Journal of Chromatography, 91 (1974) 785–794

CHROM. 7148

ANALYTICAL ISOTACHOPHORESIS

RESOLUTION, DETECTION LIMITS AND SEPARATION CAPACITY IN CAPILLARY COLUMNS

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SUMMARY

The instrument used for analytical isotachophoresis in these experiments was the LKB 2127 Tachophor. The addition of a detergent greatly increased the sharpness of the zone boundaries. The resolution of the UV detector was found to be approx. 0.1 mm zone length, corresponding to a volume of 16 nl. With leading ion concentrations of 10 m*M* and 0.5 m*M*, the detection limits were about 25 and 1–2 pmoles ATP, and the correct UV absorbance levels were reached at about 125 and 50 pmoles ATP, respectively.

Current and temperature had little influence on the separation capacity. The separation capacity as a function of capillary length, sample volume and counter-flow of leading electrolyte was also studied.

INTRODUCTION

It has been ten years since the first isotachophoretic work in a capillary column with a detector arrangement was published by Konstantinov and Oshurkova[1]. Some years later Everaerts and colleagues began publishing their work on different types of equipment[2,3].

In the present work, a newly developed instrument has been used, the LKB 2127 Tachophor, which has both a UV detector and a thermal detector, the latter with a differentiating device. Some basic properties of this instrument have been investigated, namely the resolution of the zone boundaries and the minimal sample amount detectable as well as the influences of temperature, current, capillary length and counter-flow on the separation capacity. The basic principle and construction of the Tachophor equipment have been described elsewhere[4,5].

EXPERIMENTAL

The isotachophoretic equipment used was the LKB 2127 Tachophor (LKB-Produkter, Bromma, Sweden). There are four standard capillary columns of different lengths (23, 43, 61, and 80 cm) which were used in different series of experiments.

The counter-flow pump was a Sage Model 355 syringe pump (Sage Instruments, New York, N.Y., U.S.A.).

All chemicals used were of analytical grade and used without further purification, except for Triton X-100 and polyvinyl alcohol, which were purified with a mixed-ion exchanger for the low-concentration experiments.

RESULTS AND DISCUSSION

Zone boundaries and resolution

In isotachophoresis the sample ions separate according to their net mobilities and form discrete zones with concentrations that are constant in time and homogeneous throughout each zone. During this steady state all sample ions move with the same velocity as the leading ion[6]. The boundaries between the zones are actively sharpened by an electric field that increases stepwise to compensate for the successively less mobile ions. This sharpening effect will counteract the distortion caused by diffusion and heat convection. As there will be equilibrium between these effects for each experimental set of conditions, there will be a finite sharpness at each boundary. Its width may increase from some hundredths of a millimetre towards infinity as the mobility difference over the boundary decreases towards zero. To ensure maximal theoretical detectability, the slit width of the UV detector should consequently be in the range of a few hundredths of a millimetre. Lamps and detectors available today, however, will not be able to give an acceptable signal-to-noise ratio with such a slit and therefore the slit width of the Tachophor was chosen at 0.2 mm. This means that a zone in the capillary shorter than 0.2 mm will not reach its full absorbance height on the recorder chart.

It has been found that the boundary sharpness is increased when a non-ionic detergent is present in the electrolytes in the column. In cooperation[7], it was shown that 0.05–0.2% Triton X-100 remarkably increases the sharpness of the zones. This can be seen in Fig. 1, which depicts two identical experiments without and with Triton X-100. There are four narrow UV-absorbing zones in Fig. 1b which are not sharpened enough to be detected in Fig. 1a. Further, it has been found[7] that polyvinyl alcohol has the same influence as Triton X-100. The effect of these detergents lasts for several experiments after the detergent is excluded from the leading electrolyte. A rinsing volume of 50–75 ml is needed to eliminate this effect (which indicates that a wall interaction is present).

The critical micelle concentration of Triton X-100 is reported[8] to be very low, 0.01%, which is lower than the concentrations mentioned above. The action of the detergent must be to decrease the electroendosmosis. The Teflon in the capillary is completely pure, did not show any trace of metals by emission spectral analysis, and had a smooth inner surface when examined by scanning electron microscopy.

Preliminary experiments in methanol and ethanol show no improvement in boundary sharpness by the use of Triton X-100.

It is known from many experiments that frequently very small amounts of unknown components, "markers"[9], show up as narrow zones with the UV detector (*cf.* Fig. 1b), some absorbing and some not. On the other hand, as there are always concentration steps at the zone boundaries, there are also steps in refractive index which can be used for detection[1,10] purposes. There might also be a possibility that these

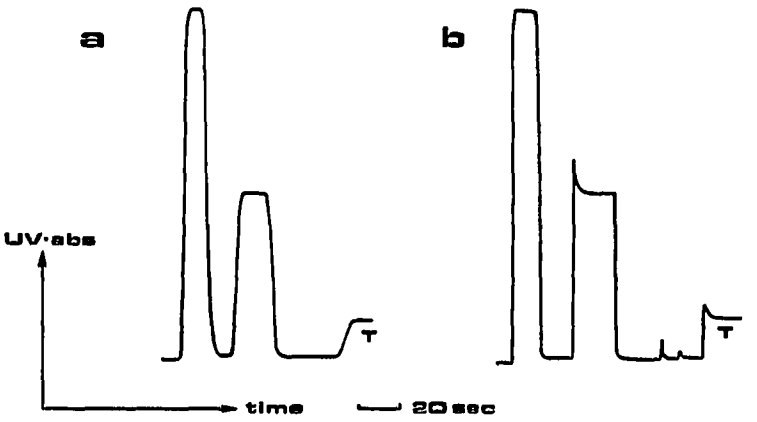

Fig. 1. Experiments (a) without or (b) with the addition of Triton X-100 to the leading electrolyte. An obvious improvement of the separation is obtained with this detergent. Leading electrolytes: 0.01 *M* HCl and 0.02 *M* histidine solution, at pH 6.0. In (b) 500 mg/l of Triton X-100 were added. Terminating electrolyte (T): 0.01 *M* phenyl acetate. The samples were approximately 0.5 μl of a 0.01 *M* solution of chromate, malonate, pyrazole-3,5-dicarboxylate, adipate, acetate and β-chloropropionate (in order of appearance). It also contained sulphate and chlorate in front of the chromate, without UV absorbance. The last three sample zones can be seen in (b) separated by two UV-absorbing markers.

steps produce diffraction effects that show up as markers in the UV pattern. However, the markers seen in our experiments are actually real zones, as can be proved from Fig. 2, where the separated zones of four sample ions are shown. In Fig. 2a there are four UV-absorbing markers (1–4) to be seen. When D is excluded from the sample (Fig. 2b), marker 3 remains but moves closer to marker 4. When B also is absent (Fig. 2c), marker 2 is found between A and C where there was no marker before, and marker 1 is no longer present. This indicates that marker 1 was an impurity present in the adipic acid sample. Marker 2 should actually be expected to show up as a shoulder on the boundary between A and C, lower than the A level. One explanation of the height of marker 2 is that as the mobilities of C and B (in Figs. 2a and b) differ less than the mobilities of C and A (in Fig. 2c) marker-zone 2 will have sharper boundaries in the latter case, thus reaching a higher concentration and absorbance. If then C is also excluded from the sample, all markers fall close to each other, as in Fig. 2d. In Fig. 2e the chart speed is increased four-fold to 12 cm/min and the three remaining markers 2, 3 and 4 are seen clearly together with 2′ and 3′, which must be markers of no or very low UV absorbance. This complete behaviour of the markers indicates that they cannot be explained as anything but real zones.

These results, however, cannot totally exclude a refractivity influence. On the other hand, experience from isotachophoretic experiments of an analogous type, where no markers were present, and also the shape of the UV cell, speak strongly against such an influence.

The resolution of the UV detector can be evaluated from Fig. 2e. The shortest marker distances, 2′–3 and 3′–4, correspond to 1.2 sec, or 0.12 mm in the column under the prevailing experimental conditions. Therefore, the resolution can be estimated as a 0.10-mm zone length. It must be pointed out, however, that the resolu-

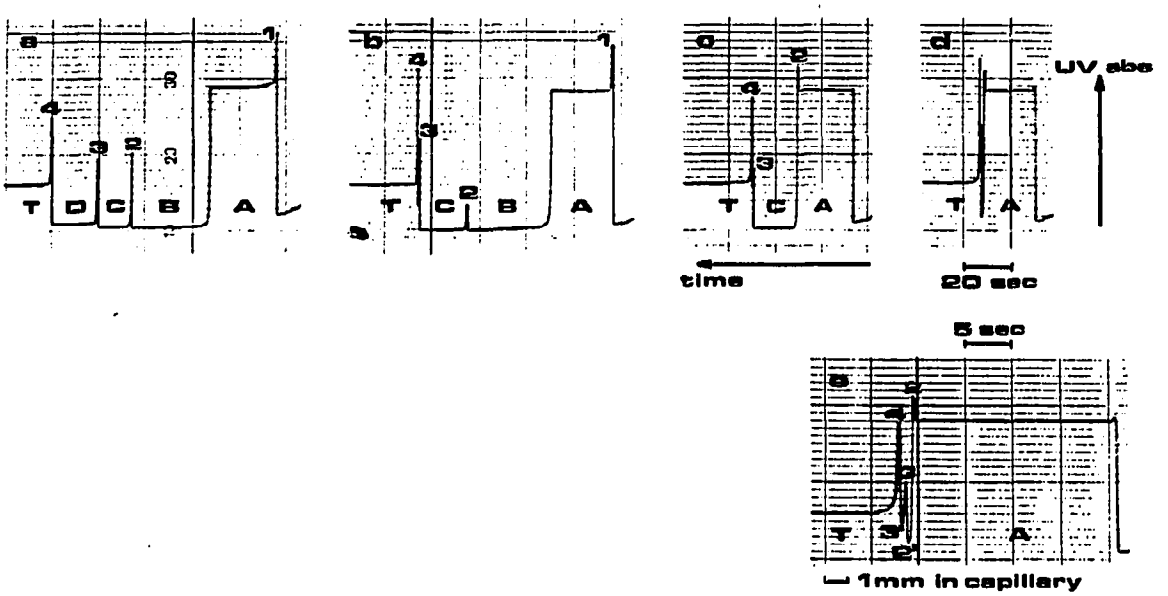

Fig. 2. Separations of four anions together with markers. Leading electrolyte: 0.01 *M* HCl, 0.02 *M* histidine and 0.1 % polyvinyl alcohol, pH 6.0. Terminating electrolyte (T): 0.01 *M* phenylacetic acid. A = maleinate, B = adipate, C = acetate, D = β-chloropropionate (*ca.* 10 nmoles each). Current, 100 μA. Temperature, 20°. Capillary length, 61 cm. Time, 25 min. For further explanation, see text. (Note that the time axis is reversed compared to the other figures.)

tion value depends upon the UV absorbance and mobilities of the adjacent zones. The resolution might be even better in more favourable cases.

In isotachophoresis, the concentration and pH of the leading electrolyte control all concentrations and pH values in the succeeding zones as a function of the mobilities of the ions involved[11]. The concentration of acetate in its zone in Fig. 2 was calculated[12] to be 0.0082 *M*. Since the volume of a 0.10-mm-long zone is about 16 nl (the I.D. of the capillary is 0.45 mm) the resolution limit for a univalent ion of net mobility close to that of acetate will be 130 pmoles.

Detection limits at common electrolyte concentrations

In order to find the minimal detectable sample amount a series of experiments was performed with ATP as the strongly UV-absorbing sample, as shown in Fig. 3. When the leading ion concentration was 0.01 *M*, which is usually the case, the minimal amount needed for detection by the UV detector was found to be about 25 pmoles. The electrolyte system was somewhat impure, as seen from the run without any ATP. (The terminator zones in Fig. 3 show a UV absorbance which, however, was not due to the capronate ions but to the UV-absorbing counter-ion creatinine[13].)

At about 125 pmoles the full UV height of the ATP zone is obtained, *i.e.* when the UV absorbance has reached a level independent of sample amount (see Fig. 3). The ATP zone can be calculated (see the last footnote to Table I) to have a pH of 4.3 with a 2.8 m*M* ATP concentration. Theoretically, the full UV absorbance should be reached for 0.2 mm zone length (*i.e.* 32 nl), which consequently corresponds to about

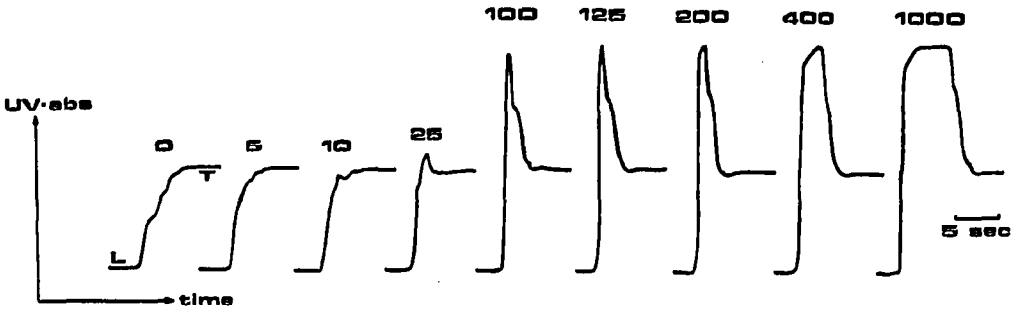

Fig. 3. Small sample amounts of ATP (in pmoles) as detected by the UV detector at the common 0.01 *M* concentration of leading ion. The leading electrolyte (L) was 0.01 *M* HCl, 0.012 *M* creatinine and 0.2 % Triton X-100, at pH 4.10. Terminating electrolyte (T): 0.01 *M* caproic acid. Current 75 μA, 1 to 5 kV in a 23-cm capillary, time 20 min.

90 pmoles. This is related to infinitely sharp zone boundaries, which means that there was a good agreement with the 125 pmoles found.

Detection limits at lower electrolyte concentrations

According to isotachophoretic theory, dilution of the leading electrolyte will also cause a dilution of all the sample zones. This in turn means a corresponding increase in zone length and consequently in sensitivity.

A number of experiments were made with a twenty-fold dilution of the leading electrolyte, *i.e.* the leading ion concentration was 0.5 m*M*. The results are shown in Fig. 4 for two different electrolyte systems. A few picomoles of ATP can be detected. The correct level of UV absorbance is reached at about 50 pmoles.

For zones of full UV absorbance the zone lengths should be twenty times longer for the sample amount shown in Fig. 4b than that in Fig. 3. As the experimental conditions differed and complicated the comparison, the calculations for the two experiments are summarized in Table I. The factor of 20 is introduced in the table in

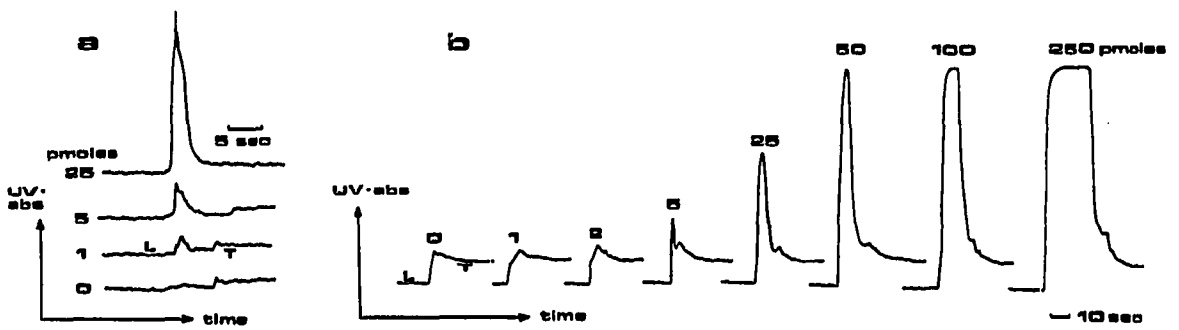

Fig. 4. Small sample amounts of ATP (in pmoles) as detected by the UV detector with the leading electrolyte diluted 20:1. (a) Leading electrolyte (L): 0.5 m*M* HCl, 1 m*M* histidine and 0.01 % Triton X-100, at pH 5.98. Terminating electrolyte (T): 0.5 m*M* phenylacetic acid. Current 10 μA, 8 to 20 kV in a 61-cm capillary, time 13 min. (b) Leading electrolyte (L): 0.5 m*M* HCl, 0.6 m*M* creatinine and 0.01 % Triton X-100, at pH 4.36. Terminating electrolyte (T): 0.5 m*M* caproic acid. Current 5 μA, 1 to 4.6 kV in a 23-cm capillary, time 15 min.

TABLE I

COMPARISON OF ZONE LENGTHS AT TWO DIFFERENT CONCENTRATIONS OF LEADING ION

	Leading ion concentration	
	0.5 mM	*0.01 M*
ATP zone	Fig. 4b, 250 pmoles*	Fig. 3, 1000 pmoles**
Zone length§ in mm	13.5	13.5§§
Zone length in mm/100 pmoles	5.40	1.35
Zone length in mm/100 pmoles, at chart speed 3 cm/min	5.40	0.337
Zone length, in mm/100 pmoles, at chart speed 3 cm/min and 100 μA	0.270	0.253
Calculated† concentration of ATP, % of leading ion concentration	25.9	28.3
Zone length, in mm/100 pmoles, normalized to 28.3 % of the leading ion concentration, at chart speed 3 cm/min and 100 μA	0.24 (7)	0.25 (3)

* pH of the leading electrolyte was 4.36, current 5 μA, chart speed 3 cm/min.

** pH of the leading electrolyte was 4.10, current 75 μA, chart speed 12 cm/min.

§ All zone lengths in Table I refer to lengths on recorder charts.

§§ The impurities were subtracted graphically before the half-width was measured.

† Calculated by the Isogen[12] programme with $pK_4 = 4.0$ and $pK_5 = 6.5$ for ATP[14]. No mobility values for ATP could be found in the literature. It was known that phosphate and ATP give equal thermal step-heights with HCl/β-alanine at pH 3.5. The phosphate data chosen were the pK values 2.12, 7.12, 12.32 and the mobility values 33, 57, $72 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1} (ref. 15). The corresponding data chosen for β-alanine were 3.55 and $29 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}. The mobilities of the ATP ions were varied until the same net mobility was obtained for ATP as for the phosphate ions. The ionic mobilities were found to be 15, 28, 39, $48 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}, which were then used for the calculation of the ATP zones in the creatinine ($pK = 4.8$, mobility $= 25 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}) systems, at pH 4.10 with 0.01 *M* chloride concentration and at pH 4.36 with 0.5 m*M* chloride concentration.

the fifth row, where the common reference current is chosen arbitrarily as 100 μA. According to the isotachophoretic theory, the ionic migration velocity will be twenty times higher when the ionic concentration is lowered twenty times, as the current through the system is constant. The zone lengths are given in identical units in the last row of Table I, and they are in good agreement with each other. It must be pointed out that the dissociation and mobility data of ATP are approximate, but the relation between the zone lengths is only slightly affected by these approximations.

Further dilution of the leading electrolyte is of doubtful value. This can be seen when a comparison of the sample amounts needed for full UV absorbance at the two different leading ion concentrations is made. At 0.01 *M* concentration (see Fig. 3) the amount was about 125 pmoles, while at 0.5 m*M* concentration (see Fig. 4) it was about 50 pmoles. This means that there was a factor of less than 3 instead of 20. Consequently, the zone boundary sharpness is considerably decreased at the lowered concentrations.

Another effect of the dilution is that impurities from the solvent, glass-ware, etc, can easily contaminate the sample and can give non-reproducible results. For instance, the sample of 25 pmole ATP in Fig. 4a was taken from a 0.01 m*M* solution, while the smaller samples were taken from a 0.001 m*M* solution. This dilution intro-

duced ions without UV absorbance (between the ATP peak and the terminator step). Some experiments were made after a further two-fold dilution to 0.25 m*M* leading ion concentration, but no improvement in detectability was found.

The study of detection limits and resolution has been restricted to the UV detector. The same investigations could be made for the thermal detector, but this is known from experience to have a resolution about fifty times lower than the UV detector. The value of the thermal detector is its general response to mobility differences, while the advantages of the UV detector are its immediate response and high resolution in zone measurements.

Separation capacity

Several separation parameters have been studied with the Tachophor: current, temperature, capillary length and counter-flow and their influence on separation capacity and resolution. These parameters have been evaluated from experiments where only the thermal detector has been used. Chloride (HCl–creatinine, pH 5.1) was chosen as the leading ion for a test system and sulphanilic acid as the terminator. The sample mixture contained fumaric, citric and succinic acids.

In the first series of experiments the maximal separable volume of the sample mixtures was determined by increasing the injected sample volume at constant concentration until mixed zones occurred. An example of mixed zones is shown in Fig. 5.

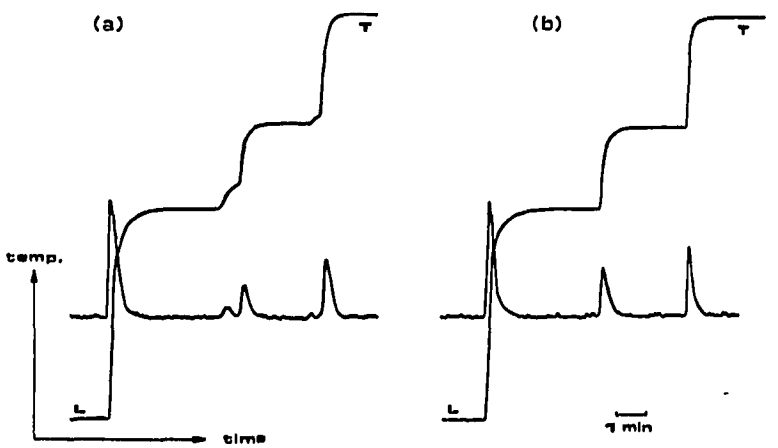

Fig. 5. Temperature detector signals from separations of different volumes of a 0.00345 *M* solution of succinic and citric acids. A volume of 20.5 μl (a) gives mixed zones, but a volume of 20.0 μl (b) gives fully separated zones. The first zone, with the lowest temperature level, is the succinate zone. The leading electrolyte (L) was 0.01 *M* HCl and 0.035 *M* creatinine at pH 5.09, with 0.01 *M* sulphanilic acid as terminator (T). The current was 100 μA, the temperature 20° and the capillary length 61 cm. A counter-flow of leading electrolyte of 0.50 μl/min was used for 5 min in both experiments.

This procedure was repeated at six different temperatures, and the result is shown in Fig. 6a. It was found that the separation capacity will increase slightly with temperature. It is known that the ionic mobilities increase 2 to 2.5% per °C, which means that the mobility differences increase to the same extent, therefore increasing the separation forces. The diffusion constant, however, also increases with the temperature,

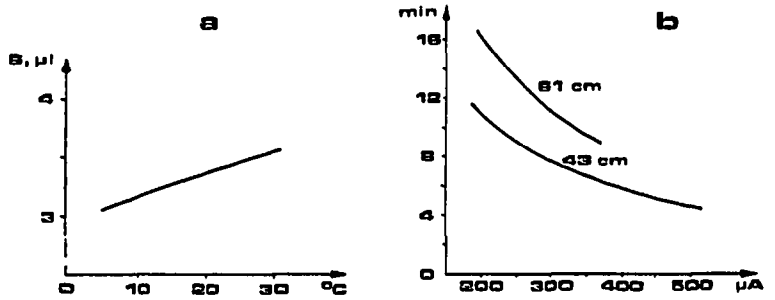

Fig. 6. (a) Separation capacity as a function of thermostat temperature. The sample (S) was a 0.007 *M* fumaric acid, 0.01 *M* citric acid and 0.01 *M* succinic acid solution. Leading electrolyte: 0.01 *M* HCl and 0.035 *M* creatinine, at pH 5.1. Terminating electrolyte: 0.01 *M* sulphanilic acid. The current was 100 μA, capillary length 50 cm. (b) Analysis time as a function of current for two different capillary lengths. The electrolytes were the same as in (a).

and therefore the variation in separation capacity is unexpected.

In order to prevent bubble formation when high power is used, the thermostat can be kept in a lower temperature range. There may also be cases when the chemicals used are more stable at lower temperatures.

Theoretically, analysis time and current through the system are directly proportional. This can be proved from Fig. 6b. The separation capacity was not affected when the current was varied from 50 to 200 μA. However, the response time of the thermal detector will limit the use of higher currents at the usual concentrations. High currents will also increase the radial non-uniformity of temperature, causing a parabolic shape of the boundaries[1], which will of course also affect the UV detector signal. On the other hand, lowering the current will decrease the electric field gradient or the separation force, and also give smaller thermal signals in addition to prolonging the analysis time. Therefore, the general current recommended is in the 50- to 150-μA range, with a leading ion concentration of 0.01 *M*.

The last set of experiments was made in order to find out the effect of capillary length and counter-flow on the separation capacity. The electrolyte system was the same as in Fig. 6, and the samples were mixtures of equal amounts of succinic and citric acids. The injected volumes of certain constant concentrations were increased until mixed zones occurred. An example is given in Fig. 5 where a capillary length of 61 cm was used. The sample concentration was 0.00345 *M* of each acid. When this is multiplied by the separable 20.0-μl sample volume, 69 nmoles are obtained. This amount can be plotted in a diagram (see Fig. 7) where this example defines one point on the curve called 2_5. The figure 2 stands for the number of turns of the capillary, which was either 1, 2 or 3 (43, 61 or 80 cm capillary length) and the subscript 5 denotes a 5-min counter-flow of the leading electrolyte, which is a method of increasing the separation capacity without increasing the column length[1,6]. It was carried out with a 500-μl syringe, connected to the leading electrolyte side of the capillary through a counter-flow inlet. Experiments were made without or with 5- or 10-min counter-flow at a rate of 0.50 μl/min, which roughly kept the zones stationary in the capillary. It is obvious that counter-flow has a considerable effect on the separation capacity and widens the range of use for a certain capillary length.

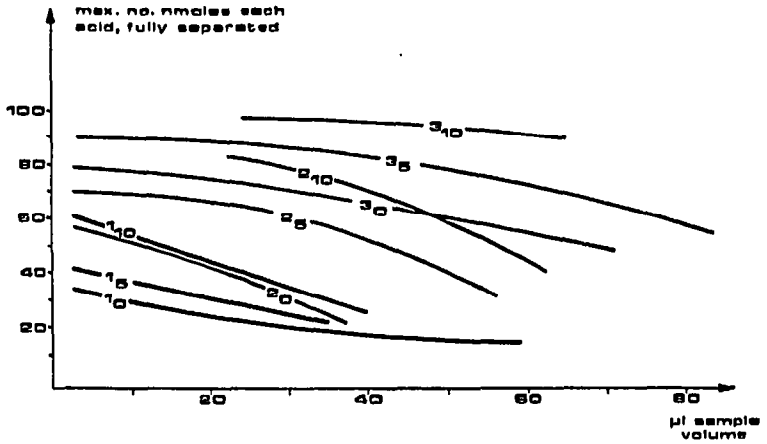

Fig. 7. Separation capacity as a function of sample volume, capillary length and counter-flow. The electrolytes were the same as in Fig. 6. The sample contained equal amounts of citric and succinic acids, but of various concentrations to allow variation of sample volumes (as demonstrated in Fig. 5). Numbers 1, 2 and 3 denote capillaries of 43, 61 and 80 cm length, respectively. The subscripts are minutes of counter-flow, adjusted to balance the migration velocity against the current used, 100 μA. The temperature was 20°.

The total volumes of the Teflon capillaries with one, two or three turns are about 70, 100 and 130 μl. This means that the curves in Fig. 7, except 1_0, can be extended to include still larger sample volumes (as well as smaller, for 2_{10} and 3_{10}). Because longer times or column lengths are used with larger sample sizes to obtain the isotachophoretic concentrations, the slopes of the curves show a decline to the right. These concentrations are, of course, identical for each acid in all experiments.

In all experiments measuring the separation capacity, no detergent was used in the leading electrolytes. Since these detergents sharpened the zone boundaries, they should also increase the separation capacity. A preliminary series of experiments with a 23-cm capillary (the other conditions as given in Fig. 6) gave an increase in separation capacity of about 40%, with sample volumes of around 1 μl.

Fig. 7 is specific to the system studied, but the general shape of these curves and their relation to one another can be generally applied and serve as a guide for other separations.

ACKNOWLEDGEMENT

The author is greatly indebted to Mr. Hans Lundin for skilled experimental assistance.

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