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Journal of Chromatography A, 838 (1999) 55–70

JOURNAL OF CHROMATOGRAPHY A

Isotachophoresis in zone electrophoresis

Ludmila Křivánková*, Pavla Pantůčková, Petr Boček

Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveří 97, 611 42 Brno, Czech Republic

Abstract

Conditions for existence of transient isotachophoresis (ITP) in zone electrophoresis are quite common. Transient ITP can either be induced by the composition of the sample or by the composition of the electrolyte system or result from the first step during capillary ITP–capillary zone electrophoresis (CZE) combination. This paper brings a comprehensive analysis of the problem and description of the effects of transient ITP on the migration time, separation efficiency and the detection sensitivity of the CZE analysis. Theoretical considerations are accompanied by model experimental examples. It is shown that in cases where transient ITP can be controlled, the effects of transient ITP can be employed for improvement of the performance of the analysis. Further, it is shown that the combination of capillary ITP–CZE is by far superior. It enables one to inject large sample volumes, to reach efficient sample clean-up, and to separate and to detect trace analytes under optimum conditions.

Keywords: Isotachophoresis–capillary zone electrophoresis; Isotachophoresis, transient; Capillary zone electrophoresis; Stacking effects

1. Introduction

Isotachophoresis (ITP) and zone electrophoresis (ZE) are electromigration separation techniques based on the same principle, i.e., the separation of ions under the influence of electric field, however, their separation regimes, accompanying effects and final stages of the separation processes are different. In ZE, the separation medium is created by a background electrolyte (BGE) composed of a co-ion migrating in the the same direction as the analytes do, and a counterion migrating in the opposite direction. The composition of the BGE is constant. When voltage is applied across the migration path, the substances migrate at different velocities corresponding to their mobilities and separate one from another. They reach the detector at a time dependent on the mobility, capillary length and voltage applied. When no additional effects (e.g., stacking) are involved, the concentration of an analyte in its zone passing the detector is always lower than that in the injected zone due to dispersive effects [1]. In ITP, the sample is injected between two electrolytes, one containing a faster co-ion with the highest mobility (leader, L) and the other containing a slower co-ion with the lowest mobility (terminator, T). During the separation process the analytes create their own zones and in the final steady state these zones are arranged in the order of their mobilities and migrate with the same velocity. The migration time of a zone is dependent not only on the mobility, capillary length and electric current but also on the amount of the leading ion and on the amount of ions migrating in preceding zones. Jumps in voltage between neighboring zones result in permanently sharp boundaries between zones and are typical for ITP. The con-

*Corresponding author. Tel.: +420-5-726-8123; fax: +420-5-4121-2113.

E-mail address: krivan@iach.cz (L. Křivánková)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00169-7

centration of an analyte in its zone is adjusted to the concentration of the leading ion and can be, in contrary to ZE, increased by several orders of magnitude [2]. For analytical purposes, the capillary versions of these methods (cITP, CZE) are employed mostly. Here, the detection of zones is performed on-line either by a non-selective detector (e.g., conductivity or potential gradient) or a selective one (e.g., UV absorbance or fluorescence). When a zone longer than the detector window is created, the detection signal creates steps with sharp boundaries and plateaus. The length of a plateau is proportional to the amount of the analyte in the sample and the step height is a qualitative parameter. When an isotachophoretic zone is shorter than the detection window of an optical detector then it gives a sharp peak resembling CZE peaks. However, neither the height of this peak nor its detection time are the qualitative parameters. On the other hand, a linear calibration curve can be obtained for the dependence of the peak height or area on the amount of the analyte in the sample until the plateau of a typical cITP step is created.

In practice, usually one of these techniques is selected for the sample analysis. However, as has been shown in several recent papers and applications [3–35], both techniques can be advantageously combined either in one capillary or in two on-line connected capillaries. Such combination offers a possibility to inject large volumes (in tens of microliters) of relatively diluted samples (10^{-7} M), to separate both micro- and macrocomponents present in the sample and to detect the macrocomponents in the cITP step, to perform clean-up and to reach the high separation efficiency and sensitivity of CZE step and thus to analyze trace components. It has been shown in cITP–CZE combination that the detection times, separation efficiency and resolution of analytes are strongly dependent on the composition of the BGE in ZE and the rules for the proper selection of the electrolyte combination for cITP and CZE step and evaluation of the records have been published [11,24,29].

In this work we would like to show that transient ITP migration in ZE mode can exist not only during the transformation process of cITP into CZE but also in the single CZE analysis induced by the composition of the sample and of the separation medium. We would like to present the conditions which must be satisfied by the system in order to induce transient ITP, and to describe and to classify the effects of transient ITP on the migration and separation performance of CZE analysis.

2. Theoretical considerations

The prerequisite for the existence of ITP in ZE is the presence of a component in the system that can play the role of either the leading or the terminating ion for the other species present in the system consisting of a sample and a BGE. To fulfill this, a component of adequate mobility has to be present in this system and its amount must be above a certain critical value in order to be effective, and the background co-ion has always to play the other role, i.e., of the terminator or of the leading ion [23]. A scheme illustrating the conditions for the existence of transient ITP in ZE is given in Fig. 1.

Providing that these conditions are fulfilled three cases of transient ITP in ZE may be distinguished:

(1) Transient ITP induced by the sample composition. Here, a macrocomponent is present in the sample and can play for some time the role of either L or T ion for sample species, the co-ion of the BGE plays simultaneously the role of T or L, respectively. The macrocomponent can either be a natural constituent of the sample or can be added purposely. The result is that analytes (microcomponents) are stacked either behind the transient L or in front of the transient T prior they reach their zone electrophoresis migration mode. For a scheme of this case, see Fig. 2.

(2) Transient ITP induced by the composition of the separation medium. Here the suitable separation medium is arranged purposely by the operator filling the capillary partially with a leading (LE) and a terminating (TE) electrolytes having higher and lower mobility than the co-ion of the BGE (C) or, in the simplified form, only with the LE when the co-ion is directly T or with the TE when the co-ion is the fastest ion in the system. For the scheme of this case, see Fig. 3.

(3) Transient ITP surviving from the ITP step in the cITP–CZE technique. This case corresponds to the two-stage analysis, on-line combination of ITP

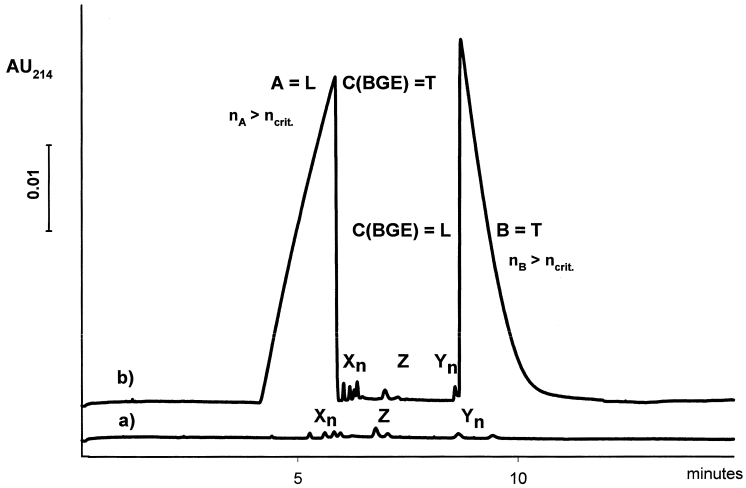

Fig. 1. Prerequisite for the existence of transient ITP in CZE. In a set of components migrating in a BGE, a fast (A) or a slow (B) component has to be present at a concentration higher than a critical value and has to play the role either of leader (L) or of terminator (T) with respect to the co-ion of the BGE which thus plays the other role, i.e., of T or L, respectively. Then, ITP conditions are fulfilled for analytes X with mobilities $u_{A=L}>u_X>u_{C=T}$ or analytes Y with $u_{C=L}>u_Y>u_{B=T}$. For analytes Z with $u_Z=u_C$ the ITP condition is not fulfilled. (a) A mixture of $1\cdot10^{-5}$ *M* analytes with effective mobilities $40>u_X>27$ (10^{-9} m^2 V^{-1} s^{-1}), $25>u_Y>19$ (10^{-9} m^2 V^{-1} s^{-1}), $u_Z\sim26\cdot10^{-9}$ m^2 V^{-1} s^{-1} separated in a BGE with the effective mobility of the co-ion, $u_C=26\cdot10^{-9}$ m^2 V^{-1} s^{-1}; (b) the analysis of the same mixture as in (a), but the concentrations of A ($u_A=39\cdot10^{-9}$ m^2 V^{-1} s^{-1}) and B ($u_B=19\cdot10^{-9}$ m^2 V^{-1} s^{-1}) are $1\cdot10^{-2}$ *M* and $5\cdot10^{-3}$ *M*, respectively.

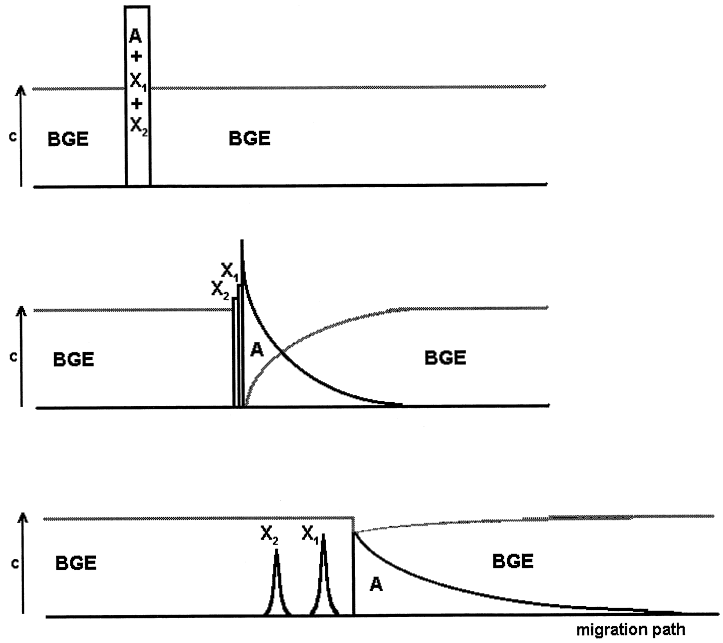

Fig. 2. Scheme of transient ITP induced by the sample composition. A, X_1, X_2, are the sample components, A acts as the transient L for X_1, X_2, with the BGE co-ion being T.

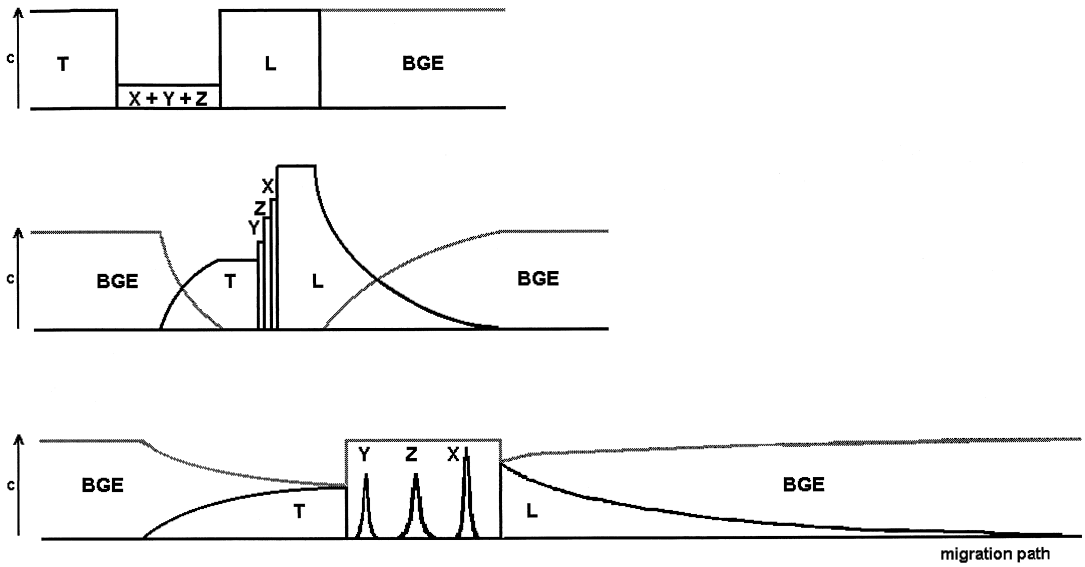

Fig. 3. Scheme of transient ITP induced by the composition of the separation medium. In addition to BGE containing the co-ion C the capillary is filled partially with L and T (or only L or only T) so that conditions for transient ITP are fulfilled for analytes for which it holds that $u_L > u_X$, u_C, $u_Y > u_T$ or $u_C > u_Y > u_T$ or $u_L > u_X > u_C$.

and CZE performed in two separate capillaries. cITP is run in the first capillary and CZE analysis is carried out in the second one. Trace analytes are pre-concentrated and pre-separated in the first capillary and a cut of important analytes accompanied with a segment of the leader or terminator enters the second capillary for the final analysis by ZE. The presence of this segment results from the fact that we do not want to lose a part of the analyzed zones and we must make the cut generously. The zone of this segment survives for a certain time during the ZE stage and this means that ITP migration continues also in the second capillary for some time. When the conditions for transient ITP are not fulfilled any more, the transient ITP is converted into ZE mode, see scheme in Fig. 4.

In all cases mentioned above, transient ITP significantly affects the analytical parameters of CZE analysis, namely, the detection time, zone dispersion, separation efficiency and sensitivity of the analysis. Obviously, the goal of the effort is to select such conditions where large sample volumes can be injected, the concentration of the analytes can be increased during the separation process, dispersion of zones can be minimized and high separation efficiency can be reached resulting in low detection limits. The prerequisite for the proper and maximum utilization of ITP effects in ZE is understanding of the transition process with the aim to select the most favorable separation conditions for one or more analytes of interest present in the sample.

3. Experimental

3.1. Instrumentation

For CZE experiments, an automated capillary electrophoresis instrument P/ACE 2100 system (Beckman, Fullerton, CA, USA) equipped with a UV detector set to 214 nm was used. Electrophoretic separations were performed in a coated capillary of 47 cm (40 cm effective length)×100 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA). The inner surface of the capillary was coated with linear polyacrylamide by the method described in Ref. [36] and modified as in Ref. [37]. Analyses of anions were performed with cathode at the injection side. The electroosmotic mobility was measured with mesityloxide with anode at the injection side and was less than $1.0 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}. The thermostatting temperature was 25°C. Samples were injected with

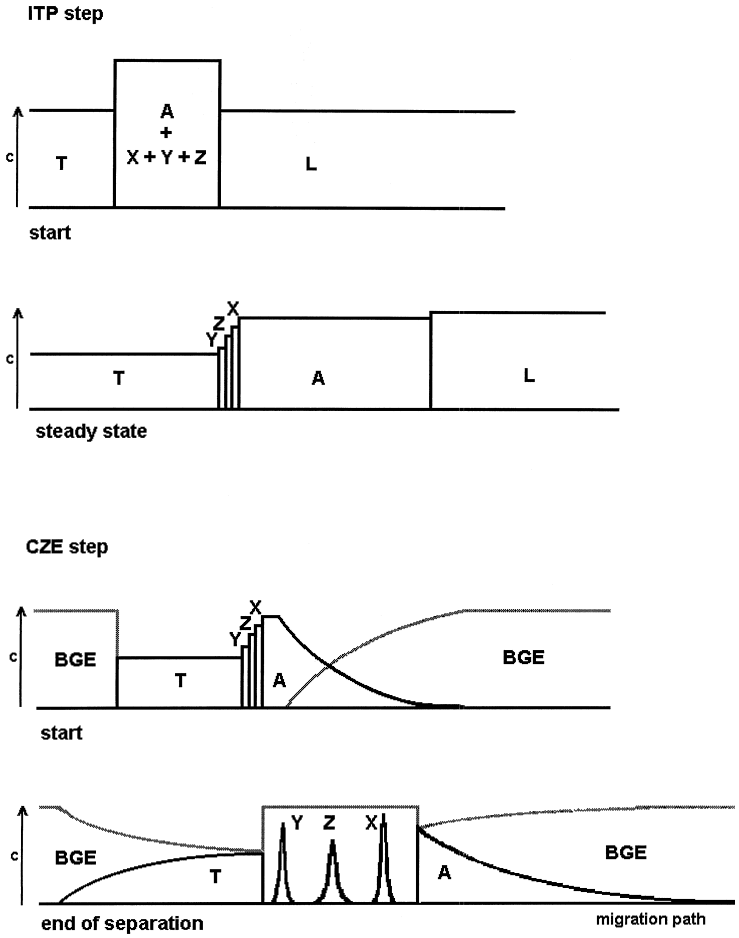

Fig. 4. Transient ITP surviving from ITP step in the cITP–CZE technique. In the first capillary, regular conditions for ITP exist. A part of the sample (here analytes A, X, Y, Z), after it had been analyzed by ITP in the first capillary, is transferred to the second one filled with a BGE together with a neighboring zone of either L or T or another zone of a macrocomponent (here A), which results in surviving of ITP mode for some time in the second capillary.

pressure of 0.5 p.s.i. (1 p.s.i.=6894.76 Pa). Between runs the capillary was washed at 20 p.s.i. for 1.5 min with the BGE .

cITP–CZE measurements were performed on a CS Isotachophoretic Analyzer ZK01 or EA 100 Villa Labeco (Spišská Nová Ves, Slovak Republic) equipped with a column switching system. Capillaries used were made from polytetrafluoroethylene or fluorinated ethylene–propylene copolymer or fused-silica. The pre-separation capillary was equipped with a conductivity detector positioned 3.8 cm from the bifurcation point, the analytical capillary was equipped with a UV (254 nm) detector. The electrolyte chambers containing the LE and the BGE were separated from electrolytes in capillaries by semipermeable Cellophane membranes.

Unless stated otherwise, cITP measurements were performed at constant current and CZE measurements (including transient ITP in ZE) at constant voltage.

3.2. Chemicals

All chemicals used were of the highest analytical purity. L-Lactic acid and ascorbic acid were from Sigma (USA), orotic acid was from Fluka (Buchs,

Switzerland), aspartic acid and histidine (His) were from Renal (Budapest, Hungary), β-alanine (β-Ala) was from Loba Feinchemie (Fishamed, Austria), hydroxypropylcellulose (HPC) was from Ega-Chemie (Steinheim/Albuch, Germany). Other chemicals were purchased from Lachema Chemapol (Brno, Czech Republic).

Deionized water prepared by trapping ions in a mixed-bed ion exchanger by an aqua purificator G 7749 (Miele, Gütersloh, Germany) was used for the preparation of all solutions.

Samples used for the study of the sample induced ITP effects were prepared by dissolving sample components in the BGE used for the analysis. pH of the samples containing increased amounts of stackers was always checked and if necessary adjusted with the counterion to the pH of the BGE. In other cases, sample components were dissolved directly in deionized water.

4. Results and discussion

The way in which the ZE migration is affected by the transient ITP, depends strongly on the role of the BGE co-ion (denoted C). In a system where only one macrocomponent is present it is crucial whether the BGE co-ion acts as L and the macrocomponent creates the zone of T, or vice versa, the BGE co-ion acts as T and the macrocomponent plays the role of L. The mobility of BGE co-ion is crucial also in the cases when two macrocomponents are present in the system and for analytes play the roles of L and T or of leaders or terminators only. In the following paragraphs, effects of transient existence of ITP in ZE separations on the migration time and zone dispersion are described. Analytes, which were or could be stacked by a transient leader present in the system (denoted A), are denoted X, analytes stacked by a transient terminator (denoted B) are described as Y. Analytes, which are not influenced by transient ITP, are denoted Z. It should be noted here that there is also a difference in the sample induced ITP and the other two cases, ITP induced by the composition of the separation medium and ITP surviving from the ITP step in cITP–CZE combination. In the sample induced ITP, ZE migration and ITP stacking proceeds simultaneously, and if the concentration and pH of the sample differ from the concentration and pH of the BGE, additional effects are involved. In the other types, pure ITP proceeds first. In the resulting steady state, the zones of the analytes are arranged according to their mobilities, and their concentrations and pHs are adjusted to the LE and hence the effect of ITP can be better predicted.

4.1. Transient ITP with one stacker and the co-ion of BGE being the terminator

4.1.1. cITP–CZE combination

As was shown and explained previously [11,24,29], in this case the migration of an analyte in the ITP mode is slower compared to the migration in ZE mode and thus the existence of transient ITP results in prolongation of detection times. The experimental example is given in Fig. 5a. The longer zone of L precedes the sample ITP stack, the longer time it takes to the analytes to reach the detector positioned at a constant distance from the injection point. This effect is more pronounced for faster analytes, which means that they stay for a longer time in ITP mode while the slower analytes leave it sooner and become thus more dispersed when passing the detector (Fig.5b). For fast analytes also substantially higher separation efficiency can be calculated (Fig. 5c).

4.1.2. ITP induced by the composition of the separation medium

Controlled conditions for transient ITP can easily be prepared so that the capillary is filled partially or totally with the LE and both electrode chambers and the rest of the capillary contain a BGE serving as TE. The dependence of the detection time on the length of the L zone is shown in Fig. 6a and is copying the course shown in Fig. 5 when the measurements are performed under constant current so as it is usual in classical cITP analyses. However, CZE analyses are usually run under constant voltage and the effect of varying current on the course of the separation is seen in Fig. 6b. Also here, the faster analytes stay for a longer time stacked in ITP and

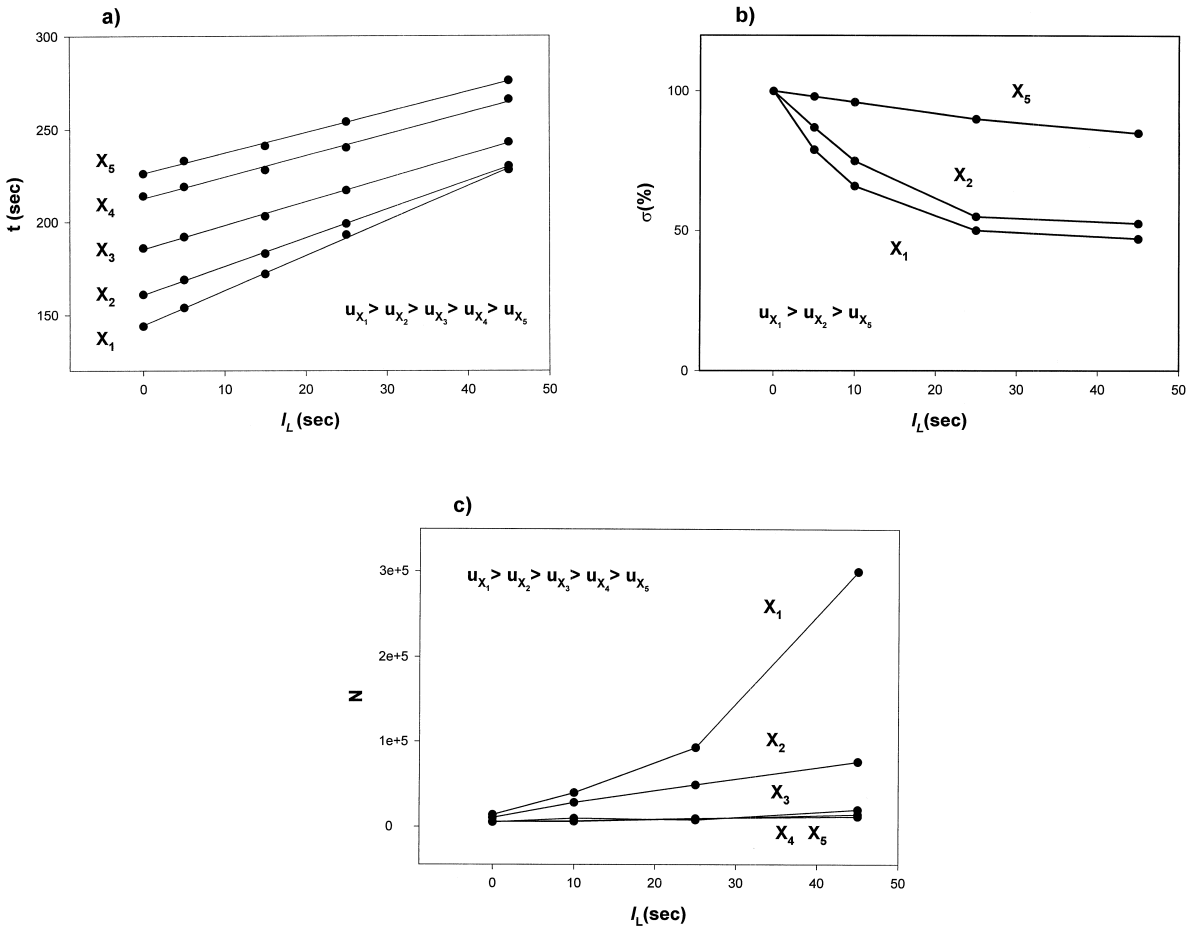

Fig. 5. Transient ITP surviving from ITP step in the cITP–CZE technique, BGE=TE. (a) Dependence of the detection time in CZE of analytes with mobilities $u=56–33\cdot10^{-9}$ m^2 V^{-1} s^{-1} on the length of L segment ($u=79\cdot10^{-9}$ m^2 V^{-1} s^{-1}) accompanying the stack of analytes into the ZE capillary filled with a BGE being terminating electrolyte, TE ($u=31\cdot10^{-9}$ m^2 V^{-1} s^{-1}) from the preceding ITP step. (b) Dependence of zone dispersion in CZE on the length of L segment. (c) Dependence of separation efficiency in CZE on the length of L segment. Measurements were performed in the cITP–CZE system, I.D. of capillaries both for cITP (19 cm effective length) and CZE (13 cm effective length) was 0.3 mm, constant current was 75 μA.

their zones are less dispersed when passing along the detector compared to the slower analytes (Fig. 6c).

4.1.3. Sample induced transient ITP

If the sample contains a macrocomponent the mobility of which is higher than the mobility of the BGE co-ion, conditions for transient ITP are created for the analytes having mobility lower than the macrocomponent and higher than the co-ion. This is illustrated in Fig. 7a and b. The effective mobility of mandelate is close to that of the co-ion and therefore it represents a ridge – for faster analytes conditions for ITP separation are created while for analytes with the mobility lower than that of the co-ion (equal to that of mandelate) only higher dispersion can be observed with increasing concentration of the macrocomponent. Analytes migrating for some time in ITP mode reach the detector later compared to the situation when the concentration of the macrocomponent is under the critical value and their limit of detection (LOD) is lower due to the concentration adjustment and smaller dispersion.

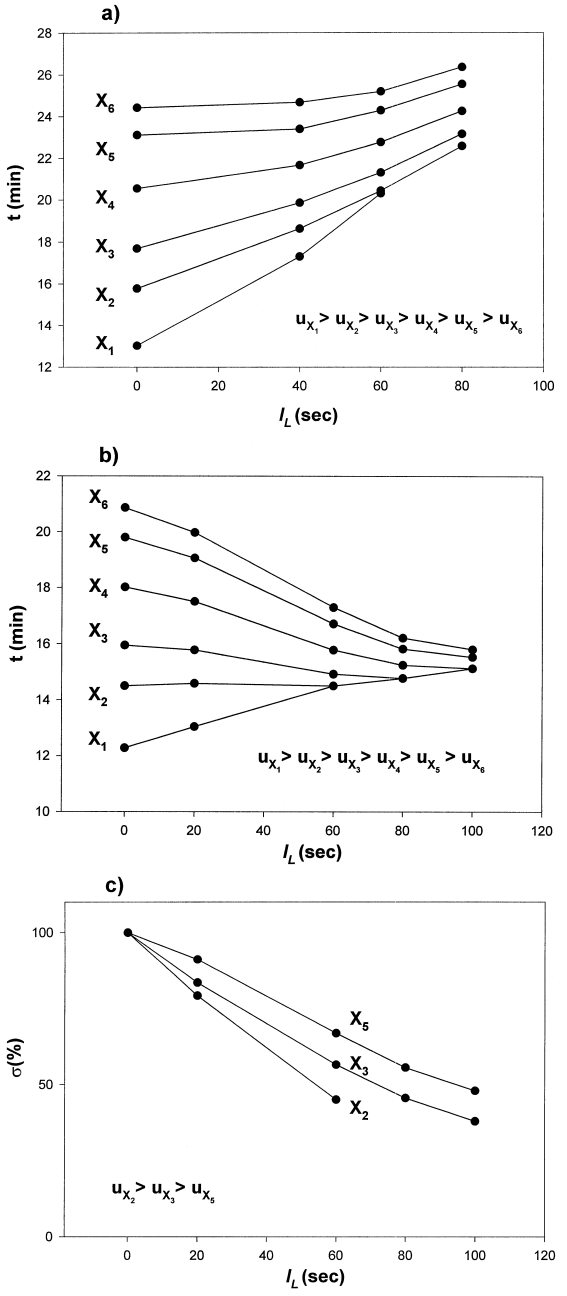

Fig. 6. Transient ITP induced by the composition of the separation medium, BGE=TE. (a) Dependence of the detection time of analytes on the length of the zone of LE at constant driving current 9 μA. (b) Dependence of the detection time of analytes on the length of the zone of LE at constant voltage 7 kV. (c) Dependence of analyte zone dispersion on the length of zone of LE, constant voltage 7 kV. $u_L = 79 \cdot 10^{-9}$ m^2 V^{-1}s^{-1}, $u_{BGE=T} = 20 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}, $u_X = 42–22 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}. BGE=20 m*M* HAc+EACA, pH 4.7, L=20 m*M* HCl+EACA, pH 4.

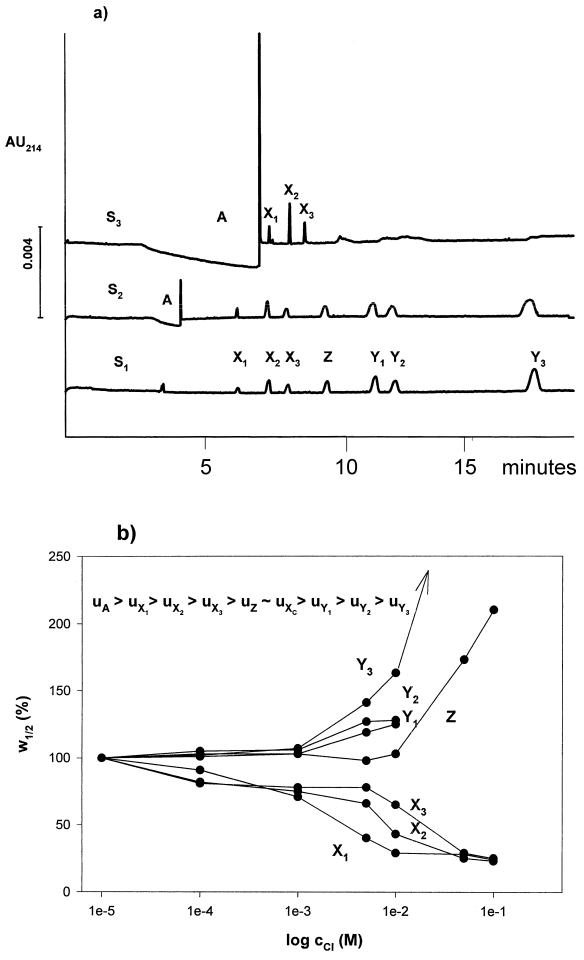

Fig. 7. Sample induced transient ITP, BGE=TE. (a) Effect of the concentration of a macrocomponent playing the role of L on the trace of analysis. Sample mixture: $S_1 = 10^{-5}$ *M* chloride (A), iodate (X_1), benzenesulfonate (X_2), *p*-toluenesulfonate (X_3), mandelate (Z), hippurate (Y_1), benzoate (Y_2) cinnamate (Y_3), $S_2 = S_1 + 5 \cdot 10^{-3}$ *M* chloride, $S_3 = S_1 + 10^{-1}$ *M* chloride. BGE=$5 \cdot 10^{-3}$ *M* lactic acid+ε-aminocaproic acid, pH 4.4, *U*=13 kV. (b) Effect of the concentration of a macrocomponent A playing the role of L on peak width of analytes from (a).

4.2. Effects of transient ITP with one stacker and the co-ion of BGE being the leader

4.2.1. cITP–CZE combination

When the co-ion of BGE serves as the leading ion, effects are quite opposite to the case described in Section 4.1.2. Transient ITP accelerates migration of analytes, the detection times of analytes are shorter

in comparison to the case when no transient ITP is present. The longer the zone of T accompanying the train of ITP zones migrating into the second capillary filled with a BGE, the shorter the detection times. The sharpest peaks can be observed for slow analytes migrating longer in ITP mode [11,24,29].

4.2.2. ITP induced by the composition of the separation medium

The same behavior of analytes can be observed as in the cITP–CZE analysis performed under constant current (Fig. 8a and b). If the difference between the conductivity of BGE and T is low, similar course of the plots with slight changes in slopes can be found in measurements at constant voltage. The shift of the detection times to shorter values is evident as well as sharpening of zones. If too long zone of T is injected as demonstrated in Fig. 8c, some slow analytes can be detected still in ITP mode stacked in one zone with high separation efficiency.

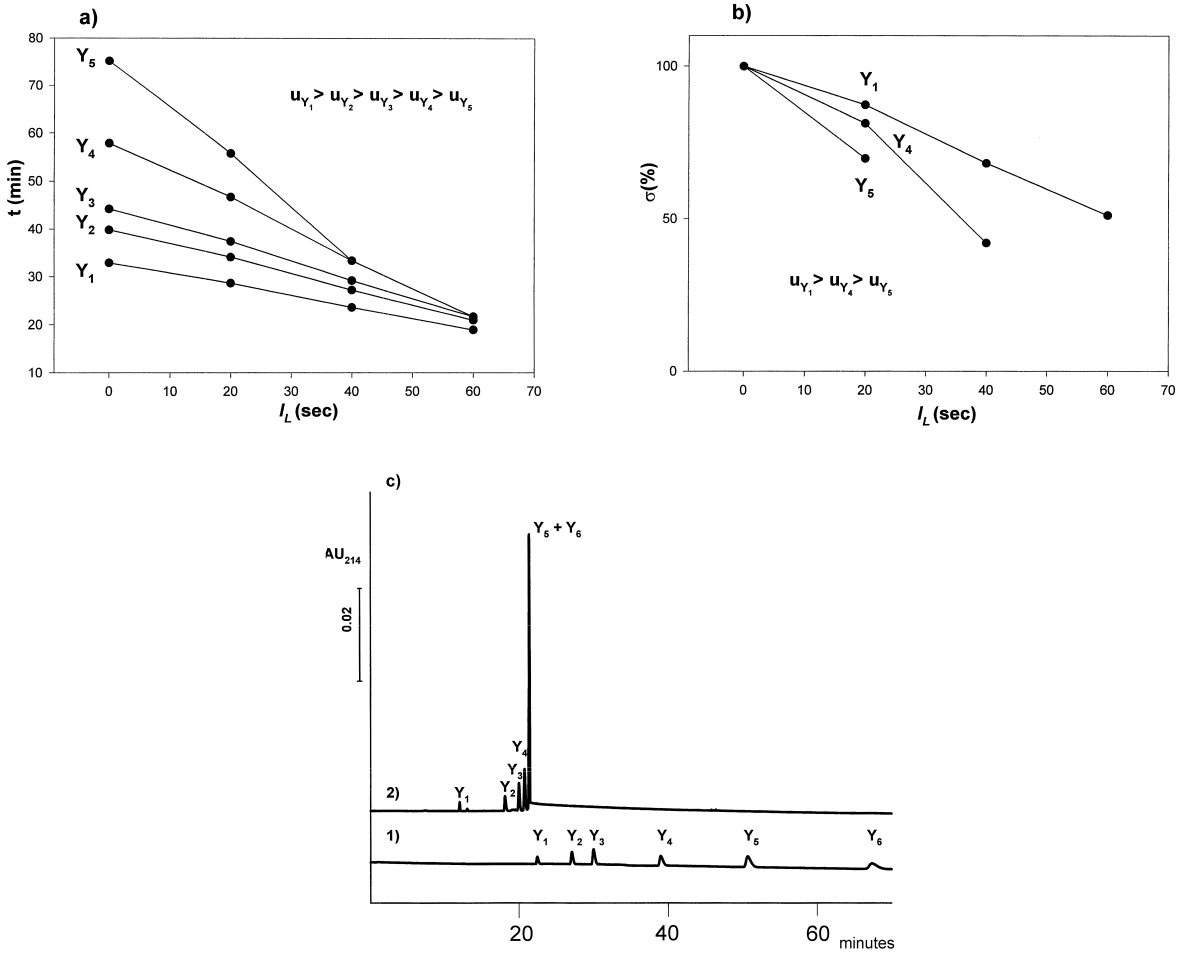

Fig. 8. Transient ITP induced by the composition of the separation medium, BGE=LE. (a) Dependence of detection time of analytes on the length of T zone at constant voltage 4 kV. (b) Dependence of peak width on the length of T zone at constant driving current 14 μA. $u_{BGE=L}=79\cdot10^{-9}$ m^2 V^{-1} s^{-1}, $u_T=20\cdot10^{-9}$ m^2 V^{-1} s^{-1}, $u_X=42–22\cdot10^{-9}$ m^2 V^{-1} s^{-1}. (c) Trace of analysis of the sample containing 10^{-4} *M* mixture of iodate (Y_1), benzenesulfonate (Y_2), *p*-toluenesulfonate (Y_3), mandelate (Y_4), hippurate (Y_5), benzoate (Y_6): (1) in BGE=LE (30 m*M* HCl+EACA, pH 4), (2) in BGE=LE with 60 s long zone of TE (20 m*M* acetic acid+EACA, pH 4.7) injected after the sample. Analytes benzoate and hippurate are still migrating in ITP mode stacked one to another and detected as one peak with high separation efficiency.

4.2.3. Sample induced transient ITP

The same tendency occurs when in a sample a macrocomponent is present having mobility lower than the BGE co-ion. For analytes with mobility values within this interval the ITP conditions are fulfilled. The closer the mobility of an analyte is to the mobility of the macrocomponent serving as T the more its zone is sharpened and passes along the detector at a time shorter compared with the situation when the conditions for ITP mode are not fulfilled, see Fig. 9.

4.3. Effects of transient ITP with two stackers – the mobility of the co-ion of BGE is higher than a terminator and lower than a leader

4.3.1. ITP–CZE combination

If in the cITP–CZE combination a BGE different from L or T is selected, three cases can occur. If the mobility of the BGE co-ion is lower than that of T, the course is the same as in the first case described in Section 4.1.1 and the co-ion acts as a real T with a short cut of the T from ITP being now a part of the stack of analytes and leaving the ITP stack as the first one. The length of the L cut is responsible for ITP surviving. The opposite case is when the co-ion has the highest mobility of the system. It acts as the real L and the rest of the leading zone cut into the second capillary together with the sample leaves the stack as the first one, the length of T zone accompanying the sample zones is responsible for ITP survival (compare Section 4.1.2). An interesting situation exists in the third case when a part both of L and T accompanies the sample stack into the second capillary filled with a BGE and the co-ion mobility is within the range of L and T mobilities. The co-ion serves for a group of analytes of higher mobility as T and for the group of analytes of lower mobility as L. Shifts in detection times and sharpening of peaks depend on the ratio of the L to T zone

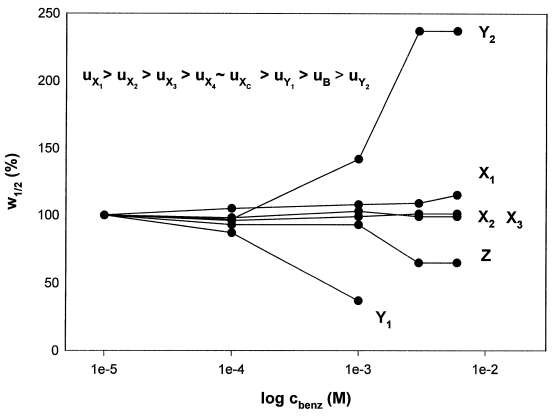

Fig. 9. Sample induced transient ITP, BGE=LE. Effect of the concentration of a macrocomponent B playing the role of T on the peak width. Sample mixture $1\cdot10^{-5}$ *M* chloride, iodate (X_1), benzenesulfonate (X_2), *p*-toluenesulfonate (X_3), mandelate (Z), hippurate (Y_1) cinnamate (Y_2) and $1\cdot10^{-5}$–$6\cdot10^{-3}$ *M* benzoate (B) playing the role of T for the analyte Y_1, for which only the condition $u_C > u_Y > u_T$ holds. BGE=$5\cdot10^{-3}$ *M* lactic acid+ε-aminocaproic acid, pH 4.4, *U*=13 kV.

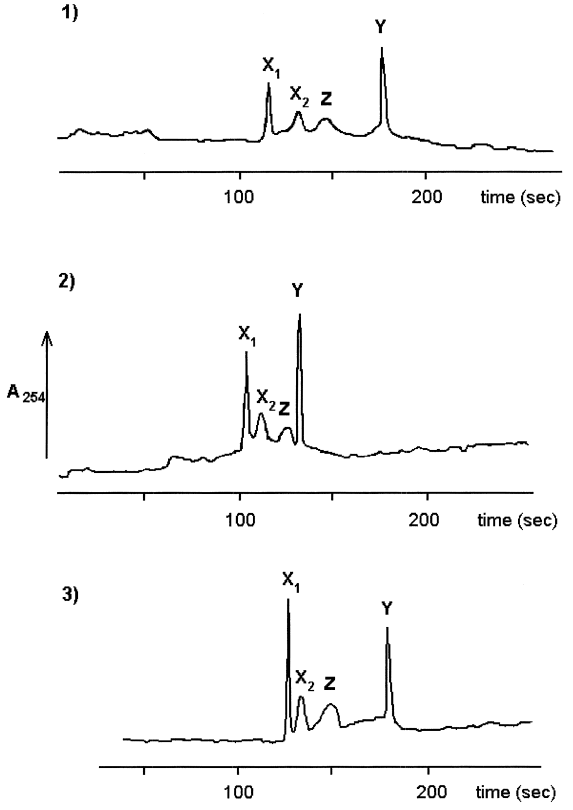

Fig. 10. Effects of transient ITP with two stackers. Transient ITP surviving from ITP step in cITP–CZE technique, $u_L > u_C > u_T$. LE: 10 m*M* HCl+His, pH 6, TE: 10 m*M* aspartic acid+His, pH 6.15, BGE: 10 m*M* acetic acid+His, pH 6.12. I_1=75 μA, I_2=100 μA, ZE capillary: polytetrafluoroethylene, 13 cm×0.3 mm I.D. Detection at 254 nm. Sample: 25 μl of $1\cdot10^{-6}$ *M* mixture of periodate (X_1), maleate (X_2), iodate (Z), benzoate (Y). (1) l_{LE}=7 s, l_{TE}=10 s, (2) l_{LE}=23 s, l_{TE}=45 s, (3) l_{LE}=23 s, l_{TE}=11 s.

length (Fig. 10). Conditions can be found when the effects of L and T are balanced and no changes in detection times can be observed. In any case, however, sharp peaks are measured for the fastest and slowest analytes of the system and no sharpening can be observed for analytes having the mobility close to the mobility of the BGE co-ion.

4.3.2. ITP induced by the composition of the separation medium

The same rules hold for the case when the capillary filled with a BGE is used for the induction of transient ITP. Both detection times and peak shapes depend on the characteristics and length of both the L and T zones (Fig. 11).

4.3.3. Sample induced transient ITP

For two macrocomponents present in the sample and fulfilling ITP conditions so that one acts as L with BGE co-ion being T and the other acts as T and the BGE co-ion is playing the role of L, the system behaves as described above and splits into two systems affecting, however, each other when detection times are taken into account (cf. Fig. 1).

4.4. Effects of transient ITP with two macrocomponents, for both of them the BGE co-ion plays the role of terminator

4.4.1. Both macrocomponents play the role of L for an analyte

From Fig. 12 it follows that for analytes for which both macrocomponents fulfill the ITP conditions being leading stackers the effects of ITP are summed. This is more evident from the table in the legend to the figure where the effects of chlorides and iodates on the peak width of benzenesulfonate are presented. The sharpening of the peak when both macrocomponents are present in the sample is given as the result of effects of macrocomponents being present in the sample individually. The effects of double concentration of chlorides as well as of the double concentration of iodates is different.

4.4.2. Only one macrocomponent plays the role of L for an analyte

In Fig. 13 the situation is demonstrated when the mobility of an analyte is between two macrocomponents that are faster than the BGE co-ion playing the

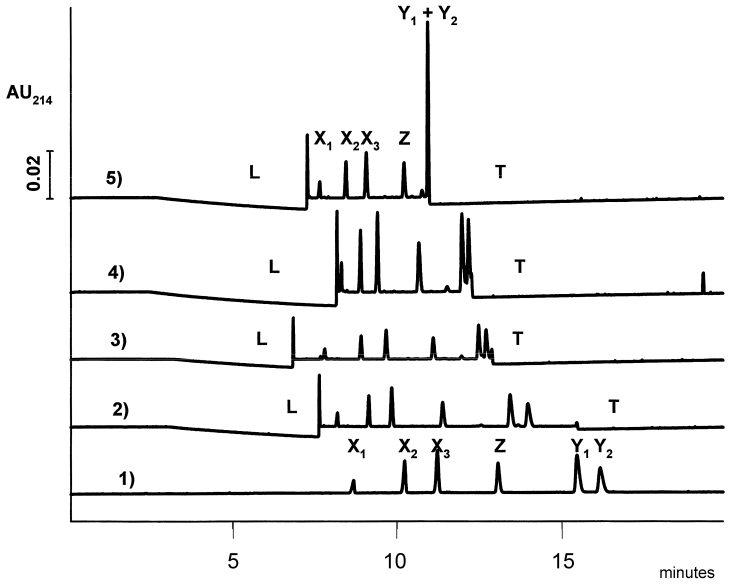

Fig. 11. Effects of transient ITP with two stackers. Transient ITP induced by the composition of the separation medium, $u_A > u_C > u_B$. Sample mixture 10^{-4} *M* iodate (X_1), benzenesulfonate (X_2), *p*-toluenesulfonate (X_3), mandelate (Z), hippurate (Y_1), benzoate (Y_2). BGE: 20 m*M* lactic acid+ϵ-aminocaproic acid, pH 4.4, U=10 kV. (1) l_{LE}=0 s, l_{TE}=0 s, (2) l_{LE}=30 s, l_{TE}=10 s, (3) l_{LE}=20 s, l_{TE}=20 s, (4) l_{LE}=40 s, l_{TE}=20 s, (5) l_{LE}=30 s, l_{TE}=30 s.

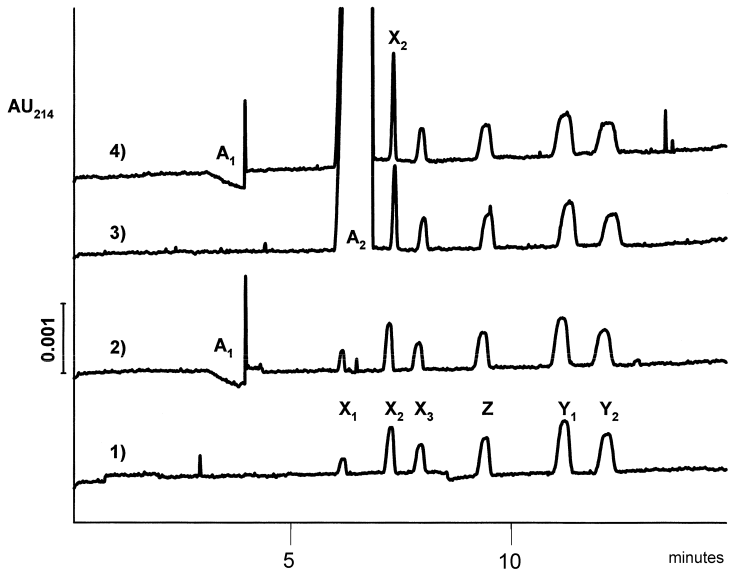

Fig. 12. Effects of transient ITP with two stackers. Sample induced transient ITP, BGE=TE, two macrocomponents present in the sample and both of them play for an analyte the role of L type stacker, $u_{A1}>u_{A2}>u_X>u_C$. Effect on zone dispersion (peak width) of analytes in the sample mixture of 10^{-5} *M* benzenesulfonate (X_2), *p*-toluenesulfonate (X_3), mandelate (Z), hippurate (Y_1), benzoate (Y_2), changing concentrations of chlorides (A_1) and iodates (A_2). BGE=$5\cdot10^{-3}$ *M* lactic acid+ϵ-aminocaproic acid, pH 4.4, *U*=13 kV.

Trace No.	$A_1=Cl^-$ (*M*)	$A_2=IO_3^-$ (*M*)	$w_{1/2\ (BS)}$ (%)
1	$1\cdot10^{-5}$	$1\cdot10^{-5}$	100
2	$2.5\cdot10^{-3}$	$1\cdot10^{-5}$	90
3	$1\cdot10^{-5}$	$2.5\cdot10^{-3}$	50
4	$2.5\cdot10^{-3}$	$2.5\cdot10^{-3}$	40
	$1\cdot10^{-5}$	$5\cdot10^{-3}$	26
	$5\cdot10^{-3}$	$1\cdot10^{-5}$	61

role of T. Here, only the faster macrocomponent chloride (A_1) fulfills for the analyte iodate (X_1) the ITP condition. The slower macrocomponent benzenesulfonate (A_2) plays for iodates neither the role of L nor of T and when present in the sample where no or low concentration of chlorides is present it leads to pronounced dispersion. Higher concentration of chlorides inducing ITP effect acts against the dispersion the more the higher is the ratio of the concentration of the two stackers. The concentration of A_1 higher by one order than the concentration of A_2 (which fulfills the ITP conditions for *p*-toluensulfonates, X_3, having the mobility lower than A_2 and higher than the BGE co-ion), eliminates fully the negative effect of the second stacker, BS, and the peak width is the same as if benzenesulfonates were present in the concentration of the analytes, see the table in Fig.13.

4.5. Summary of ITP effects expected in a sample with macrocomponent(s)

In a sample with one or more macrocomponents following ITP effects can be observed:

Simple ITP effects can occur for analytes the mobility values of which are between those of the stacking macrocomponent and the BGE co-ion, $u_A>u_{Xn}>u_C$ or $u_C>u_{Xn}>u_B$.

Enhanced ITP effects can be expected when two stacking macrocomponents fulfill the condition for the leader (or terminator) for a group of analytes, $u_{A1}>u_{A2}>u_{Xn}>u_C$ or $u_C>u_{Xn}>u_{B1}>u_{B2}$.

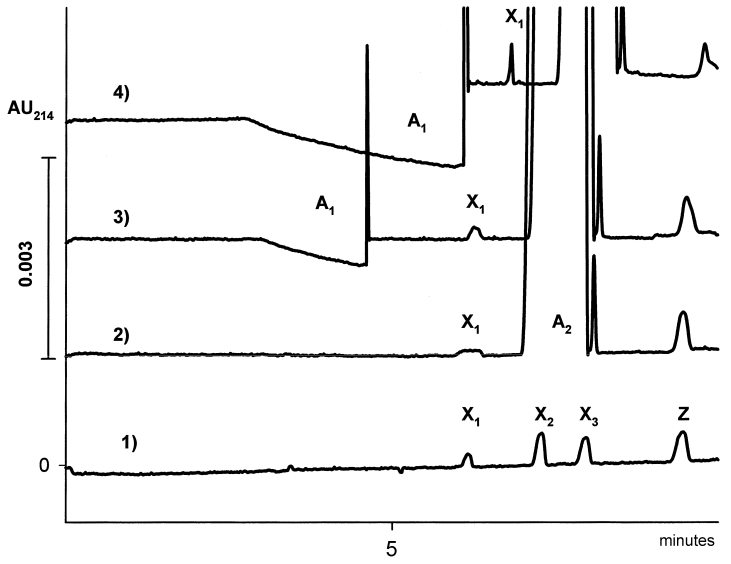

Fig. 13. Effects of transient ITP with two stackers. Sample induced transient ITP, BGE=TE, two macrocomponents faster than BGE co-ion present in the sample and only one of them plays for an analyte X the role of L type stacker, $u_{A1}>u_X>u_{A2}>u_C$. Effect on zone dispersion (peak width) of analytes in the sample mixture of 10^{-5} *M* iodate (X_1), *p*-toluenesulfonate (X_3), mandelate (Z), hippurate (Y_1), benzoate (Y_2), changing concentrations of chlorides (A_1) and benzenesulfonates (A_2). BGE=$5\cdot10^{-3}$ *M* lactic acid+ϵ-aminocaproic acid, pH 4.4, *U*=13 kV.

Trace No.	A_1=Cl (*M*)	A_2=BS (*M*)	$w_{1/2\ (IO_3^-)}$ (%)
1	$1\cdot10^{-5}$	$1\cdot10^{-5}$	100
2	$1\cdot10^{-5}$	$5\cdot10^{-3}$	308
3	$1\cdot10^{-2}$	$5\cdot10^{-3}$	131
	$5\cdot10^{-2}$	$5\cdot10^{-3}$	29
	$1\cdot10^{-2}$	$1\cdot10^{-5}$	37
	$5\cdot10^{-2}$	$1\cdot10^{-5}$	31

No ITP effects can be observed for analytes the mobility of which is out of the mobility range between the transient leader and terminator, $u_{Xn}>u_A>u_C$ or $u_C>u_B>u_{Xn}$, and for analytes the mobility of which is close to the mobility of the BGE co-ion, $u_A>u_{Xn}=u_C>u_B$. No ITP effects appear also when the macrocomponent has the mobility close to the mobility of BGE co-ion, $u_{Xn}>u_A=u_C>u_T$.

A more complicated situation with two stackers occurs when the analytes have their mobilities between the mobility of the two macrocomponents. Here only the faster macrocomponent in the case when both of them are faster than the BGE co-ion and could act as leaders, $u_{A1}>u_{Xn}>u_{A2}>u_C$ (or the slower one if they could act as terminators, $u_C>u_{B1}>u_{Xn}>u_{B2}$) has the stacking effect on these analytes and the resulting effects dependent on the ratio of amount of macrocomponents, A:B.

The ratio of the amount of the two stackers is important also in the case when one of them acts as L and the other as T for the analytes and the mobility of BGE co-ion lies between them, $u_A>u_{Xn}>u_C>u_{Yn}>u_B$.

4.6. Utilization of ITP effects

There are several ways of profiting from transient ITP effects. Large volumes of the sample can be injected into a single capillary and zones of analytes can be sharpened so that high separation efficiency and thus sensitivity of the analysis are reached. Fig. 14 demonstrates this effect for a selected analyte, X_3.

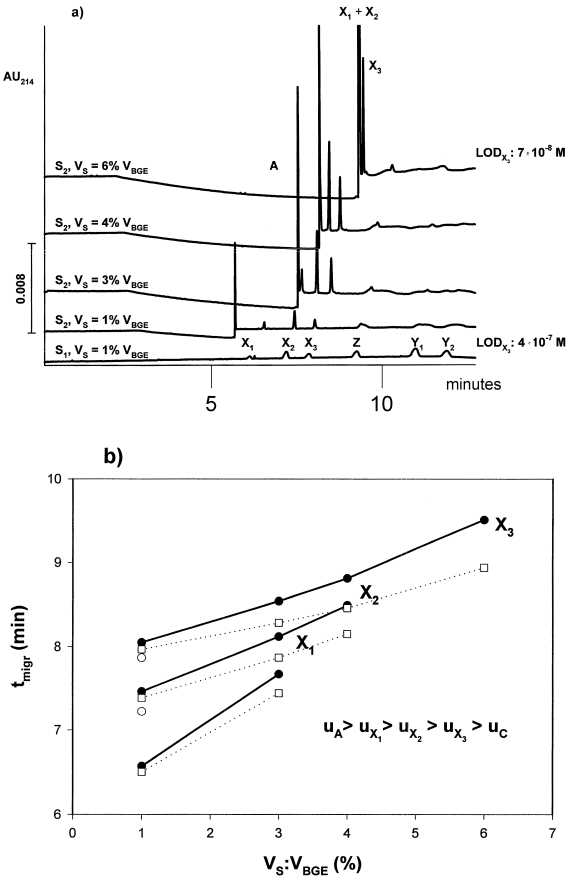

Fig. 14. Sample induced transient ITP, BGE=TE . Improving sensitivity by increasing injected sample volume. Sample mixture containing 10^{-5} *M* chloride, iodate (X_1), benzenesulfonate (X_2), *p*-toluenesulfonate (X_3), mandelate (Z), hippurate (Y_1), benzoate (Y_2), (S_1), was enriched in chlorides (A) to $5\cdot10^{-2}$ *M* concentration, (S_2), and injected up to the maximum volume (V_S) enabling resolution of *p*-toluenesulfonate, (X_3), from the preceding ITP stack of X_1+X_2. BGE=$5\cdot10^{-3}$ *M* lactic acid+ϵ-aminocaproic acid, pH 4.4, *U*=13 kV. (a) Traces of analyses, (b) effect on detection times, ○ $1\cdot10^{-5}$ *M* chloride, ● $1\cdot10^{-2}$ *M* chloride, □ $1\cdot10^{-2}$ *M* chloride, corrected migration path.

As the stacker A, chloride was chosen and its concentration in the sample was increased so as to ensure ITP stacking of X_3. Increasing the injected volume six times enabled to detect the peak of X_3 just leaving the ITP stack with the highest separation efficiency and resolution between X_3 and preceding zone of X_2 being one. It is evident that adequate prolongation of detection time of the analyte must be expected (Fig. 14b). The transition from ITP migration to ZE mode for such sensitivity enhancement was studied in more details previously [30]. It was shown for a model mixture of serum where three macrocomponents were present (stacking chlorides, phosphates and counteracting urates for hippurate, HA, being the analyte studied) that it was possible to inject up to 52% of the effective length of the capillary and to reach a LOD of $4\cdot10^{-8}$ *M* HA. In a complex matrix of real serum, however, this way of improving sensitivity failed and only 20 nl could be injected to obtain reproducible results, which increased the LOD of HA to the value $1\cdot10^{-5}$ *M*. The solution of improving sensitivity and reproducibility regardless of how the composition of the sample varied, was the technique of the combination of cITP–CZE in two connected capillaries. This technique eliminates effects of macrocomponents present in the sample on both detection time and peak area and height regardless of varying sample source and makes identification and quantification reliable (Fig. 15). A universal detector placed in front of the bifurcation part enables to identify and quantify all the separated zones longer than the detector window and to perform proper timing of switching current to the second capillary, in which the optimum composition of BGE is used with respect to the group of analytes to be analyzed. In a commercial apparatus with a common optical detection reliable and reproducible analyses of trace analytes in complex matrices (halofuginone in feed stuff [6], hippurate in serum [30], ascorbate in serum, urine and stomach fluid [33], orotate in urine [35]) were performed with the LOD in the order of 10^{-7} *M* and relative standard deviation (RSD) of detection times 1%.

5. Conclusions

Conditions for existence of transient isotachophoresis in zone electrophoresis are quite common. Transient ITP can either be induced by the composition of the sample or by the composition of the electrolyte system or result from the first step during cITP–CZE combination. In cases where the transient ITP can be controlled, the effects accompanying ITP migration can be employed for improving sensitivity of the analysis. This holds first of all for the cITP–CZE combination. Further, it holds for transient ITP

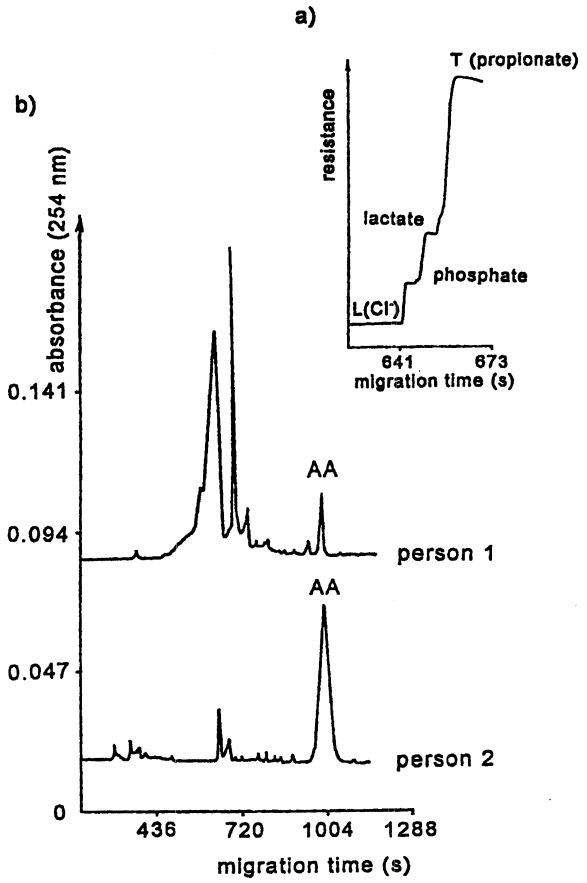

Fig. 15. cITP–CZE analysis of ascorbate in human serum. (a) Ten μl of five-times diluted untreated serum were injected into the cITP system [LE: 10 m*M* HCl+β-alanine, pH 3.3, 0.01% (w/v) HPC, TE: 50 m*M* propionic acid, capillary effective length 16 cm, I_1=300 μA] and (b) detected in the CZE system (BGE: 50 m*M* propionic acid+β-alanine, pH 3.8, capillary effective length 30 cm, I_2=80 μA). LOD: $8 \cdot 10^{-7}$ *M* ascorbate in serum. After Ref. [33], with permission.

induced by the sample or separation medium composition when samples of constant composition with respect to macrocomponents are analyzed. On the other hand, transient ITP migration can cause problems when samples with complex matrices are analyzed by CZE and the content of major sample components varies. The uncontrolled effects can result in a misinterpretation of the record as both detection times and peak shapes change in dependence on the concentration and mobility of macrocomponents present in the sample. For such a kind of samples it is evident that the best solution is to eliminate the effects of macrocomponents and to run the samples by cITP–CZE combination. This technique enables to perform efficient sample clean-up, to ensure high column hold-up in the ITP step and to separate and detect trace analytes under optimum conditions.

Acknowledgements

This work was supported by the Grant Agency of the Czech Republic, grant No. 203/96/0124, by the Grant Agency of the Academy of Sciences of the Czech Republic, grant No. 4031703, and by the Ministry of Education, grant No. VS-96021.

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