phosphogluconate; all the ions involved in the enzymatic transformation of glucose to 6-phosphogluconate. Nanomole quantities of the ions were

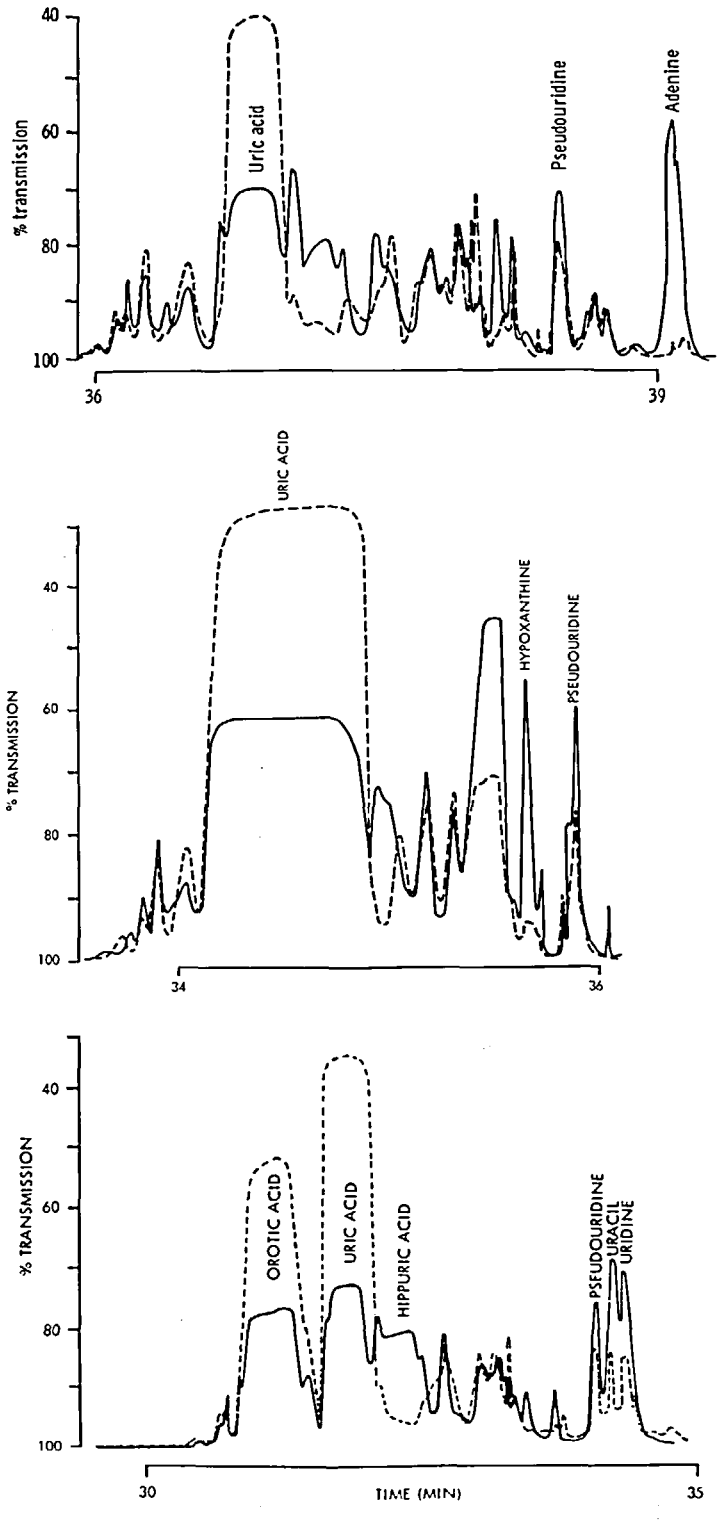


FIGURE 31. Tachophor scan of the urine of: (a) an APRT-deficient child (FD)-5$\mu\ell$ (1 in 10 dilution) were injected; (b) an HGPRT-deficient (RC)-5 $\mu\ell$ (1 in 10 dilution) were injected; (c) an OPRT/ODC-deficient child (DG)-5 $\mu\ell$ (1 in 20 dilution) were injected. (——) 254 nm; (----) 280 nm. Electrolyte system No. 24 (pH_L 6.9), Table 2. (From Sahota, A., Simmonds, A., and Payne, R. H., *J. Pharmacol. Meth.*, 2, 263 [1979]. With permission.)

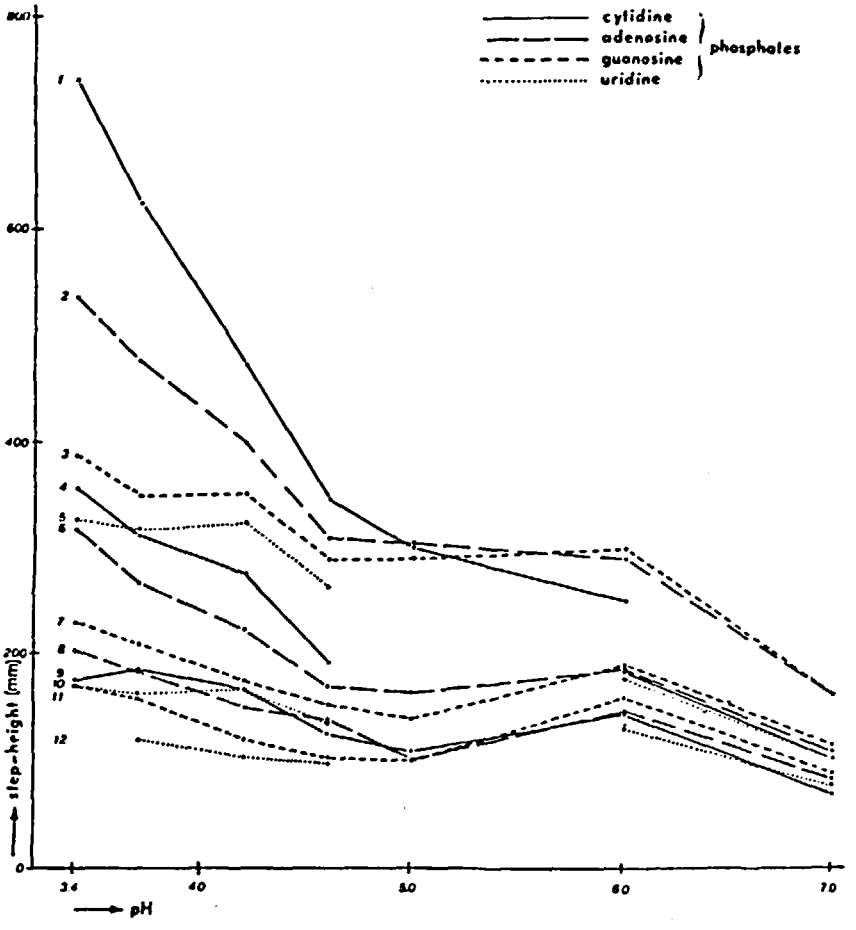


FIGURE 32. Graphical representation of the thermal step-heights, the different systems (pH), 1 = CMP; 2 = AMP; 3 = GMP; 4 = CDP, 5 = UMP; 6 = ADP; 7 = GDP; 8 = ATP; 9 = CTP; 10 = UDP; 11 = GTP; 12 = UTP. (From Beckers, J. L. and Everaerts, F. M., *J. Chromatogr.*, 71, 380 [1972]. With permission.)

separated within 30 min. Sjödin et al.[98,99] determined a great number of metabolites in muscle extracts. From those experiments it was concluded that the isotachophoretic results were in good agreement with corresponding values obtained by conventional enzymatic methods.

In the work of Dunn and Kemp,[97] the analysis of several nucleotides in perchloric acid extracts of perfused liver cells is demonstrated. The nucleotides UTP, GTP, ATP, ADP, and AMP were detected, as well as some other metabolic intermediates. The sensitivity and accuracy of the isotachophoretic method for the quantification of the adenosine phosphates in extracts of whole livers and dispersed cells compared favorably with that of conventional spectrophotometric methods.

In 1976 Gower and Woledge[68] presented in an extensive article the use of isotachophoresis for the analysis of metabolites in muscle extracts. Both methanol and perchloric acid extracts were analyzed and the compounds ATP, ADP, cAMP, AMP, NAD, IMP, inorganic phosphate, phosphocreatine, and lactate were separated. The separation of some compounds which were likely to occur in the muscle extract is shown in Figure 34. Figure 35 shows a record obtained with a real muscle extract. Identification

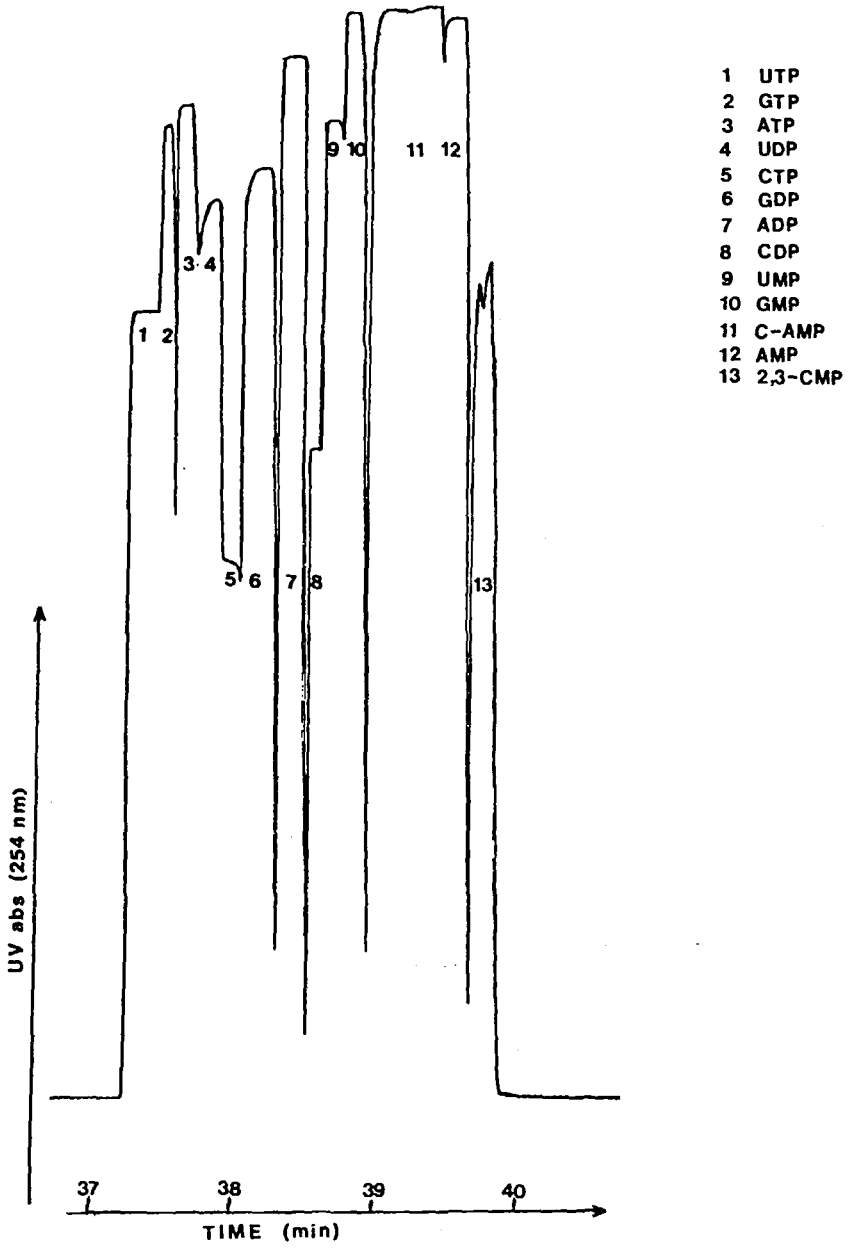


FIGURE 33. Separation of 13 common nucleotides. Electrolyte system No. 5, (pH_L = 3.9), Table 2. (From LKB Application Laboratory, *LKB Isotachophoresis News No. 1*, LKB-Produkter AB, Bromma, Sweden, 1977. With permission.)

of the zones was made simply by analyzing the extract alone and then the extract plus a standard. The purity of the zones were investigated by, for instance, comparing the results from isotachophoresis with those from other analytical methods. Both phosphocreatine and ATP were thus analyzed by enzymatic methods and the results showed very good agreement with the corresponding results from isotachophoresis (for ATP see Figure 36). Gower and Woledge stated the advantages of isotachophoresis to be that it is simple to use, does not require expensive reagents, is convenient when many

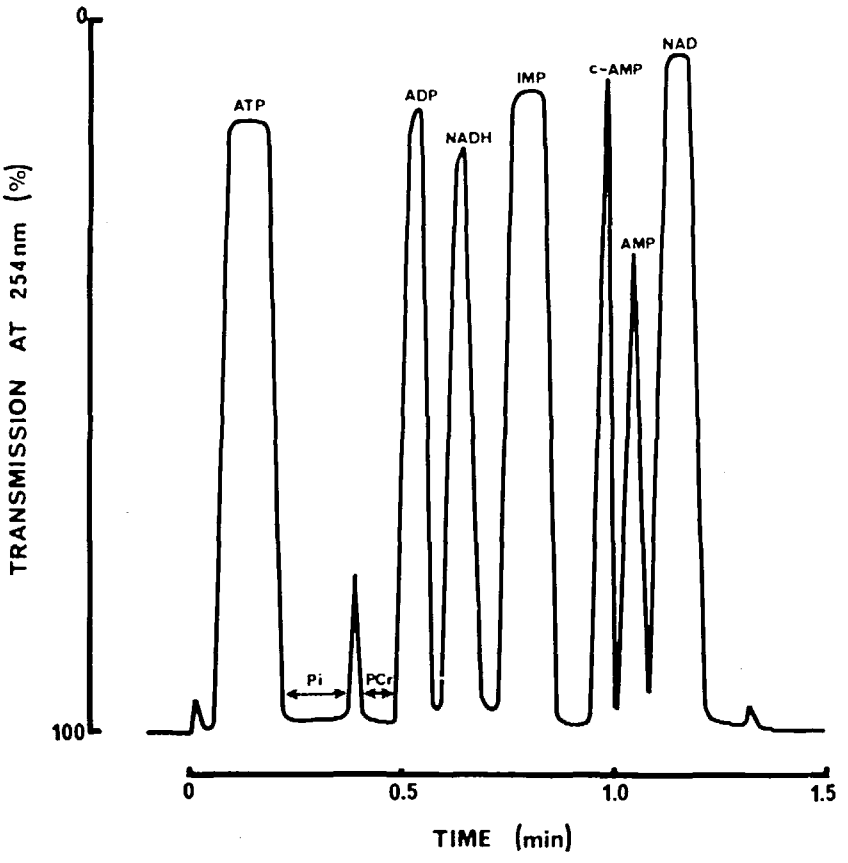


FIGURE 34. Record from a mixture of nine compounds expected in muscle extracts. The amounts of the different substances are as follows: ATP 0.8 nmol, P, 2 nmol, PCr 0.9 nmol, ADP 0.4 nmol, NADH 0.4 nmol, IMP 1 nmol, c-AMP 0.3 nmol, AMP 0.2 nmol, NAD 0.9 nmol. Electrolyte system No. 5, Table 2. (From Gower, D. C. and Woledge, R. C., *Sci. Tools*, 24, 2 [1977]. With permission.)

substances are to be measured on a few samples, is very sensitive for UV-absorbing substances, is simple to calibrate, and requires quite small samples.

In a later article, Woledge and Reilly[44] have slightly modified the conditions to improve the resolution and sensitivity when analyzing, for example, ATP, ADP, and phosphocreatine in muscle extracts. In addition, their previously used normal method for measurement of the UV records[68] has been replaced by electronic integration of logarithmic transformations of the UV transmission signal.

Surholt[148] determined the concentrations of ATP, ADP, and AMP in the body-wall musculature of *Arenicola marina* enzymatically and by means of isotachophoresis after perchloric acid extraction. The nucleotides were quantified after 12, 24, and 48 hr of experimental anaerobiosis and after electrical stimulation under aerobic and anaerobic conditions. The data obtained by the enzymatic method and by isotachophoresis corresponded very closely. The analysis of ATP in blood samples has been performed by Sakagishi[149] where the decrease of ATP content in anemia and in aged human erythrocytes was determined isotachophoretically.

In a series of recently published papers, Holloway et al.[150,151] and Brunner and Holloway[152] have utilized capillary isotachophoresis in an excellent way in following the degree of enzymatic hydrolysis of UDP-glucuronate by pyrophosphatase (E.C. 3.6.1.9) and alkaline phosphatase (E.C. 3.1.3.1). The microsomal fraction of mammalian liver

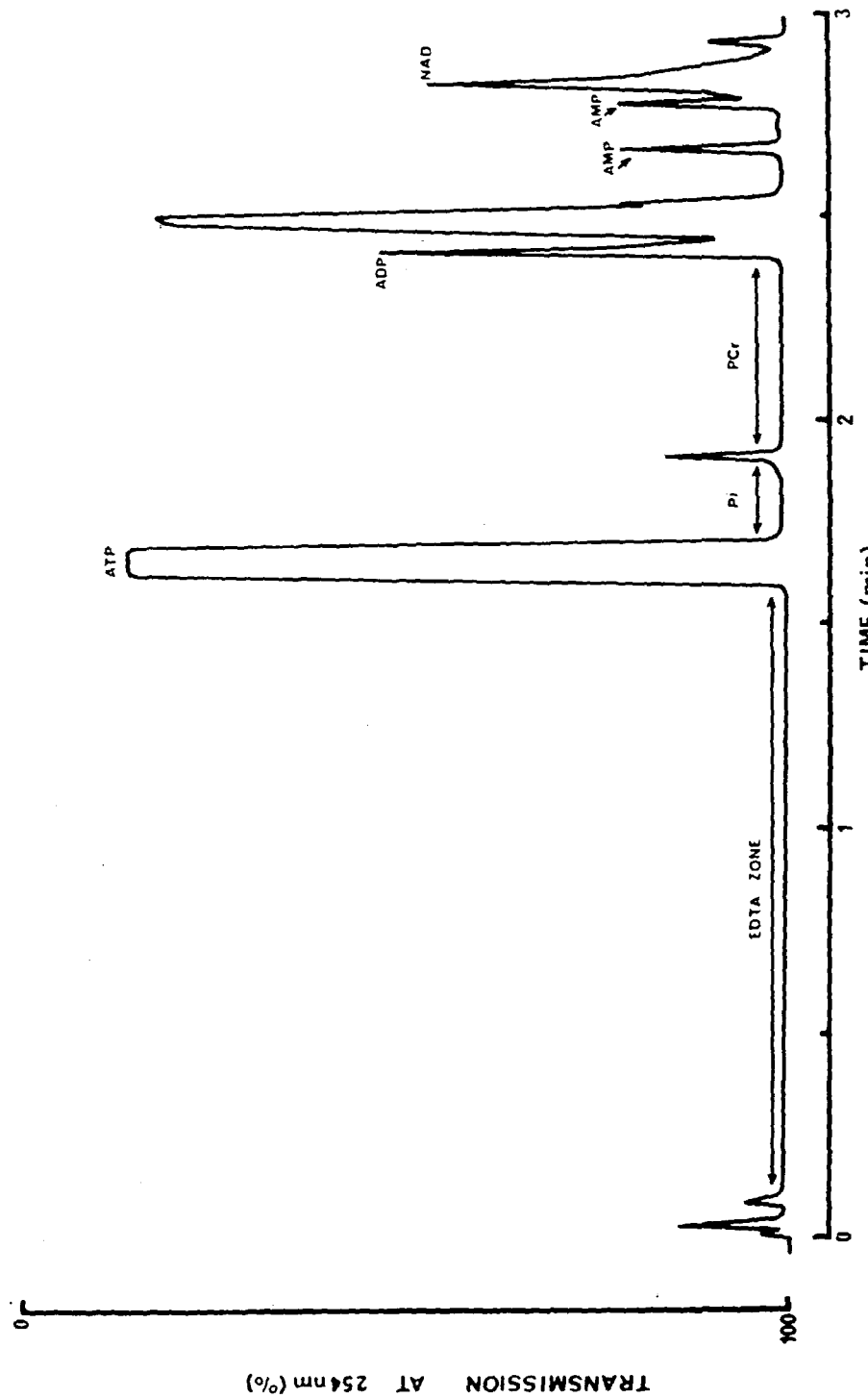


FIGURE 35. Record of typical muscle extract. 10 μ of extract were used. Electrolyte system No. 5, Table 2. (From Gower, D. C. and Woledge, R. C., *Sci. Tools*, 24, 2 [1977]. With permission.)

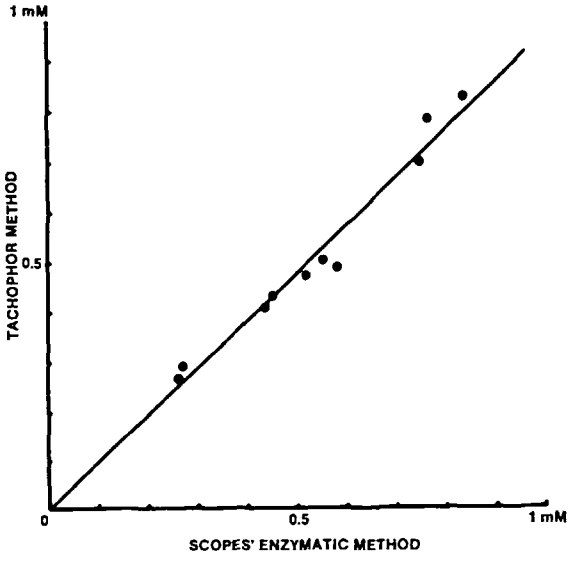


FIGURE 36. Comparison of analytical methods for ATP. Each point is for the extract of a separate muscle. The line is a regression line which has a slope of 0.98 and an intercept of −0.004 m*M*. The correlation coefficient is 0.98. (From Gower, D. C. and Woledge, R. C., *Sci. Tools*, 24, 2 [1977]. With permission.)

homogenate contains a pyrophosphatase activity which catalyses the hydrolysis of UDP-glucuronic acid to UMP and glucuronic acid 1-phosphate. A further phosphatase activity cleaves these products to uridine, phosphate, and glucuronic acid. Capillary isotachophoresis permitted the simultaneous quantitative analysis of the above-mentioned reactants and products. The sensitivity of the isotachophoretic method was sufficient for the accurate determination of 0.1 nmol of the compounds applied in a volume of 1 to 10 $\mu\ell$, i.e., for solutions of concentration as low as 10 μmol/ℓ. Holloway et al. stated the major advantages of isotachophoresis to be that a complete analysis was possible without the need for radioactively labeled compounds, and the analysis time of about 40 min was considerably less than for a conventional thin-layer chromatographic separation. Brunner and Holloway[152] have further extended the method to the assay of UDP-glucuronyl-transferase (E.C. 2.4.1.17) using paracetamol, a drug of current toxicological interest, as glucuronide acceptor. The metabolic products detected simultaneously were uridine-5′-diphosphoglucuronate, inorganic phosphate, glucoronate, uridine 5′-diphosphate, uridine 5′-monophosphate, and paracetamol glucuronide. Aspects of the enzyme kinetics of the experiments by Holloway et al. are described in section XV.K.

The degree of purity of nucleotides and related compounds is of the utmost importance when used in various experiments where the quantifying of, for example, reaction products is of interest. Kodama and Woledge[153] studied the binding of ADP to native myosin by calorimetry and found by isotachophoresis that the commercially obtained ADP showed, in a freshly prepared solution, the presence of 10 to 13% AMP and 3 to 5% ATP. The supposedly pure ADP solutions used in the binding experiments thus contained only about 84% ADP and the treatment of the binding titration data had to be processed with that fact taken into consideration.

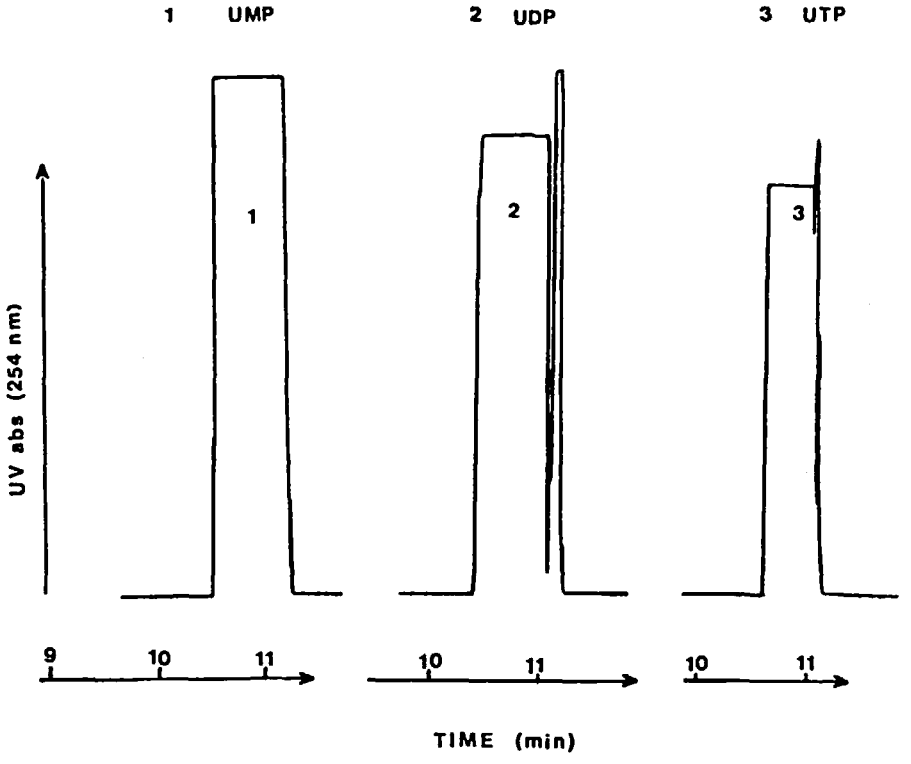


FIGURE 37. Analyses of freshly-made solutions of UMP, UDP and UTP. 10 nmol of each nucletide (2 $\mu\ell$). Electrolyte system No. 5, pH_L = 3.84, Table 2. (From LKB Application Laboratory, *LKB Isotachophoresis News No. 1*, LKB-Produkter AB, Bromma, Sweden, 1977. With permission.)

Figure 37 illustrates an analysis of the purity of commercially obtained UMP, UDP, and UTP. The analyses were made on freshly made solutions and the results showed that UDP contained about 10% UMP and that UTP showed the presence of about 10% UDP. In order to further increase the sensitivity, Wielders and Everaerts[69] and Wielders[154] developed a method of determining ADP by means of steady-state mixed-zones (see Section IX.). In this way, it is possible to determine quantitatively a few picomoles of ADP.

The purity of synthesized NAD derivatives used for enzymatic studies has been studied by Buret.[155] The derivatives 8-Br-NAD^+, N^1-(2-aminoethyl)-NAD^+ and N^1-(3-chloro-2-hydroxy-propyl)-NAD^+ were prepared and the purity checked by isotachophoresis. Figure 38 illustrates the complete separation of 8-Br-NAD^+ and NAD^+.

E. Amino Acids

The applications of isotachophoresis to amino acids is particularly interesting since in theory amino acids can be separated both as cations and as anions. It is also well known that amino acids form stable complexes with, for example, metals and aldehydes. If such a complex is formed, not only do the molecular size and solvation change, but also the pK values, and the effective mobility therefore changes in the operational system chosen. Several electrolyte systems have, therefore, been considered in the analysis of amino acids.

Konstaninov and Oshurkova[156] showed the possibility of separating amino acids as positive ions at extremely low pH values. However, later work has shown that in general

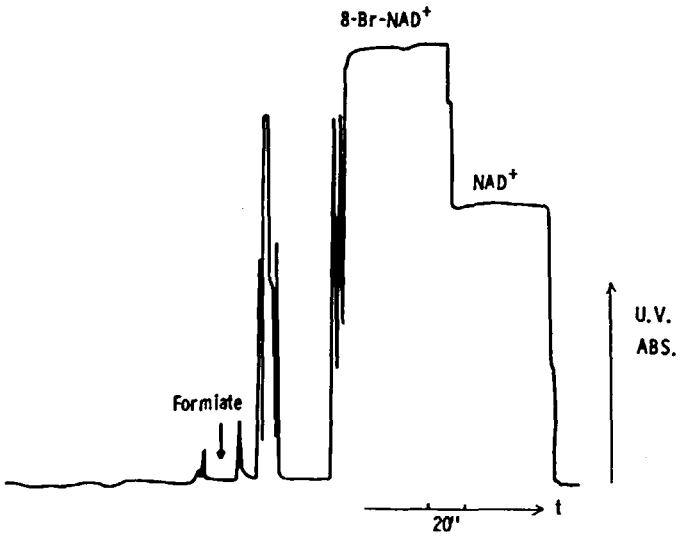


FIGURE 38. Isotachophoretic separation of 8-Br-NAD$^+$ and NAD.$^+$ Electrolyte system No. 5, $pH_L = 4.00$, Table 2. (From Buret, J., *Proc. 1st Int. Symp. Isotachophoresis*, Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980. With permission.)

the acidic and neutral amino acids are better separated as anions due to the greater differentiation of the pK values of the carboxylic groups.

Everaerts and van der Put[157] showed the possibility of treating the amino acids with formaldehyde which forms a complex with amino acids and, thereby, decreases the pK values of the amino group. In this way a leading electrolyte with a low pH could be used and still the amino acids showed sufficient negative charge. The following amino acids were separated: aspartic acid, cysteine, threonine, alanine, methionine, leucine, and valine. However, Everaerts later pointed out[16] that the aldehyde complex may not be stable and the reproducibility will be poor.

The great majority of the isotachophoretic analyses of amino acids have been performed in aqueous solution. In the work of Kopwillem and Lundin,[158] an extensive study was made of the separability and separation capacity in different electrolyte systems for the common hydrolysate amino acids. The acidic and neutral amino acids were analyzed as negative ions and the basic ones as positive ions. It was concluded that a standard mixture of the generally occurring amino acids, except for lysine and arginine, can be analyzed using two different negative electrolyte systems. Lysine and arginine were analyzed in a positive electrolyte system.

In the book of Everaerts et al.[16] a comprehensive summary of the separation of amino acids in various electrolyte systems at different pH values is given. Separation by use of complex formation and in nonaqueous solvents is also thoroughly discussed. An example of a separation in aqueous media at pH 9.2 is shown in Figure 39.

Factors affecting resolution and detection limits of amino acids have been discussed by Shiogai and Akiyama,[159] who found that the addition of up to 0.5% polyvinylalcohol to the leading electrolyte (maleic acid) improved resolution considerably. The lower detection limit with a leading electrolyte concentration of 0.5 mM was found to be about 30 pmol with potentiometric detection.

Kopwillem et al.[160] utilized the isotachophoretic analysis of amino acids in the analysis of small peptide hydrolysates. Two peptides, one undecapeptide and one decapeptide, were hydrolyzed and the amino acid composition was determined. Figure 40 shows the

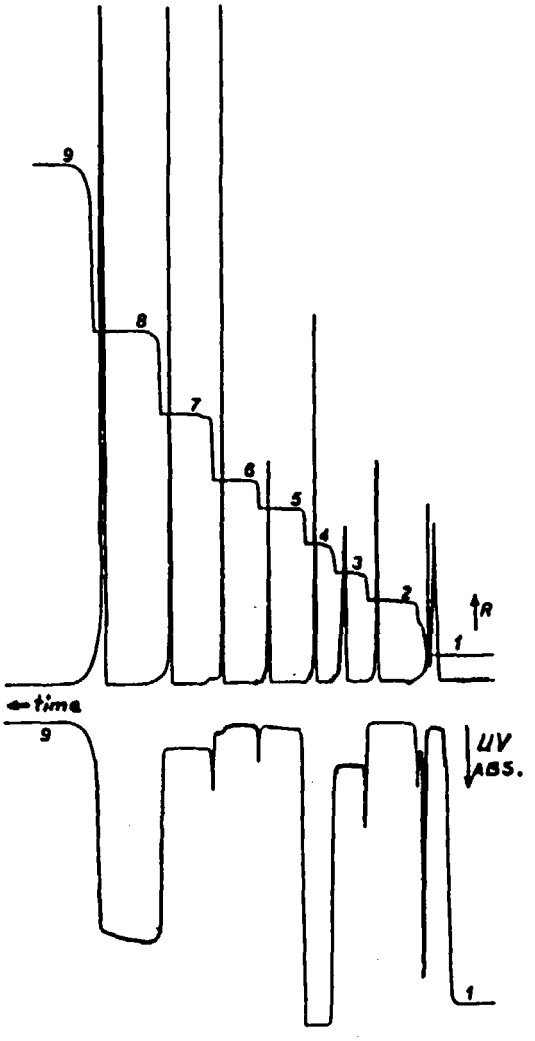


FIGURE 39. Separation of some amino acids in an operational system at pH_L 9.2 with 5-bromo-2,4-dihydroxybenzoate (0.004 *M*) as the leading ion and L-lysine as the counter-ion. Note carbonate, moving in front of L-ASP, clearly visible in the linear trace of the UV-absorption detector. The carbonate has an effective mobility in this system comparable with that of the leading ion. 1 = 5-Bromo-2,4-dihydroxybenzoate; 2 = L-ASP; 3 = L-Cys; 4 = I_2-L-Tyr; 5 = L-Asn; 6 = L-Ser; 7 = L-Phe; 8 = DL-Trp; 9 = β-Ala. R = increasing resistance (conductivity detection). (From Everaerts, F. M., Beckers, J. L., and Verheggen, Th. P. E. M., *J. Chromatogr.*, 19, 129 [1976]. With permission.)

analysis of the negatively charged amino acids in the hydrolyzed decapeptide. The ratio of Gly:Ala:Asp:Val:Leu was calculated to be 3:2:1:1:1, which was in complete agreement with the results obtained with a conventional amino acid analyzer. The basic amino acids in the hydrolysate were analyzed in a positive electrolyte system. In the same way the amino acids in the hydrolysate of an undecapeptide (1 Gln, 2 Glu, 2 Gly, 2 His, 1 Ile, 2 Leu) were determined.

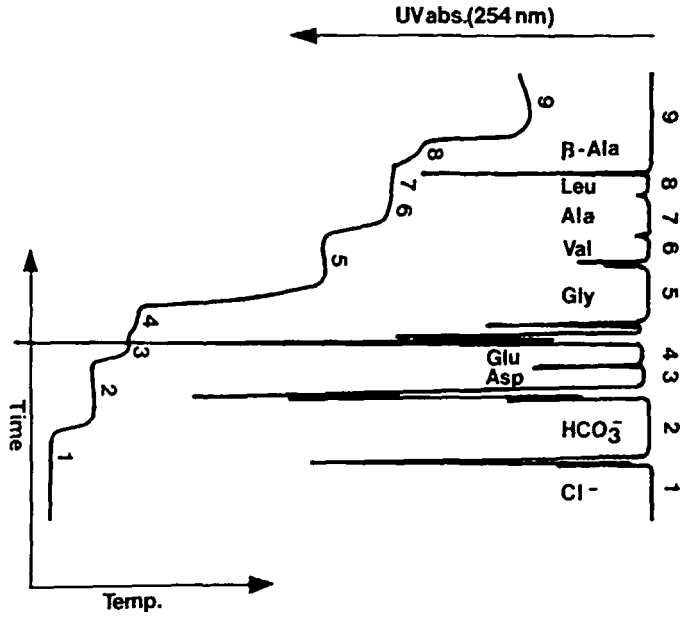


FIGURE 40. Isotachophoresis of the negatively charged amino acids in a hydrolyzed decapeptide, 11 nmol. Electrolyte system No. 36, Table 2. (From Kopwillem, A., Moberg, U., Westin-Sjödahl, G., Lundin, R., and Siervertsson, H., *Anal. Biochem.*, 67, 166 [1975]. With permission.)

Although most amino acids do not show any UV absorbance, it is still possible to utilize UV detection, as illustrated in Figure 40. This is due to the fact that in the electrolyte solutions used there exist small amounts of UV-absorbing impurities, which act as markers (spacers) in between the separated amino acids.

Robinson and Rimpler[161] recently described the isotachophoretic analysis of aspartic acid, asparagine, glutamic acid, and glutamine present in the serum of persons suffering from various metabolic disorders. The isotachophoretic determinations were compared with the results obtained by conventional ion-exchange chromatography. The values that were determined by isotachophoresis were generally somewhat higher than those received from ion-exchange chromatography. The analysis time using isotachophoresis was about 40 min for a normal serum sample. This was pointed out as a marked improvement over the ion-exchange chromatography method for "physiological" samples.

Amino acids in different kinds of food have been determined by isotachophoresis.[143] Glutamic acid in dried and seasoned tangle and amino acids in citric and tomato juices were analyzed and quantified by potentiometric detection. The isotachophoretic analysis of amino acids has also been applied to an infusion liquid[158] which contained the amino acids Thr, Met, His, Phe, Val, Leu, and Ile. The separation pattern is shown in Figure 41. The Leu and Ile zones are not fully resolved. Because of the limited number of components in the mixture, a capillary length of 62 cm could be used and the separation time was only about 30 min; this still gave sufficient accuracy in the zone length measurements used for quantification.

Recently Kodama[162] described the analysis of isovalerylglycine excreted in urine as a result of an inborn error of leucine metabolism. Isotachophoresis was found to be a rapid and very accurate method compared with the previously used gas- and thin layer chromatographic techniques. The urine was injected in a volume of 0.1 μl and without any pretreatment.

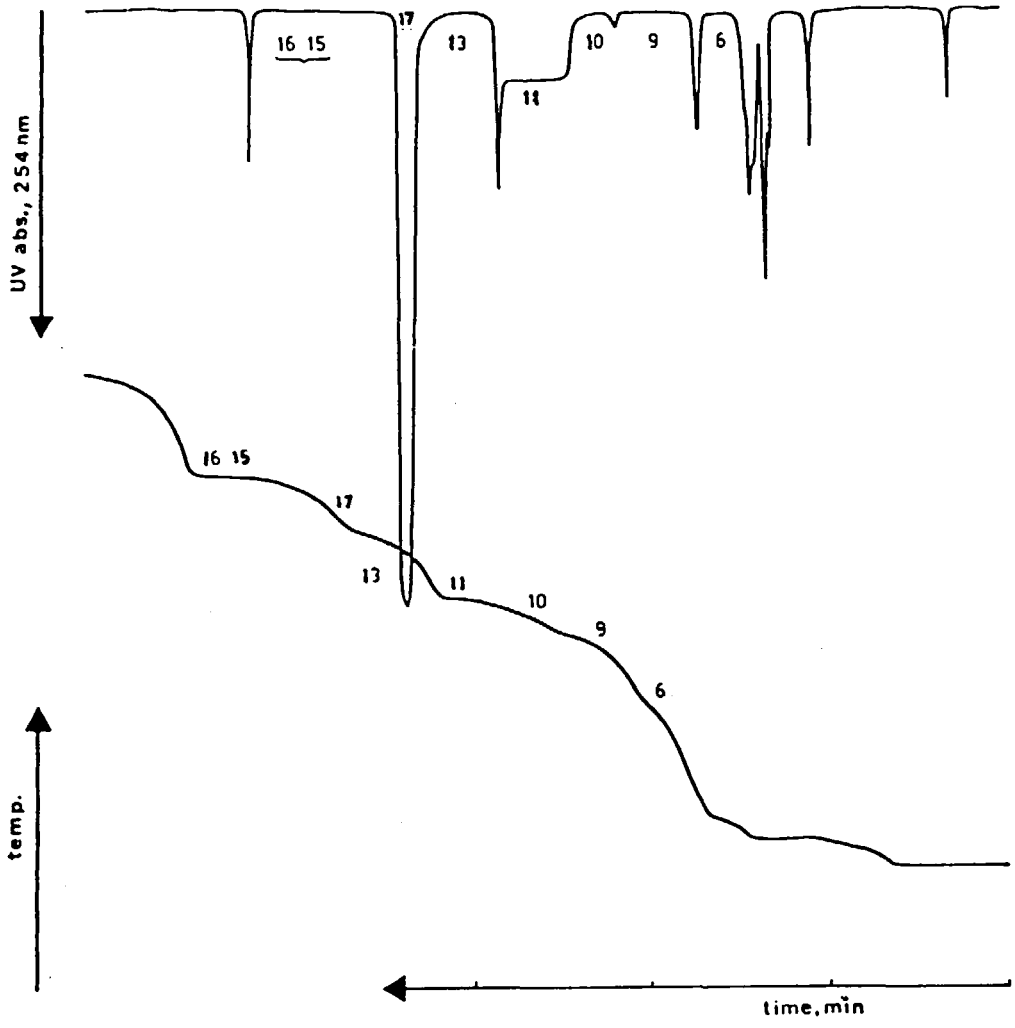


FIGURE 41. Isotachophoretic analysis of an infusion liquid, containing a limited number of amino acids. 4.6 nmol Thr (6), 8.2 nmol Met (9), 4.2 nmol His (10), 7.5 nmol Phe (11), 7.6 nmol Val (13), 1.4 nmol Trp (17), 8.2 nmol Leu (15) and 5.0 nmol Ile (16). The separation was performed within 30 min in a 62 cm long capillary at 20°C. Current during detection was 80 μA. Electrolyte system No. 36, pH_L = 9.6, Table 2. (From LKB Application Note 183, LKB-Produkter AB, Bromma, Sweden. With permission.)

The use of isotachophoresis in amino acid analysis has proven to be a good complement to the existing conventional methods. However, due to the limited dynamic range of capillary isotachophoresis, the technique should be mainly used when analyzing a small number of amino acids in a sample solution. It is also obvious that more research needs to be carried out, in particular regarding solvents or combination of solvents in which the amino acids are more soluble and where they show greater differences in mobility than they do in aqueous systems. In addition, the possibility of utilizing complex formation of amino acids might be of great value in improving separability and detectability.

F. Peptides

The application of capillary isotachophoresis to peptides has been investigated intensively during the last few years. The technique has been found to be a very rapid and

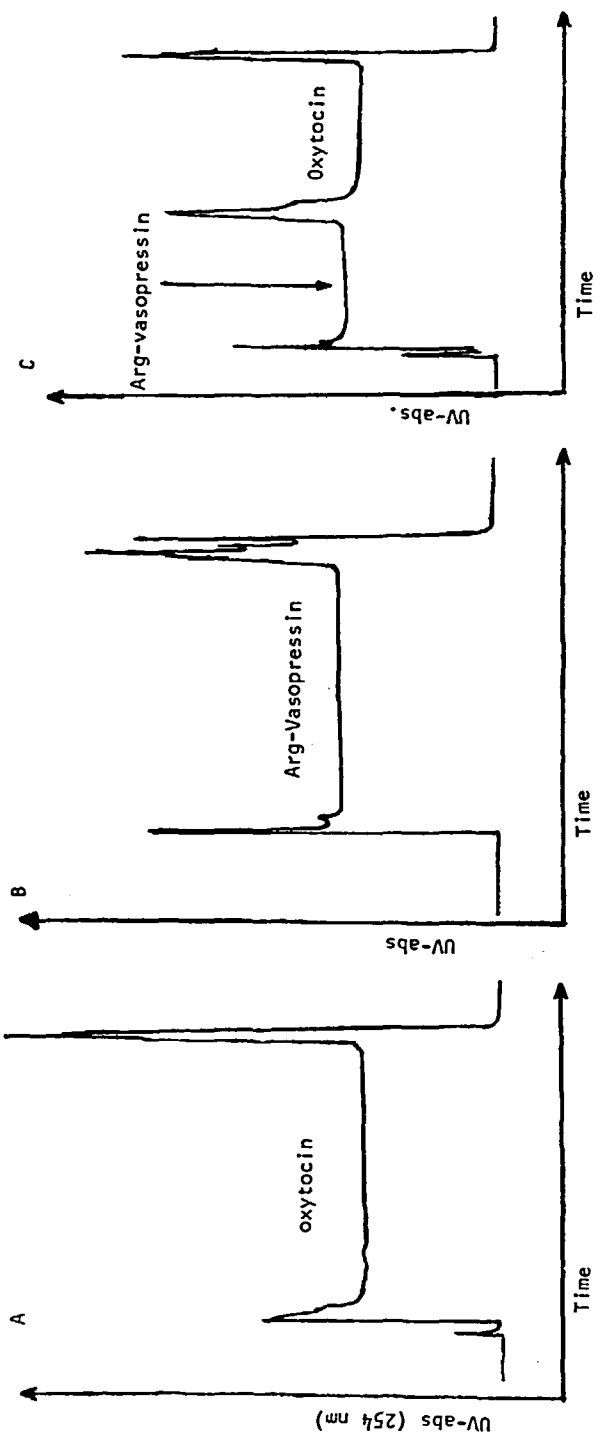


FIGURE 42. Separation of the synthesized peptides oxytocin and vasopressin. Sample: (A) Oxytocin 15 nmol (3 μℓ); B) Arg-Vasopressin 15 nmol (3 μℓ); (C) 7 nmol of each. Separation time: 15 min. Electrolyte system No. 44, pH_L = 7.0, Table 3. (From LKB Application Labatory, *LKB Isotachophoresis News No. 2*, LKB-Produkter, AB, Bromma, Sweden, 1974. With permission.)

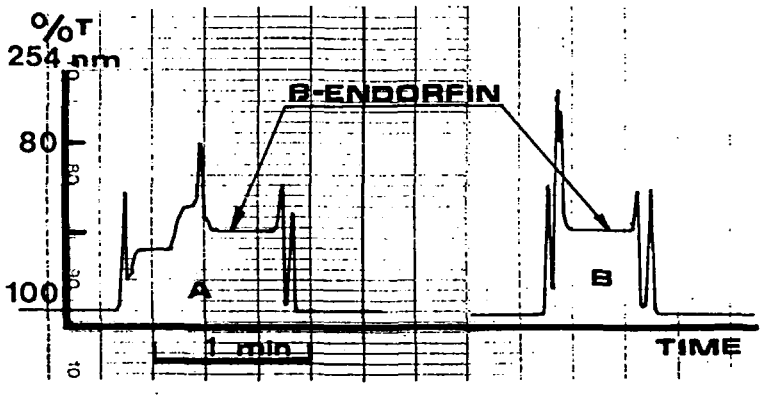


FIGURE 43. Analysis of the peptide β-endorfin after two different stages of purification. Electrolyte system No. 43, $pH_L = 4.2$, Table 3. (From LKB Application Laboratory, Bromma, Sweden.)

sensitive method for determining the purity of, for instance, synthetically prepared peptides for use in medicinal preparations. In several recent publications capillary isotachophoresis has also proven to be an excellent tool in the qualitative and quantitative analyses of peptides in physiological samples such as urine and serum. To demonstrate the resolving power of isotachophoresis in the separation of small peptides, Everaerts et al.[17] used glutathione, gly_2, gly_4, and Leu-Tyr as model substances. Miyazaki and Katoh[163] have further worked out separation conditions for 18 different peptides by varying the pH of the leading electrolyte. Physiologically important hormone peptides such as bradykinin, kallidin, Met-lys-bradykinin, and reduced and oxidized glutathione as well as angiotensins I and II were analyzed. In Reference 164 the analyses of several different hormone peptides such as oxytocin, arg-vasopressin, ACTH (adreno corticotropic hormone), somatostatin, VIP (vasoactive intestinal peptide), secretin, cholecystokinin, angiotensins I and II, bacitracin, and glutathione are described.

The analysis of the synthetized peptides oxytocin and arg-vasopressin is illustrated in Figure 42. The purity of each peptide was estimated from the ratio of the peptide zone length to the total UV-absorbing zone length and was found to be about 90%. Although the two peptides have the same basic structure and might be expected to have similar net mobilities, they can be separated quite clearly and distinguished by their different UV absorbances (Figure 42c). Capillary isotachophoresis is ideal for monitoring the development of purification schemes for peptides. This is illustrated in Figure 43, where the hormonally active peptide β-endorfin has been analyzed after two different stages of purification. Another example of isotachophoretic control of a purification procedure was given by Kopwillem et al.,[165] who followed the different steps in the synthesis of the cyclic tetradecapeptide somatostatin. It was possible to show that at least 90% of the final product was the desired hormone. The analysis time was 10 min and the analysis performed on 20 μg of peptidic material. Similar work has also been presented by Kopwillem et al. on a synthetic decapeptide and an undecapeptide obtained after the Merrifield synthesis.[160]

Martin and Hampson[166] tried to analyze insulin in methanol/water, ethanol/water, and L-ethoxyethanol/water electrolytes, but reported problems with reproducibility, probably due to adsorption to the capillary walls. Baldesten[167] recently analyzed some monocomponent or single-peak insulins isotachophoretically, and found various patterns for insulin from various sources. With the spacer technique and experiments in 6 *M* urea, it was shown that some of the inconsistency was probably due to

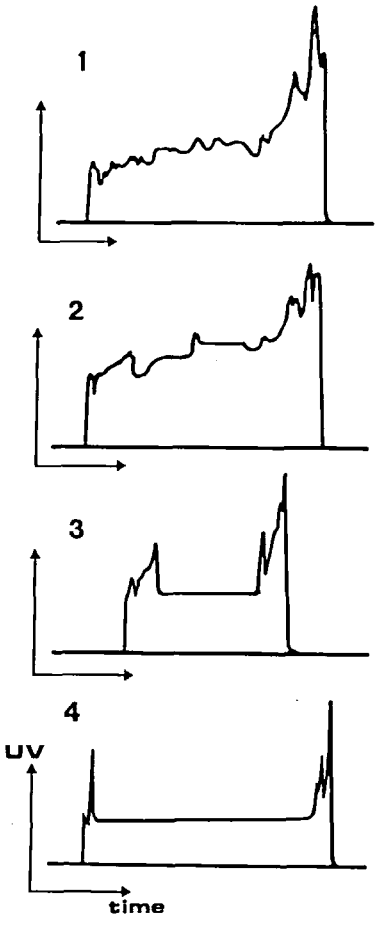


FIGURE 44. Analysis of the gastrointestinal peptide VIP during the development of purification. Sample: 30 μg. Separation time: 10 min. Electrolyte system No. 43, pH_L = 5.1, Table 3. (From Mutt, V., *Gut Hormones*, Bloom, S. R., Ed., Churchill Livingstone, Edinburgh, 1978, 21. With permission.)

polymerization of the insulin molecules. Several analyses of commercial insulins all resulted in different patterns.

Several gastrointestinal peptides have been analysed by Mutt and coworkers.[168,169] The purification of VIP (vasoactive intestinal peptide, Figure 44) shows the enrichment of the hormone during the purification procedure. In each instance, about 30 to 40 μg of material was injected and the total analysis time was about 9 min. Recently, Mutt el al.[169] have purified a hormone peptide from a porcine intestinal concentrate. The purified peptide showed an immunoreactivity similar to somatostatin. Isotachophoretic analyses showed, however, that the two peptides had different mobilities and were easily distinguished from each other although they were supposed to be very similar in structure. Heymen et al.[170] analyzed synthetic human calcitonin and spaced the peptide in between the amino acids valine and β-alanine, using a leading electrolyte with a pH of 9. The analytical result was then converted to a preparative system for purification of calcitonin from serum.

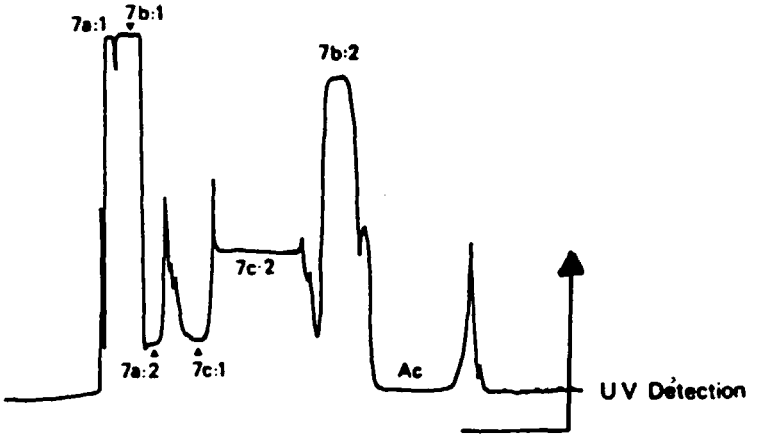


FIGURE 45. Isotachophoretic separation of a mixture of uraemic middle molecule peptides (7a:1—7c:2). Electrolyte system No. 5, Table 2. (From Zimmerman, L., Baldesten, A., Bergström, J., and Fürst, P., *Proc. Ist Int. Symp. Isotachophoresis,* Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980, With permission.)

In uremia it is assumed that toxic substances, normally excreted by the kidneys, are retained in the body fluids and exert toxic effects. It has been assumed that peptides with a molecular weight between 500 and 5000 (middle molecules) were involved in this neuropathy. Zimmerman et al.[171] separated normal and uremic plasma by ion exchange chromatography into seven or eight UV-absorbing peaks. One of these peaks, referred to as No. 7 and only found in cases of severe uremia, has been isotachophoretically separated into six subpeaks as shown in Figure 45. The mobilities of the separated compounds and the pH of the electrolyte system hinted that the pI values of the compounds lie well below pH 4. Grof and Menyhart[172] found eight components in the desalted butanol extract of uremic hemofiltrate by comparison with filtrate from patients suffering from hepatic coma and urine from healthy persons. Isotachophoretic analyses revealed one main component present in different samples and fractions from uremic patients. The dipeptide isovalerylglycine in the urine of a patient with isovaleryl acidaemia has been quantified isotachophoretically by Kodama.[162]

Snake venoms contain several peptides and, at least in some cases, it is possible to identify the species by the venom pattern. The analyses of venom from two specimens (A and B) of *Naja naja atra,* the Chinese Cobra, are illustrated in Figure 46. C and D are analyses of venoms from *N. naja siamensis,* the Siamese Cobra. Only slight differences in the UV records are discernible between the two specimens of the same subspecies. However, the difference between different subspecies are quite striking and reproducible and suitable for identification. Hendon and Tu[173] have further used the technique of isotachophoresis in the analysis of the crosslinking between specific subunits of venom peptides.

G. Proteins

In protein chemistry, electrophoresis has always been one of the most important separation methods with respect to analysis and isolation. It started with Tiselius's free boundary electrophoresis.[174] New electrophoretic separation methods for proteins have, thereafter, continually been developed, for example, zone electrophoresis, disk electrophoresis, electrofocusing, and isotachophoresis. Isotachophoresis is the latest of the electrophoretic techniques, and the application of capillary isotachophoresis to proteins has been investigated extensively only during the last 4 years.

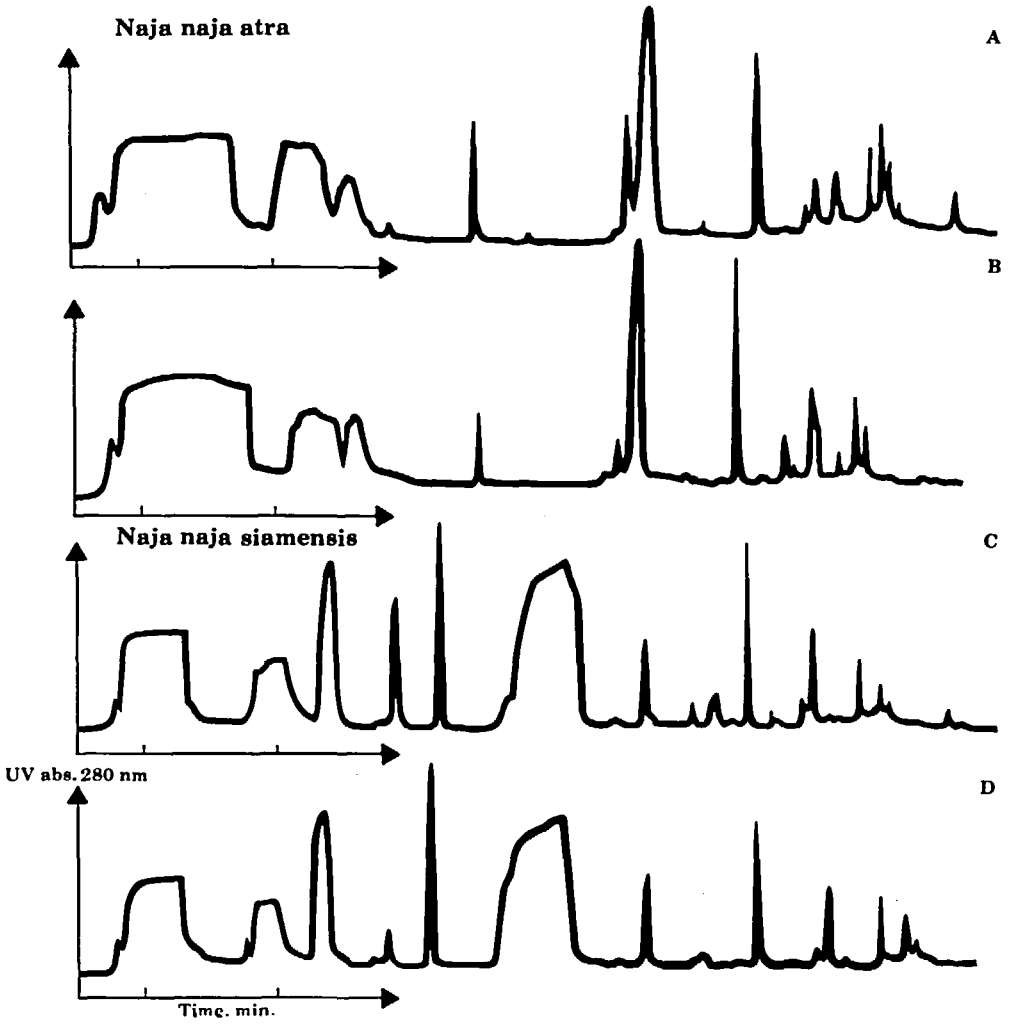


FIGURE 46. Analysis of snake venoms from two specimens of *Naja naja atra* (A and B) and two specimens from *Naja naja siamensis* (C and D). Sample: 2 μℓ whole venom (10 mg/mℓ); (LD_{50} = 5 μg/20 g in mouse) + 4 μℓ Ampholine® carrier ampholytes, pH 3 to 10 (diluted 1:100). Analysis time: 20 min. Electrolyte system No. 44, pH_L = 7.0, Table 3. (From Haast, B., Miami Serpentarium, Miami, Fla. With permission.)

Capillary isotachophoresis was previously used mainly to separate ions with relatively low molecular weights (up to about 3000 daltons). The stabilization of zones containing high molecular weight compounds in the capillary tube was limited by disturbances caused by gravity[175] and electroendosmosis. In 1967, hydroxyethylcellulose was reported to have been used to increase viscosity and to reduce electroendosmosis in isotachophoretic experiments.[14] The resolution of detectors available at that time was, however, too low to show the resulting increase in separation quality. The cellulose was, therefore, soon abandoned as an unnecessary complication. The highly resolving UV detector, however, laid the basis for more detailed studies of zone stabilization and showed the importance of reducing electroendosmosis, especially in protein analysis. Arlinger[42,176] analyzed hemoglobin and hemoglobin cyanide as model systems for protein separations. Methylcellulose was added to the leading electrolyte and good zone stabilization was obtained. In protein analysis it is of vital importance to use discrete

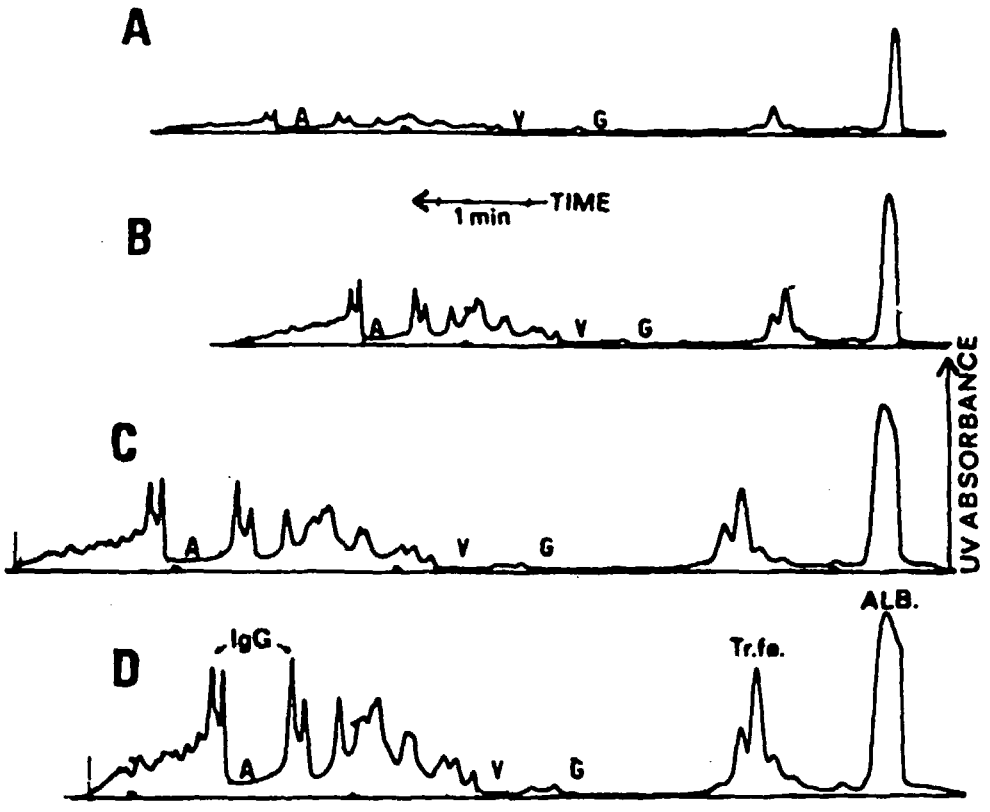


FIGURE 47. Experiments with mixtures of isolated protein fractions. Sample solution (per mℓ): 10 mg albumin, 1 mg transferrin, and 7.2 mg IgG. A. 2 μℓ of spacer solution (Ampholine®) and 0.75 μℓ of sample. B. Same as in A but 1.5 μℓ of sample. C. Same as in A but 3 μℓ of sample. D. Same as in A but 5 μℓ of sample. Electrolyte system No. 37, $pH_L = 9.1$, Table 2. (From Delmotte, P., *Sci. Tools*, 24, 33 [1977]. With permission.)

spacers or a continuous spacing mobility gradient (see Section VII.) The protein zones will otherwise follow each other in immediate contact and because of their similar UV absorbance, the UV detector will not be able to resolve the different protein zones. The influence of spacing mobility gradients on the protein zones using Ampholine® carrier ampholytes as spacer ions has been thoroughly studied by Arlinger[177] with leucoagglutinin, myoglobin, and various hemoglobin preparations as samples, and by Everaerts et al.[16] with human serum as sample.

Kjellin et al.[178-181] have demonstrated the usefulness of capillary isotachophoresis in examining cerebrospinal fluid (CSF) proteins. Samples of CSF from patients with multiple sclerosis and chronic meningoencephalitis were analyzed and the results compared with analyses of CSF from healthy persons. The pathological CSF samples showed a considerable increase of proteins in the gammaglobulin region and the albumin content also showed significant differences compared with the normal CSF samples. It was pointed out that the main advantage of isotachophoresis, in comparison with ordinary electrophoresis and electrofocusing, was that small volumes (15 to 30 μℓ) of unconcentrated CSF could be analyzed in 60 min and 10-fold concentrated CSF in 30 min.

A very important contribution to the possibility of using capillary isotachophoresis in practical applications was the work of Delmotte.[43] He reported in detail the usefulness of the technique for the separation and quantitation of complex mixtures of proteins. The

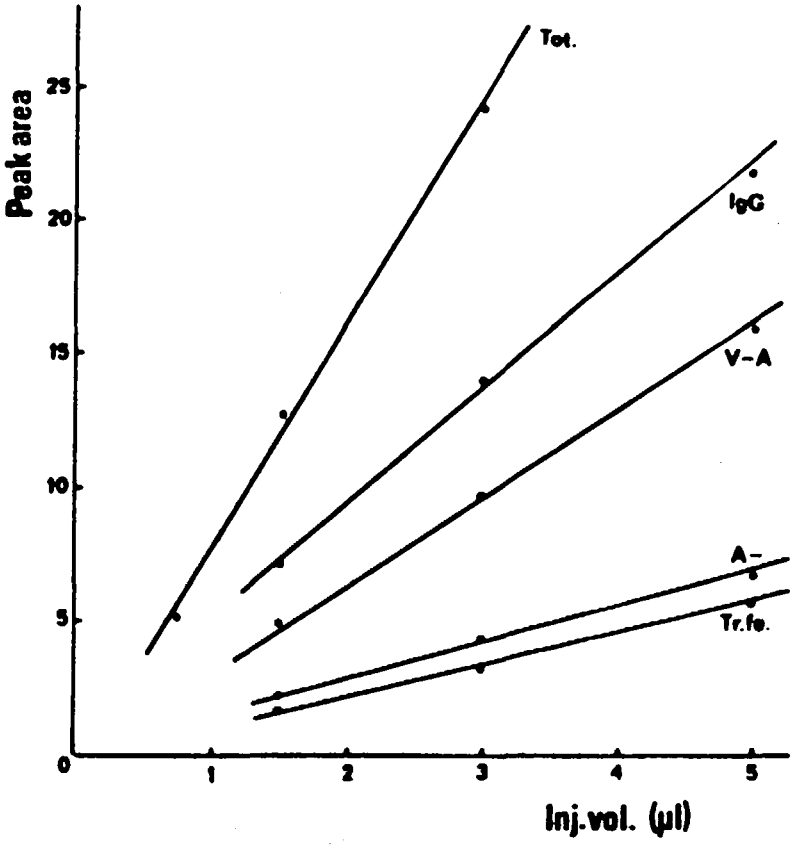


FIGURE 48. Quantitative evaluation of experiments presented in Figure 47. Plots of areas of peaks vs. injected volume of sample. Tot. = whole pattern. IgG = all gamma-globulin subfractions. V-A = all peaks between valine and beta-alanine. A− = all peaks slower than beta-alanine. Tr.fe. = transferrin subfractions. (From Delmotte, P., *Sci. Tools*, 24, 33 [1977]. With permission.)

main aim of the work was to investigate the usefulness of isotachophoresis for the study of the serum and cerebrospinal fluid gammaglobulins related to neurological diseases, but several practical and methodological problems were also approached. Delmotte described in detail the preparation of pure electrolytes in order to obtain fully reproducible results. The influence of the composition of the spacing mobility gradient on the separation pattern was studied in great detail. For this study, Ampholine® preparations of very narrow pH ranges (0.25 pH units) were prepared and used as spacer solutions, together with various amino acids which acted as discrete spacers. A series of experiments was also evaluated quantitatively by electronically integrating the area under the UV peaks. For this series of experiments, a solution of the following composition was prepared: 10 mg of human albumin, 1 mg of human transferrin, and 7.2 mg of immunoglobulin G per mℓ. The solution was submitted to isotachophoretic separation using a spacing mobility gradient solution. Increasing amounts of sample solution (0.75, 1.5, 3.0, and 5.0 µℓ) were injected. Figure 47A to D shows the separation patterns obtained. The letters A, V, and G stand for the amino acid spacers β-alanine, valine, and glycine. The plots of integrated areas against injected volume for each run are presented in Figure 48. It is evident that, even over the wide range of absolute amounts of protein injected, a virtually straight-line relationship exists between the injected

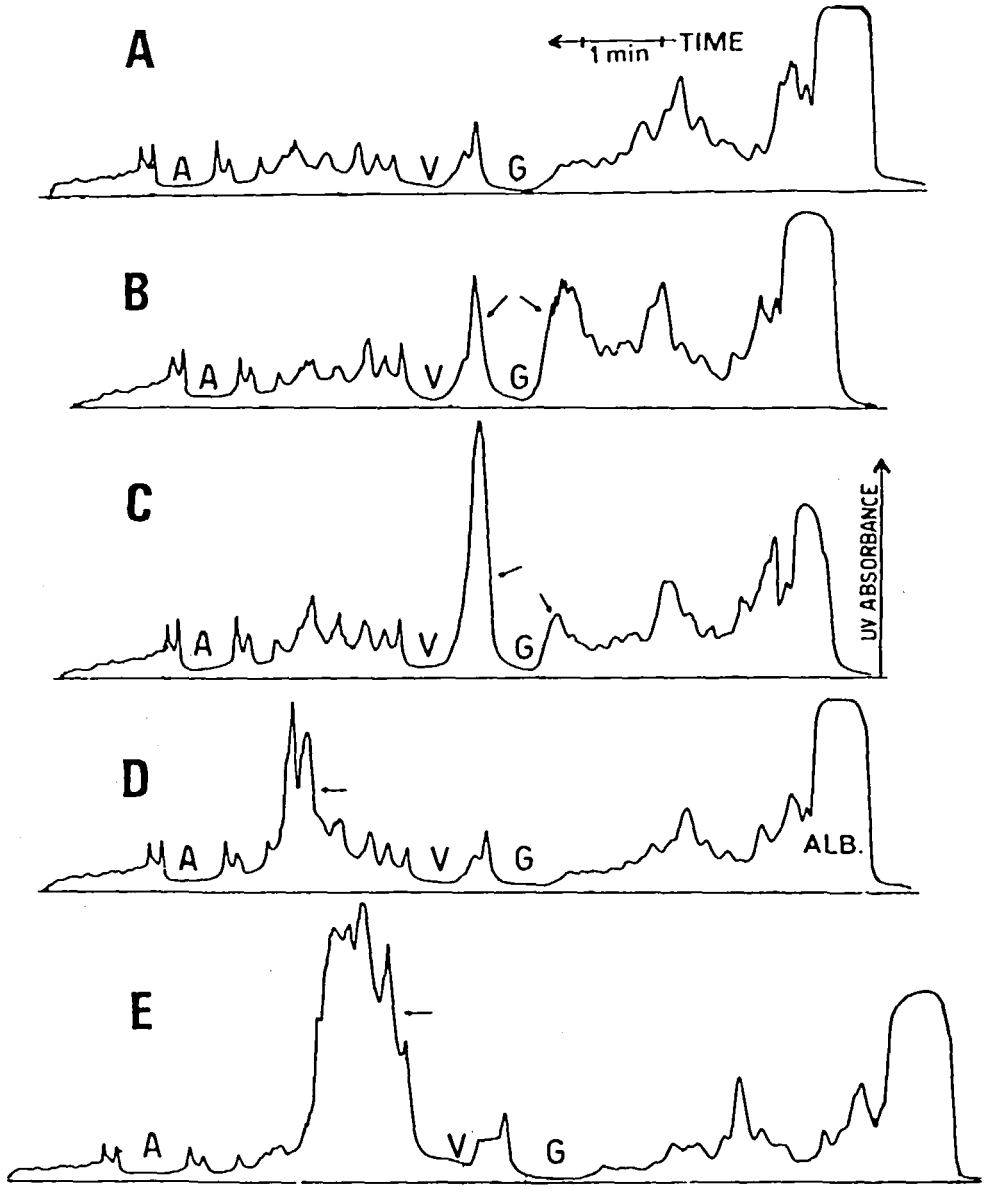


FIGURE 49. Separation patterns obtained with pathological sera. A. Normal human serum. B., C. Sera with elevated IgA content. D., E. Sera with elevated IgG content. All runs with 0.6 $\mu\ell$ of sample and 2 $\mu\ell$ of spacer solution (Ampholine®). Electrolyte system No. 37, $pH_L = 9.1$, Table 2. Analysis time: 24 min. Terminating ion: ϵ-aminocaproic acid. (From Delmotte, P., *Sci. Tools*, 24, 33 [1977]. With permission.)

quantities of proteins and the integrated areas. In another series of experiments (Figure 49), the results of capillary isotachophoresis of a normal human serum are compared with the results obtained with four sera from patients with gross abnormalities in immunoglobulin synthesis. The pattern in Figure 49A is typical of normal serum. The pattern in Fig. 49B corresponds to the serum of a patient with an immunoglobulin A gammapathy. It is evident that most of the oligoclonal immunoglobulin A in this patient's serum has a higher net electrophoretic mobility than the amino acid glycine (at the working pH of 9.1). In Figure 49C, another immunoglobulin A gammapathy is shown,

where the abnormal component has an effective mobility in between those of glycine and valine. Figures 49D and E shown the separation pattern of an immunoglobulin G gammapathy where the elevated gammaglobulin fractions have mobilities in between those of valine and β-alanine. In the last two cases, it is also noteworthy that most other immunoglobulin subfractions were strikingly reduced compared with normal serum.

By analyzing and quantifying albumin in serum and CSF, Delmotte showed the possibility of calculating the blood-CSF barrier permeability for albumin. The results obtained by isotachophoresis were in good agreement with the corresponding results obtained by radial immunodiffusion. Delmotte has further refined the method for protein analysis and several application examples are described in recent extensive works.[182,183] Examples of such applications are the analysis of CSF gammaglobulin fractions related to multiple sclerosis (Figure 8), the separation pattern of soluble proteins of the eye lens and the separation pattern of the water soluble proteins from human brain white matter. The isotachophoretic analysis of CSF proteins related to neurological diseases has been further studied in a recent paper.[184] The CSF gammaglobulins were separated as positively charged ions, in order to avoid the "injection-clog" problem, presumably caused by albumin.

As described by Delmotte,[43] amino acids are very useful as discrete spacers. Kopwillem et al.,[185] Bier et al.,[186] and Shimao[187] have described in detail the use of amino acids and peptides as discrete spacers in the analysis of human serum proteins. By using discrete spacers, the human serum proteins were separated into distinct mobility subgroups which were utilized when preparative fractionation on, e.g., polyacrylamide gel was carried out.[185] Lange[63] utilized the discrete-spacer technique when analyzing soluble brain proteins from rat hippocampus in such a way that he was able to selectively space the protein of interest between the spacer ions glycylglycine and aspartic acid. This is illustrated in Figure 9.

Hedlund and Nichelson[188] were the first to report the use of isotachophoresis in the detection of soluble immune complexes. Hedlund et al.[189] recently described the analysis of the individual subclasses of IgG purified from sera from multiple myeloma patients. When a mixture of purified IgG from each of the four subclasses is analyzed, a characteristic mobility pattern with four unique peaks is obtained (see Figure 50). An IgG of unknown subclass can be identified by the superimposition of the peak for the unknown upon one of the four characteristic peaks. The method of isotachophoresis using microgram quantities of the proteins in a volume of 1.0 μℓ was performed in 15 min.

Capillary isotachophoresis has been used to separate proteins from water extracts of dried bloodstains and dried washed hemolysates from the corresponding blood.[190] The method appears to be of value to the forensic scientist in the characterization of bloodstains.

Delmotte[43] and Bours[192] separately showed the possibility of using isotachophoresis to study eye-lens crystallins and later on jointly published an extensive report[191] on the changes in protein composition of the crystallins with age. The aim of their latest work[191] was to compare capillary isotachophoresis and electrofocusing. In the study, isotachophoresis was found to have somewhat higher resolving power and showed an increased number of separated protein components. Consequently, the isotachophoretic method provided new information on the protein composition of eye-lens crystallins.

The analysis of glycoprotein-protein fractions has been studied by Thorn et al.[193] The fractions were prepared from erythropoietin-active crude urinary protein and separated by several cycles of gel filtration and ion-exchange chromatography. The fractions obtained from chromatography were analyzed by capillary isotachophoresis and a large number of urinary protein components were separated.

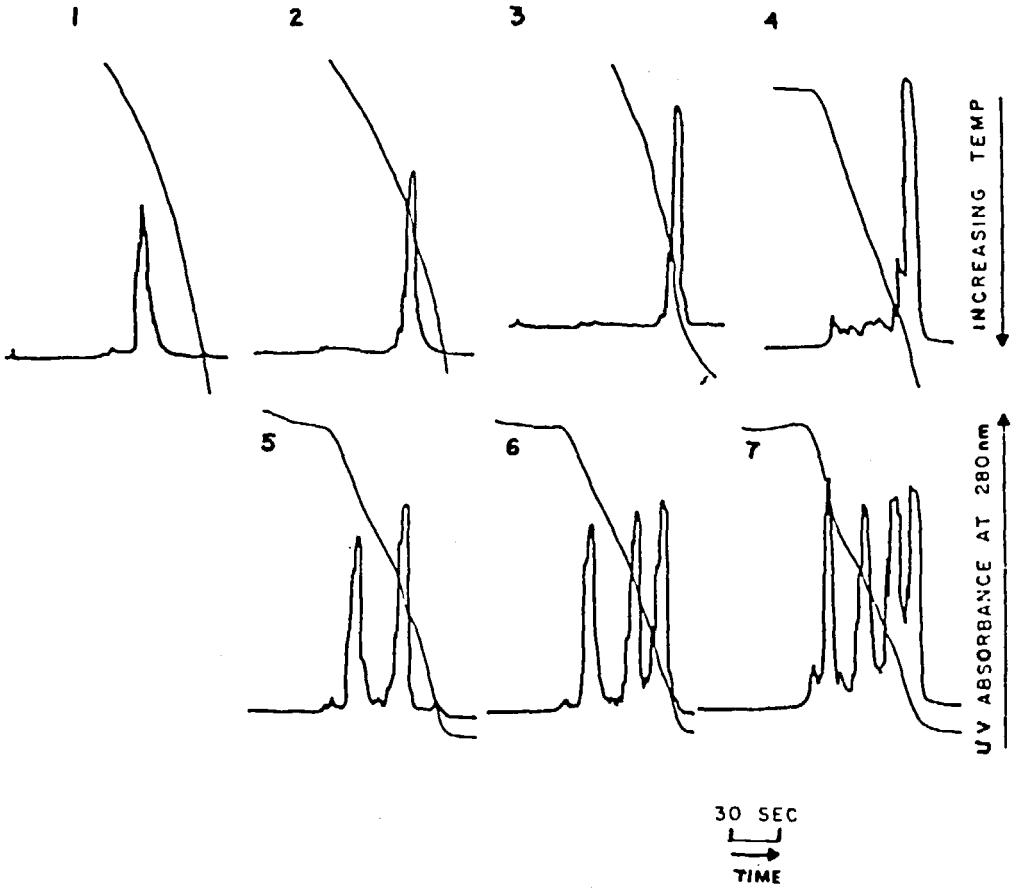


FIGURE 50. Analyses of individual human IgG subclasses. Electrolyte system No. 36, $pH_L = 8.4$, Table 2. (From Hedlund, K. W. and Nichelson, D. E., *J. Chromatogr.*, 162, 76 [1979]. With permission.)

In a study by Hendon and Tu,[173] isotachophoresis has been used in analyzing complexed rattlesnake toxins.

Everaerts et al.[17] have used pepsin as a model system in a discussion of possible denaturation effects on proteins due to high mass concentration and high field strengths in isotachophoresis.

Preparative capillary isotachophoresis (technically described in Section XIII.) has made it possible to analyze the highly resolved protein zones with further detection methods for more complete characterization. The sample zones are transferred to a moving cellulose acetate strip which can then be subjected to, for example, immunological, radioactivity, and zymogram techniques for detecting and identifying the collected sample zones.

Arlinger[194] separated transferrin and ceruloplasmin. The separated fractions, collected on the cellulose acetate strip, were then analyzed by immunoelectrophoresis by simply laying the strip directly on the agarose gel containing antibodies against transferrin and ceruloplasmin. The current was applied immediately after application of the strip in order to minimize diffusion. After electrophoresis into the antibody-containing gel, the antibody-antigen precipitates were fixed and stained according to the procedure described by Axelson et al.[195] Using the same procedure, Moberg et al.[88] separated and collected human serum proteins. Figure 51A and B shows the immunological

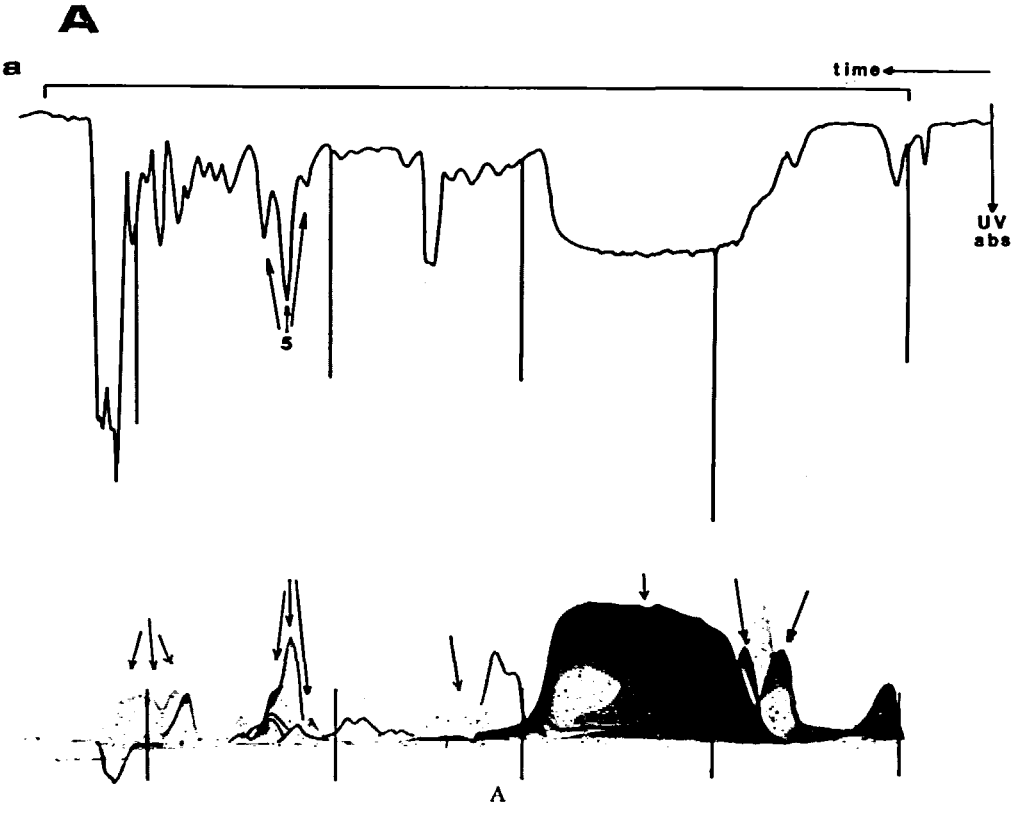


FIGURE 51. Isotachophoretic and immunological analysis of human serum proteins. 1.0 $\mu\ell$ serum and 0.5 $\mu\ell$% Ampholine® pH 6 to 8 (Fig. A) and 0.5 $\mu\ell$ 1% Ampholine® pH 7 to 9 (Fig. B). Leading electrolyte: 5 m*M* MES; 10 m*M* ammediol; 0.5% HPMC, pH_L 9.0. Terminating electrolyte: 5 m*M* EACA adjusted with $Ba(OH)_2$ to pH 10.6. Analysis time, 40 min; current during detection, 50 μA; chart speed of the sample collection strip was 2.6 cm/min; capillary length was 43 cm; monitoring wavelength 280 nm. The part of the cellulose acetate strip corresponding to the line above the UV-record (a) was cut out and applied to the agarose gel containing antibodies against whole human serum. The faintest immunoprecipition lines were drawn on the photograph. The different proteins marked in the figures were identified by the doping technique or by running against monospecific antisera 1 = orosomucoid; 2 = α_1-antitrypsin; 3 = albumin; 4 = haptoglobulin; 5 = transferrin; 6 = IgG. (From LKB Application Note 300, LKB-Produkter AB, Bromma, Sweden. With permission.)

characterization of the human serum proteins collected from the capillary isotachophoresis experiments. The sample volume was 1.0 $\mu\ell$ serum with 0.5 $\mu\ell$ 1% Ampholine®, pH 6 to 8 (experiment A) and 0.5 $\mu\ell$ 1% Ampholine®, pH 7 to 9 (experiment B). The parts of the strips corresponding to the lines at the top of the UV records (Figure 51A, B) were cut out and immunoelectrophoretically run against total anti-(human serum) (1 $\mu\ell$ antibodies/36 mℓ gel). The vertical lines on the immunopatterns correspond directly to the spikes made electronically on the UV records. The proteins were identified by running against monospecific antisera or by the doping procedure. In the latter method, a protein of interest is added in pure form to the original serum sample and causes an increase of one or several peaks in the known serum pattern.

Arlinger[194] has also demonstrated the possibility of performing specific analysis directly on the strip, using the zymogram technique.[196] The cellulose acetate strip was thoroughly treated with the reagent solution before sample collection. The sample used was cholinesterase. After the sample components had been collected on the pretreated strip (collection performed as on an untreated strip), the strip was dipped into the reagent

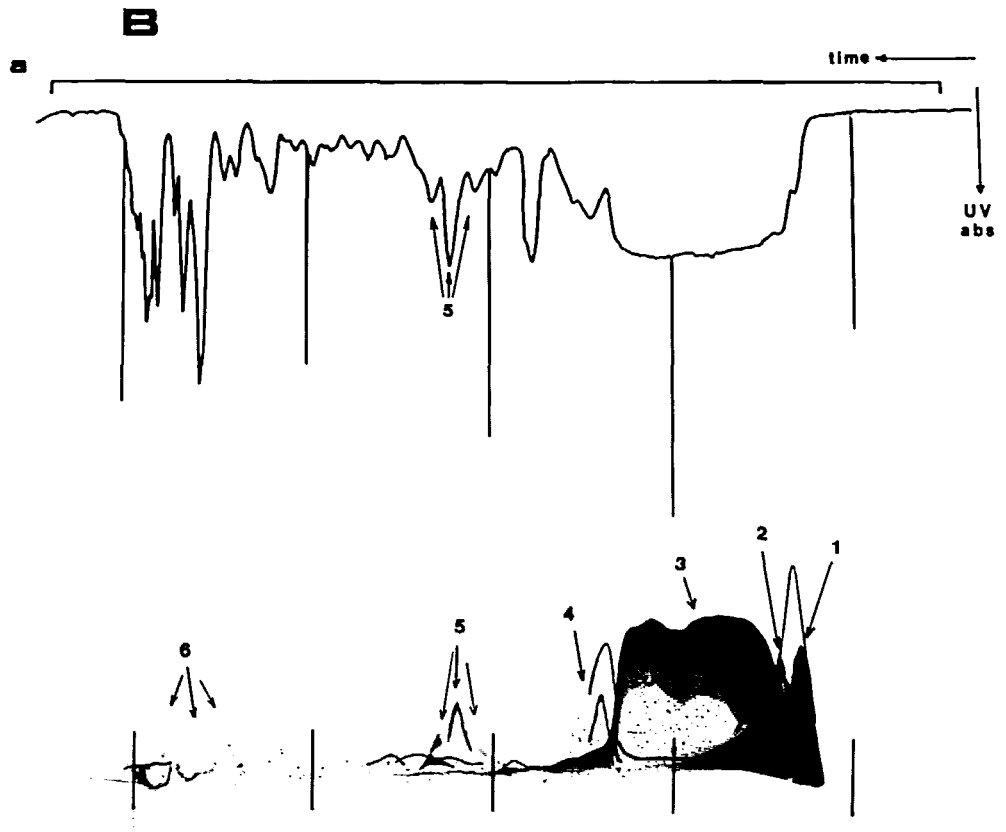


FIGURE 51B

solution and dried with a hairdryer. An intense reddish-brown band developed and was reinforced by one or two more soakings and dryings. The cholinesterase activity was found in only a small part of the whole UV-absorbing protein region. Since it can easily be found which zones contain enzyme activity, it would, thus, be an easy task to make another separation, collect the sample on an untreated strip, cut out the desired section and place it in a buffer solution in order to obtain an enzyme solution of high purity and very high specific activity. It can be concluded that the fraction-collecting possibility will widen the applicability of capillary isotachophoresis not only because it offers completely new and powerful detection and identification possibilities, but also because it makes capillary isotachophoresis a high-resolution micropreparative technique.

H. Metal Ions

The qualitative isotachophoretic determination of metal ions and other low molecular weight cationic species has been dealt with in many papers. As long ago as 1953, Longsworth[6] succeeded in separating Ca^{2+} Ba^{2+} and Mg^{2+} in a Tiselius moving-boundary apparatus. The zone boundaries were detected by means of Schlieren scanning patterns. Konstantinov and Oshurkova[8] further developed the technique and separated a large number of metal ions in a capillary tube. The zones were detected by measuring the differences in refractive index. However, it was first through the work of Beckers and Everaerts[46,197] that the isotachophoretic separation of metal ions was studied in great detail. In the initial work,[46] the separation of some cations, including the metal ions Cs^+ Rb^+ K^+ Na^+ and Li^+ was studied in water-based and methanol-based solvent systems. It was clearly shown that, for some of the compounds, the relative mobility differences

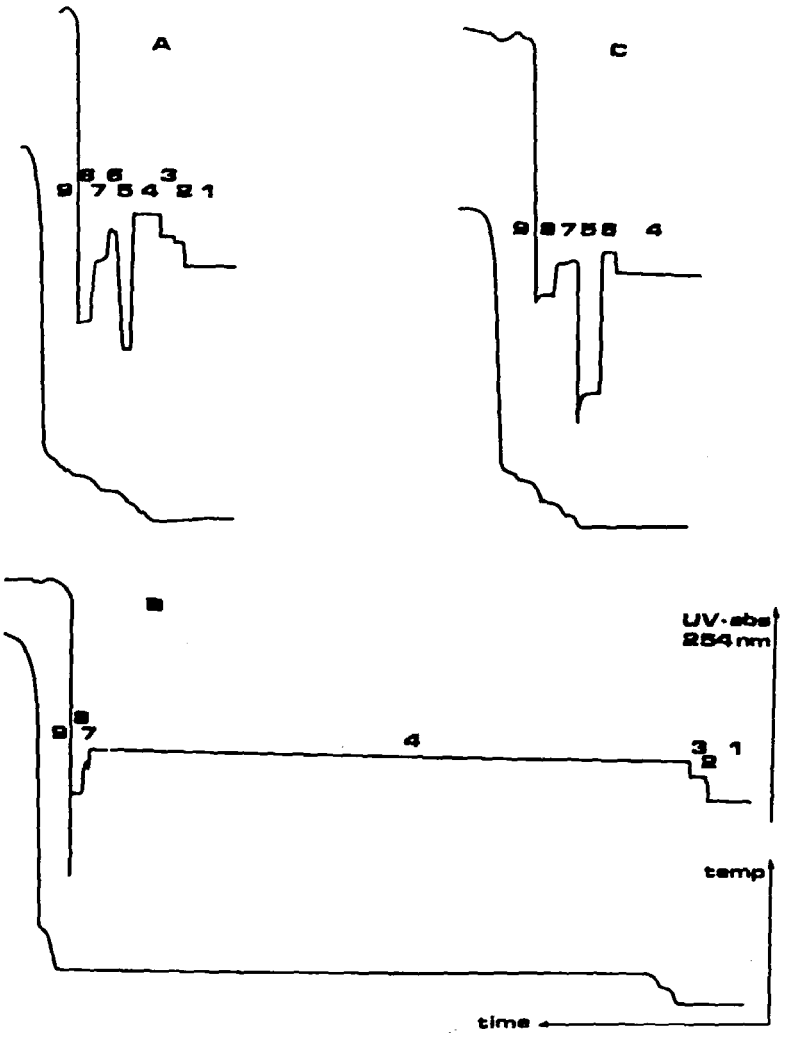


FIGURE 52. Separation of cations in methanolic systems: 1, $(CH_3)_4N^+$; 2, NH_4^+, 3, K^+; 4, Na^+; 5, Ba^{2+}, 6, Li^+; 7, Mg^{2+}; 8, Ca^{2+}; 9, Zn^{2+}. A. Sample: 1 $\mu\ell$ of a solution 0.003 *M* NH_4^+, K^+, Li^+, Ba^{2+}, Mg^{2+}, Ca^{2+} and 0.0015 *M* Na^+. Leading electrolyte: 0.01 *M* $(CH_3)_4NCl$ in methanol, saturated with sulfanilic acid. 10.5 mℓ were adjusted with about 7.5 $\mu\ell$ 10% $(CH_3)_4NOH$ to pH 4.4. Terminating electrolyte: 0.02 *M* zinc acetate in methanol. B. Sample: 1 $\mu\ell$ Seronorm serum standard solution in water, slightly acidified with acetic acid. Leading electrolyte: as in A, except for the pH which was adjusted to 4.1. Terminating electrolyte: as in A. C. Sample: 2.1 $\mu\ell$ of the same solution as in A. Leading electrolyte: 0.0089 *M* NaCl + 0.0007 *M* $NaOCOCH_3$ in methanol, saturated with sulfanilic acid, pH 5.0. Terminating electrolyte: as in A.*

changed considerably when changing solvent. In an extensive article,[197] Beckers and Everaerts studied the qualitative separation of a large number of metal ions in eight different electrolyte systems using water and methanol as solvents. The separated zones were detected by means of thermal detection. It became clear at an early stage, however,

* The pH was measured with an ordinary calomel-KCl electrode containing water. The true pH can be obtained by adding a constant factor, in these cases about −1 unit. (From Arlinger, L., LKB Application Note No. 107, LKB-Produkter AB, Bromma, Sweden, 1974. With permission.)

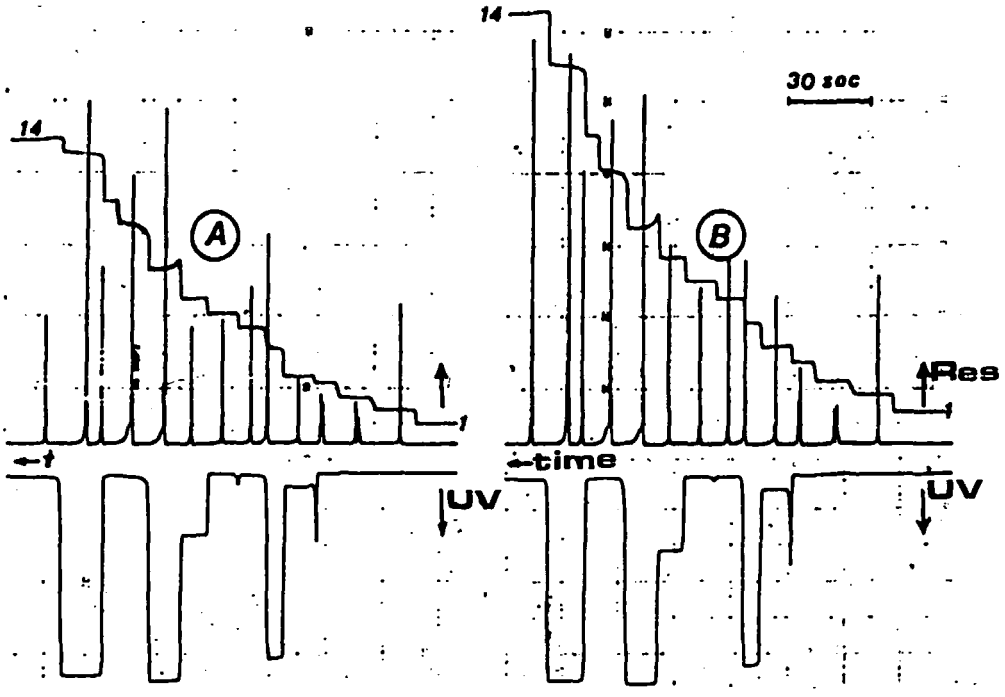


FIGURE 53. Separation of a standard mixture of cations in water (A) and in deuterium oxide (B). Peaks in sequence from 1 to 14: 1 = K^+ (leading ion); 2 = Ba^{2+}; 3 = Na^+; 4 = $(CH_3)_4N^+$; 5 = Pb^{2+}; 6 = Girard reagent P^+; 7 = $Tris^+$; 8 = histidine$^+$; 9 = creatinine$^+$; 10 = benzidine^{2+}; 11 = ε-aminocaproate$^+$; 12 = γ-aminobutyrate$^+$; 13 = aminophenzone$^+$; 14 = β-alanine$^+$ (terminating ion). R = increasing resistance. Electrolyte system No. 43, pH_L = 5.4, Table 3. (From Everaerts, F. M., Beckers, J. L., and Verheggen, Th. P. E. M., *Journal of Chromatography Library*, Vol. 6, Elsevier, Amsterdam, 1976; Everaerts, F. M., Geurts, M., Mikkers, F. E. P., and Verheggen, Th. P. E. M., *J. Chromatogr.*, 119, 129 [1976]. With permission.)

that a more sensitive detection method for the tracing of small amounts of metal ions was necessary.

In 1974 Arlinger[65] presented an ingenious way of utilizing the high resolving power of UV detection in order to detect non-UV-absorbing metal ions. The technique involved choosing a suitable counter-ion for the leading electrolyte and can be explained as follows: The concentration step between separated zones results from the step in the electric field strength at the zone boundary, which in turn is caused by the mobility differences. But field strength acts upon protons and hydroxyl ions as well as sample ions. Since the mobilities of protons and hydroxyl ions are high and their migration mechanism different from that of other ions, they must always be considered. Consequently, the concentrations of H^+ and OH^- are different on each side of the boundary and thereby produce a pH step. Since work is being done in the buffer region of the counter-ion, comparable amounts of the acidic and basic forms of the counter-ion will be used. If a counter-ion which has a large difference in molar absorptivity between the acidic and basic forms is chosen, the pH difference will give rise to an absorbance difference sufficiently large to be detectable. Arlinger[65] utilized creatinine and sulphonic acid as counter-ions. These ions have a large difference in molar absorptivity between their acidic and basic forms. Therefore their absorption will vary because of the pH steps at the zone boundaries and this will result in different UV absorbances in different zones of non-UV-absorbing sample ions. This is illustrated in Figure 52.

Everaerts et al.[16,17] have shown the use of conductometric detection in the analysis of metal ions. They have also investigated the use of deuterium oxide as solvent and found

that in the separation of certain metal ions and other cations, an improved resolution will be obtained in deuterium oxide. (See Figure 53.)

Oshurkova et al.[198] have presented conditions for the separation of $K,^{+}$ $Na,^{+}$ $Ca,^{2+}$ $Mg,^{2+}$ $Al,^{3+}$ $Mn,^{2+}$ and Fe^{3+}. The procedure was developed for use in quality control in the production of magnesium and titanium.

Dunn and Kemp[97] determined $Li,^{+}$ $Ba,^{2+}$ $Mg,^{2+}$ and Ca^{2+} quantitatively by the use of the method described by Arlinger.[65]

Capillary isotachophoresis has been used in the determination of $K,^{+}$ $Na,^{+}$ $Ca,^{2+}$ and Mg^{2+} in sylvinite ore.[199,200] The isotachophoretic results were compared with the results of various wet chemical methods. It was found that the reproducibilities of isotachophoresis and the chemical methods were virtually identical.

I. Fatty Acids

The isotachophoretic separation of fatty acids was described for the first time by Beckers and Everaerts.[201] Separation took place in an aqueous system. However, since many fatty acids have about the same effective mobility and some of them are not sufficiently soluble in water, it became obvious that another solvent was needed. Beckers et al.,[202] Beckers,[20] and Everaerts et al.[75] introduced the use of methanol as the solvent. The solubility of higher fatty acids is much better in methanol and the differences in mobility are also considerably better. In order to find the optimal pH of the leading electrolyte for the separation of fatty acids, Everaerts et al.[16] experimentally determined pK values for some saturated fatty acids in 95% methanol. It was shown that most of the pK values (in methanol solution) are about 8 and, therefore, the pH of the leading electrolyte was chosen in most cases to be about 8 to 9. Tris or Triethanolamine was used as buffering counter-ion. Everaerts et al.[16] have also shown extensively how the effective mobility of fatty acids is influenced by changing the pH of the leading electrolyte.

Unsaturated fatty acids have also been separated by isotachophoresis. In general, however, their effective mobilities do not differ sufficiently from the saturated fatty acids to enable the components of a complex mixture to be analyzed simultaneously. A possible method for the analysis of unsaturated fatty acids may involve a chemical conversion. The oxidation of the double bond generally gives a mixture of mono- and dicarboxylic acids which can be separated by isotachophoresis.[203]

Kopwillem and Eriksson[50] analyzed the unsaturated C-18 fatty acid linoleic acid and its hydroxyperoxides. In food, the quantity of peroxides is a measure of the degree of oxidation of unsaturated fatty acids, which can be correlated with the aroma of the food. The linoleic acid was partly oxidized in air and thereafter analyzed in an electrolyte system of 90% ethanol and 10% water. The separation pattern clearly showed the separation of linoleic acid from its hydroxyperoxides by the difference in thermal step height and also UV absorbance. (Figure 54).

J. Drug Analysis

Capillary isotachophoresis has proven to be an excellent tool for purity control of various drugs as well as in studying their metabolism and excretion.

The use of isotachophoresis in the quality control of various kinds of antibiotics has been described in Reference 204. Several kinds of penicillins and tetracyclines were analyzed in different electrolyte systems. Despite small difference in mobility due to similarity of structure, they were separated and quantified. Some typical quality control analyses of different penicillins are illustrated in Figure 55. Figure 56 illustrates the analysis of a tetracyline (doxycycline) in an ampoule for infusion. The ampoule for infusion also contained ascorbic acid, which can be seen as the first long UV-absorbing zone. Miyazaki and Katoh[205] have further described the qualitative and quantitative

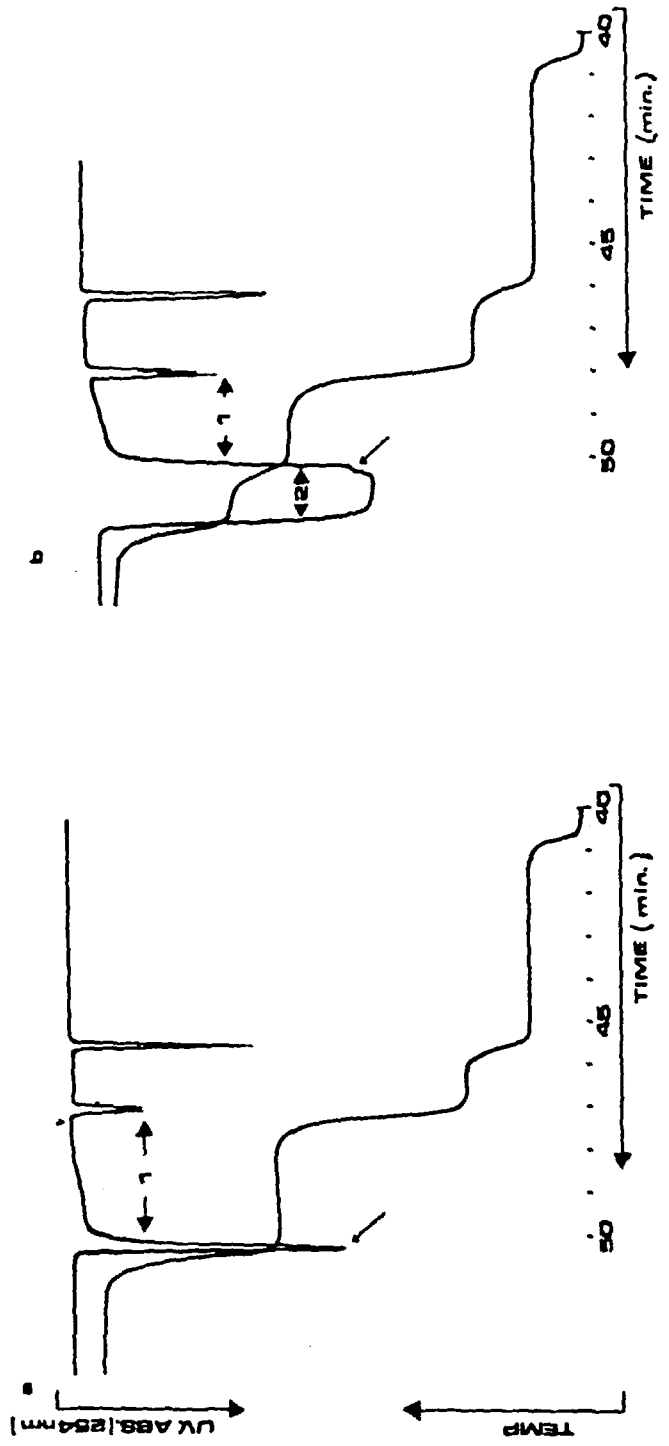


FIGURE 54. Separation of 50 nmol of linoleic acid before (a) and after (b) oxidation in the presence of soy bean lipoxygenase (zone 1 = linoleic acid, zone 2 = hydroperoxides). The leading electrolyte was 0.01 *M* TRIS and 0.2% Triton® X-100 and the terminator was 0.01 *M* cacodylic acid. All electrolytes were in a 90% ethanol-10% water medium. UV absorption (254 nm) and thermal detectors were used to detect the zones at a constant current of 50 μA. (From Kopwillem, A. and Eriksson, K., in LKB Application Note No. 111, LKB-Produkter AB, Bromma, Sweden, 1974. With permission.)

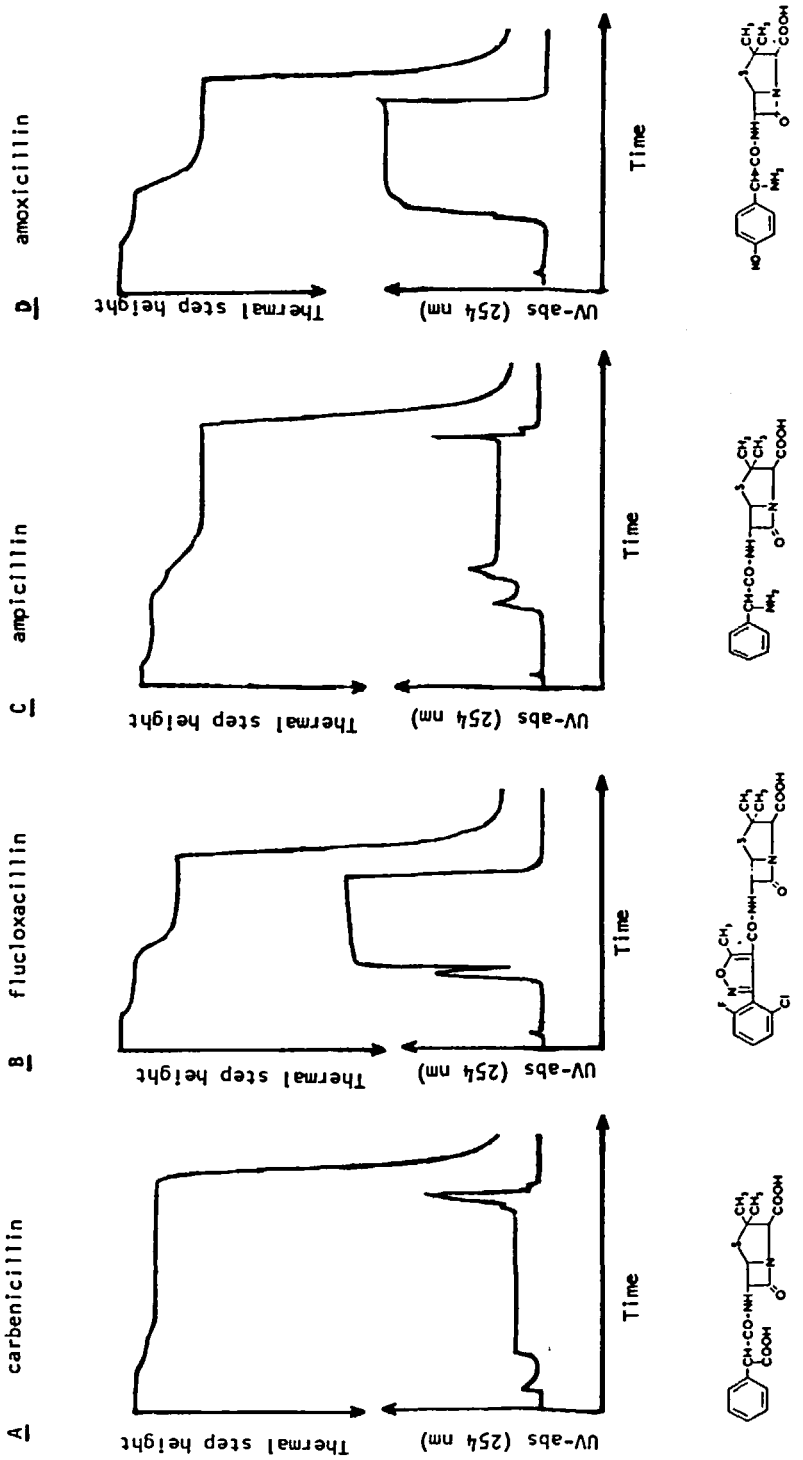


FIGURE 55. Analyses of different penicillins. Electrolyte system No. 24, $pH_L = 7.2$, Table 2. Terminating ion: β-alanine. (From LKB Application Laboratory, LKB Isotachophoresis News No. 3, LKB-Produkter AB, Bromma, Sweden, 1977. With permission.)

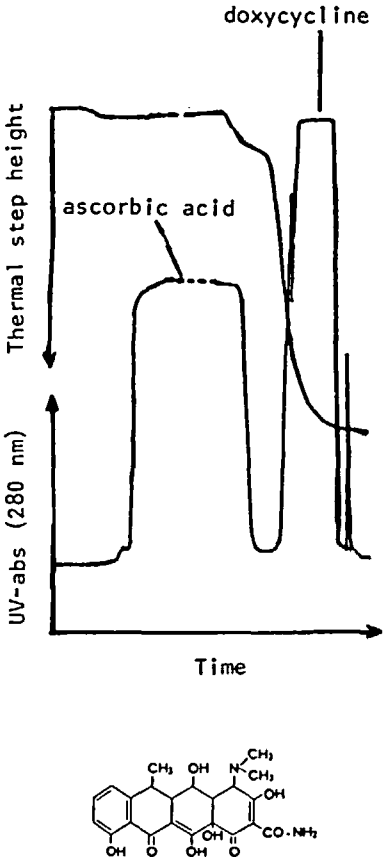


FIGURE 56. Analysis of a tetracycline (doxycycline) in an ampoule for infusion. Electrolyte system No. 24, pH_L = 7.0, Table 2. Terminating ion: β-alanine. (From LKB Application Laboratory, LKB Isotachophoresis News No. 3, LKB-Produkter AB, Bromma, Sweden, 1977. With permission.)

analyses of some drugs such as bleomycins, cephalosporins, berberin and related compounds, and glycyrrhizin.

Everaerts et al.[17] describe the analysis of aspirin (acetylsalicylate) and its oxidation products. The "pure" product contained a considerable amount of phosphate. After oxidation of the aspirin, by introducing air into a hot solution of the drug, the components phosphate, salicylate, acetylsalicylate, and acetate could be detected and quantified. In the same paper,[17] the analysis of the antiinflammatory drug, dexamethazone sodium phosphate, is shown before and after purification. The degradation of atropine has been studied by Everaerts et al.[18,206] Atropine, poorly sterilized, was analyzed and the degradation products tropane and methylamine were clearly separated from the atropine. In this way degradation can be studied as a function of time.

Water-soluble vitamins have been analyzed by capillary isotachophoresis. Everaerts et al.[18] have, for example, shown the possibility of separating vitamin B_1, B_6, and nicotinamide. The separation of vitamins B_1, B_2, B_6, and folic acid is shown in Figure 57. The sample was a commercially obtained mixture of vitamins. The

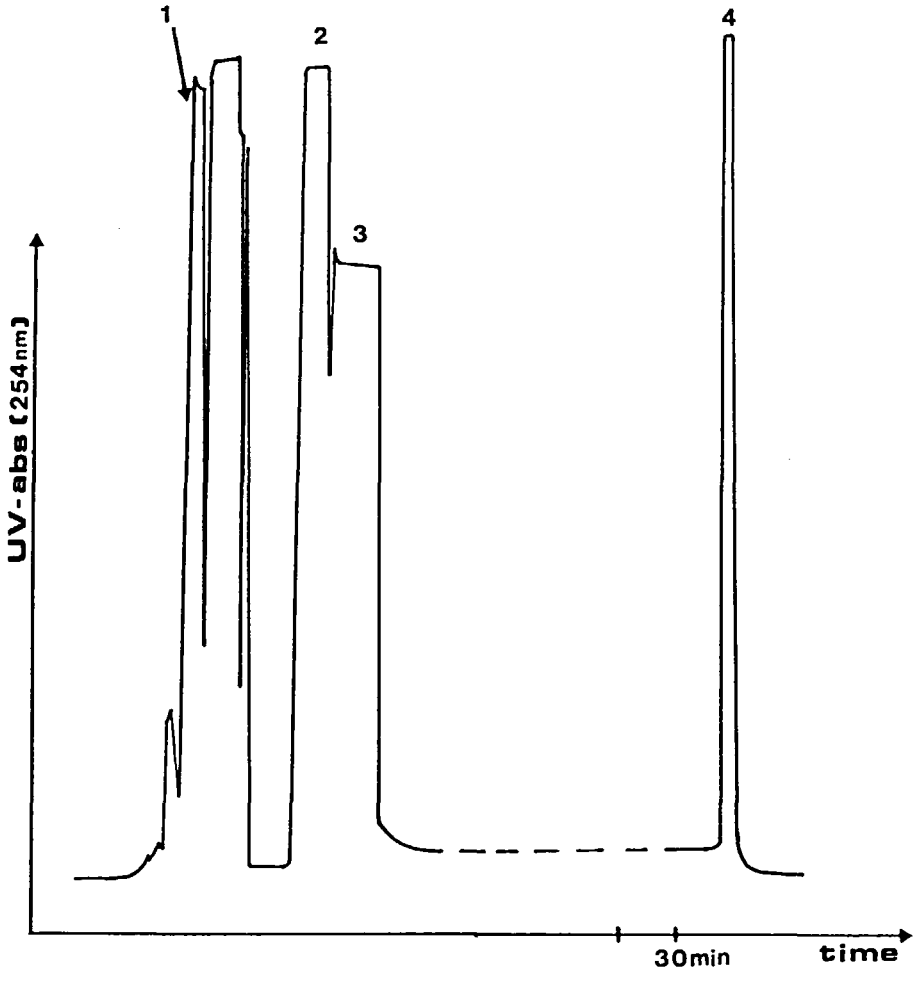


FIGURE 57. Isotachophoretic separation of vitamins B_1(2), B_2(4), B_6(3) and folic acid (1). Electrolyte system No. 24, pH_L = 8.7, Table 2. Terminating ion: ϵ-aminocaproic acid. (From LKB Application Laboratory, Bromma, Sweden.)

determination of ascorbic acid (vitamin C) has been dealt with in several articles.[110,112,113] For further details see Section XV.B.

A recent work of Flynn[207] shows the use of capillary isotachophoresis for the determination of trypanocidal arsenical drugs in aqueous solution. Figure 58 shows the isotachophoretic pattern obtained from the pentavalent arsenical sodium melarsen. The reliability of the isotachophoretic quantitation was tested by comparison with a radiodilution method and the accuracy of the isotachophoretic estimation was found to be at least of the same order as that of the dilution technique. The sensitivity of isotachophoresis was, however, about 5 to 10 times better than that of the dilution technique.[207] Several other arsenicals were also analyzed, such as 3-NO_2-4-OH-phenyl arsonic acid, phenylarsonic acid, 2-NO_2-phenylarsonic acid, 4-NH_2-phenylarsonic acid and 4-glycinamide phenyl-arsonic acid (Tryparsamide). Differential estimation of the trivalent and pentavalent forms of these compounds was shown to be possible using this technique, a separation which is not available with classical methods for the determination of arsenic.

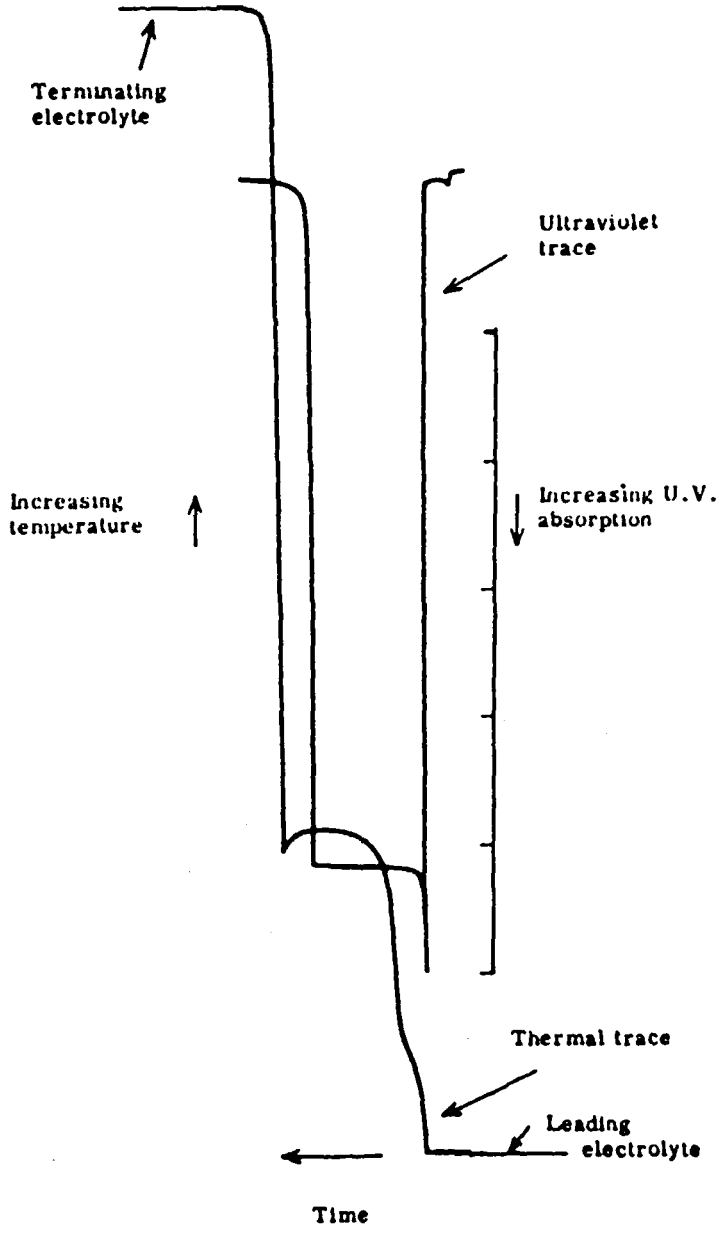


FIGURE 58. Analysis of arsenical sodium melarsen. Electrolyte system No. 24, pH_L = 8.0, Table 2. (From Flynn, I. W., *J. Pharmacol. Methods*, 2, 279 [1979]. With permission.)

The quantitative analysis of drugs in serum has been described in two recent papers by Gustavsson et al.[138] and Moberg et al.[62] The paper by Gustavsson et al. describes the quantitative determination of 5-Fluorouracil (5-FU) in serum. 5-FU is one of the most effective drugs for the palliative treatment of solid tumors in the gastrointestinal tract. Because of the low levels of 5-FU in serum, the samples were concentrated on an anion exchanger. The technique of isotachophoresis was found to be simpler than the available gas chromatographic-mass spectrometric methods and the sensitivity about the same. Down to 50 pmol of the drug was determined in serum with a methodological error of

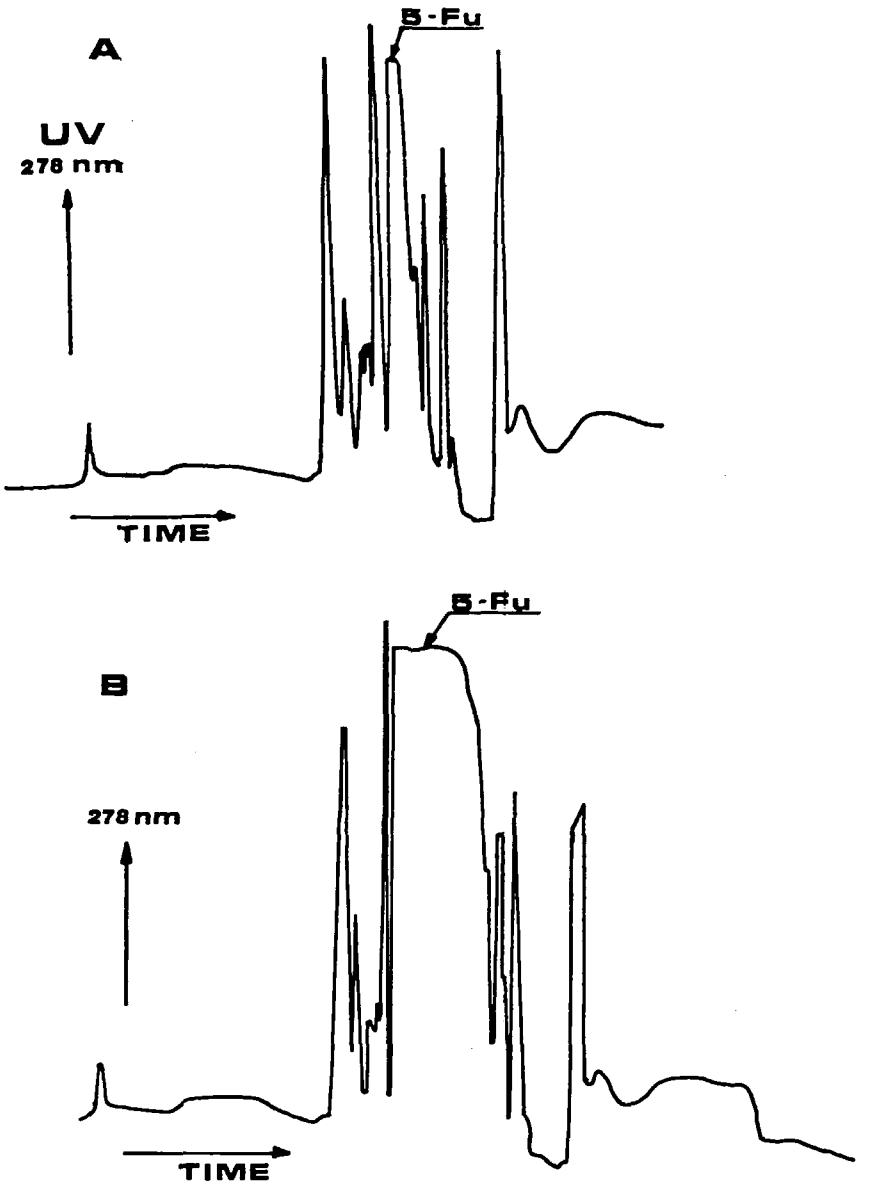


FIGURE 59. Analysis of a sample corresponding to 1 mℓ of serum from a patient treated by continuous infusion of 5-FU (15 $mg \cdot kg^{-1}$) daily. B: Analysis from the same case after addition 2 nmol of 5-FU to the original sample. Electrolyte system No. 24, Table 2. Analysis time: 20 min. (From Gustavsson, B. and Baldesten, A., *J. Chromatogr.*, 179, 151 [1979]; *Proc. Ist Int. Symp. Isotachophoresis,* Adam, A., and Schots, C., Eds., Elsevier, Amsterdam, 1980. With permission.)

±6%. The analysis time was less than 20 min. Figure 59 illustrates a typical analysis pattern of 5-FU in serum.

Moberg et al.[62] investigated the use of isotachophoresis in determining minute amounts of theophylline in plasma. Theophylline (1,3-dimethylxanthine) is a key drug in the treatment of asthma because of its effectiveness as a bronchodilator. The therapeutic value depends on the concentration in the blood. Levels of 10 to 20 μg/mℓ are considered to be therapeutic plasma concentrations. To be able to detect trace amounts

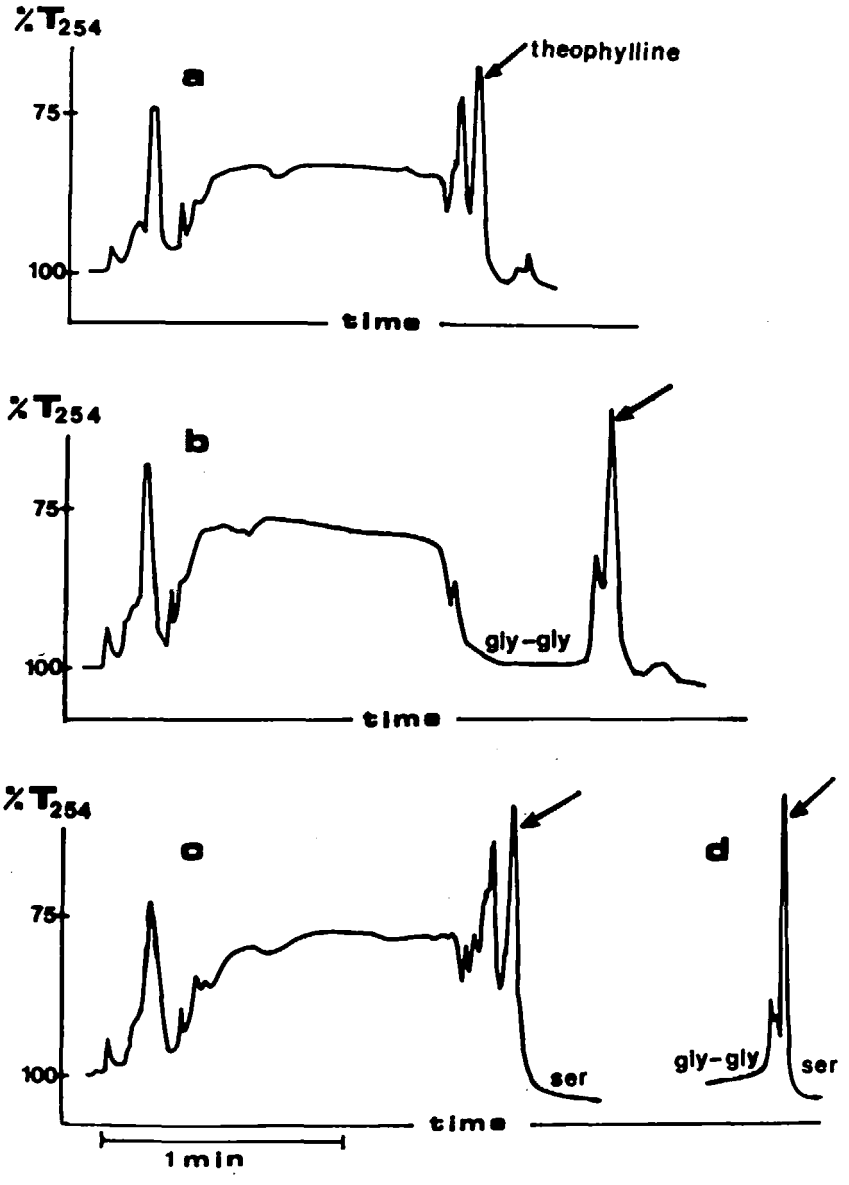


FIGURE 60. Isotachophoretic analysis of a mixture of plasma, theophylline and polyethyleneglycol. The arrow indicates the theophylline peak. For explanation, see text. Electrolyte system No. 34, Table 2. (From Moberg, U., Hjalmarsson, S.-G., and Mellstrand, T., *J. Chromatogr.*, 181, 147 [1980]. With permission.)

of theophylline in a complex mixture such as plasma or serum, it was necessary to remove interfering proteins by a precipitation step. Polyethylene glycol was used as a precipitating agent. After precipitation and centrifugation of 50 $\mu\ell$ of plasma, the clear supernatant was decanted and a few microliters were analyzed. In the electrolyte system used, related compounds such as theobromine and caffeine did not interfere. By using isotachophoresis it was possible to quantify down to 25 pmol of theophylline in plasma. The total analysis time was about 15 min, including the precipitation step. Comparison of the results with HPLC showed a good correlation. The isotachophoretic analysis of theophylline in plasma is shown in Figure 60. Figure 60a shows the analysis performed

with Cl^- as the leading ion and glycine as the terminating ion. However, in order to space apart the theophylline peak from the other components, glycylglycine was used as leading ion (Figure 60b) and serine as terminating ion (Figure 60c). In Figure 60d glycyl-glycine was used as leading ion and serine as terminating ion, producing excellent spacing of the theophylline peak from other zones. The quantity was determined by measuring the height of the peak (see also Section IX.).

The formation of a complex between a protein and a drug has been studied by Sjödahl and Hjalmarsson.[208] The interacting system studied was composed of the protein human serum albumin and the antiinflammatory drug indomethacin, 1-(p-chlorobenzyl)-5-methoxy-2-methyl-indole-3-acetic acid. By the use of isotachophoresis, it was possible to determine the number of molecules of indomethacin bound to human serum albumin (see Section XV.L.).

K. Enzyme Reactions

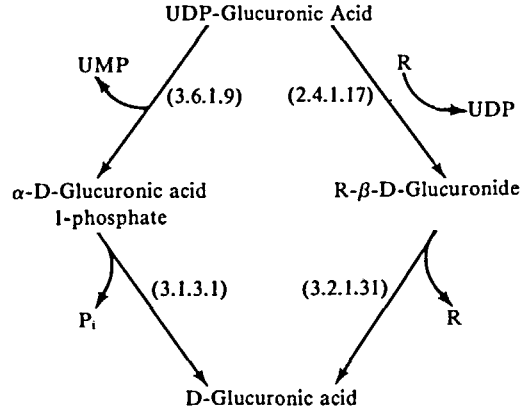
The method of isotachophoresis offers the possibility of simultaneously measuring substrate, intermediate transformation products, and final products of enzyme reactions. The instrumentation often makes it possible to analyze the reaction mixture directly without sample treatment and to analyze aliquots at any desired time in order to obtain plots of the changes in concentration of the compounds involved in an enzymatic reaction. Kopwillem[209] measured the formation of 6-phosphogluconate (6-P-G) from glucose in an enzyme preparation containing both hexokinase and glucose-6-phosphate (G-6-P) dehydrogenase activity. She worked with an electrolyte system which separates ATP, ADP, G-6-P, 6-P-G, NADPH, and NADP, and found that the extent of the formation of G-6-P, and ADP and the breakdown of ATP was linearly related to the amount of glucose in the reaction mixture. In another paper[210] Kopwillem showed that the transformation of pyruvate into succinate by a calf heart mitochondrial system could be followed by isotachophoresis. The reaction mixture containing the mitochondria could be used directly. It was possible to quantify malonate, phosphate, malate, ADP, succinate, and AMP in the reaction mixtures, and to show that the pyruvate had disappeared. Willemsen[211] studied the reaction

$$\text{Pyruvate} + \text{NADH} \underset{\text{lactate dehydrogenase}}{\rightleftharpoons} \text{NAD} + \text{lactate}$$

by isotachophoresis. All the ionic components of the reaction can be simultaneously registered. The author found that the formation of NAD and lactate showed a linear relationship with time, whereas the consumption of NADH and pyruvate was not linear during the first 30 min. However, after 40, 60, and 70 min the amount of lactate produced agreed reasonably well with the amount of pyruvate which had disappeared. The same applied for NADH and NAD. This enzyme system was also studied isotachophoretically by Everaerts and Verheggen[212] with LDH from pig heart. In the same paper they showed the conversion of glucose to glucose-6-phosphate by hexokinase from yeast followed by the conversion of glucose-6-phosphate by glucose-6-phosphate kinase. The authors state that all enzymatic conversions making use of NADP (NADPH) or ATP (ADP, AMP) may be analyzed or followed by isotachophoresis.

The most extensive study of enzyme reactions where the ions of interest have been quantified isotachophoretically has been done recently by Holloway and co-workers.[150-152] They have followed the glucuronic acid pathways from UDP-glucuronate (illustrated below). One of the routes, which involves UDP-glucuronyltransferase (EC 2.4.1.17), catalyzes the transfer of the glucuronate residue from the nucleotide to a wide range of exogenous and endogenous toxins, and serves as a detoxification mechanism, primarily in the liver. In another pathway, UDP-glucuronate serves as substrate for a hydrolytic

enzyme which is believed to be a nonspecific nucleotide pyrophosphatase (EC 3.6.1.9). A general alkaline phosphatase (EC 3.1.3.1) and the β-glucuronidase (EC 3.2.1.31) are also involved in the reaction



Capillary isotachophoresis has been applied to the complete analysis of the ions involved in these pathways. The technique has also been used to assay the UDP-glucuronyltransferase when adding paracetamol to the system.[152] Figure 61a illustrates the analysis of an incubation mixture at an early state and Fig. 61b when the reactions have proceeded for some time. From these experiments the kinetic profiles were calculated. The analysis was shown to be accurate, with as little as 1 nmol of a particular substrate or product in the reaction aliquot, but usable results were still obtained down to 0.1 nmol. It was possible to analyze the reaction mixture without pretreatment. Purified liver fractions from rabbit, rat, and pig were used as sources of enzymes.

L. Interaction Studies

Capillary isotachophoresis has proven to be a highly potent tool for the separation of compounds exhibiting very small differences in effective electrophoretic mobility. It could therefore be anticipated that upon the formation of a complex between two or more compounds, it should be possible to distinquish between the complex formed and the noncomplexed ionic species. Several interacting systems (protein/drug, protein/detergent, and protein/protein) have recently been studied.

Sjödahl and Hjalmarsson[208] studied the interacting system composed of the protein human serum albumin (HSA) and the anti-inflammatory drug indomethacin (I). This drug is frequently used for the treatment of rheumatic diseases. The HSA-I system was chosen for this approach of using capillary isotachophoresis in protein-drug complex formation studies since it was a relatively well-studied system involving a strongly protein-bound drug. Depending on the experimental techniques previously used, it has been found that indomethacin binds to four or five binding sites on the HSA-molecule. Figure 62 illustrates the UV profiles obtained from the isotachophoretic analysis of the individual components, HSA and indomethacin. By using capillary isotachophoresis, the number of binding sites on the HSA-molecule can be determined very accurately by following the titration of the binding sites on HSA with indomethacin, as illustrated in Figure 63. The significant feature of these UV profiles is the increase in peak-height with increasing amount of indomethacin. This reflects the difference between the UV-absorptivity of HSA alone and that of the complex of HSA-I_n, where $_n$ indicates the relative molar amount of bound indomethacin. Furthermore, it is evident that no or very small amounts of free indomethacin are present in the first three samples. It is concluded that, for these molar ratios, the binding of the drug to the protein is practically

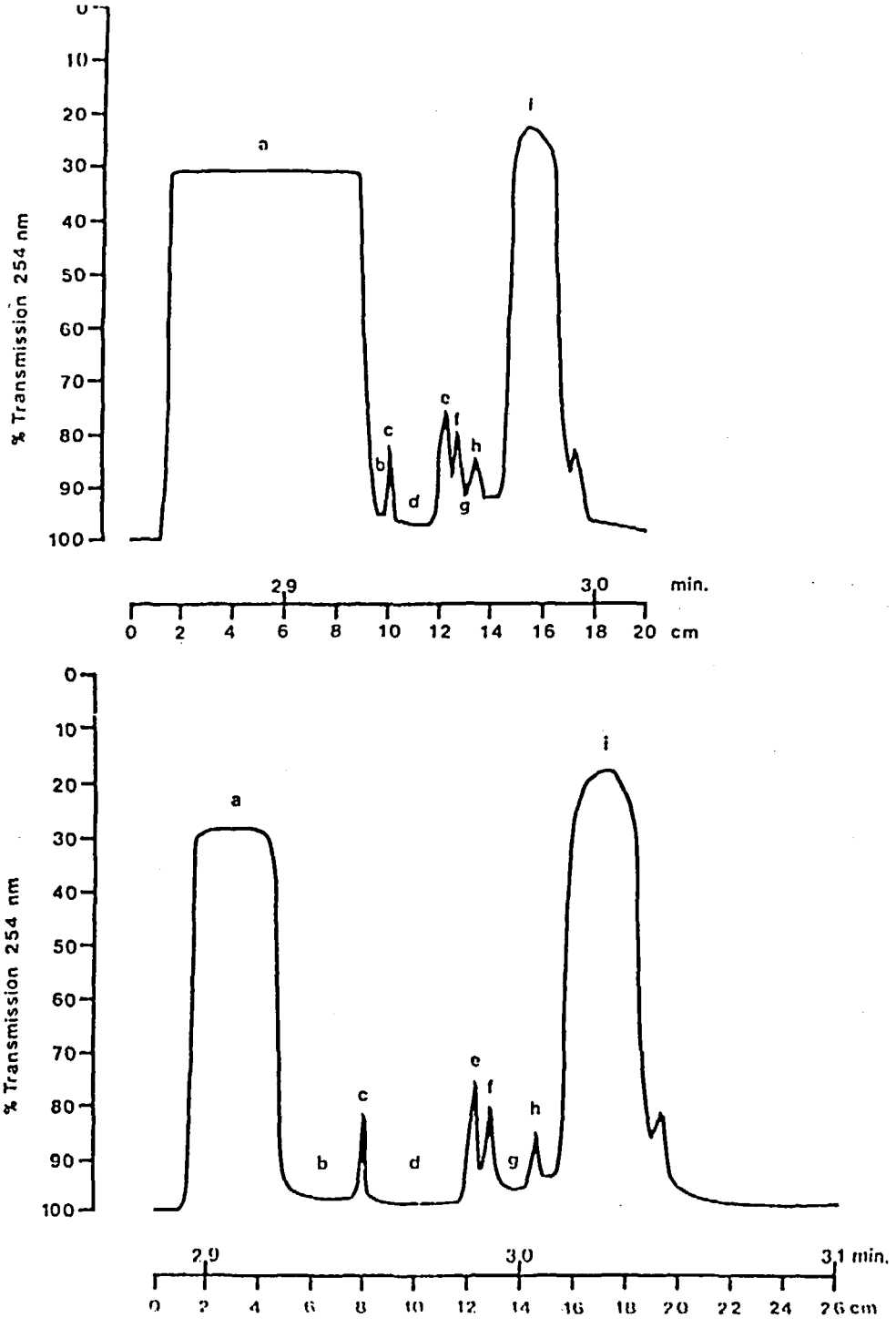


FIGURE 61. Isotachophoretic analysis showing stages of hydrolysis of UDPGA by nucleotide pyrophosphatase. The upper diagram is at an early stage of the reaction, and the lower diagram is at a considerably later stage. Peaks "a" and "i" are due to UDPGA and UMP, respectively. The nonabsorbing zones "b", "d", and "g" are due to GA-1-P, inorganic phosphate, and GA, respectively. Impurities in the system are shown by the small peaks, "c", "e", "f", and "h". The chart speed employed was 6 cm/min. Electrolyte system No. 5, pH_L = 3.89, Table 2. (From Holloway, C. J., *Proc. Ist Int. Symp. Isotachophoresis*, Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980, With permission.)

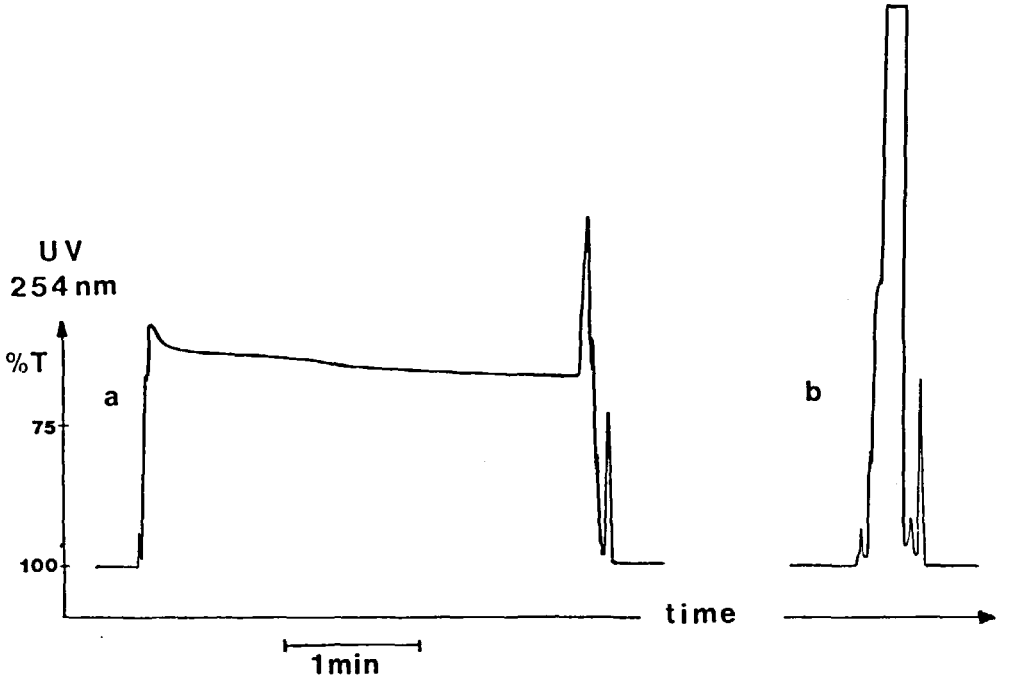


FIGURE 62. UV profiles from the isotachophoretic analysis of albumin (Fig. a) and indomethacin (Fig. b), respectively. Electrolyte system No. 36, $pH_L = 9.0$, Table 2. (From Sjödahl, J. and Hjalmarsson, S.-G., *FEBS Lett.*, 92(1), 22 [1978]. With permission.)

stoichiometric, i.e. $_n$ equals 0.7, 1.4, and 2.8, respectively. However, when molar ratio of 1:5.5 (HSA:I) is reached, a sharp indomethacin zone appears, indicating that the albumin is saturated with bound indomethacin (Figure 63d). By measuring the width of the indomethacin zone, the molar amount can be calculated from a calibration curve. Since the total amount of added indomethacin is known, the amount of bound indomethacin can be easily calculated and, thereby, the saturation ratio, which in this case was calculated to be 1:3.9 (HSA:I). This indicated the binding of four indomethacin molecules to each molecule of albumin.

In a recent article, Hjalmarsson[213] utilized the same method in studying protein-detergent interactions. The interactions of sodium dodecyl sulphate (SDS) with bovine serum albumin and ovalbumin were studied. The method made possible the very accurate determination of the number of ligands bound to the high-affinity sites of the native proteins. Bovine serum albumin was found to have seven high-affinity binding sites whereas ovalbumin in its native state was found to lack high-affinity binding sites for SDS.

The titration of the binding sites on bovine serum albumin with SDS is illustrated in Figure 64. When SDS is added to a molar ratio of 1:6.5 (bovine serum albumin:SDS), it is evident that no free SDS is present in the sample (Figure 64b). However, when a molar ratio of 1:7.4 is reached, a narrow zone of SDS appears, indicating that the albumin is saturated with bound SDS (Figure 64c). From just the first three experiments it could be concluded that each bovine serum albumin molecule binds seven SDS molecules. This binding number can, however, be verified by adding more SDS to each albumin molecule and calculating the amount of free SDS from a calibration curve. The saturation ratio was thus calculated to be 1:7.1, indicating the binding of seven SDS molecules to each molecule of bovine serum albumin.

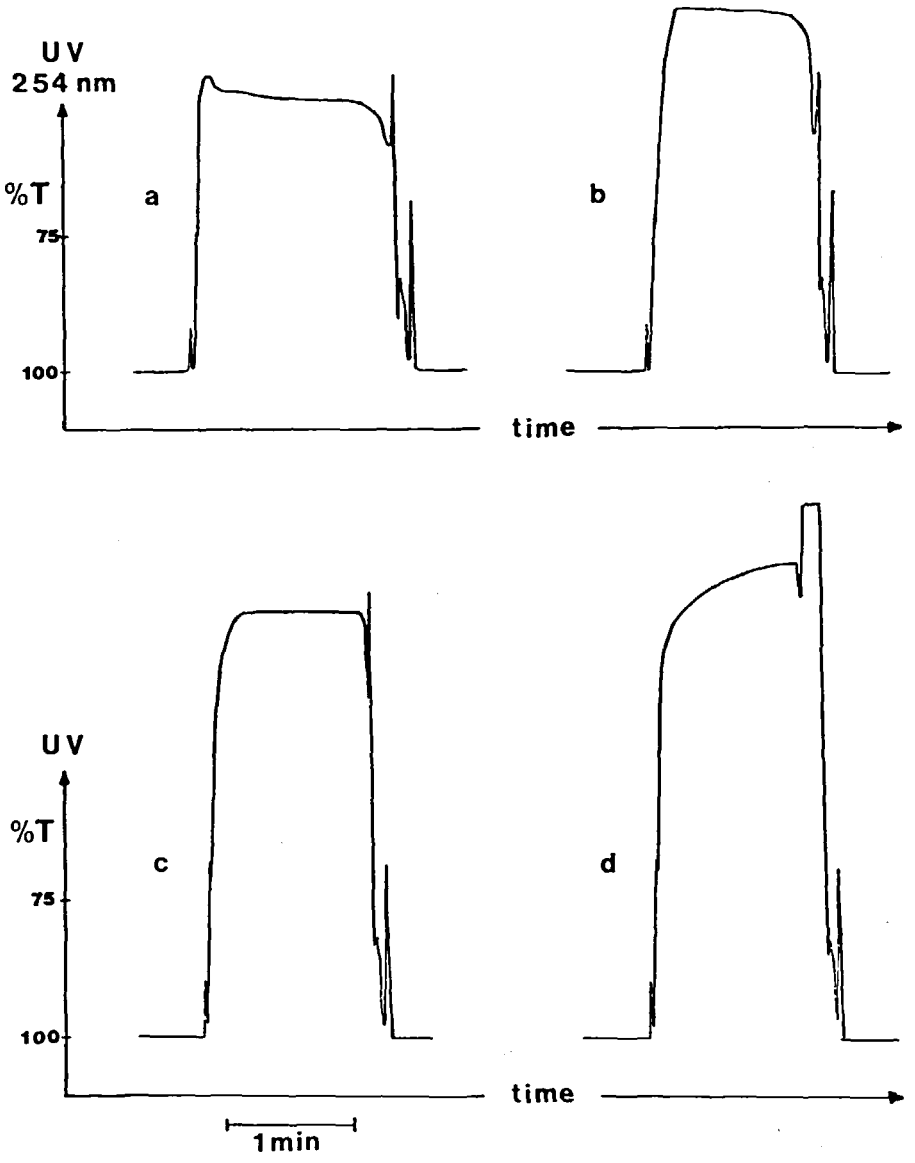


FIGURE 63. UV profiles from the isotachophoretic analysis of incubation mixtures containing various ratios of albumin to indomethacin. Ratio of albumin to indomethacin: 1:0.7 (a), 1:1.4 (b), 1:2.8 (c), and 1:5.5 (d). (From Sjödahl, J. and Hjalmarsson, S.-G., *FEBS Lett.*, 92(1), 22[1978]. With permission.)

Ovalbumin was titrated with SDS in the same way as illustrated in Figure 65. Figure 65b illustrates the UV profile of the incubated sample of ovalbumin and SDS with the molar ratio of 1:1.1. It is evident that a zone of free SDS occurs and from the corresponding zone length it is obvious that no SDS is bound to the ovalbumin. The binding values obtained by isotachophoresis are in good agreement with values obtained by other techniques such as equilibrium dialysis and nuclear magnetic resonance. The overall accuracy of the values obtained by isotachophoresis was found to be within 3 to 5%, with the main errors being in the preparation of the solutions, injection of the sample, and measurement of the zone lengths.

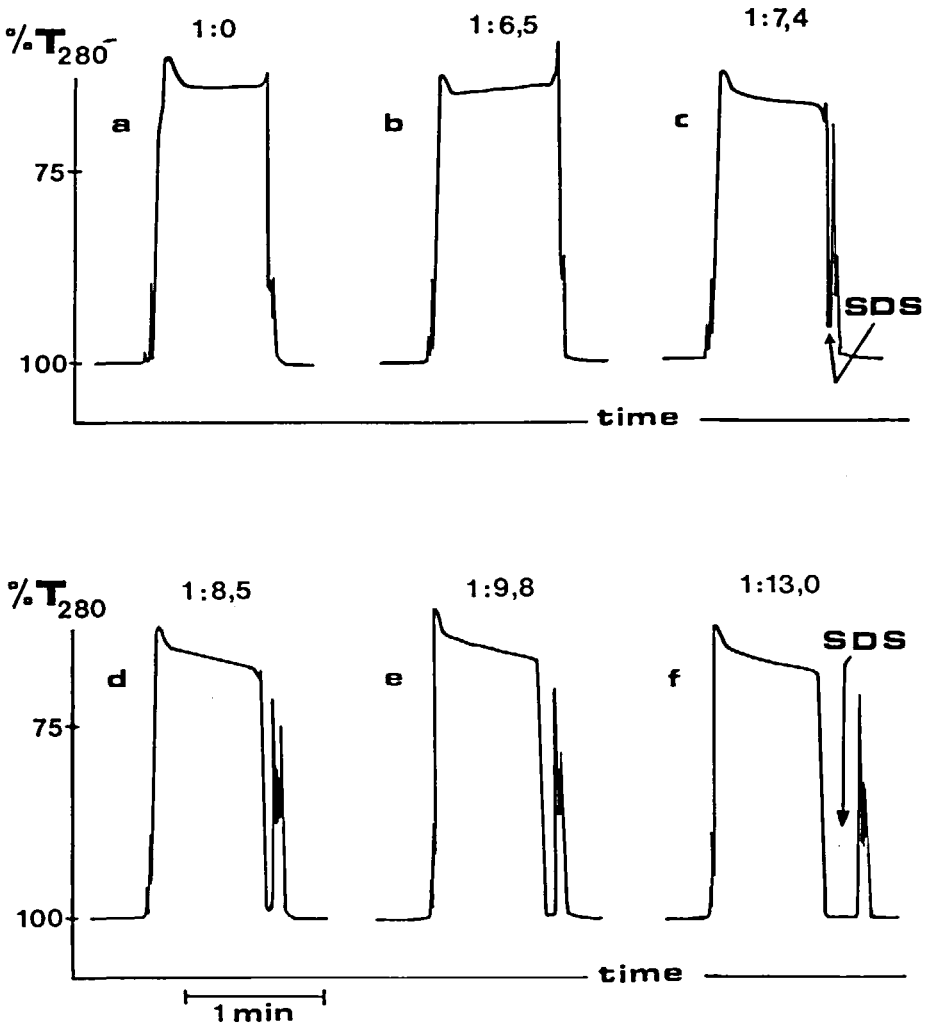


FIGURE 64. UV profiles from the isotachophoretic analyses of incubation mixtures containing various molar ratios of bovine serum albumin to SDS. Ratio of albumin to SDS: 1:0 (a), 1: 6.5 (b), 1: 7.4 (c), 1: 8.5 (d), 1: 9.8 (e), and 1: 13.0 (f). Electrolyte system No. 24, pH = 7.80, Table 2. (From Hjalmarsson, S.-G., *Biochim. Biophys. Acta*, 581, 210 [1979]With permission.)

Reactions between antigens and antibodies have been studied by Delmotte[182,183] and Hedlund et al.[188] The reaction between an antibody and an antigen can result in either the formation of an insoluble complex with no antigen remaining in solution or the formation of soluble immune complexes with free antigen left in the solution. In a series of model experiments, Delmotte and Hedlund et al. have used capillary isotachophoresis to study the formation of soluble immune complexes. Figure 66 illustrates the analyses of a mixture of albumin and an antialbumin immunoglobulin preparation. The upper tracing is the result of the injection of a pure albumin solution mixed with an equal volume of water. The middle tracing corresponds to the antialbumin immunoglobulin preparation also mixed with an equal volume of water. The lower tracing results from the injection of the supernatant from a 1:1 mixture of the albumin and the immunoglobulin solutions. From the isotachophoretic tracings, it is evident that a reaction has taken place between the antibody and the antigen. As can be seen, there is a drop in the UV-peak area

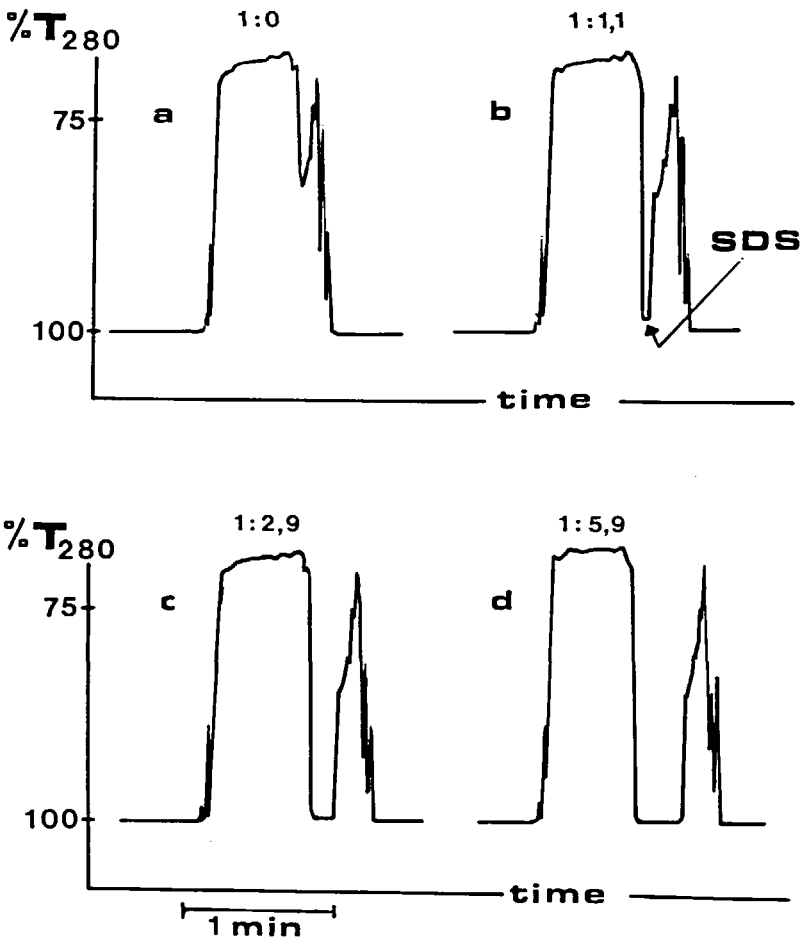


FIGURE 65. UV profiles from the isotachophoretic analyses of incubation mixtures containing various molar ratios of ovalbumin to SDS. Ratio of ovalbumin to SDS: 1: 0 (a), 1: 1.1 (b), 1: 2.9 (c), and 1: 5.9 (d). Electrolyte system No. 24, pH = 7.80, Table 2. (From Hjalmarsson, S.-G., *Biochim. Biophys. Acta*, 581, 210 [1979]. With permission.)

of the albumin zone to about 60% of its original value. In the region of the immunoglobulin mobility-subfractions, there is a substantial drop in the zone area of nearly all the fractions. In the region of mobility between the amino acid glycine and albumin, there is a strong elevation of the peak areas. This has been interpreted[183] as being due to the formation of soluble immune-complexes with mobilities lying in between those of the antigen and the antibody. Experiments like the one illustrated in Figure 66 can give valuable information in, for example, following the formation of soluble immune complexes. In addition, the percentage concentration of a specific immunoglobulin in a preparation can be quantitatively estimated and the change in peak areas of either antigen or antibody can be used to monitor an immunological reaction.

M. Miscellaneous

As has been described in the previous sections, capillary isotachophoresis is a very versatile method used in a large number of varied application areas. Some possible areas of applications have only been dealt with briefly, such as the analysis of carbohydrates,

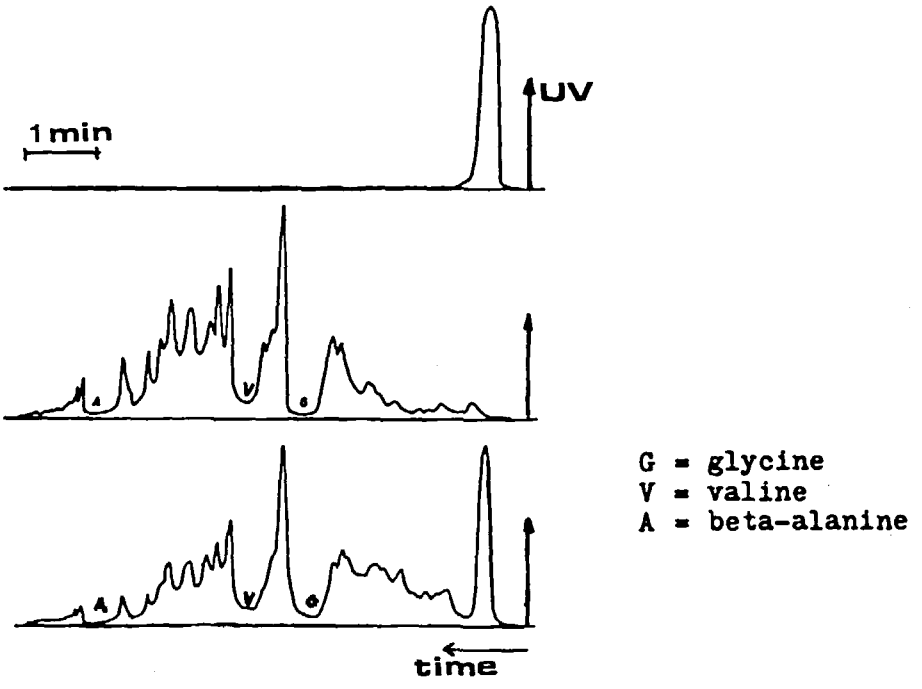


FIGURE 66. Reaction between antigen and antibody followed by isotachophoresis. Upper tracing: albumin alone; middle tracing, anti-human albumin immunoglobulin preparation alone; lower tracing, supernatant from mixture of the two previous sample solutions. G = glycine V = valine A = β-alaine Electrolyte system No 37, Table 2. (From Delmotte, P., *Electrophoresis '78*, Catsimpoolas, N., Ed., Elsevier, North-Holland, Amsterdam, 1978. With permission.)

cells, viruses, isotopes, and metal-EDTA complexes. However, investigations made with capillary isotachophoresis in these areas have given very promising results.

The isotachophoretic analysis of the anionic products formed by the homogeneous oxidation of sugar has been investigated by Everaerts and Konz.[107] Polyoxyacids obtained from the catalytic oxidation of sorbose and fructose were analyzed. The acids quantitatively determined were oxalic acid, tartronic acid, formic acid, 2-keto-l-gluconic acid, glycolic and glycerinic acids, gluconic acid, and arabonic acid.

Honda et al.[214] and Oka et al.[215] recently studied the periodate oxidation of carbohydrates under controlled conditions. Periodate oxidizes α-glycolic substances in a specific manner. Thus, periodate oxidation has been widely applied to structural studies of various carbohydrates. Basically, the method requires the determination of periodate, formate, and iodate in a reaction mixture. The most commonly used conventional methods (redox-titration, alkali-titration, spectrophotometry, potentiometry and gas-liquid chromatography) have, however, limited sensitivity or selectivity; for example, more than 1 mg of carbohydrate is required for periodate oxidation analysis.[215] This is a large amount for biologically active substances, thereby making analysis by conventional methods impossible in practice. In this regard, isotachophoresis has proven to be suitable in analyzing the reaction products (periodate, formate, and iodate) of periodate oxidation. It has been shown that the analyzable amounts of these products are less than 5 nmol, which corresponds to less than 1 μg of carbohydrates and is a value one thousandth of that required for the conventional methods of periodate oxidation analysis. Honda et al.[214] have also shown the separation of glyoxylate, glycerate, and lactate; these are all typical carboxylates which can be obtained by further oxidation and

hydrolysis of the dialdehyde fragments formed on periodate oxidation of carbohytrates.

Particles such as cells and viruses are often too large to migrate electrophoretically in the channels of a supporting medium. Therefore, electrophoresis in free solution is an alternative. The isotachophoretic analysis of cells and viruses has been described in a number of articles. Hjertén[216] showed the analysis of a crude extract of satellite tobacco necrosis virus. The isotachophoretic analyses were made in a slowly rotating tube (40 r/min) made of quartz and with a length of about 400 mm and an inner diameter of 3 mm. In this way stabilization against convective disturbances was achieved.

Bier et al.,[217] Smolka and Bier,[218] and Hinchley[219] have described the possibility of separating cells by isotachophoresis by going to a zero gravity environment. Most of the work was done with erythrocytes and a partial separation of, for example, chicken and sheep cells was achieved.

The separation of isotopes of potassium and rubidium has been described by Fiks.[220] Konstantinov and Bakulin[221] showed the separation of chlorine isotopes, and Wiedemann and Vestermark[222] separated the sodium isotopes ^{22}Na and ^{24}Na.

The separation and quantification of a number of metal-EDTA complexes has been studied by Yoshida et al.,[223] who also examined the effect on the separation when changing the pH and counter-ion of the leading electrolyte. The metal (III) complexes were found to have smaller effective mobilities than the metal (II) complexes. The relationship between the mobilities of metal (II) complexes and their molecular weights was also investigated.

Everaerts et al.[18] have shown the separation of Ni and Al present in a Raney Nickel catalyst. The two metal ions were separated as EDTA complexes.

ACKNOWLEDGMENTS

For discussions and positive criticism the authors want to express their gratitude to Dr. Lennart Arlinger. Their thanks are extended to Dr. Roger Bishop for linguistic revision, to Mrs. Iris Gustafson for the typing of this article and to Mr. Gösta Larsson for assistance with the illustrations.

REFERENCES

1. **Kohlrausch, F.,** *Ann. Physik,* 62, 209 (1897).
2. **Kendall, J.,** *Phys. Rev.,* 21, 389 (1923).
3. **Kendall, J.,** *Science,* 67, 163 (1928).
4. **Kendall, J.,** *Proc. Roy. Phil. Soc. Glasgow,* 59, 1 (1931).
5. **Kendall, J. and White, J. F.,** *Proc. Natl. Acad. Sci. U.S.A.,* 10, 458 (1924).
6. **Longsworth, L. G.,** *Natl. Bur. Stand. (U.S.), Circ.,* 524, 59 (1953).
7. **Konstantinov, B. P., Kaimakov, E. A., and Vashaya, N. L.,** *Russ. J. Phys. Chem.,* (trans.), 36, 540 (1962).
8. **Konstantinov, B. P. and Oshurkova, O. V.,** *Dokl. Akad. Nauk SSSR,* (trans.), 148, 1110 (1963).
9. **Everaerts, F. M.,** Graduation Report, University of Technology, Eindhoven, The Netherlands, 1964.
10. **Ornstein, L.,** *Ann. N.Y. Acad. Sci.,* 121, 321 (1964).
11. **Davis, B. J.,** *Ann. N.Y. Acad. Sci.,* 121, 404 (1964).
12. **Vestermark, A.,** Report from the Department of Biochemistry, University of Stockholm, Sweden, 5, 1966.
13. **Everaerts, F. M.,** Thesis, Technische Hogeschool Eindoven, The Netherlands, Eindoven, 1968.
14. **Martin, A. J. P. and Everaerts, F. M.,** *Anal. Chim. Acta,* 38, 233 (1967).
15. **Haglund, H.,** *Sci. Tools,* 17, 2 (1970).
16. **Everaerts, F. M., Beckers, J. L., and Verheggen, Th. P. E. M.,** *Journal of Chromatography Library,* Vol. 6, Elsevier, Amsterdam, 1976.
17. **Everaerts, F. M., Geurts, M., Mikkers, F. E. P., and Verheggen, Th. P. E. M.,** *J. Chromatogr.,* 119, 129 (1976).
18. **Everaerts, F. M., Mikkers, F. E. P., and Verheggen, Th. P. E. M.,** *Sep. Purif. Methods,* 6(2), 287 (1977).

19. **Routs, R. J.**, Thesis, University of Technology, Eindhoven, The Netherlands, 1971.
20. **Beckers, J. L.**, Thesis, Technische Hogeschool, Eindhoven, The Netherlands, 1973.
21. **Haglund, H.**, *Kem. Tidskr.*, 10, 38 (1976).
22. **Strongin, A. Ya., Levin, E. D., and Stepanov, V. H.**, *Bioorg. Chem.*, 2(7), 869, (1976).
23. **Neumann, G.**, *GIT Fachz. Lab.*, 20(7), 825, (1976).
24. **Ryser, P.**, Thesis, University of Bern, Switzerland, 1976.
25. **Miedziak, I. and Waksmundzki, A.**, *Wiad. Chem.*, 32(2), 69, (1978).
26. **Ball, J. and Hjalmarsson, S.**, *Electrofocusing and Isotachophoresis*, Nobusawa, K., Ed., K. Yoritsu Shuppan, Tokyo, 1978, 155.
27. **Van der Moosdijk, A.**, *De Ware (N)-Chemicus*, 6, 57 (1976).
28. **Nagayanagi, Y.**, *Am. Lab.*, 101, October, (1977).
29. **Arlinger, L.**, *Protides of Biological Fluids*, Vol. 22, Peeters, H. Ed., Pergamon Press, Elmsford, N.Y., 1975, 737.
30. **Arlinger, L.**, *J. Chromatogr.*, 91, 785 (1974).
31. **Mikkers, F. E. P., Everaerts, F. M., and Peek, J. A. F.** *J. Chromatogr.*, 168, 293 (1979).
32. **Kaniansky, D., Madajová, V., Zelenský, I., and Stankoviansky, S.**, *J. Chromatogr.*, submitted for publication.
33. **Landolt and Börnstein**, *Zahlenwerte und Funktionen*, 6 Aufl., Bd. 11, Teil 7, Springer-Verlag, Berlin, 1960.
34. **Kortüm, G., Vogel, W., and Andrussow, K.**, *Dissociation Constants of Organic Acids in Aqueous Solution*, IUPAC, Butterworths, London, 1961.
35. **Perrin, D. D.**, *Dissociation Constants of Organic Bases in Aqueous Solution*, IUPAC, Butterworths, London, 1965.
36. **Perrin, D. D.**, *Dissociation Constants of Organic Bases in Aqueous Solution*, (Suppl.,) IUPAC, Butterworths, London, 1972.
37. **Serjeant, B. P. and Dempsey, B.**, *Ionisation Constants of Organic Acids in Aqueous Solution*, IUPAC Chemical Data Series No. 23, Pergamon Press, Elmsford, N.Y., 1979.
38. **Righetti, P. G. and Caravaggio, T.**, *J. Chromatogr.*, 111, 222, (1976).
39. **Baldesten, A.**, *Sci. Tools*, 27, 1 (1980).
40. **Abramson, H. A., Moyer, L. S., and Gorin, M. H.**, *Electrophoresis of Proteins*, Reinhold, New York, 1942.
41. **Martin, A. J. P. and Everaerts, F. M.**, *Proc. R. Soc. London, Ser. A*, 316, 493 (1970).
42. **Arlinger, L.**, *Protides of Biological Fluids*, Vol. 22, Peeters, H., Ed., Pergamon Press, Elmsford, N.Y., 1975, 691.
43. **Delmotte, P.**, *Sci. Tools*, 24, 33 (1977).
44. **Woledge, R. C. and Reilly, P.**, *Proc. Ist Int. Symp. Isotachophoresis*, Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980.
45. **Fredriksson, S.**, *J. Chromatogr.*, 188, 262 (1980).
46. **Beckers, J. L. and Everaerts, F. M.**, *J. Chromatogr.*, 51, 339 (1970).
47. **Arlinger, L.**, LKB Application Note No. 67, LKB-Produkter AB, Bromma, Sweden, 1972.
48. **Everaerts, F. M. and Verheggen, Th. P. E. M.**, *J. Chromatogr.*, 91, 837 (1974).
49. **Kopwillem, A.**, LKB Application Note No. 109, LKB-Produkter AB, Bromma, Sweden, 1974.
50. **Kopwillem, A. and Eriksson, K.**, LKB Application Note No. 111, LKB-Produkter AB, Bromma, Sweden, 1974.
51. **Sugiyama, T., Sakurai, K., and Nagai, T.**, *J. Am. Chem. Soc.*, 196A (1979).
52. **Everaerts, F. M., Verheggen, Th. P. E. M., and Van De Venne, J. L. M.**, *J. Chromatogr.*, 123, 139 (1976).
53. **Akiyama, J. and Mizuno, T.**, *Bunseki Kagaku*, 24, 728 (1975).
54. **Shiogai, Y. and Akiyama, J. I.**, *Bunseki Kagaku*, 27(1), 60 (1978).
55. LKB 2127 Tachophor Instruction Manual, LKB-Produkter AB, Bromma, Sweden.
56. **Ryšlavý, Z., Boček, P., Deml, M., and Janák, J.**, *J. Chromatogr.*, 147, 446 (1978).
57. **Arlinger, L.**, *Proc. Electrofocusing Isotachophoresis Symp.*, Righetti, P. G., Ed., North-Holland, 1975, 331.
58. **Arlinger, L. and Routs, R.**, *Sci. Tools*, 17(1), 21 (1970).
59. **Svendsen, P. J. and Rose, C.**, *Sci. Tools*, 17(1), 13 (1970).
60. **Griffith, A., Catsimpoolas, N., and Kenney, J.**, *Ann. N. Y. Acad. Sci.*, 209, 457 (1973).
61. **Kopwillem, A., Merriman, W. G., Cuddeback, R. M., Smolka, A. J. K., and Bier, M.**, *J. Chromatogr.*, 118, 35 (1976).
62. **Moberg, U., Hjalmarrson, S.-G., and Mellstrand, T.**, *J. Chromatogr.*, 181, 147 (1980).
63. **Lange, P. W.**, *Proc. Ist Int. Symp. Isotachophoresis*, Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980.
64. **Hjalmarsson, S.-G.**, *Sci. Tools*, 22(3), 35 (1975).

65. Arlinger, L., LKB Application Note No. 107, LKB-Produkter AB, Bromma, Sweden, 1974.
66. Svobada, M. and Vacik, J., *J. Chromatogr.*, 119, 539 (1976).
67. Wielders, J. P. M., Thesis, University of Technology, Eindhoven, The Netherlands, 1978.
68. Gower, D. C. and Woledge, R. C., *Sci. Tools*, 24, 2 (1977).
69. Wielders, J. P. M. and Everaerts, F. M., *Electrofocusing and Isotachophoresis*, Radola, B. J. and Graesslin, D., Eds., Walter de Gruyter, Berlin, 1977, 527.
70. Holloway, C. J., Husmann-Holloway, S., Brunner, G., Trautschold, I., and Baldesten, A., *Proc. Electrophoresis '79*, Radola, B., Ed., Walter de Gruyter, Berlin, 1980, 781.
71. Rýslavý, Z., Boček, P., Deml, M., and Janák, J., *J. Chromatogr.*, 147, 369 (1978).
72. Everaerts, F. M., Verheggen, Th. P. E. M., and Mikkers, F. E. P., *J. Chromatogr.*, 169, 21 (1979).
73. Everaerts, F. M. and Routs, R. J., *J. Chromatogr.*, 58, 181 (1971).
74. Chrambach, A., Kapadia, G., and Cantz, M., *Sep. Sci.*, 7, 785 (1972).
75. Everaerts, F. M., Beckers, J. L., and Verheggen, Th. P. E. M., *Ann. N.Y. Acad. Sci.*, 209, 419 (1973).
76. Jovin, T. M., *Biochemistry*, 12, 871 (1973).
77. Jovin, T. M., *Biochemistry*, 12, 879 (1973).
78. Fredriksson, S., *Acta Chem. Scand.*, 23(4), 1450 (1969).
79. Everaerts, F. M. and Verheggen, Th. P. E. M., *J. Chromatogr.*, 53, 315 (1970).
80. Everaerts, F. M. and Verheggen, Th. P. E. M., *J. Chromatogr.*, 73, 193 (1972).
81. Haruki, T. and Akiyama, J., *Anal. Lett.*, 6, 11 (1974).
82. Stankoviansky, S., Cicmanec, P., and Kaniansky, D., *J. Chromatogr.*, 106, 131 (1975).
83. Boček, P., Deml, M., and Janák, J., *J. Chromatogr.*, 106, 283 (1975).
84. Deml, M., Boček, P., and Janák, J., *J. Chromatogr.*, 109, 49 (1975).
85. Akiyama, J. and Mizuno, T., *J. Chromatogr.*, 119, 605 (1976).
86. LKB 2127 Tachophor Data Sheet, LKB-Produkter AB, Bromma, Sweden.
87. Arlinger, L., *J. Chromatogr.*, 118 9 (1976).
88. Moberg, U., Hjalmarsson, S.-G., Arlinger, L., and Lundin, H., *Electrofocusing and Isotachophoresis*, Radola, B. J., and Graesslin, D., Eds., Walter de Gruyter, Berlin, 1977, 515.
89. Kjellin, K. G. and Siden, Å., *Adv. Exp. Med. Biol.*, p. 545, 1978.
90. Rýslavý, Z., Boček, P., Deml, M., and Janák, J., *J. Chromatogr.*, 147, 446 (1978).
91. Everaerts, F. M. and Verheggen, Th. P. E. M., *Sci. Tools*, 17(1), 17 (1970).
92. Beckers, J. L. and Everaerts, F. M., *J. Chromatogr.*, 69, 165 (1972).
93. Everaerts, F. M., *J. Chromatogr.*, 65, 3 (1972).
94. Everaerts, F. M., Mulder, A. J., and Verheggen, Th. P. E. M., *Int. Lab.*, 43, 1974.
95. Everaerts, F. M., Prosé, P., and Verheggen, Th. P. E. M., *Protides of Biological Fluids*, Vol. 22, Peeters, H., Ed., Pergamon Press, Elmsford, N.Y., 1975, 721.
96. Kopwillem, A., *J. Chromatogr.*, 82, 407 (1973).
97. Dunn, J. P. D. and Kemp, R. B., *Protides of Biological Fluids*, Vol. 22, Peeters, H., Ed., Pergamon Press, Elmsford, N.Y., 1975, 727.
98. Sjödin, B., Kopwillem, A., and Karlsson, J., *Protides of Biological Fluids*, Vol. 22, Peeters, H., Ed., Pergamon Press, Elmsford, N.Y., 1975, 733.
99. Sjödin, B., Kopwillem, A., and Karlsson, J., *Scand. J. Clin. Lab. Invest.*, 35, 699 (1975).
100. Van der Hoeven, J. S., Franken, H. C. M., Camp, P. J. M., and Dellebarre, C. W., *Appl. Environ. Microbiol.*, 35(1), 17 (1978).
101. Boček, P., Deml, M., and Janák, J., *J. Chromatogr.*, 156, 323 (1978).
102. Boček, P., Kaplanová, B., Deml, M., and Janák, J., *J. Chromatogr.*, 153, 287 (1978).
103. Boček, P., Kaplanová, B., Deml, M., and Janák, J., *Collect. Czech. Chem. Commun.*, 43(10), 2707 (1978).
104. Boček, P., Pavelka, S., Deml, M., and Janák, J., *J. Chromatogr.*, 151, 436 (1978).
105. Boček, P., Miedziak, I., Deml, M., and Janák, J., *J. Chromatogr.*, 137, 83 (1977).
106. Akiyama, J., *Dev. Biochem.*, 2, 109 (1978).
107. Everaerts, F. M. and Konz, W. J. M., *J. Chromatogr.*, 65, 287 (1972).
108. Everaerts, F. M., Mulder, A. J., and Verheggen, Th. P. E. M., *Am. Lab.*, p. 37 (1973); *Int. Lab.*, p. 43 (1974).
109. Everaerts, F. M., Prosé, P., and Verheggen, Th. P. E. M., *Protides of Biological Fluids*, Vol. 22, Peeter, H., Ed., Pergamon Press, Elmsford, N.Y., 1975, 721.
110. Baldesten, A., Hjalmarsson, S.-G., and Neumann, G., *Fresenius Z. Anal. Chem.*, 290, 148 (1978).
111. Yagi, T., Shiogai, Y., and Akiyama, J., *Shimadzu Hyoron*, 34(4), 229, (1977).
112. Kaiser, K.-P. and Hupf, H., *Dtsch. Lebensm. Rundsch.*, 75, Jahrg., Heft 10, 300, (1979).
113. Kaiser, K.-P. and Hupf, H., *Dtsch. Lebensm. Rundsch.* 75 Jahrg. Heft 11, 346 (1979).
114. Rubach, K., Breyer, C., and Kirchhoff, E., *Z. Lebensm. Unters. Forsch.*, p. 1 (1979).
115. Boček, P., Deml, M., and Janák, J., *J. Chromatogr.*, 106, 283 (1975).

116. **Boček, P., Lekova, K., Deml, M., and Janák, J.,** *J. Chromatogr.,* 117, 97 (1976).
117. **Van der Hoeven, J. S., Franken, H. C. M., Camp, P. J. M., and Dellebarre, C. W.,** *Appl. Environ. Microbiol.,* 35(1) 17 (1978).
118. **Van der Hoeven, J. S. and Franken, H. C. M.,** *Proc. Ist Int. Symp. Isotachophoresis,* Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980.
119. **Bōcek, P., Pavelka, S., Grigelová, K., Deml, M., and Janák, J.,** *J. Chromatogr.,* 154, 356 (1978).
120. **Sollenberg, J. and Baldesten, A.,** *J. Chromatogr.,* 132, 469 (1977).
121. **Vesterberg, O. and Sollenberg, J.,** *Proc. Int. Conf. in vivo Aspects Biotransformation Toxicity Ind. Environ. Xenobiotics,* Gut, I., Ed., Excerpta Medica, Amsterdam, 1977.
122. **Tschöpe, W. and Ritz, E.,** *Eur. J. Clin. Invest.,* in press.
123. **Tschöpe, W., Baldesten, A., Nowak, C., and Ritz, E.,** *Proc. Ist Int. Symp. Isotachophoresis,* Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980.
124. **Tschöpe, W., Brenner, R., Baldesten, A., and Ritz, E.,** *Determination of Urinary Oxalate by Isotachophoresis in Unprocessed Urine,* in press.
125. **Bommer, J., Ritz, E., Tschöpe, W., Waldherr, R., and Gebhardt, M.,** *Kidney Int.,* 16, 722 (1979).
126. **Schmidt, K., Bruchelt, G., and Hagmaier, V.,** *Electrophoresis '79,* Radola, B., Ed., Walter de Gruyter, Berlin, 1980.
127. **Schmidt, K., Bruchelt, G., and Hagmaier, V.,** *J. Clin. Chem. Clin. Biochem.,* 17(3), 187 (1979).
128. **Schmidt, V., Hagmaier, D., Hornig, J. P., Vuilleumier, J. P., and Rutishauser, J. P.,** *Int. Urinary Stone Conf.* Perth, Australia, 1979.
129. **Fredriksson, S.,** *J. Chromatogr.,* 188, 266 (1980).
130. **Mikkers, F., Ringoir, S., and de Smet, R.,** *J. Chromatogr.,* 162, 341 (1979).
131. **Manabe, T., Sasagawa, T., and Okuyama, T.,** *Bunseki Kagaku,* 26, 621 (1977).
132. **Hollaway, W. L. and Ball, J.,** *Int. Lab.,* 41 (1977).
133. **Simmonds, H. A., Sahota, A., Payne, R.,** *J. Clin. Chem. Clin. Biochem.,* 17(6), 441 (1979).
134. **Sahota, A., Simmonds, A., and Payne, R. H.,** *J. Pharmacol. Meth.,* 2, 263 (1979).
135. **Oerlemans, F., Verheggen, Th., Mikkers, F., Everaerts, F. M., and De Bruyn, C.,** *J. Clin. Chem. Clin. Biochem.,* 17(6), (1979).
136. **Oerlemans, F., Verheggen, Th., Mikkers, F., Everaerts, F. M., and De Bruyn, C.,** *J. Clin. Chem. Clin. Biochem.,* 17(6), (1979).
137. **Gustavsson, B. and Baldesten, A.,** *Proc. Ist Int. Symp. Isotachophoresis,* Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980.
138. **Gustavsson, B. and Baldesten, A.,** *J. Chromatogr.,* 179, 151 (1979).
139. **Gustavsson, B.,** personal communication.
140. **Kiso, Y. and Kirokawa, T.,** *Chem. Lett.,* 8, 891 (1979).
141. **Akiyama, J.,** *Dev. Biochem.,* 2, 109 (1978).
142. **Sollenberg, J. and Lundberg, E.-L.,** Bestämning av Ethylendiamin med Isotachofores, *Nord. Yrkeshygieniska Mötet i Sverige,* 28:e (1979).
143. Shimadzu IP Data Sheet, CA 198-902.
144. **Beckers, J. L. and Everaerts, F. M.,** *J. Chromatogr.,* 71, 380 (1972).
145. LKB Isotachophoresis News, No. 1, LKB-Produkter AB, Bromma, Sweden, 1977.
146. **Kopwillem, A.,** *Acta Chem. Scand.,* 27, 2426 (1973).
147. **Kopwillem, A.,** LKB Application Note No. 59, LKB-Produkter AB, Bromma, Sweden, 1973.
148. **Surholt, B.,** *Hoppe-Seyler's Z. Physiol. Chem.,* 358, 1455, 1977.
149. **Sakagishi, Y.,** *Electrofocusing and Isotachophoresis,* Nobusawa, K., Ed., K. Yoritsu Shuppan, Tokyo, 1978, 161.
150. **Holloway, C. J.,** *Proc. Ist Int. Symp. Isotachophoresis,* Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980.
151. **Holloway, C. J., Husmann-Holloway, S., and Brunner, G.,** *J. Chromatogr.,* 188, 235 (1980).
152. **Brunner, G. and Holloway, C. J.,** *Hoppe Seyler's Z. Physiol. Chem.,* 381, 1 (1980).
153. **Kodama, T. and Woledge, R. C.,** *J. Biol. Chem.* 251(23), 7499 (1976).
154. **Wielders, J. P. M.,** Thesis, University of Technology, Eindhoven, The Netherlands, 1978.
155. **Buret, J.,** *Proc. Ist Int. Symp. Isotachophoresis,* Adam, A. and Schots, C, Eds., Elsevier, Amsterdam, 1980.
156. **Konstantinov, B. P. and Oshurkova, O. V.,** *Dokl. Akad. Nauk SSSR,* 175, 113 (1967).
157. **Everaerts, F. M. and Van der Put, J. M.,** *J. Chromatogr.,* 52, 415 (1970).
158. **Kopwillem, A. and Lundin, H.,** LKB Application Note No. 183, LKB-Produkter AB, Bromma, Sweden, 1974.
159. **Shiogai, Y. and Akiyama, J.,** *Bunseki Kagaku,* 26(10), 697 (1977).
160. **Kopwillem, A., Moberg, U., Westin-Sjödahl, G., Lundin, R., and Sievertsson, H.,** *Anal. Biochem.,* 67, 166 (1975).
161. **Robinson, D. V. and Rimpler, M.,** *J. Clin. Chem. Clin. Biochem.,* 16, 1 (1978).

162. **Kodama, H.,** *J. Chromatogr.,* 163, 300 (1979).
163. **Miyazaki, H. and Katoh, K.,** *J. Chromatogr.,* 119, 369 (1976).
164. LKB Isotachophoresis News, No. 2, LKB-Produkter AB, Bromma, Sweden, 1977.
165. **Kopwillem, A., Lundin, R., and Sievertsson, H.,** LKB Application Note No. 159, LKB-Produkter AB, Bromma, Sweden, 1974.
166. **Martin, A. J. P., Hampson, F.,** *Progress in Isoelectric Focusing and Isotachophoresis,* Righetti, P. G., Ed., North-Holland, Amsterdam, 1975, 327.
167. **Baldesten, A.,** 2nd Int. Insulin Symp., Aachen, West Germany, September 4—7, 1979.
168. **Mutt, V.,** Hormone Isolation, *Gut Hormones,* Bloom, S. R., Ed., Churchill Livingstone, Edinburgh, 1978, 21.
169. **Pradayrol, L., Chayvialle, J. A., Carlquist, M., and Mutt, V.,** *Biochem. Biophys. Res. Commun.,* 85(2), 701 (1978).
170. **Heynen, G., Gaspar, S., Gysen, Ph., Adam, A., and Franchimont, P.,** *Proc. 1st Int. Symp. Isotachophoresis,* Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980.
171. **Zimmerman, L., Baldesten, A., Bergström, J., and Fürst, P.,** *Proc. 1st Int. Symp. Isotachophoresis,* Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980.
172. **Grof, J. and Menyhárt, J.,** *3rd Donau Symp. Nephrologie,* Watschinger, B., Ed., Verlag Karls Bindernagel, Friedberg (BRD), 1979, 123.
173. **Hendon, R. A. and Tu, A. T.,** *Biochim. Biophys. Acta,* 578, 243 (1979).
174. **Tiselius, A.,** *Trans. Faraday Soc.,* 33, 524 (1937).
175. **Arlinger, L.,** *Protides of Biological Fluids* Vol. 19, Peeters, H., Ed., Pergamon Press, Elmsford, N.Y., 1972, 513.
176. **Arlinger, L.,** *Biochim. Biophys. Acta,* 393, 396 (1975).
177. **Arlinger, L.,** *Proc. Electrofocusing Isotachophoresis Symp.,* Righetti, P. G., Ed., North-Holland, Amsterdam, 1975, 331.
178. **Kjellin, K. G., Moberg, U., and Hallander, L.,** *Sci. Tools,* 22, 3 (1975).
179. **Kjellin, K. G., Hallander, L., and Moberg, U.,** *J. Neurol. Sci.,* 26, 617 (1975).
180. **Kjellin, K. G.,** 5th Int. Meet. of the Int. Soc. for Neurochem., Barcelona, Spain, 1975.
181. **Kjellin, K. G.,** *Acta Neurol. Scand.,* 54, 102 (1976).
182. **Delmotte, P.,** *Electrophoresis '78,* Catsimpoolas, N., Ed., Elsevier; North-Holland, Amsterdam, 1978.
183. **Delmotte, P.,** *Proc. 1st Int. Symp. Isotachophoresis,* Adam, A. and Schots, C., Eds., Elsevier, Amsterdam, 1980.
184. **Kjellin, K. G. and Hallander, L.,** *J. Neurol.,* 221, 235 (1979).
185. **Kopwillem, A., Merriman, W. G., Cuddeback, R. M., Smolka, A. J. K., and Bier, M. J.,** *J. Chromatogr.,* 118, 35 (1976).
186. **Bier, M., Cuddeback, R. M., and Kopwillem, A.,** *J. Chromatogr.,* 132, 437 (1977).
187. **Shimao, K.,** *Electrofocusing and Isotachophoresis,* Nobusawa, K., Ed., K. Yoritsu Shuppan, Tokyo, 1978, 137.
188. **Hedlund, K. W. and Nichelson, D. E.,** *J. Chromatogr.,* 162, 76 (1979).
189. **Hedlund, K. W., Wistar, R., Jr., and Nichelson, D.,** *J. Immunol. Methods,* 25, 43 (1979).
190. **Grünbaum, B. W. and Hjalmarsson, S.-G.,** *J. Forensic Sci.,* 16, 325 (1976).
191. **Bours, J. and Delmotte, P.,** *Sci. Tools,* 26(4), 58 (1979).
192. **Bours, J.,** *Exp. Eye Res.,* 25(5), 530 (1977).
193. **Thorn, W., Weiland, E., and Wasmus, G.,** *Res. Exp. Med.,* 175, 155 (1979).
194. **Arlinger, L.,** *Electrofocusing and Isotachophoresis,* Radola, B. J. and Graesslin, D., Eds., Walter de Gruyter, Berlin, 1977, 505.
195. **Axelsen, N.H., Krøll, J., and Weeke, B., Eds.,** *A Manual of Quantitative Immunoelectrophoresis, Methods and Applications,* Universitetsförlaget, Oslo, Norway, 1973.
196. **Wadström, T. and Smyth, C. J.,** *Sci. Tools,* 20, 17 (1973).
197. **Beckers, J. L. and Everaerts, F. M.,** *J. Chromatogr.,* 68, 207 (1972).
198. **Oshurkova, O. V., Kholmogorov, S. N., and Rzhevina, L. A.,** *A. Anal. Khim.,* 30, 1276 (1975).
199. **Vodennikova, M. F., Oshurkova, O. V., and Samusik, V. P.,** *Ind. Lab. Res.,* 44(1), 13 (1978).
200. **Vodennikova, M. F., Oshurkova, O. V., and Samusik, V. P.,** *Zavod. Lab.,* 44, 11, (1978).
201. **Beckers, J. L. and Everaerts, F. M.,** *J. Chromatogr.,* 69, 165 (1972).
202. **Beckers, J. L., Everaerts, F. M., and Houtermans, W. J. M.,** *J. Chromatogr.,* 76, 277 (1973).
203. **Houtermans, W. J. M.,** Graduation Report, Eindhoven University of Technology, The Netherlands, 1972.
204. LKB Isotachophoresis News, No. 3, LKB-Produkter AB, Bromma, Sweden, 1977.
205. **Miyasaki, H. and Katoh, K.,** *Electrofocusing and Isotachophoresis,* Nobusawa, K., Ed., K. Yoritsu Shuppan, Tokyo, 1978, 173.
206. **Everaerts, F. M.,** *Proc. Anal. Div. Chem.,* 14(4), 85 (1977).
207. **Flynn, I. W.,** *J. Pharmacol. Methods,* 2, 279 (1979).

208. **Sjödahl, J. and Hjalmarsson, S. G.,** *FEBS Lett.,* 92(1), 22 (1978).
209. **Kopwillem, A.,** *Acta Chem. Scand.,* 27, 2426 (1973).
210. **Kopwillem, A.,** *J. Chromatogr.,* 82, 407 (1973).
211. **Willemsen, A. J.,** *J. Chromatogr.,* 105, 405 (1975).
212. **Everaerts, F. M. and Verheggen, Th. P. E. M.,** *Progress in Isoelectric Focusing and Isotachophoresis,* Righetti, P. G., Ed., North-Holland, Amsterdam, 1975, 309.
213. **Hjalmarsson, S.-G.,** *Biochim. Biophys. Acta,* 581, 210 (1979).
214. **Honda, S., Wakasa, H., Terao, M., and Kakehi, K.,** *J. Chromatogr.,* 177, 109 (1979).
215. **Oka, S., Hirotsune, M., and Shigeta, S.,** *Anal. Biochem.,* 98, 417 (1979).
216. **Hjertên, S.,** *Protides of Biological Fluids,* Vol. 22, Peeters, H., Ed., Pergamon Press, Elmsford, N.Y., 1975, 669.
217. **Bier, M., Hinckley, J. O. N., Smolka, A. J. K., and Synder, R. S.,** *Protides of Biological Fluids,* Vol. 22, Peeters, H., Ed., Pergamon Press, Elmsford, N.Y., 1975, 673.
218. **Smolka, A. J. K. and Bier, M.,** *Fed. Proc. Fed. Am. Soc. Exp. Biol.,* 34(3), 685 (1975).
219. **Hinckley, J. O. N.,** *Clin. Chem. (Winston-Salem, N.C.),* 20(8), 973, 1974.
220. **Fiks,** *Russ. J. Phys. Chem.,* (trans.), 38, 1218 (1964).
221. **Konstantinov, B. P. and Bakulin,** *Russ. J. Phys. Chem.,* (trans.), 39, 315 (1965).
222. **Wiedemann, B. and Vestermark, A.,** *Radiochem. Radioanal. Lett.,* 6(5), 287 (1971).
223. **Yoshida, H., Nukutsuka, I., and Hikime, S.,** *Bunseki Kagaku,* 26, 382 (1979).
224. **Akiyama, J., Mizuno, T., and Shiogai, Y.,** *Shimadzu Rev.,* 34(1), 111, (1977).