ELSEVIER

Journal of Chromatography B, 689 (1997) 13–34

JOURNAL OF CHROMATOGRAPHY B

Review

Synergism of capillary isotachophoresis and capillary zone electrophoresis

Ludmila Křivánková, Petr Boček*

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

Abstract

The combination of capillary isotachophoresis and capillary zone electrophoresis may enhance greatly the performance of analytical capillary electrophoresis with respect to both separation power and the concentration sensitivity. The concentrating effects and the separation power of isotachophoresis allow the analysis of diluted samples and the elimination of interferences due to bulk components. The separation process of zone electrophoresis enables one to resolve the stack of trace analytes and detect the resulting individual zones with high sensitivity. The transition of isotachophoresis into zone electrophoresis plays the key role in the overall performance of this hyphenated technique. This article describes the dynamics of the conversion of isotachophoresis into zone electrophoretic mode and shows that the key role is played by the segments of the leading and terminating zones from the isotachophoretic stage. The magnitude of these segments directly effects the detection time as well as the separation width of the peaks of analytes. It is shown that these effects are also important in the analyses by capillary zone electrophoresis where isotachophoresis is induced by the sample itself. Finally, the paper presents a list of recommended, user-friendly, electrolyte systems which enable one to simply predict the performance of the combination isotachophoresis–zone electrophoresis.

Keywords: Isotachophoresis; Capillary isotachophoresis

Contents

1. Introduction

The foundations of isotachophoresis (ITP) and the processes taking place during the passage of the

*Corresponding author.

PII S0378-4347(96)00339-8

electric current through the discontinuous electrolyte system were described some 100 years ago [1–3]. However, isotachophoresis as an analytical method can be thought to start in 1967 when the principles and instrumentation for the analytical capillary isotachophoresis (CITP), including on-line detection, were developed [4,5]. Since then a lot of work has been done on the theoretical description of the dynamics of the separation process [6–8], calculations of the composition of isotachophoretic zones [9] and related mobilities and dissociation constants [10]. The problems concerning the selection of electrolyte systems, zone stability, separability of substances, temperature effects, separation efficiency and resolution have also been solved [11]. For a couple of years, only laboratory-made apparatuses were available. They were used not only for the experimental verification of theoretical and methodological studies but also for a series of practical applications. Experience obtained with the laboratory-made equipment was employed afterwards in the construction of the commercially available instrumentation, offering not only single capillaries, but also a column-coupling system of two capillaries of different diameter or a device for micropreparation involving also a flow of electrolytes. The development of isotachophoresis as an independent analytical method ceased about 15 years ago when the attention of research workers was attracted by capillary zone electrophoresis (CZE), a method offering a higher speed of analysis, higher universality due to practically unlimited selection of the composition of electrolyte systems used and more sensitive types of detectors. A final significant factor enhancing the popularity of CZE is that the detection signal gives peaks, and the qualitative and quantitative interpretation of these peaks is similar to those used in chromatography [12]. In the course of time, however, practical limits of the separation efficiency and sensitivity were reached, and a marked drawback of CZE was revealed, namely the unsatisfactory detection limit resulting from the fact that it is not possible to inject sufficiently large volumes of a sample directly into the capillary. This situation explains the present come-back of isotachophoresis as a preconcentration and preseparation method for CZE. This article is a critical review of the contemporary situation in the domain of application of ITP in combination with CZE, and it should serve as a reference for beginners as well as experts in this field.

2. Basic features

In isotachophoresis, individual sample components create distinct zones migrating in the order of their mobilities, one after the other with common boundaries in between. This stack of sample zones migrates with an identical velocity which is constant on condition that a constant current is applied. From the principle of the separation process in ITP it follows that the concentration of the analytes in the sample changes during separation until it is adjusted at a defined ratio to the concentration of the leading ion (Fig. 1). The adjusted concentration of analytes in their zones is constant for a given leading- and terminating-electrolyte system, no matter whether a diluted or concentrated sample was injected. Hence the sample amount is reflected by the length (volume) of the adjusted zone. The zones are then detected as the well known isotachophoretic steps having step height (qualitative information) and step length (quantitative information). The detection time of a zone depends first of all on the composition of the leading electrolyte. The adjustment of the isotachophoretic zones is a well known effect and is usually named the concentrating effect. The concentrating effect is highly advantageous, it may concentrate diluted samples by orders of magnitude and a suitable signal from the detector can be obtained (see Fig. 2). However, if the length of the adjusted zone is shorter than the length of the detection cell used such a short zone cannot be distinguished from the preceding or following zones. This case is illustrated in Fig. 3. Fig. 3a shows the UV absorbance detection of a diluted model mixture of adenine and cytosine separated by isotachophoresis. Both zones are short. However, they are fairly well distinguished, and qualitative as well as quantitative evaluation is possible. When the model mixture is further diluted, the length of the individual zones becomes shorter than the length of the detection window (0.2 mm). The detector is not capable of distinguishing the zones and a single peak comes out of the detector and represents the sum of both zones (Fig. 3b). To

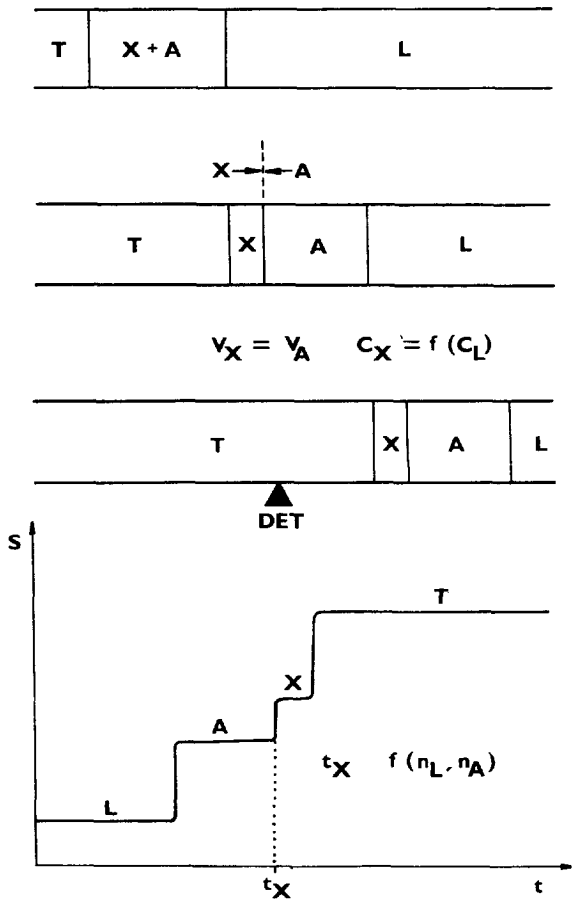

Fig. 1. Isotachophoretic separation and detection of analytes A and X. L = leading ion, T = terminating ion. Migration velocity of all the zones, v, is unique; concentration c in individual zones is adjusted in dependence of concentration of the leading electrolyte, c_L. Detection time of an analyte is dependent on the length of leading and preceding zones, i.e., on the amount of leading electrolyte and preceding analytes.

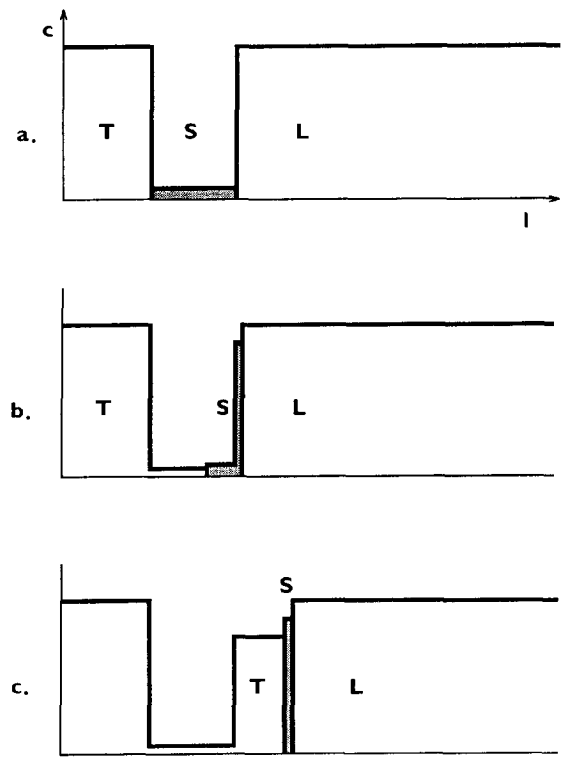

Fig. 2. Adjustment of the sample concentration to the concentration of the leading electrolyte during the ITP process. (a) Initial conditions, (b) formation of ITP zones, (c) final steady state. L = leading electrolyte; S = sample; T = terminating electrolyte; c = concentration; l = length of the migration path. After Ref. [28].

distinguish the two zones by ITP we have to find a suitable spacer, i.e., a third analyte, nonabsorbing at the selected wavelength of detection, and migrating between the short absorbing zones. Another solution is to continue with the separation in CZE mode (Fig. 3c). It follows from the principle of the zone separation process that the zones migrate with their individual velocities differing from one another and the migrating zones diverge. It is seen that with the ITP+CZE combination the concentration of analytes in the sample can be further decreased and in the same instrumental arrangement the sensitivity of detection can be improved more than a hundred times. The quantitative relationship between a single technique of CZE and the combination of ITP and CZE is shown in Fig. 4. Small volumes (nl) of rather concentrated samples had to be injected in a single CZE analysis to obtain sharp and separated peaks in capillaries of 50–100 μm I.D. An increase in the sensitivity by two orders of magnitude was reached in the same capillary and with the same detector when CZE was preceded by ITP. Due to the concentrating effect of ITP a larger volume of a more diluted sample could be injected [13]. The combination of ITP–CZE offers a strong improvement in the detection limit (Fig. 5). Isotachophoretically easily separable and detectable analytes thiamine and ferroin fail to be analyzed by single stage ITP when their amount in the sample is lower than 10^{-11} mole. The reason is that the zone lengths are shorter than

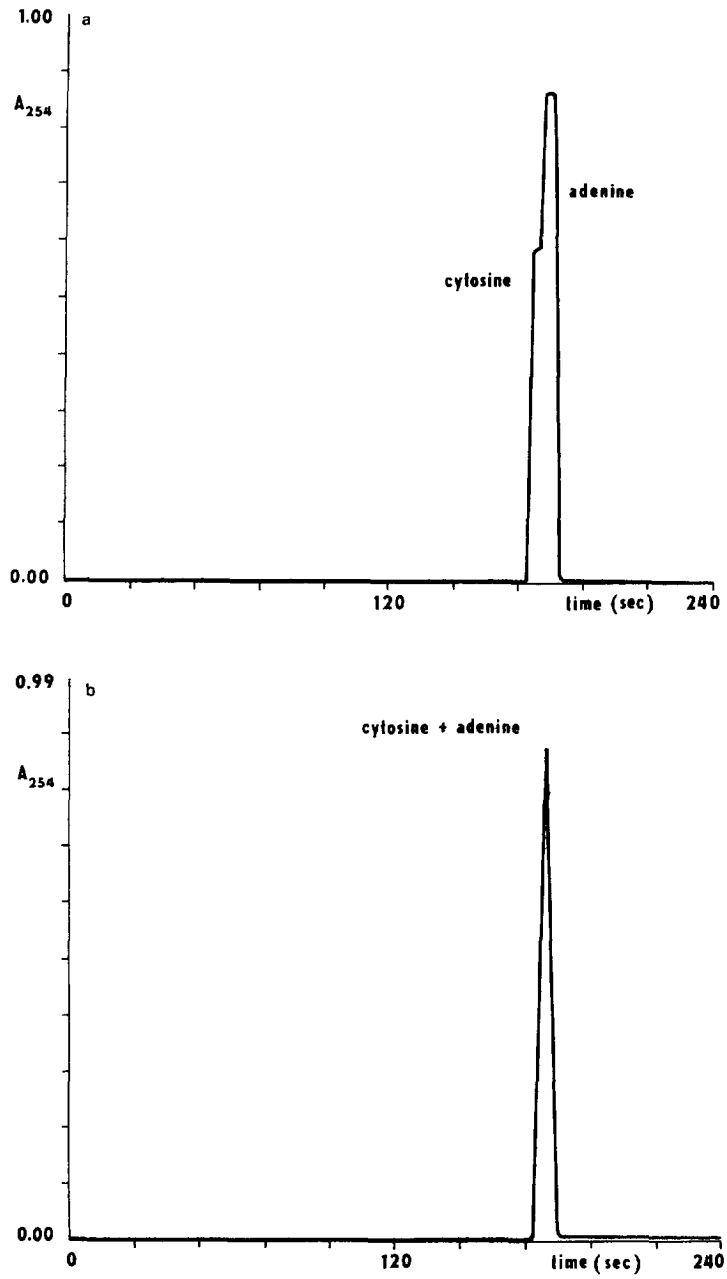

Fig. 3. Separation of adenine and cytosine by ITP–ITP (a, b) and ITP–CZE (c) combination. A Villa Labeco column coupling analyser (Spišská Nová Ves, Slovakia) was used for the measurements. The internal diameter of the second capillary made of fluorinated ethylene–propylene copolymer (FEP) was 0.3 mm, the length from the bifurcation point to the detector was 9 cm, current was 60 μA, detection was performed at 254 nm; 10 m*M* $NH_4Ac+HAc$, pH 4.7, was used as the leading electrolyte, 15 m*M* HAc was used as terminator in ITP analyses. The CZE stage was performed in the terminating electrolyte (HAc) serving as the BGE; 30 μl of (a) 1×10^{-4} *M*, (b) 2×10^{-5} *M* and (c) 1×10^{-7} *M* were analyzed.

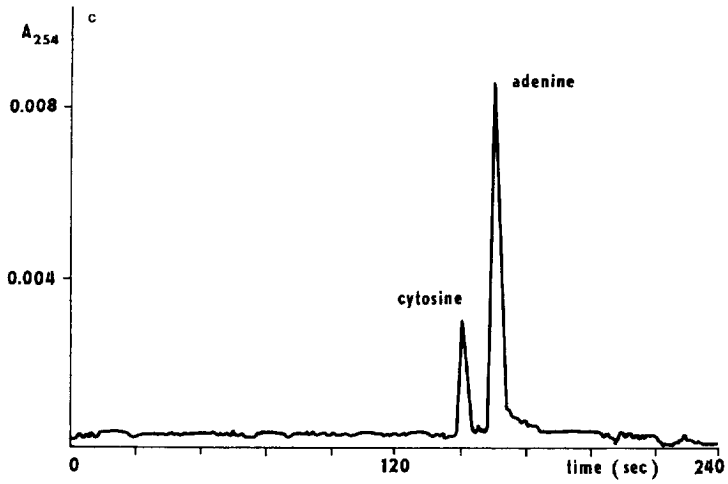

Fig. 3. (*continued*)

the detector cell. However, when the CZE stage is attached, the stack of zones splits into individual zones which are afterwards easily detected [13].

The most significant field of ITP–CZE applications can be found in complex samples especially of biological origin. This is demonstrated in Fig. 6 by an example of the analysis of hippuric acid, one of the important metabolites clinically followed in case of renal failure. By CZE, only the concentration higher than $6 \cdot 10^{-5}$ *M* could be detected at 254 nm. By using the same capillary and detection system but applying the ITP–CZE method, the levels lower than $2 \cdot 10^{-7}$ *M* could be detected under optimized separation conditions. Another example is the determination of halofuginone, HFG, in the feedstuff supplement for poultry. There is no problem in determining

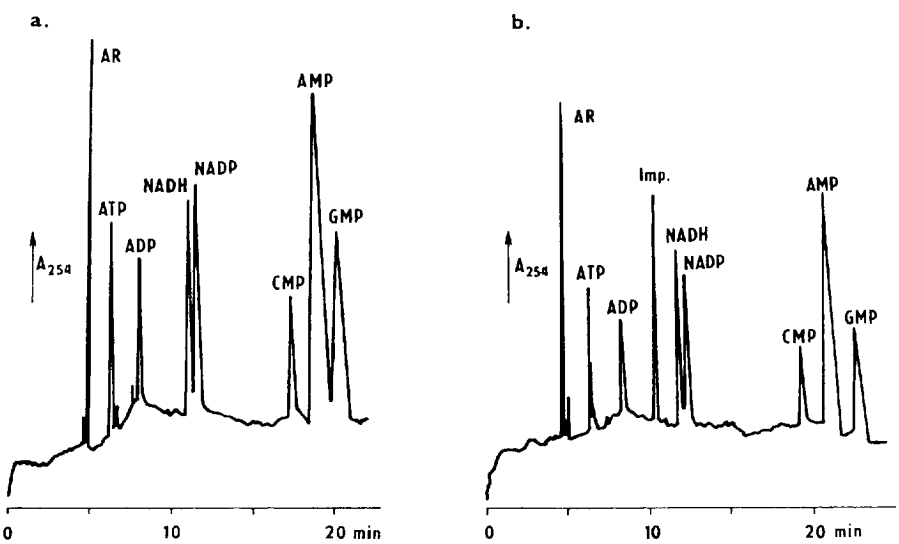

Fig. 4. Comparison of CZE and ITP–CZE separation of nucleotides. In (a) 0.02 *M* formic acid + His, pH 5.85, was used as the background electrolyte, BGE. In (b) this BGE was used as the leader and the solution of 0.005 *M* morpholinoethanesulfonic acid + His, pH 6.0 served as the terminator for ITP stage. BGE for the second stage (CZE) was the same as in (a). Sample mixture in (a) was 45 nl 3×10^{-4} *M*, (b) 10.6 μl 1×10^{-6} *M*. After Ref. [13].

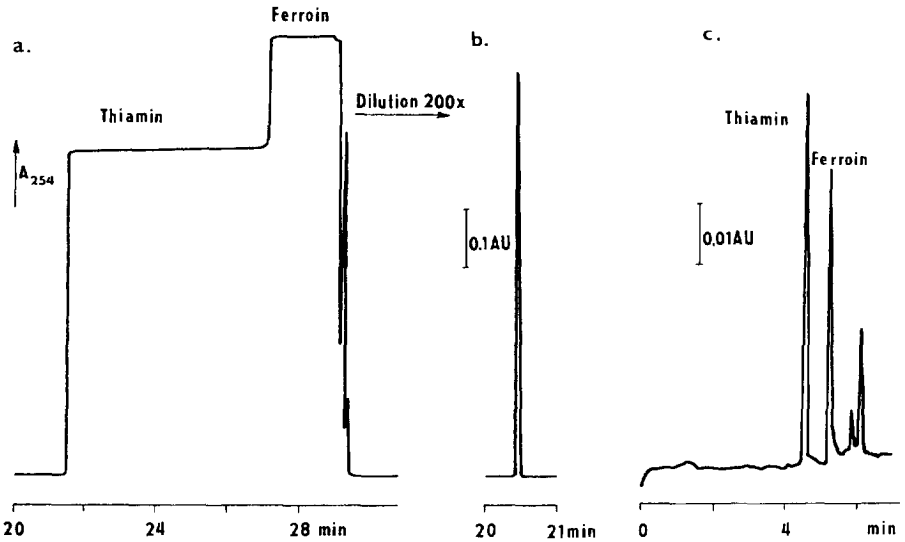

Fig. 5. Comparison of ITP and ITP–CZE separation. (a) ITP analysis of 7 nmol of thiamine and 2 nmol of ferroin; (b) ITP analysis of 35 pmol of thiamine and 10 pmol of ferroin; (c) ITP–CZE separation of 35 pmol of thiamine and 10 pmol of ferroin. Volume of the sample was 10.6 μl, leading electrolyte was 0.02 *M* sodium acetate + acetic acid, pH 3.65, terminator was 0.01 *M* acetic acid. Leading electrolyte was used as background electrolyte for CZE step in (c). After [13].

the main components of the supplement mixture, calcium and sodium, including the analytes of interest, halofuginone and vitamin B_1, by ITP (Fig. 7a). However, the concentration of halofuginone in real samples is very low compared to that of the major components, calcium and sodium. This large amount of inorganic ions prevents the injection of a larger amount of the sample in order to improve detectability. Even the application of the column-coupling system enabling separation of the major bulk components fails here. Employing the maximum sample load of the separation systems enables the detection of halofuginone and B_1 zones. However, due to the presence of a series of other absorbing microcomponents of variable composition, it is difficult to identify and quantify the zones reliably, see Fig. 7b. ITP–CZE combination is the solution – analytes are separated from calcium and sodium in the ITP step and separated into individual zones in the CZE step where they are easily identified and precisely quantified in the concentration range of 10^{-8}–10^{-6} *M* [14].

From the illustrations given above it is evident that it is the concentrating effect of ITP that manifests itself above all by improving the sensitivity of analysis by ITP–CZE combination. However, ITP–CZE combination may also bring something more – a new dimension of the separation, regardless of the fact that both separation steps are based on the differences in electrophoretic mobilities. This is documented in Fig. 8. In Fig. 8a a set of analytes is separated in an ITP electrolyte system where maleate and iodate form a stable mixed zone. When the analytes stacked by ITP are subsequently introduced into further attached capillary and separated by CZE in the same BGE, full separation was obtained (Fig. 8b). Obviously, ITP and CZE of the same species form mutually different electromigration behaviour, and one can consider their combination as two dimensional.

A scheme of an arrangement of the ITP–CZE combination and parameters commonly used in practice are given in Fig. 9. The capillary used for the ITP step must provide sufficient separation capacity and reproducible sampling of a relatively large volume of a sample. Therefore a capillary of wider diameter equipped with a sample valve is recommended for this step. A capillary of a narrower

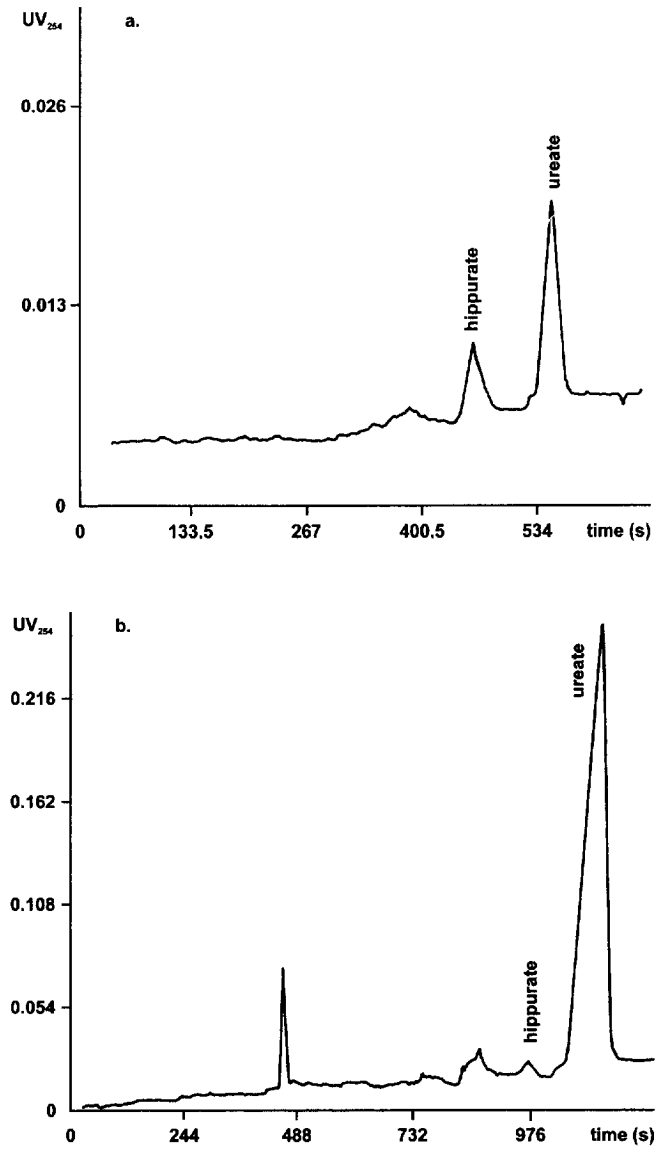

Fig. 6. Analysis of hippuric acid in serum by single CZE and ITP–CZE combination. (a) A single CZE analysis of 300 nl of untreated serum containing 1.5×10^{-4} *M* hippuric acid performed in BGE consisting of 50 m*M* morpholinoethanesulfonic acid with histidine to pH 6.2 in a FEP capillary of 0.2 mm I.D., 30 cm long with applied current 180 μA. (b) CZE step of ITP–CZE analysis of 7 μl of untreated serum with 5×10^{-7} *M* hippuric acid. Leading electrolyte: 10 m*M* HCl+histidine, pH 5.5, terminating electrolyte 10 m*M* morpholinoethanesulfonic acid. The same capillary as in (a), current 130 μA. Apparatus: A Villa Labeco column coupling analyser (Spišská Nová Ves, Slovakia).

diameter equipped with a sensitive detector is used in the CZE step and it ensures sensitivity of analysis. Both capillaries are interconnected via a bifurcation block which ensures that only a proper part of the ITP zone stack is transferred into the CZE step. This block also offers removal of the ballast from the sample. For reproducible and controlled conditions in CZE step, a tell-tale detector is indispensable [15].

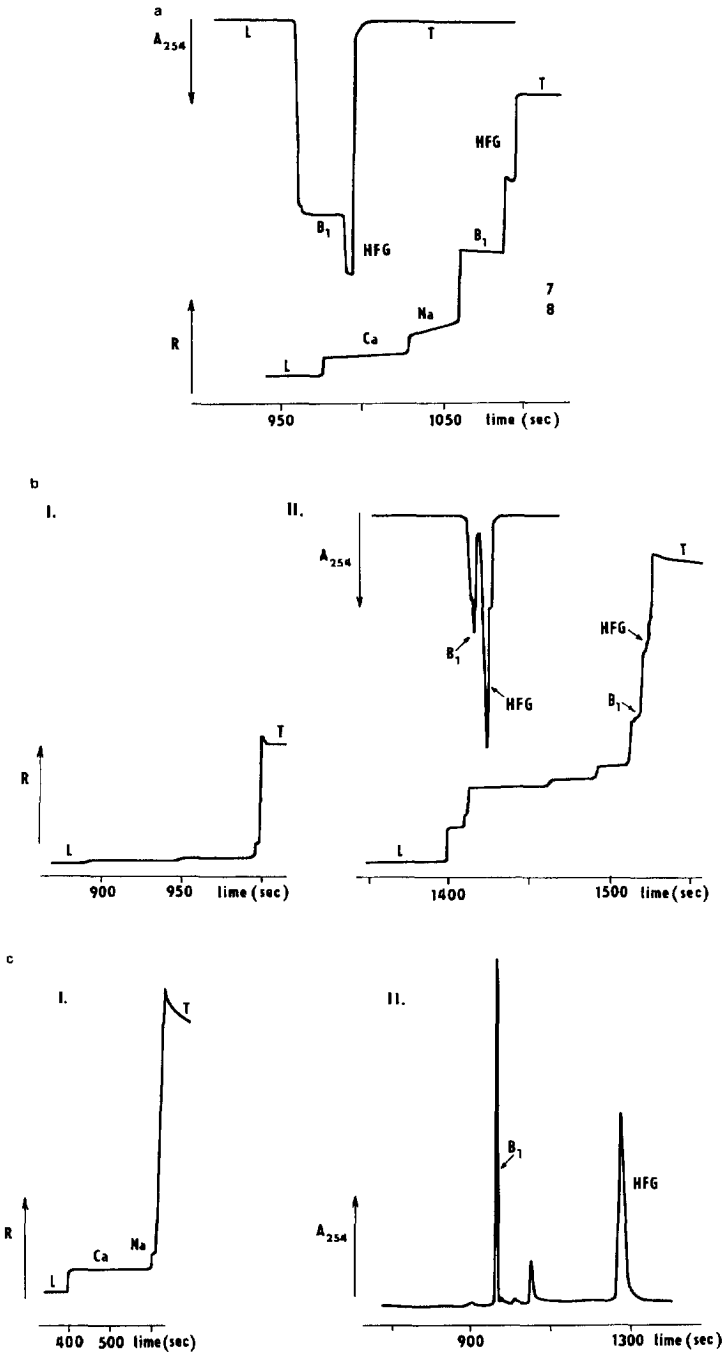

Fig. 7. Analysis of halofuginone (HFG) in the feedstuff supplement. (a) ITP analysis of a model mixture of 2 μl of 2 m*M* HFG and 5 μl of 0.76 m*M* vitamin B_1, in an electrolyte system composed of 5 m*M* KOH+MES, pH 5.7, as the leading and 10 m*M* ε-aminocaproic acid as the terminator. Constant current 20 μA, 13.5 cm long polytetrafluoroethylene capillary of 0.3 mm I.D. (b) ITP–ITP combination. I, ITP preseparation step. II, Second ITP step of ITP–ITP analysis of 10 μl of an extract of feedstuff supplement after removal of macrocomponents. Conditions as in 7a. (c) ITP–CZE combination. I, Trace of ITP step performed in 17 cm long capillary of 0.8 mm I.D., current 150 μA. II, Conductivity (*R*) and UV traces of CZE step of ITP–CZE analysis in BGE composed of 25 m*M* ϵ-aminocaproic acid adjusted with acetic acid to pH 4.0. Conditions as in 7a. After Ref. [14].

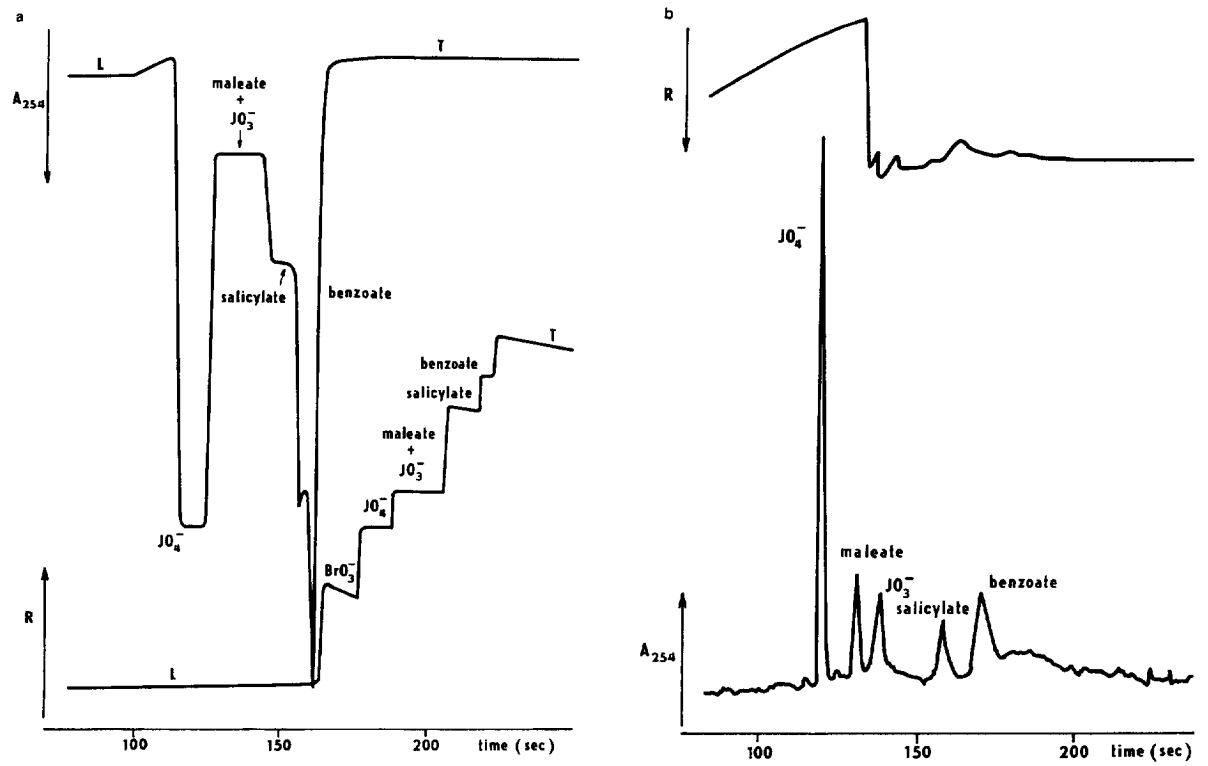

Fig. 8. ITP and ITP–CZE analysis of anions. (a) ITP analysis of 24 μl 2×10^{-4} *M* mixture of analytes was analyzed in 17 cm long PTFE capillary of 0.3 mm I.D. in 20 m*M* HCl + His, pH 6 used as the leading electrolyte and 15 m*M* aspartic acid as the terminator. (b) Trace of CZE step of ITP–CZE analysis of 24 μl 2×10^{-7} *M* mixture of analytes. Terminator from ITP step adjusted to pH 6.15 was used as BGE. The same capillary as in (a). Electric current was 100 μA in both cases.

3. Dynamics of the conversion. Transition of ITP into ZE

An important part of the analysis performed by ITP–CZE combination is the stage where a system of isotachophoretic zones of the sample components is introduced into another electrolyte system and ITP migration is transformed into ZE migration. This stage is characterized by changing either the leading electrolyte at the front, or the terminating electrolyte at the back, or changing both of them for another suitable electrolyte that would serve as the background electrolyte for CZE. This alteration starts the process in which the BGE gradually penetrates the system of ITP zones of analytes. These zones are gradually destacked and continue migrating in a rigorous zone electrophoresis mode. However, during the substitution of ITP electrolyte system with a BGE one should do it carefully to avoid losses of the sample or deterioration of the stack of sample zones. Therefore, the sample cut must be done generously and the result is that there are always residuals of L and T around the stack with sample zones at the starting position for CZE (see Fig. 10). Consequently, it is evident that the situation at the beginning of the CZE step is different from that in the single CZE and has to be described from the point of view of possible combinations of electrolytes, of the effects of temporary presence of parts of the original leading and terminating ITP zones in CZE step, and of the factors affecting identification and resolution of analytes.

Electrolyte systems for ITP–CZE combination have to ensure ITP separation in the first stage and ZE separation in the second stage. The electrolyte system for ITP has to be selected so that it ensures the migration of analytes between the fastest, leading ion, and the slowest, terminating ion. This means

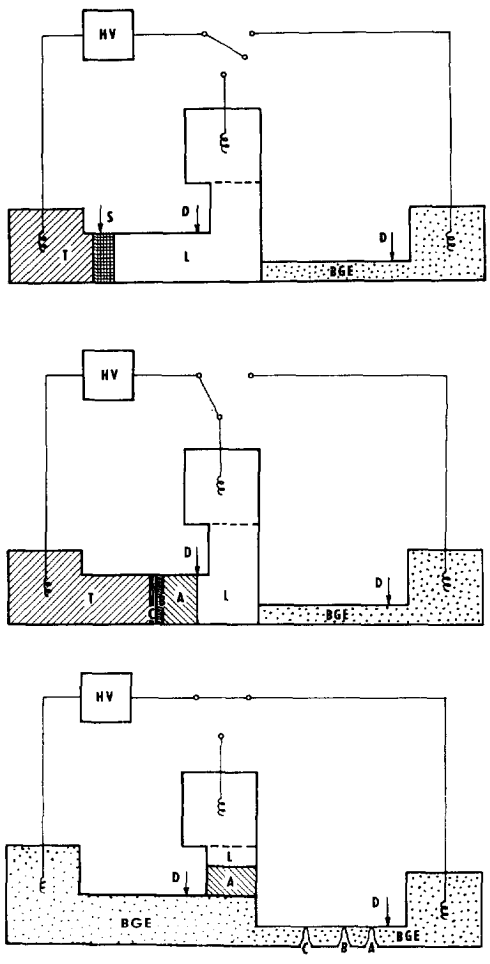

Fig. 9. Schematic arrangement of a column coupling system used for the ITP–CZE combination technique. (a) Situation before the analysis, (b) electric current passes through the circuit between leading and terminating electrolyte chambers and creates conditions for ITP analysis in the first capillary filled with proper electrolytes for ITP analysis. Analytes migrate between leading and terminating electrolytes, create zones with sharp boundaries with concentration adjusted to that of leading ion. (c) Current passes through the system of both capillaries filled with a background electrolyte and zones of analytes create diffused zones migrating with different velocities typical of CZE migration mode.

that the difference in mobilities between the leading and terminating ions determines the window for analytes that will be analyzed by ITP–CZE. The selection of BGE for CZE stage is free. However, in practice, it is advantageous when either L or T from the ITP step serve as the BGE. Of course, when necessary, other electrolytes can easily be chosen. From the point of practical handling, the system where adjusted T is used as BGE for CZE is the most user friendly. The capillaries can be filled with all electrolytes prior to analysis and no wet manipulation is needed during the measurement. It requires only proper switching of the current. We call this combination of electrolytes a T–S–T system where it depicts the system terminator sample-terminator (the other possibilities are named L–S–L and BGE–S–BGE systems, respectively), and use it here for the demonstration of transition of the ITP process into CZE migration and of effects accompanying this transformation.

The dynamics of the transition of ITP into CZE are schematically drawn in Fig. 11. The cut of a sample containing analytes A and B, and a part of the leading zone enter the second capillary dedicated for the CZE step. Immediately after switching the current the front boundary of leading zone diffuses into the BGE. It is seen, however, that for a certain period of time the ITP node of the migration of all the analytes survives until the plateau of the L zone disappears. This is why the sample zones begin to destack from the rear side. The zone of the lowest mobility leaves the ITP stack first, and the fastest analyte leaves last [15]. This process is illustrated in Fig. 12 by computer simulation and in Fig. 13 by an experimental verification. It can be pointed out that ITP–CZE combination is aimed mainly at analysis of tiny amounts of analytes. Hence, their zones create spikes only in the record of the ITP part rather than rectangular zones with a developed plateau. Such a situation is depicted in Fig. 12 and Fig. 13 where the ITP stacks present as sharp peaks fused together.

It follows from the course of the migration of an analyte in the second capillary that the simple rules describing migration in the rigorous zone electrophoretic mode are not applicable in ITP–CZE analysis. The velocity of the migration of an analyte differs in the ITP and ZE modes, and therefore the resulting detection time does not reflect only electrophoretic mobility of the analyte, but also a contribution of the temporary isotachophoretic migration. In the T–S–T system, the surviving ITP system leads to an increase in the detection time. The resulting detection time of an analyte is thus [16].

$$t_{X,r} = \frac{x_r \cdot \kappa_T}{i \cdot \bar{u}_X} + \frac{u_L - u_T}{u_L - \bar{u}_X} t_o = a_1 + b_1 t_0 \qquad (1)$$

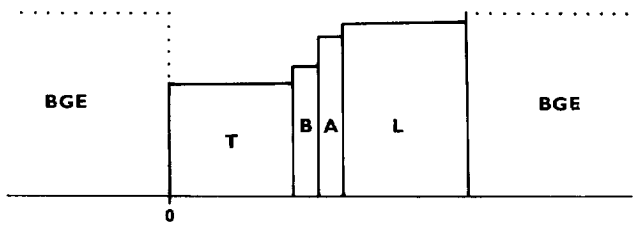

Fig. 10. Scheme of the cut from ITP step entering the CZE step in ITP–CZE combination.

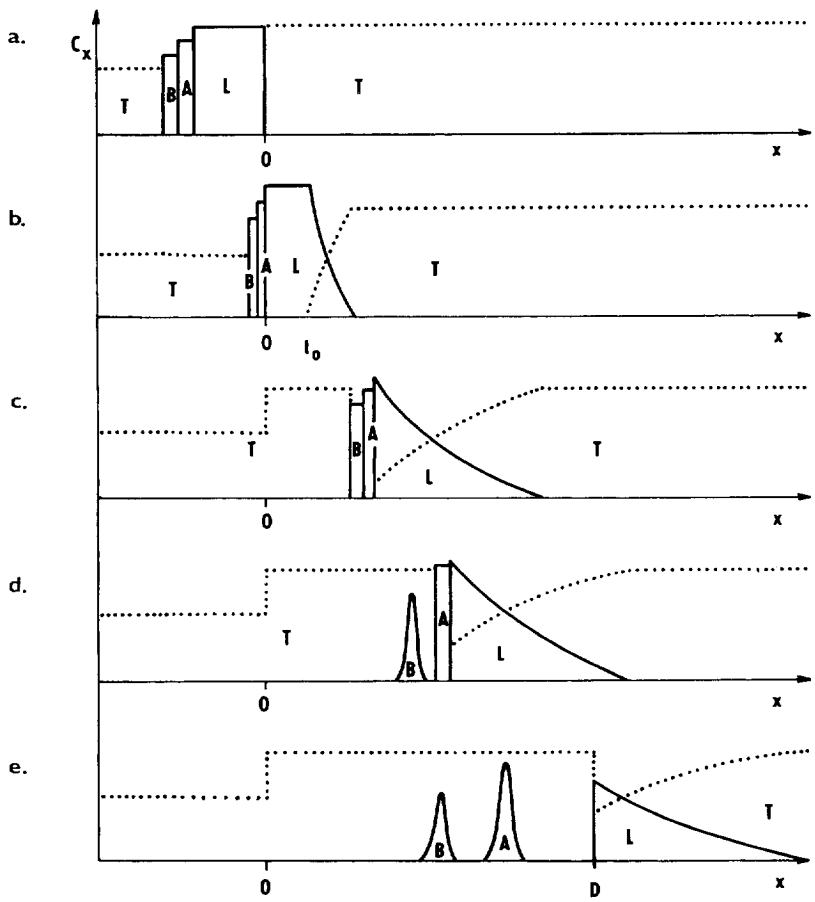

Fig. 11. Scheme of the transition of the stack of zones from isotachophoretic migration to zone electrophoretic movement in the T–S–T electrolyte system. The situations are shown at the time moments when (a) the current is switched on across to the analytical capillary, (b) the entire zone of the leading electrolyte has just migrated into the analytical capillary (c) the ITP concentration plateau of the leading zone has just disappeared, (d) the zone B has destacked while A is still migrating in the stack, (e) all the sample zones migrate in zone electrophoretic mode and the rear boundary of the leading zone just passes the detector, opening thus the time window for detection. L, leading electrolyte; T, terminating electrolyte; A,B-sample zones; x, longitudinal coordinate; c_x, concentration of a component in the separatian system; 0, interface between the analytical and preseparation capillaries; D, position of the detector. After Ref. [15].

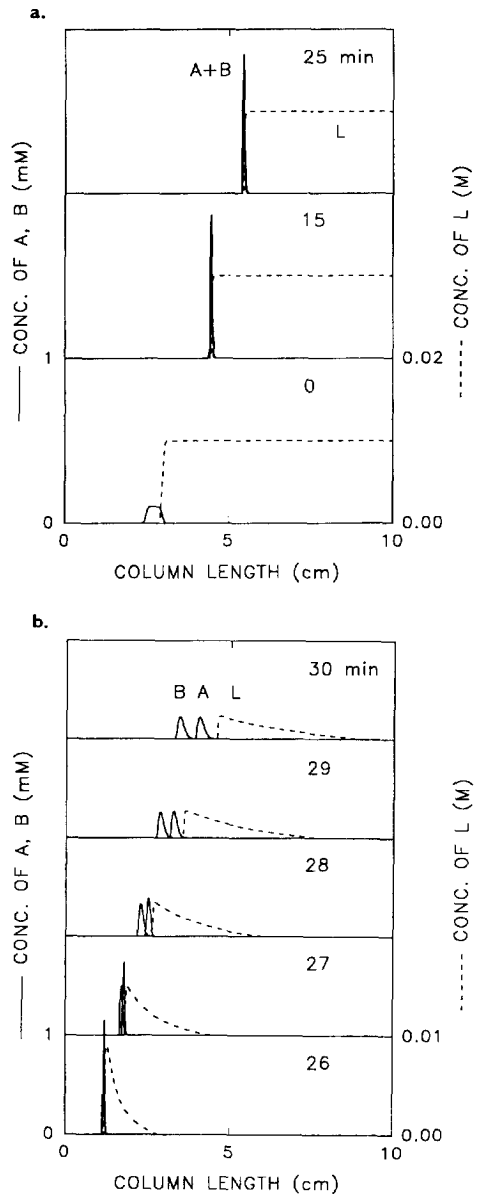

Fig. 12. Computer simulation of the separation of a pair of analytes A and B by the ITP–CZE combination in the T–S–T system. (a) Isotachophoretic step, (b) zone electrophoretic step. The concentration profiles shown correspond to the denoted time intervals. After [15].

where x_r is the distance between the detector and the inlet of the analytical capillary, κ_T is the specific conductivity of the background electrolyte (here formed by the terminator from the ITP step) in the analytical capillary and i is the electric current density in the analytical capillary. The magnitude of the segment of the leading zone introduced into the second capillary is expressed as the time period, t_o, corresponding to the migration of this segment into the analytical capillary (i.e., into the ZE stage). a_1 and b_1, are constants in a simple form of the equation predicting a linear dependence of detection time of the amount of the leading segment accompanying the sample stack into the second capillary (see Fig. 14). The theory and experiments demonstrate clearly that detection time cannot be used directly as a qualitative parameter for zone identification in practical samples since it always includes unknown variable t_o. However, the linearity of Eq. (1) enables us to find the corresponding correct values of effective mobilities with the help of internal standards (see Fig. 15), for details see Ref. [13].

In the L–S–L system, the surviving ITP migration due to the presence of a certain segment of the terminator accelerates the migration of analytes compared with the migration in rigorous zone electrophoresis. Therefore, the observed detection time decreases with the length of the terminator segment accompanying the sample stack into the second capillary, i.e., the slope of the line is negative, see Fig. 16 and Fig. 17a,b. It is evident that in the L–S–L system analytes migrate very fast, and only an indispensable amount of terminator should be allowed to migrate into the second capillary. Otherwise, slower analytes passing the detector will still be in the stack (Fig. 18).

4. Separation efficiency in ITP–CZE. Dispersion of zones in ITP–CZE

To assess the separation efficiency in ITP–CZE, it is necessary to estimate the contributions of two mutually different effects to the dispersion of zones at the moment of detection. The first stage is ITP. It follows from its principle that the zones migrate in the steady state and the dispersion of zones (the widths of the boundaries) is independent of time. The zones begin to disperse with time after they were destacked, i.e., after the transient ITP in the second capillary disappeared. Actual concentration of an

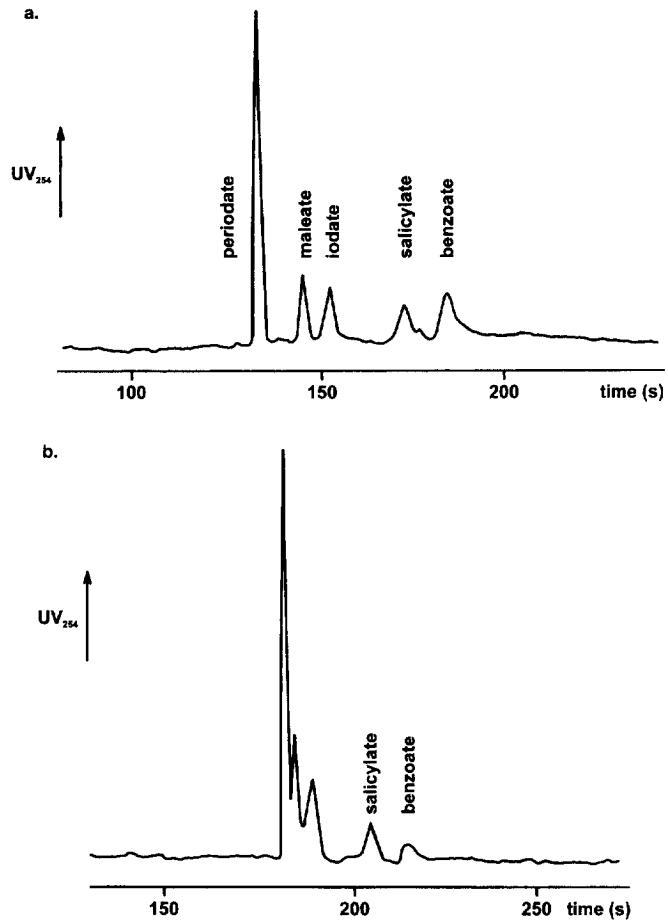

Fig. 13. Detection of the analytes separated by ITP–CZE combination performed at different stages of destacking. Leading electrolyte 20 m*M* HCl + His, pH 6, terminating electrolyte 15 m*M* aspartic acid + His, pH 6.15. This terminator was used as the background electrolyte in CZE step. Apparatus was CS Isotachophoretic Analyzer ZK 01, URVJT Spišská Nová Ves, Czechoslovakia, equipped with PTFE capillaries of 0.3 mm I.D. both for ITP and CZE step. Migration path in CZE step was 16 cm, current was 150 μA. Length of the leading zone accompanying the sample into the second capillary was (a) 9 s and all zones were detected after destacking, (b) 39 s, which enabled destacking only of the two slowest analytes.

analyte in its zone is set according to the regulation function [3] and therefore depends on the concentration of the leading electrolyte in ITP and of background electrolyte in CZE as illustrated in Fig. 19. These factors have to be taken into account in the selection of the electrolytes aimed to obtain the highest separation efficiency in ITP–CZE. At first sight, an application of a more concentrated BGE in CZE step could seem to be advantageous as the concentrating effect could ensure a narrower zone and a higher separation efficiency as it is common in rigorous ZE.

In an ITP–CZE combination the situation is somewhat different both from rigorous ITP and CZE. The adjusted zone lengths of the stacked trace analytes in ITP are short and are comparable to the width of the boundaries of the neighbouring zones. They may be considered in a realistic way as being sandwiched within the boundary (see Fig. 20). According to [17] the width of the isotachophoretic boundary is directly proportional to specific conductivity of the isotachophoretic zone and thus to its concentration, i.e., the higher the concentration of L in ITP the larger the dispersion of a zone. Time,

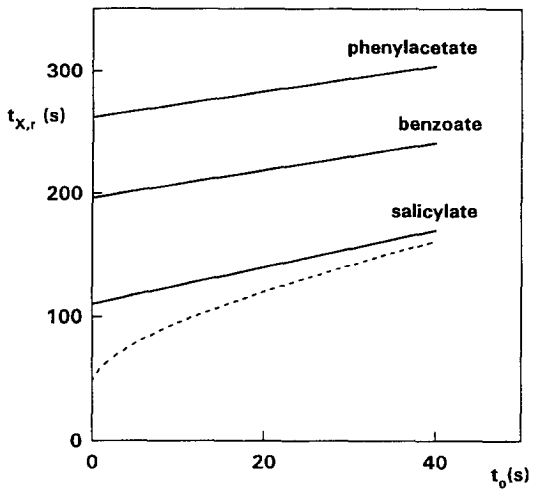

Fig. 14. Calculated dependence of detection time ($t_{X,r}$) of salicylate, benzoate and phenylacetate on the time (t_0) of migration of the leading zone into the analytical capillary in a T–S–T system composed of 0.01 *M* HCl + β-alanine, pH 3.5 (leader) and 0.01 *M* acetic acid + β-alanine, pH 4.3 (terminator, BGE). The dashed line corresponds to the detection time of the stacking rear boundary of the leading segment. After Ref. [16].

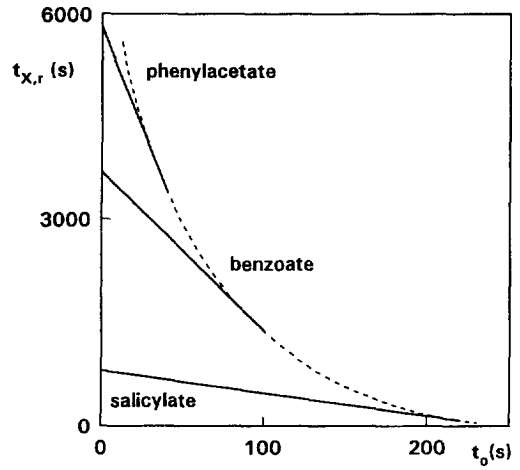

Fig. 16. Calculated dependence of detection time ($t_{X,r}$) of salicylate, benzoate and phenylacetate on the time (t_0) of migration of the terminating zone into the analytical capillary in a L–S–L system composed of 0.01 *M* HCl + β-alanine, pH 3.5 (leader, BGE) and 0.01 *M* acetic acid + β-alanine, pH 4.3 (terminator). After Ref. [16].

when the analyte leaves the stack of isotachophoretically migrating zones, is independent of L concentration [15]. Then, the zone starts to be dispersed with time which is directly influenced by the concentration of BGE in the CZE step. Subsequently, the dispersion of a zone in ITP–CZE combination at the moment of detection, $\sigma^2_{X,r}$, consists of dispersion resulting from ITP and from ZE migration,

$$\sigma^2_{X,r} = \sigma^2_{X,e} + 2D_X(t_{X,r} - t_{X,e}) \quad (2)$$

where $\sigma^2_{X,e}$ is the variance of the ITP zone at the time when it leaves the stack, $t_{X,e}$, D_X is the diffusion coefficient, and $t_{X,r}$ is the time when the zone passes along the detector, see Eq. (1), which depends on the concentration of the BGE. As a result an increased concentration of BGE has the opposite effect than simply expected from Fig. 19, i.e., it increases the

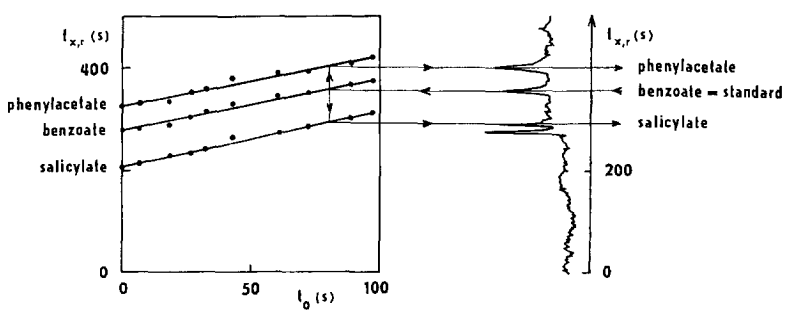

Fig. 15. Principle of the standardization method in the case of T–S–T system. Identification of analytes is based on the calibration curves for the dependence of the detection time of analytes and of an internal standard on the length of the leading electrolyte that had comigrated with the sample into the CZE step. Measurements were performed under constant current of 100 μA during the ITP mode and 50 μA during the CZE step. Leading electrolyte: 10 m*M* HCl + β-alanine, pH 3.5; ternninating electrolyte: 10 m*M* acetic acid, pH 4.3. As the background electrolyte for CZE, the terminating electrolyte from the ITP step was used. Sample: 25 μl of 1×10^{-7} *M* salicylate, benzoate and phenylacetate mixture. After Ref. [16].

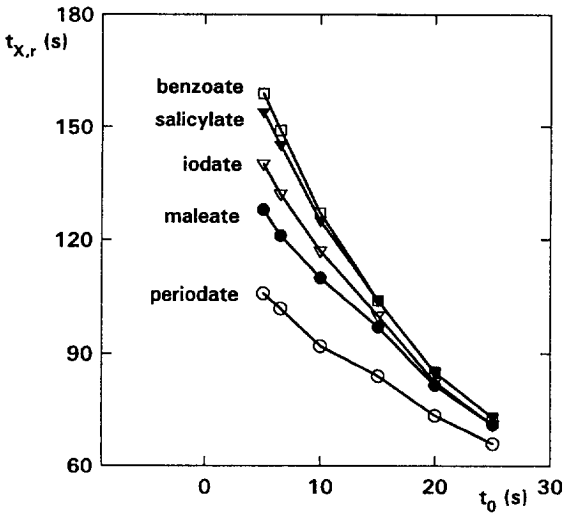

Fig. 17. Experimental verification of the dependence of the detection time ($t_{X,r}$) on the length of the terminating zone expressed as the time of its migration into the analytical capillary (t_0) filled with leading electrolyte (L–S–L electrolyte system). Measurements were performed under constant current of 75 μA during the ITP mode and 150 μA during the CZE step. Leading electrolyte: 10 m*M* HCl + histidine, pH 6.0, terminating electrolyte: 10 m*M* aspartic acid. As the background electrolyte for CZE, the leading electrolyte from the ITP step was used. Sample: 25 μl of 1×10^{-6} *M* of benzoate, salicylate, iodate, maleate and periodate. After Ref. [16].

dispersion of zones [16]. The effect of BGE concentration on the separation efficiency of salicylate in T–S–T system is illustrated in Table 1. It is seen that both the detection time, separation efficiency and dispersion of the zone are dependent on the concentration of BGE (T in this case). One should also notice that even the standard deviation of the dispersion of an isotachophoretic zone is higher in the system with the higher concentration of BGE. This situation results from the fact that a short ITP zone spreads within the ITP boundary of the neighbouring zones. It is obvious that the higher concentration of BGE brought lower separation efficiency.

Analyses shown in Fig. 21 demonstrate the difference in detection time, zone dispersion and separation efficiency when 10 and 50 m*M* terminator is used as the BGE. Obviously, the higher concentration caused broader peaks. Conversely, the more concentrated BGE resulting in longer migration times enables better resolution of analytes. This is

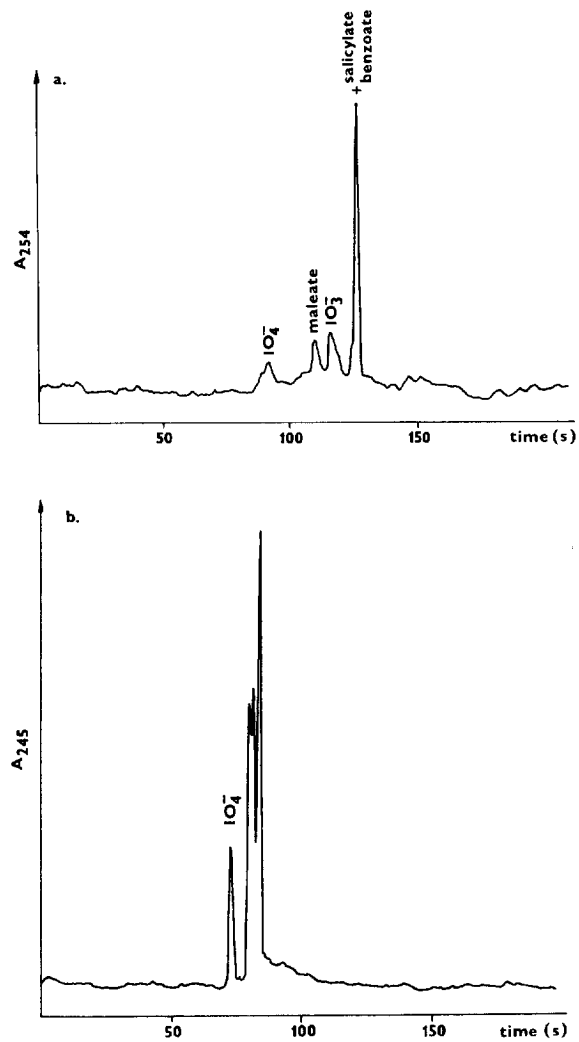

Fig. 18. ITP–CZE analysis in an L–S–L-electrolyte system. The effect of the length of the terminator segment accompanying the sample stack into the CZE step. Leading electrolyte was 10 m*M* HCl + His, pH 6, terminating electrolyte was 15 m*M* aspartic acid + His, pH 6.15. Leading electrolyte served as the background electrolyte in the CZE step. Apparatus was CS Isotachophoretic Analyzer ZK 01, URVJT Spišská Nová Ves, Czechoslovakia, equipped with PTFE capillaries of 0.3 mm I.D. for both ITP and CZE step. Migration path in the CZE step was 16 cm, current 150 μA. For ITP step and sampling into the CZE step, a current of 75 μA was applied. The length of the terminating zone accompanying the sample into the second capillary was (a) 10 s and all zones except for salicylate and benzoate were detected after destacking, (b) 30 s, which enabled destacking only of the fastest analyte.

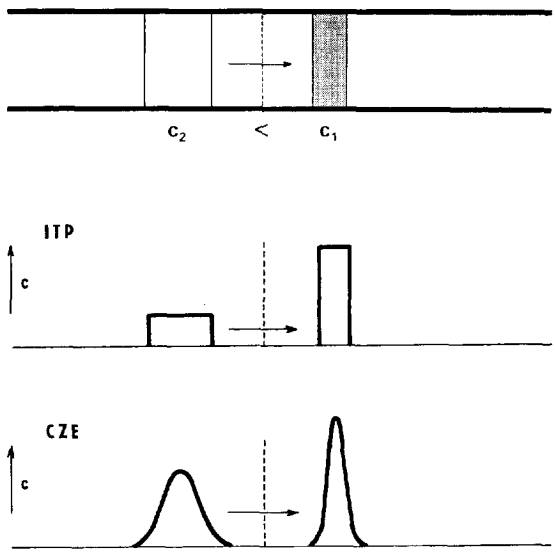

Fig. 19. Scheme of the dependence of the zone width on the concentration of leading electrolyte in ITP and background electrolyte in CZE.

due to the fact that analytes after destacking have more time to migrate separately, as can also be seen in Fig. 21, where some fast impurities were detected still in stack with salicylate in 10 m*M* BGE, while separated zones are observed in 50 m*M* BGE.

5. Sample induced transient ITP in CZE

In practice, especially in analysis of samples of biological origin, a fast ion may be present at a concentration which is several orders of magnitude higher than the concentration of analyzed species. During the analysis of such a sample, the zones of minor analytes are transiently stacked and migrate

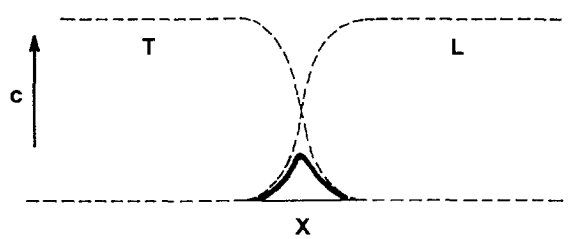

Fig. 20. Shape of a zone the length of which is comparable with the width of boundaries between normal ITP zones.

Table 1
Effect of BGE concentration on separation efficiency in the ITP–CZE technique

Parameter	Concentration of BGE	
	10 m*M*	50 m*M*
$t_{X,c}$ (s)	53.2	53.2
$t_{X,r}$ (s)	121.9	486.3
$t_{X,r} - t_{X,c}$ (s)	68.7	433.1
$\sigma_{X,c}$ (m)	$2 \cdot 10^{-5}$	$10 \cdot 10^{-5}$
$\sigma_{X,r}$	$35 \cdot 10^{-5}$	$89 \cdot 10^{-5}$
$\sigma(t)_{X,r}$	0.28	3.52
N	$108 \cdot 10^{3}$	$16.7 \cdot 10^{3}$

$\sigma(t)_{X,r}$ = Time dependent zone variance, for other parameters see text. Calculation was performed for an electrolyte system T–S–T with 10 m*M* HCl + β-alanine, pH 3.5 as the leading, and acetic acid + β-alanine, pH 4.3 as terminating electrolyte. The analyte, X, was 10^{-7} *M* salicylate, diameters of capillaries for both the ITP and CZE steps were 0.2 mm, effective length of ZE capillary was 13 cm. Current in ZE step was 50 μA, t_0 was 21 s. For 10 and 50 m*M* BGE, $t_{X,r}$ was 278 and 830 s, σ $(t)_{X,r}$ was 1.25 and 9.5, N was 49 460 and 9540, respectively.

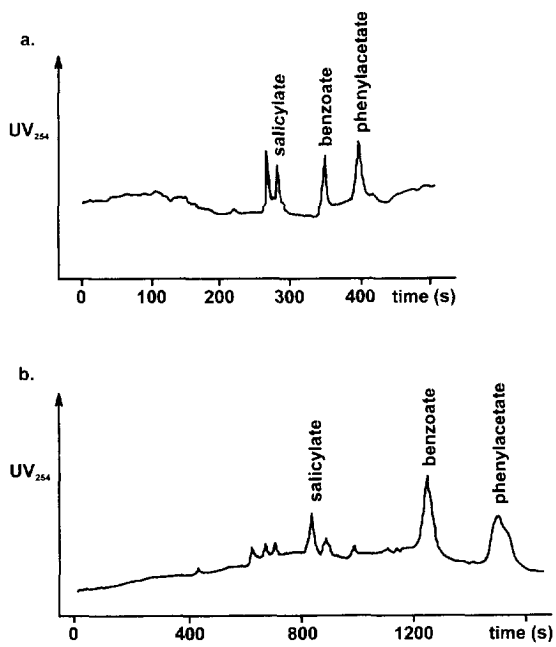

Fig. 21. Effect of the concentration of BGE on separation efficiency in ITP–CZE combination. A mixture of 10^{-7} *M* salicylate, benzoate and phenylacetate was analyzed in a T–S–T electrolyte system with 10 m*M* HCl + β-alanine, pH 3.5 as the leading, and 10 m*M* (a) and 50 m*M* (b) acetic acid + β-alanine, pH 4.3 as the terminating electrolyte. Diameters of capillaries both for ITP and CZE step were 0.3 mm, effective length of ZE capillary was 13 cm. Current in ZE step was 50 μA, t_0 was 21 s.

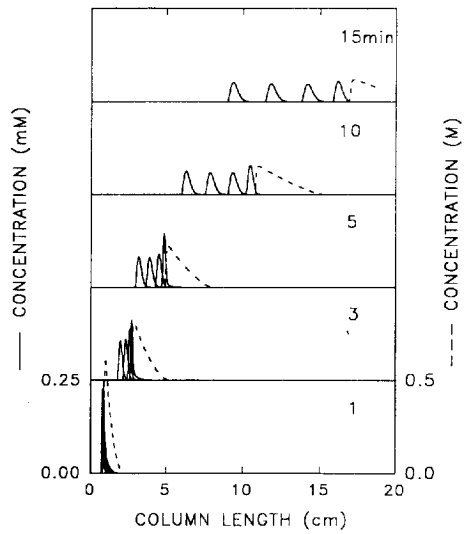

Fig. 22. Computer-simulated dynamics of the zone electrophoretic separation of four strong anions with mobilities 35, 45, 55 and 65×10^{-9} cm^2 V^{-1} s^{-1}, respectively, in the presence of a major amount of a strong anion with mobility 80×10^{-9} cm^2 V^{-1} s^{-1} in a background electrolyte containing a coion with mobility 30×10^{-9} cm^2 V^{-1} s^{-1}. The concentration profiles are shown at 1, 3, 5, 10 and 15 min of the separation process. After Ref. [18].

for some time in the ITP mode behind the zone of the bulk component. Then, this process is gradually transformed into ZE mode, as illustrated in Fig. 22 [18]. The migration behaviour of such a system resembles the second, ZE stage, of ITP–CZE technique. In the case illustrated in Fig. 22, the BGE plays for some time the role of a terminator, and chlorides from the sample serve transiently as the leading zone. Analytes which are faster than the BGE are stacked behind the zone of chlorides and leave the stack gradually according to their increasing mobilities. The analytes which are slower than BGE are not stacked at all and migrate in rigorous zone electrophoresis mode from the very beginning. The influence of increasing amounts of the stacker in a sample on migration times and peak shapes is illustrated in Fig. 23. It can be seen in Fig. 23a,b how the chlorides present in the sample form a leading zone of a transient ITP and delay the analytes, the mobility of which lies between chlorides ($79.1\cdot10^{-5}$ cm^2 V^{-1} s^{-1} [19] and caprylates ($27.4\cdot10^{-5}$ cm^2 V^{-1} s^{-1} [20], which are coions of the BGE and transiently serve as the terminator. An increased amount of chlorides, see Fig. 23c, results in such a long period of transient ITP that the fastest analytes pass the detector still being in the stack, which demonstrates itself in the form of very sharp packed peaks [21].

Generally, minor analytes in a sample can be involved in the sample-self stacking mechanism only if at least one macrocomponent of the same charge is also present in the injected mixture. The macrocomponent can serve as a temporal leading or terminating zone with BGE ion being terminator or leader, respectively. Then the analytes create a train of the stacked zones with sharp boundaries migrating isotachophoretically between the macrocomponent (stacker) and BGE. The starting situation where we can see a zone of the sample S containing bulk component A and trace component X is depicted in Fig. 24. The conditions for the sample induced stacking being effective are very complex and depend not only on the mobilities of species involved but also on their concentration in the original sample. All these variables are reflected by the migration velocities of the boundaries and species involved. One can distinguish two principal cases and formulate the following criteria [22]. For

$$v_{X,S} > v_{S/B} > v_{X,B} \tag{3}$$

the stacker is the transient terminator while for

$$v_{X,S} < v_{S/B} < v_{X,B} \tag{4}$$

the stacker is the transient leading zone. $v_{X,S}$ is the migration velocity of an analyte X in the sample zone, $v_{X,B}$ is the migration velocity of an analyte X in the BGE and $v_{S/B}$ is the migration velocity of the sharp boundary between the sample zone and BGE (Fig. 25). Obviously, the above mentioned velocities are not directly accessible from experiments, and, the criteria must be transformed with the help of mobilities and concentrations [22].

For the terminating stacker it holds

$$\bar{u}_{B,B}\frac{\bar{u}_{A,S}}{\bar{u}_{B,S}} < \bar{u}_{X,B} < \bar{u}_{B,B}, \tag{5}$$

for the leading stacker it holds

$$\bar{u}_{B,B} < \bar{u}_{X,B} < \bar{u}_{B,B}\frac{\bar{u}_{A,S}}{\bar{u}_{B,S}} \tag{6}$$

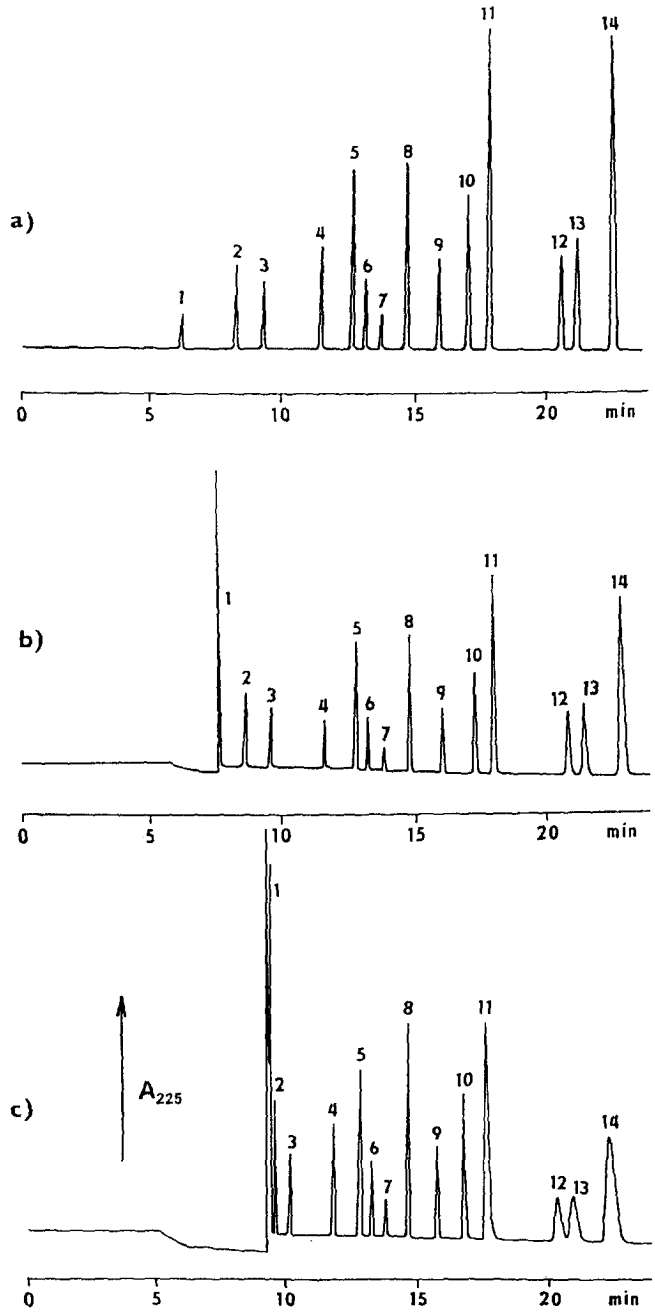

Fig. 23. Shift of migration times and change of peak shapes caused by sample induced stacking. Concentration of analytes: 0.1 m*M*, concentration of NaCl in the sample: (a) 0; (b) 100 m*M*; (c) 300 m*M*. 1–14, components of anionic model mixture. Background electrolyte was composed of 10 m*M* caprylic acid+Tris, pH 7.2. Voltage, 15 kV; hydrodynamic sampling, 5 s. After Ref. [21].

For an effective stacking the concentration of the stacker must be sufficiently high. Hence, the ratio of the concentration of the stacker and of the BGE coion in the sample, $\bar{c}_{A,S}/\bar{c}_{B,S}$, must be higher than a certain break value. This condition can be expressed explicitly by the relationship:

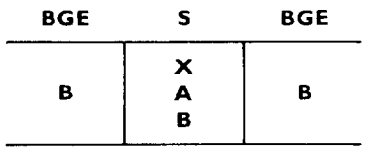

Fig. 24. Scheme of a sample for the description of the sample induced stacking. Components A and X are bulk and trace, respectively.

$$\frac{c_{A,S}}{c_{B,S}} > \left(\frac{c_{A,S}}{c_{B,S}}\right)_{br} = \frac{u_A - u_X}{u_X - u_B}\,\frac{u_B + u_R}{u_A + u_R} \qquad (7)$$

which is valid both for the leading and terminating types of stacking. Simultaneous stacking of all components of a complex sample possessing mobilities both higher and lower than the mobility of BGE coion is also possible providing that the stackers both of the leading and terminating types are present in the sample injected, as demonstrated by computer simulation in Fig. 26.

The process of the sample self stacking proceeding spontaneously in samples where the concentration of individual components substantially differ from one another can also be induced on purpose with the intention of utilizing its advantage, i.e., the adjustment of concentration in the transient ITP step. A diagram shown in Fig. 27 enables for an analyte of optional mobility to find the corresponding minimum concentration of a stacker of leading or terminating type which should be reached to bring effective stacking. This diagram can be used advantageously for creating an optimum electrolyte system when relatively large volumes of diluted samples are available [22].

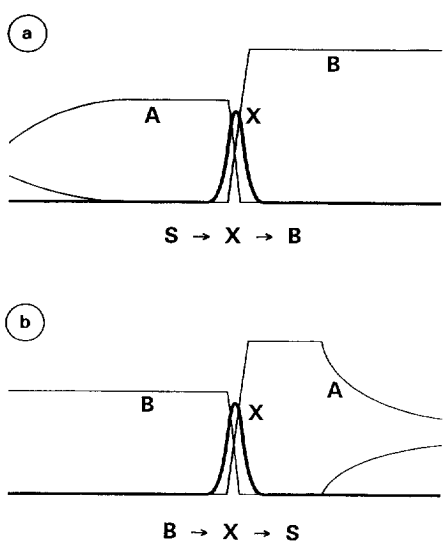

Fig. 25. Scheme of concentration profiles of sample self-stacking of (a) terminating type, (b) leading type. After Ref. [22].

Finally, it should be stressed that sample-induced stacking also brings a risk, that the zones may reach the detector earlier than they are unstacked.

Though relationships were derived enabling calculation of detection time and variance of individual zones in a model system analyzed by zone electrophoresis involving sample self-stacking [18], it is obvious that in complex samples of natural origin with varying and unknown types and amounts of stackers the evaluation is difficult. Care must be taken to ensure that all the microcomponents are detected only after destacking and identification of analytes cannot be based on detection times only.

6. Recommended electrolyte systems

The rules and recommendations summarized in this part can be used both for single ITP analysis and for ITP–CZE combination including aimed sample induced self-stacking.

First it is necessary to find the electrolyte system for the ITP step. The leading electrolyte has to possess a leading ion, the mobility of which is higher than that of any analyte. The terminating ion of the terminating electrolyte has to be the slowest one to be able to close the train of analyte zones. The pH of the leading electrolyte to which the pH values in all the following zones are adjusted, has to ensure sufficient dissociation of weak acids and bases in their own zones. Full dissociation of all analytes to be separated is usually not advantageous due to small differences in the selectivity of analytes migrating with their ionic mobilities [23].

A suitable weak acid or a weak base are generally used as terminator. However, their effective mobility must always be higher than the effective mobility of OH^- or H^+ ions otherwise the migration is incorrect and conditions for ITP migration are not fulfilled [24]. A survey of general principles of the selection of an electrolyte system for ITP and recommended counterions are given in Table 2 and Table 3.

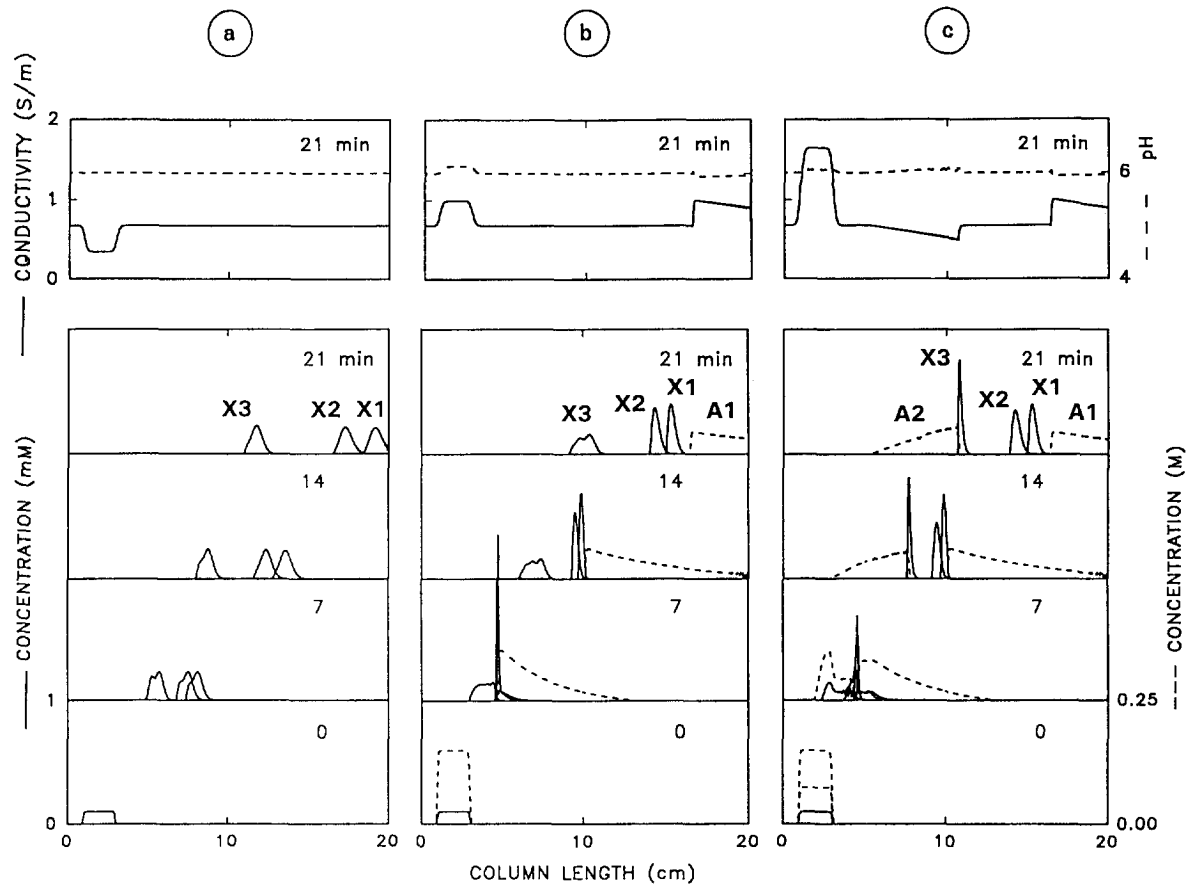

Fig. 26. Computer simulation of the effect of sample self-stacking on the zone electrophoretic separation of three minor analytes X1, X2 and X3. The concentration (lower panel) and conductivity and pH (upper panel) profiles shown, correspond to the denoted time intervals. The values used for calculations were: $\bar{c}_{B,B}=0.1$ *M*; $\bar{c}_{B,S*}=0.05$ *M*; $\bar{c}_{A1,S*}=$ (a) 0, (b) 0.15, (c) 0.15 *M*; $\bar{c}_{A2,S*}=$ (a) 0, (b) 0, (c) 0.075 *M*; $I=2000$ A m^{-2}. After Ref. [22].

For the ZE step in ITP–CZE combination, or for the sample induced stacking, either the leading or terminating electrolyte or any other electrolyte can serve as the background electrolyte. Leading or terminating electrolytes from the preceding ITP step offer simple operation, evaluation and optimization of separation efficiency and can be therefore recommended as user-friendly electrolyte combinations. However, they differ substantially in the course of destacking phase. In T–S–T system, where the BGE in CZE step is created by the terminator, detection times are longer compared to the single stage CZE: slow analytes are destacked first, they are more dispersed and more distant one from another when passing the detector, and there is a minimum risk of deteriorated detection. On the contrary, with the leading electrolyte used as BGE the detection times are shorter compared to the single stage CZE and fast analytes are destacked first. It can be recommended in the case when analytes with mobilities close to that of a leading ion are to be separated.

7. Conclusions

Capillary zone electrophoresis is one of the most rapidly developing analytical methods at present, above all due to its prompt and simple performance and evaluation. An attempt to reach higher speed of analysis, higher reproducibility, efficiency, sensitivi-

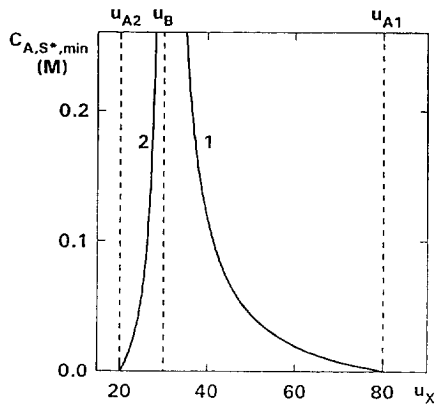

Fig. 27. Calculated dependence of the minimum necessary stacker concentration, $\bar{c}_{A,S^*,min}$, on the mobility, u_X (in 10^{-9} m^2 V^{-1} s^{-1}) of the analyte to be stacked by leading-type (curve 1) and terminating-type (curve 2) sample self-stacking. All numerical parameters were the same as used in Fig. 26. Mobility of a stacker of the leading type was 80×10^{-9} m^2 V^{-1} s^{-1}, for the terminating type it was 20×10^{-9} m^2V^{-1} s^{-1}. After Ref. [22].

ty and detectability, leads to the application of capillaries of very small diameters, temperature control of the separation process, highly sensitive detectors and to combinations with various preconcentration techniques. Using ITP as the preconcentration step either in the combination of ITP and CZE, or in the sample induced self-stacking is a natural and powerful combination. It combines a reliable analytical method enabling demanded concentration adjustment uninfluenced by the composition of the sample, with the highly sensitive, fast but simple technique of CZE offering high resolving power. By proper combination of these electrophoretic methods and by utilization of their characteristic features the expedience of both the single methods can be substantially improved. CZE with ITP preconcentration is applicable both to small ions, as demonstrated in this article, and to large molecules as proteins, as shown by e.g., Foret et al. [25,26] and Dolník et al. [27]. This article shows the ways in which to utilize the best of the isotachophoretic separation process combined with, or involved in, zone electrophoresis. It discusses suitable selection and combination of electrolytes and their influence on the resulting zone electrophoresis separation process including rules for correct evaluation of results. In the future, further improvement can be expected in the commercial instrumentation development since the limitations of the ITP–CZE lie now in this area.

Table 3
Recommended counterions for ITP separations in buffered electrolyte systems

Anionic ITP		Cationic ITP	
Counterion	pH_L	Counterion	pH_L
β-Ala	3.1– 4.1	Formate	3.2– 4.2
EACA	4.1– 5.1	Acetate	4.2– 5.2
His	5.5– 6.5	MES	5.7– 6.7
Imidazole	6.6– 7.6	Veronal	6.9– 7.9
Tris	7.6– 8.6	$AspNH_2$	8.3– 9.3
Ethanolamine	9.0–10.0	Gly	9.1–10.1

Acknowledgments

This work was supported by grants from the Grant Agency of the Czech Republic, No. 203/94/0998, and the Grant Agency of the Academy of Sciences of the Czech Republic, No. 431404.

References

[1] W. Nernst, Z. Elektrochem., 3 (1897) 308–309.
[2] W.C.D. Whetham, Phil. Trans. A, 184 (1893) 337–359.
[3] F. Kohlrausch, Ann. Phys. Chem., N.F. 62 (1897) 209–239.

Table 2
Recommended composition of electrolyte systems for ITP

Analysis	Cationic	Anionic
Leading ion 0.02–0.003 mol l^{-1}	K^+, NH_4^+, Na^+	Cl^-
Terminating ion	H^+ or weak base $\bar{u}>u_{H+}$	OH^- or weak acid $\bar{u}>u_{OH-}$
Counter ion	weak acid, $pK=pH_L\pm0.5$	weak base, $pK=pH_L\pm0.5$
Condition for ionization of analytes	$pH_L\leq pK_{BH}+1$	$pH_L\geq pK_{HA}-1$

[4] A.J.P. Martin and F.M. Everaerts, Anal. Chim. Acta, 38 (1967) 233–237.
[5] F.M. Everaerts, J.L. Beckers and T.P.E.M. Verheggen, Isotachphoresis: Theory, Instrumentation and Application, Elsevier, Amsterdam, 1976.
[6] J. Vacík and V. Fidler, Anal. Chem. Symp. Ser., 6 (1981) 19–24.
[7] R.A. Mosher, D.A. Saville and W. Thormann, The Dynamics of Electrophoresis, Verlag Chemie, Weinheim, 1992.
[8] V.G. Babskii, M. Yu. Zhukov and V.I. Yudovich, Mathematical Theory of Electrophoresis, Consultant Bureau, New York, 1989.
[9] J.L. Beckers and F.M. Everaerts, J. Chromatogr., 68 (1972) 207–230.
[10] T. Hirokawa and Y. Kiso, J. Chromatogr., 252 (1982) 33–48.
[11] P. Boček, M. Deml, P. Gebauer and V. Dolník, Analytical Isotachophoresis, Verlag Chemie, Weinheim, 1988.
[12] F. Foret, L. Křivánková and P. Boček, Capillary Zone Electrophoresis, Verlag Chemie, Weinheim, 1993.
[13] F. Foret, V. Šustáček and P. Boček, J. Microcol. Sep., 2 (1990) 229–233.
[14] L. Křivánková, F. Foret and P. Boček, J. Chromatogr., 545 (1991) 307–313.
[15] L. Křivánková, P. Gebauer, W. Thormann, R.A. Mosher and P. Boček, J. Chromatogr., 638 (1993) 119–135.
[16] L. Křivánková, P. Gebauer and P. Boček, J. Chromatogr. A, 716 (1995) 35–48.
[17] L.G. Longsworth, J. Am. Chem. Soc., 66 (1944) 449–453.
[18] P. Gebauer, W. Thormann and P. Boček, J. Chromatogr., 608 (1992) 47–57.
[19] T. Hirokawa, M. Nishino, Y. Aoki, Y. Kiso, Y. Sawamoto, T. Yagi and J. Akiyama, J. Chromatogr., 271 (1983) D1–D106.
[20] J. Pospíchal, P. Gebauer and P. Boček, Chem. Rev., 89 (1989) 419–430.
[21] R. Vespalec, P. Gebauer and P. Boček, Electrophoresis, 13 (1992) 677–682.
[22] P. Gebauer, W. Thormann and P. Boček, Electrophoresis, 16 (1995) 2039–2050.
[23] P. Gebauer and P. Boček, J. Chromatogr., 320 (1985) 49–65.
[24] P. Boček, P. Gebauer and M. Deml, J. Chromatogr., 217 (1981) 209–224.
[25] F. Foret, E. Szoko and B.L. Karger, J. Chromatogr., 608 (1992) 3–12.
[26] F. Foret, E. Szoko and B.L. Karger, Electrophoresis, 14 (1993) 417–428.
[27] V. Dolník, K.A. Cobb and M. Novotny, J. Microcol. Sep., 2 (1990) 127–131.
[28] L. Křivánková and P. Boček, Capillary Isotachophoresis, in M. Khaledi (Editor), High Performance Capillary Electrophoresis, Wiley, in press.