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ISOTACHOPHORESIS

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SUMMARY

In this paper the main principles of isotachophoretic techniques are dealt with. Some examples of electropherograms, showing both cation and anion analyses, are given. Suggestions are made for the separation in solvents different from water. Various block diagrams of equipments, in which the analyses can be carried out, are shown.

INTRODUCTION

As in chromatography, we distinguish three main principles in electrophoresis.

Zone electrophoresis and related techniques can be compared with elution techniques in chromatography.

Tiselius *free boundary electrophoresis* is a frontal analytical method and was the first to be thoroughly developed.

Isotachophoresis can be compared with a displacement technique and is discussed in this paper.

The instrument discussed here, developed for isotachophoresis analysis, can easily be used to carry out experiments with all three methods mentioned above.

Compared with the nomenclature used in chromatography for various techniques, in electrophoresis different names are used for the same techniques.

EXPERIMENTAL AND RESULTS

For simplicity, a schematic diagram of the electrophoretic instrument is given first (Fig. 1). Basically, the analytical electrophoretic equipment consists of a thin-walled narrow-hole tube, made of Teflon, glass or a common plastics material.

The experimentally determined optimal internal diameter is 0.4–0.6 mm and outside diameter 0.65–0.85 mm. The diameter is determined by the concentration of the electrolytes chosen, the current used, the temperature of the medium surrounding the capillary and other minor factors. The length of the narrow-hole tube is determined by the separation problem itself. The pair of ions most difficult to separate determines the length to be chosen.

When 10 cm is adequate for the separation of proteins, a length of 0.5–1 m is

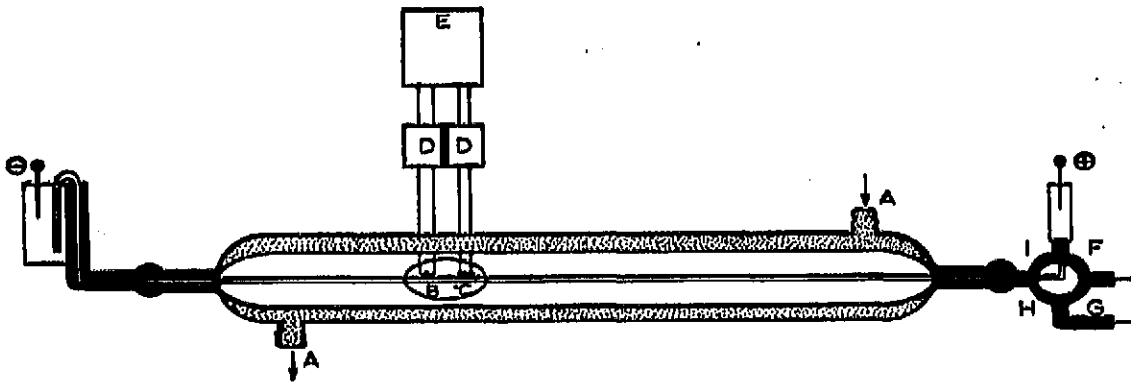

Fig. 1. Schematic diagram of the isotachophoretic equipment. A = thermostated water; B = integral thermocouple; C = differential thermocouple; D = amplifiers; E = recorder; FGHI = sample tap (HG for rinsing and refilling the capillary; FG for sample introduction; FI for filling the anode compartment; HI for running).

needed for the separation of weak acids or metals, without the use of a counterflow of electrolyte[1] and with components which differ by about 4% in mobility[2]. Also, the time for analysis depends both on the separation problem itself and on the type of detector used.

The development of new detectors makes the separation of components with smaller differences in mobility possible. When thermometric methods for detection need a zone length of about 1.5 cm, direct measurements of conductivity with specially constructed electrodes resolve zones with a length of 0.1 mm or even less.

The narrow-hole tube is connected to two reservoirs, the cathode and anode compartments. Fig. 1 shows a four-way tap between the anode compartment and the capillary tube. This tap is used as a sample tap if a cation analysis has to be carried out.

If an isotachophoretic analysis of anions is required, the following steps must be carried out.

(1) The anode compartment and capillary must be filled with an electrolyte that consists of an anion which is very mobile and a cation with buffering capacity. This electrolyte is the so-called leading electrolyte. The effective mobility of the anion in the leading electrolyte must be greater than any in the sample.

(2) The cathode compartment must contain an electrolyte with an anion with an effective mobility lower than that of any other anion in the mixture to be separated. This electrolyte is the so-called terminating electrolyte, and the anion is called the terminator.

The pH of the terminating electrolyte must be somewhat lower than the pH of the leading electrolyte, to prevent an extra contribution to the conductivity of the hydroxyl ions. The pK-value of the anion used as the terminator must be greater than the pK values of the anions in the sample and leading electrolyte. This prevents the occurrence of stable mixed zones and also prevents loss of material[3].

(3) The sample is brought between the leading electrolyte and the terminating electrolyte in the sample tap.

(4) A voltage may then be applied over the cathode and anode compartment. An electric current will pass through the system, and due to this electric field all ions will move to their corresponding electrodes.

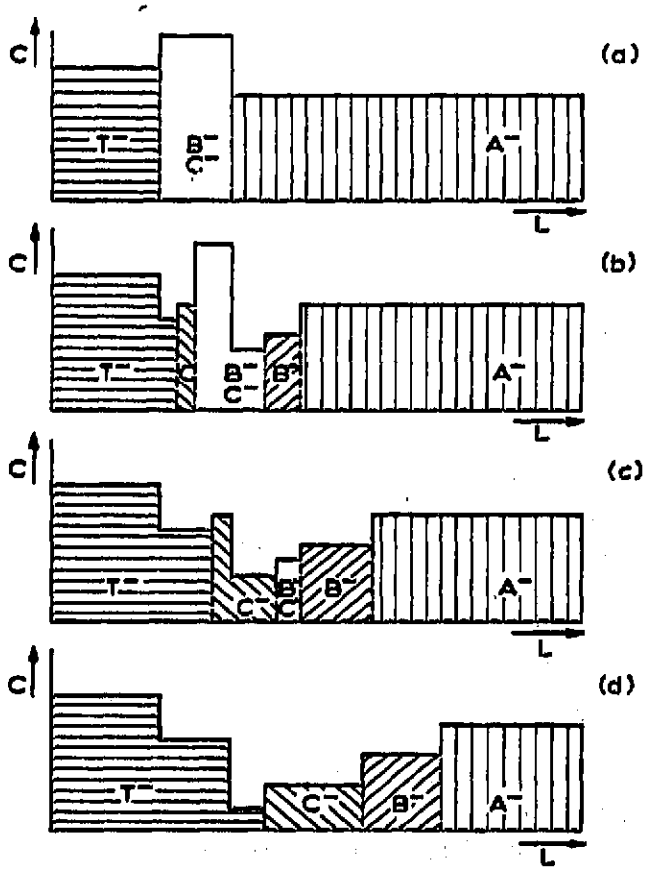

Fig. 2. Isotachophoretic separation as a function of the time for analysis. C = the concentration of the ionic part of the components considered in ion-equiv./l. L = the length of the capillary. For further explanation see text.

The electroendosmosis inside the narrow-bore tube, due to the ζ-potential of the wall, can be neglected.

The hydrodynamic flow is prevented by a membrane[4], mentioned later. A polymer or another supporting material need not be used if the separation of small ions is required, owing to the selfstabilization power of the narrow-hole tube.

For preparative analyses, a series of capillaries, mounted in parallel, can be used if no stabilizing medium is wanted. Fig. 2a shows the conditions inside the instrument in the original position. The leading electrolyte (A), the sample (BC) and the terminating electrolyte (T) can be recognized.

All concentrations are chosen to be different, as is normal at the start of an isotachophoretic analysis.

On the vertical axis, the concentration is given in g-ion/l. On the horizontal axis, the position inside the narrow-hole tube is shown.

All the diagrams in Fig. 2 together show the separation of the ions at characteristic intervals and the adjustment of the concentration of the zones to the original leading-electrolyte zone according to the Kohlraush principle[5].

Fig. 2b shows the leading-ion zone moved somewhat into the capillary. The concentration of B has already been adjusted to the concentration of the leading zone, according to the Kohlrausch regulation functions mentioned above. The concentration of B in this zone can be calculated if the concentration of the leading zone,

all mobilities and the pK values of the leading zone and zone B are known[6]. Towards the rear, a mixed zone of B and C, also adjusted to the zone in front of it, can be seen. The zones behind the sample are adjusted to the concentration of the sample itself.

Fig. 2c shows the zone of pure B enlarged. A zone of pure C, adjusted to the concentration of the mixed zone in front of it, has already been formed. This phenomenon will be shown in an actual example in Fig. 5.

In Fig. 2d, the steady-state, as finally reached, is shown. All zones inside the capillary tube consist of the ion species itself and are adjusted to the concentration of the leading electrolyte. The length of the zones will not change further if the capillary tube is of constant bore and the leading electrolyte is of constant composition.

Minor factors which will possibly disturb the zones somewhat are: pH disturbances, due to membranes or electrode reactions; temperature differences, due to the construction of the equipment used; penetration of, for instance, carbon dioxide through the Teflon wall, especially when high pHs of the leading electrolyte are applied; and electroendosmosis and hydrodynamic flow, especially when counterflow is used.

If the current chosen is constant, all zones will move with the same speed if the steady state has been reached. For this reason, we have chosen the name *Isotachophoresis*[7], which, freely translated from the classical Greek, means electrophoresis in which all zones move with the same speed. The concentration levels in the terminating electrolyte are theoretically moving only by diffusion if ideal conditions are chosen.

We can say that the separation of a sample during an isotachophoretic analysis is a free-moving boundary technique. In chromatographic terms, this means a frontal

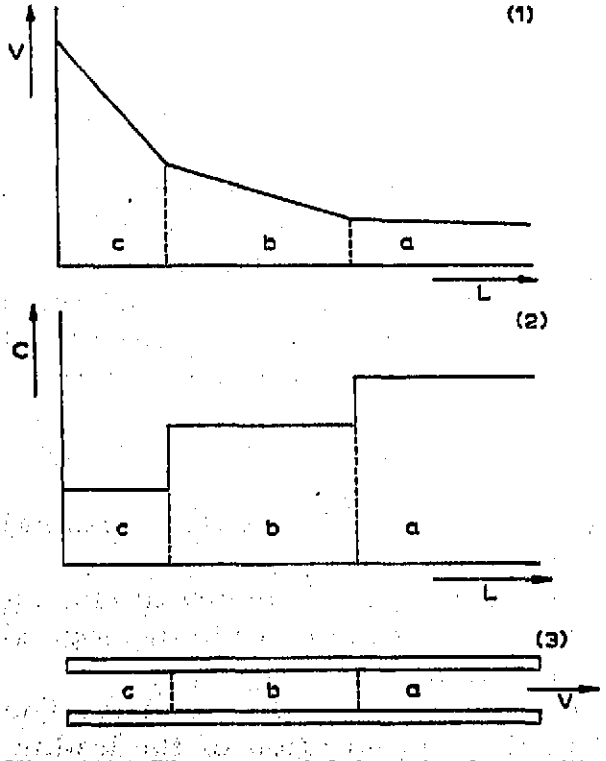

Fig. 3. (1) The potential drop in the capillary tube. (2) The corresponding ionic concentration of the compounds. (3) The zones inside the capillary tube. a = leading zone; b = intermediate zone; c = terminating zone.

analytical method with, as a result, the steady state. The zones move, in the steady state, closely together because of the electroneutrality principle: where the counterions move to their corresponding electrodes, they must be compensated electrically.

Because of the equality of speed of all zones, each zone must achieve its particular potential gradient by the difference in the effective mobility of the ion species considered. From the front towards the tail, the potential gradient is constant per zone, but increases from one zone to the other. All zones are ordered according to their effective mobility. The increment in potential gradient automatically involves a decrease in the ionic concentration of the compounds present in each zone (Fig. 3). This change in concentration can, as mentioned earlier, be calculated.

The zone boundaries in this technique are remarkably sharp, as is normal also in displacement techniques in chromatography.

If an ion *b* penetrates the zone of *a*, the potential gradient in this zone is smaller than the potential drop in the zone of *b* itself. This means that the *b* ion will move more slowly than the main velocity of the zones, and it will therefore come automatically back in the correct zone.

From the other side, if the ion *a* diffuses into the zone of *b*, it will be rejected by the higher potential gradient present in the zone of *b*. There exists a possible loss of material as discussed in ref. 3.

If the electric current is kept constant during the analysis, the difference in potential gradient means that each zone will also have a characteristic temperature. By mounting a thin-wired thermocouple or thermistor around the capillary[8], the signals derived characterize the zones. By differentiating the signals both electronically or by a differential thermocouple[8], the zone boundaries can be determined very easily.

Although an isotachopherogram looks similar to a normal gas–liquid chromatogram, the interpretation is quite different. Where the step heights give all the information about the ion species (qualitative information), the distances between successive peaks represent the zone lengths. They also give quantitative information about the ions present in the corresponding zone. The shape of the zones as finally detected depends greatly on the detector used.

Fig. 4 shows the zone boundary. The sharp curve shows the concentration profile, which can be deduced theoretically[8]. Direct measurements of the conductivity inside the capillary, with specially constructed electrodes, prove that the zones are even sharper than expected. Sometimes, however, such as in zones of HCO_3^-, other physical phenomena may disturb the sharpness. In this special case, the formation of small bubbles of carbon dioxide, which dissolve again very slowly, has been noticed. Also, the formation of clusters and even small crystals can be harmful.

The other two curves in Fig. 4 are the experimental and theoretical temperature profiles. The experimental profile was recorded with a copper–constantan thermocouple mounted around the capillary tube.

It is clear from Fig. 4 that the thermometric detection of the zones, although it is a universal and simple method, needs to be replaced by a method using a detector with a higher resolving power. Nevertheless, with thermometric detection, the systems could be studied if not too small amounts are present in the sample. The components must be present in the order of micrograms if thermometric detection is to be applicable.

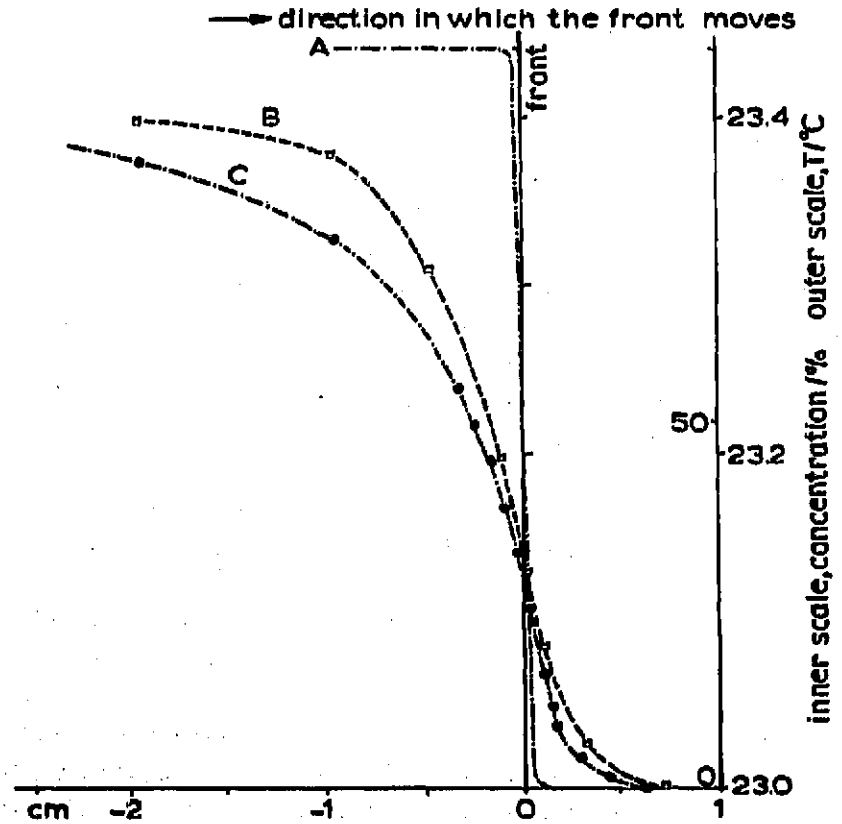

Fig. 4. The zone boundaries. A = theoretical concentration profile; B = practical profile, as registered by a thermocouple; C = theoretical temperature profile.

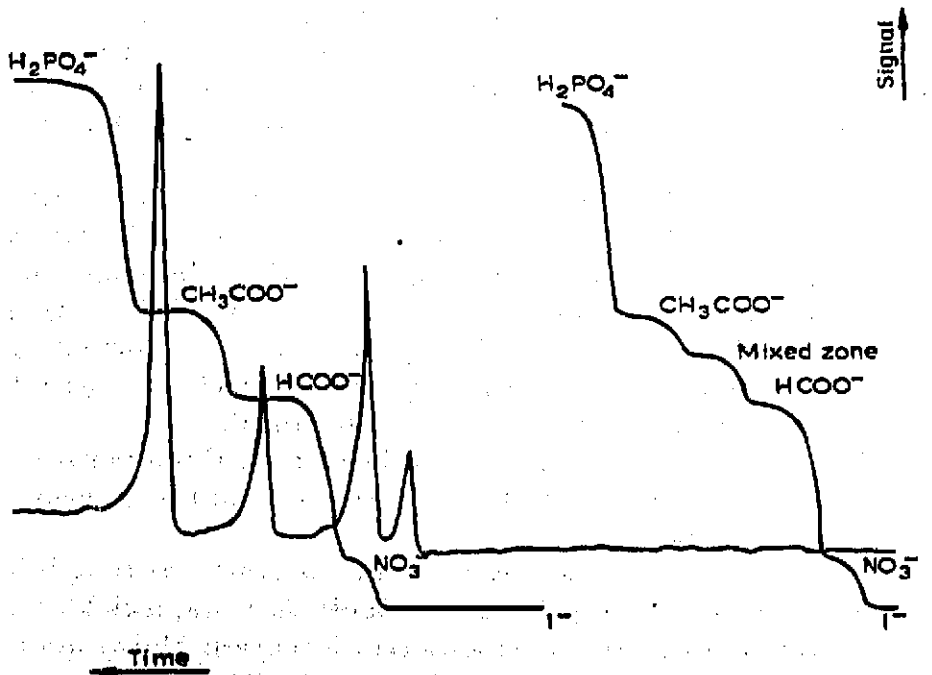

Fig. 5. Isotachophoretic separation of nitrate, formate and acetate. The recording on the right-hand side is made after 30 cm of capillary tube. The recording on the left-hand side is made after 50 cm of capillary tube. The latter isotachopherogram does not show the mixed zone.

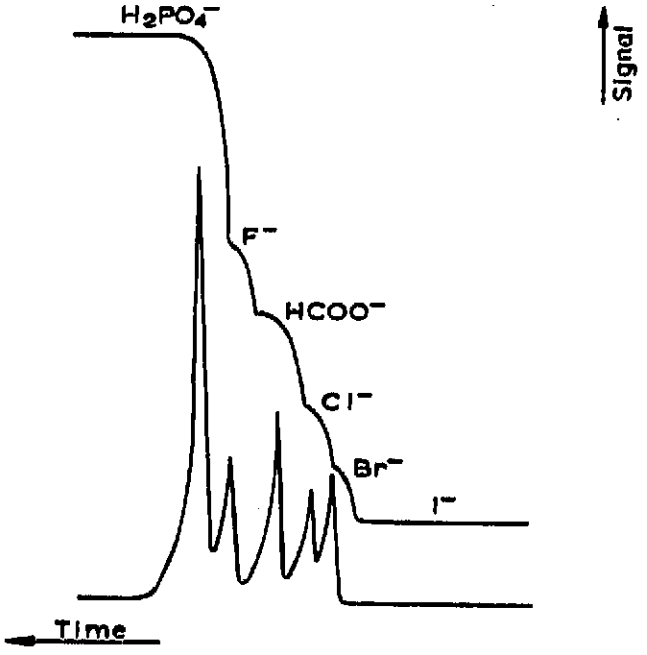

Fig. 6. Isotachophoretic separation of the halides. Formic acid is added as a reference.

Electropherograms, as finally obtained, are shown in Figs. 5 and 6. Fig. 5 shows an electropherogram of the separation of nitrate, formate and acetate. As the leading electrolyte, Tris–iodide was chosen. The terminator was the phosphate ion. Detection was carried out using thermocouples (15 μm copper–constantan wire). The thermocouples were mounted at a distance of 30 cm and 50 cm, respectively, from the injection point. The total time for analysis was 45 min, the current being stabilized at 70 μA

Fig. 5 shows clearly that the mixed zone, as detected after 30 cm, disappeared after a further 20 cm of capillary tube.

In Fig. 6, the separation of the halides is shown. Formic acid is added as a

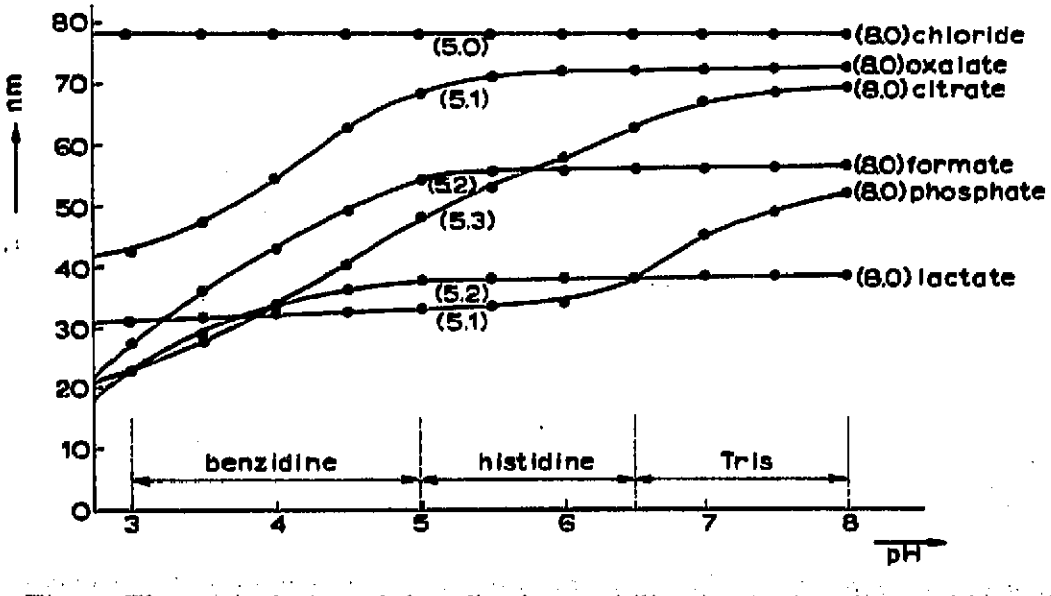

Fig. 7. Theoretical plot of the effective mobility (nm) of various acids against the pH of the leading electrolyte chosen. The calculations were made with a GE 265 computer.

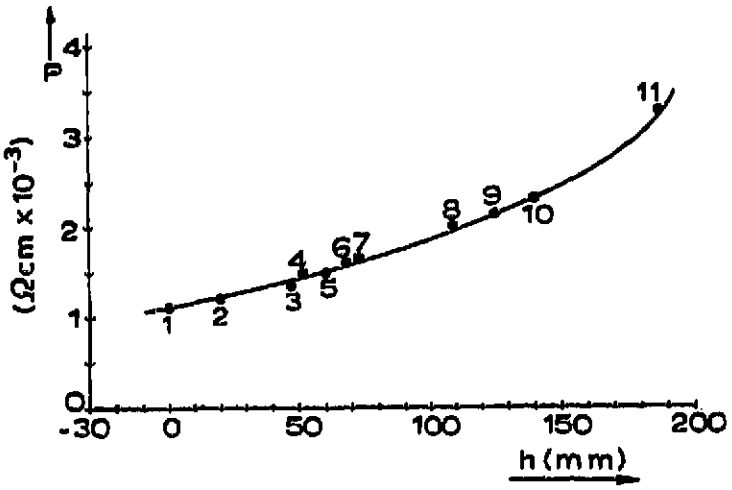

Fig. 8. Plot of the calculated conductivities of various zones against the experimental step heights found from the corresponding electropherograms. 1 = chloride; 2 = oxalate; 3 = tartrate; 4 = formate; 5 = citrate; 6 = succinate; 7 = malonate; 8 = acetate; 9 = α-hydroxybutyrate; 10 = phosphate; 11 = carbonate.

reference. If one step height is known, the ion-species can be determined from the other step heights. Sometimes, however, the difference in effective mobility is too small for a complete separation in a 1-m capillary tube. If no counterflow equipment is available, another buffer system can be chosen.

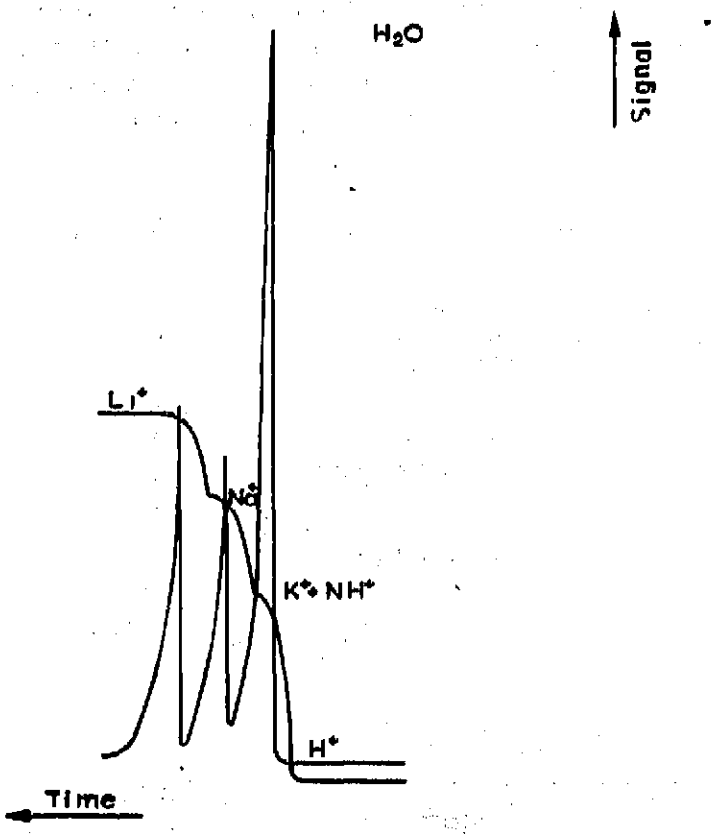

Fig. 9. Isotachopherogram of the separation of potassium, ammonium and sodium. The solvent was water. The lithium ion was chosen as the terminating ion. The hydrogen ion was used as the leading ion.

Fig. 7 shows the net mobility of some acids as a function of the pH of the leading electrolyte. If the net mobility of the ions is plotted against the actual pH of the zone, sharper curves are obtained. The values in parentheses give the pHs of the zones.

At neutral pH, phosphate will move in front of lactate. At this pH these two acids can easily be separated. At pH 6.3, the lactate and phosphate have the same effective mobility and cannot be separated. Between pH 4 and pH 6, lactate moves in front of phosphate. At pH 4 stable mixed zones of phosphate and lactate may be expected. Below pH 4, phosphate will again move in front of lactate. If the pH in anion analyses does not rise from the front towards the tail, some artifacts can exist[a].

In Fig. 8, the step heights, found experimentally from the integral curve of the isotachopherogram, are plotted against the conductivity of the zones, determined theoretically. In this curve, no correction was made for the Onsager relation and no temperature correction was applied. Measuring the mobility from such a curve means that one will obtain the effective mobility. The accuracy of such a mobility determination is about 2%.

Fig. 9 shows an isotachopherogram of the separation of some cations. In this mixture, potassium and ammonium are present. The physical properties of these ions appear to be so similar that a separation would be impossible, although a certain enrichment can be obtained. Changing the pH of the solution will not give much success, since the K values are so similar. This is only an example, and of course

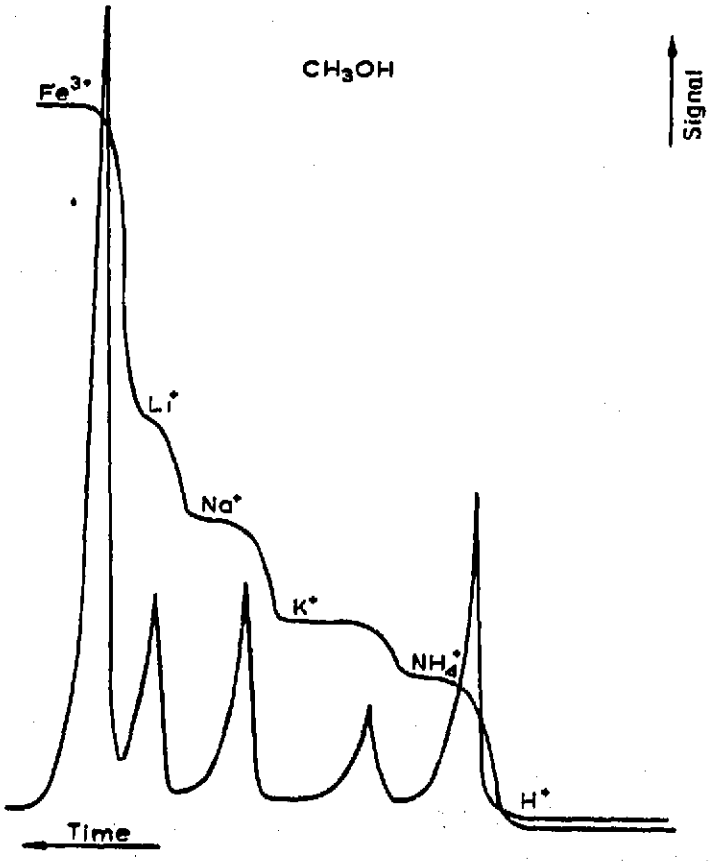

Fig. 10. Isotachopherogram of the separation of ammonium, potassium, sodium and lithium. Methanol was used as the solvent. The Fe^{3+} ion was used as the terminating ion. The H^+ ion was chosen as the leading ion.

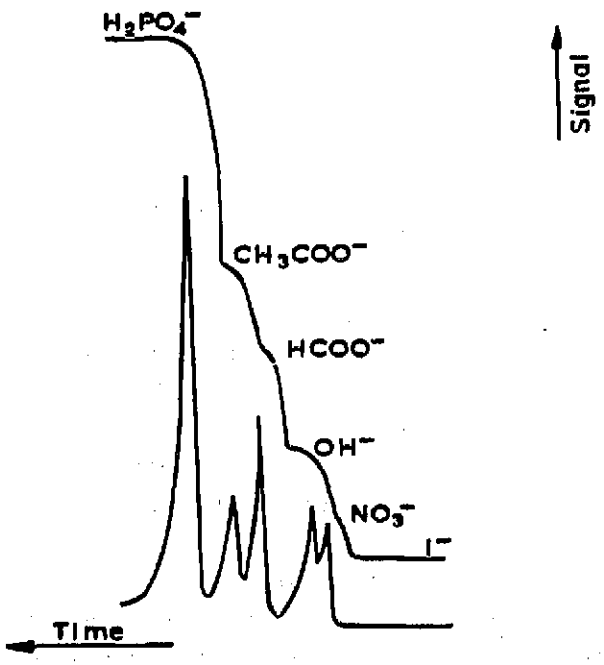

Fig. 11. Isotachopherogram of some anions. Methanol was chosen as the solvent. By the difference in solvatation, the OH^- ion is no longer the fastest ion.

others could be given. To make a complete separation possible another parameter must be changed, for instance, another solvent must be looked for. In this case, methanol is suitable.

The electropherogram shown in Fig. 10 shows a remarkable difference in the behaviour of potassium and ammonium[9]. Looking for the dependence of conductivity on concentration, it can be seen that the behaviour of potassium and ammonium ions in methanol is different from their behaviour in water. The most important influence is the solvatation of the ions by the solvent. The influence of the dielectric constant

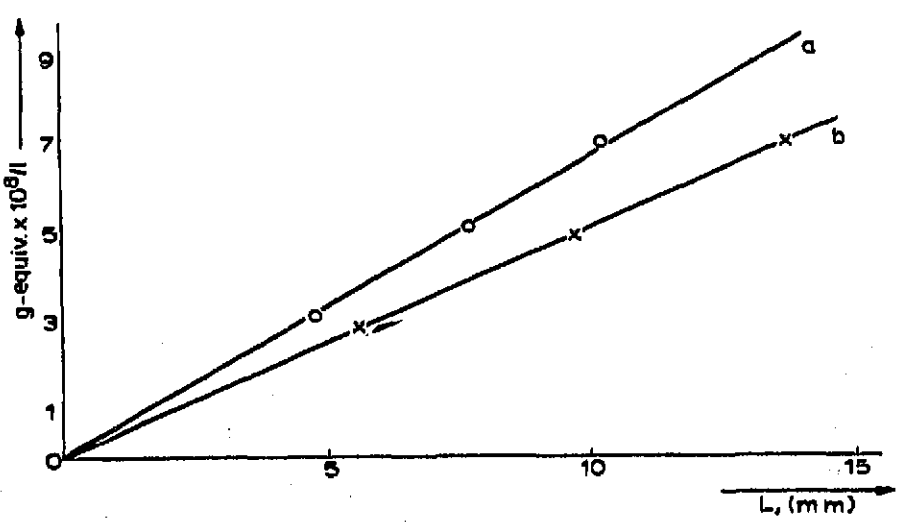

Fig. 12. Plot of the concentration of an ion species, introduced into the system, and the length found between two successive peaks in the isotachopherogram. This plot can be calculated and depends on the concentration of the leading electrolyte and the diameter of the capillary tube. a = oxalate; b = adipate.

and the dipole moment of the solvent, however, must never be neglected. Separations of metal ions can be carried out much easier in methanol than in water.

The electropherogram presented in Fig. 11 is given as a curiosity. Whereas in water the hydroxyl ion is very mobile, in methanol it is an ion moving in between the nitrate and the formate zone.

As stated above, in the steady state all zones move with the same speed. The concentrations of the zones are adjusted to the leading electrolyte zone, according to the Kohlrausch regulation functions.

If more of an ion species is introduced into the system, the distance between two successive peaks will grow; because the zone length increases with the introduction of more ionic material. Again, opposite to the case of elution gas chromatography, the distance between two successive peaks gives information about the amount of a component introduced into the system[19]. The qualitative information is obtained from the step heights, measured from the integral curves.

In Fig. 12 the relation between the concentration of an ion species in a sample

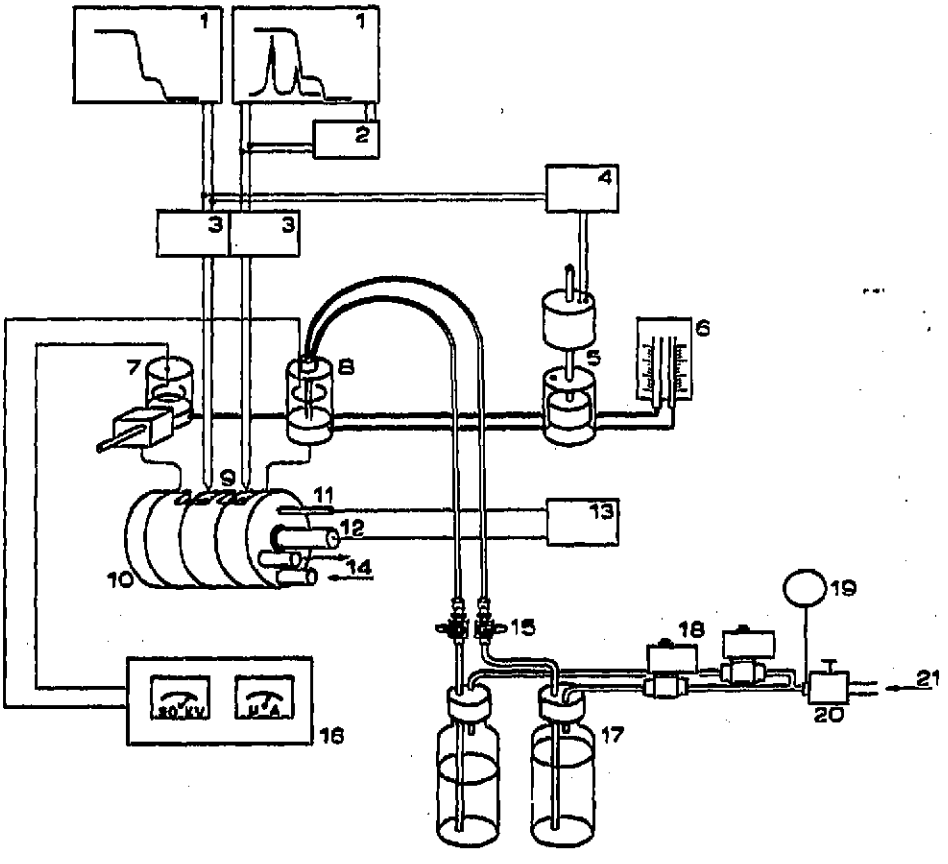

Fig. 13. Schematic diagram of the equipment used. 1 = recorders; 2 = electronic differentiator; 3 = amplifiers; 4,5 = regulator for experiments with counterflow of electrolyte; 6 = level control, if experiments with counterflow of electrolyte are carried out; 7 = injection block; 8 = counter-electrode; 9 = detector made of 15-μm copper–constantan wire; 10 = aluminium block with the capillary mounted on it in the form of a helix; 11 = platinum sensor, used for thermostating the aluminium block; 12 = electrical resistor used in the regulation of the temperature of the aluminium block; 13 = regulator for thermostating the aluminium block; 14 = prethermostated water for cooling the aluminium block; 15 = Teflon-lined Hamilton valves, used for disconnecting the narrow-bore tube from the electrolyte reservoirs and reservoirs filled with the water for rinsing; 17 = reservoir filled with leading electrolyte and water for rinsing the capillary tube; 18 = magnetic valves; 19 = manometer; 20 = pressure regulator; 21 = air pressure (2 atm).

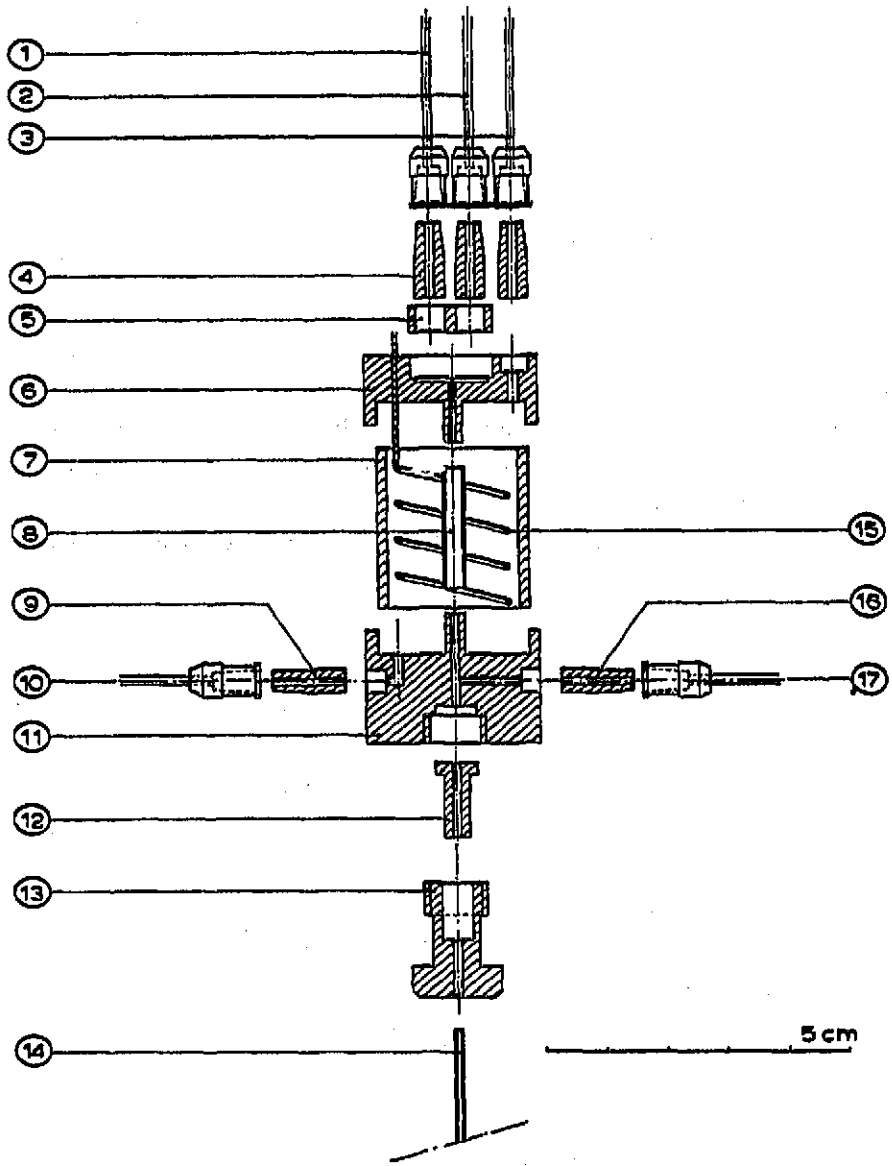

Fig. 14. Counter-electrode, containing the semi-permeable membrane. 1 = Teflon tube, connected with water reservoir; 2 = Teflon tube, connected with electrolyte reservoir; 3 = Teflon tube, connected with drain; 4 and 5 = pieces of Perspex used for connection; 6 = cover of the electrode compartment; 7 = electrode compartment; 8 = membrane; 9 = piece of Perspex used for connection; 10 = Teflon tube, connected with the electrolyte reservoir; 11 = bottom of the electrode compartment; 12 = piece of Perspex, used for fitting the capillary tube; 13 = bolt for fitting the piece 12 and the capillary tube; 14 = capillary tube; 15 = platinum electrode; 16 = piece of Perspex used for connection; 17 = Teflon tube, connected with the electrolyte reservoir of the counterflow equipment.

and the distance, found between two successive peaks in the isotachopherogram, is given. The influence of the terminator chosen is negligible. The injection was made by a sample tap, and thus a constant volume of sample was taken. Straight lines are obtained, if not too much of an ion species is introduced or if the capillary takes long enough to reach the steady state. These lines can also be calculated.

APPARATUS

A schematic diagram of the equipment used in this work is given in Fig. 13. The capillary has a length of about 1 m, an inside diameter of 0.45 mm and an outside diameter of 0.7 mm. It is made of Teflon and manufactured by HABIA (Sweden). This capillary is wound around an aluminium block in the form of a helix, pressed in a groove made in the block. A heat-sink compound is used for good thermal contact. Special compartments are made for the thermal detectors, and the capillary is adapted for electrode reservoirs on both ends. One of the electrode compartments consists of a semi-permeable membrane, to prevent hydrodynamic flow due to the difference in level between the two electrode compartments, and to reduce the electroendosmosis to negligible proportions. These electrode compartments are shown in Figs. 14 and 15.

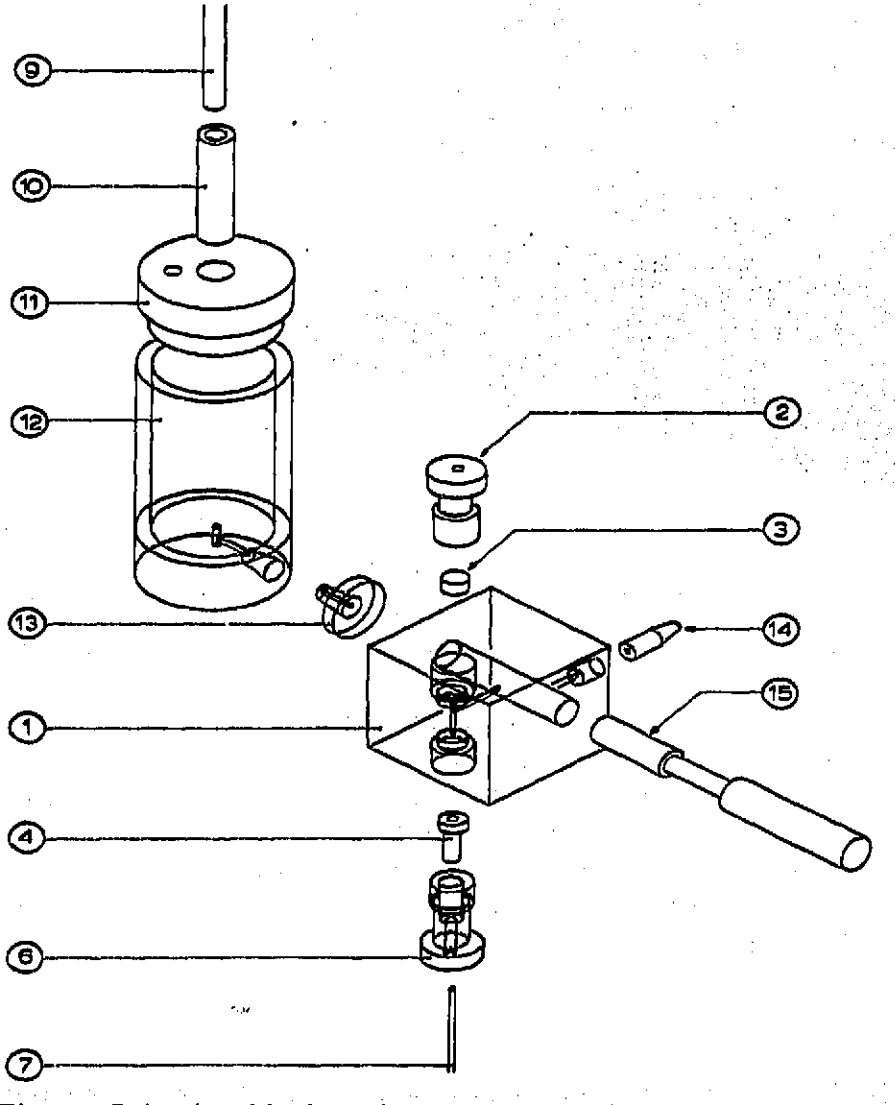

Fig. 15. Injection block and compartment for the terminating electrolyte. 1 = injection block; 2 = bolt for fitting septum; 3 = septum; 4 = piece of Perspex for fitting the capillary tube; 6 = bolt for fitting piece 4 and capillary tube; 7 = capillary tube; 9 = high-tension cable; 10 = piece of Perspex for mounting high-tension cable; 11 = cover of electrode compartment; 12 = electrode compartment; 13 = connection of electrode compartment with plunger compartment; 14 = connection towards drain; 15 = Teflon-covered plunger.

A pressure system is used for refilling and rinsing the system. More details are given in the literature[4].

The most important part of the isotachophoretic equipment is shown in Fig. 16. Instead of one capillary as before, three capillaries are mounted in parallel (Fig. 16c). Mechanically they are separated from each other. Fig. 16a shows the injection block and Fig. 16b shows the electrode compartment with the semi-permeable membrane.

A set of thermocouples is mounted below and used as detectors. The current is

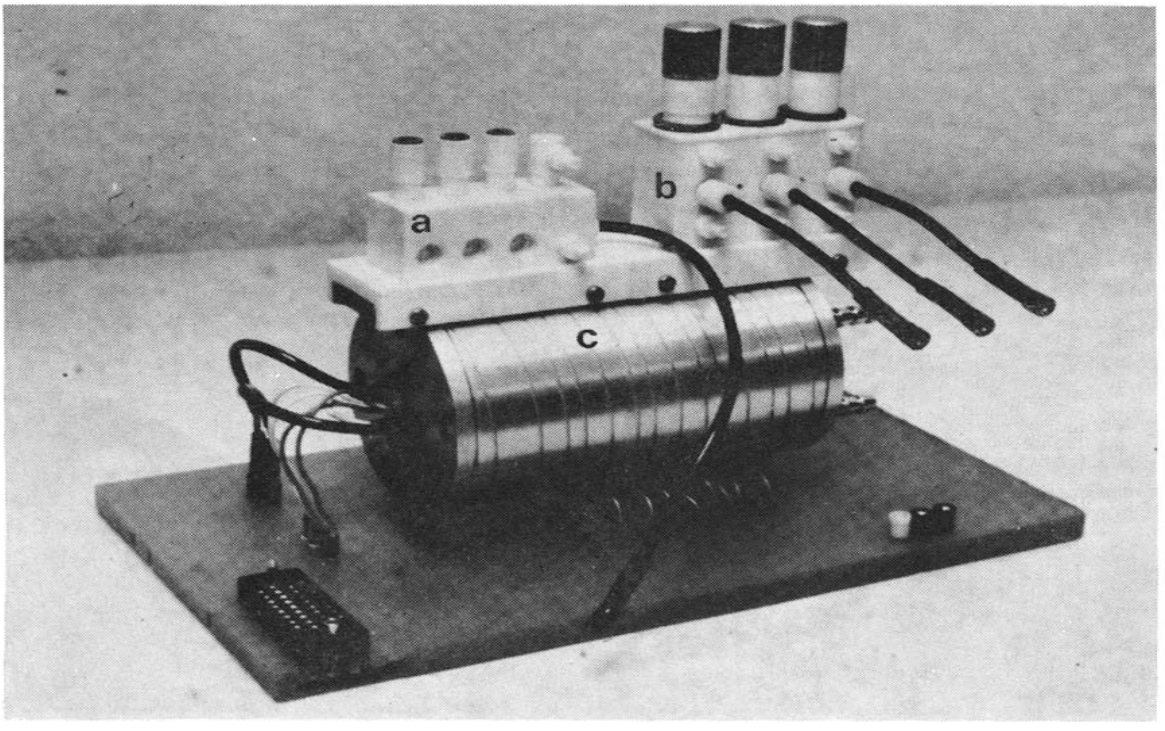

Fig. 16. The separation part of an isotachophoretic equipment. With the equipment shown, three analyses can be carried out simultaneously. a = injection block; b = electrode compartments with the semi-permeable membranes; c = aluminium block surrounded by the Teflon capillaries. The thermocouples, used as the detectors, are mounted at the bottom of the aluminium block.

kept constant in each capillary with the aid of a constant voltage supply in series with photoresistors[11]. The voltage applied to the capillary tube is regulated by a lamp mounted above the photoresistors. The amount of light is regulated by a thermocouple mounted on the capillary. The use of several types of flat membranes and arnite material (a low-temperature polymer of terephthalic acid) instead of perspex allows non-aqueous solvents to be used. The platinum resistor and loads, used for the temperature regulation of the aluminium block, can be seen on the left.

DISCUSSION

In this paper, the method of isotachophoresis is discussed and some possible equipment is shown. However, this paper is far from a complete survey of the work carried out in this field.

With this method, (weak) acids and metals can easily be analysed, and also (weak) bases.

Computer programs for the calculation of concentrations and effective mobilities are already available.

Great attention has been paid to the separation of amino acids and proteins. Although here analyses can also be carried out, the reproducibility is still poor and some phenomena have not been explained. The development of the new detectors will give new possibilities in this field.

The possibilities of using, in these closed systems, liquids other than water imply that the separation of ionic components, insoluble in water, can be achieved.

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