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Column Coupling Electrophoresis in Biomedical Analysis

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1. Introduction

Biomedical analysis is one of the most advanced areas solved in analytical chemistry due to the requirements on the analyzed samples (analyte vs. matrix problems) as well as on the overall analytical process regarding automatization and miniaturization of the analyses. Separation methods for the biomedical analysis are requested to provide high resolution power, high separation efficiency and high sensitivity. This is connected with such conditions that analytes are present in the samples in very low (trace) amounts and/or are present in multicomponent matrices (serum, plasma, urine, etc.). These complex matrices consist from inorganic and organic constituents at (very) differing concentrations and these can overlap the analyte(s) peak(s) due to migration and detection interferences. In addition, a column overloading can occur in such cases. It can be pronounced especially for the microscale separation methods such as the capillary electrophoresis (CE). Hence, it is obvious that there is the need for the sample preparation: (i) preconcentration – lower limits of detection and quantification; (ii) purification of the sample and isolation of analytes – elimination of sample matrix; (iii) derivatization – improvement of physical and/or chemical properties of the analytes, before the CE analysis in these situations to reach relevant analytical information.

Sample pretreatment can be performed either off-line (before injection of analyzed sample into the analyzer) or on-line (after the injection). The conventional separation systems (single column) use mostly external (off-line) sample pretreatment, even though this analytical approach has many limitations. These are (i) a loss of the analytes, (ii) time consuming and tedious procedure, (iii) problematic manipulation with minute amounts of the samples, (iv) problematic for automatization, (v) decreased precision of the analyses, etc. On the other hand, on-line sample pretreatment has many advantages as (i) elimination of random and/or systematic errors caused by external sample handling, (ii) simplification of an overall analytical process (less number of an external steps), (iii) reduction of the total analysis time and (iv) possibility of the automatization and miniaturization of the analytical process (routine precise microanalyses). A significant enhancement of sensitivity and selectivity is one of the main benefits of the on-line sample pretreatment. An on-line pretreatment is crucial when there are only micro amounts of the samples for the analysis and/or when analytes/samples have lower stability.

The advanced single column electrophoretic techniques (transient isotachophoresis, field-enhanced sample stacking, dynamic pH junction, sweeping, in-capillary solid/liquid phase extraction-CE, in-capillary dialysis-CE, etc.), representing the CE with the on-line (in-column) sample preparation, were described and successfully applied for trace analytes and...
less or more complex matrices in many cases (section 2). The aim of this chapter is to
demonstrate potentialities and practical applications of a column coupling electrophoresis
as another group of the on-line sample preparation analytical approaches (section 3)
enabling powerful combination of (i) electrophoretic techniques (ITP, CZE, IEF, CEC)
(sections 3.1.1 and 3.2.1), (ii) electrophoretic and non electrophoretic (liquid
chromatography, flow injection analysis, etc.) techniques (sections 3.1.2 and 3.2.2). In this
way, it should be possible to create the most complex, flexible and robust tool filling the
above mentioned requirements of the advanced analysis. Such tool and its modes are
described in this chapter with regard to the theory, basic schemes, potentialities, for the
capillary (section 3.1) as well as microchip (section 3.2) format. This theoretical description is
accompanied with the performance parameters achievable by the advanced methods
(section 4) and appropriate application examples in the field of the biomedical analysis
(section 5). For a better understanding of the benefits, limitations and application potential
of the column coupling electrophoretic methods the authors decided to enclose the short
initial section with a brief overview of advanced single column electrophoretic techniques
(section 2) that often take part also in the column coupling electrophoresis.

2. Advanced single column techniques

As it is known from the literature (Simpson et al., 2008; Bonato, 2003) CE has many
advantages (high separation efficiency, versatility, flexibility, use of aqueous separation
systems, low consumptions of electrolytes as well as minute amounts of samples). Beyond
all the advantages, conventional CE has also some drawbacks, which limit its application in
routine analytical laboratories. They include (i) relatively difficult optimization of conditions
of analytical measurements, (ii) worse reproducibility of measurements (especially when
hydrodynamically open separation systems are used where non selective flows,
hydrodynamic and electroosmotic are acting) than in liquid chromatography, (iii) low
sample load capacity and need for the external (off-line) sample preparation for the complex
matrices (measurement of trace analyte besides macroconstituent(s) can be difficult without
a sample pretreatment), and (iv) difficulties in applying several detection methods in
routine analyses (Trojanowicz, 2009).

Some of these limitations can be overcome using advanced single column techniques. They
provide (i) improved concentration LOD, (ii) automatization (external manipulation with
the sample and losses of the analyte are reduced, analytical procedure is less tedious and
overall analysis time can be shortened, labile analytes can be analysed easier) and (iii)
miniaturization of the analytical procedure (pretreating of minute amounts of the sample is
possible and effective), (iv) elimination of interfering compounds, according to the
mechanism employed. However, the sample load capacity of these techniques is still
insufficient (given by the dimensions of the CE capillaries). The advanced single column CE
techniques usually suffer from lower reproducibility of the analyses due to the complex
mechanisms of the separation which controlling can be difficult in practice. Moreover, the
capillaries with embedded non electrophoretic parts (membranes, columns, fibers, monolits)
are less versatile (Simpson et al., 2008).

2.1 Stacking electrophoretic pretreatment techniques

Stacking procedures are based on increasing analyte mass in its zone during the
electromigration process via electromigration effects, enhancing sensitivity in this way. In all
cases, the key requirement is that there is an electrophoretic component in the
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preconcentration mechanism and that the analytes concentrate on a boundary through a change in velocity. Then we can recognize (i) field-strength-induced changes in velocity (transient isotachophoresis (Beckers & Boček, 2000a), field-enhanced sample stacking (Kim & Terabe, 2003; Quirino & Terabe, 2000a), and (ii) chemically induced changes in velocity (dynamic pH junction (Britz-McKibbin & Chen, 2000), sweeping (Kitagawa et al., 2006; Quirino & Terabe, 1998, 1999; Quirino et al., 2000b)). In addition to these techniques, counter-flow gradient focusing (Shackman & Ross, 2007), electrocapture (Horáková et al., 2007), and many others can be considered as the techniques based on a combination of field-strength- and chemically induced changes in velocity offering new interesting possibilities in on-line sample preparation (mainly preconcentration).

Some of the stacking techniques (and their combinations) can provide besides (i) the preconcentration also other benefits such as (ii) an effective sample purification isolating solute (group of solutes) from undesired matrix constituents (Simpson et al., 2008) or they can be combined with (iii) chemical reaction of the analyte(s) (Ptolemy et al., 2005, 2006), simplifying overall analytical procedure in this way. The choice of on-line pretreatment method depends on the specific physical-chemical properties of the separated analytes (e.g. charge, ionization, polarity) and the sample matrices (mainly concentration). For example, an on-line desalting of a physiologic sample can be effectively accomplished by the electrokinetic removing of the fast migrating low molecular ions prior to the IEF focusing of the high molecular analytes (proteins) (Clarke et al., 1997).

2.2 Non electrophoretic pretreatment techniques
An on-line sample preparation can be carried out advantageously also combining the CE with a technique that is based on a non electrophoretic principles. Most of these approaches are based on (i) the chromatographic or extraction principles (separations based on chemical principles), but also other techniques, such as (ii) the membrane filtration, MF (separations based on physical principles), can be used. In this case, a non electrophoretic segment (e.g. extractor, membrane) is fixed directly to the CE capillary (in-line combination) (Petersson et al., 1999; Mikuš & Maráková, 2010).

In-line systems such as CEC/CZE (Thomas et al., 1999), SPE/CZE (Petersson et al., 1999) or MF/CZE (Barroso & de Jong, 1998) are attractive thanks to their low cost and easy construction. On the other hand, versatility of such systems is limited (in-capillary segment cannot be replaced). One of the main limitations of performing in-line sample preparation is that the entire sample must pass through the capillary, which can lead to fouling and/or even clogging of the separation capillary and significant decreasing of reproducibility of the analyses when particularly problematic samples (like biological ones) are used. It can be pronounced especially for the extraction techniques (created inserting a solid-phase column into capillary, where the whole analytical procedure is very complex and it includes conditioning, loading/sorption, washing, (labeling, if necessary), filling (by electrolyte), elution/desorption, separation and detection. In order to overcome these issues, on-line methods based on another way of coupling of two different techniques may be used as alternatives to the in-line systems.

3. Advanced column coupled techniques
Multidimensional chromatographic and capillary electrophoresis (CE) protocols provide powerful methods to accomplish ideal separations (Hanna et al., 2000; Křivánková & Boček, 2010).
Among them the most important ones are the integrated systems containing complementary dimensions, where different dimensions separate components on the basis of independent or orthogonal principles (Moore & Jorgenson, 1995; Lemmo & Jorgenson, 1993; Mohan & Lee, 2002). In such a multidimensional system, the peak capacity is the product of the peak capacities of each dimension (Guiochon et al., 1983). A key part in the instrumentation of the hyphenated techniques is an appropriate interface that enables to connect and disconnect two different stages (e.g. columns) reproducibly and flexibly according to the relevance and relation of the particular actions in the analytical process.

The column coupling arrangement, where two or more separation techniques are arranged into two or more separated stages, can be a very effective approach offering additional benefits to the advanced single column CE techniques and reducing some of their disadvantages. Nevertheless, the advanced mechanisms given in section 2 can also be adapted into the column coupling arrangement enhancing the effectivity and application potential of the resulting method. Two separate stages provide (i) sample preparation (preseparation, preconcentration, purification and derivatization) and (ii) analytical separation of on-line pretreated sample. The benefits of the column coupling configuration, additional to the advanced single column CE, involve (i) autonomic combination of various separation mechanisms that provide enhanced and well defined separation selectivity, and a possibility to replace easily one of the stages (ii) well defined and more effective elimination of the undesirable sample matrix components, (iii) significant enhancement of the sample load capacity (especially for the larger internal diameters of capillaries) resulting in the improved LOD, (iv) improved precision of the analyses due to well defined control of the separation mechanisms (Kaniansky et al., 1993; Kaniansky & Marák, 1990).

The most frequently used and the simplest column coupling configuration is the CE combined with another CE (CE-CE, CE-CE-CE) (Kaniansky & Marák, 1990). Hybrid column coupled techniques are based on the combination of a non electrophoretic technique with the CE, e.g. LC-CE (Pálmarsdóttir & Edholm, 1995), SPE-CE (Puig et al., 2007), dialysis-CE (Lada & Kennedy, 1997), FIA-CE (Mardones et al., 1999). They offer different separation mechanisms in comparison with the CE-CE, however, they have more demands on instrumentation. Additionally to the on-line combination of conventional column techniques (electrophoretic as well as non electrophoretic) the column coupling arrangement combining a conventional technique with an advanced one (section 2) is applicable too. These types of the column coupled techniques are discussed in detail and illustrated through the corresponding instrumental schemes for both the capillary (section 3.1) as well as microchip (section 3.2) format.

### 3.1 Capillary format

#### 3.1.1 Hyphenation of electrophoretic techniques

The hyphenation of two electrophoretic techniques in capillary format (see Fig. 1) can effectively and relatively easily (simple and direct interface) solve the problems of the sample preparation and final analysis (fine separation) in one run in well defined way, i.e. producing high reproducibility of analyses, in comparison to the single column sample preconcentration and purification approaches (section 2). Moreover, the CE performed in a hydrodynamically closed separation system (hydrodynamic flow is eliminated by semipermeable membranes at the ends of separation compartment) with suppressed electroosmotic flow (EOF), that is typically used in the CE-CE configuration, has the advantage of (i) the enhanced precision due to elimination of the non selective flows (hydrodynamic, electroosmotic), and (ii) enhanced...
sample load capacity (30 μL sample injection volume is typical) due to the large internal diameter of the preseparation capillary (800 μm I.D. is typical) (Kaniiansky & Marák, 1990; Kaniiansky et al., 1993). The commercially available CE-CE systems have a modular composition that provides a high flexibility in arranging particular modules in the separation unit. In this way, it is possible to create desirable CE-CE combinations such as (i) ITP-ITP, (ii) ITP-CZE, (iii) CZE-CZE, etc., capable to solve wide scale of the advanced analytical problems (see Fig. 1). Although such combinations require the sophisticated instrument and deep knowledge in the field of electrophoresis, the coupled CE methods are surely the most effective way how to take/multiply benefits of both CE techniques coupled in the column-coupling configuration of separation unit. The basic instrumental scheme of the column coupled CE-CE system shown in Fig. 1 is properly matching with hydrodynamically closed CE modes where effective electrophoretic mobility is the only driving force of the separated compounds. On the other hand, when additional supporting effects such as counterflow, electroosmotic flow etc. must be employed, appropriate modifications of the scheme in Fig. 1 are made. Such modified instrumental schemes are attached into the sections dealing with IEF or CEC coupled techniques (3.1.1.3 and 3.1.1.5) that are principally applicable only in hydrodynamically open CE mode (Mikuš et al., 2006; Danková et al., 2001; Busnel et al., 2006).

Fig. 1. CE-CE method in column coupling configuration of the separation units for the direct analysis of unpretreated complex matrices sample, basic instrumental scheme. On-line sample preparation: removing matrices X (ITP, CZE), preseparation (ITP, CZE) and/or preconcentration (ITP, stacking) and/or derivatization (with stacking) of analytes Y, Z in the first CE stage (column C1). Final separation: baseline separation of Y and Z in the second CE (ITP, CZE) stage (column C2). Reprinted from ref. (Tekeľ & Mikuš, 2005), with permission. C1 – preseparation column, C2 – analytical column, B – bifurcation block for coupling C1 and C2, D – positions of detectors.

### 3.1.1.1 ITP-CZE

Although all electrophoretic methods can be mutually on-line combined, the biggest attention was paid to the ITP-CZE coupling, introduced more than 20 years ago by
Kaniansky (Kaniansky & Marák, 1990). The analytical benefits of the ITP-CZE combination have been already well documented (Fanali et al., 2000; Danková et al., 2001; Kvasnička et al., 2001; Valcárcel et al., 2001; Bexheti et al., 2006; Beckers, 2000b; Krivánková et al., 1991; Krivánková & Thormann, 1993; Krivánková & Boček, 1997a).

An on-line combination of ITP with CZE appears to be promising for alleviating some of the following practical problems (Kaniansky & Marák, 1990):

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

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

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

A characteristic advantage of the ITP-CZE combination is a high selectivity/separability obtainable due to the CZE as the final analytical step. Hence, the ITP-CZE method can be easily modified with a great variety of selectors implemented with the highest advantage into the CZE stage enabling to separate also the most problematic analytes (structural analogs, isomers, enantiomers). The ITP-CZE methods with chiral as well as achiral CZE mode have been successfully applied in various real situations (Mikuš et al., 2006a, 2008a, 2008c; Danková et al., 2001; Marák et al., 2007; Kvasnička et al., 2001).

The most frequently used ITP-CZE system works in the hydrodynamically closed separation mode that is advantageous for the real analyses of multicomponent ionic mixtures because of the best premises for enhancing sample load capacity (enables using capillaries with very large I.D.). Such commercial system is applied with just one high-voltage power supply and three electrodes (one electrode shared by the two dimensions), see Fig. 1. The electric circuit involving upper and middle electrode (electric field No. 1) is applied in the ITP stage while upper and lower electrode (electric field No. 2) is applied in the CZE stage. For the separation ITP-CZE mechanism see chronological schemes in Fig.2. The focused zone in the first dimension (ITP) is driven to the interface (bifurcation point) by only electric field No. 1. The cut of the zone of interest in the ITP stage is based on the electronic controlling (comparison point) of the relative step height (\(R_{sh}\), a position of the analyte between the leading and terminating ion, it is the qualitative indicator depending on the effective mobility of the analyte) of the analyte, see Fig. 3. The conductivity sensor (upper D in Fig. 1, D-ITP in Fig.2) serves for the indication of the analyte zone. This is very advantageous because such indication is (i) universal and (ii) independent on other comigrating compounds (sample matrix constituents migrating in the ITP stage) and therefore independent on sample composition. The electric circuit is switched and electric field No. 2 (upper and lower electrode) is applied in an appropriate time (this time is set electronically depending on requirements of the composition of the transferred plug) after the indication of the analyte zone passing through the upper D. From this moment the all ITP zones are directed to the CZE stage for the final separation and detection. It is possible to carry out
one or more cuttings depending on the zones of interest and/or interfering matrix constituents present in the sample. The interface between the separation solutions in the ITP and CZE capillary is free (without any mechanical restraint) but mixing of the electrolytes is eliminated (with the exception of diffusion) by suppressing all non selective flows (hydrodynamic, electroosmotic) in the system. This is advantageous by an easy construction and elimination of dead volumes in the separation system (Olvecká et al., 2001; Kaniansky et al., 2003).

Fig. 2. ITP sample clean up for CZE with the closed separation system (without any supporting non selective flow). (a) Starting arrangement of the solutions in the capillaries; (b) ITP separation with the analyte (A) trapped into the boundary layer between the zones of front (M1) and rear (M2) spacers; (c) end of the run in the ITP capillary followed by an electrophoretic transfer of the analyte containing fraction to the CZE capillary (by switching the direction of the driving current); (d) removal of the sample constituents migrating behind the transferred fraction (by switching the direction of the driving current); (e) starting situation in the separation performed in the CZE capillary (the direction of the driving current was switched); (f) separation and detection of the transferred constituents in the CZE capillary. BF = bifurcation region; C1, C2 = the ITP and CZE separation capillaries, respectively; D-ITP, D-ZE = detection sensors in the ITP and CZE separation capillaries, respectively; TES = terminating electrolyte adapted to the composition of the sample (S); TITP = terminating electrolyte adapted to the composition of the leading electrolyte solution; A = analyte, i = direction of the driving current. Reprinted from ref. (Kaniansky et al., 2003), with permission.
The principle of this hyphenated technique consists from well-defined preconcentration (concentration LODs could be reduced by a factor of $10^3$ when compared to conventional single column CZE) and preseparation (up to 99% or even more interfering compounds can be isolated (Danková et al., 1999)) of trace analytes in the first, wider, capillary (isotachophoretic step) and subsequently a cut of important analytes accompanied with a segment of the matrix, leading or terminator enters the second, narrower, capillary for the final separation by CZE (Fig. 2, Fig.3). 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 a cut generously. The zone of this segment survives for a certain time during the CZE stage and this mean that ITP migration continues also in the second capillary for some time and it influences strongly the results of the analysis, especially the detection times of analytes used for identification of the analytes in CZE separations (Busnel et al., 2006; Gebauer et al., 2007; Mikuš et al., 2006a). From this is clear that it is important in an ITP-CZE combination to choose suitable electrolyte systems and find the optimum time to switch the current from the preseparation capillary to the separation capillary (Křivánková et al., 1995).

The ITP-CZE technique appeared to be very useful especially for the common universal detectors producing relatively low concentration LODs (UV-VIS photometric detector). It is because such method provides probably one of the most acceptable ratio simplicity-cost: universality-concentration LOD in comparison to other column coupling methods and detection systems. This suggestion is supported by many advanced applications of the ITP-CZE-UV method in the pharmaceutical and biomedical field (Marák et al., 2007; Mikuš et al., 2008a, 2008b, 2008c, 2009). Jumps in voltage (conductivity) between neighboring zones result in permanently sharp boundaries between zones (Fig. 3) that is extremely convenient for the conductivity detection in ITP. Although convenient to the
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detection of the ITP zones, conductivity detection technique has a limited applicability in the CZE separations (often measurements of small conductivity changes due to the zones on a relatively high conductivity background of the carrier electrolyte) (Olvecká et al., 2001; Kaniansky et al., 2000).

3.1.1.2 ITP-ITP
The ITP-ITP combination represents the simplest possibility how to combine CE techniques. For the general instrumental scheme valid also for ITP-ITP, see Fig. 1. In the ITP-ITP mode both preseparation (wider) and analytical (narrower) capillaries are filled with (i) the same leading electrolyte (one-dimensional ITP) or (ii) different electrolytes (two-dimensional ITP) (Flottmann et al., 2006; Bexheti et al., 2006; Mikuš et al., 2006b; Kubáček et al., 2006a, 2006b, 2007). The ITP separation in a concentration cascade, introduced into conventional CE by Boček et al. (Boček et al., 1978) enhances the detectabilities of the separated constituents from the response of the conductivity detection due to well-known links between the concentration of the leading electrolyte and the lengths (volumes) of the zones (Marák et al., 1990)
The first ITP stage of the ITP-ITP combination can apply all benefits as they are described for the ITP stage of the ITP-CZE combination in section 3.1.1.1. On the other hand, the ITP-ITP technique can take the highest advantage of the hyphenation with the MS detection (Tomáš et al., 2010). It is because of an intrinsic feature of ITP to produce pure analyte zones, i.e. those in which the analyte is accompanied only with counter ion, in the isotachophoretic steady state. In this way, the maximum response of the MS detector can be obtained for the analyte. Therefore, the ITP-ITP-MS hyphenation seems to be one of the most promising methods for the fully automatized biomedical analyses such as pharmacokinetic studies, metabolomics, etc. An economic aspect of the ITP-ITP-MS method in comparison with the HPLC-MS method for the ionic compounds is apparent.

3.1.1.3 ITP-CEC
Another approach in the column coupled electrophoresis is the use of ITP sample focusing to improve the detection limits for the analysis of charged compounds in capillary electrochromatography (CEC). Besides this, the on-line isotachophoretic stage can serve also for a loadability enhancement (due to a large inner diameter of the ITP capillary). Both of these effects are then responsible for a dramatic reduction of the sample concentration detection limits through simultaneous acting of (i) large volume injection and (ii) analyte stacking (Mazereeuw et al., 2000).
In the ITP-CEC combination (Fig. 4), the open ITP mode must be applied because of the demands of the second stage (CEC) that is based on the EOF action. A coupled-column set-up can be used, in which counterflow ITP focusing is performed, and the separation capillaries are connected via a T-junction. For the schematic representation of the ITP-CEC procedure see Fig. 5. From the application point of view, the first ITP stage is advantageous especially for the injection of large volumes (tens of microliters) of diluted samples. When a very large sample is introduced, however, the focusing time of the sample often exceeds the migration time to the outlet of the ITP capillary. By applying a hydrodynamic counterflow (applicable in the hydrodynamically open CE systems) the ITP focusing will continue while extending the migration towards the outlet of the ITP capillary. Although the hydrodynamically open CE systems have the advantage of application of the supporting flows (counterflow, electrosmotic), it must be realized that
the reproducibility of cutting and also overall analysis is generally lower than in the hydrodynamically closed CE systems due to the fluctuations of the non selective flows in the separation system.

Fig. 4. Schematic representation of the ITP-CEC set-up. Right scheme: Schematic representation of the ITP-CEC-UV set-up with a (P) programmable capillary injection system, (D) UV-VIS absorbance detector, (A) amperometer and (T) laboratory made polyethylene T-piece. Untreated fused-silica capillaries of 220 μm I.D. (1 and 2) and 75 μm (3) are used. Left scheme: Schematic representation of the entire ITP-CEC-MS set-up. The electrospray needle with the sheath flow contains the CEC column, which is directly connected with the electrospray. The spray is directed towards the inlet capillary of the interface on the SSQ 710 mass spectrometer (MS). HV is the electrospray power supply. Reprinted from ref. (Mazereeuw et al., 2000), with permission.

The first ITP stage of the ITP-CEC combination can apply all benefits as they are described generally for the ITP stage of ITP-CZE combination in section 3.1.1.1. In ITP-CEC, the ITP sample clean-up effect is extremely important for enhancing reproducibility of CEC especially when injecting complex biological samples. The CEC stage of the ITP-CEC technique can take a high advantage of the hyphenation with the UV-VIS or MS detection, for the schemes of the experimental setups see Fig. 4. It is pronounced in the situations when the selectors interfering with the detection must be used in the separation system in order to establish the required selectivity. Immobilization of such selectors in the CEC column prevents their entering into the detector cell resulting in the elimination of the detection interferences. In this way, the maximum response of the UV-VIS or MS detector can be obtained for the analyte. Hence, the ITP-CEC combination seems to be a powerful tool for the on-line selective separation, sensitive determination and spectral identification of chiral compounds and various other isomers and structurally related compounds (i.e. “problematic” analytes) present in complex ionic matrices. The ITP-CEC-MS hyphenation seems to be one of the most promising methods for the fully automatized biomedical chiral analyses such as enantioselective pharmacokinetic studies, metabolomics, etc. (Mazereeuw et al., 2000).
3.1.1.4 CZE-CZE

CE separation system with tandem-coupled columns, i.e. CZE-CZE makes possible, within certain limits, splitting a CZE run into a sequence of the separation and detection stages (for the general instrumental scheme valid also for CZE-CZE, see Fig. 1). Therefore, the carrier electrolyte employed in the first (separation) stage of the run could be optimized with respect to the resolution of an analyte from a complex (biological) matrix. In this way, a very significant “in-column” clean-up of the analytes from complex ionic matrices can be reached in the separation stage of the tandem by combining appropriate acid-basic (pH) and complexing (selectors) conditions. Due to this, the detection (e.g. spectral) data could be acquired in the detection stage of the tandem with almost no disturbances by matrix co-migrants (Danková et al., 2003).

The carrier electrolyte employed in the second (detection) stage could be chosen to reach favourable conditions in the acquisition of detection (e.g. spectral) data while maintaining the resolution of the analyte from matrix constituents as achieved in the separation stage.
Such two-dimensional systems reduce probability of component overlap and improve peak identification capabilities since the exact position of a compound in a two-dimensional electropherogram is dependent on two different separation mechanisms (Sahlin, 2007).

The CZE-CZE combination can be set to achieve a remarkable selectivity. On the other hand, it is considerably less sensitive than the ITP-CZE combination due to the absence of stacking capability of the basic CZE technique. It can be overcome, fortunately, replacing a basic CZE technique by an advanced one (e.g. stacking). The CZE-CZE technique is favorable for the hyphenation with various detection techniques (e.g. spectral, electrochemical) because it makes possible splitting of the CZE run into a sequence of the separation and detection stages (Danková et al., 2003).

3.1.1.5 IEF-CZE, -CGE

Arduous proteomics tasks require techniques with high throughput and high efficiency in order to screen a certain proteome expression and to monitor the effects of environmental conditions and time on the expression. There seldom is, at present, a single separation mode sufficient enough to deal with such complex samples. CE is a significant tool for the separation of proteins and peptides (Dolník & Hutterer, 2001). To finish complicated separation jobs, great efforts have been concentrated on the development of 2D CE (Yang et al., 2003b). IEF, CGE and CZE are the most effective electrophoretic techniques for zwitterionic compounds, therefore the on-line combination of these techniques is of the highest importance for the protein analysis with perspectives of their automatization and miniaturization (Kaniantsky et al., 2000; Chen X. et al., 2002; Kvasnička et al., 2001).

When performing isoelectric focusing, one can fill the total volume of a capillary with sample solution. It can be expected that the detection sensitivity of the hyphenated system benefits from the concentration effect of the first dimension of IEF. This feature holds advantage over other CE modes such as CZE, CGE, micellar electrokinetic capillary chromatography (MECC), and capillary electrochromatography (CEC). Practically, IEF has a power to concentrate analytes up to several hundred folds in a capillary (Shen et al., 2000). Such a condensed and shortened analyte plug in a capillary is appropriate for sample injection to other CE modes. Therefore IEF is a proper candidate for the first dimension in a multi-dimensional CE system. Apparently, this will improve the sensitivity for mass detection. It is advantageous over those systems in which IEF was utilized as the second dimension. Nevertheless, the sensitivity of UV absorbance suffers from the necessity of the CAs involved in IEF. Of course, isotachophoresis (ITP) as a pretreatment tool for CZE separation also has a concentration effect (Kaniantsky et al., 1999). ITP is carried out based on the mobility differences of ions and, IEF, based on different pIs of ampholytic molecules.

Capillary isoelectric focusing (IEF) and capillary zone electrophoresis (CZE) can be on-line hyphenated by a dialysis interface to achieve a 2D capillary electrophoresis (CE) system, i.e. IEF-CZE (Fig. 6), as it was demonstrated by Yang et al. (Yang et al., 2003b). The system was used with just one high-voltage power supply and three electrodes (one cathode shared by the two dimensions). The focused and preseparated (according to differences in the isoelectric points of the analytes) zones in the first dimension (i.e. the IEF) were driven to the dialysis interface by electroosmotic flow (EOF), besides chemical mobilization from the first anode to the shared cathode. Zero net charged analyte molecules focused in the first dimension are recharged in the interface (I_2 in Fig. 6) according to the pH of the altered buffer. The semi-permeable property of the interface ensures that macromolecules of...
ampholytic analytes remain in the separation channel. In the second dimension (i.e. the CZE), the preseparated zones were further separated (according to the ratios of charge and mass, i.e. electrophoretic mobility) and driven by an inverted EOF, which originated from the charged layer of a cationic surfactant adsorbed onto the inner wall of the capillary. It can be concluded that the 2D IEF-CZE system possesses higher resolving power than each of the single modes. This protocol of the 2D CE system endues the interface with durability and makes for convenient performance. To reduce the dead volume, it is necessary to match the inner diameter of the hollow fiber to that of the capillaries. The tangent surfaces of these units should be made even and smooth.

![Fig. 6. Construction of 2D IEF-CZE.](image)

A two-dimensional capillary isoelectric focusing–capillary gel electrophoresis (IEF-CGE) system is another modification of the technique based on on-line combination of IEF with zone electrophoresis (Yang et al., 2003a). It also can be accomplished just with one high-voltage power supply and three electrodes. Chemical mobilization can be utilized to drive the sample zones of the first dimension. To actualize 2D IEF-CGE performance, coated and gel-filled capillaries are needed to eliminate the undesired EOF. In a gel-filled capillary the emergence of bubbles is tedious. From this point of view, it is valuable to exploit a more convenient and robust 2D CE system such as IEF-CZE (as illustrated above).

### 3.1.2 Hyphenation of electrophoretic and non electrophoretic techniques

Lately there were introduced into CE several hybrid on-line sample preparation techniques that are still in development as there is a big effort (i) to simplify usually a very complex
instrumental arrangement and simultaneously (ii) to ensure the enhancement of the compatibility within and reproducibility of the procedure. The column coupled non electrophoretic stages include (i) chromatography (Pálmarsdóttir & Edholm, 1995; Pálmarsdóttir et al., 1996, 1997), (ii) SPE extraction (Puig et al., 2007), (iii) dialysis (Lada & Kennedy, 1997), and (vi) flow injection analysis (FIA) (Mardones et al., 1999). A great potential of the hybrid on-line sample preparation techniques is given by their complementarity that enables to cumulate positive effects and/or overcome the weak points of the individual sample preparation techniques. In addition, these techniques, likewise to CE-CE, can be simultaneously combined also with stacking effects or chemical reaction in order to enhance further overall analytical effect as it is demonstrated in the following sections. From the practical point of view, the following sections are starting with the on-line implementation of FIA because the flow injection principles and instrumental procedures/arrangements are widely applied also for the effective integration of other non electrophoretic techniques (SPE, LC, dialysis) with CE.

3.1.2.1 FI-CE

The concept of flow injection analysis (FIA) was introduced in the mid-seventies. It was preceded by the success of segmented flow analysis, mainly in clinical and environmental analysis. This advance, as well as the development of continuous monitors for process control and environmental monitors, ensured the success of the FIA methodology (Trojanowicz et al., 2009; Lü et al., 2009). A combination of CE with a flow injection (FI) offers a great scale of sample preparation and the most frequently it is used for the on-line implementation of chemical reactions. The technique of combined flow injection CE (FI-CE) integrates the essential favorable merits of FI and CE. It utilizes the various excellent on-line sample pretreatments and preconcentration (such as cloud point extraction, SPE, ionexchange, DPJ and head-column FESS technique, analyte derivatization) of FI, which has the advantages of high speed, accuracy, precision and avoiding manual handling of sample and reagents. Therefore, the coupling of FI-CE is an attractive technique; it can significantly expand the application of CE and has achieved many publications since its first appearance (Mikuš & Maráková, 2010).

Fig. 7. Typical FI manifold used for the derivatization of the analytes and their on-line introduction into the CE system. Reprinted from ref. (Mardones et al., 1999), with permission.

A high potential of the FI-CE method in automatization of sample derivatization and subsequent separation was demonstrated by Mardones et al. (Mardones et al., 1999). The
derivation reaction for carnitine as the model analyte was carried out on a FI system coupled with the CE equipment via a programmable arm (Valcárcel et al., 1998). The arrangement is shown in Fig. 7. The derivation reaction (FMOC-Cl) is introduced directly into the loop of the injection valve (IV) when load position is selected, while the sample is introduced into the system and it is mixed with the buffer (carbonate). Then, valve is switched to the injection position allowing the mixing of sample-buffer and reagent solution. In this position the flow is stopped for a defined time in the reactor loop (390 cm), which is introduced into the thermostatic bath (50°C). Finally, the reaction mixture is introduced via the mechanic arm into the CE system.

The third generation of flow-injection (laboratory-on valve, lab-on-valve or LOV) allows scaling-down sample and reagent volumes to the 10–20 μL range, while waste production is typically 0.1–0.2 mL per assay (Solich et al., 2004). These facts make LOV an ideal tool for on-line coupling with CE systems (Kulka et al., 2006).

3.1.2.2 SPE-CE

The new trends in the coupling between SPE–CE are focused on several strategies, one of which involves developing new materials to increase the retention and selectivity of some analytes. In this sense the increasing use of materials such as immunoaffinity sorbents has been shown to overcome the problem of selectivity especially when complex samples are analysed. The use of molecular imprinted polymers (MIP) could be also an attractive alternative and further development is expected in this area in the near future. Carbon nanostructures also seem to be very promising materials which are in the first stages of development and so more research is expected in this field (Puig et al., 2007).

Extraction techniques now play a major role for sample preparation in CE. These techniques can be used not only for reconstitution of the sample from small volumes but also for sample purification in complex matrices and desalting for very saline samples that would interfere with the electrophoretic process (e.g. FESS requires low conductivity sample). Considerable progress has been made towards the coupling of solid phase extraction (SPE) with a subsequent electrophoresis while coupling of liquid phase extraction (LLE) with electrophoresis is less used. Before coupling the SPE and CE, the appropriate SPE conditions for trapping and eluting the test compounds must be investigated. The breakthrough

Fig. 8. Schematic diagram of the three types of interfaces for on-line SPE–CE coupling: (a) vial interface; (b) valve interface; (c) T-split interface. Reproduced with permission from (a) Stroink et al. (Stroink et al., 2003), (b) Tempels et al. (Tempels et al, 2007) and (c) Puig et al. (Puig et al., 2007).

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volumes, desorption efficiency and desorption volume must be studied too. Typical approaches of the on-line coupling of SPE with CE, advantageous by a high flexibility and variability of extraction volumes, are based on the use of a vial, valve or T-split interfaces. Schematic diagram of these types of interfaces for on-line SPE-CE coupling are shown in Fig.8.

An on-line SPE-CE approach based on a Tee-split interface was demonstrated by Puig et al. (Puig et al., 2007). The Tee-split interface is required for the on-line coupling of SPE-CE and to allow an injection volume that is suitable for CE analysis because the SPE elution volume is considerably larger than the maximum volume that can be injected into the CE capillary. Using this interface, a part of the SPE elution plug is injected while the rest of the sample is flushed to waste. Depending on the matrix, however, the sample must be appropriately pretreated prior to the injection into the first stage (i.e. SPE). As plasma is a relatively complex sample, the introduction of a pretreatment step (protein precipitation) prior to injection was necessary to prevent clogging of the SPE column.

For various specific purposes where chemical reaction and preconcentration must be involved simultaneously (e.g. in case of peptide mapping), the on-line coupling of microreactor (with an immobilized-enzyme), SPE preconcentrator and CE can be applied (Bonneil & Waldron, 1999). The problems related to the preconcentrator, such as reversal of EOF at low pH, can be eliminated by designing the on-line system in such a way that the preconcentrator is not part of the separation capillary, unlike most configurations reported in the SPE-CE literature. Consequently, the preconcentrator should not interfere with the separation process. Benefits of the on-line microreactor-SPE-CE system include (i) sensitivity (several hundred-fold preconcentration factor can be achieved) for the analyte products isolated in very small quantities from complex (biological) samples, (ii) avoiding conventional experimental steps that are quite long, labor intensive and require a lot of sample handling. Such system can be reused for several samples with acceptable reproducibility and relatively short analysis time. On the other hand, a loss of separation efficiency can be observed that is induced by the multiple-valve design of the system and dispersion of the desorption plug.

Another way of the integration of chemical reaction to the SPE-CE is the lab-on-valve (LOV) interface. The automatic minicolumn SPE preconcentration in LOV module coupled on-line with the CE equipment was proposed for the separation and quantification of mixtures of target analytes in very diluted samples (Jiménez & de Castro, 2008). This method can be applied with or without an on-line analyte derivatization depending on requirements. So that the complex derivatization-SPE-CE method integrates several different working principles such as (i) flow injection with chemical reaction, (ii) preseparation and preconcentration with non electrophoretic (extraction) principles, (iii) final separation with electrophoretic principles and detection of the separated zones. The usefulness of the LOV interface for the on-line coupling with a CE instrument interfaced by the appropriate manifold was reflected in excellent concentration LODs and linear dynamic ranges obtained.

Solid-phase microextraction (SPME) is interesting and alternative technique because it is simple, can be used to extract analytes from very small samples and provides a rapid extraction and transfer to the analytical instrument. Moreover, it can be easily combined with other extraction and/or analytical procedures, improving to a large extent the sensitivity and selectivity of the whole method (Lord & Pawliszyn, 2000; Ouyang & Pawliszyn, 2006; Saito & Jinno, 2003; Fang et al., 2006a, 2006b). Even though SPME is becoming an attractive alternative to using SPE, its use in combination with CE is still rather
limited. Such coupling has not been widely used because of its inherent drawbacks regarding the low injection volumes typically required in CE (which are crucial to obtaining good separation efficiency) and also because the different sizes of the separation capillaries usually used for CE and the SPME fibers (Liu & Pawliszyn, 2006). Moreover, SPME suffers from limited choice of selectivity in comparison with SPE since only few stationary phases are available (Puig et al., 2007).

3.1.2.3 LC-CE

When biological samples have to be analyzed, additional sample pretreatment prior to the SPE step may be needed to remove compounds that jeopardize an effective analyte concentration (or even block the SPE column) and the subsequent CE analysis. Sample pretreatment prior to SPE can be achieved by carrying out a preceding separation. Generally, sample analysis with on-line multidimensional separation systems can be performed using a comprehensive or a heart-cut approach. The comprehensive approach results in the analysis of the complete sample in all subsequent dimensions, whereas the heart-cut approach analyzes only a small part of the pre-separated sample in the second separation step. The comprehensive approach demands a slow preceding separation compared to the subsequent separation in order to accomplish analysis of the complete sample in all dimensions. Typical examples of such comprehensive systems are the on-line size exclusion chromatography (SEC)-CE systems and reversed phase LC–CE systems developed in the group of Jorgenson (Bushey & Jorgenson, 1990; Lemmo & Jorgenson, 1993; Moore Jr. & Jorgenson, 1995; Hooker & Jorgenson, 1997), which are coupled by various interfaces. These systems do not concentrate the chromatographic fractions prior to introduction into the CE system, which reduces the sensitivities of the total systems. Efforts to integrate such a focusing step would imply the need for an even slower preceding separation step to create time for sample trapping in a SPE column, washing and desorption of the concentrated fraction and sample introduction into the CE system. In practice, a comprehensive multidimensional system with a focusing step seems almost impossible, unless a number of columns are integrated into the system in a parallel fashion to enable “parking” of the LC fractions. In that case, the LC fractions are stored in the focusing step on various SPE columns and can be sent to the CE system at any convenient moment. The heart-cut approach is less demanding and is best suitable for target analysis. In the case of a heart-cut approach for an on-line system, it is also easier to integrate a concentration step between the preceding and the final separation step because there are no time constraints. Stroink et al. (Stroink et al., 2003) coupled SEC-SPE with CE through a vial-type interface for the quantitative analysis of enkephalins in cerebrospinal fluid (CSF). The SEC dimension separated the sample in a protein and a peptide-containing fraction. This resulted in a relatively large volume of the peptide fraction (about 200μL), requiring a subsequent SPE step prior to CE analysis to obtain acceptable LODs. Tempels et al. (Tempels et al., 2006) developed an on-line SEC-SPE-CE system with a Tee-split interface (Fig. 9) for the isolation, concentration and separation of peptides or other lower molecules in biological fluids (such as CSF). The SEC dimension served for the fractionation of the sample so that a fraction having required molecular weight could be easily selected (here, proteins were discarded). The small SPE column provided effective sample preconcentration using small desorption volumes (425 nL). The Tee-split interface enabled on-line injection of the concentrated analytes into the CE system without disturbing separation efficiency.
Fig. 9. Schematic diagram of the on-line SEC–SPE–CE system with the Tee-split interface. The on-line SEC–SPE–CE system was built in three distinct parts: a SEC, a SPE and a CE part. The SEC part consisted of a pump (pump 1), a valve (valve 1) for introduction of sample, a SEC column, and a UV detector (detector 1). The SPE part comprised a pump (pump 2), a micro valve (valve 3) for introduction of acetonitrile, and a SPE column. Valve 2 functioned as a selection valve to direct a fraction of solvent A towards the SPE column or to detector 1. The CE part of the complete system is framed. Lengths of capillaries are shown in italics (cm). The CE part consisted of a CE system with a build-in photodiode array detector. The CE and SPE parts were connected by a micro Tee with a void volume of 29 nL. The SEC part was filled with solvent A, whereas in the SPE and CE parts BGE was used. Reprinted from ref. (Tempels et al., 2006), with permission.

Although LC-CE coupling is technically much more difficult than CE-CE, because it has to be accompanied by collection, evaporation and reconstitution of fraction isolated by LC, some of these actions can be eliminated implementing an advanced CE stage (with a concentration capability) into LC-CE. Micro-column liquid chromatography (MLC) can be used on-line with an advanced (stacking) CE for sample purification and concentration allowing injection of microliter volumes into the electrophoresis capillary (Bushey & Jorgenson, 1990; Pálmarsdóttir & Edholm, 1995). By using the double stacking procedure with assistance of the backpressure almost complete filling of the electrophoresis capillary is possible without significant loss of CZE separation performance. The combined system has a much greater resolving power and peak capacity than either of the two systems used independently of each other.

3.1.2.4 Dialysis-CE

Microdialysis is a widely accepted sampling and infusion technique frequently used to sample small molecules from complex, often biological, matrices (Adell & Artigas, 1998; Chaurasia, 1999). In the microdialysis, small molecules are able to diffuse across the dialysis membrane into the probe, while large molecules, such as proteins and cell fragments, are excluded. This is the sample cleanup provided by the microdialysis. On-line microdialysis-CE assays for neurotransmitters to date have been most successful for easily resolved analytes such as glutamate and aspartate (Thompson et al., 1999; Lada et al.,
However, the efficiency and peak capacity of high-speed CE separations are often not high enough to resolve complex mixtures. Recently, improvements in injection technique and detection limits have improved separation efficiency (Bowser & Kennedy, 2001). In the microdialysis, the minimum volume required for analysis often determines the rate at which the dialysate can be sampled. On-line microdialysis-derivatization-CE-LIF assays as proposed by Lada et al. (Lada et al., 1997) (for the instrumental scheme see Fig. 10) eliminate fraction collection. The separation capillary was coupled to the reactor capillary via a flow-gated interface which allowed dialysate samples to be automatically injected onto the separation capillary. This elimination of fraction collection, combined with the high mass sensitivity of LIF or electrochemical detectors, makes sampling rates on the order of seconds possible (Thompson et al., 1999; Lada et al., 1997, 1998). The microdialysis-CZE-LIF system with on-line derivatization has the advantage of simultaneously obtained high relative recoveries and good temporal resolution with (in-vivo) microdialysis sampling for the real biological system (brain) (Lada et al., 1997).

Fig. 10. Diagram of the microdialysis:CZE-LIF system with on-line derivatization. Reprinted from ref. (Lada et al., 1997), with permission.

3.2 Microchip format

Developments in the fields of microfluidics and microfabrication during the last 15 years have given rise to microchips with broad ranges of functionality and versatility in the areas of bioanalysis such as clinical applications (Li & Kricka, 2006) and chiral separations (Belder, 2006). Microfluidic devices such as microchips can provide several additional advantages over electromigration techniques performed in capillary format. The heat dissipation is much better in chip format compared with that in a capillary and therefore higher electric fields can be applied across channels of microchip. This fact enables, along with a considerably reduced length of channels, significant shortening of separation time (millisecond analysis time is possible to achieve, see e.g. (Belder, 2006)). Sample and reagent consumption is markedly reduced in microchannels. Hence, microchip capillary electrophoresis (MCE) can provide a unique possibility of ultraspeed separations of microscale sample amounts. Applicable are both electrophoretic (Gong & Hauser, 2006; Belder, 2006) as well as electrochromatographic modes (Weng et al., 2006).

In practice, however, the resolution achievable in MCE devices is often lower compared to that obtainable in classical CE utilizing considerably longer separation capillaries. In order
to obtain sufficient resolution in MCE, different strategies have been used (Belder, 2006), such as (i) enhancing the selectivity of the system as much as possible (changing type and amount of selector, adding coselector, etc.), (ii) using of folded separation channels, the column length can be extended without enlarging the compact footprint of the device, (iii) using coated channels, internal coatings improve separation performance by the suppression of both analyte wall interaction and electroosmosis. A use/combination of above mentioned tools applicable in MCE gives a better chance for real-time process control and for multidimensional separations and makes the MCE to be powerful tool in real applications (pharmaceutical, biomedical, etc.). Sample pretreatment has been recognized as another significant barrier to higher levels of integration. Other accompanied problems in real applications of basic MCE are as follows: The detection volume of microfluidic devices due to the channel dimension and the sampling amounts is rather small, which would impact the detectable concentration. Fabricating a microchip with a large detection volume can be easily performed, but the separation efficiency is usually insufficient (Hempel, 2000). Another way is to inject a long sample band and then stack it into a narrow zone using on-line preconcentration techniques prior to separation (Chien, 2003). In such case, not only the preconcentration but also sample clean-up can be simultaneously carried out. Therefore, further considerable enhancement of analytical capabilities can be achieved in the MCE format using advanced single or multiple channel configurations. Practically all of the advanced principles, effects and techniques described in previous sections (2 and 3.1) are applicable also in microchip format. The most effective advanced MCE approaches are briefly presented in this section.

3.2.1 Hyphenation of electrophoretic techniques

The column coupling (CC) configuration of the separation system is more compatible with microfluidic devices than capillary electrophoresis (Bergmann et al., 1996), since the manufacturing process is the same for simple and coupled channel chips (Huang et al., 2005). The on-line coupling of sample pretreatment systems to MCE have a great interest because it allows the automatization of the analytical process (from sample preparation to data treatment), which is a current trend in analytical chemistry (Ma et al., 2006; Cho et al., 2004).

When we consider the sample amounts currently handled in conjunction with the separations on MCE it is clear that direct couplings of the sample pretreatment procedures to the separation stages of the analysis are almost inevitable (Kaniansky et al., 2003). For the above mentioned purposes the electrophoretic pretreatment methods are mostly used and it is important that they provide, mainly: (i) different separation mechanism in the pretreatment and separation stages of the analysis; (ii) an electrophoretically driven removal of the matrix constituents from the separation system on the pretreatment (to desalt the sample and reduce the number of the sample constituents); (iii) processing an adequate amount of the sample (to make the analyte detectable in the separation stage of the analysis); (iv) a nondispersive transfer of the analyte after the pre-treatment to the separation stage.

3.2.1.1 ITP-ZE

ITP-ZE (ZE, zone electrophoresis) performed on microchip is the most frequently used configuration similarly to the ITP-CZE in capillary format. It is because of the robustness and application potential of the microchip ITP-ZE. ITP and ZE, as the basic electrophoretic methods, differ in the sample loadabilities, spatial configurations of the separated constituents, concentrating effects, and in part in applicabilities for particular categories of
the analytes they make tools that can be effectively on-line combined on the column coupling chip in two general ways (Kaniiansky et al., 2000; Wainright et al., 2002; Bodor et al., 2002) (i) ITP, concentrating the sample constituents into a narrow pulse is intended, mainly, as a sample injection technique for ZE; (ii) ITP, while concentrating the analyte and some of the matrix constituents into a narrow pulse, serves mainly as a sample clean-up technique and removes a major part of the sample matrix from the separation system before the final ZE separation. For the separation mechanism of ITP-ZE in microchip format see Fig. 2, that is principally the same for the capillary and microchip format. MCE provided with the column-coupling (CC) configuration of the separation channels for the ITP-ZE separations is illustrated in Fig. 11. Different volumes of the sample channels (S1, S2) serve for a low or large volume injection depending on analyte and matrix concentration. At this scheme, the contact conductivity detector is used, nevertheless, other common detectors such as UV-VIS absorbance photometric detector, and especially LIF detector can be successfully applied, see e.g. (Belder, 2006).

Fig. 11. MCE provided with the column-coupling (CC) configuration of the separation channels. CC poly(methylmethacrylate) chip provided with the conductivity detection cells. Details: C3 = terminating electrolyte channel; S1 and S2 = 9000 and 950 nL sample injection channels, respectively; W = an outlet hole from the chip channels to a waste container; C1 = first separation channel (3050 nL volume; 76x0.2x0.2 mm (length, width, depth)) with a platinum conductivity sensor (D1); C2 = second separation channel (1680 nL volume; 42x0.2x0.2 mm) with a platinum conductivity sensor (D2). Reprinted from ref. (Kaniiansky et al., 2003), with permission.

3.2.1.2 ITP-GE

ITP-GE is proposed for the special category of separations where high molecular compounds are separated from each other in presence or absence of matrix constituents (Huang et al., 2005). A microchip for integrated ITP preconcentration with GE separation enables to decrease the detectable concentration of biopolymers such as sodium dodecyl sulfate (SDS)-proteins. Each channel of the chip is advantageously designed with a long sample injection channel to increase the sample loading and allow stacking the sample into a narrow zone using discontinuous ITP buffers. The preconcentrated sample is separated in GE mode in sieving polymer solutions. All the analysis steps including injection, preconcentration, and separation of the ITP-GE process are performed continuously, controlled by a high-voltage power source with sequential voltage switching between the analysis steps. Without deteriorating the peak resolution, the integrated ITP-GE system can result in a decreased detectable concentration of tens-fold compared to the GE mode only. The picture of the ITP-GE microchip and the protocol of the ITP-GE procedure on the microfluidic device are illustrated in Fig. 12.
Fig. 12. (a) Glass microchip developed for ITP-GE separation, consisting of three separation elements; (b) protocol of the ITP-GE procedure on the microfluidic device. S: sample; SW: sample waste; B: background electrolyte; L: leading electrolyte; T: terminating electrolyte. 1) B and T loading; 2) S and L injection—S at ground, SW at high voltage; 3) stacking—T at ground, B (well 6) at high voltage; 4) separation—B (well 5) at ground, B (well 6) at high voltage. The electrodes not in use float. Channel lengths are expressed in mm. Reprinted from ref. (Huang et al., 2005), with permission.

3.2.1.3 ITP-ITP

Undoubtedly, the use of MCE can be extended advantageously to 2-D ITP separations (Ölkecká et al., 2001; Kaniansky et al., 2000). CE chip provided with the column-coupling (CC) configuration of the separation channels and corresponding scheme of the equipment for the ITP-ITP separations are the same as those ones for ITP-ZE illustrated in Fig.11. ITP-ITP with the tandem-coupled separation channels makes possible a complete resolution of various analytes, even the structurally related compounds (such as enantiomers). However, this can lead only to a moderate extension of the concentration range within which such analytes can be simultaneously quantified that is pronounced especially for the microfluidic devices such as MCE. The best results in this respect can be achieved by using a concentration cascade of the leading ions in the tandem coupled separation channels. Here, a high production rate, favored in the first separation channel, is followed by the ITP migration of the analytes in the second channel under the electrolyte conditions enhancing their detectabilities. This enables to separate structurally related analytes with their higher concentration ratios, and similarly, trace analyte besides higher concentration of matrix ions (Ölkecká et al., 2001).

3.2.1.4 ZE-ZE

In a ZE-ZE on-line combination, different separation mechanisms are implemented via appropriate compositions of the BGE solutions placed into the separation channels prior to the ZE run. Column switching provides means that significantly enhance resolving power attainable in the ZE separations performed on the CC chip. These, mainly include (i) on-
column sample purification of the multicomponent and/or high salinity samples and (ii) different separation mechanism applicable in the coupled channels (2D features). Undoubtedly, a very reproducible transfer process, a well defined and highly efficient removal of the matrix constituents from the separation compartment and the use of different separation mechanisms in the channels are features that makes column switching ZE on the CC chip a very promising tool for a miniaturized analysis of multicomponent samples. The ZE-ZE based MCE operating with a hydrodynamically closed separation system, makes a separation platform for highly reproducible migrations of separated constituents (Kaniansky et al., 2004; Sahlin, 2007; Hanna et al., 2000). The same instrumentation and channel arrangement as used for ITP-ZE, ITP-ITP or ITP-GE can be also applied for ZE-ZE (of course, with appropriate electrolyte systems).

3.2.2 Hyphenation of electrophoretic and non electrophoretic techniques

3.2.2.1 Extraction techniques

An interesting focus of research is the emergent development of microchips in the field of the chip-based SPE-CE. However, the research in this field is mainly centered in the manufacturing process, so the application of such microdevices is rather limited. In the coming years research in this field will focus on exploring the potential of chip-based SPE-CE for its application in the analysis of real samples (Puig et al., 2007, 2008). SPE is the most attractive way of coupling extraction with CE and, especially, MCE, particularly as it can provide significant improvements in sensitivity without the use of electrokinetic injection (Puig et al., 2007, 2008; Bertoncini & Hennion, 2004). A potential of the affinity extraction in a chip format for a comprehensive proteomic analysis was demonstrated by Slentz (Slentz et al., 2003). This paper reports a system for three-dimensional chip electrochromatography (for the scheme of the chip, see Fig.13). The steps involved include (i) chemical reaction (enzymatic digestion), (ii) affinity extraction (selection of e.g. histidine-containing peptides), and (iii) CEC separation (reversed-phase capillary electrochromatography of the selected peptides). Fluidic manipulations including loading media, sample injection, and sample elution can be successfully performed by voltage manipulation alone.

![Fig. 13. Scheme of the column used and SEM of the microfabricated frit (frit B) and head of the collocated monolithic support structure (COMOSS) column. Reprinted from ref. (Slentz et al., 2003), with permission.](www.intechopen.com)
3.2.2.2 Membrane techniques

In bioanalytical applications, separation protocols based on molecular size are commonly required to extract small analytes from the sample matrix or clean up the macromolecular samples. Typical processes such as filtration (Broyles et al., 2003) and dialysis (Xu et al., 1998; Jiang et al., 2001) had been initially implemented upon microchips. Analytes can also be concentrated by inducing a velocity change due to their size by physically restricting their movement. This has traditionally been most easily performed with large molecules, such as proteins and DNA (Yu et al., 2008). Implementation of nanoporous media (nafion membrane, anionic hydrogel plug, etc.) in microchips (MCE is dominant in this field) has led to a number of interesting developments where the concentration of much smaller molecules is possible (Holtzel & Tallarek, 2007; Dhapeshwarkar et al., 2008; Long et al., 2006).

Porous membranes are ideal candidates for on-chip sample separation. Various researchers had attempted to integrate polymeric membranes to enhance the functionality of microfluidic devices. By sandwiching a dialysis membrane between two polycarbonate chips, Smith and co-workers (Xu et al., 1998) developed a microdialysis device to cleanup biosamples in ESI MS. In a similar approach, Lee and co-workers (Jiang et al., 2001; Gao et al., 2001) built some microfluidic devices with PVDF membranes for protein digestion, drug screening, and residue analysis of contaminants. Zhang and Timperman (Zhang Y. & Timperman, 2003) put a polycarbonate ultrafiltration membrane within the 3-D microchannel to act as a concentrator for fluorescein and FITC-labeled peptides. More recently, Bohn and co-workers (Kuo et al., 2003a, 2003b; Cannon et al., 2003; Tulock et al., 2004) reported several multilayer microfluidic structures with nanocapillary array interconnects; they studied the fluidic flow properties inside these devices and then utilized them for gated analyte injection and fraction collection in microchip electrophoresis. In most of the previous works, the membrane was insularly used as a dialyzer, a concentrator, or an injection valve.

On the other hand, the use of commercially available nanoporous membrane as a sample filter/concentrator prior to electrophoretic separation in a single microfluidic device has been shown by Long et al. (Long et al., 2006). These multilayer devices (Fig. 14) consist of a small piece of thin polycarbonate track-etched (PCTE) membrane (10 nm pore diameter) sandwiched between two PDMS monoliths with embedded microchannels and can work in two opposite operational modes. In the first, they can be used for selective injection and determination of small analytes from a complex sample matrix which contains particles or large molecules. For determination of small molecules, a “filter-CE” device layout shown in Fig. 14a can be used. Alternatively, they can be used for purification and preconcentration of macromolecular analytes by removing small interfering contaminants from the sample stream. For this purpose, “concentrator-CE” device shown in Fig. 14b can be utilized. Because of the membrane isolation, material exchange between two fluidic layers can be precisely controlled by applied voltages. Because the membrane is hydrophilic, the nanopores are filled with solutions by capillarity and can serve as an electrokinetic valve between two fluidic layers (present in upper and lower channels).

In another work, Foote et al. (Foote et al., 2005) fabricated a porous silica membrane incorporated into a microchip to perform on-line preconcentration of SDS-proteins before GE separation. Despite of significant improvement of sensitivity in this method, rather complicated microfluidic manipulations were necessary. Here, a microchip for integrated
ITP preconcentration with GE separation can be a simpler alternative to the membrane filtration-modified MCE devices for the decreasing of the detectable concentration of biopolymers as it was demonstrated in section 3.2.1.2.

![Diagram](image1)

**Fig. 14.** Layout and dimensions of the “filter-CE” device (a) and “concentrator-CE” device (b), these multilayer devices consist of a small piece of nanoporous membrane (gray area) sandwiched between the upper (dotted line) and lower (solid line) PDMS layers. The microchannels were about 40 µm deep, 100 µm or 50 µm wide for upper and lower layers, respectively, and the size of the membrane was about 4 mm x 8 mm. (c) Photograph of the “concentrator-CE” device. (d) 3-D schematic diagram of the intersection region. The upper and lower channels are separated by a thin membrane. Reprinted from ref. (Long et al., 2006), with permission.

4. **Performance parameters of column coupling electrophoresis**

Limit of detection (cLOD) and limit of quantification (cLOQ) are usually calculated as the ratio of standard deviations of y-intercepts of regression lines (s_a) and the slopes of the regression lines (b) multiplied by factor 3.3 (cLOD) or 10 (cLOQ). The obtained values of cLOD and cLOQ by using electrophoretic methods in the column coupling arrangement clearly indicate significantly higher sensitivity in comparison to the single column CZE and therefore, favor the use of these hyphenated techniques (comparing with single column ones) in ultra trace quantitative determination of various analytes present in different multicomponent matrices.

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Linearity of the calibration lines is indicated by the values of the corresponding correlation coefficient ($r$) and coefficient of determination ($r^2$). Column coupled techniques show acceptable linearity, in many cases better as it is in single column systems. Moreover, a linear dynamic range of the analytes can be considerably extended because of an elimination of major matrix constituents, higher sample load capacity, and sample preconcentration. Precision (the repeatability) is usually expressed via relative standard deviation (RSD) of (i) peak areas measured within the concentration range of calibration line and/or (ii) migration times of analytes. Hydrodynamically closed separation systems contributed to high precisions of both the migration and quantitation data in comparison with the flow electrophoretic and non electrophoretic systems. It is because of a fluctuation of the flow velocity in the separation system contributing to a dispersion of the data. Accuracy (expressed via relative error, RE) is evaluated through the recovery test of the analytes from relevant matrices (dosage form, urine, blood, etc.) at different concentration levels. Recovery is evaluated by spiking of blank complex matrix (dosage form, urine, blood, etc.) and water samples with an analyte at different concentration levels and comparing resulting peak areas of the analyte obtained in the different matrices (spiked complex matrix vs. spiked water). An on-line sample pretreatment can considerably enhance the recovery and accuracy of the method due to well controlled sample preparation procedure, minimization of the analyte losses, and effective elimination of the interfering matrix constituents. The main performance parameters of particular column coupled methods are introduced in the Table 1 for a quick overview, what these electrophoretic techniques offer when using them in biomedical analysis.

<table>
<thead>
<tr>
<th>Method (Detection)</th>
<th>LOD/LOQ (ng/mL/LOQ)</th>
<th>Linearity ($r$)</th>
<th>Precision\a (RSD, %)</th>
<th>Recovery (%), (matrix)</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITP-CZE (DAD)</td>
<td>9.3/28.3</td>
<td>0.99989</td>
<td>-</td>
<td>104.7 (urine)</td>
<td>Robustness &lt;6%</td>
<td>Mikuš et al., 2009</td>
</tr>
<tr>
<td></td>
<td>6.5/9.7</td>
<td>0.99995-0.99998</td>
<td>0.58 - 2.79</td>
<td>99.25-100.15 (urine)</td>
<td>Robustness &lt;2.3%</td>
<td>Mikuš et al., 2008b</td>
</tr>
<tr>
<td></td>
<td>9.3 and 10.4</td>
<td>0.99989</td>
<td>-</td>
<td>98.9 - 99.5 (urine)</td>
<td>Robustness &lt;6.5%</td>
<td>Mikuš et al., 2008a</td>
</tr>
<tr>
<td></td>
<td>5.2 and 6.8</td>
<td>0.99965-0.99980</td>
<td>-</td>
<td>100.28-101.16 (urine)</td>
<td></td>
<td>Marák et al., 2007</td>
</tr>
<tr>
<td></td>
<td>4.8, 1.1, 3.2</td>
<td>0.9994</td>
<td>3.95 - 4.54</td>
<td>94.4 - 96.8 (urine)</td>
<td>Robustness &lt;5%</td>
<td>Mikuš et al., 2006</td>
</tr>
<tr>
<td></td>
<td>250/830</td>
<td>0.998</td>
<td>-</td>
<td>107.8 - 113.4 (serum)</td>
<td></td>
<td>Budáková et al., 2009</td>
</tr>
<tr>
<td>Method (Detection)</td>
<td>LOD (LOQ)</td>
<td>Linearity (r)</td>
<td>Precisiona (RSD, %)</td>
<td>Recovery (%), (matrix)</td>
<td>Remarks</td>
<td>References</td>
</tr>
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</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>0.16 µM (blood) 0.11 µM (serum) 0.47 µM (urine)</td>
<td>-</td>
<td>3.5 - 11.4</td>
<td>-</td>
<td>-</td>
<td>Pantůčková et al., 2010</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>150 ng/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Elimination of matrix &gt;95%</td>
<td>Kaniansky et al., 1993</td>
</tr>
<tr>
<td>ITP-CZE (DAD)</td>
<td>0.2 µM</td>
<td>0.9991 - 0.9998</td>
<td>3 - 5</td>
<td>-</td>
<td>Elimination of matrix 60-90%</td>
<td>Danková et al., 2001</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>0.3 µM</td>
<td>0.9996</td>
<td>7.27</td>
<td>-</td>
<td>-</td>
<td>Procházková et al., 1999</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>90-150 ng/mL</td>
<td>&gt;0.998</td>
<td>8</td>
<td>-</td>
<td>Procházková et al., 1998</td>
<td></td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>0.7 µM</td>
<td>&gt; 0.999</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>Křivánková et al., 1997b</td>
</tr>
<tr>
<td>ITP-CZE (DAD)</td>
<td>1.5 ng/mL</td>
<td>-</td>
<td>0.27 - 0.63</td>
<td>-</td>
<td>Elimination of matrix &gt;99%</td>
<td>Danková et al., 1999</td>
</tr>
<tr>
<td>ITP-CZE (MS)</td>
<td>1 µM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Peterson et al., 2003</td>
</tr>
<tr>
<td>ITP-CZE (CON)</td>
<td>2.5x10³ and 8.5x10³ ng/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Budáková et al., 2007</td>
</tr>
<tr>
<td>ITP-ITP, ITP-CZE (CON)</td>
<td>4.8 - 20.5 µM (ITP-ITP) 3.7 - 14.6 µM (ITP-CZE) (LOQ 14.7 - 45.7 µM (ITP-ITP) 11.4 - 42.6 µM (ITP-CZE)</td>
<td>0.9979 - 0.9998 (ITP-ITP) 0.9986 - 0.9996 (ITP-CZE)</td>
<td>&lt;4</td>
<td>95.3 - 97.4 (ITP-ITP) 96.5 - 97.7 (ITP-CZE) (urine)</td>
<td>-</td>
<td>Bexheti et al., 2006</td>
</tr>
<tr>
<td>ITP-ITP (CON)</td>
<td>4.8 - 20.5 µM (LOQ 2 - 27 µM)</td>
<td>0.9980 - 0.9990</td>
<td>0.3 - 7.2</td>
<td>84.5 - 100.6 (serum)</td>
<td>-</td>
<td>Hercegová et al., 2000</td>
</tr>
<tr>
<td>ITP-ITP (CON)</td>
<td>100 µM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Mikuš et al., 2003</td>
</tr>
<tr>
<td>ITP-ITP (MS)</td>
<td>1.10⁴ µM</td>
<td>-</td>
<td>&lt;1</td>
<td>-</td>
<td>-</td>
<td>Tomáš et al., 2010</td>
</tr>
<tr>
<td>ITP-ITP (DAD)</td>
<td>2.7 - 3.7 µM</td>
<td>0.9953 - 0.9970</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Flöttmann et al., 2006</td>
</tr>
<tr>
<td>ITP-ITP (CON)</td>
<td>1.0Q 4.1x10¹ and 5x10³ ng/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Sádecká &amp; Netriová, 2005</td>
</tr>
<tr>
<td>ITP-CEC (UV, MS)</td>
<td>-</td>
<td>0.9849 - 0.9980</td>
<td>13.1</td>
<td>-</td>
<td>-</td>
<td>Mazareeuw et al., 2000</td>
</tr>
<tr>
<td>Method (Detection)</td>
<td>LOD (LOQ)</td>
<td>Linearity (r)</td>
<td>Precision* (RSD, %)</td>
<td>Recovery (%) (matrix)</td>
<td>Remarks</td>
<td>References</td>
</tr>
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</tr>
<tr>
<td>CIEF-ITP-CZE (UV)</td>
<td>-</td>
<td>-</td>
<td>5-10</td>
<td>-</td>
<td></td>
<td>Mohan &amp; Lee, 2002</td>
</tr>
<tr>
<td>CZE-CZE (DAD)</td>
<td>3.5 µM (218nm), 0.4 µM (280nm)</td>
<td>0.9996 - 0.9998</td>
<td>-</td>
<td>93 – 98 (urine)</td>
<td></td>
<td>Danková et al., 2003</td>
</tr>
<tr>
<td>CZE-MEKC (UV)</td>
<td>-</td>
<td>-</td>
<td>1.7 - 4.0 (migration times)</td>
<td>-</td>
<td></td>
<td>Sahlin, 2007</td>
</tr>
<tr>
<td>Microdialysis-CZE (LIF)</td>
<td>0.02 and 0.04 µM</td>
<td>&gt;0.9998</td>
<td>2.5 - 4.9 (peak heights)</td>
<td>-100 (caudate nucleus)</td>
<td></td>
<td>Lada &amp; Kennedy, 1997</td>
</tr>
<tr>
<td>SPE-CE (UV)</td>
<td>-</td>
<td>-</td>
<td>0.5 - 5.2 (migration times)</td>
<td>-</td>
<td></td>
<td>Bonneil &amp; Waldron, 2000</td>
</tr>
<tr>
<td>SPE-CE (UV)</td>
<td>100 ng/mL</td>
<td>0.9828 - 0.9879</td>
<td>8.1 - 11.9</td>
<td>57 – 90 (plasma)</td>
<td></td>
<td>Puig et al., 2007</td>
</tr>
<tr>
<td>SEC-SPE-CE (DAD)</td>
<td>100 ng/mL</td>
<td>&gt;0.9950</td>
<td>10 (peak heights)</td>
<td>65 (cerebrospinal fluid)</td>
<td></td>
<td>Tempels et al., 2006</td>
</tr>
<tr>
<td>MLC-CZE (UV)</td>
<td>-</td>
<td>0.9998</td>
<td>0.7 - 3.5</td>
<td>-</td>
<td></td>
<td>Pálmarsdóttir &amp; Edholm, 1995</td>
</tr>
<tr>
<td>Chips</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITP-ZE (CON)</td>
<td>(LOQ 1.10^3-5.10^3 ng/mL)</td>
<td>0.9542 - 0.9999</td>
<td>6.7 - 11.8</td>
<td>-</td>
<td></td>
<td>Ölvecká et al., 2003</td>
</tr>
<tr>
<td>ITP-ZE (CON)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ölvecká et al., 2004</td>
</tr>
<tr>
<td>ITP-GE (LIF)</td>
<td>-</td>
<td>0.9992</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Huang et al., 2005</td>
</tr>
<tr>
<td>ITP-ZE (LIF)</td>
<td>4.10^7 µM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Wainright et al., 2002</td>
</tr>
<tr>
<td>Membrane filtration-ZE (LIF)</td>
<td></td>
<td>14.6 (plasma) and 8.9 (cells)</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Long et al., 2006</td>
</tr>
</tbody>
</table>

* RSD of peak areas
LOD=limit of detection, LOQ=limit of quantification, r=correlation coefficient, RSD=relative standard deviation, CON=conductivity detection, UV=spectral UltraViolet detection, DAD=diode array detection, LIF=laser fluorescence detection, MS=mass spectrometry, ITP=capillary isotachophoresis, CZE=capillary zone electrophoresis, CE=capillary electrophoresis, ZE=zone electrophoresis, CEC=capillary electrochromatography, MEKC=micellar electrokinetic chromatography, CIEF=capillary isoelectric focusing, CGE=capillary gel electrophoresis, GE=gel electrophoresis, SEC=size exclusion chromatography, CLC=column liquid chromatography, SPE=solid phase extraction.

Table 1. Performance parameters of column coupling electrophoretic methods
5. Applications of column coupling electrophoresis

The column coupled methods, described in previous sections (3 and 4) concerning the theory (principles, experimental arrangement, benefits, limitations) and performance parameters (LOD, precision, recovery/accuracy, etc.), have been used in many applications. Capillary (CE) and microchip (MCE) formats were taken into consideration. CE and MCE determinations of various biologically active compounds in model (spiked samples) as well as real biological matrices, employing an on-line coupling of the electrophoretic methods with other electrophoretic or non electrophoretic methods, are listed in Table 2. In this table we tried to emphasize briefly the most important features and characteristics of the applications of column coupling electrophoresis in capillary as well as in chip arrangement. This table is further supported by the text where other relevant parameters and explanations are included for the real bioapplications and proteinc analysis.

<table>
<thead>
<tr>
<th>Method (Detection)</th>
<th>Analyte</th>
<th>Matrix</th>
<th>Type of application</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITP-CZE (CON)</td>
<td>Valproic acid</td>
<td>Serum</td>
<td>Biomedical (Therapeutic drug monitoring)</td>
<td>Budáková et al., 2007</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>Lamotrigine</td>
<td>Serum</td>
<td>Biomedical (Therapeutic drug monitoring)</td>
<td>Budáková et al., 2009</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>5-methyltetrahydrofolate</td>
<td>Blood, serum and urine</td>
<td>Biomedical</td>
<td>Pantůčková et al., 2010</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>Sulphanilate, 3,5-dinitrosalicylate</td>
<td>Urine (spiked)</td>
<td>Model</td>
<td>Kaniansky et al., 1993</td>
</tr>
<tr>
<td>ITP-CZE (DAD-spectral informations)</td>
<td>Orotic acid</td>
<td>Urine</td>
<td>Biomedical (biomarker analysis)</td>
<td>Danková et al., 2001</td>
</tr>
<tr>
<td>ITP-CZE (DAD-spectral informations)</td>
<td>Amlodipine</td>
<td>Urine</td>
<td>Biomedical (pharmakokinetick study)</td>
<td>Mikuš et al., 2009</td>
</tr>
<tr>
<td>ITP-CZE (DAD-spectral informations)</td>
<td>Amlodipine (enantiomers)</td>
<td>Urine</td>
<td>Biomedical (pharmakokinetick study)</td>
<td>Mikuš et al., 2008b</td>
</tr>
<tr>
<td>ITP-CZE (DAD-spectral informations)</td>
<td>Celiprolol</td>
<td>Urine</td>
<td>Biomedical (pharmakokinetick study)</td>
<td>Mikuš et al., 2008a</td>
</tr>
<tr>
<td>ITP-CZE (DAD-spectral informations)</td>
<td>Pheniramine (enantiomers)</td>
<td>Urine</td>
<td>Biomedical (metabolic study)</td>
<td>Marák et al., 2007</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>Pheniramine, dimethindene, dioxopromethazine (enantiomers)</td>
<td>Urine</td>
<td>Biomedical (metabolic study)</td>
<td>Mikuš et al., 2006</td>
</tr>
<tr>
<td>Method (Detection)</td>
<td>Analyte</td>
<td>Matrix</td>
<td>Type of application</td>
<td>References</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------</td>
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<td>------------</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>Orotic acid</td>
<td>Urine</td>
<td>Biomedical (biomarker analysis)</td>
<td>Procházková et al., 1999</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>L-ascorbic acid</td>
<td>Serum, urine, stomach fluid</td>
<td>Biomedical</td>
<td>Procházková et al., 1998</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>Hippurate</td>
<td>Serum</td>
<td>Biomedical (biomarker analysis)</td>
<td>Křivánková et al., 1997b</td>
</tr>
<tr>
<td>ITP-CZE (DAD)</td>
<td>Tryptophan</td>
<td>Urine (spiked)</td>
<td>Model</td>
<td>Danková et al., 1999</td>
</tr>
<tr>
<td>ITP-CZE (UV)</td>
<td>2,4-dinitrophenyl labeled norleucine, tryptophan</td>
<td>Urine (spiked)</td>
<td>Model</td>
<td>Fanali et al., 2000</td>
</tr>
<tr>
<td>ITP-CZE (MS)</td>
<td>Angiotensin peptides</td>
<td>Aqueous</td>
<td>Model</td>
<td>Peterson et al., 2003</td>
</tr>
<tr>
<td>ITP-ITP, ITP-CZE (CON)</td>
<td>Amino bisphosphonate</td>
<td>Urine (spiked)</td>
<td>Model</td>
<td>Bexheti et al., 2006</td>
</tr>
<tr>
<td>ITP-ITP (CON)</td>
<td>Antirheumatic drugs</td>
<td>Serum (spiked)</td>
<td>Model</td>
<td>Hercegová et al., 2000</td>
</tr>
<tr>
<td>ITP-ITP (CON)</td>
<td>Cystine</td>
<td>Urine (spiked)</td>
<td>Model</td>
<td>Mikuš et al., 2003</td>
</tr>
<tr>
<td>ITP-ITP (MS)</td>
<td>Vitamins</td>
<td>Blood</td>
<td>Biomedical</td>
<td>Tomáš et al., 2010</td>
</tr>
<tr>
<td>ITP-ITP (DAD)</td>
<td>Homovanilic acid, vanillylmandelic acid</td>
<td>Urine (spiked)</td>
<td>Model</td>
<td>Flottmann et al., 2006</td>
</tr>
<tr>
<td>ITP-ITP (CON)</td>
<td>Naproxen and its metabolites</td>
<td>Urine</td>
<td></td>
<td>Sádecká &amp; Netriová, 2005</td>
</tr>
<tr>
<td>ITP-CEC (UV, MS)</td>
<td>Cationic low molecular mass compounds (neostigmine, salbutamol, fenoterol)</td>
<td>Plasma, urine (spiked)</td>
<td>Model</td>
<td>Mazareeuw et al., 2000</td>
</tr>
<tr>
<td>CIEF-CGE</td>
<td>Hemoglobin</td>
<td></td>
<td></td>
<td>Yang et al., 2003a</td>
</tr>
<tr>
<td>CIEF-ITP-CZE</td>
<td>Tryptic digest proteins</td>
<td>Extract of proteins</td>
<td>Model</td>
<td>Mohan &amp; Lee, 2002</td>
</tr>
<tr>
<td>CZE-MEKC (UV)</td>
<td>drugs</td>
<td>Urine</td>
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<td>Zhang et al., 2010</td>
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<td>CZE-CZE (DAD-spectral information)</td>
<td>Orotic acid</td>
<td>Urine</td>
<td>Biomedical (biomarker analysis)</td>
<td>Danková et al., 2003</td>
</tr>
<tr>
<td>Method (Detection)</td>
<td>Analyte</td>
<td>Matrix</td>
<td>Type of application</td>
<td>References</td>
</tr>
<tr>
<td>-------------------</td>
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<td>------------</td>
</tr>
<tr>
<td>CZE-MEKC (UV)</td>
<td>Tryptic digest of bovine serum albumin</td>
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<td>Model</td>
<td>Sahlin, 2007</td>
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<td>Rat caudate nucleus (in vivo)</td>
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<td>Lada &amp; Kennedy, 1997</td>
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<td>Model</td>
<td>Bonneil &amp; Waldron, 2000</td>
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<td>SPE-CE (UV)</td>
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<td>Model</td>
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<td>Model</td>
<td>Tempels et al., 2006</td>
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<td>Terbutalin (enantiomers)</td>
<td>Plasma</td>
<td>Model</td>
<td>Pálmarsdóttir &amp; Edholm, 1995</td>
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<td>Serum</td>
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<td>Ölvecká et al., 2003</td>
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<td>Proteins</td>
<td>Aqueous</td>
<td>Model</td>
<td>Ölvecká et al., 2004</td>
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<td>ITP-GE (LIF)</td>
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<td>Aqueous</td>
<td>Model</td>
<td>Huang et al., 2005</td>
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<td>Model</td>
<td>Cho et al., 2004</td>
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<td>Long et al., 2006</td>
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<td>Peptides</td>
<td>Extract of proteins</td>
<td>Model</td>
<td>Slentz et al., 2003</td>
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</table>

CON= conductivity detection, UV=spectral UltraViolet detection, DAD=diode array detection, LIF=laser fluorescence detection, MS=mass spectrometry, ITP=capillary isotachophoresis, CZE=capillary zone electrophoresis, CE=capillary electrophoresis, ZE=zone electrophoresis, CEC=capillary electrochromatography, MEKC=micellar electrokinetic chromatography, CIEF=capillary isoelectric focusing, CGE=capillary gel electrophoresis, GE=gel electrophoresis, SEC=size exclusion chromatography, CLC=column liquid chromatography, SPE=solid phase extraction, EDTA=ethylenediaminetetraacetic acid.

Table 2. Applications of column coupling electrophoretic methods
5.1 Capillary arrangement

5.1.1 Analysis of drugs and biomarkers in clinical samples

*ITP-CZE*. In our recent works (Mikuš et al., 2006a, 2008a, 2008b, 2008c, 2009; Marák et al., 2007) we illustrated possibilities of ITP-EKC method combined with diode array detection (DAD) for the direct achiral (celiprolol, CEL, amlodipine, AML) as well as chiral (amlodipine, AML, pheniramine, PHM, dimethinden, DIM and dioxoprometazine, DIO) quantitative determination of trace drugs in clinical human urine samples, see an example in Fig.15. ITP, on-line coupled with EKC, served in these cases as an ideal injection technique (high sample load capacity, preseparation and preconcentration) producing analyte zone suitable for its direct detection and quantitation in EKC stage. Spectral DAD, used in our works, in comparison with single wavelength ultraviolet detection enhanced value of analytical information (i) verifying purity (i.e., spectral homogeneity) of drug zone (according to differences in spectrum profiles when compared tested and reference drug spectra) and (ii) indicating zones/peaks with spectra similar to the drug spectrum (potential structurally related metabolites). Very good selectivity was achieved by using a negatively charged carboxyethyl-β-cyclodextrin (CE-β-CD) as a chiral selector for enantioseparation and determination of trace (ng/mL) antihistaminic drugs (PHM, DIM, DIO) present in urine (Mikuš et al., 2006a, 2008c; Marák et al., 2007). Charged chiral selector provided significantly different affinity towards the analytes on one hand and sample matrix constituents on the other hand; enabling the analytes can be transferred into the analytical stage without any spacers and multiple column-switching even if accompanied by a part of sample matrix constituents detectable in analytical stage. This analytical approach enabled us to obtain pure zones of the drugs enantiomers (without the need of the sample pretreatment). DAD spectra of PHM metabolites were compared with the reference spectra of PHM enantiomers (Marák et al., 2007; Mikuš et al., 2008c) and a very good match was found which indicated the similarities in the structures of enantiomers and their metabolites detected in the urine samples. This fact was utilized for the quantitative analyses of PHM metabolites in the urine samples by applying the calibration parameters of PHM enantiomers also for PHM metabolites. Spectra obtained by DAD helped with the identification of analytes even having the similar structures but it was necessary that their peaks were resolved. The on-line coupled ITP-EKC technique was used also for the pharmacokinetic studies of CEL (Mikuš et al., 2008b) and AML (Mikuš et al., 2008a, 2009) in multicomponent ionic matrices. In order to control a reliability of the results, we utilized spectral data from DAD (evaluation of purity of separated analyte zone; confirmation of basic structural identity of the analyte). A great advantage of the ITP-EKC-DAD method was a possibility to characterize electrophoretic profiles of unpretreated (unchanged) biological samples and, by that, to investigate drug and its potential metabolic products with higher reliability.

The increase of the sensitivity, by applying ITP preconcentration before the final CZE separation, was necessary for a determination of orotic acid in human urine (Procházková et al., 1999; Danková et al., 2001). Procházková et al. showed, that this method was suitable for determination of orotic acid also in children’s urine samples (conventional CZE method failed in this application) and they reached very high reproducibility of analyses (effective clean-up of the sample). Danková et al. increased in their work 3-4 times the amount of urine ionic constituents loadable on the ITP-CZE separation system in comparison with the work of Procházková et al. Moreover, DAD detection served in this work also for identification of the analyte by UV spectra, even though the analyte was present at very low concentration level.
Fig. 15. ITP-EKC-DAD method for the direct sensitive determination of enantiomers in unpretreated complex matrices sample with spectral characterization of electrophoretic zones. 3D traces were obtained combining electrophoretic (EKC) and spectral (DAD) data where the spectra were scanned in the interval of wavelengths 200-400 nm. (a) 3D trace illustrating the whole EKC enantioseparation of pheniramine and its metabolites in the online pretreated clinical urine sample (spectra of matrix constituents, well separated from the analytes, are pronounced), (b) detail on the 3D spectra showing the migration positions of pheniramine enantiomers (E1 and E2) and their structurally related metabolites (M1 and M2). The spectrum of the little unknown peak marked with the asterisk differed from the pheniramine spectrum significantly and, therefore, it was not considered as a pheniramine biodegradation product. The urine sample was taken 8.5 hours after the administration of one dose of Fervex (containing 25 mg of racemic pheniramine) to a female volunteer and it was 10 times diluted before the injection. The separations were carried out using 10 mM sodium acetate - acetic acid, pH 4.75 as a leading electrolyte (ITP), 5 mM ε-aminocaproic acid - acetic acid, pH 4.5 as a terminating electrolyte (ITP), and 25 mM ε-aminocaproic acid - acetic acid, pH 4.5 as a carrier electrolyte (EKC). 0.1% (w/v) methyl-hydroxyethylcellulose served as an EOF suppressor in leading and carrier electrolytes. Carboxyethyl-β-CD (5 mg/mL) was used as a chiral selector in carrier electrolyte. Reprinted from ref. (Marák et al., 2007), with permission.
A comparison of two types of CE instrumentation, single CZE and commercially available ITP-CZE, used for the determination of hippuric acid in serum was demonstrated by Krivánková et al. (Krivánková et al., 1997b). Results obtained in the single-capillary methods (ITP and CZE) were comparable and were limited both by the sensitivity of the detector used and by the load capacity of the system. This work pointed out decreasing of concentration LOD (cLOD 7.10⁻⁷ M was two-orders of magnitude lower by using ITP-CZE method in comparison with single column CZE). The sample volumes that could be injected using this combined technique were up to 10³ orders of magnitude higher in the case of natural biological samples than those that could be analyzed in a single capillary CZE technique. Excellent reproducibility of migration times (R.S.D. less than 1%) and resistance to changes in the matrix composition enabled the determination of HA in serum not only for patients suffering from renal diseases but also for healthy individuals.

Fig. 16. (a) Conductivity trace of the analysis of 1µL undiluted blood. LE: 10mM ammonium acetate pH 7.8, TE: 20mM acetic acid pH 3.5. (b) Selected ion monitoring of the ions in the ITP zones of undiluted blood. Reprinted from ref. (Tomáš et al., 2010), with permission.
CZE-CZE. Danková et al. (Danková et al., 2003) showed also the analytical potentialities of CZE in the separation system with tandem-coupled columns to the spectral identification and determination of orotic acid (OA) in urine by diode array detection (DAD), coupled to the separation system via optical fibers. A very significant “in-column” clean-up of OA from urine matrix was achieved in the separation stage of the tandem by combining a low pH (2.8) with complexing effects of electroneutral agents [α- and β-cyclodextrins, poly(vinylpyrrolidone) and 3-(N,N-dimethyldecylammonio)propanesulfonate]. Due to this, DAD spectral data of OA was acquired in the detection stage of the tandem with almost no disturbances by matrix co-migrants.

ITP-ITP. Tomáš et al. (Tomáš et al., 2010) have modified the commercial coupled column isotachophoresis system for direct connection to an ion trap mass spectrometer. Although identification of individual zones is possible with the help of standard substances, selected ion monitoring of the individual masses in the electrospray-MS signal provided additional means for identification. The instrumentation was tested for determination of vitamins in whole blood analysis (see Fig.16) and separation of tryptic peptides. The main advantage of large bore ITP system with fluoropolymer based columns which was used in this work was the possibility to inject crude samples, such as urine or blood, with minimum or no sample pretreatment. In many cases injections of 10 μL or higher sample volumes result in sensitivities with cLOD in the range of 10^{-10} M.

Microdialysis-CE. A fully-automated method for monitoring thiols (glutathione and cysteine) in the extracellular space of the caudate nucleus of anesthetized rats (in vivo) using microdialysis coupled on-line with CZE with laser-induced fluorescence detection (dialysates were derivatized on-line) was investigated (Lada & Kennedy, 1997). This system allowed to obtain high relative recoveries (nearly 100%) and high temporal resolution (high mass sensitivity of CZE-LIF permits frequent sampling) simultaneously for multiple thiols present in the brain.

5.1.2 Analysis of proteins

ITP-CZE. Comprehensive ITP-CZE was successfully coupled to electrospray ionization orthogonal acceleration time-of-flight mass spectrometry using angiotensin peptides as model analytes (Peterson et al., 2003). ITP-TOF-MS alone was adequate for the separation and detection of high concentration samples. The problems (ion suppression and discrimination) can occur when lower analyte concentrations are analysed because mixed zones or very sharp peaks are formed. This problem was effectively overcome by inserting a CE capillary between the ITP and TOF-MS.

CZE-MEKC. Capillary zone electrophoresis at two different pH values has been developed to perform a comprehensive two-dimensional capillary electrophoresis separation of tryptic digest of bovine serum albumin using CZE followed by MEKC (Sahlin, 2007). Two-dimensional systems reduced probability of component overlap and improved peak identification capabilities since the exact position of a compound in a twodimensional electropherogram is dependent on two different separation mechanisms.

CIEF-CGE. An on-line two-dimensional CE system consisting of capillary isoelectric focusing (CIEF) and capillary gel electrophoresis (CGE) for the separation of hemoglobin (Hb) was reported by Yang et al. (Yang et al., 2003a). After the Hb variants with different isoelectric points (pIs) were focused in various bands in the first-dimension capillary, they were chemically mobilized one after another and fed to the second-dimension capillary for further separation in polyacrylamide gel.
Fig. 17. (A) CIEF separation of cytochrome c digest in a single capillary setup. Capillary: HPC coating, 37 cm x 50 \( \mu \)m ID x 192 \( \mu \)m OD; sample, 0.1 mg/mL cytochrome c digest in 2% Pharmalyte pH 3–10 and 0.38% N,N,N',N'-tetramethylethylenediamine; anolyte, 0.1 M acetic acid at pH 2.5; catholyte, 0.5% w/w ammonium hydroxide at pH 10.5; electric field strength, 500 V/cm; hydrodynamic mobilization; detection, UV absorbance at 280 nm, 7 cm from cathodic end. (C) Early fraction of acidic peptides (pI 3.6–3.9) analyzed by transient CITP-CZE in a 2-D separation system. Reprinted from ref. (Mohan & Lee, 2002), with permission.

**CIEF-tITP-CZE.** A microdialysis junction was employed as the interface for on-line coupling of capillary isoelectric focusing with transient isotachophoresis-zone electrophoresis in a two-dimensional separation system for the separation of tryptic proteins (Fig.17) (Mohan & Lee, 2002). This 2-D electrokinetic separation system combined the strengths of sample loading and analyte preconcentration in CIEF and CITP with high resolving power provided by isoelectric focusing and zone electrophoresis. Many peptides which have the same isoelectric point had different charge-to-mass ratios and thus different electrophoretic mobilities in zone electrophoresis. In comparison with chromatographic systems, electrokinetic separations require no column equilibration and offer further reduction in protein/peptide adsorption through the use of polymercoated capillaries.

**SPE-CE.** An on-line system allowing digestion of the protein, followed by preconcentration, separation and detection of the tryptic peptides of insulin chain B, cytochrome c and
β-casein at sub-micromolar concentrations were developed by Bonneil and Waldron (Bonneil & Waldron, 2000) to minimise the sample handling. Despite fairly good reproducibility of the maps, the resolution and efficiency were poor compared to conventional CE. It was mainly because of backpressure generated by the preconcentrator, small internal volumes of the micro-tee, separation capillary and 60-nl injection loop, which led to inconsistent transfer of the elution plug into the separation capillary. To minimize the backpressure effect, elution plug injection should be made at the lowest pressure possible or by electroosmosis (the use of a separation buffer with moderate to high pH).

Fig. 18. Electropherogram of (a) CSF spiked with des-Tyr^1-[d-Ala^2-d-Leu^5]-enkephalin (1) and [Met^5]-enkephalin (2), each present at 0.5 μg/mL, and (b) unspiked CSF using the on-line SEC-SPE-CE system. Sample volume, 20 μL; split ratio, 1:40; analysis voltage, −20 kV. Reprinted from ref. (Tempels et al., 2006), with permission.

SEC-SPE-CE. An on-line coupled size exclusion chromatography (SEC) has been shown to be effective tool for removing potentially interfering proteins and permitted reproducible solid-phase extraction (SPE) and capillary electrophoresis (CE) in the analysis of peptides in biological fluids (enkephalins in cerebrospinal fluid-CSF), see Fig. 18 (Tempels et al., 2006). This method was shown to be effective enough for the determination of exogenous enkephalins (present in the low μg/mL range) in CSF or plasma, but for endogenous enkephalins (present in the low ng/mL range) sensitivity improvement would still be needed.

5.2 Microchip arrangement
5.2.1 Analysis of drugs and biomarkers in clinical samples
Membrane filtration-MCE. The multilayer MCE device consisting of a small piece of thin polycarbonate track-etched (PCTE) membrane (10 nm pore diameter) sandwiched between two PDMS monoliths with embedded microchannels serves for the speed microscale sample filtration (clean-up) and preconcentration of the complex samples composed of low and high molecular compounds (Long et al, 2006). This approach has been effectively applied in rapid determination of reduced glutathione in human plasma and red blood cells without any off-chip deproteinization procedure (Fig. 19).
Fig. 19. Electropherograms of (a) human plasma and (b) red blood cell lysate injected across a 10 nm pore diameter membrane without any off-chip deproteinization procedure. The separation buffer was 100 mM TBE (pH 8.4). The injection time was 2 s, $V_{\text{inj}} = 800$ V, $V_{\text{sep}} = 1500$ V. Reprinted from ref. (Long et al., 2006), with permission.

5.2.2 Analysis of proteins

**ITP-ZE.** Ölvecká et al. (Ölvecká et al., 2004) demonstrated the potential of their CC chip for highly sensitive analysis of proteins using the online ITP–ZE combination method. The aim of the ITP step in this work was restricted mainly to the concentration of proteins before their ZE separation and conductivity detection. ITP and ZE cooperatively contributed to low- or sub-μg/mL concentration detectabilities of proteins and their quantitations at 1-5 μg/mL concentrations.

**IEF-ZE.** A two-dimensional electrophoresis platform, combining isoelectric focusing (IEF) and zone electrophoresis (ZE), was established on a microchip for the high-throughput and high-resolution analysis of complex samples (separation of the digests of bovine serum albumine and proteins extracted from E. coli) (Cong et al., 2008). During the separation, peptides were first focused by IEF in the first dimensional channel, and then directly driven into the perpendicular channel by controlling the applied voltages, and separated by ZE.

**ITP-GE.** A microchip for online combination of ITP with gel electrophoretic separation was developed to decrease the detectable concentration of SDS-proteins (Huang et al., 2005). Without deteriorating the peak resolution, this system provided a 40-fold increase of the sensitivity, saved analysis time and simplified the instruments for SDS-proteins analysis when compared to the gel electrophoresis mode (see Fig.20).
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Fig. 20. ITP-GE (B) versus GE (A) mode of SDS-protein complexes analysis in the sieving matrix of 10% dextran on microchip. Peak identification: 1, carbonic anhydrase (124 µg/mL); 2, ovalbumin (20 µg/mL); 3, BSA (50 µg/mL); 4, conalbumin (60 µg/mL). Reprinted from ref. (Huang et al., 2005), with permission.

SPE-MCE. The study involved trypsin digestion, affinity extraction of histidine-containing peptides, and reversed-phase capillary electrophromatography of the selected peptides in a single polydimethylsiloxane chip was described by Slentz et al. (Slentz et al., 2003). Copper (II)-immobilized metal affinity chromatography 5µm-particles have been introduced into the chip. Frits have been fabricated in order to maintain the beads, with collocated monolithic support structures (COMOSS). They were able to trap particulate contaminants ranging down to 2µm in size. Fig. 21 presents the on-chip separation of fluorescein isothiocyanate-labeled bovine serum albumin digest (A) before and (B) after affinity extraction.

Fig. 21. On-chip separation of fluorescein isothiocyanate-labeled bovine serum albumin digest (A) before and (B) after affinity extraction. Reprinted from ref. (Slentz et al., 2003), with permission.

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6. Conclusion

This thematic chapter of the scientific monograph indicates, as expected, that there is not available any universal method capable to solve all the analytical problems. On the other hand, this work clearly shows that the advanced on-line coupled systems are characterized by a capability to solve individual groups of very complex analytical tasks (trace analyte, structurally related analytes, high concentration ratio matrix:analyte, detection interferences, unstable substances, minute sample amounts, in-vivo applications, and various combinations of these problems). Moreover, they allow an elimination of external sample handling that is favorable for the automatization and miniaturization of the analytical procedure. All the categories of on-line column coupled methods provide one or more interfaces for the autonomous, flexible, and well defined/controlled performance of different analytical techniques. Nevertheless, the particular categories of on-line column coupled methods are differing from each other by their specific features and analytical potentialities. An on-line column coupling of CE-CE is advantageous especially because of a simple instrumentation and control of the analytical process, as well as good compatibility of combined separation (electrolyte) systems. On the other hand, an implementation of different separation mechanisms, reflected in an enhanced selectivity of the methods, and possibilities to process larger sample volumes can be counted among typical benefits of an on-line column coupling of CE with non electrophoretic techniques. Very interesting and promising alternative, compromising several analytical aspects, is the hydrodynamically closed CE-CE mode employing capillaries with higher internal diameters as employed in the conventional (hydrodynamically open) mode. Such closed mode has an advantage of higher sample load capacity and obtainable reproducibility of the measurements that are the parameters of a high importance for the real applications of the analytical method. On the other hand, hydrodynamically closed CE-CE systems are limited in the applicability of various supporting electrophoretic (e.g. electroosmotic flow) and non electrophoretic (e.g. pressure counterflow) effects and therefore the achieving of desired separation selectivity can be more difficult. Moreover, here are several critical parameters with respect to a deterioration of the separation efficiency such as capillary size (internal diameter), driving current/voltage, and electrolyte systems that must be very carefully selected and optimized. Therefore, the selection of the method will be determined by particular demands of the analysis. An appropriate selection of the method should then make possible to achieve favorable performance parameters (validation data) while maintaining all benefits of the given method. In such a case, the method can be fully accepted for a routine use in a given advanced application area.

Another future direction concerns the development of analytical microsystems, which is currently one of the major challenge in analytical chemistry and may play a role in the future of life science oriented research and development. The main incentives in miniaturization include a reduction of reagents and samples consumption, increased analytical performance, shorter analysis time, and high-throughput. The overall goal is progression towards a µ-total analysis system (µTAS), whereby chemical information is periodically transformed into an electronic or optical signal, where analysis is carried out on a micrometer scale using centimeter-sized glass or plastic chips. However, samples from biological extracts will always be complex and target analytes at trace-levels. With respect to the potentialities of the advanced CE separation systems, as illustrated also in this chapter, there is/will be thus a great current and future interest in adapting the advanced on-line electrophoretic and non electrophoretic techniques to a micrometer scale.
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8. References


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In all different areas in biomedical engineering, the ultimate objectives in research and education are to improve the quality life, reduce the impact of disease on the everyday life of individuals, and provide an appropriate infrastructure to promote and enhance the interaction of biomedical engineering researchers. This book is prepared in two volumes to introduce a recent advances in different areas of biomedical engineering such as biomaterials, cellular engineering, biomedical devices, nanotechnology, and biomechanics. It is hoped that both of the volumes will bring more awareness about the biomedical engineering field and help in completing or establishing new research areas in biomedical engineering.

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