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DETERMINATION OF SUBSTANCES AT LOW CONCENTRATIONS IN COMPLEX MIXTURES BY ISOTACHOPHORESIS WITH COLUMN COUPLING

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SUMMARY

A system is discussed that makes use of two PTFE narrow-bore tubes with different internal diameters. In the pre-separation narrow-bore tube, which has the larger internal diameter, a high pre-separation current is permitted. At a well defined distance from a conductivity detector (mounted in this pre-separation narrow-bore tube) the final separation narrow-bore tube, which has the smaller internal diameter, is coupled to the pre-separation narrow-bore tube via a T-piece. The smaller internal diameter of the final narrow-bore tube permits work at a higher current density during final separation and detection by means of conductivity and UV absorption detectors. The zones of interest can easily be separated from the sample zones, migrating isotachophoretically in the pre-separation narrow-bore tube via the detector.

There are several advantages over conventional isotachophoretic equipment: (i) a high sample load is permitted; (ii) the analysis time is increased negligibly; (iii) high ratios of concentrations between the sample species are permitted; and (iv) different operational systems can be applied in separation and preseparation compartment.

INTRODUCTION

The development of modern analytical techniques has made possible the qualitative and quantitative determination of substances present at low concentrations. Specially sensitive and/or specific detectors have been developed and are commercially available, and the available equipment makes use of a variety of separation principles.

The substances of interest are often present in solution together with numerous other substances at higher concentrations and sample pre-treatment procedures, such as extraction, derivatization or column techniques, are widely discussed in the literature.

In isotachophoretic analyses also various methods can be used to obtain reli-

able qualitative and quantitative information about sample components present in low concentrations. Two main methods for the determination of small amounts of substances will be discussed. A larger sample can be introduced into the equipment and the sensitivity or selectivity of the detection is improved. Furthermore sample pre-treatment procedures can always be applied. One possibility is the creation of longer zone lengths (*i.e.*, the quantitative measure in isotachophoresis) by simply introducing a larger amount of sample. It must be remembered that the concentrations of the sample constituents migrating isotachophoretically between the leading and the terminating zones are related to the concentration of the leading zone. For complete separation of a larger amount of sample introduced into conventional isotachophoretic equipment, a greater tube length[1,2] or a counter flow of electrolyte[1] can be utilized to avoid overloading the narrow-bore tube (assuming that an optimal diameter of the narrow-bore tube has already been chosen[3], together with a suitable concentration of leading electrolyte). An excessively low concentration (<0.001 *M*) of leading electrolyte is inadvisable because the choice of pH ranges suitable for the selection of operational systems is lowered. This is due to the contribution made by H^+ and OH^- to the separation process if work at very low or very high pH is considered.

A longer narrow-bore tube means that a higher potential is needed. In the equipment used in our laboratories[1], the diameter of the narrow-bore tube is generally 0.2 mm and its length 20 cm. The end-voltage is often about 15 kV. This means that, for purely practical reasons, little more than doubling the length of the narrow-bore tubes can be permitted.

If long narrow-bore tubes are applied, sometimes the H^+ or OH^- ions originating from the counter-electrode compartment can influence the analysis. Therefore for longer analysis times precautions must be taken, *i.e.*, the counter-electrode compartment must be filled with a buffer solution instead of distilled water. The use of a counter flow of electrolyte as an alternative to elongation of the narrow-bore tube is also seldom satisfactory, for a number of reasons:

(i) a relatively long analysis time[1] is needed and the effective elongation of the narrow-bore tube is about 60%;

(ii) the maximal counter flow of electrolyte can be applied as soon as the stationary state has been reached;

(iii) in a long run, the ionic impurities present in the chemicals and solvents applied often disturb or obscure the final result.

Moreover, a relatively large difference between the effective mobility of the ionic species of interest and that of its isotachophoretic neighbours is needed for a complete and reproducible separation in the selected length of the narrow-bore tube.

The major benefit from using a counter flow of electrolyte compared with elongation of the narrow-bore tube is the low end-voltage needed for a complete separation. Moreover, the equipment need not be re-assembled between analyses. Because a counter flow of electrolyte is not applied during the detection, the reproducibility of the analyses is comparable with that of analyses carried out without a counter flow of electrolyte.

The use of a continuous sampling system[4,5] does not provide further possibilities for the introduction of larger amounts of sample in comparison with elongation of the narrow-bore tube or the use of a counter flow of electrolyte. In our experience, electroendosmotic pumps[6] (used in the continuous sampling system) give

less consistent results than well constructed membrane pumps[7]. This means that even longer analysis times can be expected and a greater difference in the mobilities of ionic species migrating in adjacent zones is necessary. A much simpler solution for the introduction of a larger amount of sample is to mount a pre-separation compartment (*e.g.*, of a conical type) between the injection block and the narrow-bore tube of 0.2 mm I.D. In our equipment this compartment can be easily removed and replaced with a cylinder with a different bore (*e.g.*, 0.2 mm). All of the advantages of using a counter flow of electrolyte are retained. The increment in the final potential is almost negligible. As in experiments with a counter flow of electrolyte, impurities in the electrolyte systems[2] may obscure the final result and again longer analysis times can be expected. Because there is no disturbance of the zone profiles (as in experiments with a counter flow of electrolyte), the final time needed for the complete separation of a given sample is much shorter. Of all possibilities discussed, the use of a well adjusted pre-separation compartment is the most preferable. Without complicated regulating techniques or a high potential, the same results can be achieved.

Another possible means for introducing a larger amount of sample is to use the column switching system, a method with greater potential than simply increasing the maximal load capacity. This technique will be discussed extensively later, but is mentioned here because it is cited in Table I (A).

The results in Part B of Table I for the determination of small amounts of ionic substances by isotachophoresis can be obtained by making the detection more sensitive and/or specific (selective). The detectors can be mounted on or in the separation compartment or can be used after the isotachophoretic separation[8,9]. If specific detectors are mounted on or in the separation compartment, specific components can be detected quantitatively down to the picomole level[10;11]. If substances to be separated are present in a mixture at a low concentration and these substances have a high molar absorption coefficient for UV light (or light of a specific wavelength), an ionic species that has an effective mobility matching that of the substance of interest can be selected. This ionic species does not absorb light at the wavelength chosen. The two ionic species (with and without light absorption) migrate together in a mixed zone (steady-state mixed zone) if the pH of the mixed zone is only slightly different from that of the individual ionic species migrating as pure zones. If, however, the pH is very different, non-ideal mixed zones are obtained, even if the ratio of the concentrations varies only slightly. In isotachophoresis, it is possible for two ionic species to be separated in consecutive zones with sharp zone boundaries, while their effective mobilities are identical, because the zone pHs are different. By measuring the integrated UV absorbance of the steady-state mixed zone, a substance present in that mixed zone can be analysed quantitatively and qualitatively, even at the picomole level. Although the method is sensitive, finding the correct operational conditions is time consuming, especially if various substances require analysis simultaneously. In practice, large differences in the concentrations of the substance to be analysed and other ionic species 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. No special operating conditions are required if the "UV spike" method[10] is used. Again, only substances that are detected with a selective detector can be analysed. Assume a zone is present that has a UV ab-

sorption. It migrates isotachophoretically and the step height found in the UV absorption trace provides a qualitative measure of the ionic species considered. When the zone length is too small, the step height value in the UV absorption trace is not reached: a UV spike is formed. If the UV-absorbing ionic species is sandwiched between two non-UV-absorbing ionic species, the peak height of the spike gives quantitative information. Also, picomole levels are easily measured[10].

The use of sample pre-treatment is more or less universally applicable to all separation techniques, if direct analysis fails. Selective extraction procedures, derivatization or the use of columns packed with ion-exchange resins are often recommended. Although sample pre-treatment is often time consuming, it can be a useful adjunct to a separation method. It can be used in all systems (I–VIII) listed in the Table I, where the most important features are compared.

Special attention will now be paid to the column switching system.

TABLE I

SURVEY OF POSSIBILITIES FOR DETERMINING SMALL AMOUNTS OF IONIC MATERIAL BY ISOTACHOPHORESIS

The use of an optimal I.D. of the narrow-bore tube in I, II, IV, V, VII and VIII is assumed. ΔV_{tot} = total potential gradient between cathode and anode compartments at the end of the analysis; t_{anal} = analysis time needed for the isotachophoretic analysis, including detection; $t_{total} = t_{anal}$ + sample pre-treatment and time needed for specific detection after analysis; Φ_{high} = high ratio of concentrations of sample constituents; rep. = reproducibility of both qualitative and quantitative information; sel. = selectivity for one sample component; c.e. = complexity in handling and or construction of the equipment; f.o.s. = flexibility in using operational systems. + = Good; ○ = neutral; − = bad.

Conditions	*No.*	*Method*	ΔV_{tot}	t_{anal}	t_{total}	Φ_{high}	*f.o.s.*	*rep.*	*sel.*	*c.e.*
A: The sample load is increased; the minimal detectable amount does not change	I	Increasing the length of the narrow-bore tube[1]	−	−	−	○	○	+	○	+
	II	Counter flow of electrolyte[1,7]	+	−	−	○	○	+	○	−
	III	Continuous sampling system[4,5]	+	−	−	○	○	○	○	−
	IV	Pre-separation compartment (*e.g.*, conical)	+	−	−	○	○	+	○	+
	V	Column coupling system	+	+	○	+	+	+	+	−
B: The minimal detectable amount is decreased, *e.g.*, via the selectivity of the detection system	VI	Off-column detectors[8,9]	+	+	○	+	−	○	+	−
	VII	"UV spike" method[10]	+	+	+	+	○	○	○	+
	VIII	Steady-state mixed zone method[11]	+	+	+	+	○	○	+	+
	IX	Sample pre-treatment in general	+	+	○	+	+	+	+	+

INSTRUMENTATION

The T-piece

The coupling of the two columns to be used for the pre-separation and the final separation by isotachophoresis is shown schematically in Fig. 1. The narrow-

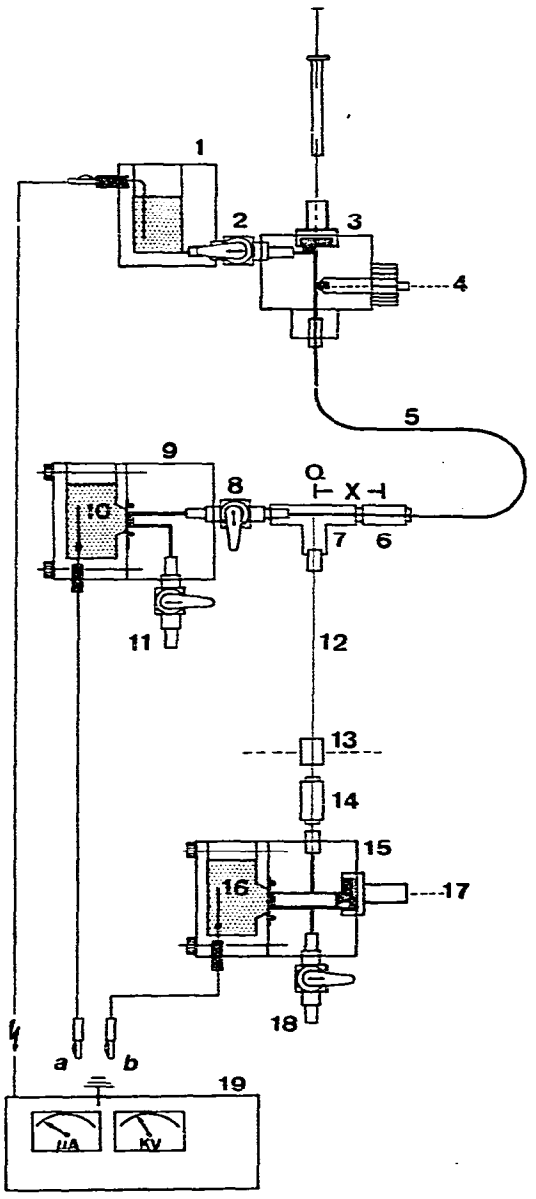

Fig. 1. Device for column coupling in isotachophoresis. 1 = Compartment filled with terminating electrolyte; 2 = PTFE-lined valve (1MM1; Hamilton, Bonaduz, Switzerland); 3 = injection block with septum; 4 = connection towards drain; 5 = pre-separation narrow-bore tube (0.8 mm I.D.); 6 = conductivity 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 semi-permeable membrane; 10 = counter electrode, immersed in doubly distilled water to be used in mode a (pre-separation); 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 semi-permeable 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).

bore tube, applied as a pre-separation compartment, has an I.D. of 0.8 mm and the separation compartment has an I.D. of 0.2 mm. The electric current is *ca.* 350 μA for the pre-separation (operational system: Table II), and *ca.* 25 μA for the final separation (operational systems: Tables II and III).

TABLE II

OPERATIONAL SYSTEM AT pH 6, SUITABLE FOR ANIONIC SEPARATIONS

Solvent: water.

Purification: morpholinoethanesulphonic acid (MES) is recrystallized. Hydroxyethylcellulose (HEC) is purified with a mixed-bed ion exchanger (Merck V).

Parameter	*Electrolyte*	
	Leading	*Terminating*
Anion	Cl^-	MES*
Concentration	0.01 *N*	*ca.* 0.01 *N*
Counter ion	$Histidine^+$	$Tris^+$
pH	6	*ca.* 6
Additive	0.2% HEC	None

* For the experiment shown in Fig. 8 glutamic acid was used as terminating ion.

TABLE III

OPERATIONAL SYSTEM AT pH 3, SUITABLE FOR ANIONIC SEPARATIONS

Solvent: water.

Purification: β-alanine (BALA) is recrystallized. Hydroxyethylcellulose (HEC) is purified with a mixed-bed ion exchanger.

Parameter	*Electrolyte*	
	Leading	*Terminating*
Anion	Cl^-	Glutamate
Concentration	0.01 *N*	*ca.* 0.07 *N*
Counter ion	BALA	$Histidine^+$*
pH	3	*ca.* 6*
Additive	0.2% HEC	none

* The terminating ion comes from the pre-separation narrow-bore tube, which is buffered with $histidine^+$–histidine.

The apparatus (Fig. 1) consists of an injection block (3) with an electrode compartment (1); a pre-separation narrow-bore tube (5); a T-piece (7); the final separation compartment (12) and two electrode compartments (9, 15) in which semi-permeable membranes of cellulose-polyacetate are used. Details of the mounting of the various narrow-bore tubes with the other pieces of the apparatus are given in ref. 1. Also included in Fig. 1 are two conductivity detectors (6, 14) and one UV absorption photometric detector (13). The first conductivity detector registers the pre-separation. Because the zones (under carefully chosen operational conditions) migrate with equal and constant velocity, the time needed for a zone boundary to reach the point in the T-piece at which the separation compartment is connected is constant and readily determined. Thus, once a zone has been registered by the conductivity detector (6),

the moment at which it reaches the narrow bore of 0.2 mm is known. Hence the zone or zones of interest can be easily isolated, even if they are not migrating consecutively. Because high concentrations of mobile ionic species are present in many samples (*e.g.*, Cl^-, Na^+, ClO_4^-) the zone(s) of these ionic species can quickly pass the narrow bore in the T-piece. The zones of interest are further analysed and detected in the 0.2-mm tube at approximately equal current density, but at a lower electric current. An average time necessary for a stack of zones originating, *e.g.*, from biological fluids, to migrate from the injection point to the narrow bore in the T-piece is *ca.* 6 min. As soon as a zone of interest reaches the narrow bore in the T-piece, the electric current is lowered from 350 to 25 μA and passed through the final separation column (12). In the separation compartment (12), a relatively high current density can be permitted and still the temperature increase is negligible[3]. The length of this narrow-bore tube can be chosen to suit individual requirements. For the experiments presented later, the length is selected so that the zones have *ca.* 8 min to migrate from the T-piece to the detectors (13, 14).

The valve for column coupling

The apparatus used is comparable to that shown schematically in Fig. 1, except that instead of the T-piece a disc-type valve is used. As soon as the passage of the zones is registered by the conductivity detector (6), the time is again selected at which the zone(s) enter(s) the bore of the valve. At this moment the valve can be switched to a position in which the trapped zones can be further separated into a narrow-bore tube of much smaller internal diameter. The trap valve is particularly applicable to collection of material for sensitive off-column techniques.

In this paper, we are mainly concerned with the results obtained using the T-piece. Handling of the trap valve is more complicated, especially as a relatively high pressure is needed for filling the narrow-bore separation compartment (I.D. 0.2 mm), compared with the filling of the pre-separation compartment. Because the valve must be a close fit, the pressure on the small column remains and the separation already obtained in the pre-separation compartment is adversely affected after the valve has been switched. This can be easily demonstrated if dyes are applied as sample species. Moreover, the use of off-column techniques, such as liquid scintillation counting and specific colour reactions, will not be dealt with in this paper.

EXPERIMENTAL

A series of experiments were performed with the apparatus shown in Fig. 1. Both the pre-separation and the separation narrow-bore tubes may be filled with the same leading electrolyte (operational system: Table II), but different leading electrolytes can also be applied and separation can be effected according to pK values, instability constants or activity (operational systems at different concentrations) and solvation. An example will be given of the quantitative and qualitative determination of ascorbic acid in fruit juices. Fig. 2 shows the analysis of 0.75 μl of natural orange juice* (diluted 10-fold with doubly distilled water) in an isotachophoretic apparatus with a narrow-bore tube (20 cm $\times$ 0.2 mm I.D.). This apparatus was equip-

* This is not the maximal load for the apparatus.

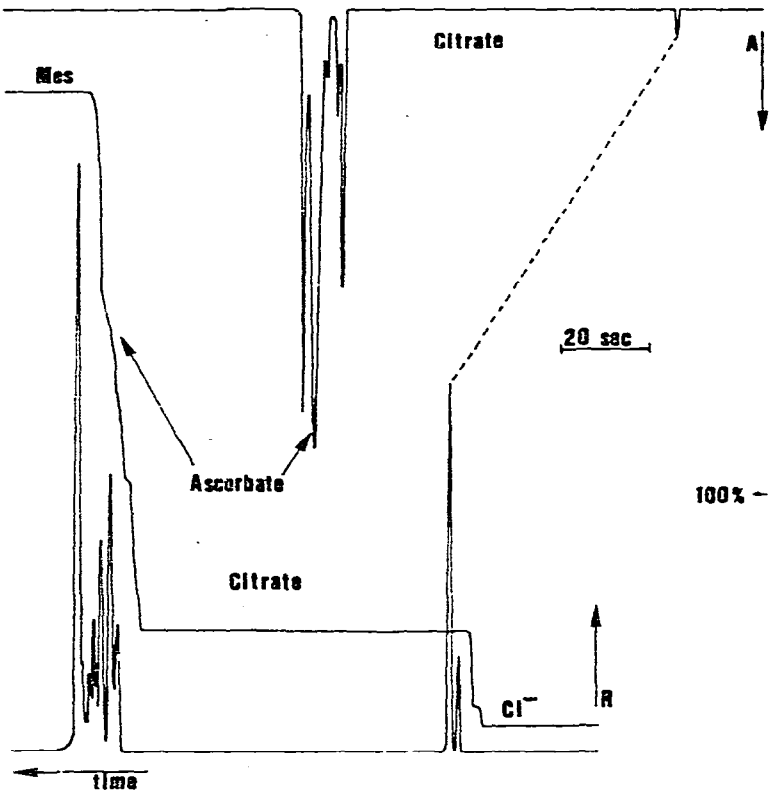

Fig. 2. Isotachophoretic separation of orange juice, carried out in the operational system at pH 6 (Table II) in conventional[1] isotachophoretic equipment.

ped with a photometric detector (256 nm) and a conductivity detector[1]. The time for analysis was *ca.* 15 min. The main part of the isotachopherogram (Fig. 2) is occupied by the citrate zone. Because ascorbic acid absorbs strongly at 256 nm and is spaced by two non-UV-absorbing ionic species, its presence can be readily observed in the linear trace of the photometric detector. Its presence can be verified by the addition of ascorbic acid to the juice and its effect on the length of the marked zone (Fig. 2). For practical purposes, this UV spike can be used for quantitative evaluation via the "UV spike" method[10]. If a greater accuracy is required and a calibration graph (zone length *versus* amount of ascorbic acid) is used, a longer zone is desirable. If double the amount of juice is injected and analysed, zone elongation will be two-fold; about double the time for analysis is needed to reach the steady state and little more practical information will be obtained. To extend the tube more than three times is unacceptable, because the end-voltage of more than 30 kV is not practical in ordinary analytical laboratories. The use of a counter flow of electrolyte (Table I, No. II) and the use of a conical pre-separation compartment (Table I, No. IV) gives low end-voltages but still involve long analysis times. Because of the complexity of the mixture, an off-column detector (Table I, No. VI) does not give correct information. The "UV spike" method can be used (Table I, No. VII), but an error of at least 10% in quantitative evaluation can be expected. The steady-

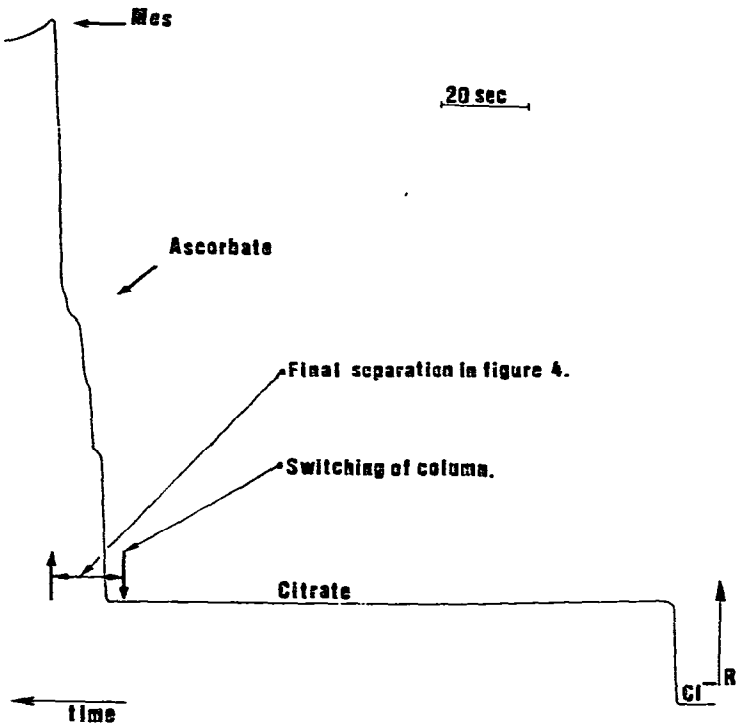

Fig. 3. Registration of the isotachophoretically separated substances (anions) in orange juice by the conductivity detector (Fig. 1; component 6). An arrow indicates the moment at which the equipment is switched from mode a into mode b (Fig. 1). The isotachophoretic pre-separation and separation were performed at pH 6 (Table II). The time from injection to switching of column was *ca.* 8 min.

state mixed zone method (Table I, No. VIII) is elaborate and has therefore not been confirmed. An adequate sample pre-treatment (Table I, No. IX) has not been devised for isotachophoresis.

The same orange juice (1 μl) was also injected into the equipment with coupled columns. The result, as obtained from the conductivity detector, mounted in the pre-separation tube of 0.8 mm I.D. (Fig. 1, component 6), is shown in Fig. 3. This analysis has been also performed in the operational system at pH 6 (Table II) with a stabilized current of 250 μA* (Fig. 1, mode a). The zone boundary of citrate reached the detector about 8 min after injection. After about 2 min 20 sec the citrate zone was allowed to pass the narrow bore (0.2 mm) in the T-piece (Fig. 1, component 7). Next, a stabilized current of 25 μA* was passed through the narrow-bore separation compartment (I.D. 0.2 mm) (Fig. 1, mode b). A small zone of citrate was trapped, although this is not necessary for the determination. Under the conditions described for the analysis shown in Fig. 2 (the analysis of orange juice in conventional isotachophoretic equipment[1]), the citrate zone that had passed the bore should have contributed for about 23 min to the isotachopherogram. The result, as registered by

* The choices of 250 and 25 μA were made for practical reasons: the current-stabilized power supply is equipped with a switch through which a 10-fold adjustment of the electric current can be selected.

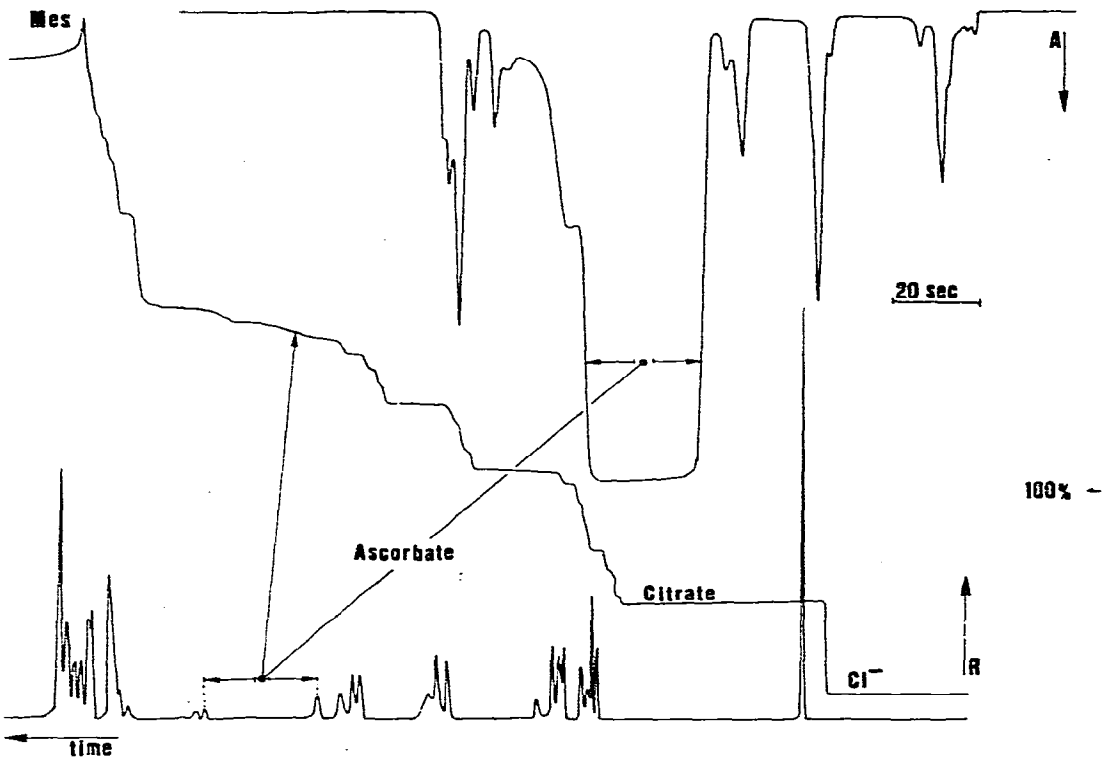

Fig. 4. Registration via photometric (256 nm) and conductivity detectors (Fig. 1) of the trapped zones (Fig. 2) from orange juice, isotachophoretically separated. Both the pre-separation and separation narrow-bore tubes were filled with the operational system at pH 6 (Table II). Full qualitative and quantitative information is obtained for the zone of interest (ascorbate). For a comparable result, the length of the narrow-bore tube in conventional[1] equipment would have to be elongated about 6-fold. The total analysis time (including the time needed for pre-separation) was about 15 min.

the photometric detector (256 nm) and the conductivity detector on the trapped zones (see Fig. 3, zones between arrows), is given in the Fig. 4. The total analysis time, *i.e.*, the time for pre-separation, separation and detection, was about 15 min. Full qualitative and quantitative information could be obtained from both the conductivity and photometric detector for the ascorbate zone. Using the calibration graph (Fig. 5) with the analysed orange juice, the ascorbate content was 586 mg/l. Of course, many more zones of the orange juice could be determined, because without problems the zone length can be easily increased by a factor of 4, and the total time for analysis is still comparable to that on conventional equipment (Fig. 2).

A comparable result, as shown in Fig. 4, could be obtained if:

(i) a *ca.* 6 times longer narrow-bore tube is used with conventional equipment[1] (V_{end} = *ca.* 70 kV), and an analysis time of about 90 min should be obtained;

(ii) a counter flow of electrolyte in a conventional[1] equipment is used (V_{end} = *ca.* 12 kV), and an analysis time of about 150 min should be obtained;

(iii) a conical pre-separation compartment is mounted on the conventional equipment[1] (V_{end} = *ca.* 14 kV), and an analysis time of about 90 min should be obtained.

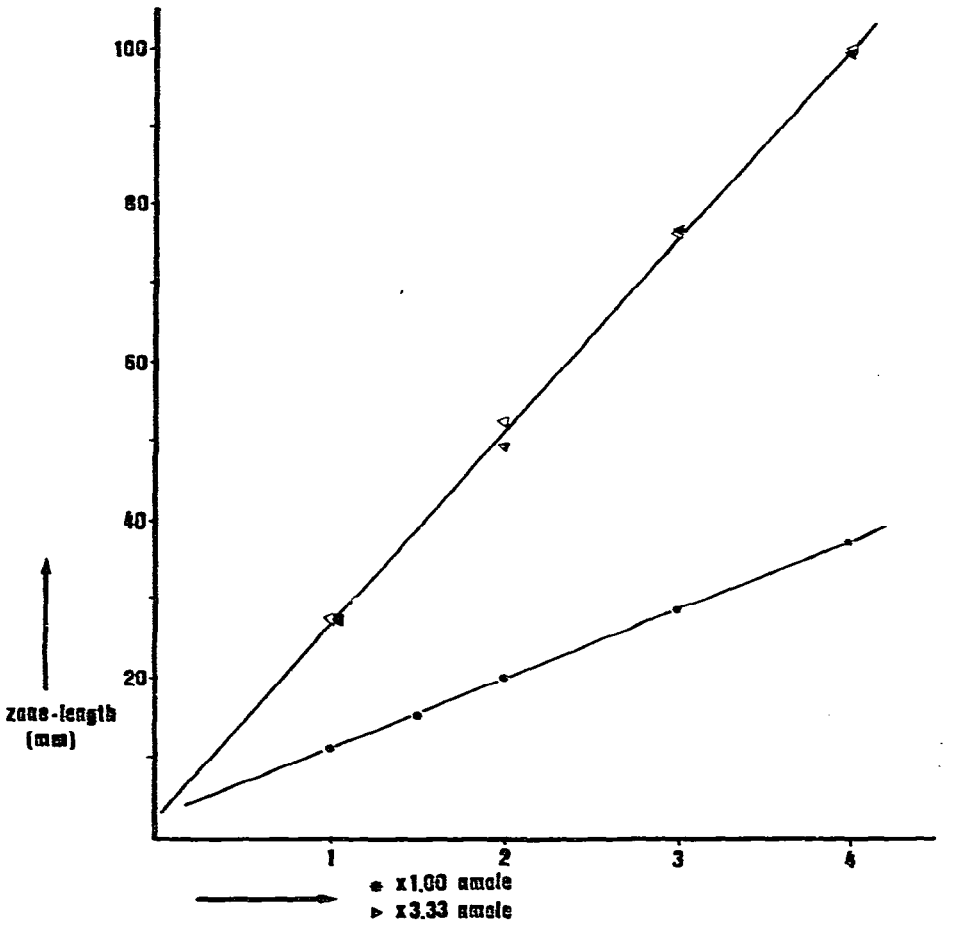

Fig. 5. Calibration graphs for ascorbic acid (▽, obtained from analysis in conventional[1] equipment, 0.2 mm I.D.; ▼, obtained in the apparatus in Fig. 1) and hippuric acid (●, obtained from the coupled columns). A solution of $5 \cdot 10^{-4}$ *M* hippuric acid dissolved in a solution of 0.2 *M* NaCl in water was injected. The excess of chloride was allowed to pass the narrow bore in the T-piece at 350 μA.

The optimal choice of electric current in the pre-separation tube and the separation tube, together with the optimal length of both tubes, will be dealt with further under Discussion. As has been shown in Figs. 2, 3 and 4, with column coupling small amounts of ionic substances in biological fluids can easily be determined. The accuracy in the quantitative evaluation of a trapped zone is as good as the quantitative evaluation of zones of equal length in conventional isotachophoretic apparatus. Two calibration graphs are given in Fig. 5. The zone lengths of ascorbic acid as a function of the amount introduced in conventional equipment and the device with coupled columns are compared, and the agreement is good. This is surprising, because in experiments using dyes* it was observed that small amounts

* In the pre-separation compartment long zones of dye were studied.

of the dye were not trapped and left behind in the T-piece. A negligible amount of the stacked zone may flow in its original direction, so not all of the dye enters the separation narrow-bore tube. The stabilized electric current in mode b (Fig. 1) must not be exactly zero in the bore of 0.8 mm, behind the bore of 0.2 mm in the T-piece. The point Q (Fig. 1) will reach a high potential in the course of the analysis, owing to terminating ions finally entering the separation tube, and by this means the electric resistance increases considerably. Although the counter electrode is disconnected at the moment Q is at high potential, part of the electric current is lost due to a leak. During the time in which a zone is trapped, however, Q is at moderate potential and, moreover, during the analysis no current leak could be measured.

Another possible explanation for a small amount of a long zone of the dye being left in the pre-separation compartment is as follows. In mode b the electric current in the pre-separation compartment is 10 times smaller than the electric current in mode a. This means that the self-correction of the zone boundary, a characteristic if isotachophoretic analyses, is reduced. Thus, more diffuse zones can be expected in the pre-separation compartment, during trapping.

Also, a solution of hippuric acid ($5 \cdot 10^{-4}$ *M*, dissolved in 0.2 *M* sodium chloride solution) has been analysed in the device with coupled columns (Fig. 5). As might be expected[2], the swamping amount of Cl^- does not influence the final result. The increase in analysis time is only a few minutes, because the chloride zone is allowed to pass the narrow bore (0.2 mm) in the T-piece at high speed. It should be noted that, as is often the case, the calibration graphs (Fig. 5) do not pass zero, and this is not the result of coupling the columns. If impurities are present in the chemicals and/or solvents, these impurities can form steady-state mixed zones with the substances to be analysed. In such instances an intercept on the abscissa is observed. On the other hand, intercepts with the ordinate can also be expected if, for example, owing to irreversible complex formation, denaturation or precipitation during the isotachophoretic separation, the zone length of a substance decreases as a function of time. Of course, components can be found for which the calibration line passes through zero.

Fig. 6 shows the result of separation of human urine (1 μl) performed using the device with coupled columns. Both narrow-bore tubes were filled with the operational system at pH 6 (Table II). No sample pre-treatment was used. The time needed in the pre-separation tube was *ca.* 11 min and the total time for analysis was *ca.* 19 min. The substances indicated could be determined directly using calibration lines, with the exception of the nicotinate zone for which the "UV spike" method was necessary. Of course, further studies are needed to elucidate the other, unidentified zones.

A major benefit of using the device with coupled columns is its flexibility in the use of different operational systems within an individual run. As discussed extensively in ref. 1, there are many means of influencing the separation parameter in isotachophoresis. It is outside the scope of this paper to consider in too much detail the factors that influence the effective mobility of a given substance. It should be remembered that the effective mobility can be influenced through changes in the concentration of the leading electrolyte, LE (separation according to activity coefficients), the pH of the LE (separation according to dissociation constants), the addition of complexing agents to the LE (separation according to instability con-

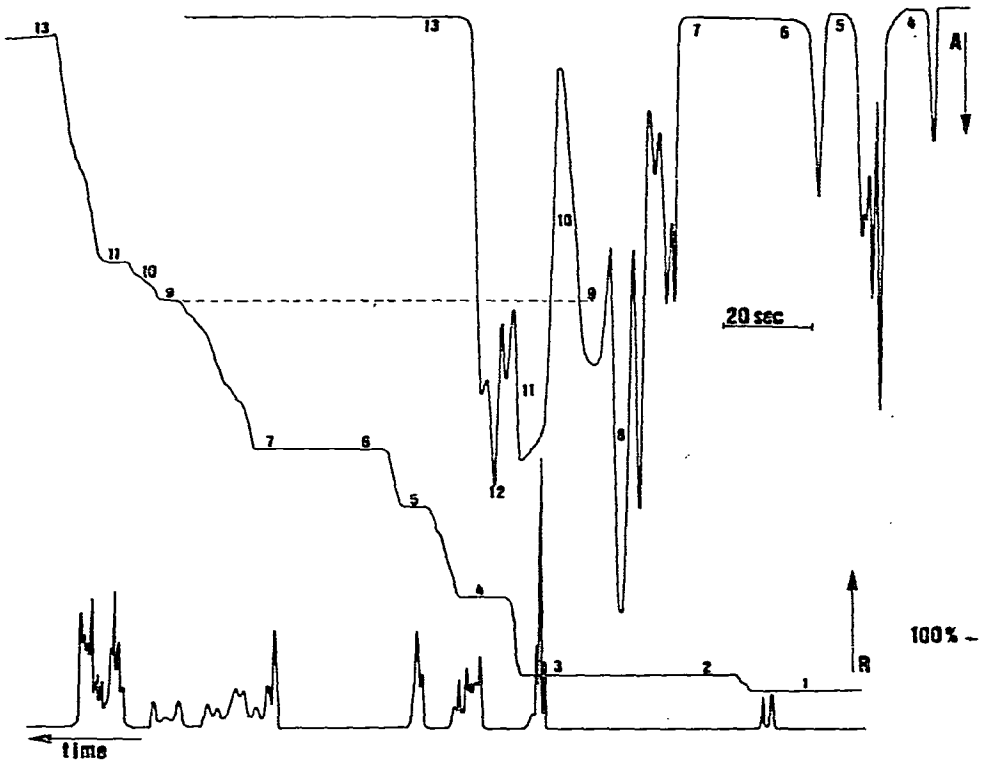

Fig. 6. Isotachophoretic separation of human urine in the operational system at pH 6 (Table II) in both the pre-separation and separation narrow-bore tubes. 1 = Chloride; 2 = sulphate; 3 = nitrate; 4 = citrate; 5 = acetate; 6 = phosphate; 7 = lactate; 8 = ascorbate; 9 = hippurate; 10 = glucuronate; 11 = urate; 12 = nicotinate; 13 = MES.

stants) and the choice of solvent for the LE. In the device with coupled columns, the simplest choice is the use of different concentrations of LE in the pre-separation and final separation narrow-bore tubes. The zones of substances to be separated are elongated not only by the ratios of the inner diameter of the tubes chosen, but also by the different concentrations of the electrolytes in these tubes. Moreover, separation according to activity coefficients is seldom selected. If different concentrations are applied, a higher value for i_2 and a lower value for i_1 can be chosen (Fig. 7; see also Discussion).

An example will be given of the use of two totally different operational systems in the pre-separation and final separation narrow-bore tubes. If this choice is made, first the final separation compartment and then the pre-separation compartment need to be filled with LE. This must be done to prevent the LE in the pre-separation compartment from having no definite composition. We have chosen the separation based on dissociation constants, *i.e.*, two LEs are selected with different pH values. These operational systems are given in Tables II and III. Glutamic acid was used as the terminator. A mixture of phosphate and lactate required analysis in a solution in which formic acid was present in high concentration.

In Fig. 8a a separation is shown of this mixture of anions using conventional

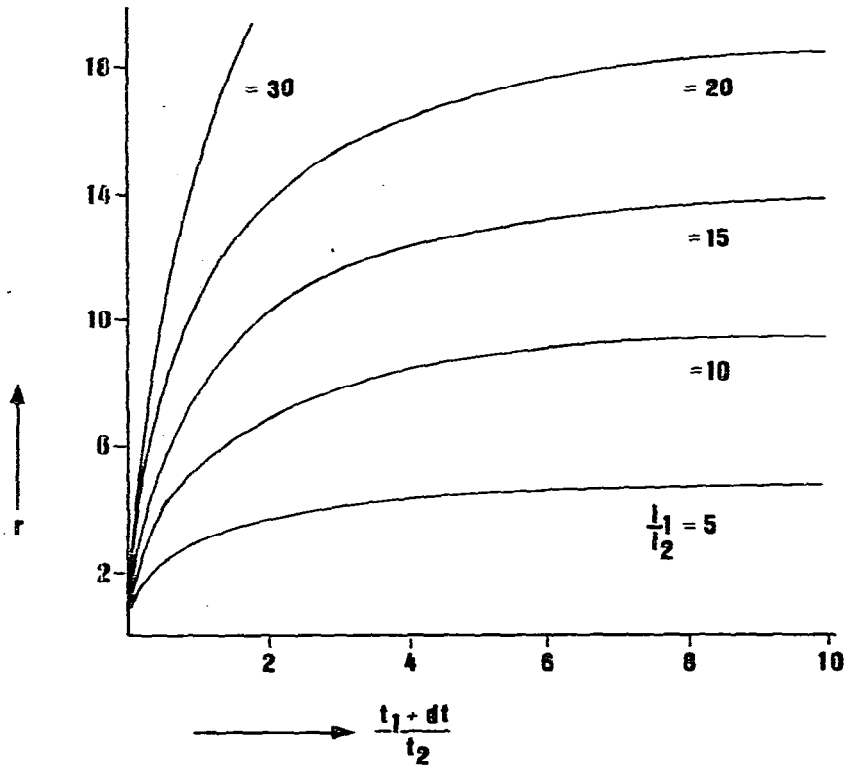

Fig. 7. Ratio of time in the pre-separation narrow-bore tube ($t_1 + dt$) to time in the final separation narrow bore tube (t_2) *versus* the gain factor, r. The ratio $i_1:i_2$ is used as a parameter. The figure shows that for optimization a large ratio of $i_1:i_2$ is desirable while the ratio of $(t_1 + dt):t_2$ should be small. For further explanation see text.

equipment[1]. The LE used is given in Table II. The apparatus comprises a tube (20 cm × 0.2 mm I.D.), equipped with a photometric detector (256 nm, slit 0.1 mm) and a conductivity detector (10-μm Pt–10% Ir electrodes). Phosphate and lactate migrate in the same zone. Neither the conductivity nor the photometric detector indicates the boundary between lactate and phosphate* and therefore no qualitative and quantitative information is obtained. Phosphate and lactate can be easily separated at low pH, *e.g.*, in the operational system at pH 3 (Table III). In the example given, however, the formate ion will have an effective mobility such that

$$m_{\text{eff.phosphate}} > m_{\text{eff.formate}} > m_{\text{eff.lactate}}$$

Although phosphate, formate and lactate can be separated in the operational system at pH 3, the concentration of formate is so large that a mixed zone can be expected with phosphate[2].

Fig. 8b shows separation of the mixture in the device with coupled columns.

* Lactate and phosphate migrate at pH 6 in individual zones, owing to the difference in pH in the sample zone[2].

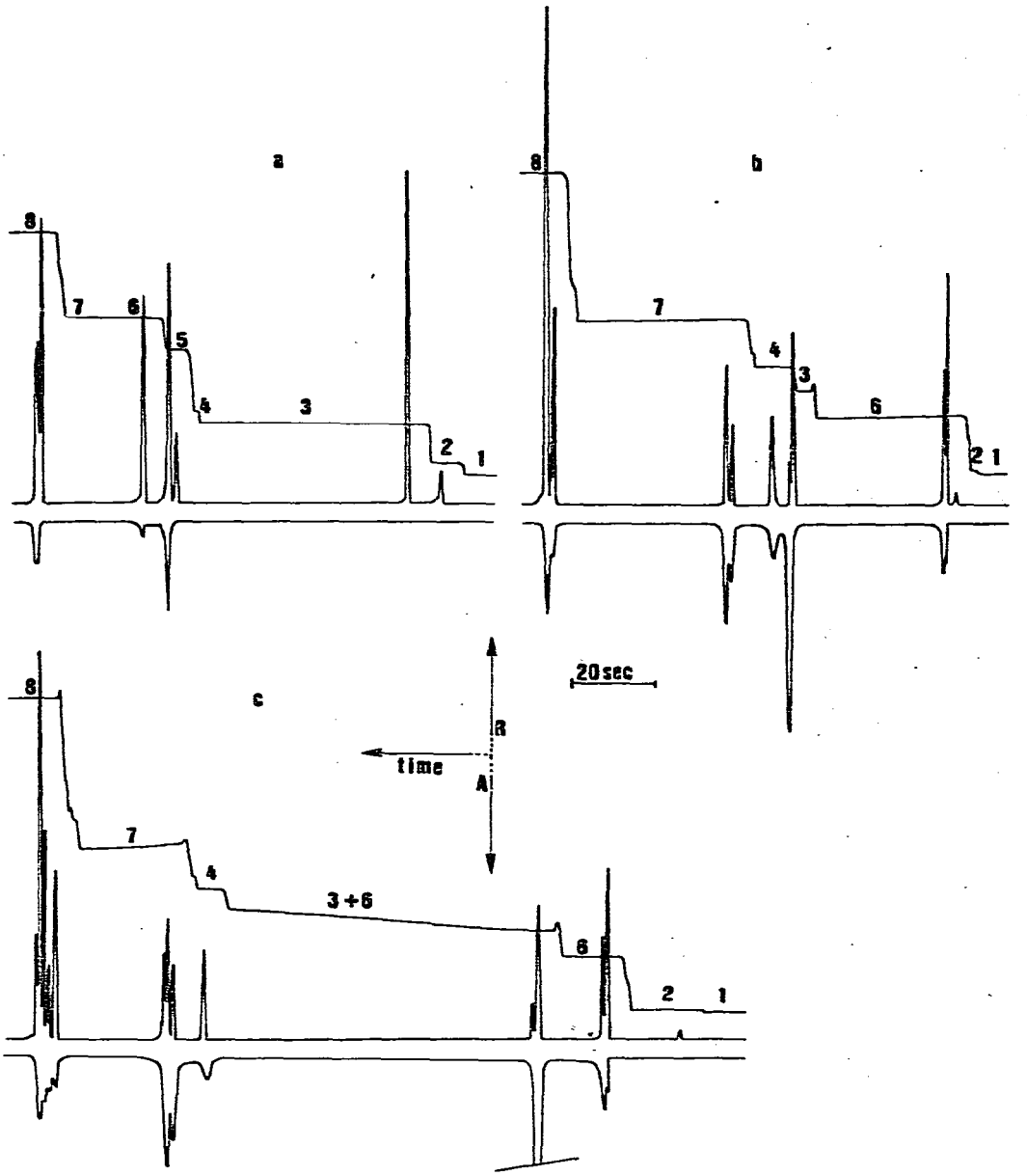

Fig. 8. Separation of a model mixture in the device with coupled columns. Pre-separation compartment filled with the leading electrolyte at pH 6 (Table II), glutamic acid used as terminator. The separation compartment was filled with the leading electrolyte at pH 3 (Table III). (a) Separation using conventional[1] equipment, in which only the operational system is at pH 6 (Table II). (b) Separation in the device with coupled columns, in which the pre-separation compartment is filled with the operational system at pH 6 (Table II) and the final separation narrow-bore tube is filled with the operational system at pH 3 (Table III). The zones of phosphate and lactate (6 and 7) are trapped. Citrate and acetate are used as indicator zones (see text). (c) Separation in the device with coupled columns again making use of both operational systems as listed in the Tables II and III, if all the zones between leading and terminating zone are trapped (non-ideal trapping). 1 = Chloride; 2 = sulphate; 3 = formate; 4 = citrate; 5 = acetate; 6 = phosphate; 7 = lactate; 8 = glutamate. In analyses (b) and (c) three times more sample is injected than in (a).

The pre-separation tube was filled with LE at pH 6 (Table II). The final separation was performed at pH 3 (Table III). To improve trapping of the phosphate–lactate zone, samples of acetate and citrate were added to the mixture. At pH 6 the sequence of the zones is chloride, formate, citrate, acetate, phosphate–lactate, glutamate. At pH 3 the sequence of the zones is chloride, phosphate, formate, citrate, lactate, glutamate, acetate. Again, citrate and acetate are used as indicator zones. As soon as these zones are detected by the conductivity detector in the pre-separation compartment, the time is ascertained at which the system is switched from mode a to mode b (Fig. 1). For complete trapping a long zone of acetate is practical*, especially as this zone is left behind in the terminator at pH 3. Citrate was added to demonstrate that the trapping was complete, as it has a lower effective mobility than acetate at pH 6 and lower than lactate at pH 3. Thus, if citrate is present in the final isotachopherogram, the phosphate–lactate zone must be completely trapped. Of course, for normal analysis no indicator zones are necessary, as can be seen in Fig. 5.

Fig. 8b gives the result of pre-separation, trapping and final separation when three times more mixture was injected than in the analysis shown in Fig. 8a. Full qualitative and quantitative information are obtained for lactate and phosphate. The citrate zone is present and the acetate zone has disappeared. If different operational systems are used, better separations can be achieved, based on instability constants, because it is easier to find a terminator that is not affected in its effective mobility by the ligand used than to find a terminator not affected by a change in pH. In principle, only those substances which are pure and chemically stable and have low mobility and a high pK_a value can be recommended as terminators. An ideal terminator for a separation at pH 6, therefore, has too small an effective mobility at pH 3.

Fig. 8c shows the separation of the same mixture shown in Fig. 8a and b. The zones between the LE and the terminator are all trapped after the pre-separation at pH 6. Zone 2 (sulphate) was used as the indicator zone for trapping. The poor result is simply explained: at pH 6 formate is in front of phosphate, at pH 3 phosphate migrates in front of formate and the time available was insufficient for phosphate to run through the formate zone and to form a pure zone. Therefore, a mixed zone is seen as the steady state has not been reached. These effects must be taken into consideration if combinations of operational systems are contemplated.

DISCUSSION

Column coupling in isotachophoresis widens the field of application, without requiring much more complex equipment construction and handling. Compared with other isotachophoretic equipment, the accuracy in the evaluation of data for qualitative and quantitative purposes is excellent. As with conventional equipment, highly sensitive or selective detectors can be used in the device with coupled columns, because the equipment consists essentially of the conventional equipment while making use of a specially constructed pre-separation compartment. We shall now compare the device using coupled columns with a conventional device, *i.e.*, with a narrow-bore tube (20 cm × 0.2 mm I.D.).

* Switching of the column is not yet automatic.

Assume the electric current in the pre-separation tube is i_1 (during the pre-separation), the time to reach the bore of 0.2 mm in the T-piece is t_1, the time for an unwanted zone to pass this bore is dt, the current in the final separation narrow-bore tube is i_2 (during the final separation) and the time to reach the detector is t_2. Then, the gain in analysis time for a complete isotachophoretic separation of comparable samples can be expressed as

$$\Delta t = (t_1 + dt)(i_1 i_2^{-1} - 1) \quad (1)$$

A practical example with optimal values for use in the device with coupled columns is as follows: $i_1 = 350\ \mu A$; $i_2 = 25\ \mu A$; $t_1 = 6$ min; and $t_2 = 8$ min.

Suppose a sample consists of citrate and ascorbate, as shown in Figs. 2, 3 and 4, and $dt = 3$ min for the citrate zone. We are now able to compare the time for ascorbate analysis (t_A) as obtained from the device with coupled columns (ideal trapping), or the time needed to trap the whole stacked sample between the leading zone and the terminating zone [non-ideal trapping, *i.e.*, $dt_{citrate} = 0$, (t_B)], with the analysis time in conventional equipment using a stabilized electric current of 25 μA throughout the experiment (t_C): $t_A = 17$ min; $t_B = 56$ min; and $t_C = 134$ min.

Using eqn. 1, we obtain three possibilities, A, B and C:

(A) Conventional equipment, compared with ideal trapping ($\Delta t_{C \to A}$). $\Delta t_{C \to A} = 117$ min; $t_1 = 6$ min; and $dt = 3$ min.

(B): Conventional equipment, compared with non-ideal trapping ($\Delta t_{C \to B}$). $\Delta t_{C \to B} = 78$ min; $t_1 = 6$ min; and $dt = 0$ min.

(C) Ideal trapping and non-ideal trapping ($\Delta t_{B \to A}$). $\Delta t_{B \to A} = 39$ min; $t_1 = 0$; and $dt = 3$ min.

Fig. 7 shows the contribution of the ratio $(t_1 + dt):t_2$ in the device with coupled columns as a function of the gain (r), with $i_1:i_2$ as a parameter. For the above sample given, where $t_1 = 6$ min, $t_2 = 8$ min, $dt = 3$ min, $i_1 = 350\ \mu A$ and $i_2 = 25\ \mu A$, $r = 7.88$ (*i.e.*, $t_C:t_A$).

The factor r can be expressed either as gain in analysis time or as gain in potential needed for a given separation.

Fig. 7 shows that a long t_1 is inadvisable and the major benefit must be found in the greater $i_1:i_2$ ratio. The ratio of the inner diameters of the pre-separation and final separation narrow-bore tubes, therefore, plays an important role. For practical purposes the inner diameters of 0.2 and 0.8 mm chosen allow a ratio of approximately 14. The diameter of the final separation narrow-bore tube can be reduced to 0.15 mm and that of the pre-separation compartment can be easily increased. It must be remembered that the electric current during the final separation is much smaller and the effect of zone sharpening in the pre-separation compartment is reduced correspondingly. Because this effect is also a function of the ratio of the mobilities of two isotachophoretic neighbours, the optimal ratio of the diameters of the pre-separation compartment and the final separation compartment is also dictated by the substances to be analysed.

A further benefit of using the device with coupled columns is the increase in maximal load capacity[2] (n_a). For the example given, 11.5 times more sample can be introduced compared with conventional equipment. In other words, although the sensitivity of the detector is not lowered and no sample pre-treatment is used, in

a given analysis time (*i.e.*, comparable) the minimal detectable concentration of a given substance is in fact lowered by a factor of 11.5.

If the concentration ratio of the LEs in the pre-separation and final separation tubes is chosen as 2, this factor would be 23. Thus, column coupling in isotachophoresis can be used for the determination of substances at low concentration levels, even if a high concentration ratio of the sample constituents exists.

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