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Isotachophoresis as an on-line concentration pretreatment technique in capillary electrophoresis

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ABSTRACT

One of the major disadvantages of capillary electrophoresis (CE) is its limited loadability. Therefore, the on-line coupling of isotachophoresis (ITP) and CE was studied with regard to its potential for the improvement of the minimum concentration that can be measured by CE. Based on the concentrating and separating power of ITP, detection limits could be lowered by at least two orders of magnitude. Especially for biological samples containing proteins, it appeared that in non-treated capillaries the electromigration characteristics are hardly influenced when isotachophoretic pretreatment is applied. The potential of ITP–CE coupling is illustrated by the analysis of *o*-phthaldialdehyde and fluorescein isothiocyanate derivatives of a number of amino acids.

INTRODUCTION

Capillary electrophoresis (CE) is a highly efficient miniaturized separation technique with remarkable resolving power. The technique is based on differences in electrophoretic mobilities. Typical capillary dimensions are between 20 and 100 μm in diameter and a length of 50–100 cm, resulting in a volume of a few microlitres. As a consequence, the loadability of the system is limited to injection volumes of 1–30 nl. This poor loadability is a severe drawback in CE and puts high demands on the detection. Although impressive detection limits in the (sub)attomole range [1–6] have been reported, the corresponding measurable sample concentrations are still too high (10^{-7}–10^{-6} mol/l) to allow trace-level determinations of, *e.g.*, drugs in plasma.

Another limitation of CE is the poor performance in the analysis of complex biological matrices. For bioanalysis based on CE, a sample pretreatment for concentration and isolation of the analyte is essential. Isotachophoresis (ITP) is a capillary separation technique also based on the differences in electrophoretic mobilities [7–9], but this technique has the advantage of much higher loadability, *e.g.*, microlitres instead of nanolitres in CE. In addition, ITP is a concentration technique for trace components and a dilution technique for major constituents of the sample. The combination of these features makes ITP in principle an ideal technique for sample treatment. The use of ITP for sample pretreatment in CE has already been described [10–14]. Especially the extended study of Kaniansky and Marak [10] gives

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a good impression of the potential of combined ITP–CE. The diameters used in their study were 300 μm for both ITP and the CE capillary. Although, owing to the heat generation in the capillary, such large diameters are in principle not favourable in CE, the results achieved were impressive.

In contrast to the previous techniques [10–14] in which the ITP preconcentration and CE separation were performed in capillaries with identical dimensions, we have developed a coupled system which involves two separate capillaries with optimum dimensions for each process. Using this approach it is easier to use smaller diameters for the CE capillary allowing higher voltages without disturbing the plug flow of electroosmosis.

The degree of the concentration effect of ITP can be derived [15] from the Kohlrausch equation:

$$C_L/C_A = [\mu_L/(\mu_L + \mu_R)]\,[(\mu_A + \mu_R)/\mu_A] \quad (1)$$

where C_L is the molarity of the leading buffer, C_A the analyte concentration and μ the electrophoretic mobility (the subscript R refers to the counter ion). From this equation it can be seen that the final concentration of the analyte is proportional to the molarity of the leading buffer. Thus the equation can be written as

$$C_A = C_L K \quad (1a)$$

where K is a proportionality factor. In the case of similar mobilities, K will have a value of about 1. Eqn. 1a clearly demonstrates the tremendous concentration potential of ITP. The result of the ITP process is that major components are diluted but also, much more important, that trace compounds are concentrated.

In this paper, the coupling of an ITP system with a CE system enabling the determination of level concentrations that cannot be determined by direct injection in CE is described.

EXPERIMENTAL

Materials

Sodium borate and barium hydroxide were purchased from Merck (Darmstadt, F.R.G.). N-(2-acetamido)-2-aminoethanesulphonic acid (ACES) from Serva (Heidelberg, F.R.G.), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), histidine (His), threonine (Thr), alanine (Ala), valine (Val), lysine (Lys), arginine (Arg), fluorescein isothiocyanate (FITC) and *o*-phthaldialdehyde (OPA) from Aldrich (Steinheim, F.R.G.) and mercaptoethanol from Fluka (Buchs, Switzerland). The OPA reagent was a mixture of 100 mg of OPA, 100 μl of mercaptoethanol and 10 ml of borate buffer (5 mmol/l, pH 9.5) and was made freshly every day.

Equipment

The ITP system was a commercial system (Isotachophor; LKB, Bromma, Sweden). The fused-silica capillary used for CE was obtained from SGE (North Melbourne, Australia). The UV detector used for the detection of the OPA derivatives in CE was a variable-wavelength instrument (Spectroflow 757; Kratos, Ramsey, NJ,

U.S.A.), equipped with a laboratory-made on-capillary detection cell with a volume of 8 nl. Detection of the FITC derivatives was based on laser-induced fluorescence (LIF) using an air-cooled argon-ion laser (Model 161 C; Spectra Physics, San Jose, CA, U.S.A.) lasing at 488 nm.

A 100-kV power supply was used for both ITP and CE (Gamma, Model RR 100–1.5 P; High Voltage Research, Coimex, Hattem, The Netherlands). Electropherograms were registered on a flat-bed recorder (Model 40; Kipp & Zonen, Delft, The Netherlands).

Coupling of ITP and CE

The ITP system was modified by inserting a fused-silica capillary into the ITP PTFE capillary as close as possible to the UV cell. In this way the ITP system is hardly influenced by the coupling to CE. Also, the CE part of the coupled system continues to perform like a separate CE system. The only difference for the CE system is that the electrode of the leading electrolyte buffer vial of the ITP system, which is connected with earth, is acting as the CE anode (Fig. 1). For such a set-up a reversible power supply is advantageous.

Isotachophoresis

In the ITP sample pretreatment mode, 5 mmol/l borate buffer (pH 9.5) was used as the leading buffer and 5 mmol/l ACES (pH 10.0) as the terminating buffer. The ACES buffer was adjusted to pH 10 with barium hydroxide to avoid interference from carbonate from the air because of the alkaline pH. After thorough cleaning of the system by rinsing with sodium hydroxide, hydrochloric acid and ethanol, samples of 1–50 μl can be injected into the ITP system with a 100-μl injection syringe. A voltage of about 8 kV was applied over the capillary (61 cm $\times$ 500 μm I.D.). ITP was stopped when the zone of interest reached the detector.

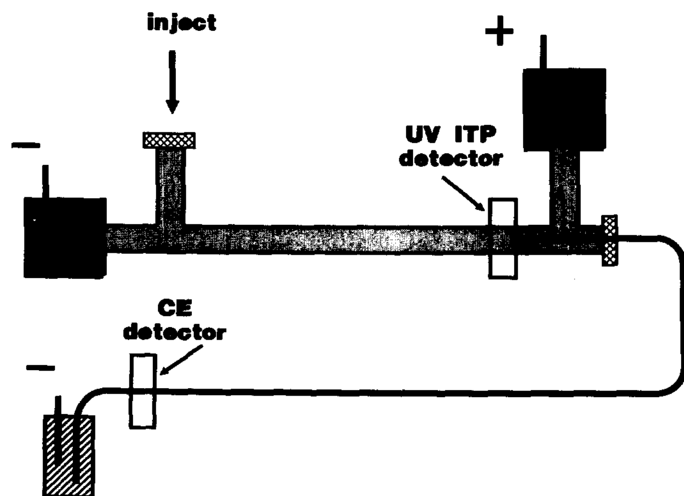

Fig. 1. Scheme of coupled ITP–CE system.

Injection into capillary electrophoresis system

Injection into the CE system was effected by electrokinetic injection, positive displacement injection or by a combination of both techniques depending on the concentration and the number of analytes.

Electrokinetic injection

After a sample zone has reached the beginning of the CE capillary, the high voltage of the ITP system is switched off. Then the power supply of the CE system is switched on for 5 s at 5 kV, whereafter the ITP capillary is flushed with the leading buffer in the direction of the ITP injection port. This is done by sucking with an injection syringe through the ITP injection port. The high voltage is raised to the running voltage of 25 kV during the cleaning in order to prevent back-migration of the analytes.

Positive displacement injection

After the compounds of interest have reached the beginning of the CE capillary, a few microlitres of the terminating buffer are injected into the ITP system. This causes a positive displacement of the sample zones passing the entrance of the CE capillary, During flushing of the ITP capillary the high voltage is switched on in order to prevent back-migration as described for electrokinetic injection.

Capillary electrophoresis

After the injection is completed the electrophoretic run is started, applying 25 kV over the 75 cm × 50 μm I.D. capillary. The electrophoresis is performed in a continuous buffer system using the leading buffer of the ITP system.

o-Phthaldialdehyde derivatization

From a mixture of amino acids containing Asp, Glu, Gly, His, Thr, Ala, Val, Lys and Arg, several dilutions were made, varying from 10 μg/ml to 1 mg/ml. To 100 μl of these dilutions, 100 μl of OPA reagent were added, then the mixture was vortex mixed for 3 s and immediately injected. The serum samples that were analysed were derivatized without any prior sample pretreatment. Volumes of 100 μl of serum were mixed with 100 μl of borate buffer (pH 9.5, 0.02 mol/l) and 100 μl of OPA reagent. The extra borate buffer was added to compensate for the strong buffer capacity of the serum.

Fluorescein isothiocyanate derivatization

To 50 μl of a Phe solution with a concentration of 5 μg/ml, 100 μl of FITC solution (1 mg/ml) in borate buffer (0.01 mol/l, pH 9.5) were added. The derivatization mixture was allowed to stand for overnight in order to complete the reaction.

RESULTS AND DISCUSSION

Fig. 1 shows the scheme of the coupled ITP–CE system. The fused-silica CE capillary was inserted into the ITP capillary as close as possible to the detector cell without affecting the optical path. This can easily be seen during insertion by observing the baseline of the UV detector. It should be realized that, by inserting an open CE

capillary, the possibility of hydrodynamic flow and thus during ITP a real electroosmotic flow has been created. This can be circumvented by the use of a CE capillary with an inside diameter such that the ratio of the diameters of the ITP and CE capillary is at least 10. Care should be taken that no hydrodynamic forces caused by differences in liquid levels will initiate a flow in the capillaries. Using an ITP PTFE capillary of I.D. 500 μm and an CE fused-silica capillary of I.D. 50 μm, it appeared that the electroosmotic flow through the CE capillary during the ITP run can be neglected.

Apparently this manner of coupling results in an inflexible system, but in practice it appeared to improve the reliability of the complete system considerably. Injections into the CE system are done without moving the CE capillary, a buffer vial or a sample vial. Only the buffer or the sample itself is flushed through the system while the capillaries remain in a constant configuration with respect to each other.

Two modes of injection are predominantly used in CE, *viz*., hydrodynamic and electrokinetic injection. Positive displacement injection is in fact a hydrodynamic mode of injection by the displacement of a well defined liquid volume. The reproducibility, characterized by the relative standard deviation (R.S.D.), was 4.2% (n = 5), which is superior to that of electrokinetic injection with an R.S.D. of 7.6% (n = 5). The reproducibility of electrokinetic injection is also significantly improved, because all injections are made from an environment of constant molarity resulting from the ITP process.

In practice, it appeared that a combination of electrokinetic injection and positive displacement offers the best performance. In fact, most of the time both methods are used in combination because by inserting the syringe into the ITP system in order to flush the ITP system, a small displacement injection is made. In principle this is the minimum injection that always has to be made. This volume can be increased by either injecting some buffer to increase the displacement or by doing an electrokinetic injection.

In order to study the characteristics of the ITP system, OPA derivatives of different amino acids were used as test compounds. In Fig. 2 the isotachopherogram of

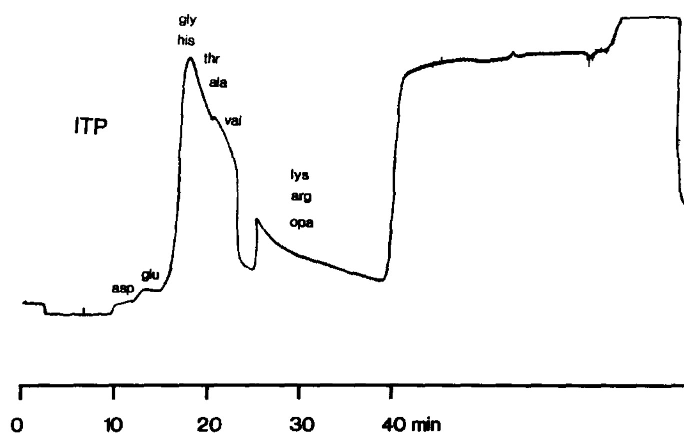

Fig. 2. Isotachopherogram of a test mixture of OPA-derivatized amino acids. Injection: 25 μl of an amino acid mixture with each amino acid at a concentration of about 1 ng/ml Applied voltage: 8 kV. Detection: UV absorbance at 254 nm. For other conditions, see text.

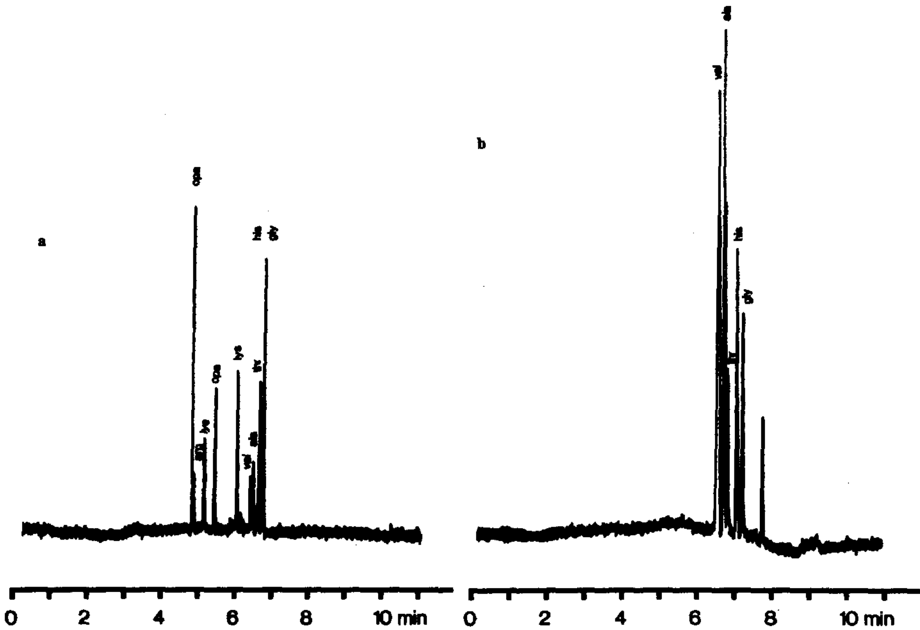

Fig. 3. (a) Single capillary electropherogram of some OPA-derivatized amino acids. Electrokinetic injection (5 kV, 5 s) of a test mixture with each amino acid at a concentration of about 100 ng/ml. Applied voltage: 25 kV. Detection: UV absorbance at 234 nm. For other conditions, see text. (b) Coupled ITP–CE of OPA-derivatized amino acids. ITP injection: 25 ml of a test mixture with each amino acid at the concentration of about 1 ng/ml. Injection in CE electrokinetic (5 kV, 5 s). Applied voltage: in ITP 8 kV and in CE 25 kV. For other conditions, see text.

such a mixture is depicted. Amino acids with an extra amino group, *e.g.*, Arg and Lys, migrate much more slowly than the other acids, probably because at the pH chosen these acids have electrophoretic mobilities that are even slower than the terminating buffer, implying that migration is not based on ITP but on CE, as recently suggested by Beckers and Everaerts [16]. From the time scale, it can be concluded that the ITP run is time consuming, which is caused by the length of the applied capillary and the molarity of the buffers. It must be emphasized that the time window in which the amino acids occur corresponds to an extremely small volume. Starting with amino acid concentrations of 10 μmol/l and applying a leading buffer of 10 mmol/l, in principle ITP will result in an effective volume reduction of a factor of 1000. With an applied injection volume of 25 μl this means that each component will have a volume of about 25 nl. On the other hand, this also explains the limited quality of the ITP trace, as the detection volume is large in comparison with the small zones. Owing to these small dimensions it is possible to inject the complete mixture of all components into the CE system, although of course a split injection is made. The splitting ratio is determined by the ratio of the cross-sectional areas of the ITP capillary and the CE capillary.

The following calculation illustrates the concentration effect of the total system, including the split during injection into the CE capillary. According to eqn. 1, ITP of an analyte solution (1 μmol/l) in a leading buffer concentration of 5 mmol/l results in

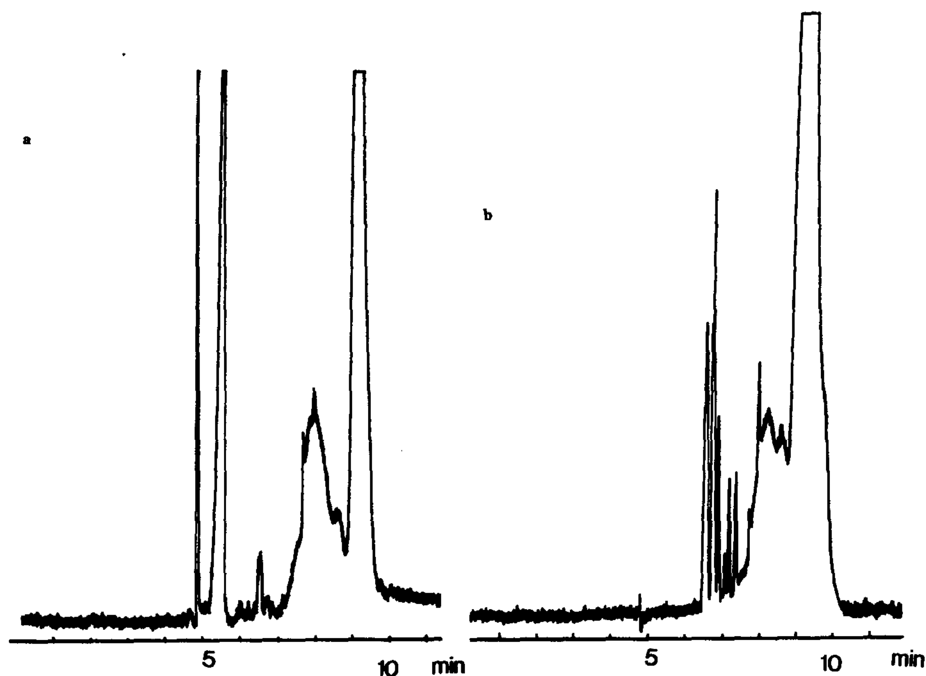

Fig. 4. (a) Single capillary electropherogram of an OPA-derivatized sample of serum spiked with some amino acids. Electrokinetic injection (5 kV, 5 s). Applied voltage: 25 kV. Detection: UV absorbance at 234 nm. For other conditions, see text. (b) Coupled ITP–CE of an OPA-derivatized sample of serum spiked with some amino acids. ITP injection: 25 μl of derivatized serum. Injection in CE: electrokinetic (5 kV, 5 s). For other conditions, see text.

a concentration factor of about 5000. A 50-μl injection will result in a zone of 10 nl with a concentration of about 5 mmol/l.

Because of the differences in the I.D. of the two capillaries, a split is made of 1:100, so an absolute volume of 100 pl is injected with a concentration of about 5 mmol/l. In single CE an injection of about 500 nl has to be made to obtain precisely the same amount injected, which will totally overload the system and will make analysis nearly impossible. These aspects of ITP–CE are illustrated in Figs. 3 and 4. Fig. 3a shows the electropherogram of a mixture of a number of amino acids derivatized with OPA, as obtained with single CE, and Fig. 3b is the result of the coupled ITP–CE system. These two figures clearly demonstrate the concentration effect of ITP, as almost no signal can be observed in single CE, whereas the peaks in the ITP–CE run can be quantified easily.

Fig. 4a shows the electropherogram of a serum sample spiked with OPA derivatives of amino acids. Owing to the instability of such derivatives, serum was spiked with relatively high concentrations (0.01–0.1 mmol/l). As the ITP process demands nearly 40 min, endogenous levels of the amino acids (0.01–0.7 mmol/l) could hardly be detected after derivatization with OPA, because the degradation of low concentrations is faster than that of high concentrations. As it was not the intention to develop an amino acid assay for serum samples, but only to explore the potential of this

combined technique, we accepted the spiking of the amino acids in serum. It can be seen from Fig. 4a that in CE analysis the ratio between the rapidly migrating amino acids and the slower serum constituents migrating as an unresolved peak is unfavourable with regard to amino acid analysis. Low concentrations will be affected considerably by this unresolved peak. However, in the coupled system it appears that this ratio has been improved considerably, as can be seen in Fig. 4b. The amino acids have been concentrated by a factor of at least 100, whereas the total amount of the other serum constituents is about the same as in single CE. In fact, the trace amounts of the amino acids have been concentrated while the major components have been diluted, thus enhancing the selctivity of the total system. This example shows the selectivity of the coupled method clearly.

Although in this configuration, with the CE capillary inserted in the ITP capillary, only a small fraction of an ITP zone is transferred to the CE capillary, it must be realized that by applying a concentration-sensitive detector (UV absorbance and fluorescence in this study) the full benefit of ITP concentration of an analyte will be obtained, in combination with favourable CE diameters allowing high voltages. Only in those instances where a mass flow-sensitive detector (*e.g.*, mass spectrometer) is applied will this splitting procedure result in corresponding lower signals.

Quantitative aspects

To demonstrate the quantitative aspects of the method, a calibration plot was constructed. Because the stability of the OPA derivatives is limited we chose FITC derivatives that have fluorescence characteristics that are almost perfectly compatible with the 488-nm lasing line of the argon-ion laser. Fig. 5 shows the electropherograms obtained by single CE and ITP–CE. Although a mixture of only four amino acids was derivatized, many degradation peaks can be observed, hampering trace analysis [17].

A calibration graph was constructed by analysing dilutions of the FITC derivative of Phe at concentrations ranging from 3.5 to 350 μmol/l. In CE the analyte was introduced by electrokinetic injection at 5 kV for 5 s. The correlation coefficient was 0.9999 and the function of the curve was $y = (0.569 \pm 0.004)x + (-1.054 \pm 0.513)$. Another series of dilutions were made ranging from 0.175 to 35.0 μmol/l. From these dilutions a calibration graph was made using on-line ITP as sample pretreatment. The correlation coefficient of this curve was 0.999 and the function of the curve was $y = (-11.15 \pm 0.14)x + (2.52 \pm 1.79)$. The calibration graphs demonstrate that the coupled system can be used quantitatively in a concentration range lower than the minimum detectable amount in separate CE.

Limitations

For optimum injection into the CE system, the ITP zone has to be detected in the ITP system preferably in an identifying way, as matrix compounds can influence the retention time of the various zones. Of course, this is a limitation of ITP. On the other hand, it must be realized that ITP is a concentration technique for trace components, whereas CE for each compound is a dilution technique comparable to chromatography. This implies that by application of similar detection systems, observation of a compound in ITP is not guarantee that this compound will also be observed in CE. When the zone length is smaller than the aperture of the detector cell in the ITP capillary, such a zone will be difficult to detect. In such an event the use of additives functioning as a marker or even as a spacer will be helpful.

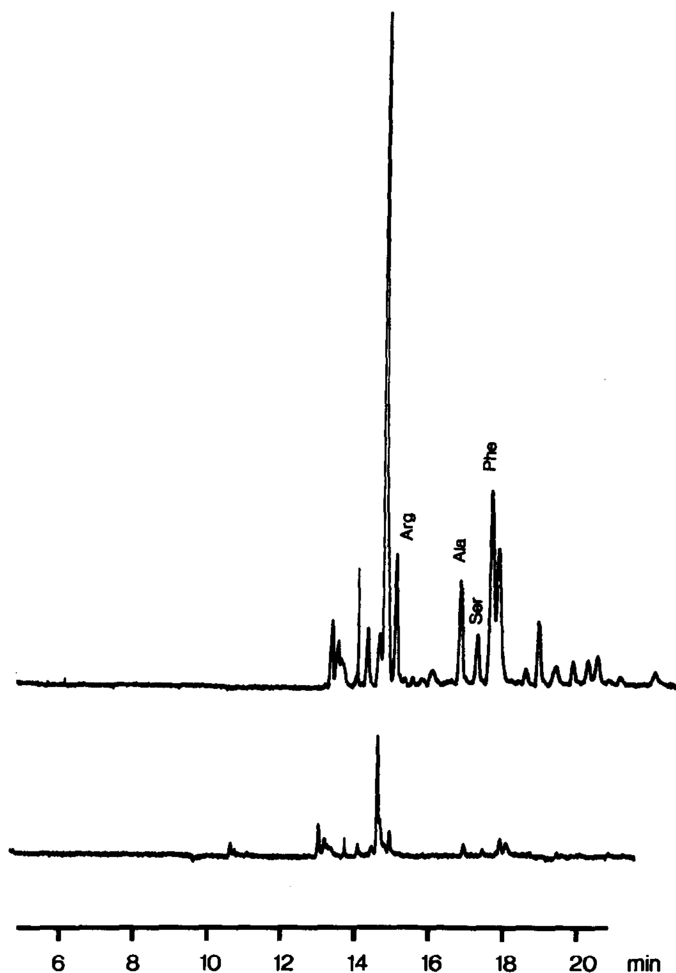

Fig. 5. Electropherograms of an FITC derivatization mixture of some amino acids. Lower trace: single CE. Conditions: electrokinetic injection (5 kV, 5 s) of 10 mmol amino acid–FITC derivative mixture. Applied voltage: 25 kV. Upper trace: coupled ITP–CE. Conditions: ITP injection, 25 μl of derivatization mixture, followed by CE electrokinetic injection (5 kV, 5 s). Applied voltage: in ITP 10 kV and in CE 25 kV. Detection: laser-induced fluorescence at 488/514 nm.

Fig. 3 shows that not all amino acids present in a single CE run also appear in a coupled ITP–CE run. An explanation can be derived from the isotachopherogram of the single ITP system given in Fig. 2. The order of migration is exactly the opposite of that in CE. This is caused by the fact that in ITP the analytes migrate towards the anode and in CE towards the cathode. An injection from the beginning of the sample therefore does not include the less mobile ions.

Another limitation of coupled ITP–CE is that a compromise has to be made between the optimum buffer system for the ITP system and for the CE system. In the combined method one buffer initially acts as a leading buffer in ITP and finally acts as a supporting buffer in CE. For the separation of derivatized amino acids a better

resolution can be obtained when a higher concentration of borate buffer can be used in CE. However, when borate buffers with concentrations higher than 5 mmol/l are used in ITP the heat generation becomes problematic and the analysis times will be long. In the ITP system the use of Tris buffers results in good performance, but is disappointing in the CE system. As a consequence, a buffer has to be chosen that works acceptably in both systems, which is usually not the best buffer for either system.

CONCLUSIONS

ITP can be combined with CE resulting in a gain in sensitivity of at least a factor 100, which is merely limited by the equipment applied such as the detector in the ITP system. The additional selectivity of the coupled ITP–CE system gave promising results concerning the problems of CE with biological matrices.

A limitation of the coupled system is that a compromise has to be made concerning the buffer system that is used in the overall system between analysis time and resolution in both the ITP and the CE stages.

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