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Capillary Isotachophoresis

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Introduction

Isotachophoresis (ITP) is one of the fundamental electrophoretic separation techniques, where charged constituents are separated in an electric field due to their differences in their electrophoretic mobilities.

The moving boundary electrophoretic experiments and theoretical developments were the forerunners of isotachophoresis. Even by 1923, Kendall and Crittenden had described separation of some metals and acids by – as they called it – the 'ion migration method', which was in fact isotachophoresis. They concluded that ion concentrations were in accordance with the Kohlrausch regulation function. In 1942, Martin did his first experiments on what he called 'displacement electrophoresis' as an analogue of displacement chromatography. In 1963, Everaerts and Martin started their work on isotachophoresis. Up to 1970 several names had been used for what Kendall had called the ion migration method: these included the 'moving boundary method', 'displacement electrophoresis', 'steady-state stacking', and 'ionophoresis'. In 1970, Haglund introduced a name, based on the characteristic feature of the electrophoretic technique, namely the equal velocity of the sample zones in the steady state: isotachophoresis (ITP). The basic theory and early development in the field of isotachophoresis was described in 1976 by Everaerts in his fundamental book. An outline of the development of isotachophoresis is given in **Table 1**. Some advances in isotachophoresis are described in detail below.

ITP in Closed Systems

Up to 1990, ITP was carried out in commercial apparatus in 200–500-μm i.d. narrow-bore plastic capillaries and with closed systems, i.e. no electroosmotic flow (EOF) occurred.

Basic Theory

Under the influence of an applied electric field, E, ionic species will move towards the electrode with a migration velocity, v, of:

$$v = m \times E \qquad [1]$$

where m is the effective mobility of an ionic species. The effective mobility depends on various factors, for example, the ionic radius, shape and charge of the ion, degree of dissociation, pH, dielectric constant and viscosity of the solvent, and temperature.

Typically, ITP is performed with a constant current and it is not possible to separate cations and anions in the same run (unidirectional isotachophoresis).

It is characteristic of ITP that the sample to be separated is injected between two different electrolyte solutions. The first solution (the leading electrolyte) contains an ion (the leading ion) with the same charge

Table 1 Development of isotachophoresis

Year	*Development of:*		*Attributed to:*
1897	Regulation function		Kohlrausch
1930	Moving boundary electrophoresis		Tiselius
1942	Displacement electrophoresis		Martin
1968	Capillary tube apparatus for isotachophoresis		Verheggen, Everaerts
1970–1989	ITP in closed systems, 200–500 μm i.d. narrow-bore plastic capillary with minimized EOF		
	Column-coupling ITP		Everaerts
	1970–1980	Thermometric, conductometric, potentiometric and UV detection	Everaerts
	1981	Refractometric detection	Bresler
	1981	Offline ITP–MS	Kenndler
	1983	Radiometric detection	Kaniansky
	1984	Fluorimetric detection	Reijenga
	1984	Amperometric detection	Kaniansky
	1985	Absorption spectra	Hanibalová
1989	ITP in open system, 100 μm i.d. fused-silica capillary with EOF, online ITP–MS		Udseth
1990	ITP in open system, 25–50 μm i.d. fused-silica capillaries		Thormann
1990	Online ITP–CZE (column coupling)		Kaniansky
1991	Offline ITP–PIXE		Hirokawa
1993	Bidirectional ITP		Hirokawa
1995	Raman spectroscopic detection		Walker

sign as that of the sample ions, but with an effective mobility higher than that of the fastest moving sample ion. The second solution (the terminating electrolyte) contains an ion (the terminating ion) with the same charge sign, but with an effective mobility slower than that of the slowest moving sample ion. The polarity of the electric field has to be such that the leading ion migrates to the electrode that is placed on the same side of the sample as the leading electrolyte. After application of an electric field to the system, each ionic species will have a different migration velocity according to eqn [1] and hence the isotachophoretic process starts.

The process of isotachophoresis may be divided into two parts. In the first part, the separation of the ions proceeds and the migration velocity of the individual ions in the mixed zones is different. In the second part (in the steady state) the ions have already separated from one other and all move with the same velocity, v:

$$\mathrm{v} = m_{\mathrm{L}}E_{\mathrm{L}} = m_iE_i = m_{\mathrm{T}}E_{\mathrm{T}} \qquad [2]$$

where L is the leading ion, i is the ith ion and T is the terminating ion.

A schematic representation of the cationic and anionic modes in ITP experiments without EOF is given in **Figure 1**(A,B). As the ionic species are arranged in order of decreasing effective mobilities, the electric field strengths increase on the terminating ion side.

The increase in the electric field strength in the consecutive zones induces the zone-sharpening effect. When a zone has attained the steady state, the boundary will not broaden further, which is in contrast to zone electrophoresis, where the peaks are broad owing to adsorption and diffusion. This effect can easily be explained. If an ion diffuses into a preceding zone, where the electric field strength is lower than the value that corresponds to its velocity, its velocity will decrease according to eqn [2], and it will be overtaken by its own zone. If an ion diffuses into a zone with a higher electric field strength, then it will obtain a higher migration velocity according to eqn [2], until it reaches its own zone.

It is characteristic for the steady state that the concentration of each component is adjusted to the value following from the Kohlrausch regulation function in the form:

$$c_i = c_{\mathrm{L}}\frac{m_{\mathrm{L}} + m_{\mathrm{R}}}{m_{\mathrm{L}}} \times \frac{m_i}{m_i + m_{\mathrm{R}}} \times \frac{z_{\mathrm{L}}}{z_i} \qquad [3]$$

where R is the common counterion, Z_i is the ionic charge. In the steady state, the concentration C_i of the ith ion is always adjusted to a certain value depending only on the concentration of the leading electrolyte C_{L} and on the mobility of the ions i, L and R. From the analytical point of view this is a very important feature of ITP. It can be concluded that for a given set of experimental conditions, the zone length is a direct measure of the amount of an ion present in the zone. Another important consequence of these properties is the concentration effect of isotachophoresis. In fact, a species more concentrated in the original sample is diluted during the separation and, a species originally too dilute is concentrated during the separation.

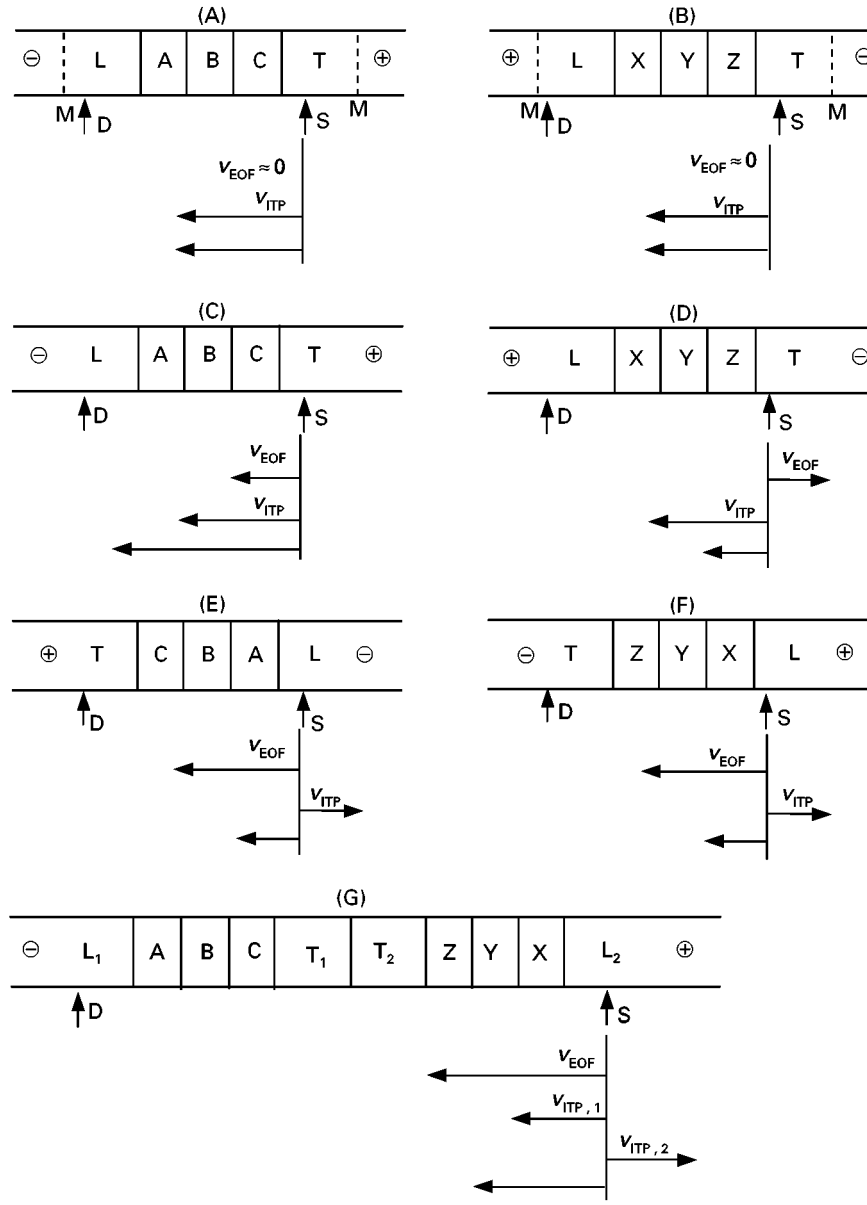

Figure 1 Schematic representation of the two modes in unidirectional ITP experiments without EOF: (A) the cationic separation of a mixture of cations A, B, and C with $m_A > m_B > m_C$; (B) the anionic separation of a mixture of anions X, Y, and Z with $m_X > m_Y > m_Z$. The four modes in unidirectional ITP with EOF: (C) the cationic cations as in (A); (D) the anionic anions as in (B); (E) the reversed cationic; (F) the reversed anionic; (G) Schematic representation of the bidirectional ITP; separation of mixture of cations A, B and C and anions X, Y and Z. L_1 = leading cation; T_1 = terminating cation; L_2 = leading anion; T_2 = terminating anion. Only steady state is presented. S = sample inlet; D = detector position, L = leading ion, T = terminating ion, v_{EOF} = velocity of EOF; v_{ITP} = isotachophoretic velocity; $\leftarrow$ or $\rightarrow$ = net velocity; M = semipermeable membrane. For further explanation, see text.

In ITP, the response is usually recorded against time with a detector placed at the end of capillary (**Figure 2**).

The identity of a species is characterized by the effective mobility (or a quantity proportional to the effective mobility). This is usually the response of the universal detector. It is called the height (step height) or the relative height (relative step height, rsh) of the zone, and is given by the relation:

$$\mathrm{rsh}_i = \frac{h_i - h_L}{h_T - h_L} \qquad [4]$$

where h_i is the step height of the compound, h_L is the step height of the leading ion and h_T is the step height of the reference ion (usually the terminating ion)

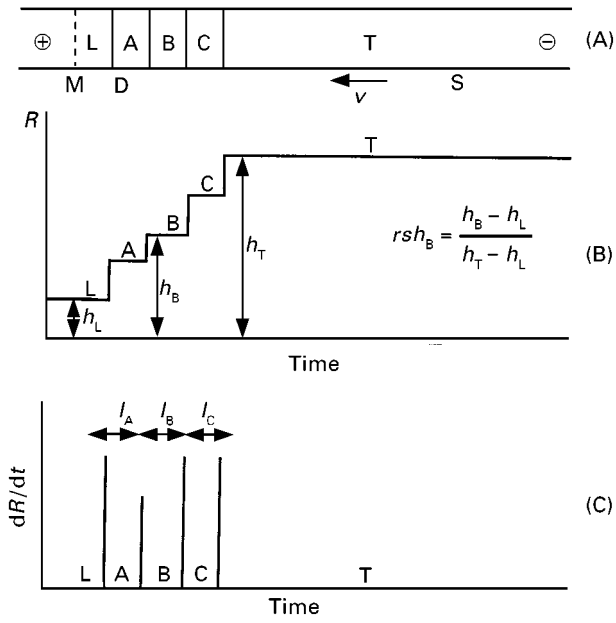

Figure 2 Graphical representation of response R from universal detector [(B) linear; (C) differential] for the different anions A, B, and C, moving in the steady state of an isotachophoretic analysis (A). L = leading anion; T = terminating anion; S = sample inlet; D = detector position; M = semipermeable membrane. For further explanation, see text.

(**Figure 2**B). The values obtained in this way are then compared with those of standard species measured under the same experimental conditions.

The quantification is in general simplified by differentiating the signals and measuring the distance between the inflection point (**Figure 2**C). The zone lengths l_i are directly proportional to the number of ions (n_i): $l_i = K_i n_i$. The constant K_i depends on the equipment and the current used. A universal calibration constant (the response factor RF, eqn [5], which is independent of the diameter of the capillary, construction of the universal detector and driving current using during detection, has been introduced. For each component, the RF depends only on the concentration of the leading electrolyte:

$$\mathrm{RF} = \frac{l \times I}{|z| \times F \times Q} \qquad [5]$$

where l is the zone length (seconds), I is the driving current (amps), $|z|$ is the charge of the ion (equiv mol^{-1}), F is Faraday's Number (coulombs equiv^{-1}) and Q the amount injected (mol).

Based on the mathematical models for isotachophoresis described, computer programs have been set up for calculation of the parameters of the different zones. Unfortunately, only a few schemes can be used for simulating capillary isotachophoresis at realistic current densities without causing either severe oscillations or unexpected program termination.

Online Coupling of ITP with CZE (Column-Coupling Instrumentation)

Column-coupling instrumentation (see below) of the separation unit for ITP as described by Everaerts has been shown to be suitable for online coupling of ITP and CZE. The extensive studies of Kaniansky and Marák give a good impression of the potential of combined ITP–CZE.

The online combination of ITP and CZE is a very effective tool for increasing the separation capability and sensitivity of CZE. It is characterized by isotachophoresis in the first capillary followed by online transfer of the sample cut into the second capillary where zone electrophoresis proceeds.

In principle, there are three ways of performing an ITP–CZE combination technique as far as the electrolyte systems are concerned. The simplest way is to use the terminating electrolyte as the background electrolyte (BGE) for CZE; the second possibility is to use the leading electrolyte as BGE and the third possibility is to use a totally different BGE.

ITP has the advantage of much higher loading volumes, e.g. microlitres instead of nanolitres in CE. In addition, ITP is a concentration technique. The combination of these features makes ITP, in principle, an ideal technique for sample pretreatment. In ITP–CZE, a 10^4-fold concentration increase can be achieved, and this even for a component present in a 10^5-fold excess of the matrix.

ITP in Open Systems

Since the early 1990s, commercial instruments for CZE have been available generally with open-tubular fused-silica capillaries with an inner diameter between 20 and 100 μm, together with an on-column detector placed towards one end of the capillary. As this apparatus can be used for ITP it was of interest to study the possibilities for ITP in open systems. If ITP experiments are performed in open-tubular fused-silica capillaries, the negative surface charge of untreated fused-silica causes an EOF towards the cathode. This EOF will influence the ITP system and four different modes can be observed. In **Figure 1**(C), the cationic ITP mode is shown. The EOF will generally act in the direction from the anode to the cathode and as a result the cationic ITP system will be pushed towards the cathode with a higher velocity compared with cationic experiments in closed systems. In

Figure 1(D), the anionic ITP mode is shown. This mode can be applied if the velocity of the leading ion is greater than that of the EOF during the whole experiment. Only in this case will anions with mobilities slower than that of the EOF also migrate to the anode according to the isotachophoretic condition. The reversed cationic mode (**Figure 1**E) can be applied if there is a reversed EOF (e.g. using coated capillaries or additives to the electrolyte) with a velocity greater than that of the cationic system. Here the cathode must be placed at the sample inlet end and the anode at the detector end. Although the ITP separation takes place in the direction of the cathode, there will be a net velocity of the ITP system in the direction of the detector end and components will be detected in a reversed order compared with a normal cationic ITP system. In **Figure 1**(F), the reversed anionic mode is presented. Here the anode is placed at the sample inlet end, the cathode at the detector end and components will be detected in a reversed order compared with a normal anionic ITP system.

As the velocity of the EOF is extremely important in the migration behaviour of ITP systems, much effort must be put into controlling EOF. The velocity of the EOF strongly depends on the choice of the leading and terminating electrolyte and it also varies with the composition of the sample. Moreover, the velocity of the EOF continuously changes during the analysis and is first determined by the composition of the leading electrolyte and finally by that of the terminating electrolyte. Varying EOF velocities cause irreproducible migration times and zone length and the results of quantitation are erroneous. The addition of methylhydroxyethylcellulose to the electrolytes and sample largely suppresses the EOF in order to improve quantitation. In spite of the addition of methylhydroxyethylcellulose, the reproducibility of the zone lengths with time is poor, and an internal standard is, therefore, needed. Hence the reproducibility in ITP quantitative analysis in open systems is a problem similar to that in electrophoresis. Generally, closed systems are to be preferred to open systems for quantitative analysis.

The presence of an EOF, however, facilitates the development of bidirectional ITP for the simultaneous determination of anionic and cationic components. In bidirectional ITP, the leading electrolyte for cations must be simultaneously the terminating electrolyte for anions, and vice versa the leading electrolyte for anions must be the terminating electrolyte for cations. That is, the counterions (cations) coexisting with the leading anions play the role of the terminating cation, and the counterions (anions) coexisting with the leading cations play the role of the terminating anion. In a fused silica capillary in the presence of a cathodic EOF, cationic sample trains can be detected with a detector placed towards the cathodic end of the capillary. However, anionic species can be detected only at $pH > 6$. At $pH > 6$, the velocity of the EOF is greater than that of the anionic ITP system and hence the anions migrate slowest since they are attracted to the anode, but are still carried by the EOF towards the cathode (**Figure 1**G).

Instrumentation for ITP

Separation Capillary

The actual separation takes place in a PTFE (polytetrafluoroethylene) or a silica capillary. The separation capacity can be increased by extending the length of the capillary, but the analysis time and the maximum voltage required also increases. From the instrumental point of view, the column-coupling system (**Figure 3**) frequently used today has led to significant progress. It consists of a pre-separation unit with a capillary of larger diameter (e.g. 0.8 mm) equipped with the detector and bifurcation block, to which an analytical capillary of small diameter (e.g. 0.3 mm) is connected. At the beginning of the analysis, the driving current passes through the pre-separation capillary only. The detection system in the first capillary is employed to evaluate analysis. In addition, it provides the information necessary to control the transfer of the analytes into the second capillary and the removal of the sample constituents which are led out of the separation compartment after the first stage. At a suitable moment, the driving current is switched so that it passes through the analytical capillary and thus introduces the required sample zones into this capillary where further separation takes place. Column coupling enables use of different leading electrolytes in the pre-separation and analytical capillaries, thereby influencing the subsequent separation, separation of mixtures containing components in ratios up to 1 : 1000 without increasing the voltage and without prolonging the analysis time, and application of ITP in combination with CZE.

Electrode Chamber, Electrodes and Power Supply

The capillary is connected on each side to an electrode chamber provided with a platinum electrode. In closed systems, the chamber, filled with the leading electrolyte, is connected to the capillary via a semipermeabile membrane. The terminator chamber is connected via a multiway switching valve, which is open in the course of the analysis. In open systems, the ends of the capillary are placed in electrolyte reservoirs (electrode chamber).

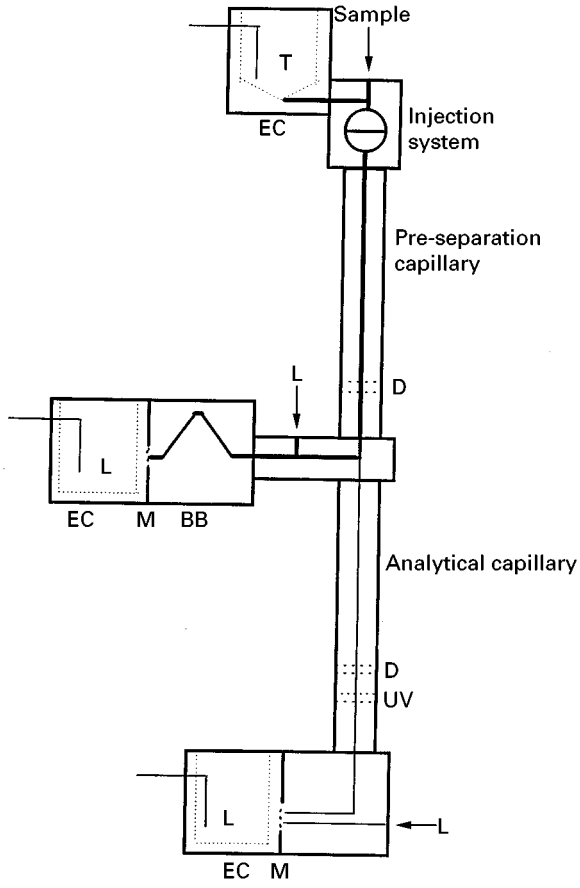

Figure 3 Column-coupling isotachophoretic system. EC = electrode compartment; BB = bifurcation block; D = conductivity detector; UV = UV detector; L = leading electrolyte; T = terminating electrolyte; M = semipermeable membrane.

A high-voltage power supply capable of delivering 500 μA at up to 20–30 kV d.c. is needed. The constant current regulation of the power supply must be extremely well designed.

Injection System

In closed systems, the sample can be introduced by a microsyringe through a septum or by a multi-port valve system. In open systems, the sample can be vacuum-aspirated or loaded electrokinetically.

Detection

The first universal online detector was the thermocouple detector. Owing to its low sensitivity, the thermocouple detector was replaced with universal contact detectors, which sense the electrical resistance or potential gradient in the zones. The disadvantage of contact detectors is polarization of the sensing electrodes. To solve this problem, a universal contactless high-frequency conductivity detector was proposed in the 1970s. Detection based on differences in the refractive index of various zones was introduced in 1981. The disadvantage of this system was the necessity of working with high electrolyte concentrations, which resulted in slow analysis.

In 1991, McDonnell and Pawliszyn developed a new refractive index detector for ITP consisting of a He–Ne laser or a laser diode and photodiode position sensor. The direction of the beam is deflected when it passes through the refractive index gradient produced by the sample zone. By using this detector, a few nanomoles of sample can be detected. The development of a selective UV-absorption detector for ITP had been an important contribution to the development of ITP in the 1970s. The UV detector is now a common component of commercial apparatus. In most cases only the wavelengths 254 and 280 nm have been utilized for detection. Arlinger had shown in 1974 that a UV detector could be applied as a pseudo-universal detector. UV-absorbing counterions were used, for which the molar absorption was pH-dependent. As each zone has its own defined pH and concentration, the pH and concentration difference gave rise to an absorbance difference sufficiently large to be detectable.

Sometimes it can be advantageous to use a UV-absorbing spacer in order to make the detection of consecutive zones of nonabsorbing ionic species possible. In some instances it is possible to detect boundaries between two consecutive non-UV-absorbing zones because of the trace amounts of UV-absorbing impurities which are present in most electrolytes and which concentrate as markers between the separated non-UV-absorbing zones. Great attention has been paid to development of new selective detectors for ITP, to facilitate the identification of compounds in the detected zones. Sensing of absorption spectra in isotachophoretic zones is one of the possibilities. Fluorimetric detection is a highly sensitive method. In ITP, the equipment designed initially for the dual-wavelength UV detection has been employed for fluorimetric zone detection. Zones of fluorescing compounds or of compounds quenching counterion fluorescence can be detected.

In 1991, Hirokawa introduced a new specific detection method for metal ions. He used an offline combination of ITP and particle-induced X-ray emission (PIXE), which is a multi-elemental method with high sensitivity. As the method is based on the characteristic X-rays emitted by target elements, it has a high specificity for the determination of the elements even if they are not separated. Radiometric detection of compounds labelled with a radioactive isotope is a specific method. Its principle is the detection of the radiation emitted from the labelled

compound zone passing the window of Geiger–Müller tube. Electrochemical detection, owing to its high sensitivity and specificity, is widely used in liquid chromatography. Its direct use in ITP is hindered by the presence of the driving electric field. To minimize disturbances due to the driving current, post-column amperometric detection has been employed. The separated constituents are hydrodynamically transported from the separation compartment into the detection cell. The hydrodynamic transport causes the dispersion to increase, therefore, the resolving power of post-column detection is lower in comparison, with for example, the conductivity detector. However, this disadvantage can be outweighed by its inherent selectivity and/or sensitivity.

The online combination of ITP with mass spectrometry was first demonstrated in 1989. The ITP/MS interface is based on electrospray ionization. Separations were conducted in open-tubular untreated fused-silica capillaries. The interface requirement of strong electroosmotic flow did not significantly degrade separations and both high sensitivity (limit of detection 10^{-9} mol L^{-1}) and high resolution can be obtained. Recently, Walker has demonstrated that a fibreoptic Raman probe can be used to obtain real-time intracapillary Raman spectra during ITP. Even at 2×10^{-5} mol L^{-1} initial concentration, Raman spectra were obtained at a good signal-to-noise ratio.

Preparative Procedures in Isotachophoresis

Capillary isotachophoretic analysers can be used for preparative purpose in a discontinuous arrangement only. Once the separation has been performed, the analysis is discontinued and the analysed compound zone is isolated by using a microsyringe, a specially designed fractionating valve placed at the end of the separation capillary or a counterflow of leading electrolyte (**Figure 4**).

Continuous free-flow isotachophoresis (**Figure 5**) was developed to fractionate large-scale samples continuously. The separation field of continuous free-flow isotachophoresis is typically a thin film of fluid flowing between two parallel plates. An electric field is applied perpendicular to the flow direction. The leading and terminating electrolytes and the sample solution are continuously supplied with a multifold peristaltic pump into one end of the electrophoretic chamber and are collected with a multifold pump at the other. The leading and terminating electrolytes used for the electrode compartments circulate by pumps during migration. A dialysis membrane isolates the separation chamber from the electrode compartments.

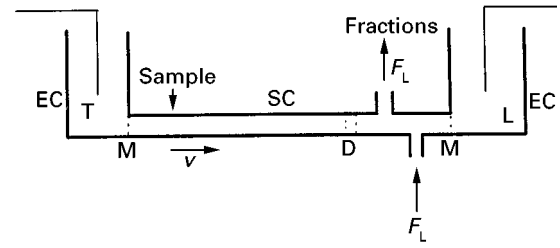

Figure 4 Capillary preparative isotachophoresis with a counterflow of leading electrolyte. EC = electrode compartment; SC = separation capillary; D = detector, M = semipermeable membrane; L = leading electrolyte; T = terminating electrolyte; F_L = counter flow of leading electrolyte.

In recycling electrophoresis, in order to increase the electric charge applied to the sample, the fraction from each channel are continuously reinjected into the inlet port of the separation chamber. This instrumentation allows a high throughput and complete separation of the injected sample. Typical operation is batchwise, in contrast to continuous free-flow isotachophoresis.

Future Developments

Isotachophoresis underwent major development in the years 1970–1990. Over the last ten years CZE has occupied the major part of both the theory and applications of electrophoresis. Despite this, capillary isotachophoresis has kept its position as a special technique with unique features. Concentrating and

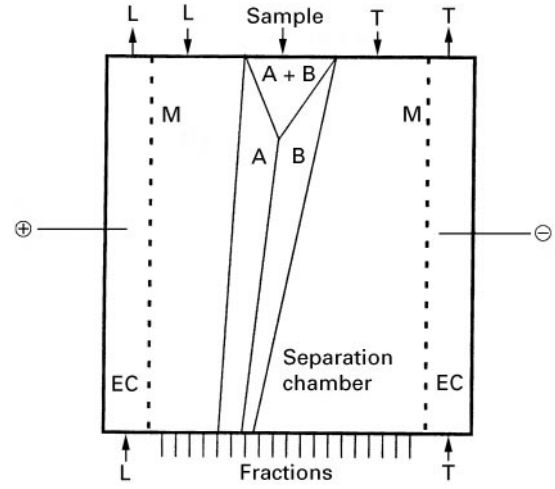

Figure 5 Continuous free-flow isotachophoresis. EC = electrode compartment; M = semipermeable membrane; L = leading electrolyte; T = terminating electrolyte; sample = mixture of A and B.

zone sharpening make it possible to obtain, in particular cases, much better results than when using CZE. Most promising is the combination of ITP with CZE where ITP serves as a preconcentration and pre-separation step for analysis of samples with complex matrices. Unfortunately, there is only one manual ITP–CZE system still commercially available.

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Cellulose Acetate

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The introduction of zone electrophoresis, pioneered by Konig in 1939, played a crucial role in the progress of electrokinetic separations. With this technique, molecules migrate as zones with sharp boundaries in a supporting medium immersed in a buffer solution under the application of an electric field. Zone electrophoresis was quickly found to be superior in performance to Tiselius's original technique of moving boundary electrophoresis and replaced it entirely – to be superseded in turn by displacement electrophoresis and isoelectric focusing (IEF). Interestingly, the term 'zone electrophoresis' was first suggested by Tiselius himself.

Kohn first used cellulose acetate (CA) as a supporting medium for zone electrophoresis in 1957, as a superior substitute for plain filter paper. Since then, CA has been used in many electrophoretic protocols, for both research and clinical investigations (**Table 1**). Nowadays CA electrophoresis is a widespread technique.

In this article we explain what CA is and why it is used in electrophoresis. This is followed by a brief overview of the uses of CA in various electrophoretic contexts. Finally, some recent and innovative applications of CA in electrophoretic protocols are discussed.

General Concepts

Preparation of CA

CA sheets employed in electrophoresis are made of a molecular matrix, similar in structure to a sponge but a thousand times smaller. This matrix is obtained by letting acetic anhydride react with cellulose and dissolving the product in an organic solvent, that can evaporate quickly. After letting the solvent evaporate in closely-controlled conditions of temperature and humidity, a highly permeable matrix is obtained with a uniformly distributed microporosity. The spatial volume of the pores may account for 80% of the total matrix size, ensuring ideal permeation by any

Table 1 Historical sequence of main applications of CA to electrophoretic protocols in different areas of research and clinical investigations

Year	*Application*
1957	CA is used as an electrophoretic support (Kohn)
1971	Application to conventional electrophoresis of white cell and red cell enzymes (Meera Khan)
1975	Application to isoelectric focusing of alpha-1-antitrypsin in human serum and 6–phosphogluconate dehydrogenase (Harada)
1984	Application to counterflow affinity isotachophoresis of antigens in biological fluids with low protein contents (Abelev and Karamova)
1992	Introduction of CA for protein transfer from polyacrylamide gels
1993	Introduction of protocols for reusing CA