Journal of Chromatography, 545 (1991) 283–297
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 145

Isotachophoresis in open systems

Problems in quantitative analysis

M. T. ACKERMANS*, F. M. EVERAERTS and J. L. BECKERS
Laboratory of Instrumental Analysis, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven (The Netherlands)

ABSTRACT

So far, isotachophoretic (ITP) analyses have been carried out in commercially available or laboratory-made ITP instruments, generally in closed systems. At present several instruments are commercially available, originally designed for capillary zone electrophoresis with open capillaries, and these instruments can also be used for ITP. If ITP experiments are carried out using such apparatus, however, an electroosmotic flow (EOF) will act on the ITP system. The velocity of the EOF strongly varies with the choice of the leading electrolyte and terminating electrolyte and also the composition of the sample. Hence the reproducibility in quantitative analyses is a serious problem. For quantitative experiments at least an internal standard must be used to correct for undesirable fluctuations in the EOF and irreproducible injections. Better results can be obtained by effectively suppressing the EOF by using additives such as methylhydroxyethylcellulose. Results of quantitative experiments using the Beckman P/ACE System 2000 HPCE are presented, showing some of the problems in quantitative analyses with ITP in open capillaries.

INTRODUCTION

Generally, electrophoretic equipment can be used for all electrophoretic modes, *viz.*, for isotachophoresis (ITP), zone electrophoresis (ZE), moving boundary (MB) and isoelectric focusing (IEF). The choice of the electrolytes in the capillary and electrode compartments determines which electrophoretic mode is applicable, *e.g.*, for the ZE mode a background electrolyte and for ITP a leading and terminating electrolyte will be used.

So far, ITP has usually been carried out in laboratory-made [1] or commercially available apparatus with closed systems, *i.e.*, no electroosmotic flow (EOF) acts. At present, commercial apparatus for CZE is available, generally with open capillaries. Because this equipment can also be used for ITP, it is of interest to investigate the possibilities of ITP in open systems.

Hjerten *et al.* [2] carried out displacement electrophoresis experiments in glass capillaries, which were coated to suppress the EOF and adsorption of the solutes on the tube wall. Udseth *et al.* [3] reported experiments with tandem ITP–mass spectrometry and showed that the separation was not disturbed by the EOF. They recog-

0021-9673/91/$03.50

nized that during the analysis the velocity of the EOF is not constant and is first determined by the composition of the leading electrolyte and finally by that of the terminating solution. Thormann [4] studied the impact of electroosmosis on zone formation and displacement and described anionic and cationic separations in open capillary systems. Recently Beckers *et al.* [5] adapted a mathematical model for the calculation of parameters for the different zones in ITP without EOF, for application to ITP with EOF, and it was concluded that these models are identical because the effects of the EOF cancel each other in all equations.

Four modes can be distinguished in ITP with EOF, *viz.*, the anionic (AM) and cationic modes (CM) and the reversed-anionic (RAM) and reversed-cationic modes (RCM). For the reversed modes the isotachophoretic velocity is directed away from the detector but the isotachophoretic system has a net velocity in the direction of the detector owing to the large velocity of the EOF. In the reversed modes the components are detected in a reversed order, *i.e.*, first the terminator, then the sample components with increasing mobilities and finally the leading zone [4,5].

Whether a specific mode can be applied depends on the velocity of the ITP system and of the EOF. Generally, the velocity of the EOF changes during ITP analyses because the capillary is filled more and more with the terminating solution, which has an EOF different from that of the leading electrolyte. Therefore, the ITP system sometimes comes to a standstill if during the analyses the velocity of the EOF counteracts the isotachophoretic velocity and increases until the net migration of the ITP system becomes zero.

In this paper we present data concerning quantitative experiments and discuss some problems in quantitative analyses.

EXPERIMENTAL

For all ITP experiments in open systems the Beckman P/ACE System 2000 HPCE (Palo Alto, CA, U.S.A.) was used, applying UV detection at 254 nm for all cationic and at 214 nm for all anionic analyses. All experiments were carried out at 25°C applying a constant direct current, using an original Beckman capillary of 57 cm, with a distance between injection and detection of 50 cm and an I.D. of 75 μm. For further information concerning the apparatus, see ref. 6.

For ITP experiments in the cationic mode (CM) the capillary is filled with the leading electrolyte L, while the terminating solution T must be present at the inlet of the apparatus. The sample solution is introduced between L and T by pressure injection. The detector and cathode are placed at the outlet and the anode at the inlet. For ITP experiments in the anionic mode (AM) the cathode is placed at the inlet side and the anode at the outlet. The capillary is filled with the leading electrolyte and the terminator is present at the inlet.

For all ITP experiments with closed systems, a laboratory-built apparatus, with conductivity and UV detectors, was used as described previously [1]. In this apparatus, the closed system is obtained by shielding the separation capillary from the open electrode compartments with semipermeable membranes. Note that a PTFE capillary tube (0.2 mm I.D.) is used, in contrast to the fused-silica capillaries used in the Beckman apparatus.

RESULTS AND DISCUSSION

On applying ITP in closed systems, all ITP zones move with an equal velocity if the steady state is reached. As all zone concentrations are adapted to that of the leading zone, according to the Kohlrausch condition, a linear relationship is obtained between zone length and amount of sample injected. Another approach in quantitative determinations is to use the response factor [7], *RF* (C/mol), representing the slope of the calibration graph of the product of the zone length *l* (s) and applied constant electric current (A) *versus* the amount of sample (mol).

When using ITP in open systems, an EOF acts on the ITP system. Generally, the velocity of the EOF changes during ITP analyses because the capillary is filled with different electrolytes during the analyses as the terminator is migrating into the capillary. Thus the zones do not have the same velocity passing the detector and the detected zone length depends on the velocity of the EOF at the moment of detection. Non-linear calibration graphs are the result. In order to work quantitatively in ITP in open systems, the behaviour of the EOF must be understood.

Velocity of the electroosmotic flow

To obtain an impression of the velocity of the EOF we can fill, by pressure injection, the capillary alternately with the chosen electrolyte and a mixture of this electrolyte and a UV-absorbing component, without electrophoretic mobility, such as mesityl oxide (MO). Monitoring the absorbance of the equidistant EOF marker bands in the chosen electrolyte during the ITP experiment gives an idea of changes in the EOF velocity.

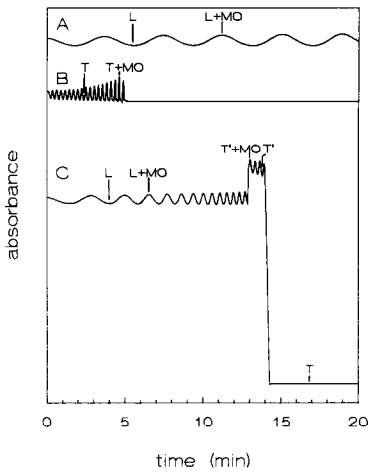

Fig. 1. Part of the electropherograms obtained by applying a constant electric current of 1.5 μA for (A) a leading electrolyte of 0.01 *M* sodium nicotinate at pH 5.4, (B) a terminating electrolyte of 0.01 *M* GABA–nicotinate at pH 5 and (C) an ITP system with a leading electrolyte L of 0.01 *M* sodium nicotinate at pH 5.4 and a terminating electrolyte T of 0.01 *M* GABA–nicotinate at pH 5. The terminating solution T′ is the modified terminating solution according to Kohlrausch's law. The electrolyte in the capillary was alternately mixed with equidistant bands of mesityl oxide (MO) in order to indicate variations in the velocity of the EOF.

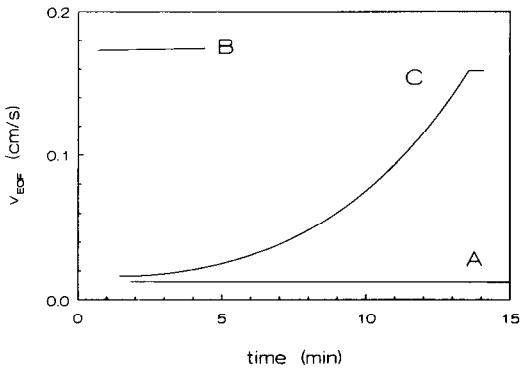

Fig. 2. Calculated velocities of the EOF, using the time intervals between the MO peaks, for the electropherograms in Fig. 1 as a function of time. The EOF velocity of (C) the ITP system varies between the values of (A) the leading and (B) the terminating electrolyte, although not linearly.

As an example to demonstrate the change in EOF during ITP experiments, in Fig. 1 the electropherograms are given for the migration in the cationic mode of (A) the leading electrolyte of 0.01 *M* NaOH at pH 5.4 adjusted by adding nicotinic acid, (B) the terminating electrolyte of 0.01 *M* γ-aminobutyric acid (GABA) at pH 5 adjusted by adding nicotinic acid and (C) the ITP experiment with the leading electrolyte sodium nicotinate and terminator GABA–nicotinate. The three experiments were carried out at a constant electric current of 1.5 μA. To visualize the change in the velocity of the EOF, we filled the capillary alternately with sodium nicotinate or GABA–nicotinate (22 s, pressure injection) and a mixture of sodium nicotinate (or GABA–nicotinate) with 0.001 *M* MO (3 s, pressure injection). From the number of MO peaks over the separation length from the inlet to the detector the distance between the equidistant peaks can be calculated and using the differences in time between the adjacent peaks the average velocity at that time can be calculated.

In Fig. 2 the calculated velocities of the EOF as function of time are given for the situations in Fig. 1. It can be clearly seen that if the capillary is filled with one electrolyte (A and B) the velocity is constant, although this velocity is much greater with GABA–nicotinate because the voltage gradient is higher at the same electric

TABLE I

VOLTAGE *V*, MIGRATION TIMES *t*, VELOCITIES *v* AND MOBILITIES $m \cdot 10^5$ OF THE EOF FOR THE LEADING ELECTROLYTE SODIUM NICOTINATE AND SEVERAL TERMINATING SOLUTIONS

Electrolyte	pH	Counter ion	*V* (kV)	*t* (min)	*v* (cm/s)	*m* (cm^2/V s)
0.01 *M* NaOH	5.4	Nicotinic acid	2.24	80.64	0.010	26.30
0.01 *M* GABA	5.0	Nicotinic acid	23.44	5.23	0.159	38.75
0.01 *M* GABA	3.5	Formic acid	2.37	119.05	0.007	16.84
0.01 *M* HIS	5.0	Nicotinic acid	3.41	59.52	0.014	23.40
0.01 *M* HIS	6.7	MES	13.89	6.46	0.129	52.94

current and the mobility of the EOF is larger owing to the low ionic concentration of the terminating solution at pH 5 (the pK value of GABA is 4.03).

If an ITP experiment is carried out (Fig. 2C) in the first instance, the velocity of the EOF is comparable to that of the original leading electrolyte L. During the analysis the terminator T is migrating into the capillary behind the leading electrolyte and the velocity of the EOF varies strongly with time until a value is reached that is comparable to that of the original terminating solution of the whole capillary if filled with that solution T. The velocity of the EOF varies during the ITP experiment, between the values for the pure leading and the terminating solutions, but this relationship is not linear. Between the leading and terminating solutions we can see the adapted terminating solution T′ according to Kohlrausch's law (Fig. 1C). The last MO peak coincides with the concentration boundary between adapted terminating T′ and original terminating T solution.

In Table I the applied voltages and migration times, velocities and calculated mobilities of the EOF are given for the leading electrolyte sodium nicotinate and several terminating solutions applied with a constant electric current of 1.5 μA. In fact the mobility of the EOF does not differ much for sodium nicotinate and GABA–nicotinate, but it must be remembered that the voltage gradients in the ITP system differ considerably between the leading and terminating solutions so that the velocity of the EOF increases very rapidly if the terminating solution fills the capillary.

From Figs. 1 and 2, it can be concluded that the velocity of the EOF changes

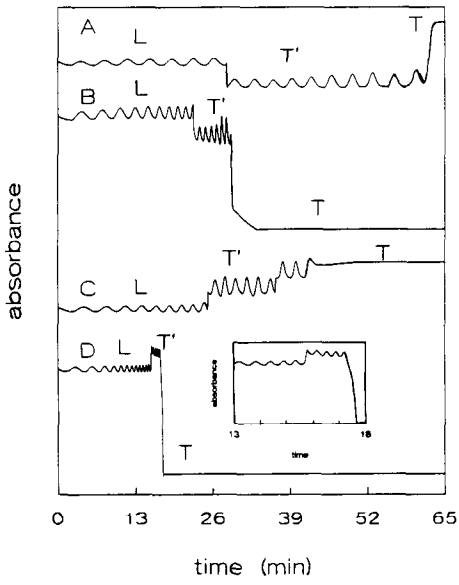

Fig. 3. Electropherograms for a leading electrolyte of sodium nicotinate at pH 5.4 applying as terminating solutions (A) 0.01 M HIS–nicotinate at pH 5, (B) 0.01 M HIS–MES at pH 6.7, (C) 0.01 M GABA–formate at pH 3.5 and (D) 0.01 M GABA–nicotinate at pH 5. Electric current, 1.5 μA. The inset shows part of the electropherogram D from 13 to 18 min. It can be clearly seen that different terminating solutions in ITP experiments can cause strong variations in the velocity of the EOF (note the varying time intervals between the MO bands), resulting in different times of analysis.

during ITP experiments because the capillary contains more than one electrolyte. Because it is essential that the EOF is constant during detection in order to carry out quantitative ITP, we considered further the influence of both the effect of the composition of the terminating and sample solutions and the effect of an EOF-suppressing additive, in order to choose an optimum ITP system.

Effect of different terminators. The variation in EOF is caused by the presence of more than one solution migrating in the capillary. To demonstrate what the effect of different terminating solutions is, in Fig. 3 the electropherograms (CM) are given for a leading electrolyte of 0.01 *M* sodium nicotinate at pH 5.4, alternately mixed with 0.001 *M* MO, and applying as the terminator (A) 0.01 *M* histidine (HIS) at pH 5 adjusted by adding nicotinic acid, (B) 0.01 *M* histidine at pH 6.7 adjusted by adding 2-(N-morpholino)ethanesulphonic acid (MES), (C) 0.01 *M* GABA at pH 3.5 adjusted by adding formic acid and (D) 0.01 *M* GABA at pH 5 adjusted by adding nicotinic acid. In Fig. 4 all calculated velocities of the EOF as a function in time are given.

It can be clearly seen that by applying (A) a terminating solution of histidine (pK = 6.04) with a relatively high mobility at a pH at which a high ionic concentration is present (pH < pK), a nearly constant velocity of the EOF can be obtained during the ITP experiment (see Fig. 4A). For (B) with the same terminator at low ionic concentration (pH > pK), the voltage gradient in the original solution will be very high, causing a high end-velocity of the EOF and thus a strong variation in the velocity of the EOF during the analysis (see Fig. 4B). On carrying out similar experiments with the terminator GABA (pK = 4.03) at pH (C) 3.5 (pH < pK) and (D) 5 (pH > pK), similar effects can be obtained as with histidine as the terminator. On applying the terminator GABA–formate at pH 3.5, a step in the T′ zone could be observed. Repeating this experiment applying GABA–nicotinate at pH 3.5 also gave a double T′ zone. Although not understandable, it might be a moving pH boundary between

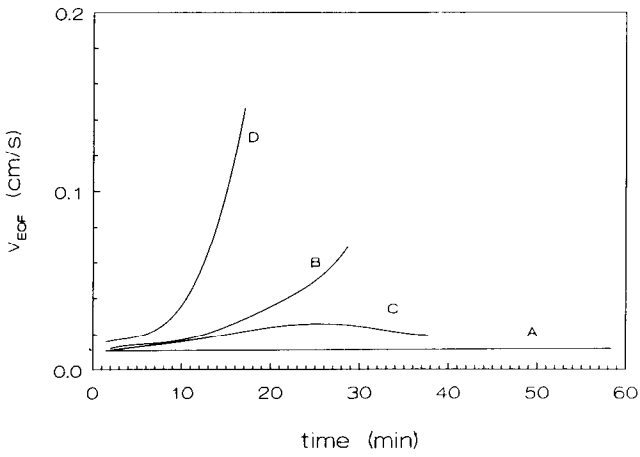

Fig. 4. Calculated velocities of the EOF, using the time intervals between the MO peaks, for the electropherograms in Fig. 3 as a function of time. The (B,D) EOF velocity of the ITP system varies strongly if the pH of the terminator is higher than the pK value of the terminator (low ionic concentrations and thus a high EOF) compared with (A,C) terminator solutions at a pH < pK.

the adapted T′ zone and the original terminating solution T as suggested by Hirokawa *et al.* [8].

From Fig. 3 it can clearly be concluded that using terminators with a low effective mobility and/or at low ionic concentration, strongly varying values of the EOF are the result. It should be remembered that sample ionic species will be found between the L and T′ zones, and that EOF velocities at that moment determine whether reproducible quantitative determinations are possible. The time of analysis also varies strongly depending on the terminator applied.

Effect of sample solutions. To demonstrate the effect of sample solutions on the velocity of the EOF during ITP experiments, we used the leading electrolyte 0.01 *M* sodium nicotinate at pH 5.4 and the terminating electrolyte 0.01 *M* HIS–nicotinate at pH 5, for which a fairly constant EOF can be expected.

In Fig. 5 the electropherograms (CM) obtained by injecting a sample of 0.0025 *M* lithium nitrate with increasing pressure injection times are given. It is clear that the injection of large sample zones causes strongly increasing EOF velocities, so that the migration times decrease substantially and the results of quantitative analyses are erroneous. This effect will be much greater when using sample solutions at a lower ionic strength. It should be remembered that the velocity of the EOF increases strongly because the mobility of the EOF is larger at low ionic strength and the voltage gradient over the original diluted sample zone is much larger.

Suppressed EOF. From the foregoing, it can be concluded that the velocity of the EOF varies strongly with the compositions of the terminating and sample solutions. Varying EOF velocities cause irreproducible migration times and zone lengths and hence the results of quantitative determinations are erroneous. To work quantitatively with ITP in open systems in an appropriate way, the velocity of the EOF must be controlled or eliminated.

It is well known that the EOF can be suppressed by adding surface-active substances to the electrolyte system, such as methylhydroxyethylcellulose (MHEC). A problem is that if MHEC is used as an EOF suppressor, the MO peaks in the leading electrolyte cannot be used as an EOF indicator as they no longer move.

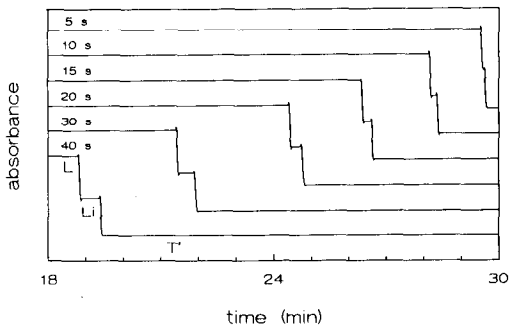

Fig. 5. Electropherograms with a leading electrolyte (L) of 0.01 *M* sodium nicotinate at pH 5.4 and a terminator of 0.01 *M* HIS–nicotinate at pH 5 for several pressure injection times of a solution of 0.0025 *M* lithium nitrate (Li). Increasing zone lengths of the introduced sample result in decreasing times of analysis owing to an increasing velocity of the EOF. Electric current, 1.5 μA.

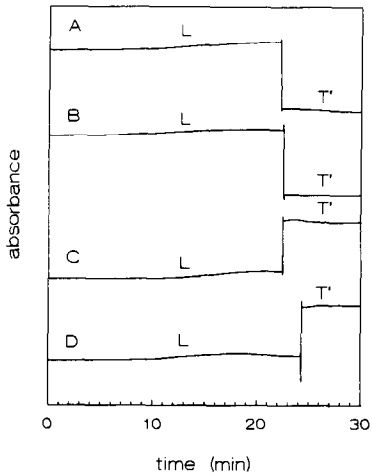

Fig. 6. Electropherograms for a leading electrolyte of sodium nicotinate at pH 5.4 applying as terminating solutions (A) 0.01 *M* HIS–nicotinate at pH 5, (B) 0.01 *M* HIS–MES at pH 6.7, (C) 0.01 *M* GABA–formate at pH 3.5 and (D) 0.01 *M* GABA–nicotinate at pH 5. To all solutions 0.05% of MHEC was added. It can be clearly seen that, compared with the electropherograms in Fig. 3, different terminating solutions in ITP experiments with suppressed EOF show a fairly constant velocity of the EOF, resulting in reasonably constant migration times. Electric current, 3 μA.

However, the migration times for a leading electrolyte in ITP should be constant, independent of the choice of the terminator. In Fig. 6 the electropherograms for the same electrolyte systems as used in Fig. 3 are given, but 0.05% of MHEC was added to all solutions. Although the migration times are not identical they are fairly similar, indicating that the EOF is nearly suppressed, *i.e.*, the velocity of the EOF must be constant, resulting in, however, longer times of analysis (note that only the adapted T′ zones are detected).

Another possibility to check the effect of MHEC on the EOF is the relationship between the voltage over the capillary and time of analysis. These relationships are given in Fig. 7A for the electrolyte systems used for Fig. 3 (different terminators without MHEC) and in Fig. 7B for the electrolytes systems used for Fig. 6 (the same terminators with MHEC).

It can be clearly seen that with addition of MHEC (Fig. 7B) linear relationships are obtained, in contrast to those obtained without MHEC (Fig. 7A), especially for cases B and D. The decrease in case C (Fig. 7A) corresponds to the migration of the unmodified terminator solution into the capillary. In the next section, the results of quantitative experiments, applying several electrolyte systems, are compared with and without the addition of MHEC.

Quantitative analysis

Although the UV detector of the Beckman apparatus is not a universal detector, it can be used in a reasonably universal way in ITP by applying a UV-absorbing counter ionic species because the concentration of the sample ions differ considerably according to Kohlrausch's law. Owing to the electroneutrality equation the concen-

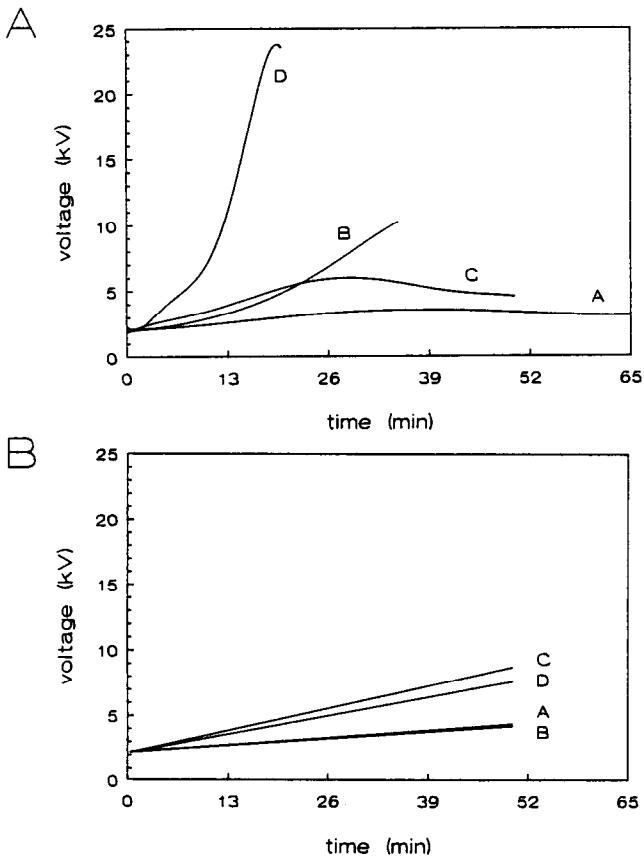

Fig. 7. Relationship between voltage drop over the capillary tube and time of analysis (A) for the electrolyte systems used for Fig. 3, without MHEC, and (B) the same electrolyte systems with MHEC as used in Fig. 6. All data are recalculated to an electric current of 1.5 μA. With the additive MHEC linear relationships are obtained in all instances.

tration of the buffering counter ions also varies from zone to zone, causing different UV absorbances. The same principle can be used in CZE applying a UV-absorbing carrier ion [9].

In Fig. 8 the electropherograms (CM) obtained using a laboratory-made ITP apparatus with (A) a conductivity and (B) a UV detector and (C) using the Beckman apparatus with a UV detector are given. The leading electrolyte was 0.01 *M* sodium nicotinate at pH 5.4 and the terminating electrolyte was 0.01 *M* GABA–nicotinate at pH 5. These electropherograms show clearly that generally the UV detector is applicable for non-UV-absorbing components. Note the similarity between the two UV signals.

Effect of suppressed EOF in quantitative ITP. In order to study the effect of suppressing EOF in ITP in open systems, we measured the zone lengths *versus* the amount of sample by varying the pressure injection time for a concentration of $2.5 \cdot 10^{-3}$ and $1 \cdot 10^{-2}$ *M* of histidine with the leading electrolyte 0.01 *M* sodium nicotin-

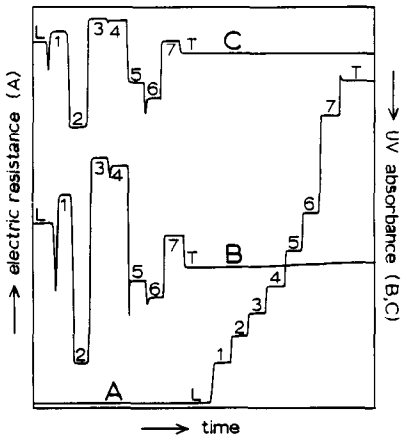

Fig. 8. Electropherograms obtained with (A) a conductivity detector and (B) a UV detector with a laboratory-made ITP apparatus with a closed system and (C) a UV detector with the Beckman P/ACE System 2000 HPCE. The leading electrolyte was 0.01 *M* sodium nicotinate at pH 5.4 and the terminator was 0.01 *M* GABA–nicotinate at pH 5. The electric current was 1.5 μA for the Beckman and 5.5 μA for the laboratory-made ITP instrument. Note the similarity between the two UV signals. The sample composition was 0.00125 *M* of (1) lithium, (2) Girard reagent P, (3) TRIS, (4) HIS, (5) creatinine, (6) *o*-phenylenediamine and (7) ε-aminocaproic acid. Injection, 1 μl for the laboratory made ITP instrument and 5 s pressure injection for the Beckman apparatus.

ate at pH 5.4 using the terminators 0.01 *M* GABA–formate and 0.01 *M* GABA–nicotinate at pH 3.5 and 5, respectively, without and with the addition of 0.05% of MHEC to all electrolyte solutions. All experiments were carried out with an electric current of 1.5 μA.

In Fig. 9A all measured zone lengths as a function of the sample amount (in *M* s units, *i.e.*, molarity multiplied by injection time in seconds) are given without the addition of MHEC. The zone lengths using the terminator GABA–nicotinate at pH 5 are much shorter than those of GABA–formate at pH 3.5 owing to its higher EOF velocity (effect of the low ionic concentration in the original terminating solution). For both terminating solutions the zone lengths decrease considerably with the low concentrations of histidine applying longer injection times, owing to the sample solution effect on the EOF velocity.

It can be concluded that the influence of the composition of both the terminating solutions and the sample solutions on the EOF velocity makes quantitative analyses difficult.

In order to compare the effect of the EOF *versus* the suppressed EOF in quantitative ITP, we repeated all experiments after adding 0.05% of MHEC to all electrolyte solutions. In Fig. 9B the measured zone lengths as a function of the amount of sample are given for these experiments.

A comparison between Fig. 9A and B shows that the zone lengths for $1 \cdot 10^{-2}$ *M* histidine for both terminators with MHEC are much longer than those for the systems without MHEC. The zone lengths for $2.5 \cdot 10^{-3}$ *M* histidine are longer than those for the systems without MHEC, although for GABA at pH 5 not quite a linear relationship could be obtained, identical with those for the $1 \cdot 10^{-2}$ *M* solutions.

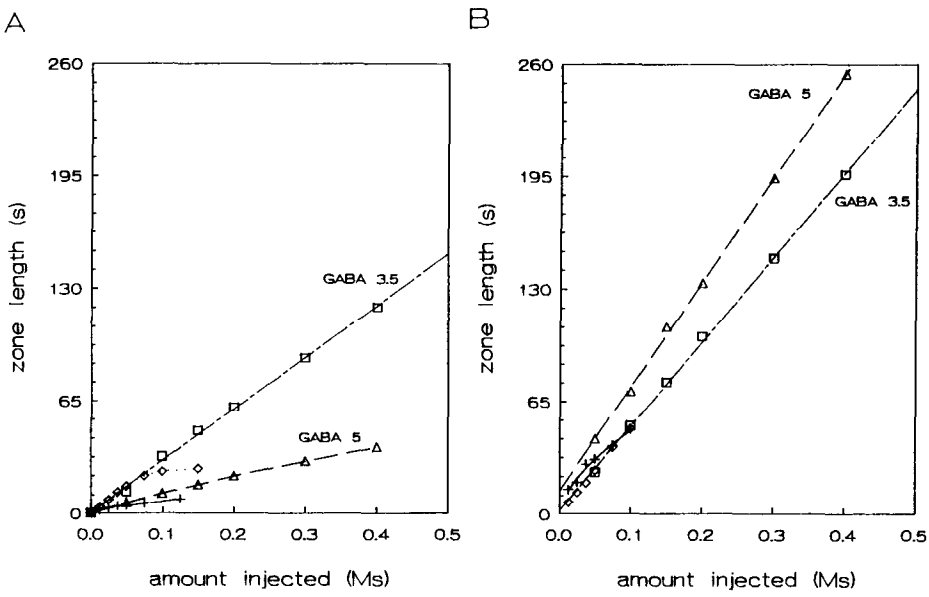

Fig. 9. Relationship between measured zone length (s) and injected amount of histidine (M s) using the terminator 0.01 M GABA at pH 3.5 by adding formic acid and a sample of (□) 0.01 M and (◇) 0.0025 M histidine and the terminator 0.01 M GABA at pH 5.0 by adding nicotinic acid with a sample of (△) 0.01 M and (+) 0.0025 M histidine, (A) without and (B) with the addition of 0.05% of MHEC to all electrolyte solutions.

From Figs. 9A and B it can be concluded that although the effect of the composition of the terminating solutions on the EOF by the addition of MHEC can be suppressed for the greater part, the influence of the ionic strength of the sample solution cannot be eliminated completely.

Reproducibility in quantitative ITP in open systems. In order to demonstrate the reproducibility of quantitative ITP in open systems, we determined five times a calibration graph (CM) for lithium, tris(hydroxymethyl)aminomethane (TRIS), HIS and ε-aminocaproic acid (EAC) in sodium nicotinate at pH 5.4, applying three different terminators, *viz.*, (A) GABA–nicotinate at pH 5, (B) GABA–formate at pH 3.5 and (C) acetic acid at pH 3, injecting six different amounts of the mixture of the sample components with pressure injection times of 5, 10, 15, 20, 30 and 40 s. To all solutions 0.05% of MHEC was added. Data for the calibration graphs are given in Table II.

Although all the calibration graphs were fairly linear (the average regression coefficient is 0.9989), the absolute values of the slopes differ by up to about 12%, and large differences arise for the systems with different terminators. If we calculate the slope relative to that of histidine, the differences are much smaller. For example, the first two values of the slope of the calibration graph for lithium in system A are 870.94 and 934.11 whereas the relative slopes are 1.263 and 1.264.

Two important conclusions can be drawn from Table II. First, by applying ITP in open systems absolute values of zone lengths can never be handled and it is essential to work with at least an internal standard. An extra advantage is that by applying an internal standard, the fluctuations in injected volume are eliminated. Second, the

TABLE II

SLOPES OF CALIBRATION GRAPHS FOR LITHIUM, TRIS, HIS AND EAC AND AVERAGE SLOPE AND STANDARD DEVIATIONS OF THESE VALUES AND THOSE FOR SLOPES RELATIVE TO HISTIDINE

Leading electrolyte, 0.01 *M* sodium nicotinate (pH 5.4); terminating electrolyte, (A) 0.01 *M* GABA–nicotinate (pH 5.0), (B) 0.01 *M* GABA–formate (pH 3.5), (C) acetic acid (pH 3). To all electrolytes 0.05% of MHEC was added. Constant current, 1.5 μA. All calibration graphs were determined by pressure injection of 5, 10, 15, 20, 30 and 40 s of the sample solution (all ionic concentrations were 0.002 *M*).

Sample	Slope (arbitrary units)			Relative slope		
	A	B	C	A	B	C
Lithium	870.94	442.50	464.03	1.263	0.833	0.861
	934.11	435.44	477.18	1.264	0.854	0.850
	911.06	458.32	454.26	1.279	0.839	0.846
	946.03	461.53	459.68	1.360	0.860	0.872
	876.85	456.56	452.03	1.320	0.860	0.854
Average	907.80	450.87	461.44	1.297	0.849	0.857
S.D.	29.90	10.10	8.92	0.038	0.011	0.009
TRIS	730.82	509.18	530.38	1.060	0.959	0.984
	789.47	499.03	544.62	1.068	0.979	0.970
	754.09	525.41	507.03	1.059	0.962	0.944
	769.82	501.53	491.59	1.106	0.934	0.932
	707.29	518.09	505.50	1.065	0.975	0.955
Average	750.73	510.65	515.82	1.072	0.962	0.957
S.D.	28.84	9.94	19.04	0.018	0.016	0.019
HIS	689.47	531.06	538.82	1.000	1.000	1.000
	739.18	509.79	561.32	1.000	1.000	1.000
	712.38	546.12	537.24	1.000	1.000	1.000
	695.79	536.82	527.41	1.000	1.000	1.000
	664.12	531.15	529.24	1.000	1.000	1.000
Average	700.19	530.99	538.81	1.000	1.000	1.000
S.D.	24.92	11.93	12.09			
EAC	678.79	540.21	563.38	0.985	1.017	1.046
	723.06	505.94	577.24	0.978	0.992	1.028
	690.62	569.32	574.59	0.969	1.042	1.070
	681.91	540.44	550.06	0.980	1.007	1.043
	657.74	561.35	551.68	0.990	1.057	1.042
Average	686.42	543.45	563.39	0.981	1.023	1.046
S.D.	21.27	21.98	11.24	0.007	0.023	0.013

use of different terminators can give totally different relative slopes, although reproducible with time. For further experiments we used as the terminator acetic acid at pH 3.

Quantitative ITP in open systems. In order to study the usefulness of the relative slopes in quantitative ITP, we determined the relative slopes for several ionic species in both a cationic and an anionic ITP system. Further, we compared the determined relative slopes in open systems with those in closed systems and theoretical values.

TABLE III

THEORETICAL VALUES OF SLOPES AND EXPERIMENTALLY DETERMINED VALUES FOR ITP IN CLOSED SYSTEMS AND OPEN SYSTEMS (IN DUPLICATE) FOR SEVERAL CATIONIC COMPONENTS IN COMPLEX MIXTURES RELATIVE TO HISTIDINE

Leading electrolyte 0.01 *M* KOH at pH 5.4 adjusted by adding nicotinic acid; terminator, acetic acid at pH 3; both containing 0.05% of MHEC; constant current, 3 μA.

Sample	Theoretical	Closed	Open (1)	Open (2)
Barium	1.377	1.321	1.349	1.315
Creatinine	0.888	0.888	0.897	0.881
EAC	1.084	1.066	1.025	1.006
GABA	1.135	1.000	1.019	1.039
HIS	1.000	1.000	1.000	1.000
Lithium	0.836	0.863	0.866	0.855
OPDA[a]	–	0.940	0.906	0.922
Sodium	0.740	0.713	0.685	0.643
TRIS	1.005	0.993	0.986	0.976

[a] *o*-Phenylenediamine.

In Table III all values are given for cationic species determined with a leading electrolyte of 0.01 *M* KOH at pH 5.4 adjusted by adding nicotinic acid and the terminator acetic acid at pH 3, both containing 0.05% of MHEC. MHEC was also added to all sample solutions. The calibration graphs were measured by injecting five different amounts of the sample components with pressure injection times of 10, 20, 30, 40 and 50 s and an applied direct current of 3 μA.

From Table III, it can be concluded that ITP in open systems can be applied in a reproducible way for cations, although special care must be taken in the choice of the electrolyte system. We also carried out numerous experiments with GABA at pH 5 and 3.5 as terminator and here the relative slopes varied much more with time, although the regression coefficients were good.

More problems with the reproducibility were encountered on applying an anionic system, because the systems in the AM seem to be much more sensitive to fluctuations in EOF.

In Table IV all values are given for anionic species with the leading electrolyte 0.01 *M* HCl at pH 6 adjusted by adding histidine and the terminator 0.02 *M* HIS at pH 6.43 adjusted by adding MES, both containing 0.05% of MHEC. With the addition of MHEC the anionic species could be measured in the normal AM. We already discussed earlier [5] that without the addition of MHEC most anionic systems do not move in the anionic mode owing to the counter action of the EOF.

To illustrate the importance of suppression of the EOF caused by the sample composition, we give in Table IV the theoretical relative slopes, the experimentally determined relative slopes in closed systems and those for open systems (in duplicate) for (A) an anion mixed with the standard without MHEC, (B) mixtures of several anions without MHEC and (C) mixtures of anions with MHEC. The calibration graphs were measured by injecting five different amounts of the sample components with pressure injection times of 10, 20, 30, 40 and 50 s.

From Table IV, it can be concluded that most values for the closed system fit

TABLE IV

THEORETICAL VALUES OF SLOPES AND EXPERIMENTALLY DETERMINED VALUES FOR ITP IN CLOSED SYSTEMS AND OPEN SYSTEMS (IN DUPLICATE) FOR SEVERAL ANIONIC COMPONENTS (A) MIXED WITH THE STANDARD AND (B) IN COMPLEX MIXTURES AND (C) IN COMPLEX MIXTURES WITH MHEC RELATIVE TO FORMATE

Leading electrolyte, 0.01 *M* HCl at pH 6.0 adjusted by adding histidine + 0.05% MHEC; terminating electrolyte, 0.01689 *M* histidine at pH 6.43 adjusted by adding MES + 0.05% MHEC. Constant current, 3μA; wavelength of UV detector, 214 nm.

Sample	Theoretical	Closed	Open					
			A	A	B	B	C	C
Acetate	1.12	1.16	1.16	1.08	1.06	1.06	0.97	1.01
Adipate	2.09	2.06	1.94	1.98	2.16	2.16	2.20	2.07
Benzoate	1.25	1.28	1.34	1.35	1.37	1.49	1.28	1.28
Benzylaspartate	1.44	1.58	1.77	1.78	2.03	2.29	1.79	1.71
Chlorate	0.95	0.99	0.99	1.01	1.15	1.19	0.99	0.99
Enanthate	1.37	1.41	1.40	1.54	1.42	1.68	1.47	1.55
Formate	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Malonate	1.80	1.80	1.82	1.89	1.90	2.02	1.88	1.54
Propionate	1.19	1.21	1.26	1.29	1.09	0.98	1.14	1.18

the theoretical values reasonably well. The measured values (A) for anionic components mixed with the standard formate are not too bad, although that for benzyl aspartate is too high. The measured values (B) for mixtures of several anionic components are often too high. It must be remembered that although 0.05% of MHEC was added to the leading and terminating electrolytes in order to suppress the EOF, no MHEC was added to the sample mixtures. For small injected amounts it can be expected that the effect of the sample on EOF will be minor but especially long injection times with dilute samples can cause serious variations in the EOF. Because the EOF is oppositely directed to the ITP migration velocity, longer sample zones can be expected. The measured values (C) for mixtures with MHEC are much better.

CONCLUSIONS

It is concluded that although the UV detector is a selective detector, it can also be used for non-UV-absorbing components using a UV-absorbing counter ion. In contrast to ITP in closed systems, in open systems an EOF will act on the ITP system causing an extra displacement of the system. The velocity of the EOF changes continuously if the capillary contains more than one electrolyte. Terminating and sample ions with low mobility, especially at a low ionic concentration, accelerate the EOF considerably, so that quantitative analyses are meaningless.

The addition of MHEC to the electrolyte solutions suppresses the EOF for the greater part, so that linear relationships between sample amounts and zone lengths can be obtained. In spite of the addition of MHEC, the reproducibility of the zone lengths with time is poor and depends on the 'state' of the capillary, an internal standard is necessary for quantitative analyses. Different terminating electrolytes can cause differences in relative slopes.

It can be stated that quantitative ITP in open systems is only possible if precautions are taken to suppress the EOF in an effective way. The addition of, *e.g.*, MHEC to the sample solution is very important, especially in the AM. In the AM the reproducibility seems to be more troublesome. The use of other EOF-suppressing agents can probably give better results. Generally, closed systems are to be preferred to open systems for quantitative ITP.

ACKNOWLEDGEMENT

The authors express their gratitude to the State Institute for Quality Control of Agricultural Products (RIKILT, The Netherlands) for financial support of this investigation.

REFERENCES

1 F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis–Theory, Instrumentation and Applications* (*Journal of Chromatography Library*, Vol. 6), Elsevier, Amsterdam, 1976.
2 S. Hjerten, K. Elenbring, F. Kilar, J. Liao, A. J. C. Chen, C. J. Siebert and M. Zhu, *J. Chromatogr.*, 403 (1987) 47.
3 H. R. Udseth, J. A. Loo and R. D. Smith, *Anal. Chem.*, 61 (1989) 228.
4 W. Thormann, *J. Chromatogr.*, 516 (1990) 211.
5 J. L. Beckers, F. M. Everaerts and M. T. Ackermans, *J. Chromatogr.*, 537 (1991) 429.
6 V. P. Burolla, S. L. Pentoney and R. Zare, *Am. Biotechnol. Lab.*, 7(10) (1989) 20.
7 J. L. Beckers and F. M. Everaerts, *J. Chromatogr.*, 470 (1989) 277.
8 T. Hirokawa, K. Nakahara and Y. Kiso, *J. Chromatogr.*, 463 (1989) 51.
9 J. L. Beckers, F. M. Everaerts and M. T. Ackermans, *J. Chromatogr.*, 537 (1991) 407.