Sensing in Electroanalysis

Vol. 6 (K. Kalcher, R. Metelka, I. Švancara, K. Vytřas; Eds.), pp. 357–368.

ISBN 978-80-7395-434-5 (printed); 978-80-7395-435-2 (on-line)

Capillary Electrophoresis in Multidimensional Separation Techniques For Analysis of Natural Compounds in Complex Matrices (A Review)

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Abstract: In past decades, capillary electromigration separation methods, based on the differences of migration velocities in high intensity electric field, have been established as the routine analytical techniques suitable for the analyzes of both ionized and non-ionized compounds. The improvement in sensitivity of detection lead to discoveries of new substances presented at trace levels in biological, environmental and food samples. For determination of such compounds, separation methods with high resolving power are needed, which leads to the rapid development of multidimensional separation methods. The article presented here reviews some experimental aspects and applications of capillary electrophoresis in the multidimensional separation systems, focused mainly to its combinations with liquid chromatography. Capillary electrophoresis is being applied in multidimensional systems either in both or presumably in the second dimension due to the speed of the analysis and injected volumes of samples. Advantages and drawbacks of the off-line and on-line experimental setups are discussed. Applications of two-dimensional liquid chromatography-capillary electrophoresis for the separation of naturally occurring compounds are discussed with special emphasis on the possibilities of improvement of the detection sensitivity using in-capillary preconcentration techniques.

Keywords: Capillary electrophoresis; Micellar electrokinetic capillary chromatography; Two-dimensional separations; Orthogonality; On-line preconcentration; Natural compounds; Review.

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Introduction

Improvement of sensitivity of both electrochemical and spectrometric detection in recent years led to the discoveries of new substances presented at the trace level in biological and environmental samples.

The complexity of such matrices, containing compounds of either natural or man-made origin, pushes the research in analytical chemistry towards the development of separation techniques capable to resolve and to determine hundreds of compounds in the analysis time in range of minutes or tenths minutes. To rapidly improve the number of resolved compounds, conventional separation techniques such as gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE) are being coupled in two-dimensional (2D) or even multidimensional separation systems.

In such LCxLC systems, the techniques with different separation mechanisms have to be used, and they are practically achieved by the combinations of different columns and mobile phases types in both dimensions. The uncorrelated "orthogonal" separation selectivities are difficult to realize in two LC modes, because the interactions of analytes with stationary and mobile phases are rarely based only on a single factor and the secondary interaction mechanisms must be taken into account when developing 2D LC separations [1]. Moreover, mobile phases should be fully miscible and low elution strength solvent should be preferably applied in the first separation dimension. The most useful combination of phase systems utilizes reversed-phase (RP) separations in both dimensions, which, however, show more or less correlated selectivities and a lower than maximum peak capacity is usually achieved. Ion-exchange based or normal phase systems — in fact, coupled with RP separations less frequently — are suitable for separation of ionic compounds [2], or low-polar compounds [3], respectively.

Electromigration separation techniques in the capillary configuration are presumably highly orthogonal to the reversed-phase liquid chromatography. While RP-LC separates analytes according to the differences in hydrophobicities, capillary zone electrophoresis (CZE), which is the most spread mode of CE, separates analytes mainly on the basis of charge, and to a lesser degree on size [4]. Other modes of separation of analytes under the influence of high intensity electric field, like micellar electrokinetic capillary chromatography mode suitable for separation of non-ionized compounds, can also possess high degree of orthogonality in comparison to the certain RP-LC phase systems [5].

In recent years, 2D methods employing capillary electrophoresis operated in different modes in both dimensions have been also introduced for separation of highly complex samples, mainly in the field of proteomics, peptidomics and other types of bioanalyzes [6,7]. Although the field of 2D CE is growing rapidly and represents one of the major research directions in development of electromigration separation techniques for forthcoming years, to our best knowledge it has not been applied for separation of natural compounds yet.

In this article, recent advances in the development of 2D separation methods and systems are reviewed, when the particular attention is devoted to the methods and procedures having employed capillary electrophoresis in combination with liquid chromatography for separation of natural compounds in environmental, food and metabolite samples.

Experimental Setups Used For 2D LC-CE Separations

Capillary electromigration separation methods in 2D systems are applied solely in the second dimension for the separation of the sample fractions eluted from LC column. Analytical LC with either conventional columns or capillary microcolumns would allow more concentrated fractions to be collected as the injected sample volumes are in the order of magnitude larger in LC (microliters) in comparison to CE (nanoliters). The collection of fractions from CE is more complicated and also their volume is much smaller than in LC.

First step in the optimization of the 2D LC-CE separation method is the choice of the experimental setup. In the simpler approach, effluent from LC column can be collected off-line and then reanalyzed using CE method. In the on-line approach, which has been introduced by Bushey and Jorgenson [4] and further improved by Jorgenson's group [8-12], fractions are directly transferred to CE capillary for the second-dimension analysis. Both approaches have some advantages and limitations, which are summarized in the Table I.

The compromise between the off-line and on-line instrumental design represents automated off-line setup, which has been introduced recently by our group [5]. In this approach (shown on the left side of Fig. 1), the LC is coupled with the CE using an autosampler in the CE instrument as the fraction collector interface. The outlet of the LC column is connected to the autosampler via an electronically controlled six-port switching valve, which is programmed to collect the fractions of the effluent from LC column during the analysis in the first dimension into the vials with glass inserts.

The autosampler is rotated in the period corresponding to the fraction collection time, while the number of fractions to be stored is dependent on the number of free positions in the autosampler. The fractions are stored until the LC analysis run is finished. After the end of the LC step, the valve is switched into the second position, redirecting the effluent to the waste while the LC column is re-equilibrated to the initial conditions. Then, collected fractions are consequently reanalyzed in the CE separation dimension.

Table I: *Comparison of different types of 2D LC-CE experimental setups.*

Setup	Advantages and Benefits	Drawbacks and Limitations
On-line connection	• speed of the analysis • high throughput • easy automation	• fast analysis in the 2nd dimension • dimensions of separation column and capillary must be compatible • no reanalysis of fractions possible • special instrumentation needed • mobile phase must be compatible with background electrolyte
Off-line connection	• commercial instruments can be used • reanalysis of collected fractions • manipulation with fractions (derivatization, preconcentration) • no need for matching of column /capillary dimensions • no restrictions in number and volume of fractions	• longer analysis time • possible contamination/loss of sample fractions during manipulation • difficult to automate

On-line connection of the LC to the CE can be operated in comprehensive mode, where all fractions pass through the both separation methods without loss of the resolution during fraction transfer step. Such a type of the analysis can provide complete characterization of original sample, as the 2D chromatogram shows all compounds presented in the sample, in the best case baseline separated. If only primary information about the sample is needed or if the target compound has to be separated from the complex mixture, heart-cut mode can be selected, where only part of the LC effluent is transferred to the CE and reanalyzed. An example of experimental setup utilized for the heart-cut 2D LC-CE analysis based on a commercial instrumentation is shown on the right side of Fig. 1. In this arrangement, the special cassette for capillary electrophoresis/mass spectrometry coupling, which enables connection of the separation capillary outlet to the external and electronically controlled four-port two-position switching valve. The internal loop of the valve rotor (typical volume 60 or 200 nL) is used for selecting of the appropriate fraction eluted from the LC column, and for re-injecting it to the second dimension CE separation method. After injection of the sample fraction to the CE, the voltage is applied across to the capillary (valve body is grounded and serves as the inlet electrode), meanwhile the LC analysis continues or, alternatively, is being re-equilibrated to the initial LC conditions [13].

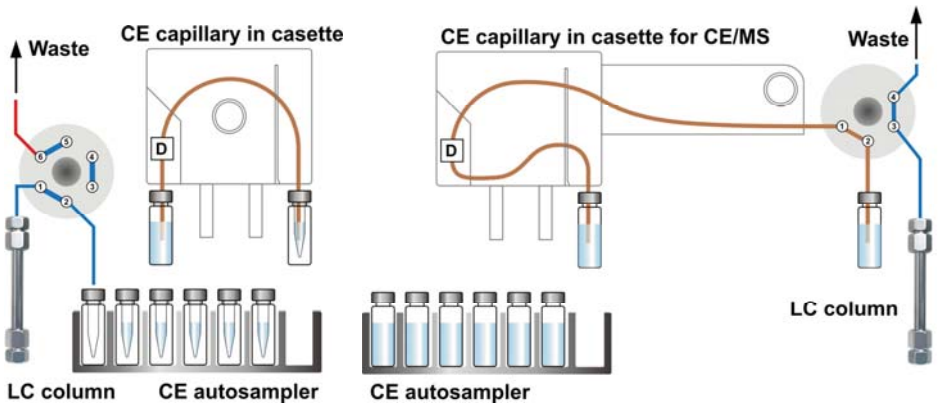

Fig. 1: *Experimental setups for two-dimensional separations combining liquid chromatography with capillary electrophoresis.*
Schematic illustration with the automated off-line (**left** side) and heart-cut on-line (**right** side) experimental setup of connection of liquid chromatography to capillary electrophoresis.

Application of 2D LC-CE Methods for Analysis of Natural Compounds

As described in preceding text, the main application area of 2D separations employing CE in either second or both dimensions is in analyses of proteins and peptides. Although the separation of other types of compounds by the 2D LC-CE is less common, the number of publications focused on this topic slightly has been increasing in recent years [5,13-21].

Separation of neutral components of traditional Chinese medicines by using the 2D LC-CE system has been reported by Zhang et al. [14,15]. For separation of the neutral compounds in the second dimension, micellar electrokinetic chromatography has been applied. The conditions, i.e. the background electrolyte composition, the capillary length and the strength of the electric field applied across the capillary were optimized to achieve the best separation in the second dimension. The micro-LC separation has been coupled on-line with the CE using a new design of the gating interface based on the effluent stacking-injection. Performance of the 2D method was demonstrated by the separation of medicines liquorice and Cheng-Qi-Tang, consisted of the three raw materials, *Rheum officinale Baill*, *Citrus aurantium L.* and *Magnolia officinalis Rehd. et Wils*.

A new approach solving a problem of the long separation time in the second dimension of the 2D LC-CE system was presented by Ehala et al. [16], introducing a new type of interface for coupling of the both methods and having proposed the measurement procedure called "stroboscopic sampling".

This flow injection analysis-like approach is based on the repeating of injection of sample to the first LC separation dimension, while with each run, different sample fraction is transferred to the second CE dimension. By changing the time delay between the first and the second dimension sampling from the hold-up time to the last peak retention time of the sample at the first dimension column, all the analyte zones will be subjected to the separation in the second dimension and the comprehensive 2D analysis becoming possible.

In the LC step, RP and ion-chromatography was used for the separation of organic acids and phenolic compounds, while the capillary zone electrophoresis in 20 mmol/L sodium tetraborate buffer in the second dimension provided fast separation in the range of five minutes and overall time of the analysis in the range of several hours. The proposed experimental setup also puts quite stringent requirements for the equipment reproducibility.

Another application of the 2D LC-CE method for the separation of naturally occurring compounds was shown by Garcia-Villalba et al. [17]. The HPLC-CE system was applied for the analysis of olive oil samples. The method involves semi-preparative column with octadecylsilica gel stationary phase in the first dimension, where the isolation of fractions from commercial extra-virgin olive oils were made and the composition of the isolated fractions was determined using the CE coupled to the time-of-flight mass spectrometer equipped with an orthogonal electrospray interface. The authors identified some compounds, which have never been described before in the olive oils, and some of the fractions have been quantified and isolated to make the *in vitro* studies of their anti-carcinogenic properties.

In the results recently published by our group, the 2D separations combining LC in the first dimension with the micellar electrokinetic chromatography mode of the CE in the second separation dimension have been employed for the analysis of natural antioxidants presented in samples of beverages [5,18,19]. We have compared both experimental approaches, i.e. automated off-line setup suitable for the full characterization of natural compounds in beverages [5] and the heart-cut on-line connection of the capillary micro-LC with the micellar electrokinetic chromatography for the separation of selected fractions, which are difficult to separate in the RP-LC [18]. Separation of the phenolic acids and flavonoids using these two techniques are depicted on Figs 2 and 3.

In the off-line setup, the narrow bore LC column with the polyethylenglycol silica stationary phase has been employed with the advantage of low concentration of acetonitrile, which is sufficient to elute and separate all the sample compounds in several minutes. The automated setup also enables sufficiently effective separation of all the fractions collected from the LC in the second CE method.

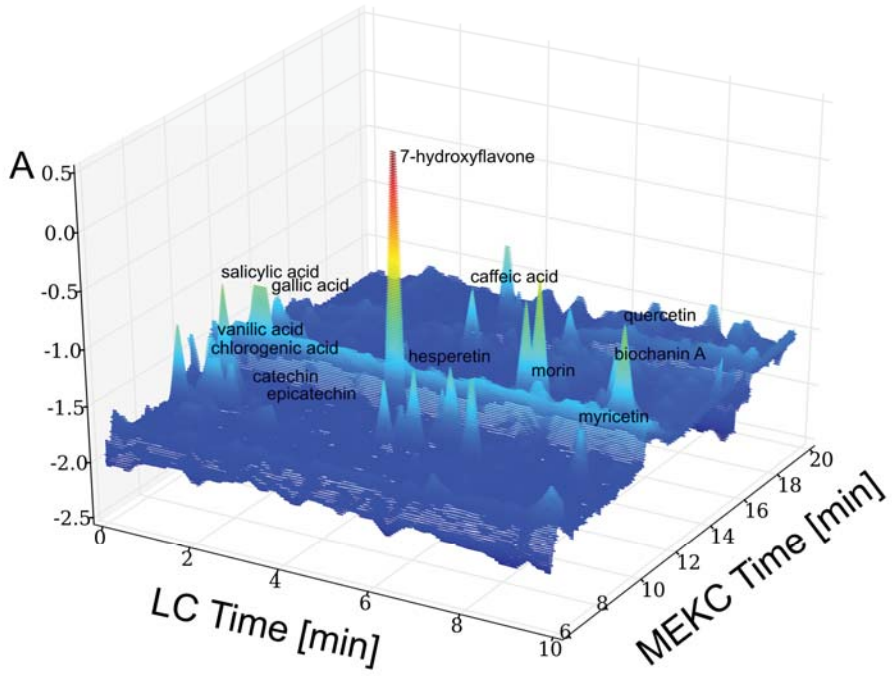

Fig. 2: *Separation of phenolic acids and flavonoids using 2D method combining liquid chromatography with micellar electrokinetic capillary chromatography.*

LC 1st dimension conditions: Discovery HS PEG column, 3 μm, 50 mm long x 2.1 mm i.d., gradient elution, 1 % (v/v) to 20 % (v/v) acetonitrile in 0.2 % formic acid in 10 min, 0.4 mL/min, 40°C.
CE 2nd dimension conditions: 25 mmol/L borate buffer pH 9.05 with 35 mmol/L sodium dodecyl sulfate, 25 μm i.d. capillary with extended 125 μm light path cell, 48 cm total length, 40 cm to the detector, UV detection at 280 nm, 20 kV applied voltage, 25°C. Fractions of 100 μL transferred between both methods using experimental setup shown on the left in Fig. 1.

The resulting 2D record contains all compounds presented in the original sample and peak capacity as high as five hundred can be generated, which is almost twice of the number achieved in the 2D comprehensive LCxLC [20]. The main disadvantage of these techniques is the overall analysis time in the optics of lengthy hours required; however, the analysis may run unattended overnight.

The heart-cut on-line approach was applied for the separation of phenolic acids and flavonoid natural antioxidants presented in the beer samples. Here, capillary microcolumns with octadecyl- and octylsilica porous shell particles were used, providing fast separation of the sample compounds in the first LC dimensions.

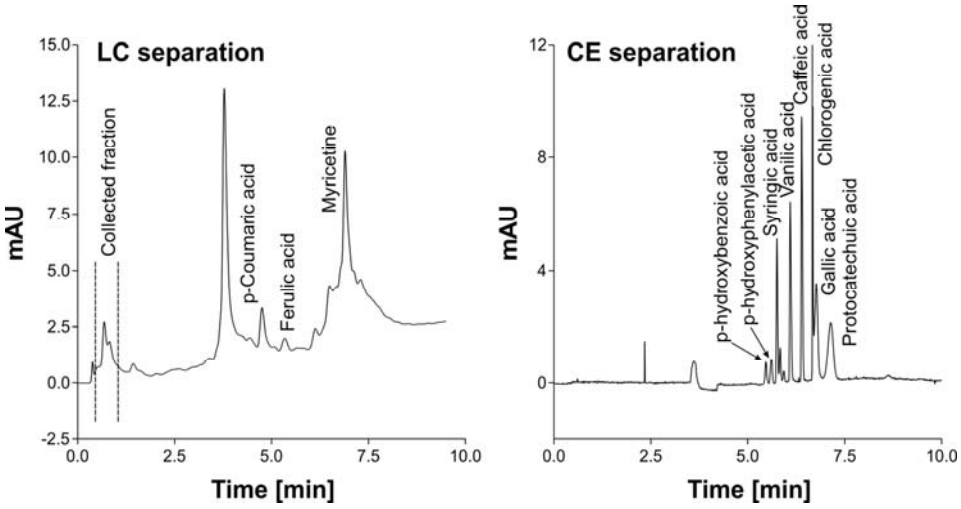

Fig. 3: *Separation of phenolic acids and flavonoids in beer sample using 2D heart-cut on-line method combining micro-LC with micellar electrokinetic capillary chromatography.*

LC 1st dimension conditions: Ascentis Express C8 microcolumn, 2.7 μm porous shell particles, 50 mm long x 200 μm i.d., gradient elution, 0 min - 1 % (v/v) acetonitrile in 0.2 % formic acid, 2-4 min – 8 % acetonitrile, 8 min – 60 % acetonitrile, 3 μL/min, ambient temperature, detection UV at 220 nm.
CE 2nd dimension conditions: 25 mmol/L borate buffer pH 9.05 with 35 mmol/L sodium dodecyl sulfate, 50 μm i.d. capillary, 72 cm total length, 39 cm to the detector, UV detection at 220 nm, 20 kV applied voltage, 25°C. On-line connection of both methods according to the setup shown on the right side of fig. 1, volume of heart-cut fractions 60 nL.

In RP-LC, the retention of strongly polar compounds on alkylsilica stationary phases is usually low even at the pH yielding non-dissociated form of the compounds, which leads to their insufficient separation. On the other hand, when transferred to the CE capillary, such compounds can be separated very efficiently according to the differences in their own mobilities in the electric field, and/or by the partitioning equilibria with micelles. The heart-cut on-line 2D system can therefore provide fast characterization of both low polar compounds separated in the LC step and of strongly polar compounds separated in the CE (Fig. 3).

Improvement of the Sensitivity of 2D LC-CE Methods. Although the 2D separation methods combining LC with CE offers high orthogonality and peak capacity for the separation of complex samples, it usually lacks sensitivity of detection, mainly due to the injection of only a small part of the fractions collected from the LC to the second dimension CE separation.

In the recent years, possibilities of the application of in-capillary preconcentration methods prior to the CE analysis have been extensively studied [18,19,21,22]. The sweeping preconcen-tration method can significantly improve the limits of detection and sensitivity of natural antioxidants using the off-line 2D LC-CE separation method and it can increase the volume of fractions injected to the CE capillary. It is essential especially when the dimensions of LC column and CE capillary are too much different and thus incompatible for on-line connection [19]. The efficiency of sweeping preconcentration step is dependent on the composition of the mobile phase transferred from the first dimension.

We have shown that the 10 mmol/L ammonium acetate pH 3.0 mobile phase component can be replaced by the 0.2 % (v/v) formic acid, which is more suitable matrix for the sweeping, without considerably changed selectivity of the LC separation. By studying the effects of the injected sample zone length to the CE capillary on the resolution of critical pairs of the compounds, we have demonstrated that injection time can be increased ten times compared to the typical injection time required for filling of 1% of the total volume of the separation capillary. The limits of detection of the corresponding method had decreased approximately five times, ranging from 17.1 to 89 μg/L for the set of 24 analyzed phenolic acids and flavonoid natural antioxidants.

Another example of the utilization of in-capillary preconcentration in the 2D separation methods could be seen in work presented by Terabe's group [21,22]. Authors applied dynamic pH junction and sweeping preconcentration methods for the improvement of sensitivity of detection of the 2D micro-LC-CE separation of metabolites of *Bacillus subtilis* [21] and field-amplified sample stacking technique for the analysis of metabolites of *Escherichia coli* [22]. Field-amplified stacking is based on the preparation of sample in low conductivity solution in comparison to the background electrolyte, which leads to the higher electric field strength in the sample zone and the analytes present in the zone are migrating faster than in the background electrolyte.

When they reach the boundary between the sample zone and the background electrolyte, analytes are slowed down and focused into the narrow zones. The optimized conditions allows detecting of metabolites in the concentration range of 2-183 ng/mL and the composition of background electrolyte was also suitable for the direct coupling of the 2D LC-CE method with the mass spectrometer operated in selected ion monitoring mode and used it as the third dimension for identification of metabolites co-migrating in CE separation.

Conclusions and Prospects

Capillary electrophoresis applied in the two-dimensional separation systems offers many advantages in very high separation efficiency and in high orthogonality between each separation modes or with respect to the liquid chromatography. The 2D approaches and the respective methods are being widely applied in biological analyzes, while the importance of such methods for analysis of the naturally occurring compounds is also growing quite rapidly. However, there is a question which research orientation in this very promising field can be expected in the future.

One of the possible trends could be the coupling of capillary electrophoresis in real multidimensional systems (with more than two-coupled methods for the separation), as has been shown some years ago [23], but, up until now, without a continuation.

The other directions and trends will be most likely in the more substantial application of the electrokinetic phenomena for increasing the sensitivity of 2D methods. Significantly more research work is also needed to improve the reliability and robustness of 2D methods employing capillary electrophoresis, which has hitherto hindered their wider applicability in analytical practice.

Acknowledgements

The work was supported by the Ministry of Education, Youth and Sports of the Czech Republic, project No. MSM0021627502. P.Č. would like to acknowledge the financial support from the Czech Science Foundation, project No. 203/09/P199.

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