Journal of Chromatography, 169 (1979) 11–20

CHROM. 11,442

HIGH-PERFORMANCE ZONE ELECTROPHORESIS

F. E. P. MIKKERS, F. M. EVERAERTS and Th. P. E. M. VERHEGGEN

Department of Instrumental Analysis, Eindhoven University of Technology, Eindhoven (The Netherlands)

(Received September 1st, 1978)

SUMMARY

An experimental approach to high-performance zone electrophoresis is given. It is shown that dispersion can be well controlled by the use of narrow-bore tubes made of chemically and electrically inert materials. The asymmetric concentration distributions that are frequently obtained in free zone electrophoresis are the result of migrational dispersion. This asymmetry only can be suppressed by the application of very small amounts of sample. High-performance separations with UV and conductimetric detection are shown. The time of analysis can be reduced to a few minutes by selecting the appropriate operational conditions. Plate heights smaller than 10 μm can easily be obtained.

INTRODUCTION

Many of the problems in the development of electrophoresis can be reduced to convection and detection. In experimental practice there are three alternative approaches to alleviate the problem of convection. One emphasizes the use of additional force fields, *e.g.*, gravitational or electromagnetic, to eliminate the disturbing influence of convection[1,2]. In a second, more practical approach, stabilizing media such as paper, cellulose acetate or gels are used[3]. Although by this approach convection can be effectively suppressed, it inherently introduces an interaction between the solutes of interest and the anticonvective medium. Such interactions may well be beneficial in many applications, but are generally not desirable. The third approach, which has proved to give a satisfactory solution to obtaining a stable electrophoretic performance, is the use of the anticonvective "wall effect"[4]. Hence stability can be obtained by decreasing the ratio of the cross-section of the separation compartment to its surface area. In moving boundary electrophoresis and isotachophoresis, such configurations have been applied successfully, mainly by using narrow-bore tubes made of chemically and electrically inert materials[5–12].

Zone electrophoresis in narrow-bore tubes has attracted less attention, although several suggestions about its feasibility have been made[13–15]. Hjertén[13] performed zone electrophoresis in tubes of quartz glass coated with methylcellulose and used UV detection. The adverse effect of the relatively large inner diameter was reduced by

rotating the separation compartment about its longitudinal axis. Although the operational conditions seem complex, he clearly showed the feasibility of the technique.

Everaerts and Hoving-Keulemans[14] used an isotachophoretic instrument equipped with a thermometric detector. With PTFE capillary tubing they were able to perform zone electrophoretic separations and to detect highly asymmetric zones. A detailed study was made by Virtanen[15], who employed potentiometric detection. The performance of his equipment was poor, however, as the experiments were extremely sensitive to disturbances and required a trained and dedicated operator[15].

Giddings[16] evaluated theoretically the ultimate capabilities of zone electrophoresis by introducing the HETP concept. Provided that a low dispersive performance can be achieved, he suggested that plate heights down to 10 μm should be possible. Up to now this limit of performance has never been reached, but the use of capillary configurations seems to be promising in this respect. In analogy with modern chromatographic methods, the low load capacity of capillary systems places high demands on detection. For moving boundary electrophoresis and isotachophoresis a satisfactory solution has been given. In zone electrophoresis, however, a higher sensitivity is needed. Moreover, there are some methodological problems[17] that may hamper the development of high-performance zone electrophoresis (HPZE).

In zone electrophoresis, a sample zone is eluted by a carrier electrolyte, of which the carrier constituent has the same charge as the sample constituents to be separated. It has been shown that with a low diffusional performance concentration distributions in zone electrophoresis have very characteristic forms[17]. This form depends mainly on the effective mobility of the sample constituent relative to that of the carrier constituent, *i.e.*, r_C. A sample zone that contains a sample constituent with a relative mobility that is greater than unity will have a diffuse leading side, whereas the back of the zone will be sharp. The reverse holds for sample constituents with an effective mobility less than that of the carrier constituent.

When a non-scanning detector is used, it can easily be shown that the electrical gradient profile for an $r_C > 1$ configuration is given by[17]

$$\frac{E^Z(x_{det}, t)}{E^S} = \sqrt{\frac{t_{det}}{t}} \tag{1}$$

for $t_{det} < t < t_{end}$, where E^Z and E^S are the electrical field strength in the sample zone and in the carrier electrolyte, respectively, and x_{det} is the distance at which the detector is located.

The time interval at which the first sample constituent reaches the point of detection is given by t_{det}. The time at which the last sample constituent reaches the point of detection, t_{end}, follows from a mass balance:

$$n_C = A \int_0^\infty c_C^Z(x_{det}, t)\, dt \tag{2}$$

where n_C is the amount of sample and A is the cross-sectional area of the separation compartment. Analogous relationships can be obtained for other configurations. It should be stressed that concentration distributions and electrical gradient profiles have different forms and that there is a difference between time-based and distance-based distributions[17].

EXPERIMENTAL

All experiments were performed in the electrophoretic equipment developed by Everaerts *et al.*[12]. The separation compartment was formed by a PTFE narrow-bore tube of I.D. 0.2 mm and O.D. 0.35 mm. Samples were introduced by means of a microlitre syringe or a specially constructed four-way injection valve with a volume of 0.7 μl and I.D. 0.3 mm. Potential gradient detectors were used in the conductance mode[12] and UV absorption (254 nm) was converted electronically into absorbance. The direct and constant electrical driving current was taken from a modified Brandenburg (Thornton Heath, Great Britain) high-voltage power supply. All chemicals used were of analytical-reagent grade or additionally purified by conventional methods. The operational systems and conditions are given in Table I.

TABLE I

OPERATIONAL SYSTEMS

MES = 2-(N-morpholino)ethanesulphonic acid; HEC = hydroxyethylcellulose, Polysciences Inc., Warrington, Pa., U.S.A., Cat. No. 5568.

Parameter	*System No.*		
	1	*2*	*3*
Carrier constituent	MES	Acetic acid	Acetic acid
Carrier concentration (M)	0.01	0.005	0.1
Counter constituent	Histidine	Histidine	γ-Aminobutyric acid
pH	6.05	6.02	4.00
Additive	0.1% HEC	0.1% HEC	0.1% HEC
Inner diameter (mm)	0.2	0.2	0.2
Electrical driving current (μA)	20	30	100
Temperature (°C)	22	22	22

RESULTS AND DISCUSSION

In Fig. 1 an electrical gradient profile of a chloride zone, migrating in operational system 1 (Table I), is shown. From the theoretical profile, given in Fig. 1 by the dotted line, and the experimental profile it must be concluded that the theoretical model is in close agreement with experimental practice. It should be emphasized that the theoretical model[17] was developed for monovalent strong electrolytes. Operational system 1 (Table I) is well buffered, as both the carrier constituent and the counter constituent have pK_a values that are close to the pH of the carrier electrolyte. Any pH shift in this system is unlikely and therefore the theoretical considerations will apply. From Fig. 1 we conclude that the dispersive factors, other than electrophoretic migration, are well controlled and have a negligible influence on the concentration distribution. Owing to the high relative mobility of the sample constituent and its concentration, an appreciable inhomogeneity in the electrical field occurs, resulting in stable zone boundaries. It can be calculated[17] that under the given operational conditions diffusional dispersion becomes important at the picomole level for the amount sampled. Theoretically, it has been shown that t_{det} is independent of the amount sampled, as is experimentally confirmed by the right-hand side of

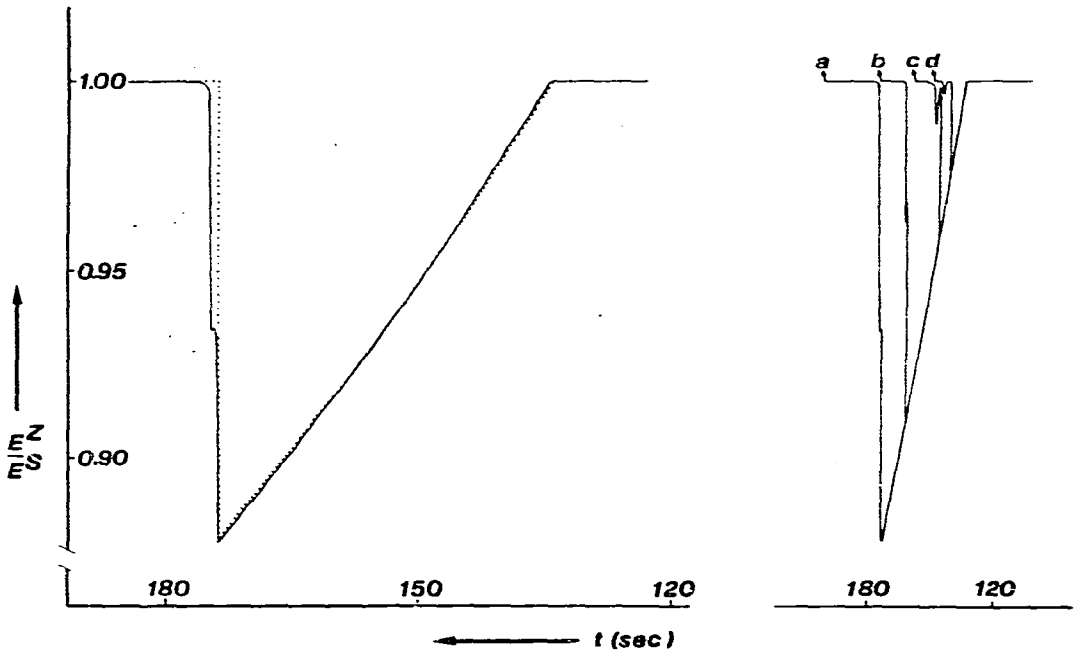

Fig. 1. Electrical gradient profile of a chloride sample zone, migrating in operational system 1 (Table I). Solid line, measured profile; dotted line, theoretical profile. (a) $707 \cdot 10^{-12}$ mole Cl^-; (b) $354 \cdot 10^{-12}$ mole Cl^-; (c) $70.7 \cdot 10^{-12}$ mole Cl^-; (d) $21.2 \cdot 10^{-12}$ mole Cl^-. E^Z/E^S = electrical field strength in the sample zone relative to that in the carrier electrolyte; t (sec) = time of analysis.

Fig. 1 and the data in Table II. Decreasing the sample load has no effect on the shape of the concentration distribution and only t_{end} is varying, as this time is determined by the amount sampled and the generated distribution function.

From the data in Table II, it follows that neither the peak height nor the peak width is linearly related to the amount sampled. Assuming that the non-diffusional model is valid, it follows from eqns. 1 and 2 that

$$n_C = \frac{A}{a_C}\left(t_{end} - \frac{2}{3}\sqrt{\frac{t^3_{end}}{t_{det}}} - \frac{1}{3}t_{det}\right) \quad (3)$$

TABLE II

RETENTION DATA

C.V. = Coefficient of variation.

No. of determinations	*Sample load (mole × 10^{-12})*	*Peak width*		*Peak height*		t_{end}		t_{det}	
		sec	*C.V. (%)*	*mm*	*C.V. (%)*	*sec*	*C.V. (%)*	*sec*	*C.V. (%)*
3	707	39.1	0.2	1230	0	172.4	0.3	133.3	0.2
4	500	32.4	0.3	1094	0.4	166.1	0.4	133.7	0.1
4	354	27.4	0.3	908	0.2	162.2	0.2	134.8	0.4
4	225	21.8	0.2	732	0.5	154.9	0.1	133.1	0.3
4	70.7	11.8	0.3	427	1.0	146.2	0.3	134.4	0.4
4	21.2	7.53	0.4	243.5	1.0	140.7	0.3	133.2	0.6
3	7.07	6.14	0.1	165.2	3.0	138.7	0.2	132.6	0.1

where a_C is a constant that is determined by the concentration of the carrier constituent and all constituent mobilities[17]. The data in Table II fit eqn. 3 with a correlation coefficient of 0.99969. Again, it must be concluded that the experiments performed were non-diffusional.

In those instances in which somewhat blurred concentration distributions are obtained, *e.g.*, when injection is made with a microlitre syringe, it is advisable to use the peak area. As the difference in specific conductance between a sample zone and the carrier electrolyte is directly related to the concentration of the sample species[17], it is obvious that the conductance-based peak area must be used. The detection as shown in Fig. 1 is, however, essentially not on a conductance base and therefore the signals have to be converted. It can be derived that, when dealing with moderate peak widths, the electrical field strength area in a triangular approximation is linearly related to the amount sampled. Fig. 2 shows the calibration graph for the sample constituent chloride; the correlation coefficient for the line is 0.99968.

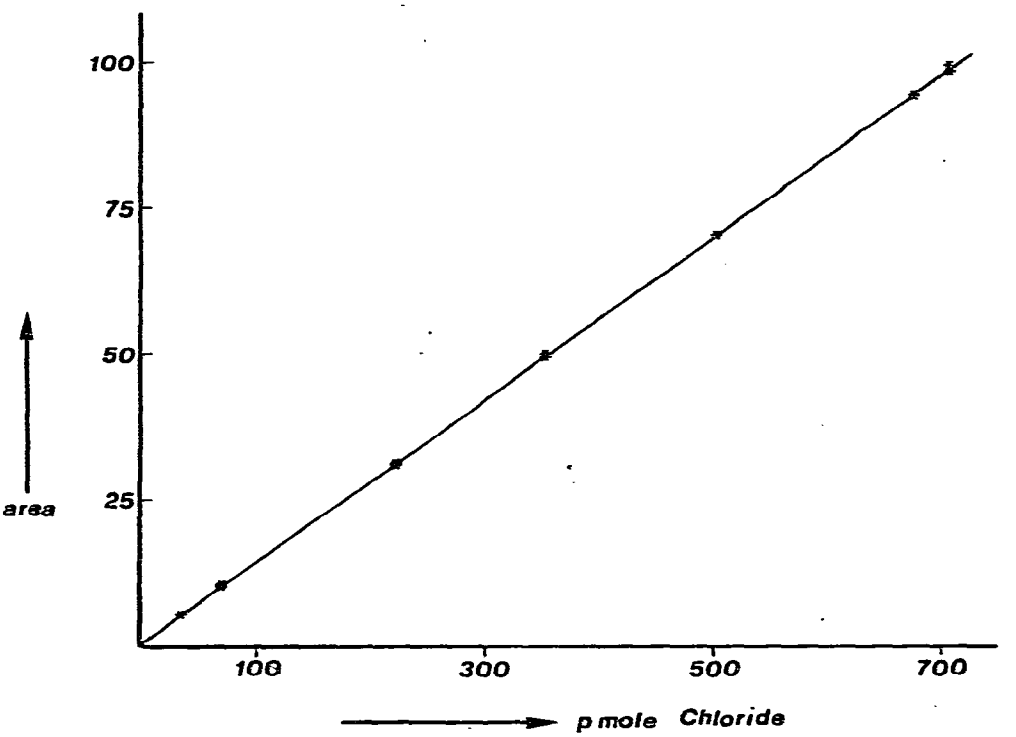

Fig. 2. Calibration graph for chloride determinations.

For identification purposes a retention time has to be defined. As the time-based centre of gravity of the concentration distribution is strongly affected by the sample load, this retention time can be used only at low sample loads. For practical reasons generally the time of the peak maximum will be preferred. At higher sample loads an appropriate correction function can be used. The use of a constant voltage instead of a constant driving current may be favourable in this respect.

From Fig. 1 it can be seen that the sample contained not only chloride, but also an impurity, which was identified as sulphate. At a high load of sample it is difficult to decide whether separation is complete or not, but resolution is easily obtained when the amount sampled is low.

According to theoretical considerations[17], a sample constituent with a mobility that is smaller than that of the carrier electrolyte must migrate with a sharp leading front, whereas the back of the zone must diffuse. From Fig. 3B it can be seen that the distribution of propionate conforms with theory when acetate is used as the carrier constituent. In Fig. 3A the distribution of formate, detected under the same operational conditions [system 2 (Table I)], is given. It should be noted that for $r_C > 1$, *i.e.*, formate, t_{det} is independent of the amount sampled, whereas for $r_C < 1$, *i.e.*, propionate, no independence exists. The result of the analysis of a mixture of the two separands is shown in Fig. 3C. It can be seen that neither the retention behaviour nor the distribution function of the more mobile sample constituent (formate) is affected by the presence of the second sample constituent. The reverse is not true, however. Therefore, Fig. 3 emphasizes the complex nature of retention in zone electrophoresis. This complexity may hamper the handling of multicomponent samples in which large concentration differences occur. Although asymmetric concentration

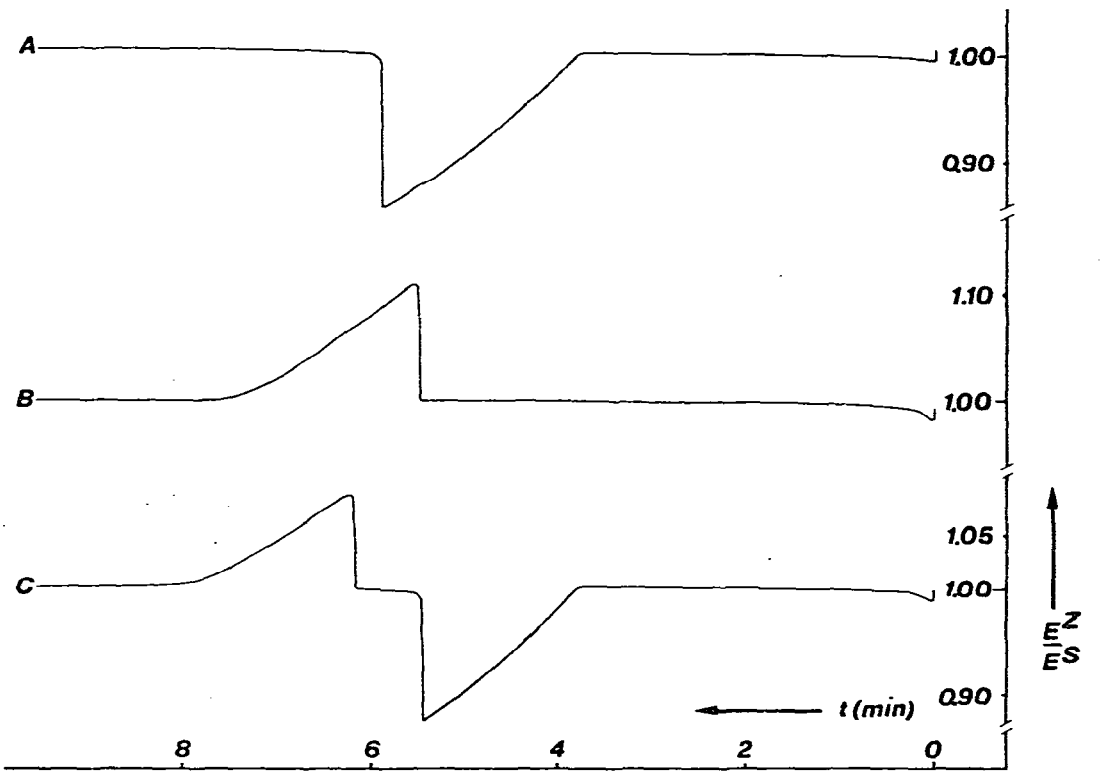

Fig. 3. Zone electrophoretic separation of formate and propionate in operational system 2 (Table I). (A) formate, $3.5 \cdot 10^{-9}$ mole; (B) propionate, $3.5 \cdot 10^{-9}$ mole; (C) formate + propionate, each $2.33 \cdot 10^{-9}$ mole. E^Z/E^S = electrical field strength in the sample zone relative to that in the carrier electrolyte; t (min) = time of analysis.

distributions are frequently measured in free zone electrophoresis, they also can occur when anticonvective media such as gels or cellulose acetate are used[18]. Using capillary systems, however, they seem to be more pronounced because most forms of non-migrational dispersion can be well controlled. In fact, the high asymmetry of the separations shown in Figs. 1 and 3 gives an indication of the low dispersive performance of the equipment.

As has already been discussed, the initial sample width, Δl_0, should be minimized. The adverse effect of a relatively large Δl_0 can often be decreased by the concentrating capabilities of the electrolyte system. The left-hand side of Fig. 4 shows a separation of three sample constituents, analysed in operational system 3 (Table I). The relatively high concentration of the carrier constituent and the fact that the sample constituents were not dissolved in the carrier electrolyte guarantees a concentration of the sample over the stationary boundary between the sampling and the separation compartment. As a result, a good separation is obtained. A comparable result can be obtained by disc electrophoresis[19,20] by choosing a suitable stacking electrolyte. The right-hand side of Fig. 4 shows the sample, but now dissolved in the carrier electrolyte. In this instance the electrolyte system has no concentrating capabilities and the adverse effect of the relatively long sample width is clearly visible.

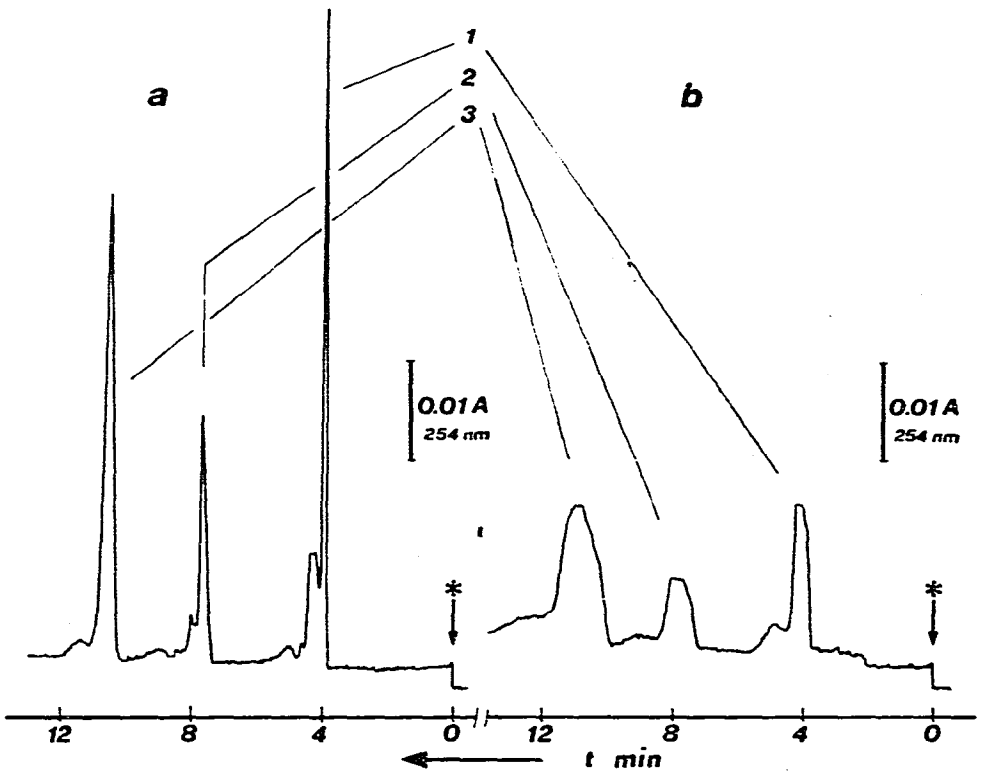

Fig. 4. Zone electrophoretic separation of three anionic sample constituents with UV detection, using operational system 3 (Table I). (1) Sulphanilic acid, $70 \cdot 10^{-12}$ mole; (2) 5-bromo-2,4-dihydroxybenzoic acid, $140 \cdot 10^{-12}$ mole; (3) adenosine-5'-monophosphoric acid, $35 \cdot 10^{-12}$ mole. t (min) = time of analysis. (a) Constituents dissolved in water; (b) constituents dissolved in carrier electrolyte.

It should be noted that UV absorbance detection was carried out with conventional equipment[12], in which there is only an optical path length of 0.2 mm available. Increasing this path length will give a high sensitivity to this detection system. Noting that for an optimal retention behaviour very small amounts of sample have to be applied[17], it follows that the high sensitivity and the specific nature of UV detection are very attractive.

The advantage of a non-specific detection system follows from Fig. 5, where a separation of a 16-component sample is shown in operational system 1 (Table I). Because only 17.5 pmole of each constituent were injected, reasonably symmetric distributions are obtained. From the separation shown in Fig. 5, it follows that the molar response[17] of the conductimetric detector decreases with increasing retention time. Moreover, divalent constituents have a considerably higher response than monovalent constituents. For a quantitative evaluation at this low concentration, the integrated peak area can be used directly. The peak maxima can be used most conveniently for retention times, because they are fairly independent of the amount sampled. As their reproducibility is very high, as can be seen from Table III, they can be used for identification.

Giddings[16] predicted that the minimal plate height that can be obtained in zone electrophoresis is about 10 μm. According to Fig. 5 and the data in Table III, this

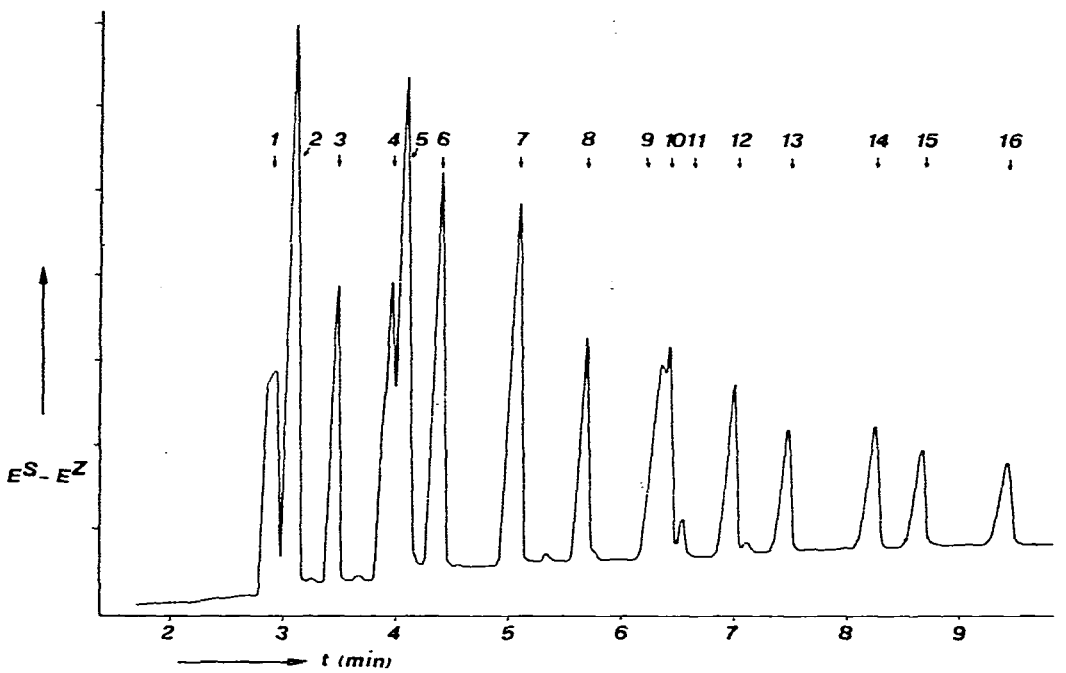

Fig. 5. Zone electrophoretic separation of a 16-component sample. 1 = Chloride; 2 = sulphate; 3 = chlorate; 4 = malonate; 5 = chromate; 6 = pyrazole-3,5-dicarboxylate; 7 = adipate; 8 = acetate; 9 = propionate; 10 = β-chloropropionate; 11 = unidentified; 12 = benzoate; 13 = naphthalene-2-monosulphate; 14 = glutamate; 15 = enanthate; 16 = benzyl-DL-aspartate. Sample load: $17.5 \cdot 10^{-12}$ mole for each constituent. $E^S - E^Z$ = difference in electrical field strength between the carrier electrolyte and the sample zone. t (min) = time of analysis.

TABLE III

PERFORMANCE CHARACTERISTICS

Length of separation compartment = 20 cm. C.V. = Coefficient of variation (n = 6).

Constituent	*No.*	*Retention time*		*HETP* (μm)	*N*
		sec	*C.V. (%)*		
Chloride	1	—	—	—	—
Sulphate	2	185.3	0.8	38	5300
Chlorate	3	208.2	0.8	20	10,000
Malonate	4	236.3	0.2	—	—
Chromate	5	244.1	0.6	22	9200
Pyrazole-3,5-dicarboxylate	6	262.6	0.8	18	11,000
Adipate	7	304.2	0.9	18	11,000
Acetate	8	339.4	0.9	8.7	23,000
Propionate	9	376.9	0.9	—	—
β-Chloropropionate	10	382.7	0.6	—	—
Benzoate	12	416.4	0.6	7.1	28,000
Naphthalene-2-monosulphonate	13	443.2	0.9	5.6	36,000
Glutamate	14	486.9	1.3	6.3	32,000
Enanthate	15	514.0	1.0	5.6	36,000
Benzyl-DL-aspartate	16	558.0	1.0	5.9	34,000

minimal value is easily obtained for several sample constituents. As there is still some asymmetry left, it must be concluded that at a lower sample load even better HETPs can be obtained. This, however, will place even higher demands on the detection system, which essentially was developed for isotachophoresis and moving boundary electrophoresis[12]. Using eqns. 1 and 3 and the data in Table II, it follows that 1 pmole of chloride, analysed in operational system 1 (Table I), will cause a maximal deviation of the baseline signal of 0.5%. As most constituents have a lower response than chloride, an even higher stability is generally required. At a very high sensitivity, *e.g.*, 0.01%, the conductivity signal often shows a poor reproducibility and time-dependent instabilities, which are not of electronic origin. As a result, an irreproducible drift of the baseline, signal wander and ghost peaking occur. These effects are due mainly to electrochemical effects, adsorption and the occurrence of impurities. Temperature effects have only a minor influence[18]. Signal stability causes less problems when UV detection is used. Noting that in the conventional equipment[12] an optical path length of only 0.2 mm is available, a substantial increase in sensitivity can be achieved by increasing this length. Other advantages of UV detection, *e.g.*, the selectivity and the compatibility with electrophoretic gradient elution techniques, and its inherent disadvantages are obvious.

From the separations shown in Figs. 4 and 5, it must be concluded that HPZE at a low concentration level is not only possible but also gives reproducible and reliable results in a short analysis time. The design of the equipment[4,12] and the use of narrow-bore tubes guarantees a low dispersive performance and allows simple and easy operation. On the other hand, Figs. 1 and 3 emphasize the problematical character of zone electrophoresis considering retention behaviour, especially when large concentration differences of the sample constituents occur.

REFERENCES

1 A. Kolin, *J. Appl. Phys.*, 25 (1954) 1442.
2 S. Hjertén, *Ark. Kemi*, 13 (1958) 151.
3 J. R. Sargent and S. G. George, *Methods in Zone Electrophoresis*, BDH Chemicals, Poole, Great Britain, 3rd edn., 1975.
4 Th. P. E. M. Verheggen, F. E. P. Mikkers and F. M. Everaerts, *J. Chromatogr.*, 132 (1977) 205.
5 B. P. Konstantinov and O. V. Oshurkova, *Sov. Phys. Tech. Phys.*, 11 (1966) 693.
6 F. M. Everaerts, *Thesis*, Eindhoven University of Technology, Eindhoven, 1968.
7 L. Arlinger and R. Routs, *Sci. Tools*, 17 (1970) 1.
8 J. Vacik and J. Zuska, *Chem. Listy*, 66 (1972) 416.
9 T. Haruki and J. Akiyama, *Anal. Lett.*, 6 (1974) 24.
10 S. Stankoviansky, P. Cicmanec and D. Kanianski, *J. Chromatogr.*, 106 (1975) 131.
11 P. Bocek, M. Deml and J. Janak, *J. Chromatogr.*, 106 (1975) 283.
12 F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis*, Journal of Chromatography Library, Vol. 6, Elsevier, Amsterdam, Oxford, New York, 1976.
13 S. Hjertén, *Thesis*, University of Uppsala, Uppsala, 1967.
14 F. M. Everaerts and W. M. L. Hoving-Keulemans, *Sci. Tools*, 17 (1970) 25.
15 R. Virtanen, *Thesis*, Helsinki University of Technology, Otanieme, 1974.
16 J. C. Giddings, *Separ. Sci.*, 4 (1969) 181.
17 F. E. P. Mikkers, F. M. Everaerts and Th. P. E. M. Verheggen, *J. Chromatogr.*, 169 (1979) 1.
18 M. H. M. Vervoordeldonk, *Graduation Report*, Eindhoven University of Technology, Eindhoven, 1977.
19 L. Ornstein, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321.
20 T. M. Jovin, *Biochemistry*, 12 (1973) 871.