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Review

Preparative procedures in isotachophoresis

Takeshi Hirokawa* and Yoshiyuki Kiso☆

Applied Physics and Chemistry, Faculty of Engineering, Hiroshima University, Kagamiyama 1, Higashi-Hiroshima 724 (Japan)

ABSTRACT

Preparative procedures of isotachophoresis (ITP) for free solutions are reviewed from the practical viewpoints of the separability and the preparative efficiency (*i.e.*, the sample amount processed in unit time). The theoretical background of ITP separations and the principle of the fractionation technique in various apparatus are detailed; these are helpful in obtaining good separation efficiency and for estimating the preparative efficiency. The instrumentation for preparative ITP is reviewed, covering capillary-type ITP (cITP), continuous free-flow ITP (cffITP) and recycling ITP (rITP). Typical applications of cITP and rITP are briefly introduced. In conclusion, micropreparative cITP is valuable for further analysis in combination with other highly sensitive analytical methods (coupled techniques). On the other hand, cffITP and rITP are useful for preparative purposes to obtain up to several grams of pure ionic substance in 1 day.

CONTENTS

1. INTRODUCTION

Isotachophoresis (ITP) is a powerful separation technique for the analysis and preparation of ionic substances [1–3]. Although the analytical utility of ITP has been widely accepted, its preparative use is still limited, especially when the separation field does not utilize solid support media.

This review has two aims: to characterize

* Corresponding author.
☆ Present address: Hijiyama Women's College, Ushita-shinmachi, Hiroshima 732, Japan.

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various preparative ITP apparatuses for free solutions, and to summarize the theoretical background of ITP separations, which may be useful both in understanding the operational principles of the apparatus and in estimating the preparative efficiency. Today, capillary electrophoresis is attracting many researchers who are seeking compensating techniques for liquid chromatography. This review is intended to give guidelines to those who are interested in preparative ITP.

From a practical viewpoint, preparative efficiency (*i.e.*, the sample amount processed in unit time) is very important. When a continuous free-flow apparatus is used, as detailed later, up to several grams of pure ionic substance can be fractionated daily. However, it should be noted that the processable amount of a sample varies depending on the apparatus used, the sample itself and the operational electrolyte system used (leading and terminating electrolytes). Maximum preparative efficiency is obtained at maximum separation efficiency of the sample components, which does not depend on the apparatus used but on the differences in electrophoretic mobilities among the sample components which can be regulated by the operational electrolyte system.

As a theoretical approach is always important in order to avoid performing time-consuming trial-and-error experiments, ITP separation and its separation efficiency are described in detail first. Next, instrumentation for preparative ITP is reviewed, covering capillary-type ITP (cITP), continuous free-flow ITP (cffITP) and the newly developed recycling ITP technique (rITP).

2. THEORETICAL

In order to obtain good separation efficiency in ITP, the operational electrolyte system should be optimized to give appropriate differences among the effective mobilities of separands. However, as the pH and ionic strength of the separated zones in ITP are different from each other, evaluation of the effective mobility needs a computational procedure [1–5]. Consequently, optimization of the separation in ITP is not straightforward in comparison with zone electrophoresis.

Fortunately, the separation field of ITP can be regarded as a free electrolyte solution. This allows a precise theoretical simulation of zone properties [4,5], such as the concentration of the sample and the pH buffer, the effective mobilities, the conductivity of the zones, the qualitative and quantitative indices and the resultant isotachopherograms. A table of isotachophoretic qualitative and quantitative indices simulated for 287 ions in the pH range 3–10 may be useful for separability assessment [4]. A similar table for 73 amino acids and peptides was recently reported [5].

The isotachophoretic process is bidirectional in principle, as pointed out by Thormann *et al.* [6] and recently reconfirmed by us [7]. That is, isotachophoretic stacking zones of anionic and cationic components in a sample can be formed simultaneously in a separation tube when a suitable electrolyte system is chosen. Leading electrolytes for a cationic analysis and an anionic analysis can be combined as a catholyte and an anolyte of such a bidirectional electrolyte system, although the pH difference between the anolyte and the catholyte is not arbitrary [7]. Obviously, the use of bidirectional ITP has the merit that concentrated zones of anions and cations can be simultaneously analysed and fractionated.

2.1. *Isotachophoretic separation*

2.1.1. *Capillary-type isotachophoresis*

cITP is mainly used for analytical purposes owing to its high separability. cITP is also useful for preparative purposes, although the amount of sample separable is not so large (0.01–1 μmol). The separation field is typically a capillary of I.D. 0.2–0.5 mm, where no solid medium is used.

Assume a two-component sample (S) containing separands A and B. The sample solution is injected at the boundary between the leading (L) and terminating (T) electrolytes, as shown in Fig. 1a.

At the initial stage of migration a homogeneous mixed zone (M) of the separands is formed as shown in Fig. 1b, where the separands

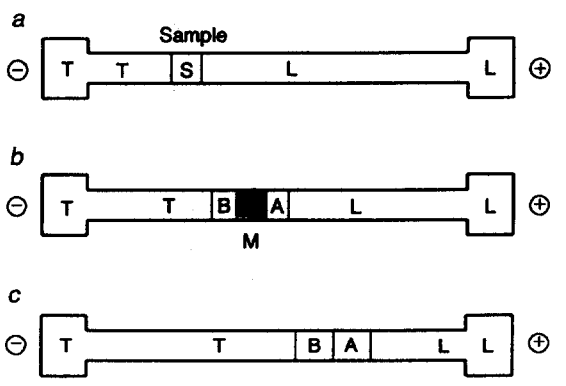

Fig. 1. Separation process in capillary-type isotachophoresis. (a) Before migration (sample injection); (b) separation process forming mixed zones; (c) complete separation (steady state). L = Leading zone; S = injected sample solution; A, B = sample zones; M = mixed zone; T = terminating zone.

A and B migrate with different velocities (v_A, v_B) under the same potential field. Since $v_A > v_B$ in the mixed zone, the faster component A forms its pure zone in the leading side of the mixed zone, and the other component B forms its pure zone in the terminating side of the mixed zone (Fig. 1b). Fig. 1c shows the ITP steady state, where ITP zones migrate with the same speed [8]. The zones are kept in a stack with no mixed zone and they are partitioned by sharp boundaries with a "self-sharpening" effect.

The sample concentration in the steady-state zone is uniquely determined by the concentration of the leading ion (C_L^t) and the effective mobilities of the leading ion and the sample ion ($\bar{m}_L > m_A > \bar{m}_B > \bar{m}_T$). As a result, the zone length of each component is proportional to its amount [1–3]. The following relationship is valid among the total equivalent concentrations of the samples (C^t):

$$C_L^t > C_A^t > C_B^t > C_T^t \quad (1)$$

When $C_L^t = 10$ mM, $C_T^t \approx 5$ mM or less, depending on the effective mobility of terminator. Eqn. 1 shows that dilute components in a sample are concentrated according to Kohlrausch's regulating function [9] and, conversely, the concentrated components are diluted during migration. When the components migrate between the leading and terminating zones, the recovery of sample components is 100% in principle. Such a separation mode is worthwhile especially for preparative purposes. In comparison with zone electrophoresis, for example, the available sample concentration is very high.

On the other hand, the stack configuration of separated samples suggests a high current efficiency of ITP. That is, the electric current is carried by the leading, sample and terminator ions and there are no background ions except for the counter ions. Hence ITP is a high-performance preparative method because of the high resolution, high current efficiency and high concentration of the separated zone. The latter two ensure an efficient throughput.

2.1.2. *Continuous free-flow isotachophoresis*

Two-dimensional separation is often used with solid support media. Most investigations involving preparative ITP report the use of solid support media such as gel slabs. It is obvious, however, that such a type is not suitable for continuous processing on a large scale.

cffITP was developed to fractionate large-scale samples continuously: the separation field of cffITP is typically a thin film of fluid flowing between two parallel plates, where no solid medium is used. An electric field is applied perpendicular to the flow direction. The leading and terminating electrolytes and the sample solution are continuously admitted into one end of the electrophoretic chamber and are fractionated at the other.

Fig. 2 illustrates the separation process in cffITP, where the sample contains two components, A and B. All the ITP separation process observed in cITP (Fig. 1) is seen during the sample residence time. That is, separation process shown in Fig. 1a, b and c correspond to a, b and c, respectively, in Fig. 2.

In cffITP, the migration velocities of samples are not constant during the residence time, in contrast to cITP. This is obvious from the fact that the current density in the separation chamber decreases from the inlet port towards the outlet because of the development of the terminating zone with a low specific conductivity.

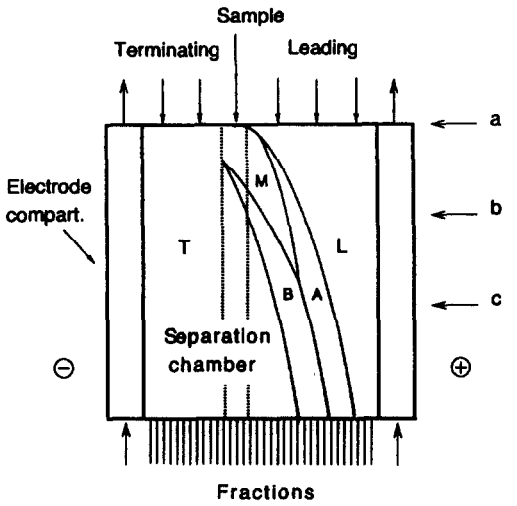

Fig. 2. Separation process in cffITP. (a) Before migration; (b) transient state with mixed zone M; (c) steady state. Abbreviations as in Fig. 1.

The boundary velocity varies depending on the local current density.

As estimated from Fig. 2, the positions of the sample zones at the exit are dependent on the flow-rate of the fluid in the separation chamber and the electric field strength applied. The flow-rate and the field strength should be constant during fractionation. Even when this condition is satisfied, the separability of cffITP is not as high as that of cITP, as there is always a hydrodynamic flow of the operational electrolyte and sample solution. To make matters worse, the potential field is weakest at the exit. This situation can be improved by applying a counter flow of the leading electrolyte to compensate for isotachophoretic migration. This is also effective in increasing the quantity of electric charge.

Mathematical modelling and computer simulation of cffITP have been intensively investigated by Ivory [10] and Bier *et al.* [11]. Kašička and Prusík [12] have proposed a simple method.

2.2. Preparation efficiency and separation efficiency

Preparation efficiency (P_{eff}) can be defined as the sample amount processed in unit time. It consists of two factors, as follows:

$$P_{eff}\,(\text{mol h}^{-1}) = S_{eff}\,(\text{mol C}^{-1}) \cdot Q\,(\text{C h}^{-1}) \qquad (2)$$

where S_{eff} is separation efficiency of the separands, which is defined as amount of sample separable after passing a unit quantity of electric charge, and Q is the quantity of charge supplied in unit time. S_{eff} does not depend on the apparatus used, but depends on the differences in electrophoretic mobilities among the sample components, which are determined by the properties of the sample and the electrolyte system used. On the other hand, Q is closely dependent on the apparatus used.

S_{eff} for a given sample component can be evaluated theoretically [13–16]. Using an appropriate relationship [16], S_{eff} of a sample containing two strong electrolytes (A and B) can be approximated as

$$S_{eff} = \frac{n_A}{it_{res,M}} = \frac{m_A}{F(m_A + m_Q)} \cdot \frac{\bar{m}_A - \bar{m}_B}{\bar{m}_A + \bar{m}_B} \qquad (3)$$

where n_A is the molar amount of the component A, i the migration current, $t_{res,M}$ the resolution time of the mixed zone M, m_A and m_Q, the mobility of ion A and the counter ion as the pH buffer, $\bar{m}_A$ and $\bar{m}_B$ the effective mobility of ions A and B at the transient state and F the Faraday constant.

Obviously, from eqn. 3 it can be concluded that m_Q should be small and the mobility difference $\bar{m} - \bar{m}_B$ should be large so as to obtain high S_{eff}. An electrolyte system should be chosen to maximize S_{eff}. It should be noted that in the case of a sample containing weak electrolytes the separation efficiency from eqn. 3 may be overestimated, depending on the electrolyte pH.

As estimated from eqn. 2, the preparation efficiency (P_{eff}) can be increased by increasing the quantity of electric charge supplied in unit time (Q). P_{eff} of cffITP is larger than that of cITP, because the cross-section of the separation chamber in cffITP is much larger than that in cITP and the migration current can be increased. Even in cITP, the use of a large-bore tube allows a high migration current and consequently a large P_{eff}. It should be noted that disturbances due to heat convection become serious when a large-bore tube is used. A 5 mm I.D. column,

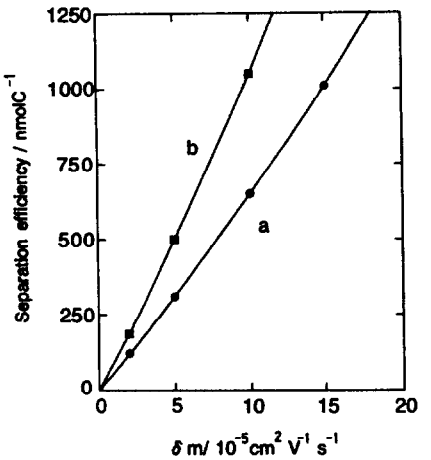

Fig. 3. Separation efficiency (S_{eff}) simulated for two-component samples of strong electrolytes. $\delta m = \bar{m}_A - \bar{m}_B$. m_A = (a) $60 \cdot 10^{-5}$ and (b) $30 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}. Leading electrolyte, 10 m*M* HCl buffered by histidine (pH 6.0).

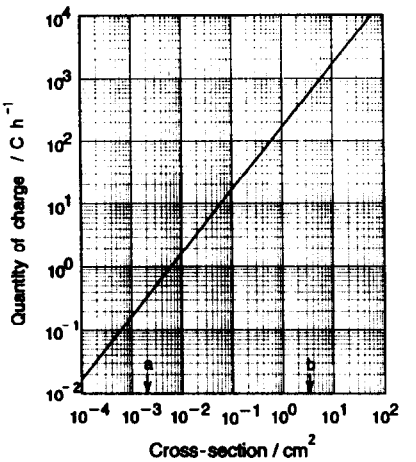

Fig. 4. Quantity of charge supplied per hour (Q) *versus* cross-section of the separation field. (a) cITP separation tube (0.5 mm I.D.); (b) cffITP separation chamber (height 50 cm and thickness 0.5 mm).

however, can be successfully used in our experience.

Fig. 3 shows the separation efficiency strictly simulated for two-component samples of strong electrolytes [15,16], where $m_A = 60 \cdot 10^{-5}$ and $30 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} and m_B is in the range from m_A to $m_A + 20 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}. The leading electrolyte used in simulation is 10 m*M* HCl buffered by histidine (pH = 6.0, $m_Q = 29.6 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}). For example, when m_A is $60 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} and m_B is $55 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}, the separation efficiency is 663 nmol C^{-1}. It should be noted, however, that if other components are present in the sample, the separability is reduced depending on the amount and mobility of the co-existing components [16] (a composition effect).

Fig. 4 shows the quantity of charge supplied per hour (Q) *versus* cross-section of the separation field. Figs. 3 and 4 can be conveniently used for the estimation of preparative efficiency as follows: the capillary I.D. and migration current typically used in cITP are 0.5 mm and 100 μA, respectively; Q is 0.36 C h^{-1} and the current density is 50.9 mA cm^{-2}. Then, the preparative efficiency of cITP for the model mixture ($m_A = 60 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}, $m_B = 55 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}) is calculated as follows:

$$P_{eff} = 663 \text{ (nmol C}^{-1}) \cdot 0.36 \text{ (C h}^{-1}\text{)}$$
$$= 239 \text{ (nmol h}^{-1}\text{)}.$$

If the same current density is assumed for cffITP (a separation chamber having a height of 50 cm and a thickness of 0.5 mm), the migration current is calculated as 127.3 mA and Q is 458 C h^{-1}. The preparative efficiency of cffITP is estimated as follows:

$$P_{eff} = 663 \text{ (nmol C}^{-1}) \cdot 458 \text{ (C h}^{-1}\text{)}$$
$$= 304 \text{ (μmol h}^{-1}\text{)}$$

Obviously, P_{eff} in cffITP is 1000 times larger than that in cITP owing to the large cross-section of the separation field.

It should be noted that in cITP the length of the separation tube determines the quantity of electric charge supplied in a run. With a selected electrolyte system, the amount of sample separable depends linearly on the quantity of charge applied before detection. In practice, it depends on the load of the leading electrolyte (*i.e.*, the volume of the separation tube multiplied by the concentration of the leading ion) [13,14]. Therefore, a long separation tube should be used in cITP and a separation chamber with a large volume is necessary in cffITP. For example, under the above experimental condition, a 1.4 m

long capillary (0.5 mm I.D.) is necessary to supply 0.36 C in 1 h.

3. INSTRUMENTATION

3.1. Capillary-type apparatus

In addition to direct cutting of the capillary section contained the target of interest [17], preparative methods in cITP can be classified into three types, as shown in Fig. 5.

Fig. 5a shows the preparative ITP system reported by Arlinger [18] for the fractionation of the entire sample zones. This system was applied in the LKB (Bromma, Sweden) Tachofrac (1983). The zones were swept gradually by a counter flow of a leading electrolyte (*e.g.*, 3μl min^{-1}) on applying a migration current, and the fractions were fixed on a cellulose acetate strip. The separated zone was pushed out through a T-branch by the counter flow and it was continuously fixed on the strip by electric spray phenomena. The linear velocity of the counter flow was set to be only a few per cent higher than the isotachophoretic migration velocity. This was intended not to dilute the sample by the leading electrolyte. The fractions on the strip can be measured by using immunological and radioactive methods. The zymogram technique can be used directly on the strip. The fractions have to be eluted and then they are analysed by the different methods.

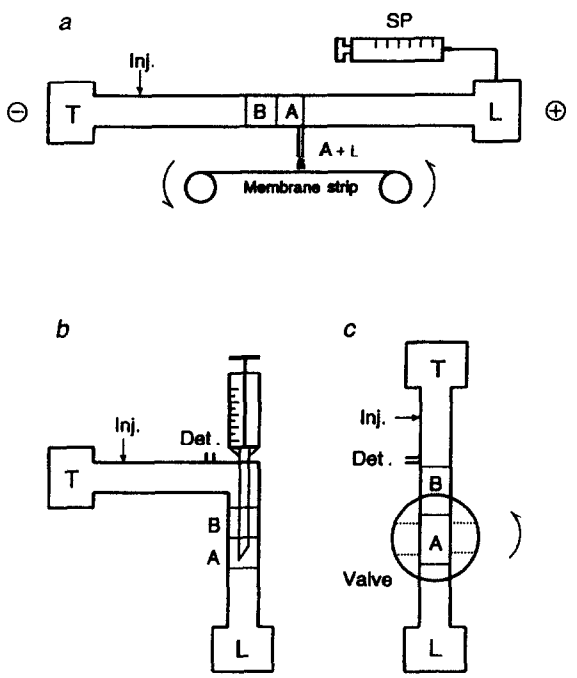

Fig. 5. Preparative methods in capillary-type isotachophoresis. (a) Counter-flow method; (b) microsyringe method; (c) fractionation valve method. A, B = Samples; L = leading electrolyte; T = terminating electrolyte; Inj = sample injection port; SP = counter-flow pump; Det = detector.

Fig. 5b shows another method, reported by Kobayashi *et al.* [19], in which the separated sample zone was discontinuously isolated by using a microsyringe. They reported a potential gradient detector (PGD) with a sample removal port to fractionate the target zone immediately after the tail of the zone was detected by the PGD. Although the method was not intended for the successive fractionation of the entire sample zones, the operational facility is notable. This technique was applied in IP-1B and IP-2A instruments (Shimadzu, Kyoto, Japan).

Fig. 5c shows the other discontinuous fractionation technique, using a specially designed fractionating valve placed at the end of the separation capillary [20]. After trapping the target zone in the valve, the zone was flushed out.

Recently, a dropwise fractionation method was developed utilizing a counter flow technique [21]. A schematic diagram of the apparatus is shown Fig. 6. When the sample zone is pushed out from a T-branch, an electric spray effect is usually observed owing to electrostatic force.

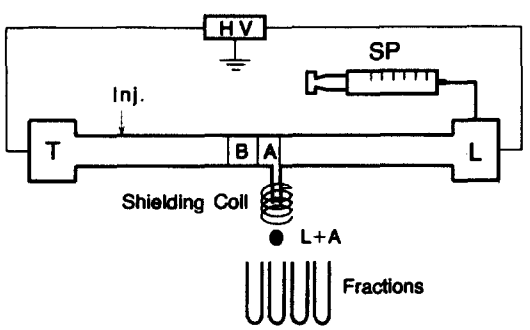

Fig. 6. Modified Arlinger's method for fractionation in capillary-type ITP. HV = High-voltage power supply; other abbreviations as in Fig. 5.

This can be a convenient interfacing technique but it disturbs dropwise fractionation. The electric spray and fluctuations of the dropping course due to electrostatic force were suppressed by a very simple electrostatic device. As shown in Fig. 6, the pushed-out fraction is surrounded by a copper coil, which is connected to a nozzle. The fractions were collected directly into small test-tubes on the fraction collector through the coil. By using this technique, complete recovery of the mobile components in the injected samples was possible with minimum risk of loss and contamination. It should be noted, however, that mixing of adjacent sample zones could not be avoided in principle. The average volume of one drop was *ca.* 5 μl and the deviation was estimated to be ±10%. A few nanomoles of the sample components were contained in each drop. The concentration of samples in the fractionated drops or the amount of the target in each fraction was adjustable by changing the flow-rate of the leading solution. A typical flow-rate was *ca.* 12 μl min^{-1}, which is much higher than in the Arlinger-type apparatus.

In addition, the separation tube used was a series of four separation tubes (I.D. 5–0.5 mm) in order to increase the separable sample amount [21]. The tube of 5 mm I.D. was made of acrylic resin and the maximum injectable sample volume was 2.5 ml.

3.2. Continuous free-flow apparatus

As no solid medium is used in free-flow electrophoresis (FFE), the most important point with the instrumentation is the stabilization of the separated zones for any electrophoresis mode. Unstable zones may be caused by an unstable operational electrolyte and sample flow, heat convection, density-driven flow, electroosmosis, etc. Bier *et al.* [22] and Thormann and Moscher [23] summarized several different types of instrumentation which were made to stabilize the zones. A flat-type FFE system is treated in this review, although there are several different approaches using different geometries, such as a thin film between parallel plates or a cylindrical laminar flow between two coaxial cylinders.

Continuous FFE apparatus utilizing a thin, flowing fluid was originally designed by Hannig [24] for zone electrophoresis. Prusik *et al.* [25,26] and Wagner and co-workers [27–29] designed and constructed similar apparatus, suggesting that several modes of electrophoresis can be used. At present, the FFE apparatus designed by Wagner *et al.* is commercially available as the Elphor Vap22 continuous electrophoresis apparatus from Bender and Hobein (Munich, Germany). Using this apparatus, up to several grams of pure substances can be prepared daily, although the amount depends closely on the properties of the sample and the electrolyte system used.

3.2.1. Continuous free-flow isotachophoresis

Fig. 7 illustrates the electrolyte circuits of the Elphor Vap22 FFE system operated in cffITP mode. The effective size of a typical separation chamber (SC in Fig. 7) was 10 cm wide × 50 cm high × 0.5 mm thick. Sample solution (S) was supplied with a multi-fold peristaltic pump (PER1) together with a leading electrolyte (L1) and a terminating electrolyte (T1). Ninety fractions were collected by means of a fraction collector (FR) at the exit of the separation chamber. For fraction collection and making a

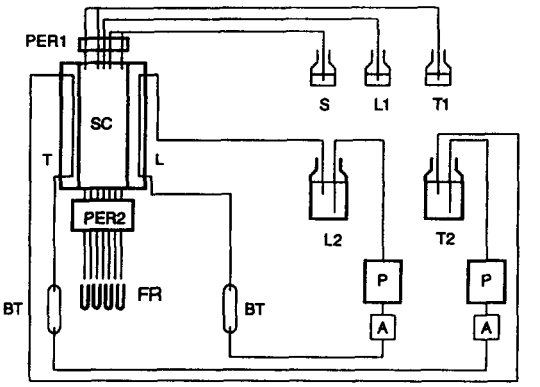

Fig. 7. Electrolyte circuits of a free-flow isotachophoresis instrument (Elphor Vap22). SC = Separation chamber; L = leading electrolyte and electrode compartment; T = terminating electrolyte and electrode compartment; PER1 = peristaltic pump to feed sample and electrolyte; PER2 = 90-fold peristaltic pump for fractionation; FR = fraction collector; S = sample solution reservoir; L1, L2 = leading electrolyte reservoirs; T1, T2 = terminating electrolyte reservoirs; P = electrolyte circulation pump; A = flow-rate adjuster; BT = bubble trap.

vertical flow of fluid, a 90-fold peristaltic pump (PER2) was used. The flow-rate was variable in the range 0.3–100 ml h^{-1}. The sample residence time is variable in the range 1–40 min. High flow-rates and small residence times allow stable flow and consequently stable positions of zones.

The volumes of leading and terminating electrolytes (L2 and T2) used for the electrode compartments (L and T) were each 2 l and they were circulated by pumps (P) during migration. A dialysis membrane isolates the separation chamber from the electrode compartments. The electrolyte solutions may be denatured (*e.g.*, the pH may change) after a few hours of operation, depending on the current. The separation chamber can be thermostated in the range 4–25°C. The heat dissipation allowed is up to 1200 W. The separation behaviour can be monitored by using a position-scanning UV detector (Elphor Scan), which can be set near the exit of the separation tube.

To fractionate pure components, the positions of the separated zones at the end of the separation chamber should be highly stable. The positions are dependent on several factors such as the electric field strength, temperature of the electrolytes, flow-rate and sample and buffer compositions. As these factors are closely correlated with each other, careful control is needed. Especially when the mobility differences among the sample components are small, sufficient residence time and separation distance are necessary. For this purpose, a larger separation chamber or a counter-flow technique should be used, as reported by Prusik *et al.* [25,26] (a 50 cm × 50 cm square chamber with a thickness of 0.5 mm was used). A counter-flow technique to compensate for electrophoretic migration was introduced first by Preetz *et al.* [30]. Later it was modified to a large-scale instrument by Prusik *et al.* [25,26]. The effect of a counter flow is very similar to that in recycling isotachophoresis described in the next section.

3.2.2. *Recycling isotachophoresis*

In recycling electrophoresis, in order to increase the electric charge applied to the sample, the fractions from each channel are continuously reinjected into the influent 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 cffITP.

Fig. 8 shows a schematic diagram of an automated rITP apparatus developed by Sloan *et al.* [31,32]. A recycling free-flow focusing apparatus (Model RF3; Protein Technologies, Tucson, AZ, USA) was also used for rITP [33,34].

The separation field designed by Sloan *et al.* is a thin film (0.75 mm) of flowing fluid between two parallel plates in a 35 cm long rectangular chamber having a width of 5.5 cm (volume 14.44 ml). The cross-section of the separation field is 2.6 cm^2, therefore Q is comparable to that of the Elphor Vap22. Arrays of 48 inlet and outlet ports define the bottom and top, respectively, and

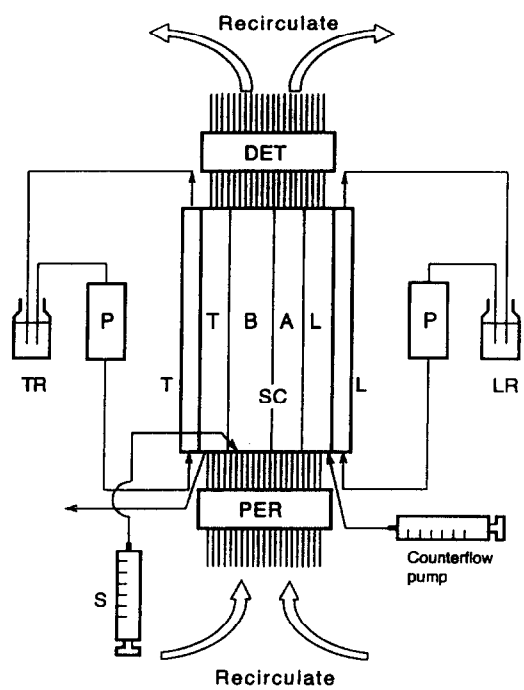

Fig. 8. Recycling isotachophoresis instrument. SC = Separation chamber; L = leading electrolyte and electrode compartment; T = terminating electrolyte and electrode compartment; LR = leading electrolyte reservoir; TR = terminating electrolyte reservoir; PER = peristaltic pump to circulate sample and electrolytes; S = sample injector; P = electrolyte circulation pump; DET = boundary position detector.

dialysis membranes isolate the separation chamber from the electrode compartments.

In Fig. 8, the sample components A and B are completely separated (steady state). At the transient state, a mixed zone AB exists between zones A and B as in Fig. 1b. They are separated gradually while recycling. The fluid residence time in the separation chamber is a few seconds. This allows a remarkable stability owing to fluid dynamics. On the other hand, the short residence time prevents complete separation in a single pass through the separation chamber. In rITP, recycling is essential to achieve a steady state. In order to cancel the electrophoretic migration and keep the positions of the sample zones constant, a counter-flow technique is utilized. Typically the counter-flow pumping rate did not exceed 1.5–3.0 ml min^{-1}. The rate is computer controlled, taking into account the positions of the sample zones.

4. APPLICATIONS

Comprehensive reviews by Boček and coworkers [35,36] on analytical ITP [35,36] are very useful for obtaining an overview of ITP and for surveying the substances treated by ITP techniques. Recent new developments were summarized by Thormann and Moscher [37] and Gebauer *et al.* [38]. In order to avoid overlap, two typical applications by using cITP and rITP are introduced here.

4.1. Capillary-type isotachophoresis

As discussed, cITP is useful for micropreparative purposes. In addition to the purification of a small amount of sample by fractionation, preparative cITP is useful for the determination of unknown constituents in combination with different analytical methods (coupled techniques). In such a case, cITP is regarded as one of the pretreatment techniques with high selectivity for further characterization and identification. Especially for the analysis of a complex sample by cITP, conventional detection methods such as conductimetric and optical detection are occasionally not sufficient. For further analysis in combination with a different analytical method, an on-line combination is most convenient, although off-line interfacing is also useful.

As cITP fractions are concentrated, the amount of sample in the fractions is sufficient for highly sensitive analytical methods, such as HPLC [39], MS [40,41] and UV–Vis spectrometry [21]. We have developed an ITP–particle-induced X-ray emission (PIXE) technique [42], which is very useful for studying the separation behaviour of metal ions. PIXE is a multielemental analytical method with high sensitivity [43]. As it is based on the characteristic X-rays emitted by target elements, it has high specificity for the determination of the elements even if they were not separated. The amount of sample necessary for ITP–PIXE depends on the abundance of the target element, but it is usually of the order of submicrograms.

A crude rare earth chloride (a primary product from a monazite) was selected to demonstrate the analytical utility of the ITP–PIXE technique. The major elements were La, Ce, Pr and Nd (total abundance *ca.* 95% of total rare earth elements) and the minor elements were Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Y (total abundance *ca.* 5%). The ratio of the amount of Ce, with the maximum abundance, to that of Yb, with the minimum abundance, was >10 000.

In order to determine the trace rare earth elements Ho, Er, Tm, Yb and Lu, *ca.* 1.8 mg of sample dissolved in 200 μl of water was separated and analysed by ITP–PIXE [44]. The sample contained 638 μg of rare earth elements. The micro-preparative analyser used was the capillary type made by us [21]. The quantity of charge applied during the separation was *ca.* 10 C. After removing the zones of the major elements La, Ce, Pr and part of the Nd, the zones containing the other minor elements were fractionated. Fig. 9 shows the isotachopherogram observed and the constituents of the fractions and the amount evaluated by PIXE method. Twenty fractions were obtained in 582 s (29.1 s per fraction). As shown in Fig. 9, even the very trace element Lu was determined. The amount was 6 ng, which was 0.036 mass% of the injected sample. The amount of Sm was 16.6 μg (0.94 mass%).

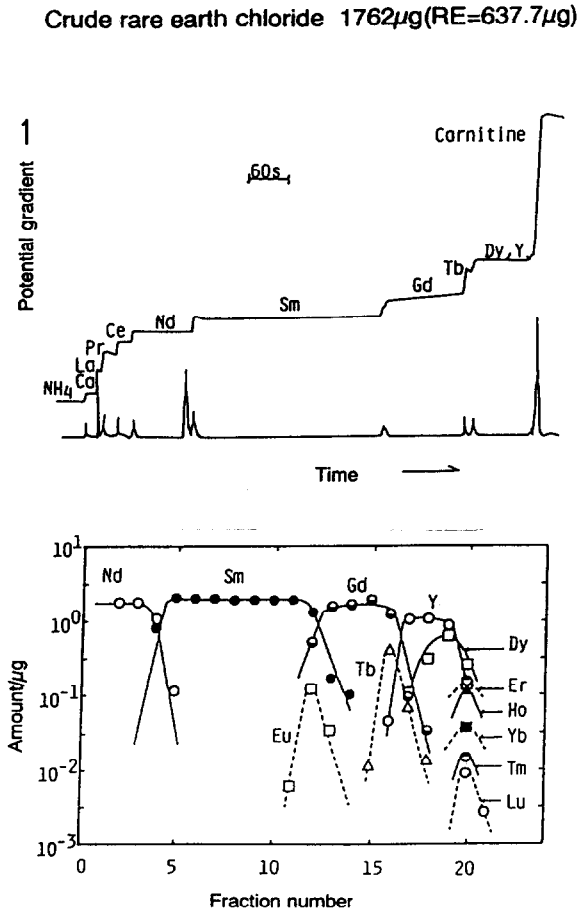

Fig. 9. Observed isotachopherogram of crude rare earth chloride (1762 μg) and analytical result for the 20 fractions obtained by the PIXE method. Not all of the sample was fractionated (see text). The quantity of charge integrated was *ca.* 10 C. The leading electrolyte was 20 m*M* ammonia solution containing 10 m*M* α-hydroxyisobutyric acid as the complex-forming reagent. The pH was adjusted to 4.8 by adding acetic acid. The terminating electrolyte was 10 m*M* carnitine hydrochloride.

For the determination of metal ions in solution, there are several convenient methods such as atomic absorption spectrometry and inductively coupled plasma atomic emission spectrometry. These methods are highly sensitive and useful for low-concentration samples. However, the sample volume required is as large as 10 ml or more. In addition, there is sometimes serious spectral interference, which can be avoided only by separation.

4.2. Recycling isotachophoresis

FFE techniques are important especially for the purification of products in biotechnology (proteins, etc.) because of high separability and throughput. Seemingly, the instability of separated zones which adversely affects purity can be most reasonably reduced in rITP.

Fig. 10 shows the analytical results for the

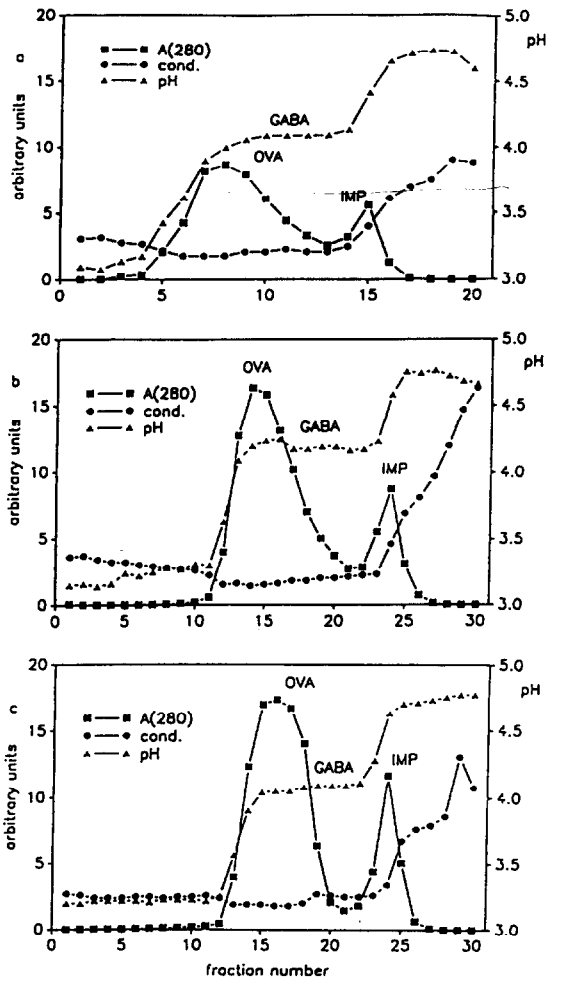

Fig. 10. Analytical results for the fractions obtained by rITP [33]. (a) 310 mg of ovalbumin (OVA) and 9.1 mg of γ-aminobutyric acid (GABA), Rotofor, 54 C (60 min); (b) 710 mg of OVA and 14 mg of GABA, RF3 without counter flow (180 C, 60 min); (c) with counter flow (346 C, 112 min). The leading electrolyte (right-hand side) was 10 m*M* potassium acetate–acetic acid (pH 4.75) and the terminating electrolyte was 10 m*M* acetic acid.

fractions obtained in rITP experiments for ovalbumin (OVA) and γ-aminobutyric acid (GABA) [33]. Fig. 10a was obtained by using a Rotofor (Bio-Rad Labs., Richmond, CA, USA) at 4.5 rpm. The separation field of the Rotofor is a screen-segmented laminar flow between cylinders. The samples were 310 mg (6.9 μmol) of OVA and 9.1 mg (88.2 μmol) of GABA and the quantity of charge applied was 54 C (60 min). Fig. 10b was obtained by rITP using an RF3 without a counter flow. The samples were 710 mg (15.8 μmol) of OVA and 14 mg (136.8 μmol) of GABA and the quantity of charge applied was 180 C (60 min). Fig. 10c was obtained for the same amount of sample with a counter flow after an additional 52 min. The integrated charge was 346 C. Accordingly the quantity of charge supplied in unit time (Q) was calculated to be 185 C h^{-1}.

The applied charge was varied in these experiments as follows: (a) 0.17 C mg^{-1} OVA and 5.9 C mg^{-1} GABA, (b) 0.25 C mg^{-1} OVA and 12.9 C mg^{-1} GABA and (c) 0.49 C mg^{-1} OVA and 24.7 C mg^{-1} GABA. Obviously, the quality of separation was improved with increase in the applied charge. The separation efficiency (S_{eff}) for OVA and GABA was estimated from Fig. 10c to be 45.6 and 395 nmol C^{-1} and the preparative efficiency (P_{eff}) was 8.4 and 73 μmol h^{-1}, respectively. Hence 9 g of OVA will be obtained if the apparatus is operated throughout the day.

In addition, the simulated pH of the GABA zone was 4.04 (observed, 4.1 from Fig. 10c), the concentration was 5.3 mM and the effective mobility was $14.3 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}. The SIPS program was used for the simulation [4,5].

5. CONCLUSIONS

Although the preparative efficiency of cITP is not so high and a batch processing cannot be avoided, preparative cITP is still an important technique because of its high separability. Additionally, preparative cITP is useful as the pretreatment technique in coupled analytical methods owing to its concentrating ability and the separability.

For the purification of biotechnological products on a large scale, it seems that rITP shows the highest preparative performance at present. This is because the controllability of the zone position is high in principle and recirculation allows a complete separation of whole sample.

If ITP is carried out under microgravity, better efficiency may be obtained, because a separation chamber with a large cross-section (*i.e.*, a thick film of fluid) is allowed. This has been exemplified by isoelectric focusing in space [45]. Considering the restrictions accompanying space experiments, however, construction of a large-scale apparatus for rITP may be more efficient and reasonable.

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