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Separation possibilities of three-dimensional capillary electrophoresis

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ABSTRACT

Separation possibilities of three-dimensional (3D) capillary electrophoresis (CE) were studied in this work. They were demonstrated using phthalic acid as a model analyte and human urine as a complex ionic model matrix. Complexity of the selected problem ordering from several facts, such as (i) analyte present on a trace concentration levels, (ii) analyte present in multicomponent matrix and (iii) analyte migrating in the region of electropherogram in which is naturally present the majority of ionizable organic compounds (i.e. potential interfering compounds). 3D tandem was created by (i) isotachophoresis (ITP) preseparation stage (first), (ii) ITP analytical stage (second), and (iii) capillary zone electrophoresis (CZE) analytical stage (third). Comparison of 2D and 3D CE employing two and three different CE stages, respectively, revealed considerably enhanced separation capability of the 3D CE system. Although no single ITP was sufficient for the effective sample pretreatment, the mutual combination of these two ITP steps do it. The proposed ITP tandem was based on the different migration pattern of two spacers–analyte–matrix constituents under different acid-base conditions (*pH* 3.1 vs. pH 4.5 in ITP1 and ITP2, respectively), that can be, generally, very effective tool for acidic compounds present in multicomponent ionic matrices. Besides the enhanced separation selectivity/sample clean-up, the 3D CE method kept benefits of the hydrodynamically closed separation system with enhanced sample loadability, such as excellent (i) reproducibility of the measurements and (ii) concentration detection limits. Hence, this study clearly demonstrated great potentialities of 3D CE in the solving even the most advanced separation problems as can be found in bioanalytical field.

1. Introduction

Although the technologies in electromigration separation field have matured in the past decades, the development of new, even more advanced separation systems still belongs to the more challenging tasks in separation science and analytical chemistry. Multidimensional (or capillary coupling or tandem) capillary electrophoresis (CE) belong to such advanced systems providing enhanced analytical possibilities to the single capillary CE systems, as comprehensively reviewed by Mikuš and Maráková [1]. The comprehensive two-dimensional capillary electrophoresis belongs to the group of techniques where a complex sample is fractionated and each fraction is then subjected to a second dimension separation. Such comprehensive two-dimensional systems are generally very convenient for detail evaluation of very complex separation profiles (e.g. profiling metabolites), and structure of biopolymers (e.g. monitoring peptides from proteinic digest), as it was demonstrated in several works, see e.g. the capillary liquid chromatography–CE method [2] and the capillary high-performance liquid chromatography-microchip electrophoresis method [3], respectively. In contrast, there is also a literature on two-dimensional capillary electrophoresis techniques that are advantageous by providing heart-cut analysis, where a fraction is isolated in the first dimension and subjected to a second dimension. This approach is of value for the analysis of a specific analyte within a complex matrix.

An on-line combination of two CE techniques, i.e. two-dimensional (2D) CE, proposed by Kaniansky and Marák [4], can effectively solve problems of (i) sample preparation (preseparation, preconcentration, clean-up) and (ii) final analysis (fine separation and sensitive detection) in one run and in well defined way that have great impact on the effectivity of the overall analytical procedure [5,6]. CE performed in the hydrodynamically closed separation system (hydrodynamic flow is eliminated by semi-permeable membranes at the ends of separation compartment) enabling to use wide bored capillaries (with enhanced sample loadability) can provide additional benefits to the conventional CE systems employing narrow bored capillaries, reflecting in the real applicability. These benefits are (i) enhanced reproducibility of the measurements (because of elimination of non-selective flows, namely hydrodynamic and electrophoretic), (ii) considerably improved concentration limit of detection (LOD) (because of

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large volume sample injection following by in-capillary stacking) [7,8]. The CE performed in a hydrodynamically closed separation system and employing wide bored capillaries can be easily implemented into CE systems operating with coupled capillaries. The proposed and commercially available 2D CE systems have usually modular composition that provides (i) high flexibility in arranging particular moduls in the separation unit, creating desirable CE–CE combinations (e.g. ITP–ITP, ITP–CZE and CZE–CZE), (ii) high variability of the injection volumes, and, by that, (iii) enhanced capability to solve wide scale of real advanced analytical problems [9–16].

Capillary isotachophoresis (ITP) coupled on-line with capillary zone electrophoresis (CZE) provides the most significant CE tool applicable for the trace analytes and complex ionic matrices and therefore it is the most frequently used 2D CE system, see Ref. [1] and references cited therein. Nevertheless, in specific cases there still could be requirement to improve separation selectivity. Moreover, it could be accompanied simultaneously with additional issues such as not to prolong capillary (to keep separation efficiency), not to use selector (to prevent detection interferences), etc. One of the approaches to meet such specific demands could be based on employing an additional CE dimension. Three-dimensional (3D) CE was described for the first time and used only by Hanna et al. [17]. Although the authors demonstrated successful design, construction and evaluation of performance parameters of the 3D CE system, no separation example illustrating essential role of the 3D system in comparison to the 2D CE system has been presented in the literature so far. The aim of our work was to present benefits of the 3D CE system over the 2D CE system in the advanced separation field. Phthalic acid as a model analyte and urine as a multicomponent ionic model matrix were chosen for this purpose in order to simulate potentialities of 3D CE in bioanalytical field.

2. Material and methods

2.1. Instrumentation

A modular capillary electrophoresis analyzer EA-103 (Villa-Labeco, Spišská Nová Ves, Slovakia), assembled in the capillary-coupling configuration of the separation units, was used in this work for performing the ITP–ITP–CZE runs. The 3D CE instrumental scheme is shown in Fig. 1. The system consisted of the following main subunits: (i) three-dimensional CE separation unit, (ii) high voltage supply, (iii) conductivity detectors (on the first and second capillary) and (iv) UV absorbance photometric detectors (on the second and third capillary). These subunits are the same as described in Ref. [17] unless otherwise stated.

Electrode compartments with hydrodynamically (membrane) closed connecting channels to the separation compartment (Villa-Labeco) were employed. The samples were injected by a 30 μl internal sample loop of the injection valve of the analyzer and spiked by Hamilton micro-syringes. Flushing of capillaries between analyses was made with disposable plastic syringes. The ITP preseparation (first) capillary was provided with an 800 μm I.D. FEP capillary tube of a 90 mm length and a contactless conductivity detector. The ITP analytical (second) capillary was provided with a 300 μm I.D. FEP capillary tube of a 90 mm length and a contact conductivity detector along with UV absorbance photometric detector. The CZE analytical (third) capillary was provided with a 300 μm I.D. FEP capillary tube of a 160 mm length and a UV absorbance photometric detector. The photometric detectors Sapphire 2082 (ECOM, Prague, Czech Republic) were set at a 254 nm wavelength. Both photometric detectors were connected to the capillary module via optical fibers. The signals from the detectors were evaluated by ACES software (Villa-Labeco).

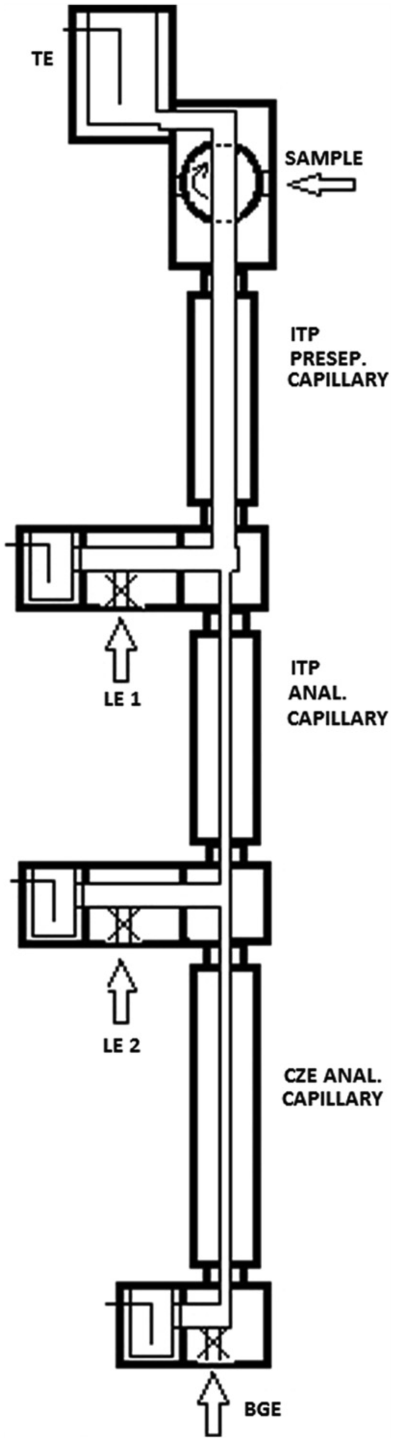

Fig. 1. The 3D CE instrumental scheme for the modular hydrodynamically closed separation system combining ITP–ITP–CZE stages.

Prior to the use, the capillaries were not treated by any rinsing procedures to suppress an electroosmotic flow (EOF). A dynamic coating of the capillary wall by means of methylhydroxyethylcellulose (MHEC 30,000; Serva, Heidelberg, Germany) present in leading and background electrolyte solutions served for this purpose. The separating electrolytes in the capillaries were replaced by the fresh ones between each run. The CE analyses were carried out in the anionic regime of the separation (i.e. anodic movement of the analytes) with direct injections of the samples. The experiments were performed in constant current mode at 20 °C. The driving currents applied were 300 μA (preseparation ITP), 60 μA (analytical ITP), and 40 μA (analytical CZE).

2.2. *Chemicals and samples*

The electrolyte solutions were prepared from chemicals obtained from Merck (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany), and Fluka (Buchs, Switzerland) in water demineralized by a Rowapure-Ultrapure water purification system (Premier, Phoenix, Arizona, USA). All chemicals used were of analytical grade. The solutions of the electrolytes were filtered before use through disposable membrane filters of a 0.8 μm pore size (Millipore, Molsheim, France).

The standard substance of phthalic acid was obtained from Sigma-Aldrich.

2.3. *Procedures for sample and standard solution preparations*

The standard stock solution of phthalic acid was prepared in demineralized water at the concentration of 100 ppm. Working standard solutions were made by an appropriate dilution of the standard stock solution with demineralized water.

The urine samples (morning urines) were obtained from 20 male and female volunteers of different age, collected in the interval of 6 weeks, frozen (−18 °C) immediately after the sampling and kept in the freezer and dark until the use. Each sample was thawed out just before its final dilution, (spiking), and injecting into the sampling loop of the electrophoretic analyzer. Working urine solutions were made by spiking human urine with the standard stock solution of phthalic acid (blank urine without spiking) following by an appropriate dilution (50-fold) with demineralized water.

3. Results and discussion

3.1. *Two-dimensional ITP–CZE approach*

In the first part of our work, the modular electrophoresis analyzer EA-103 was used in two-dimensional configuration of the separation units. This 2D CE method was developed for the advanced analytical task, i.e. separation of phthalic acid as a model analyte in human urine as a complex ionic model matrix. Complexity of this problem ordering from several facts, such as (i) analyte present on a trace concentration levels, (ii) analyte present in multicomponent matrix and (iii) analyte migrating in the region of electropherogram in which is naturally present the majority of ionizable organic compounds (i.e. potential interfering compounds). The analyte, having the migration properties similar to the majority of matrix compounds for the given biological sample, was selected for our work in order to examine true separation potential of the proposed 3D method and clearly show the differences between 2D and 3D systems.

The ITP step was integrated with CZE step in this method. ITP was optimized with respect to the effective sample pretreatment while CZE was developed for the optimum final (analytical) separation. Moreover, the buffer constituents (hydrochloric acid, alanine, ε-aminocaproic acid, histidine, acetic acid), concentration of leading and carrier cations (leading anion: 5–20 mmol/l, carrier anion: 10–40 mmol/l), pH (3.1–4.5 of leading electrolyte, 4.0–6.0 of background electrolyte), and driving currents (ITP: 50–500 μA, CZE: 10–100 μA) in the ITP–CZE separations were optimized to ensure (i) appropriate compatibility of both CE stages, (ii) sufficient buffering capacity of the electrolytes, (iii) minimization of dispersion effects, (iv) high separation efficiency, (v) maximum resolution (phthalic acid vs. urine matrix constituents) and (vi) short analysis time. Optimum pH values of the electrolytes were chosen with respect to the sufficient ionization of the analyte, and to obtain different migration pattern of the analyte vs. spacers in ITP1 (pH 3.1) and ITP2 (pH 4.5). In this way, the sample pretreatment effectivity of ITP depending on pH was clearly visible. Lower pH minimized the migration of interfering compounds as only very few (strongly ionizable) compounds can migrate under such conditions. pH value (4.4) of the background electrolyte was chosen mainly with respect to the maximum resolution of the analyte from the matrix interfering compounds. Lower conductivity of the electrolytes {provided by lower concentrations of leading (10 mmol/l) and carrier (20 mmol/l) anions} was chosen for lower Joule heating/termal dispersion. Reasonable buffering capacity of the electrolytes was maintained with these concentrations too. The appropriate mobility matching of acetic acid as carrier anion and analyte ensured the elimination of electromigration dispersion. Currents (ITP1 300 μA, ITP2 60 μA, CZE: 40 μA) were chosen as a compromise between the analysis time (decreased with higher currents) and production of Joule heating/ termal dispersion (increased with higher currents). Optimized separation systems for the ITP preseparation and CZE analytical stages are given in Table 1.

The ITP preseparation (ITP1 in Table 1) is illustrated in Fig. 2. The isotachopherogram clearly illustrates a migration position of phthalic acid that is defined by two spacers (formate, citrate). This migration configuration ensured an efficient sample pretreatment (preconcentration and elimination of the majority of the matrix constituents) and a reproducible transfer of the well defined part of the sample (phthalic acid plus accompanied matrix constituents) into the CZE stage. Electrophoreogram from the analytical CZE separation (CZE in Table 1) of the isotachophoretically (ITP1 in Table 1) pretreated sample is illustrated in Fig. 3a. It is clearly visible from this figure that despite the on-line sample pretreatment and two-dimensionally influenced selectivity (ITP1–CZE) it is not possible to achieve complete (baseline) separation of phthalic acid from the rest of the matrix constituents. For the comparison, similar unsatisfactory results concerning the resolution of the analyte from the rest of the matrix constituents were obtained also when combining a modified ITP preseparation step (ITP2 in Table 1) with the same CZE step (CZE in Table 1), as it can be seen in Fig. 3b. Therefore, other approaches for increasing separation selectivity and separability have to be investigated.

3.2. *Three-dimensional ITP–ITP–CZE approach*

In the second part of our work, the modular electrophoresis analyzer EA-103 was used in three-dimensional configuration of the separation units for increasing separation selectivity and, by that, separability in comparison with the 2D system. The same

Table 1
Electrolyte systems for the optimized 2D and 3D CE methods.

Parameter	ITP1	ITP2	Parameter	CZE
Solvent	water	water	Solvent	water
Leading anion	Cl^-	Cl^-	Carrier anion	AcH
Concentration (mmol/l)	10	10	Concentration (mmol/l)	20
Counter ion	Alanine	EACA	Counter ion	EACA
Concentration (mmol/l)	12	20	Concentration (mmol/l)	20
pH	3.1	4.5	pH	4.4
EOF suppressor	MHEC	MHEC	EOF suppressor	MHEC
Concentration (%, w/v)	0.05	0.05	Concentration (%, w/v)	0.05
Terminator	AcH	AcH		
Concentration (mmol/l)	20	20		

EOF–electroosmotic flow; MHEC—methylhydroxyethylcellulose; EACA–ε-aminocaproic acid and AcH–acetic acid.

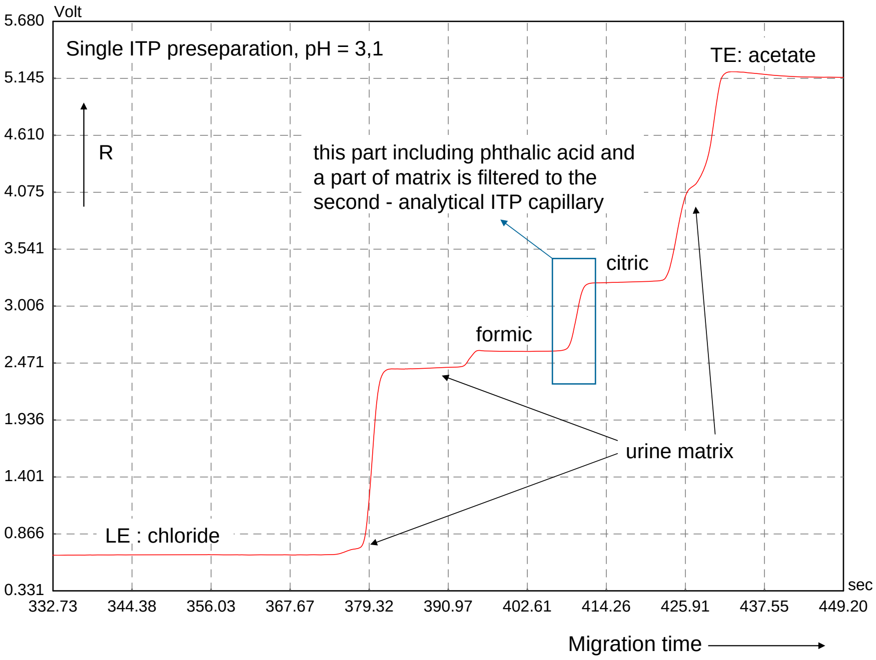

Fig. 2. Preseparation ITP stage of the ITP-CZE method. Isotachopherogram from the separation of phthalic acid in the urine sample. Urine was diluted 50-fold with demineralized wated and spiked with phthalic acid (20 ppm in original urine). In addition, formic acid (50 mg/l) and citric acid (75 mg/l) were added to the sample too, serving as spacers (boundary) for the zone of phthalic acid. The marked part of the sample only was transferred and separated in the second capillary. Electrolyte system "ITP1" given in Table 1 was used for the separation. For other working conditions Section 2.

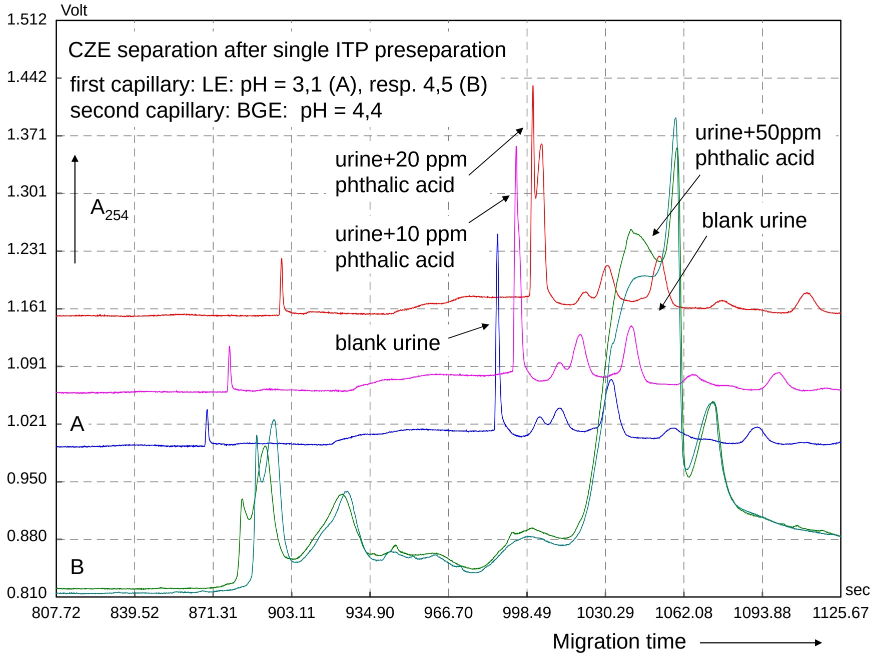

Fig. 3. Analytical CZE stage of the ITP–CZE method. (a) Electropherograms from the final separation of phthalic acid in the on-line ITP1 (pH 3.1) pretreated urine sample (for the ITP1 profile see Fig. 2). (b) Electropherograms from the final separation of phthalic acid in the on-line ITP2 (pH 4.5) pretreated urine sample. Electropherograms (blank and spiked trace for each pH) were obtained analyzing the same human urine sample. Unmarked peaks in the CZE profiles represent the unidentified urine matrix constituents transferred into the CZE stage after the single ITP sample pretreatment. Electrolyte system "CZE" given in Table 1 was used for the separation. For other working conditions see Fig. 2 and Section 2.

separation problem was applied for the 3D CE method. In comparison with the 2D systems used in Section 3.1., the 3D system described in this section was based on the combination of two ITP steps with CZE step. It was expected that the combination of two ITP steps with the different electrolyte composition and pH could enhance the sample pretreatment/preseparation and, consequently, the final CZE separation of the analyte.

Optimization of the separation conditions in the 3D system was based on the creation of mutually compatible CE stages. In the 3D system the appropriate compatibility of the electrolytes was ensured (i) using the same leading anion (chlorides), terminating anion (acetate) and their concentrations (10 mmol/l of chlorides, 20 mmol/l of acetate) in both ITP stages, and (ii) using the same counter ion (ε-aminocaproic acid in ITP2 and CZE), its concentration (20 mol/l in ITP2 and CZE) and close *pH* values (4.5 in ITP2 vs. 4.4 in CZE) in the ITP2 and CZE stages. The double sample pretreatment effect was tested with different migration patterns of the analyte vs. spacers in the combined ITP stages. The maximum effect (maximum resolution of the analyte) was achieved combining *pH* 3.1 in ITP1 vs. *pH* 4.5 in ITP2 vs. *pH* 4.4 in CZE (for the optimum composition of the electrolyte systems see Table 1). The optimization of the particular CE stages was analogical as in Section 3.1.

Comparing to the ITP1–CZE system (Fig. 3a), the additional ITP step (ITP2) served for the fine (analytical) separation of the isotachophoretically pretreated sample. The additional acid-basic equilibrium provided significant changes in the migration order of migrants in the second ITP step so that a well defined part of the matrix constituents (including the interfering compounds) was bordered by the spacers (formate and citrate) and removed from the separation system. On the other hand, the analyte with a reduced part of matrix constituents left the position bordered by the spacers (i.e. migrated outside of the spacer boundaries) and could be transferred into the CZE step for the final separation and detection without the interfering compounds. It can be generalized that the mechanism provided by the ITP tandem, based on the combination of acid-base equilibria accompanied with changes in migration order and electronic selection of sample fragment, can be essential/especially useful for the finest separation of acidic/basic compounds in multicomponent ionic matrices. Although the ITP preseparation (ITP1) was the same as illustrating in Fig. 2, its combination with the additional analytical ITP separation (ITP2), illustrated in Fig. 4, was prerequisite for accomplishing the baseline resolution of phthalic acid from the residual matrix constituents in the analytical CZE stage, as it can be seen in Fig. 5. Hence, the analyte peak obtained in the 3D CE system was suitable for its qualitative and quantitative evaluation. On the other hand, the analyte peak obtained in 2D is significantly or completely (depending on *pH* of ITP electrolyte) overlapped with matrix interfering compounds so that its identification (especially in lower concentrations) and quantification (in every case) is entirely impossible. Therefore, the proposed 3D method provides crucial, not only incremental, effect, i.e. decrease in interference of an impurity with this target analysis, and a significant improvement in the separability by increasing the CE dimensionality can be clearly seen comparing the final CZE traces of the 2D and 3D CE systems in Figs. 3 and 5, respectively.

The experiments showing several biological replicates (i.e. different urine samples, obtained from 20 male and female volunteers of different age) were carried out in order to demonstrate the effectivity of the 2D (ITP1+ITP2) sample pretreatment depending on the variability of the multicomponent ionic matrices. Three representative profiles are shown in Fig. 6. The experiments revealed/confirmed the crucial role of the 3D CE method in the highly reproducible producing of the sample fragments that: (i) contained only limited number of well separated peaks of similar intensities (a risk of CZE capillary overloading/peak overlapping is minimized) and (ii) were free of any compounds interfering with the analyte and preventing its further analysis. It means, although various biological samples contain different impurities, no additional significant interfering compound (in the migration position of the analyte) arose in the double on-line pretreated sample fragment, and the separation pattern of this fragment (including preserved resolution of the analyte) was maintained regardless of variability of the original samples. These findings demonstrated the true separation/sample preparation potential of the 3D CE system and outlined its role among the multidimensional techniques in the advanced separation field.

Besides enhanced separation capabilities of the proposed 3D CE method there are also other aspects for its preference in practical use. In this special case the improved separability was achieved by mere additional acid-basic equilibrium so that no selector was necessary. Such selector-free CE systems can be advantageous for their hyphenations with various powerful detectors (e.g. mass spectrometers). Benefits of the hydrodynamically closed CE systems with enhanced sample loadability, such as concentration LOD or precision, were not negatively influenced by the increased dimensionality. The concentration

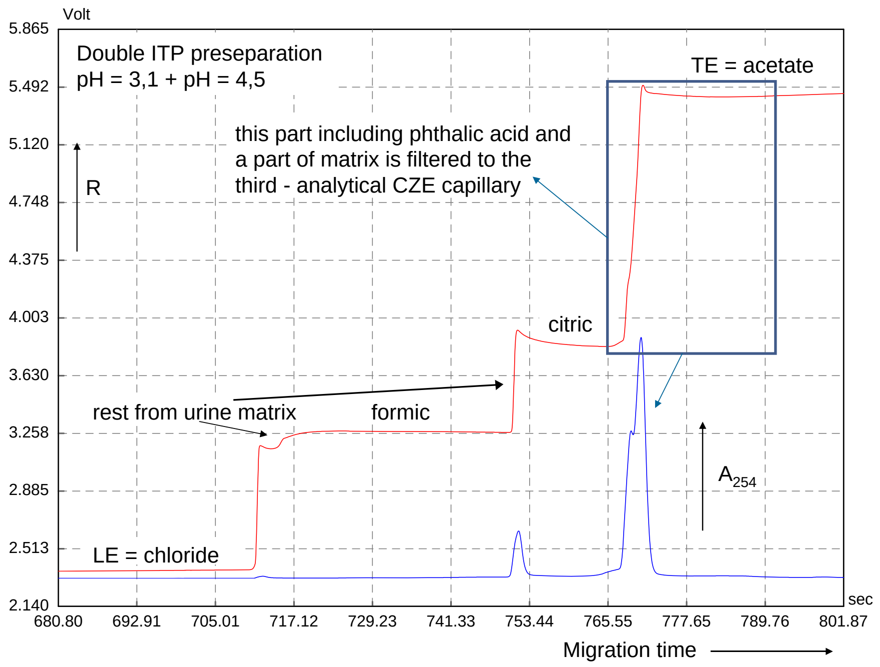

Fig. 4. Analytical ITP stage of the ITP–ITP–CZE method. Isotachopherogram from the fine separation of phthalic acid in the on-line ITP pretreated urine sample (as in Fig. 2). In this stage, boundaries for the zone of phthalic acid were changed to citrate and acetate. The marked part of the sample only was transferred and separated in the third capillary. Electrolyte system "ITP2" given in Table 1 was used for the separation. For other working conditions see Fig. 2 and Section 2.

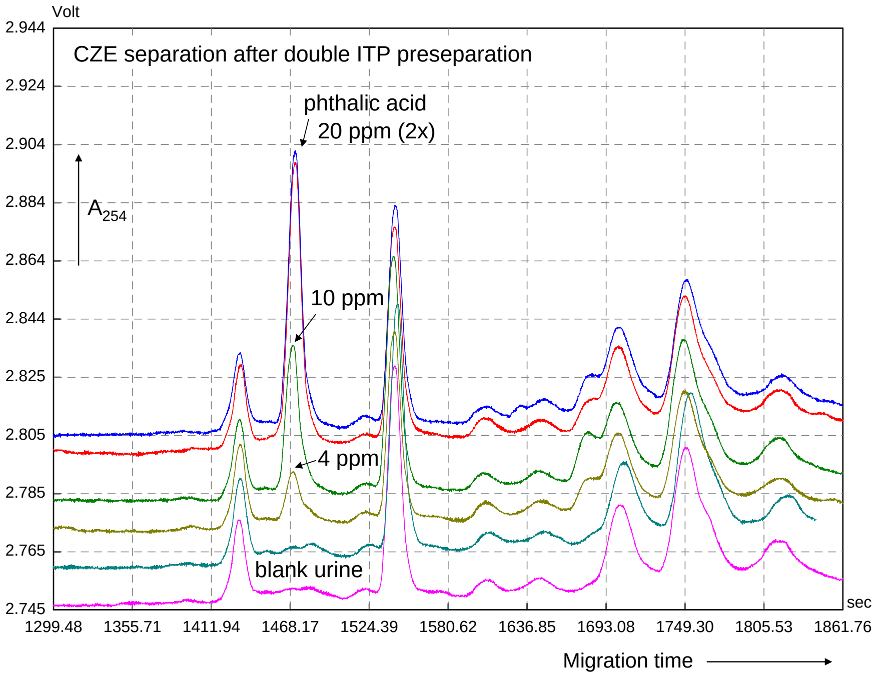

Fig. 5. Analytical CZE stage of the ITP–ITP–CZE method. Electropherograms from the final separation of phthalic acid in the on-line ITP pretreated/preseparated urine sample (see the isotachopherogram in Figs. 2 and 4). The electrophoretic profiles represent a urine blank and its corresponding spiked traces. Unmarked peaks in the CZE profiles represent the unidentified urine matrix constituents transferred into the CZE stage after the double ITP sample pretreatment. Electrolyte system "CZE" given in Table 1 was used for the separation. For other working conditions see Fig. 2 and Section 2.

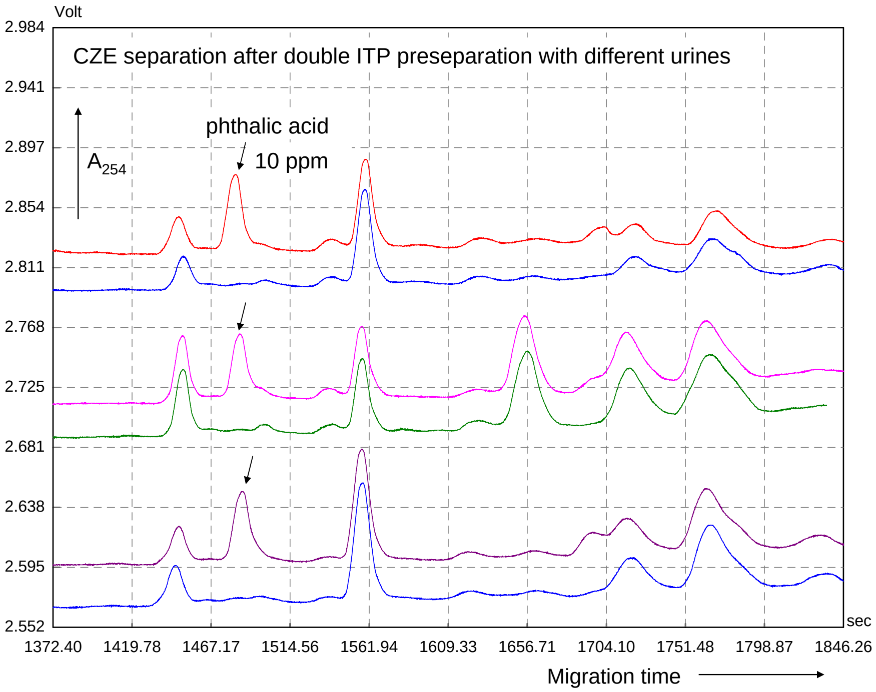

Fig. 6. Analytical CZE stage of the ITP–ITP–CZE method (the method as in Fig. 5). Three different profiles (blank and spiked trace for each one) represent three different human urine samples obtained from three different volunteers. Unmarked peaks in the CZE profiles represent the unidentified urine matrix constituents transferred into the CZE stage after the double ITP sample pretreatment. Electrolyte system "CZE" given in Table 1 was used for the separation. For other working conditions see Fig. 2 and Section 2.

LOD ($S/N=3\sigma$) of phthalic acid in the reference sample (water) was 150 ppb while it was 400 ppb for the urine samples, repeatability of the peak areas of phthalic acid for the six consecutive runs was below 2%, and repeatability of the migration times of phthalic acid peaks for the six consecutive runs was below 1% using the ITP–ITP–CZE–UV method. Although the overall analysis time is a bit higher in the 3D system than in corresponding 2D system, this increase is not so dramatic from practical point of view, and, moreover, it is compensated by the full separation of the analyte (that is the crucial requirement). For example, in the proposed ITP1–ITP2–CZE method, where particular analysis times are ca. 8, 6 and 15 min, respectively, the increase of overall analysis time due to adding the second ITP step is only ca. 20%. The second ITP step is generally shorter than the first one where the abundant major matrix constituents are migrating and removing, therefore, the tandem ITP arrangement can be advantageous for the sample pretreatment also in practical applications (reasonable analysis time plus more effective sample pretreatment).

4. Conclusion

It can be concluded from the results obtained in the present work that the 3D CE method in comparison with 2D CE enables (i) finer (better defined) selection of the zone of interest for its transfer into the analytical CE stage, i.e. more effective sample clean-up, (ii) considerable enhancement of the separation selectivity reflecting in changes in the migration order being prerequisite for the consecutive analytical CE separation and detection and (iii) enhanced flexibility and variability of combination of the electrophoretic stages spreading the application range.

Although no single ITP was sufficient for the effective sample pretreatment, the mutual combination of these two ITP steps do it. The proposed ITP tandem was based on the different migration pattern of two spacers–analyte–matrix constituents under different acid-base conditions (*pH* 3.1 vs. *pH* 4.5 in ITP1 and ITP2, respectively) that effectively eliminated the matrix constituents in the sample fragment transferred into the next (CZE) separation stage. Such ITP tandem can be, generally, very effective tool for acidic compounds present in multicomponent ionic matrices. Of course, the type of spacers and *pH* values must be carefully selected and tested for a given analyte and matrix.

These benefits of the proposed 3D method are accompanied by other ones, typical for the hydrodynamically closed CE systems with enhanced sample loadability employing capillary-coupling instrumental arrangement as published in related works previously. Hence, the ITP–ITP–CZE approach is proposed as an advanced alternative to the ITP–CZE one enabling to solve very complex tasks in pharmaceutical and biomedical analysis regarding (i) complexity of the matrices, (ii) concentration levels of the analyte(s) and (iii) hyphenations with detection systems. Understandably, the increased dimensionality of CE reflects in increased demands on skills in electrophoresis theory in order to combine different buffer systems/CE stages appropriately, i.e. to reach expected effect(s) with required compatibility of the systems.

Despite of some increased demands of the 3D CE system (selection of compatible electrolytes, higher electrophoretic run time, skills of the analysts), it has several very important benefits also when comparing to the hybrid multidimensional separation systems such as HPLC–CE, SPE/LPE–CE, Microdial–CE, etc. Ones of the most important are (i) relatively cheap and simple instrumentation/configuration, and (ii) naturally good compatibility of the particular dimensions (as all of them are based on the same, i.e. electrophoretic, principles). Hence, the 3D CE systems have a great potential for their miniaturization (microfluidic devices), commercial use, and advanced applications. It clearly highlights the importance of further research and development of the 3D CE systems and continued study of their potentialities.

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