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INDIRECT PHOTOMETRIC DETECTION IN CAPILLARY ZONE ELECTROPHORESIS

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SUMMARY

An indirect photometric detection method is described which is based on the use of an absorbing co-ion as the principal component of the background electrolyte. The zones of non-absorbing ionic species are revealed by changes in light absorption due to charge displacement of the absorbing co-ion. Theoretical considerations are given for selecting a suitable absorbing co-ion to achieve a high sensitivity of detection.

The role of electromigration dispersion is illustrated by experiments and the effects of the differences in the effective mobilities of sample ions and that of the absorbing co-ion are discussed. The highest sensitivity can be achieved for sample ions having an effective mobility close to the mobility of the absorbing co-ion. In such a case, the concentration of the sample component in its migrating zone can be high while electromigration dispersion is still negligible. The useful dynamic range of the detection is then limited by the linearity and noise of the detector, the former parameter being given mostly by the shape of the on-column detection cell. The best sensitivities can be obtained in low-concentration background electrolytes containing a co-ion with high absorption at a given detection wavelength.

It is shown that indirect photometric detection can be useful for detecting substances that have no optical absorption in the UV and/or visible region, provided that the composition of the background electrolyte is selected correctly.

INTRODUCTION

Sensitive and reliable universal detection of all migrating zones in capillary zone electrophoresis (CZE) is of key importance for the utilization of this technique in practice.

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In capillary isotachophoresis[1], potential gradient and conductivity detectors are currently used for this purpose. In CZE[2], the use of potential gradient[3] and conductivity[4–6] detectors is limited to ionic species that have an effective mobility that is substantially different to that of the background electrolyte co-ion. When this difference decreases, the detector signal also decreases and it can often be masked by the noise generated by electrochemical reactions on the sensing electrodes of the detector cell[4]. Therefore, selective optical detectors are currently used here in either the absorbance[7] or the fluorescence mode[8]. Promising results were reported also with electrochemical[9] and mass spectrometric[10] detection. The possibility of using a selective optical detection system as a universal detector is offered by the utilization of the indirect photometric mode. Indirect photometric detection has already been well adopted in ion chromatography[11,12] and, by monitoring the counter ion, it has also been used as a universal detector in isotachophoresis[13].

In CZE, indirect fluorescence detection has been used to monitor the migration of zones of some amino acids[14], nucleotides, iodate, hydrogencarbonate and lysozyme[15]. The reported detection limits are impressive, mainly owing to the high intensity of the excitation laser beam and small inner diameter (15 μm) of the separation capillary used. In this paper we propose a method for the universal indirect detection of zones in CZE based on absorption photometric monitoring of a suitable absorbing co-ion which is the principal component of the background electrolyte.

THEORETICAL

The excellent separation properties of CZE are due mainly to the low dispersive performance of the equipment. In the optimum limiting case, the dispersion of the migrating zones is determined only by diffusion, initial sample pulse width and Joule heat[16,17]. In practice, however, dispersion due to sorption phenomena[17–19] and electromigration dispersion[4,20] contributes significantly to the dispersion of migrating zones. The latter type of dispersion is closely related to the detection. It always occurs during the migration of sample ions which possess effective mobilities different to that of the background electrolyte co-ion; the higher the concentration of the sample component in its zone, the more pronounced is the electromigration dispersion. The electromigration dispersion is different for different ions, and the method of suppressing it is to keep the solute concentrations in their zones sufficiently lower than the concentration of the background electrolyte (BGE).

Obviously, the supprssion of electromigration dispersion by lowering the concentration of the solutes in their zones places greater demands on the detection sensitivity and limits the useful concentration range of detection in CZE. Generally, electromigration dispersion is considered to be negligible when the concentration of the solute ions is two orders of magnitude lower than that of the BGE co-ion[4,18].

The absorbance detectors currently used in CZE exhibit a noise level of *ca.* $1 \cdot 10^{-4}$ absorbance units (A.U.) and their useful dynamic concentration range covers roughly three orders of magitude, as the upper limit of linearity is *ca.* 0.1 A.U. This upper limit is given mainly by the shape of the on-column detector cell, which is exclusively of circular cross-section in present practice. Here the Lambert–Beer law does not hold true in the form derived for a cell with plane parallel windows.

The situation is shown schematically in Fig. 1. The light beams delimited by the

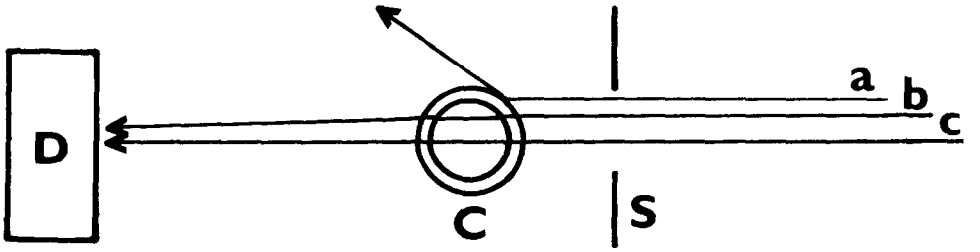

Fig. 1. On-column absorbance detection in capillaries. For details, see text.

slit S strike the capillary C at different positions. The part of the radiation denoted by a is refracted by the wall of the capillary and is lost. The beams b and c pass through the solution inside the capillary and serve for the detection. Their path lengths are, however, different. Hence, for each beam i a special equation can be written in the form

$$I_i = I_{0i} \cdot 10^{-\varepsilon c d_i} \tag{1}$$

where I_i is the intensity of the ith beam, ε is the molar absorption coefficient of a sample of concentration c and d_i is the optical path length of the ith beam. To obtain explicitly the mean intensity of the radiation detected by the detector D, integration is necessary and the resulting absorbance is not a linear function of the concentration c. A more detailed numerical treatment of this problem can be found in the literature[21]. However, for low values of the exponent $\varepsilon c d_i$, eqn. 1 can be expanded into a series and, by neglecting higher terms, it can be derived that for the intensity I detected by the detector

$$I = I_0 (1 - 2.3\, \varepsilon c \bar{d}) \tag{2}$$

where $\bar{d}$ is the mean optical path length in the capillary ($\bar{d} \approx 0.6$ I.D.). Eqn. 2 can be used in practice to describe the attenuation of the light beams up to *ca.* 0.1 A.U. At higher absorbance, the detected and registered peaks are already significantly distorted owing to the non-linearity of the detector.

When considering a capillary of I.D. 100 μm filled with a solution of a solute having $\varepsilon = 10\,000$ l mol^{-1} cm^{-1}, the corresponding admissible maximum concentration is about 10^{-3} M. It is interesting that according to eqn. 2, the detector to be used in capillary techniques does not need to be equipped with a logarithmic converter.

Returning to the mutual relationship between the detection linearity and electromigration dispersion, it follows that if indirect photometry is to be used for detection in CZE, and if simultaneously the concentrations of solutes in the zones should be 100 times lower than that of the BGE co-ion, then the useful dynamic decrease in the BGE absorbance due to the migration of a zone (useful signal) would be only 0.001 A.U. When using a photometer with a noise level of 0.0001 A.U. the resulting signal-to-noise ratio is only 10, which is too low for practical use. Hence, another means of suppressing electromigration dispersion must be found which is based on the selection of a co-ion with a mobility close to those of sample components.

In such a case, the electromigration broadening of zones during the migration is negligible even if the concentrations of solutes reach the concentration of the BGE co-ion. It should be stressed here that this method of suppression of the electromigration dispersion is of key importance as it is advantageous both for detection (direct or indirect) and for achieving high separation efficiencies (number of theoretical plates). Concerning the selection of the counter ion of the BGE, species of low mobility should be selected to ensure a low conductivity of the BGE and hence prevent excessive Joule heating during the analysis.

Another practical hint concerns the sample injection. It is convenient to inject a low-concentration sample which is not mixed with the BGE[4]. In this instance the concentration effect applies across the stationary boundary and the narrow sample pulse obtained facilitates an increase in both the sensitivity of detection and the separation power. Of course, the Joule heat limits the injection of low-concentration (low-conductivity) samples by possible overheating at the point of injection.

EXPERIMENTAL

Equipment

The experiments were carried out in fused-silica capillaries of 130 μm I.D. kindly supplied by Dr. Doupovec (Physical Institute, Slovak Academy of Sciences, Bratislava, Czechoslovakia) and of 100 μm I.D. deactivated fused-silica capillaries (Chrompac International, Middelburg, The Netherlands). One end of the capillary was connected to the electrode vessel via a block of Perspex, equipped with a Hamilton valve, enabling the capillary to be rinsed and filled after each analysis with the help of a syringe. The other end of the capillary served for sample introduction and was held in a mechanical moving arm for easy movement of the capillary orifice from the electrode vessel to the raised sample vial for hydrodynamic injection and back for the analysis. A laboratory-made power supply delivering up to 14 kV and 100 μA was used to drive the separation.

The zones separated in a 130 μm I.D. fused-silica capillary that was 46 cm long (42 cm to the detection cell) were detected by a single-beam UV detector from a Tachophor 2127 ITP analyser (LKB, Bromma, Sweden) with the aid of a previously described fibre-optic on-line detection cell[5].

In some experiments a Varian 2550 variable-wavelength detector was used. In this instance the original flow cell was replaced by a holder made of hard black PVC, which held the 100 μm I.D., 40 cm long (30 cm to the detector) capillary tightly in the optical path of the detector.

Chemicals and electrolytes

All chemicals were of analytical-reagent grade, supplied by Fluka (Buchs, Switzerland). Distilled water was deionized on a mixed-bed ion exchanger.

Two types of background electrolytes were used. The first contained 0.02 *M* benzoic acid as the UV-absorbing anion and was titrated with histidine to pH 6.2; 0.1% Triton X-100 was added to this BGE to suppress the electroosmotic flow. The second BGE contained a lower concentration of the anion with a higher UV absorbance and consisted of 0.007 *M* sorbic acid titrated with histidine to pH 6.2. The elimination of electroosmosis by the addition of Triton X-100 failed in this instance

and, therefore, no additive was used. In both instances the concentration of the BGE was selected so that its absorbance was the limit of the detector linearity.

All values of mobilities were taken from published isotachophoretic data[22].

RESULTS AND DISCUSSION

The ranges of linear response of the detectors used were determined by filling the capillary with standard solutions of benzoic acid. The plot of signal *vs.* concentration of benzoic acid for the LKB detector is shown in Fig. 2. It can be seen that undistorted peaks can be recorded for a detector signal up to 250 mV. The noise level was *ca.* 2 mV. Similar plots were obtained with the Varian 2550 detector. This double-beam detector is equipped with a logarithmic converter and the response was linear up to 0.08 A.U. The noise level was lower than 0.0001 A.U., which is an order of magnitude better performance than that of the single-beam LKB detector.

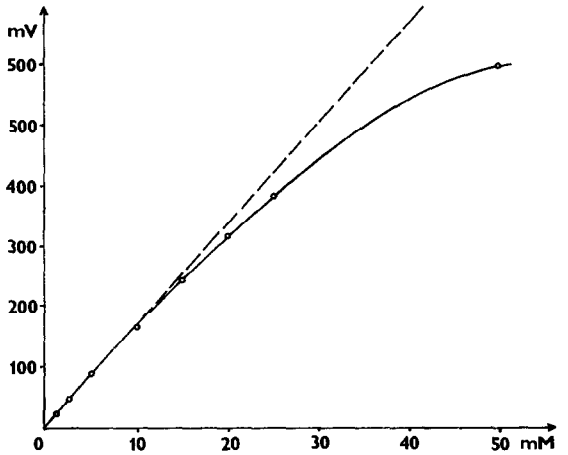

Fig. 2. Detector response *vs.* concentration of benzoic acid inside the 130 μm I.D. capillary.

In Fig. 3 the separation of fourteen model anions in BGE I with both indirect and direct photometric detection is shown. The model sample composition covers a wide mobility range from chloride ($u = 79.08 \cdot 10^{-5}$ $cm^2V^{-1}s^{-1}$) to glucuronate ($\bar{u} = 25.4 \cdot 10^{-5}$ $cm^2V^{-1}s^{-1}$). The effective mobility of the UV-absorbing BGE co-ion (benzoate) is $32 \cdot 10^{-5}$ $cm^2V^{-1}s^{-1}$ at this pH.

The electroosmotic flow was suppressed by the presence of 0.1% Triton X-100 in the BGE I and its magnitude was determined from the migration times of individual zones. The resulting electroosmotic mobility was low, being *ca.* $1 \cdot 10^{-4}$ $cm^2V^{-1}s^{-1}$ in the cathodic direction.

As expected, highly mobile anions provide broad peaks with a diffuse front and sharp rear boundary and a low detector response. As the mobility of migrating anions decreases, the detector response increases and the peaks become narrower.

The best signal is obtained for the zone of hydroxyisobutyrate (HIBA), which

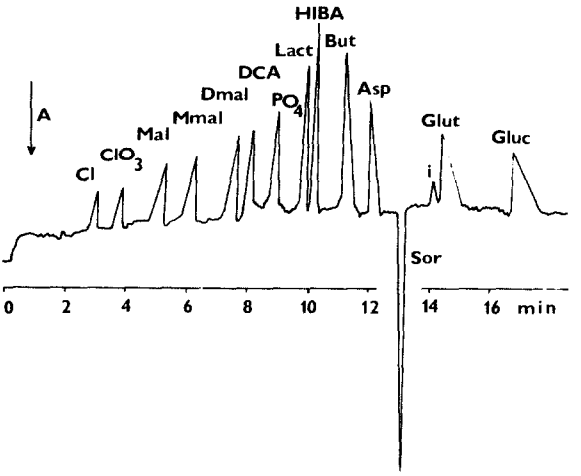

Fig. 3. CZE separation of anions in 130 μm I.D. capillary with indirect photometric detection with a modified LKB Tachophor detector operating at 254 nm. BGE I: 0.02 *M* benzoic acid–histidine at pH 6.2 + 0.1% Triton X-100. Driving current: 35 μA at 13 kV. Abbreviations: Mal = malonate; Mmal = methylmalonate; Dmal = dimethylmalonate; DCA = dichloroacetate; Lact = lactate; HIBA = hydroxyisobutyrate; But = butyrate; Asp = aspartate; Sor = sorbate; i = impurity; Glut = glutamate; Gluc = glucuronate.

has roughly the same effective mobility as benzoate. The slow ions form zones with a sharp front and diffuse rear boundary. Owing to the longer time of migration these zones are broader than the zones of fast ions.

As the noise of the LKB detector was *ca.* 0.001 A.U., a further increase in the sensitivity can be expected with the detector having lower noise. This is demonstrated by Fig. 4, where the separation of thirteen non-UV-absorbing ions was performed in a 100 μm I.D. capillary with the Varian 2550 spectrophotometric detector. Although a 3-fold lower concentration of the sample in comparison with previous experiments

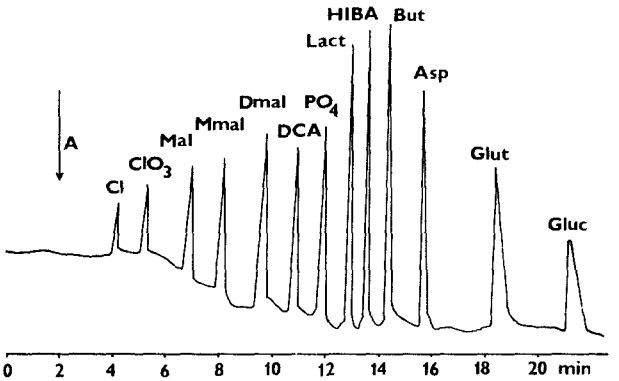

Fig. 4. CZE separation with indirect photometric detection using a Varian 2550 double-beam spectrometric detector operating at 254 nm. The 100 μm I.D. capillary was filled with BGE I. Driving current: 20 μA at 10 kV. For details, see text.

was injected here, a better signal-to-noise ratio was still obtained. On the other hand, a new problem arose here with the baseline drift, which was found to fluctuate with the BGE absorption owing to Joule heating during the analysis. For this reason the use of thinner capillaries seems to be advantageous, where theoretically also better separation efficiencies should be obtained[17,23].

Concerning the separation efficiency, it seems to be of interest to illustrate how it is limited by electromigration dispersion. In Fig. 5, the number of theoretical plates is plotted *vs* the difference between the mobility of the BGE co-ion and that of a sample species. Obviously, the number of theoretical plates reaches its maximum for ionic species having effective mobilities close to that of the co-ion. For greater differences in mobilities the separation efficiency decreases strongly.

Apparently, the use of BGE I provides satisfactory results, however, some conditions should be mentioned. The use of a relatively high concentration of BGE enables the electroosmosis to be reduced substantially by the simple addition of Triton X-100. On the other hand, the low absorbance of benzoate is the reason why the sensitivity of indirect detection is low in comparison with that of the direct detection of highly absorbing substances. This can result in peak masking even by trace UV-absorbing components in the sample. Such a situation is demonstrated in Fig. 6, where the original model mixture was enriched with *o*-aminobenzoate (OAB) and picrate at concentrations of $2 \cdot 10^{-4}$ *M*. Obviously the concentrations of both species added were three times lower than those of other sample components. It can be seen that the aspartate peak is partly overlaped by OAB and seems to be much sharper, and

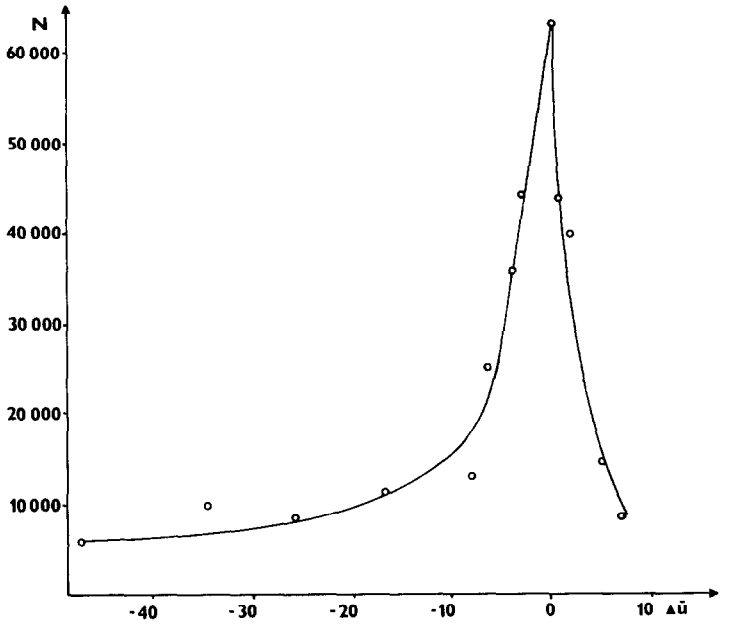

Fig. 5. Effect of electromigration dispersion on the separation efficiency. *N* is the number of theoretical plates, Δu is the difference $\bar{u}_c - \bar{u}_i$ (10^{-5}cm^{2}V^{-1}s^{-1}), where i and c are sample ionic species and co-ion, respectively.

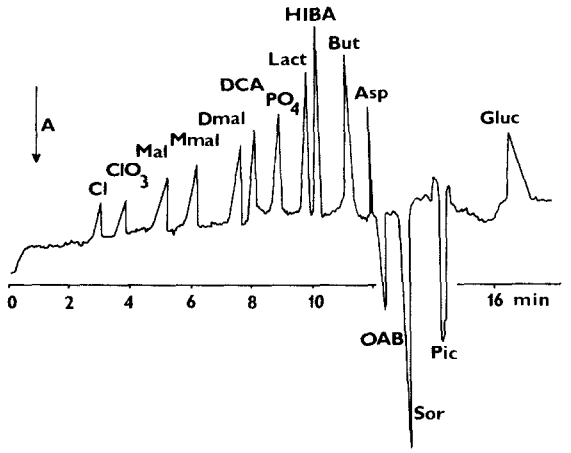

Fig. 6. Indirect photometric detection with peak masking by strongly absorbing trace components in the sample. The concentrations of *o*-aminobenzoate (OAB) and picrate (Pic) were $2 \cdot 10^{-4}$ *M*. The concentrations of other components were $6 \cdot 10^{-4}$ *M*. Other conditions as in Fig. 3.

the peak corresponding to glutamate disappeared completely owing to migration of picrate in the same zone.

The sensitivity of detection can be greatly increased by using a low-concentration but highly absorbing co-ion in BGE, namely, BGE II containing $7 \cdot 10^{-4}$ *M* sorbic acid. This solution has a low ionic strength and with the given instrumental arrangement strong cathodic electroosmotic flow was observed. The use of additives, *e.g.*, Triton X-100 did not suppress electroosmosis significantly. Therefore, no additives were used in further experiments and cathodic electroosmosis was utilizd to drive sample components through the detection cell. The separation of the model mixture of anions, driven cathodically by electroosmosis, is shown in Fig. 7.

The first detected positive peaks belong to potassium and lithium originating

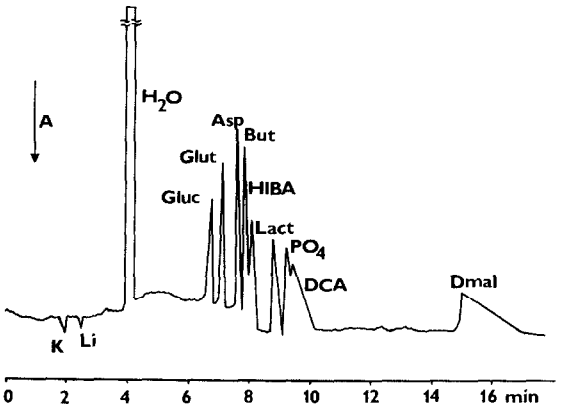

Fig. 7. CZE separation of anions with indirect photometry in low-concentration BGE II consisting of $7 \cdot 10^{-4}$ *M* sorbic acid–histidine at pH 6.2 with no additives. Driving current: 2 μA at 13 kV. LKB detector. For details, see text.

from the potassium phosphate and lithium lactate used for the sample preparation. The large rectangular peak of water transported by electroosmotic flow corresponds to the volume of sample injected and can be used as an electroosmotic marker.

The value of electroosmotic mobility, *i.e.*, the term $\varepsilon\zeta/\eta$, where ε, ζ and η are the permitivity of the BGE, the zeta potential and viscosity of the BGE, respectively, in the Helmholtz–Smoluchovski equation was found to be *ca.* $60 \cdot 10^{-5}$ $cm^2V^{-1}s^{-1}$. Obviously, the first peaks of anionic sample components correspond to low-mobility anions, whereas the highly mobile anions with electrophoretic velocities comparable to or higher than the electroosmotic velocity are not detected at all.

After the start of the analysis the sample ions migrate across the concentration boundary between the BGE and sample solution. As the sample concentration is low here ($2 \cdot 10^{-5}$ *M* of each ionic species), the migrating species are first concentrated across the above-mentioned boundary into a narrow sample zone in which their concentrations are adjusted to the values fulfilling the Kohlrausch regulation function[24]. Hence a sharp starting sample zone is created which aids positively the detection of sample components. During the following migration (superposition of electroosmosis and electrophoresis), however, zones containing ions with effective mobilities different from that of the sorbate ion are broadened by electromigration dispersion. The sharpest zones with the best detector response again provide anions with a mobility close to that of the BGE co-ion (sorbate).

The detection sensitivity in this system is roughly 50 times better than that in the previous instance and the detection limit even with a single-beam detector approaches 0.5 pmol injected.

Finally, we should mention an important practical aspect of the utilization of electroosmotic flow for driving the sample species along the separation path. The magnitude of the electroosmotic flow is strongly dependent on the history of the capillary used. This is demonstrated in Fig. 8, which shows the separation of a sample

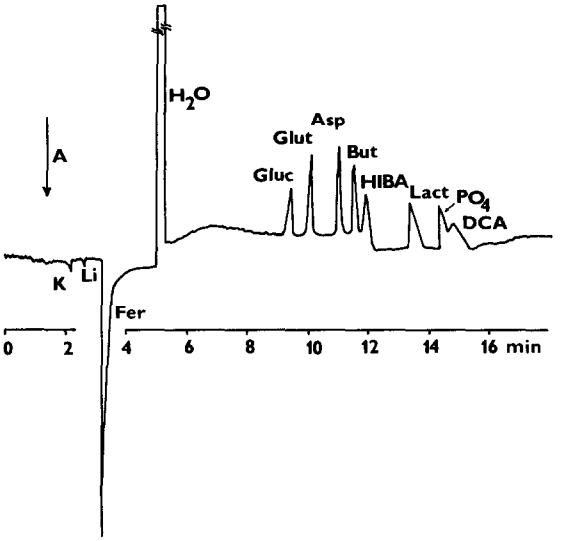

Fig. 8. Variation of migration times due to changes in electroosmotic flow caused by the sorption of the sample component. Fer = Ferroin. Other conditions as in Fig. 7.

to which 10^{-6} *M* ferroin was added. The sorption of ferroin on the capillary wall manifests itself not only by the tailing of the ferroin peak but also by a substantial decrease in the electroosmotic flow, which led to longer migration times and loss of the dimethylmalonate zone.

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