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ISOTACHOPHORESIS IN NARROW-BORE TUBES

INFLUENCE OF THE DIAMETER OF THE SEPARATION COMPARTMENT

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SUMMARY

The importance of the inside diameter of the separation compartment of electrophoretic equipment in which tubes are used in achieving optimal stabilization against convective disturbances and for limiting the temperature increase is demonstrated. Both from a theoretical point of view and experimentally, smaller inside diameters than are commonly used are recommended. Results obtained with thermometric, conductivity and UV absorption detectors in narrow-bore tubes with inside diameters of *ca.* 0.2 mm are compared.

INTRODUCTION

In electrophoretic experiments, optimal stabilization against convective disturbances is nearly always desired. This stabilization can be achieved by, amongst other methods, decreasing the ratio of the cross-section of the separation compartment to its surface area, assuming that other disturbing phenomena can be well controlled.

Obviously, circular cross-sections, because of their high symmetry, are preferable to rectangular cross-sections. This is especially important when dealing with quantitative aspects. Annular cross-sections have limited applicability from a practical point of view and we shall therefore confine ourselves to circular cross-sections only.

The dimensions of the narrow-bore tubes are determined mainly by the material of which they are constructed and the detection systems applied. So far these dimensions have hardly been discussed in the literature. At the time the first reproducible isotachophoretic experiments were carried out in our laboratory¹ the only suitable material available was Pyrex glass. The optimal diameters, which are still applied in almost all instruments²⁻⁵, were found to be I.D. 0.4-0.6 mm and O.D. 0.7-1.0 mm. No semi-permeable membrane was used. In order to prevent a hydrodynamic flow between the electrode compartments, the viscosity of the electrolyte in the separation compartment had to be increased.

A small diameter of the narrow-bore tube therefore necessitates a high pressure

for rinsing and refilling the separation compartment. Moreover, the electroendosmotic flow at low (centimolar) concentrations was found to be far from negligible when using glass tubes with inside diameters smaller than 0.4 mm. If the concentration of the electrolytes is increased, relatively high current densities can be applied and the electroendosmotic flow can be neglected, even in narrow-bore tubes with a very small inside diameter⁶. The diameter of the narrow-bore tube can thus be decreased without the necessity for taking special precautions except as regards the pressure for rinsing and refilling the separation compartment. From an analytical point of view, however, high concentrations of the electrolytes are less important, because the absolute amounts of the sample compounds increase and many components are no longer soluble.

In early work only a thermocouple was used as the detector, the resolution and response of which is determined mainly by the wall thickness⁷ and the thermal conductivity of the material of which the narrow-bore tube is made. The use of Pyrex glass narrow-bore tubes made it impossible to decrease the thickness of the wall significantly because thin-walled tubes are very fragile. The ratio of the cross-section to the surface area also could not be decreased significantly, because for a full qualitative and quantitative analysis using a thermometric detector scarcely any signal remains at the current density chosen.

In order to permit a better understanding of the resolution, response and sharpness of a zone boundary in isotachophoretic analyses, we shall first summarize a few basic features. In isotachophoretic experiments, consecutive zones all have a characteristic temperature, potential gradient, pH and conductivity, once the steady state has been reached. Apart from the so-called transition boundary between the zones, in which two ions of consecutive zones are mixed, we always have to cope with the profiles of the zone boundaries. The transition boundary is determined mainly by the operational parameters such as the difference in the effective mobilities of the constituents involved. Convective disturbances may increase this transition boundary. The profiles of the zone boundaries are due mainly to the temperature differences between the centre of the tube and the wall of the tube and between the zones mutually. The viscosity of the electrolyte⁸ and the additive used to increase this viscosity also play a role. In specific cases, the contributions of the electroendosmotic flow, adsorption on the wall and diffusion into the wall on the profile cannot be neglected. Suppose the zone boundary has an "infinite" sharpness, *i.e.*, the transition boundary can be neglected. The final registration may be poor, however, because the resolution of the detector is low, the response of the detector is low (*e.g.*, thermal *versus* conductivity detection) or the profile of the zone boundary is large. The various profiles (concentration, temperature and pH) need not be identical, because they are affected by different parameters.

A decrease in the inside diameter, at a constant current density, will decrease the temperature difference between the centre of the tube and the wall, and the temperature profile will thus also decrease. Moreover, the absolute increase in temperature is smaller, which reduces the convective disturbances. The resolution therefore increases, assuming that the response is sufficiently high. The addition of non-ionic soluble polymers reduces the convective disturbances, which are caused mainly by the difference in temperature. By this means an increase in resolution is obtained, again assuming that the response is sufficiently high. The addition of surface-

active compounds decreases the electroendosmotic flow, if any, and therefore also increases the resolution. Surface-active compounds may also improve the quality of the material of the instrument that is in direct contact with the electrolytes. By this means, adsorption on the wall and/or diffusion into the wall is reduced, which increases the resolution.

The availability of PTFE tubing made it easier to control the dimensions of the narrow-bore tubes more effectively. Together with the development of detectors with higher resolution and response than those of the thermometric detector, a better choice of dimensions of the narrow-bore tube is possible.

It is obvious that the temperature inside a conductivity probe made of a cylinder (8 mm diameter) of acrylic is lower than that in a narrow-bore tube made of PTFE with a similar inside diameter and a much smaller outside diameter, surrounded by air. The properties of the gas surrounding the narrow-bore tube have a great influence on the final temperature of the electrolytes in the separation compartment. The exact temperature inside the conductivity probe is difficult to measure, but we can state that the temperature profile is blurred.

EXPERIMENTAL AND RESULTS

The basic equipment has been described extensively by Everaerts *et al.*⁹. For experiments with thermometric detectors and comparative temperature measurements, PTFE narrow-bore tubing (Habia, Breda, The Netherlands) with approximate I.D.s of 0.4, 0.2 and 0.1 mm and corresponding O.D.s of 0.7, 0.4 and 0.2 mm was used. The diameters were found to be more constant if the narrow-bore tubes were prepared by stretching a tube with a larger inside diameter over a wire that has the inside diameter required for the tube. The inside diameter obtained was determined by measuring the length of a mercury thread at various points in the tube and weighing the mercury used.

The thermocouples (30 μm copper, 25 μm constantan) were made by electrically welding the twisted wires over a length of *ca.* 0.5 mm using a spark discharge from a capacitor (1000 μF , 25 V). The twisted section was kept under a reducing medium (*e.g.*, methanol) in order to prevent oxidation of the wires by oxygen in the air during the discharge. The junction obtained was round (diameter *ca.* 40 μm). After stretching the thermocouple, it was mounted around a narrow-bore tube with only one turn, making use of an elastic glue to which some solvent had been added in order to decrease its viscosity. Care was taken to ensure that besides the measuring point hardly any contact was made between the thermocouple wires and the narrow-bore tube. The reference junction was mounted on a probe of a quartz crystal thermometer (Hewlett-Packard, Avondale, Pa., U.S.A., Type 2801 A) and was protected with a heat-sink compound. By this means, absolute temperatures at the narrow bore tube could always be measured.

The narrow-bore tubes were clamped between an injection block and a counter electrode compartment, provided with a cellulose polyacetate semi-permeable membrane. These compartments are basically made of Perspex (acrylic) and are described in detail elsewhere⁹. The narrow-bore tube was surrounded by a Perspex tube (I.D. 30 mm) for thermostating the air around the tube. This Perspex tube was further surrounded by a weak PVC tube in which thermostated oil was circulated. The

ambient gas temperature could be kept constant to better than 0.1°. In all experiments the mode of operation was vertical.

The conductivity and UV absorption detectors have been discussed elsewhere⁹. The construction of conductometric cells of I.D. 0.2 mm with equiplanar-mounted electrodes is rather difficult. Moreover, the cell constant varies considerably. Therefore, conductimetric probes of the potential gradient type⁹ have been constructed. The distance between the electrodes was 0.1 mm, the material of construction being platinum-iridium (9:1). The linearity of the electronics in the range 10 k Ω –10 M Ω (the normal operating condition for experiments with centimolar solutions) was ensured with detectors with I.D. 0.4 and 0.2 mm; the accuracy was better than 0.2%.

The UV absorption detector was equipped with a slit of diameter 0.3 mm for the narrow-bore tube of I.D. 0.4 mm and diameter 0.1 mm for the narrow-bore tube of I.D. 0.2 mm. All experiments were carried out in the operational system listed in Table I.

TABLE I

OPERATIONAL SYSTEM AT pH 6, SUITABLE FOR ANIONIC SEPARATIONS

MES = morpholinoethanesulphonic acid; TRIS = tris(hydroxymethyl)aminomethane; HEC = hydroxyethylcellulose.

	<i>Electrolyte</i>	
	<i>Leading</i>	<i>Terminating</i>
Anion	Chloride	MES*
Concentration	0.01 <i>N</i>	<i>ca.</i> 0.01 <i>N</i>
Counter ion	Histidine	TRIS
pH	6	<i>ca.</i> 7
Additive	0.3% HEC**	None

* Purified by recrystallization.

** Purified by shaking the 2% solution with a mixed-bed ion exchanger and filtering.

Thermal step heights of several components were measured in narrow-bore tubes of different sizes over a wide range of current densities. Some of the results, carried out with chloride and morpholinoethanesulphonic acid (MES) anions are shown in Fig. 1. A survey of some other anions is given in Table II, where the correct dimensions of the narrow-bore tube are also given.

Assuming a thermal balance of the parabolic type, a plot of log ΔT against log J (J , current density) should give a straight line with a slope of 2. The experimental values, shown in both Fig. 1 and Table II, are in good agreement with theory, but the slope differs significantly from the theoretical value of 2, for several reasons: (1) there is a cooling effect due to the thermocouple mounted; (2) there is a change in the effective mobility of the ions, due to the difference in temperature of a zone at different current densities; and (3) there is a thermal expansion of the narrow-bore tube, especially at high current densities and with low effective mobilities of the ions involved.

To establish the significance of the first factor in a narrow-bore tube of I.D. 0.45 mm and O.D. 0.81 mm for chloride-histidine (Table I) at 80 μA ($J = 0.050$

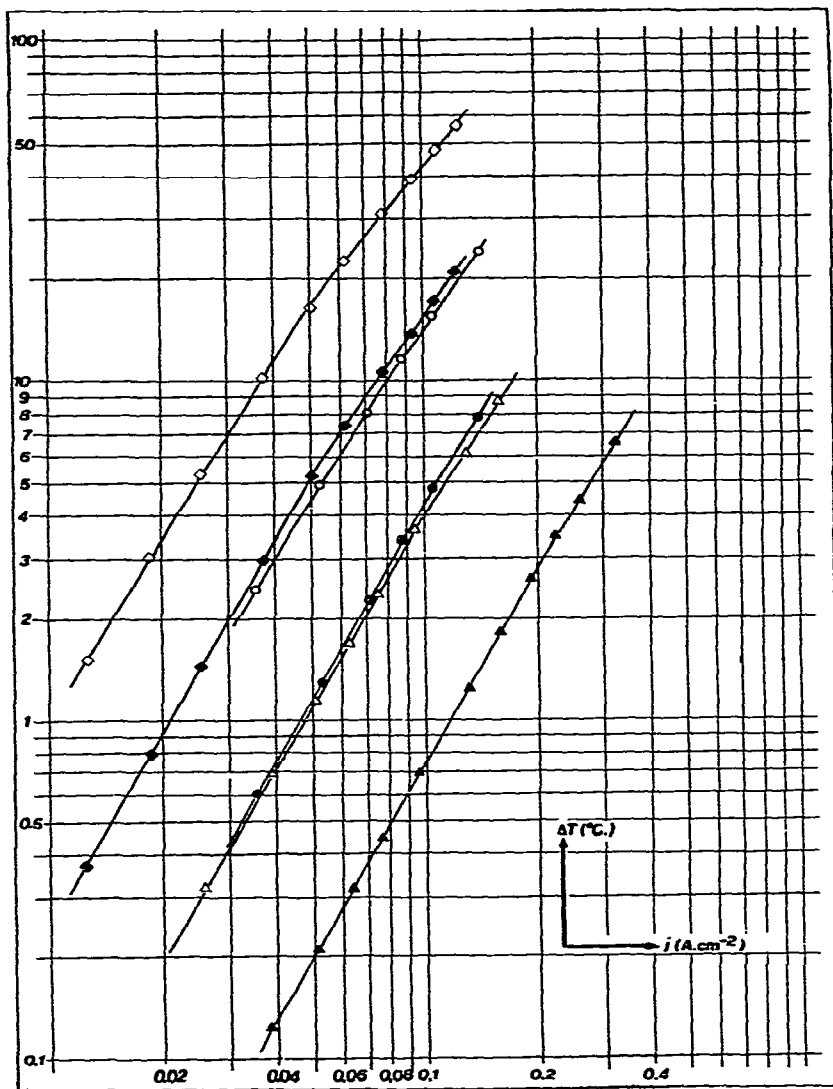


Fig. 1. Relationship between the temperature, measured by a thermocouple at the outside of a narrow-bore tube, and the current density applied. ▲, Chloride (0.10 mm I.D.; 0.20 mm O.D.), ●, chloride (0.19 mm I.D.; 0.40 mm O.D.), ■, chloride (0.45 mm I.D.; 0.81 mm O.D.); Δ, MES (0.10 mm I.D.; 0.20 mm O.D.); ○, MES (0.19 mm I.D., 0.40 mm O.D.); □, MES (0.45 mm I.D.; 0.81 mm O.D.). The experiments were carried out in the operational system listed in Table I.

$\text{A}\cdot\text{cm}^{-2}$), the following experiment was performed. After the current had been switched on and equilibrium of the thermal signal had been attained, a hydrodynamic flow was permitted in the direction of the counter electrode compartment. The increase in the thermal signal, due to the fact that leading electrolyte with a higher temperature (not cooled by the thermocouple wire) displaces the leading electrolyte with a lower temperature, was about 3.8%. Hence the thermal capacity of the thermocouple

TABLE II

SLOPES OF THE STRAIGHT LINES OBTAINED IF $\log J$ IS PLOTTED AGAINST $\log \Delta T$ FOR VARIOUS COMPONENTS MIGRATING ISOTACHOPHORETICALLY IN NARROW-BORE TUBES WITH DIFFERENT DIMENSIONS

J is the current density and ΔT is the difference between the temperature of the wall of the narrow-bore tube and that of the ambient gas, measured with a thermocouple mounted on the outside of the narrow-bore tube.

Component	Slope		
	I.D. = 0.10 mm, O.D. = 0.20 mm	I.D. = 0.19 mm, O.D. = 0.40 mm	I.D. = 0.45 mm, O.D. = 0.81 mm
Chloride	1.92	1.92	1.90
Acetate	1.92	1.92	1.76
Glutamate	1.92	1.90	1.72
MES	1.90	1.80	1.68
ACES*	—	1.78	1.60

* ACES = Acetaminoethane sulfonic acid.

mounting influences the final temperature of the various zones. The correction for the temperature dependence of the mobilities for this case is *ca.* 3% for the chloride zone. The correction for the thermal expansion of the narrow-bore tube ($J = 0.050 \text{ A} \cdot \text{cm}^{-2}$) is only about 0.8% if the terminator MES is used, and can therefore be neglected. The influence of temperature on the pK values, the activity coefficients and the thermal conductivity of the solvent has not been taken into consideration.

The deviations in the straightness of the lines at high current densities are thus caused mainly by the temperature dependence of the mobilities and the cooling effect of the thermocouple mounting.

The influence of the inside diameter of the narrow-bore tube on the final temperature of the various zones is considerable, as shown in Fig. 1. Theory now predicts that for a constant current density and constant wall thickness, ΔT can be expected to be a square function of the internal diameter. For example, $J = 0.095 \text{ A} \cdot \text{cm}^{-2}$ in a narrow-bore tube of I.D. 0.1 mm gives an increase in temperature of *ca.* 0.75° , while in a narrow-bore tube of I.D. 0.45 mm it will give an increase of 15° . Experi-

TABLE III

MEASURED AND CALCULATED¹⁰ TEMPERATURES FOR THE LEADING AND TERMINATING ELECTROLYTES (TABLE I) IN TWO NARROW-BORE TUBES, INDICATING THE TEMPERATURE PROFILE, AT A CURRENT DENSITY OF $J = 0.095 \text{ A} \cdot \text{cm}^{-2}$

T_s = temperature of the surrounds (ambient gas temperature); T_m = temperature as measured by the thermocouple at the outside of the narrow-bore tube; T_w = calculated temperature at the inside of the wall of the narrow-bore tube; T_c = calculated temperature at the centre of the narrow-bore tube.

I.D. of narrow-bore tube (mm)	Composition of electrolyte	T_s	T_m	T_w	T_c
0.2	Cl ⁻ (histidine H ⁺)	22.0	26.3	26.4	26.5
	MES ⁻ (histidine H ⁺)	22.0	34.0	34.2	34.7
0.45	Cl ⁻ (histidine H ⁺)	22.0	35.0	35.2	35.7
	MES ⁻ (histidine H ⁺)	22.0	64.0	64.8	66.8

mentally, in the last example a difference in temperature of 14° was determined. For further information, Fig. 1 should be examined. These results indicate that, as far as possible, a reduction in the diameter of the electrophoretic separation column should be sought.

Apart from the gain in the temperature profile (some relevant temperatures are summarized in Table III), a smaller bore is preferable for many other purposes, such as quantitation, theoretical studies, times of analysis and the fact that slower terminators (for many thermally labile components such as proteins) can be applied. Although it is preferable to have a smaller difference in temperature between the various zones, it may occur that a given pair of ions in an operational system can be separated only as a result of the difference in temperature. So far little attention has been paid to temperature programming in electrophoretic analyses.

The influence of temperature on the conductivity of the various zones in isotachopheretic experiments is also far from negligible, as shown in Fig. 2. The effect

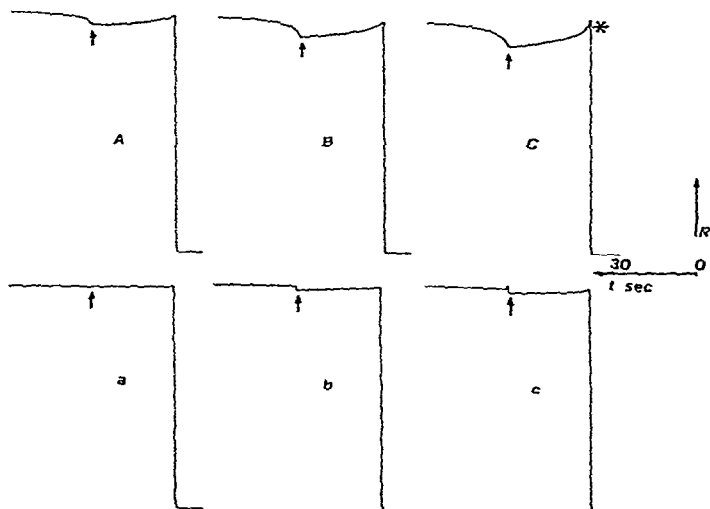


Fig. 2. Isotachopherograms (leading electrolyte/terminating steps) obtained from an a.c. conductivity detector with a potential gradient probe of I.D. 0.4 mm (A, B, C) and I.D. 0.2 mm (a, b, c) at various current densities. A, a, $J = 0.064 \text{ A}\cdot\text{cm}^{-2}$; B, b, $J = 0.080 \text{ A}\cdot\text{cm}^{-2}$; C, c, $J = 0.095 \text{ A}\cdot\text{cm}^{-2}$. R = increasing resistance; t = increasing time. The small arrows indicate the moment at which the electric current is switched off. *, overshoot.

of temperature on the conductivity is especially noticeable for the transition between the leading and terminating electrolytes. If sample zones are present, sandwiched between the leading and terminating zones, more time is available for the detector to warm up and hence the overshoot (marked with an asterisk in Fig. 2) is not as clearly visible, although the influence on the qualitative information is present.

In Fig. 2, isotachopherograms (chloride-MES boundaries) obtained from a potential gradient probe (a.c. mode) with a bore of I.D. 0.4 mm (A, B, C) and a bore of I.D. 0.2 mm (a, b, c) under identical J values: 0.064 (A, a), 0.080 (B, b) and $0.095 \text{ A}\cdot\text{cm}^{-2}$ (C, c) are compared. At the lowest J value a difference of about 4% can still be found if the probe of I.D. 0.4 mm is used.

Hardly any difference is found with the probe of I.D. 0.2 mm. It needs no further explanation that especially for theoretical studies of, for example, complex formation and mobility determination or the influence of activity or solvation, this result is very important. Assuming that the analyses are carried out at current densities of up to $0.064 \text{ A} \cdot \text{cm}^{-2}$, the qualitative information is no longer a function of the current density and the sample injected (warming up of the detector) and is better than 1% (electronics of the a.c. conductivity detector).

Apart from the diameter, the gas surrounding the narrow-bore tube was also found to be important. If the narrow-bore tube was surrounded by hydrogen an initial temperature difference from the ambient gas temperature was found to be *ca.* 1.9° for the leading electrolyte and *ca.* 7.0° for the terminating electrolyte (Table I). J was $0.050 \text{ A} \cdot \text{cm}^{-2}$ and the dimensions of the narrow-bore tube were I.D. 0.45 mm and O.D. 0.81 mm. Under similar conditions, a temperature difference of 5.2° for the leading electrolyte and 16.0° for the terminating electrolyte were measured when nitrogen (or air) surrounded the narrow-bore tube. If kerosene was used as the medium surrounding the narrow-bore tube, negligible signals remained for a complete qualitative (and/or quantitative) determination with thermometric detection. Carbon dioxide (which has an even smaller thermal conductivity than nitrogen) cannot be applied, because it easily passes through the PTFE wall. Carbon dioxide dissolves in the electrolytes and disturbs the isotachopheretic analysis, migrating as HCO_3^- or CO_3^{2-} , assuming that the pH of the leading electrolyte is sufficiently high (even at pH 6).

Because of the above effects, more care must be taken if operational systems are applied at high pH, not only during the preparation of the various electrolytes, under nitrogen, and with the addition of, *e.g.*, barium hydroxide to the terminating electrolyte.

Another effect was examined visually during experiments with the narrow-bore tubes of I.D. *ca.* 0.4 and 0.2 mm. Poly(vinyl alcohol) (Mowiol), purified by running a concentrated solution over a mixed-bed ion exchanger, was added to the electrolyte at a concentration of 0.05%. In the narrow-bore tubes of I.D. *ca.* 0.4 mm a clear convective disturbance appears at about $130 \mu\text{A}$ ($J = 0.081 \text{ A} \cdot \text{cm}^{-2}$), for instance at the zone boundary of bromophenol blue-morpholinoethane sulphonate in the operational system at pH 6 (Table I). These disturbances could be suppressed by addition of 0.3% of hydroxyethylcellulose (Polysciences, Warrington, Pa., U.S.A.), also purified by using a mixed-bed ion exchanger and filtering*, at this current density.

These convective disturbances did not appear at comparable current densities in narrow bore-tubes of I.D. *ca.* 0.2 mm, owing to the smaller differences in temperature between the various zones, the gain in temperature profile and the stabilization by the decrease in the ratio of the cross-section to surface area.

Some isotachopherograms are shown of analyses carried out in narrow-bore tubes smaller than those conventionally used. Fig. 3 shows an isotachopheretic separation of a standard mixture of anions in the operational system listed in Table I, carried out in a narrow-bore tube of I.D. *ca.* 0.1 mm. An ordinary thermocouple (copper-constantan) was used as the detector ($J = 0.114 \text{ A} \cdot \text{cm}^{-2}$).

* The polymer solution must be filtered in order to remove solid particles, which migrate under the influence of the electric field and disturb the registration, especially with a UV absorption detector.

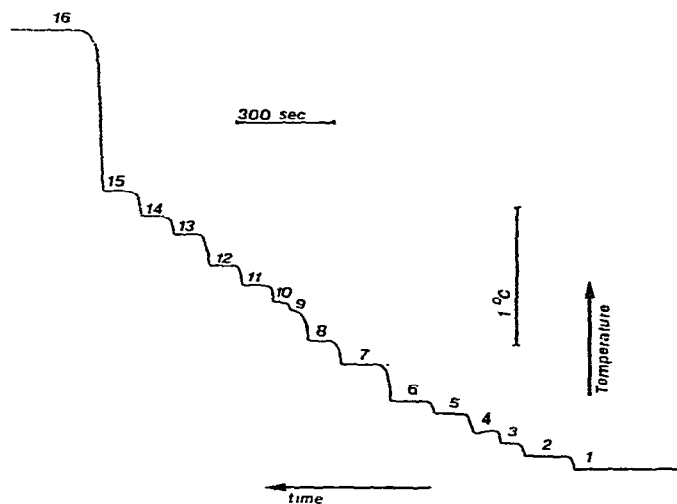


Fig. 3. Isotachopherogram of a test mixture of anions obtained using the operational system listed in Table I. The registration was effected with a thermocouple, mounted around a narrow-bore tube of O.D. 0.2 mm and I.D. 0.1 mm, $J = 0.114 \text{ A} \cdot \text{cm}^{-2}$. 1 = Chloride; 2 = sulphate; 3 = chlorate; 4 = chromate; 5 = malonate; 6 = pyrazole-3,5-dicarboxylate; 7 = adipate; 8 = acetate; 9 = β -chloropropionate; 10 = propionate; 11 = benzoate; 12 = naphthalene-2-sulphonate; 13 = glutamate; 14 = enanthate; 15 = benzyl-*dl*-aspartate; 16 = morpholinoethanesulphonate.

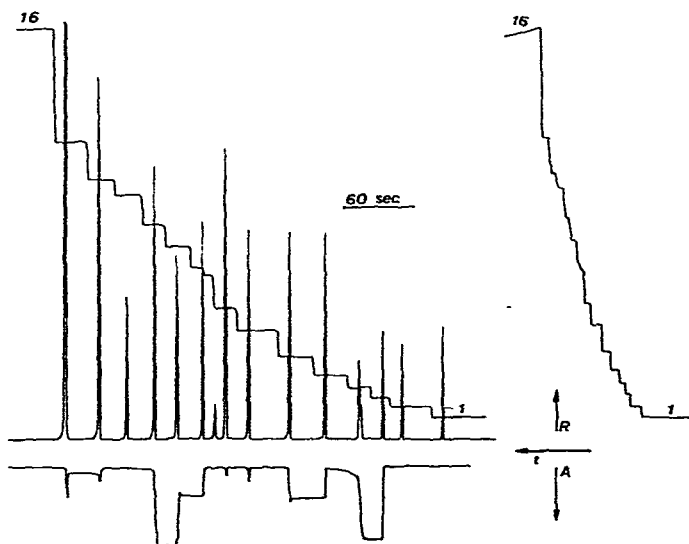


Fig. 4. Isotachopherogram of the test mixture of anions, as shown in Fig. 3, obtained using the operational system listed in Table I. The left-hand isotachopherogram was obtained in a narrow-bore tube of I.D. ca. 0.2 mm, and the right-hand isotachopherogram in a narrow-bore tube of I.D. ca. 0.4 mm. A conductivity detector (a.c. mode) and an UV absorption detector were applied. Equal amounts of sample were injected. R = increasing resistance; A = increasing UV absorption; t = increasing time. $J = 0.080 \text{ A} \cdot \text{cm}^{-2}$.

It is rather difficult to effect a satisfactory thermometric registration for this mixture of anions, making use of a narrow-bore tube of I.D. *ca.* 0.4 mm. Up till now no set of ions could be found, assuming that the concentration ratio was chosen correctly, that could be detected via a conductivity or UV detector once separated isotachophoretically, if the separation could not be registered with a thermometric detector.

Fig. 4 shows the test mixture of anions (Fig. 3) separated isotachophoretically in the operational system listed in Table I, using conductivity detectors (a.c. mode) with potential gradient probes of 0.2 mm (left-hand side) and 0.4 mm (right-hand side) as detectors. In the left-hand analysis, a registration is also shown that was obtained with a UV absorption detector (254 nm) with a slit width of 0.1 mm (in both experiments, $J = 0.080 \text{ A} \cdot \text{cm}^{-2}$). The total amounts of all ions in the test mixture were identical in each experiment, which indicates once more the importance of reducing the inside diameter of the narrow bore tube. For both the conductivity and UV absorption detectors the resolution is improved if a smaller diameter of the narrow-bore tube is chosen (more spikes due to impurities are visible). The reproducibility of the step heights (qualitative information) found in the linear trace from the conductivity detector was better than 1% and was no longer a function of J .

Fig. 5 shows the standard mixture of anions separated isotachophoretically in the operational system listed in Table I ($J = 0.064 \text{ A} \cdot \text{cm}^{-2}$) using a conductivity detector (a.c. mode) with a potential gradient probe of I.D. 0.2 mm. The registration was effected with a potentiometric recorder with zero suppression. The total scale could be enlarged five times. The reproducibility of the linear trace, which shows that the conductivity in the various zones is not exactly the same throughout a zone (for instance, in the zone of chromate and benzyl-*dl*-aspartate), was found to be almost 100%.



Fig. 5. Isotachopherogram of the test mixture of anions (Fig. 3) obtained using the operational system listed in Table I, carried out in a narrow-bore tube of I.D. *ca.* 0.2 mm. The registration was effected with a conductivity detector (a.c. mode) with a potential gradient probe of I.D. 0.2 mm. The potentiometric recorder was equipped for zero suppression (for this analysis, five times). At the arrow (off), the electric current is switched off. R = increasing electric resistance. $J = 0.064 \text{ A} \cdot \text{cm}^{-2}$.

CONCLUSION

From these experiments, we recommend the use of narrow-bore tubes with an inside diameter smaller than the *ca.* 0.4 mm commonly used, especially for analytical purposes. Even for preparative isotachopheresis, a series of narrow-bore columns, mounted in parallel, is worth considering, instead of increasing the inside diameter. Undesirable temperature increases, especially if thermally labile components are of interest, can be prevented.

The use of soluble linear polymers, which decrease the convective disturbances, is also recommended. The concentration of these polymers depends on, amongst other factors, the molecular weight, the type of polymer, the diameter of the narrow-bore tube and the current density applied.

Owing to the construction of our injection system and the way our conductivity detector is constructed, we found a narrow-bore tube with an I.D. of *ca.* 0.2 mm to be almost optimal.

The advantages of decreasing the inside diameter of the narrow-bore tubes are:

(1) smaller amounts of sample are needed for full qualitative and quantitative separation;

(2) the qualitative information is no longer a function of the current density;

(3) the temperature differences between the various zones are smaller;

(4) the total time for analysis can be decreased, if required;

(5) terminators with a lower effective mobility can be applied.

Whether or not the diameter can be decreased further in the future depends on, amongst other factors, the electroendosmosis, the availability of current-stabilized power supplies that enable one to work at low electric currents ($I < 10 \mu\text{A}$) and better injection systems.

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