Journal of Chromatography, 498 (1990) 191–204
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 992

ON-LINE COUPLING OF CAPILLARY ISOTACHOPHORESIS WITH CAPILLARY ZONE ELECTROPHORESIS

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(Received August 22nd, 1989)

SUMMARY

The on-line combination of capillary isotachophoresis (CITP) with capillary zone electrophoresis (CZE) in the column-coupling configuration of the separation unit was studied. The inherent concentrating power of the ITP stage was effective in achieving a high volume reduction of the injected sample so that the ZE separations with 2–3 μm plate heights could be achieved in 300 μm I.D. capillary tubes for nitrophenols and 2,4-dinitrophenyl-labelled amino acids taken as model analytes. Very reproducible migration times (0.3–1.2% relative standard deviations for 138–350-s migration times) and a high precision of sample injection (0.9–5.1% relative standard deviations for 8-pmol amounts of the analytes) could be typically achieved. Removal of the separated macroconstituents from the separation compartment after the ITP stage prevented column overloading in the ZE stage so that low detection limits could easily be achieved for the analytes (*ca.* 10^{-7} mol/l for a 5-μl injection volume by using a photometric detector) even in a 10^5-fold excess of a sample macroconstituent.

INTRODUCTION

Zone electrophoretic separation is associated with an inherent concentration adaptation of the separated constituents[1–4]. It has been shown that this adaptation process can be advantageously utilized in capillary zone electrophoresis (CZE) to reduce the initial volume of the injected sample[2–4] and, thus, positively influence the efficiency of separation.

The use of a discontinuous buffer system is another alternative for concentrating the sample constituents before their CZE separation[5]. This alternative, analogous to that proposed by Ornstein[6] and Davis[7] for ZE separations in polyacrylamide gels, is in fact a sequential use of isotachophoretic (ITP) concentration with ZE separation.

0021-9673/90/$03.50

It is apparent that such an approach can be beneficial in CZE in general. From a practical point of view, however, tandem coupling of the capillary columns as used in capillary isotachophoresis (CITP) with a concentration cascade of the leading electrolytes[8] and/or its functional equivalent consisting of a capillary column, a sample valve [applicable for the stacking (ITP) phase] and a microsyringe injection block (ref. 9, p. 210) are probably more convenient instrumental solutions than a single capillary column successively filled with a desired sequence of the electrolyte solutions[5].

The column-coupling configuration of the separation unit for ITP (CC-ITP) as first described by Everaerts *et al.*[10] was also shown to be suitable for work with discontinuous buffer systems (see ref. 3, p. 142). In addition, it enables interfering sample constituents to be removed from the separation compartment after the separation in the first (preseparation) column so that mainly the constituents of primary analytical interest are subjected to a final separation in the second column. As such a configuration seems desirable in an on-line combination of CITP with CZE, we preferred the use of the column-coupling configuration of the separation unit in this feasibility study. Our interest in this combination of basic electrophoretic techniques was stimulated by the following facts:

(i) ITP is a separation technique with a well defined concentrating power while the separands migrate stacked in sharp zones, *i.e.*, it can be considered as an ideal sample injection technique for ZE.

(ii) In some instances the detection and quantitation of trace constituents separated by ITP in a large excess of matrix constituents may require the use of appropriate spacing constituents. Such a solution can be very beneficial when a limited number of the analytes need to be determined in one analysis (see, e.g., ref. 11). It becomes less practical (a search for suitable spacing constituents) when the number of trace constituents to be determined in one analysis is high.

(iii) In CZE, high-efficiency separations make possible a multi-component analysis of trace constituents with close physico-chemical properties. However, the separations can be ruined, *e.g.*, when the sample contains matrix constituents at higher concentrations than those of the trace analytes.

(iv) An on-line combination of CITP with CZE appears to be promising for alleviating some of the above practical problems.

This paper presents the results of introductory work obtained with instrumentation intended mainly for CITP. Nitrophenols and 2,4-dinitrophenyl derivatives of amino acids served as model analytes.

EXPERIMENTAL

Instrumentation

A CS Isotachophoretic Analyzer (VVZ PJT, Spišská Nová Ves, Czechoslovakia) that enabled work in the column-coupling mode to be performed was used. The separation unit was assembled from components from the manufacturer and also from those developed in this laboratory.

Laboratory-made columns provided with 0.30 mm I.D. capillary tubes (O.D. $\approx$ 0.65 mm) made of fluorinated ethylene–propylene (FEP) copolymer were employed in both separation stages. The capillary tubes were placed in compartments that allowed efficient dissipation of heat produced on passage of current.

The first column was 10 cm long and was provided with a conductivity sensor[12] to monitor the ITP phase and to provide information necessary for proper switching of the columns by the analyser controller. The second column (ZE stage) was 28 cm long and was provided with a laboratory-made on-column UV–VIS photometric detector with a design similar to that described elsewhere[13]. Detection was performed at 405 nm and a rectangular slit of the detector (0.25 mm in height) was placed 20 cm downstream of the bifurcation point (see Fig. 1).

The samples were injected with the aid of a sampling valve[14] either by a 15-μl internal loop or with the aid of a 10-μl 701 N microsyringe (Hamilton, Bonaduz, Switzerland). When required, both injection alternatives were combined.

An HP 3390 A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.) served for peak-area and migration-time measurements in the ZE stage. The data provided by the integrator (peak-height and peak-area ratio) served for the calculation of the column efficiency for the studied separands as described[15].

Chemicals

Chemicals used for the preparation of the electrolyte solutions were obtained from Serva (Heidelberg, F.R.G.), Sigma (St. Louis, MO, U.S.A.) and Lachema (Brno, Czechoslovakia) and were purified by conventional methods. Hydroxyethylcellulose 4000 (HEC) and methylhydroxyethylcellulose 30000 (m-HEC) served as anticonvective additives in the electrolyte solutions. A stock aqueous solution containing 0.5% (w/v) of each of the derivatives was purified on a mixed-bed ion exchanger (Amberlite MB-1; BDH, Poole, U.K.).

Water from a Rodem-1 two-stage demineralization unit (OPP, Tišnov, Czechoslovakia) was further purified by circulation through laboratory-made polytetrafluoroethylene (PTFE) cartridges packed with Amberlite MB-1 mixed-bed ion exchanger. The electrolyte solutions were prepared from freshly recirculated water.

Nitrophenols and 2,4-dinitrophenyl derivatives of amino acids (see Table I) were obtained from Serva and Sigma. The sample solutions were prepared from stock solutions of these preparatives [1 mg dissolved in 1 ml of water–methanol (1:1)]. Sulphate and naphthalene-1,3,6-trisulphonate (NTS) were added at $2 \cdot 10^{-4}$ mol/l concentrations to the sample solutions to prevent adsorption losses of the analytes during sample handling and to suppress their adsorption in the separation compartment during the analysis[11].

RESULTS AND DISCUSSION

Isotachophoresis as a sample injection technique for zone electrophoresis

The on-line combination of CITP with CZE as studied in this work can be divided into several well defined separation phases (Fig. 1). In experiments to investigate the capabilities of ITP as a sample injection technique for ZE, the overall separation scheme consisted of a sequence of three of these phases (successively a, b and e in Fig. 1). In this way, we could separate and concentrate the sample constituents in the ITP stage, remove sulphate and the main part of NTS (see Experimental) after this stage from the separation compartment and separate the transferred sample fraction (the remainder of NTS and the analytes) by ZE in the second column. Part of the injected NTS was transferred into the ZE stage to minimize adsorption of the analytes on the wall of the capillary tube[11,16].

TABLE I

NITROPHENOLS AND 2,4-DINITROPHENYL-LABELLED AMINO ACIDS USED AS MODEL ANALYTES

No.	*Name*[a]	*Abbreviation*
1	DNP-L-cysteic acid	DNP-CYS-SO_3H
2	DNP-L-aspartic acid	DNP-ASP
3	DNP-DL-glutamic acid	DNP-GLU
4	2,6-Dinitrophenol	2,6-DNP
5	2,4,6-Trinitrophenol	2,4,6-TNP
6	2,4-Dinitrophenol	2,4-DNP
7	DNP-glycine	DNP-GLY
8	DNP-L-alanine	DNP-ALA
9	DNP-L-serine	DNP-SER
10	DNP-DL-α-aminobutyric acid	DNP-AABA
11	DNP-L-asparagine	DNP-ASP-NH_2
12	DNP-L-valine	DNP-VAL
13	DNP-DL-methionine	DNP-MET
14	DNP-L-leucine	DNP-LEU
15	DNP-DL-ethionine	DNP-ET
16	DNP-DL-α-aminocaprylic acid	DNP-AACA
17	DNP-γ-aminobutyric acid	DNP-GABA
18	DNP-ε-aminocaproic acid	DNP-EACA

[a] DNP = Dinitrophenyl.

The driving current in the ITP stage (150 μA) was chosen as an experimental optimum considering a rapid ITP separation, sharp zone boundaries and the disturbing role of thermal effects[17]. An optimum value of the driving current for the ZE

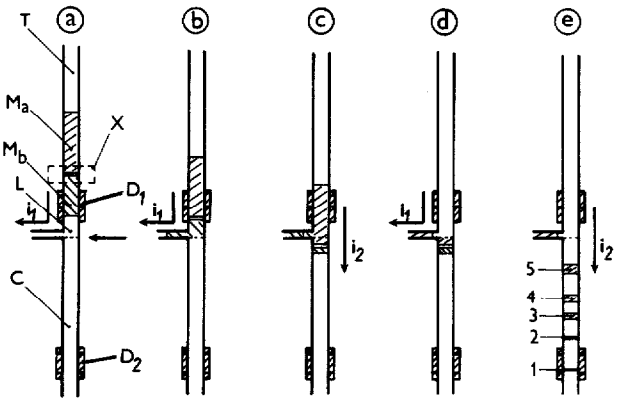

Fig. 1. Schematic illustration of the separation phases in combining ITP with ZE in the column-coupling configuration of the separation unit. (a) ITP separation in the first column (ITP stage); (b) removal of matrix constituent(s), M_b, from the separation compartment; (c) transfer of the sample fraction containing the analyte(s), X, into the second column (ZE stage); (d) removal of matrix constituent(s), M_a, from the separation compartment; (e) ZE separation in the second column (ZE stage). L, T = leading and terminating zones, respectively; C = carrier electrolyte; D_1, D_2 = detectors for ITP and ZE stages, respectively; i_1,i_2 = directions of the driving currents; 1–5 = symbols for the separated constituents in the ZE stage. The arrow on the right in (a) indicates the bifurcation point.

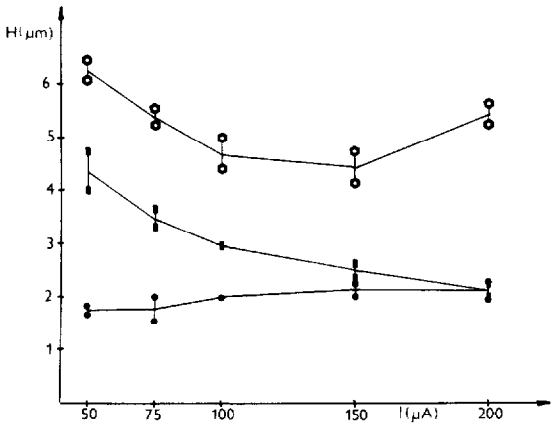

Fig. 2. Dependences of plate height (H) on the driving current (I). The plotted data were obtained for 10-pmol amounts of (○) DNP-ASP, (■) DNP-GLY and (○) DNP-EACA in the operational system listed in Table II. The H values were calculated from the data provided by a computing integrator (see Experimental).

stage was found from experimentally obtained dependences of the heights equivalent to a theoretical plate (H) on the driving current as shown in Fig. 2. A 150-μA driving current was estimated as an optimum for the ZE stage. The values of the driving currents for both stages were higher in comparison with (via current densities) to the values used in CITP and CZE in capillary tubes of similar I.D.[1–4,9,14]. Preliminary experiments revealed that this was possibly due to improved heat dissipation from the capillary tubes in comparison with the column design currently used in our laboratory[14].

An electropherogram (Fig. 3) obtained from the separation of a model mixture

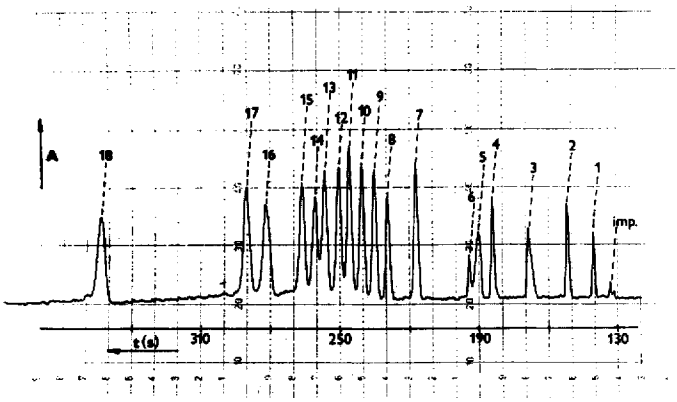

Fig. 3. Electropherogram from the separation of a mixture of nitrophenols and DNP-labelled amino acids. A 5-μl volume of the sample containing the constituents at $2 \cdot 10^{-6}$ mol/l concentrations was injected with a microsyringe. The separation was carried out in the operational system described in Table II (except the pH value of the carrier electrolyte was 5.3). The driving currents were 150 μA in both stages. The total analysis time was slightly less than 10 min. A = increasing light absorption; t = analysis time in the ZE stage; imp = unidentified impurity from the sample. For peak assignments, see Table I.

of anionically migrating constituents (Table I) under the working conditions employed clearly shows the high separation efficiences achieved. In this instance, the detected constituents were present in a sample solution at $2 \cdot 10^{-6}$ mol/l concentrations and were injected in a 5-μl volume with a microsyringe. As expected, none of these constituents was detectable by the conductivity detector under the conditions employed in the ITP stage (see also Fig. 4). As the conductivity detector allowed the detection of isotachophoretically migrating zones as short as 0.2 mm, we can assume that this value corresponded to a maximum length of the sample fraction of interest transferred to the ZE stage. In other words, it represented less than a *ca.* 14-nl injection volume into the 14-μl capillary tube employed for the ZE separation. The ratio of these volumes indicates a very positive role of ITP in achieving the injection volume required for a highly efficient ZE separation[1,2,18]. Experiments carried out with anionically migrating dyes revealed that no visible loss of the sharpness of the sample zone in the bifurcation point (see Fig. 1) occurred. However, this could be due to the fact that a small part (0.5 mm in length) of the leading zone was transferred with the focused dyes into the ZE stage. Hence ITP sharpening effect[9] could be responsible for the suppression of the partial zone dispersion in the bifurcation point (see also below).

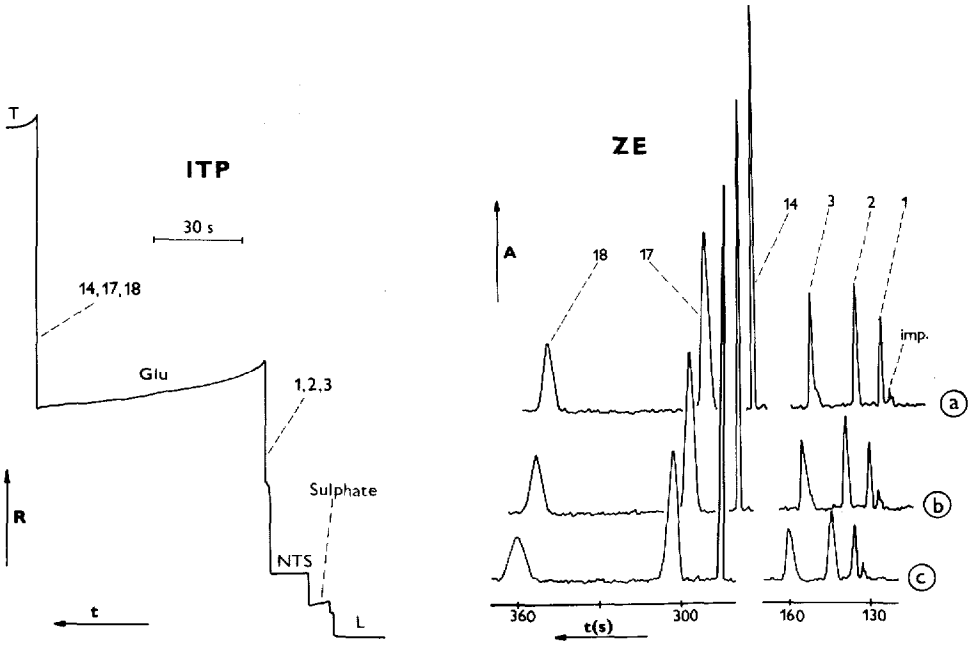

Fig. 4. Influence of interruption of the driving current on the separation efficiency in the ZE stage. The driving current was switched into the ITP stage to remove a sample macroconstituent (glutamate). 95% of the injected glutamate [see the isotachopherogram (ITP) from the conductivity detector in the ITP stage] was removed from the separation compartment after the ITP stage. Electropherograms (ZE) were obtained under the following conditions: (a) 70-s removal step; (b) as in (a) but after constituents 14, 17 and 18 had been transferred into the ZE stage the driving current was disconnected for 490 s; (c) as in (b) but the driving current was disconnected for 1400 s. L, T = leading and terminating zones, respectively. *A*, *t* and R = increasing light absorption, time and resistance, respectively. For the peak assignments see Table I and for working conditions see Table II.

Reproducibilities of migration times, quantification and separation efficiences

A series of experiments were carried out to measure the reproducibilities of the migration times of the studied constituents under the working conditions employed in the ZE stage (Table II). As the ITP separations are very reproducible when the factors influencing the migration velocities are controlled, a very reproducible transfer of the sample fraction of interest into the ZE stage was expected. In addition, for the ZE separations we employed a carrier electrolyte with a sufficient pH buffering capacity (some of the separands were moderately weak acids with pK_a values in the range 4–5). As all precautions concerning uncontrolled movement of the solution in the separation compartment[19] were also taken, high reproducibilities of the migration times (Table III) were achieved. The relative standard deviations of the migration times are in very good agreement with those obtained by CZE in a 0.2 mm I.D. capillary tube[2,3] in which the sample was injected with the aid of a sampling valve.

Mean H values and their reproducibilities obtained in the above series of experiments are summarized in Table III. In general, these values indicate the high separation efficiences achieved. When the electropherograms in Figs. 3 and 6 are compared, it can be seen that the higher H value for 2,4,6-TNP was due to 2,4-DNP (originating from the labelled amino acids) migrating almost unresolved from 2,4,6-TNP. It can also be seen that an unidentified impurity migrating in the front side of the DNP-GLU zone was responsible for a higher H value of this constituent. Although a search for optimum separating conditions in the ZE stage was not the aim of this work, a small pH difference of the carrier electrolytes as used in the run in Fig. 3 and in the remainder of experiments (Figs. 4–6) indicated that at a lower pH we could expect a better resolution of 2,4-DNP and 2,4,6-TNP.

The results published recently by Foret *et al.*[20] show that under our experimental conditions (hydrodynamically closed system) dispersion due to electroosmosis

TABLE II

OPERATIONAL SYSTEM

Discrete spacing constituents: sulphuric, chloric, tartronic, tartaric, malonic, citric, succinic, glutaric, phosphonomethyliminodiacetic, adipic, acetic, dichloroacetic, trichloroacetic, β-bromopropionic, malonic monoethyl ester, iminodiacetic, aspartic, butyric, N-acetylserine, hydroxyethyliminodiacetic, glutamic, succinic monoisopropyl ester, N-acetylleucine, α-aminoadipic and α-aminopimelic acids.

Parameter	*Electrolyte*[a]		
	ITP		*ZE: carrier*
	Leading	*Terminating*	
Solvent	H_2O	H_2O	H_2O
Anion	Cl^-	MES	MES
Concentration (mM)	20	10	50
Counter ion	HIS	HIS	HIS
pH	5.5	~5.5	5.5
Additive	HEC; m-HEC	–	HEC; m-HEC
Concentration (%, w/v)	0.1;0.1	–	0.1;0.1

[a] HEC = hydroxyethylcellulose; HIS = histidine; m-HEC = methylhydroxyethylcellulose; MES = morpholinoethanesulphonic acid.

TABLE III

MIGRATION TIMES AND SEPARATION EFFICIENCIES IN THE ZE STAGE (n = 9)

H = height equivalent to a theoretical plate (HETP); $\bar{t}_r$ = average migration time; s_r = relative standard deviation; n = number of data points.

Constituent		*Migration time*		*HETP*	
No.	*Abbreviation*	$\bar{t}_r$ *(s)*	s_r *(%)*	H *(μm)*	s_r *(%)*
1	DNP-CYS-SO_3H	138.4	0.99	2.00	19
2	DNP-ASP	150.3	0.75	2.42	12
3	DNP-GLU	167.1	0.99	5.29	22
4	2,6-DNP	183.4	1.24	2.16	30
5	2,4,6-TNP	190.0	0.59	5.64	23
7	DNP-GLY	215.5	0.41	2.88	5
8	DNP-ALA	227.96	0.46	2.19	18
9	DNP-SER	233.7	0.37	2.73	12
10	DNP-AABA	239.2	0.42	3.27	4
11	DNP-ASP-NH_2	244.5	0.37	3.02	4
12	DNP-VAL	249.2	0.40	3.24	6
13	DNP-MET	255.4	0.39	3.23	6
14	DNP-LEU	259.51	0.35	3.59	7
15	DNP-ET	265.1	0.40	3.18	7
16	DNP-AACA	281.13	0.40	5.00	5
17	DNP-GABA	289.1	0.34	3.91	4
18	DNP-EACA	352.0	0.30	4.22	7

can be dominant. Although the addition of HEC and m-HEC to the electrolyte solutions was a very efficient way of suppressing electroosmosis[21], its experimental comparison with other alternatives[21,22] seemed desirable. In our experiments, ad-

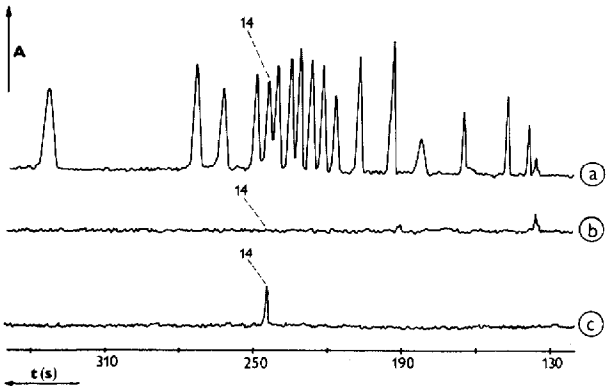

Fig. 5. Role of "ITP" sharpening effect on the detectability of the constituents in the ZE stage. (a) ZE separation of a model mixture containing the separated constituents at $2 \cdot 10^{-6}$ mol/l concentrations (5-μl injection volume); (b) same sample as in (a) except the concentrations of the separands were $8 \cdot 10^{-8}$ mol/l; (c) same as in (b) except the sample contained, in addition, glutamate at $8 \cdot 10^{-3}$ mol/l; 95% of the glutamate was removed after the ITP stage and the remaining part was transferred into the ZE stage to improve the detectability of DNP-LEU (the peak corresponds to 400 fmol of the analyte). The working conditions are described in Table II. The analysis times were *ca.* 10 min.

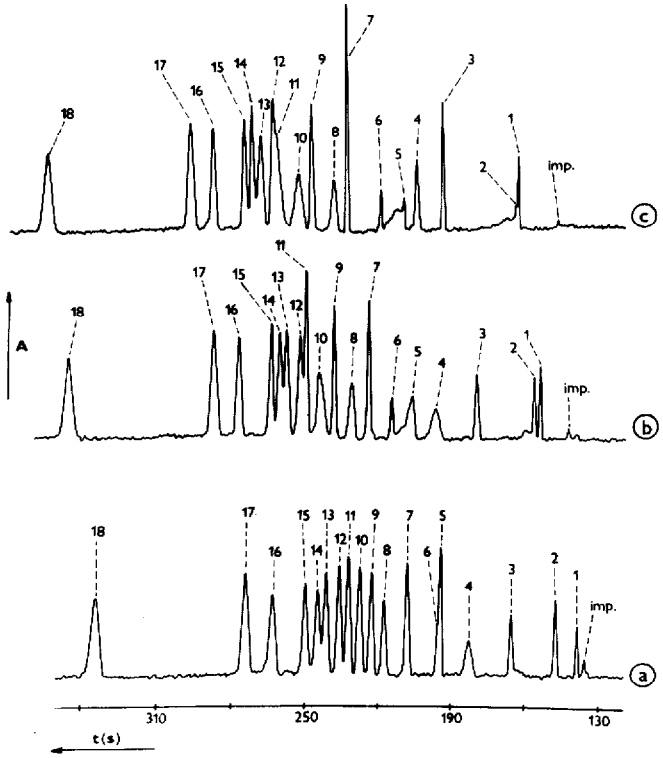

Fig. 6. Influence of a mixture of constituents undetectable in the ZE stage on the separation of nitrophenols and labelled amino acids. (a) 5-μl sample volume containing nitrophenols and 2,4-dinitrophenyl-labelled amino acids at $2 \cdot 10^{-6}$ mol/l concentration; (b) as in (a) except the sample contained, in addition, a 25-component mixture of discrete spacers (each at $4 \cdot 10^{-5}$ mol/l); (c) as in (b) except the concentrations of the spacing constituents were $8 \cdot 10^{-5}$ mol/l. *A*, *t* = increasing light absorption and time, respectively; imp = unidentified impurity from the sample. For the peak assignments see Table I and for working conditions see Table II. The composition of the mixture of discrete spacers is given in Table II. The analysis times were *ca.* 10 min.

sorption of the separated constituents was minimized by a dynamic coating of the surface with triply negatively charged NTS. In this respect other alternatives[18,22,23] should also be examined to find the optimum solution for minimizing these undesired dispersive phenomena.

The calibration lines (Table IV) were evaluated for 0.5–5-μl volumes of the sample solution containing the analytes at $2 \cdot 10^{-}6$ mol/l concentrations (1–10 pmol injected). The lowest injected amounts were *ca.* double the estimated detection limits (*ca.* 600–800 fmol). As the response of the detector (transmittance scale) deviated from linearity by more than 5% for 12-pmol amounts of the analytes, we were restricted in our experiments only for the lowest quantifiable concentrations. However, it is necessary to note that 50–80-pmol amounts of the analytes gave zones with migration times identical with those obtained for lower concentrations and with no observable electromigration dispersion[1–3], indicating that a wider concentration

TABLE IV

PARAMETERS OF THE REGRESSION EQUATIONS ($y = a + bx$) AND CORRELATION COEFFICIENTS FOR THE ANALYTES PRESENT IN THE SAMPLES IN 1–10-pmol AMOUNTS

n = Number of data points; r = correlation coefficient; x = sample volume (μl) with $2 \cdot 10^{-6}$ mol/l concentrations of the analytes; y = peak area (thousands of counts of the integrator).

Constituent		a^a	b^a	r	n
No.	*Abbreviation*				
1	DNP-CYS-SO_3H	28	94	0.9869	21
2	DNP-ASP	25	164	0.9947	20
3	DNP-GLU	5	185	0.9896	18
4	2,6-DNP	37	167	0.9877	20
5	2,4,6-TNP	92	198	0.9725	18
7	DNP-GLY	59	308	0.9977	18
8	DNP-ALA	22	231	0.9836	18
9	DNP-SER	25	343	0.9958	17
10	DNP-AABA	50	375	0.9980	17
11	DNP-ASP-NH_2	43	429	0.9985	17
12	DNP-VAL	39	401	0.9971	17
13	DNP-MET	41	410	0.9956	17
14	DNP-LEU	27	356	0.9946	17
15	DNP-ET	48	383	0.9933	17
16	DNP-AACA	76	427	0.9832	17
17	DNP-GABA	31	409	0.9882	17
18	DNP-EACA	74	464	0.9860	17

[a] Thousands of counts of the integrator.

TABLE V

REPRODUCIBILITY OF THE DETERMINATION OF 8-pmol AMOUNTS OF THE ANALYTES (n = 4)

$\bar{y}$ = average peak area (thousands of counts of the integrator); s = standard deviation (thousands of counts of the integrator); s_r = relative standard deviation; n = number of data points.

Constituent		$\bar{y}$	s	s_r
No.	*Abbreviation*			*(%)*
1	DNP-CYS-SO_3H	388.0	19.7	5.1
2	DNP-ASP	713.5	21.4	3.0
3	DNP-GLU	765.3	13.0	1.7
4	2,6-DNP	733.8	7.5	1.0
5	2,4,6-TNP	908.3	25.7	2.8
7	DNP-GLY	1324.3	37.7	2.8
8	DNP-ALA	965.7	29.2	3.0
9	DNP-SER	1429.3	25.1	1.8
10	DNP-AABA	1596.3	41.4	2.6
11	DNP-ASP-NH_2	1797.5	32.3	1.8
12	DNP-VAL	1683.0	15.1	0.9
13	DNP-MET	1746.8	18.9	1.1
14	DNP-LEU	1512.5	34.5	2.3
15	DNP-ET	1671.0	29.0	1.7
16	DNP-AACA	1931.0	53.1	2.8
17	DNP-GABA	1734.3	51.7	3.0
18	DNP-EACA	2014.5	41.4	2.1

range is applicable for quantitative analysis by using measurements on the absorbance scale.

Reproducibilities of the determinations of 8-pmol amounts of the analytes (4-μl injection volume) are listed in Table V. The relative standard deviations of the peak areas clearly show that by using microsyringe injection in conjunction with ITP focusing of the separands, the precision in the sample introduction was at least comparable to those achieved by advanced injection systems for CZE in 50–200-μm columns[24,25] with comparable separation efficiencies. These results also suggest that by using ITP as a sample introduction technique for ZE, problems associated with the injection of samples from extremely small sample volumes[24,26] could be alleviated, *e.g.*, by using a microsyringe for low nanolitre volumes[27] for sample handling and injection of the sample into the ITP stage.

Removal of the matrix constituents after the ITP stage

The sample solutions used in the above experiments contained sulphate and NTS as macroconstituents which were removed from the separation compartment after the ITP stage. The sample fractions of analytical interest were transferred into the ZE stage after a short interruption (*ca.* 3 s) in the delivery of the driving current between the phases b and e (see Fig. 1) to switch the columns by the controller of the analyser via a relay. This interruption in the delivery of the driving current had no detectable effect on the efficiency of the ZE separation, as the time was considerably shorter than the migration times. However, as discussed above, in these instances ITP zone sharpening compensated for any dispersion of the zones. From the schematic illustration in Fig. 1 it can be seen that by using a complete sequence of the separation phases the sample fraction transferred into the ZE stage in phase c does not migrate while a less mobile matrix constituent is led out of the separation compartment. Obviously, it is desirable to remove this constituent within the shortest possible time in order to minimize the dispersions of the transferred zones by diffusion. On the other hand, there are inherent restrictions concerning the maximum applied driving current in the ITP stage[9]. The electropherograms in Fig. 4 were obtained in experiments aimed at studying the consequences of such an interruption in the driving current on the ZE separation. Here, the transferred sample fractions were present in the second column for various times before the driving current for the final ZE separation (phase e in Fig. 1) was applied. The interruption of the driving current necessary to remove 95% of the injected glutamate (a in Fig. 4) had no observable influence on the separation efficiencies of constituents 1, 2 and 3 or on their migration times. On the other hand, considerably longer interruptions of the driving current [corresponding to the removal of 8-fold (run b) and 21-fold (run c) larger amounts of glutamate] led to easily detectable decreases in the efficiencies of separation.

As very high reproducibilities in the ITP stage were typical, we could remove more than 99% of the injected glutamate and the migration times of the transferred sample constituents agreed within random error with those obtained for the sample without glutamate (see Table III). Also, no visible differences in the peak shapes were detected in these experiments. When 5% amounts of the injected glutamate were transferred with the labelled amino acids into the ZE stage, a sharp DNP-LEU peak was typical (see Fig. 4) and the migration times of the constituents migrating behind the glutamate zone in the ITP stage (14, 17 and 18) were higher in comparison with

the values obtained without glutamate. Such a behaviour is an obvious consequence of the electromigration dispersion of the glutamate zone in the ZE stage[1–3] accompanied by a strong ITP sharpening effect of the adjacent DNP-LEU zone. As the ZE separations were also reproducible under these conditions, differences in the migration times of constituents 14, 17 and 18 were constant from run to run. These facts led us to investigate the role of the sharpening effect in improving the detectability of the separands in the ZE stage. The electropherograms in Fig. 5 obtained from these experiments clearly illustrate this possibility. For example, in this particular instance the detection limit for DNP-LEU was improved *ca.* 5-fold in comparison with the separations without transferred glutamate. It should be also mentioned that in this way the analyte could be detected with confidence in a sample containing a 10^5-fold excess of glutamate (c in Fig. 5).

In practical situations, the constituents of analytical interest can be present in the samples containing a large number of matrix constituents at concentrations comparable to or slightly higher than those of the analytes. Here, the matrix constituents form very short zones (0.5–1 mm) detectable in the ITP stage by the conductivity detector. Although detectable in the ITP stage, their removal after this stage as described above can be almost impossible, especially when the number of both the analytes and matrix constituents is high. In an ideal case, *i.e.*, with the analytes migrating spaced by the matrix constituents (undetectable in the ZE stage), we could expect sharp peaks of the analytes in the ZE separation with the migration times being influenced by their different injection times and by the electromigration dispersions of the zones of the matrix constituents. However, a time- and labour-consuming search for a suitable operational system seems unavoidable in order to achieve such a goal.

The electropherograms in Fig. 6 illustrate some problems that can be encountered in such instances. In these experiments a model mixture of the analytes was separated with various amounts of a 25-component sample of discrete spacing constituents for the ITP separations of anions at pH 5.5 (Table II). It can be seen that the presence of the spacing constituents in the sample led to sharpening of some zones (Nos. 1, 3, 4 and 7), their "dilution" (Nos. 2, 5 and 10), loss (Nos. 11 and 12) or improvement of the resolution (No. 6). It is apparent that whereas some of the analytes were injected as sharp pulses, another forming mixed zones (spread along the spacing constituents) in the ITP phase gave wide injection pulses. In spite of the fact that a detailed investigation of all factors influencing the ZE separation in such an instance was not made, it appears that the injection of smaller sample amounts or the use of more concentrated electrolyte solutions are, in practice, the simplest alternatives for solving problems of this kind. Here, the choice is not straightforward as the former alternative introduces higher demands on the detection system and the latter can lead to problems associated with thermal effects.

CONCLUSIONS

The results clearly show promising analytical capabilities of on-line coupling of CITP with CZE. As could be expected, a well defined concentration adaptation of the sample constituents in the ITP stage was effective in reducing the volume in which they were transferred into the ZE stage. For example, a 10^2–10^3 volume reduction

was typical under our experimental conditions on injecting 0.5–15-μl sample volumes with the aid of a microsyringe or a sample valve. Thus, by using a microsyringe sample injection, very small sample volumes can be sufficient for the analysis and as no sample splitting is necessary[28] it can be completely transferred for the ZE analysis. It is also important that in this way very reproducible migration times in the ZE stage could be achieved while the precision of the sample injection remained high.

A sample clean-up in the ITP stage can be effective, at least, in removing sample macroconstituents so that the sample volume need not be reduced to avoid column overloading in the ZE stage. It is apparent that in this way it is possible to improve the concentration detection limits for the constituents of analytical interest in CZE. In addition, as the ITP stage also provides analytically important information, an overall evalution of the analysis can be based on the data obtained in both stages in one run.

Our experiments suggest that a defined electromigration dispersion can be effective in improving the detection limits in the ZE stage. However, further research in this respect is necessary to investigate the possibilities of this approach and problems that can occur in its practical use.

Some restrictions concerning the choice of the electrolyte systems (the terminating constituent migrating in the ZE stage) and a need to use a more advanced construction of the instrument are apparent disadvantages of the studied combination of basic electrophoretic techniques. However, only comparative experiments with "pure" techniques can provide more relevant information in this respect.

The use of such a combination in electrophoretic separations with electroosmotic transport of the electrolyte solution in the separation compartment[29] or in electrokinetic chromatography with micellar solutions[30] seems possible. A development of appropriate instrumentation, preferably with well defined control of the electroosmotic flow is necessary, however.

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