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TRENDS

Two-dimensional capillary electrophoresis-mass spectrometry (CE-CE-MS): coupling MS-interfering capillary electromigration methods with mass spectrometry

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Abstract
Electromigration separation techniques often demand certain compounds in the electrolyte to achieve the required selectivity and efficiency. These compounds, including the electrolyte itself, ampholytes, polymeric compounds for sieving, complexing agents, tensides, etc. are often non-volatile. Thus, interference with the electrospray ionization process is a common issue, impeding direct coupling of such electrolyte systems to mass spectrometry. Still, several options exist to obtain mass spectra after separation, including offline fractionation, alternative ionization, dilution, or the change to volatile constituents. In the first part of this article, these methods are discussed. However, all of these options are a compromise of separation performance and sensitivity of mass spectrometric detection. Two-dimensional capillary electrophoresis-mass spectrometry (CE-CE-MS) systems represent a promising alternative to the aforementioned challenges, as they allow the use of existing methods with best separation performance in combination with sensitive mass characterization. In this context, the second part of this article is dedicated to the advantages, limitations, and applications of this approach. Finally, an outlook towards future developments is given.

Keywords Capillary electrophoresis · Electrospray ionization · Two-dimensional separation · Interference-free mass spectrometry · Pharmaceutical analysis · 2D interface

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Abbreviations

2D	Two dimensional
AA	Ascorbic acid
ACE	Affinity capillary electrophoresis
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photo ionization
ASA	Acetylsalicylic acid
BGE	Background electrolyte
CD	Cyclodextrin
CE	Capillary electrophoresis
CSE	Capillary sieving electrophoresis
CIEF	Capillary isoelectric focusing
CZE	Capillary zone electrophoresis
ESI	Electrospray ionization
ICP	Inductive-coupled plasma
mAb	Monoclonal antibody
MALDI	Matrix-assisted-laser desorption/ionization
MEKC	Micellar electrokinetic chromatography
MS	Mass spectrometry
SDS	Sodium dodecyl sulfate

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Introduction

Electromigration techniques such as capillary electrophoresis (CE) enable highly selective and efficient separation for a variety of ionic compounds. Since its introduction in the early 1980s, CE has emerged as a powerful analytical routine tool in the fields of environmental analysis [1, 2], forensic analysis [3], food analysis [4], and bioanalytical analysis [5]. Electromigration includes several techniques such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), capillary sieving electrophoresis (CSE), micellar

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electrokinetic chromatography (MEKC), and affinity capillary electrophoresis (ACE).

The most common mode of operation is CZE. Separation in CZE occurs according to different electrophoretic mobilities of ions based on their charge-to-size ratio in an electric field. Typical analytes span from small to large molecules, such as proteins including monoclonal antibodies (mAbs) [6]. The selectivity is mainly given by pH (charge of the analyte) and the background electrolyte (BGE). It can be altered by the type of solvent (non-aqueous capillary electrophoresis). Furthermore, additives are often used to modify the mobility, e.g., cyclodextrines can be applied for enantiomeric separation (chiral CZE) or affinity interaction can be studied by adding, e.g., antigens.

By adding a (pseudo)gel, molecules with similar charge-to-size ratio but different size can be separated referred as capillary sieving electrophoresis (CSE). This principle is widely applied for DNA sequencing [7] and protein separation [8] (after complexing with sodium dodecyl sulfate (SDS)). In CIEF, proteins and peptides are separated in a pH gradient formed in a capillary after applying an electric field. The pH gradient is formed utilizing an acidic anolyte, a basic catholyte, and ampholytes in the BGE. In MEKC, analytes are separated by the use of a pseudostationary phase consisting of micelles.

Mass spectrometry is a major technique for the characterization and identification of analytes separated in gas or liquid phase. Electrospray ionization (ESI) is an efficient way to transfer analytes separated in liquid phase into the mass analyzer, especially ionic species as separated in CE. Efficient ESI premises the absence of non-volatile constituents, which often can be achieved in liquid chromatography (LC) without compromising separation. However, most capillary electromigration techniques (as discussed above) require dedicated compounds in the electrolyte for selective separation, compatible with optical detection (UV-Vis absorption, fluorescence) but not with ESI-MS. Nevertheless, possible solutions to obtain online mass spectra from such electromigration techniques exist and are discussed in this paper.

Strategies for direct coupling of MS-interfering capillary electromigration methods to mass spectrometry

Capillary electrophoresis coupled with mass spectrometry (CE-MS) has become a powerful routine tool for analysis of a broad range of analytes. In recent years, several applications about CE-MS have been published highlighting its diversity and importance, including metabolite and intact protein analysis [9–12]. In these applications, almost entirely volatile BGEs have been used.

The pH is the most important parameter for selectivity in CZE. The availability of volatile BGE systems in CZE, covering almost the entire pH range, enables the direct coupling of these methods to ESI-MS. Most common BGEs for CZE-MS applications are formic acid (HFA) and acetic acid (HAc) for low pH values and their respective ammonium salts for acidic to neutral pH values. Other volatile buffers for higher pH ranges include ammonium carbonate ($(NH_4)_2CO_3$) as well as ammonium hydroxide (NH_4OH). The mentioned volatile buffer electrolytes cover the total pH range. However, the type of BGE and possible additives can also influence the selectivity in CE separations, which can limit their applicability to CZE-MS. The separation of anionic active ingredients and their degradation products in effervescent tablets using different BGEs is shown in Fig. 1. In this case, only the highly ESI-interfering 100 mM tricine electrolyte enables the complete separation of all analytes. Both, the use of a lower concentrated tricine and the use of volatile acetate- or formate-based BGE are insufficient in this regard. The low number of different electrolytes suitable for CZE-MS does not only restrict the versatility of the separation itself, but also the possible use of preconcentration techniques such as transient-isotachophoresis.

CE modes such as CIEF (ampholytes, anolyte, catholyte), chiral CZE (cyclodextrins), and MEKC (surfactants) often rely on ESI-interfering electrolyte compounds. For these techniques, the complete exchange of the BGE system, avoiding non-volatile or ESI-interfering compounds is often very difficult preserving the original separation efficiency and selectivity. Nevertheless, e.g., for MEKC, volatile [13, 14] or at least semi-volatile [15] surfactants can be used in certain applications. If this most straightforward approach using volatile electrolytes is not accessible, other options need to be considered.

Another approach is the use of BGEs with low concentrated interfering compounds. Still, a compromise between MS compatible conditions and separation performance of the CE method has to be made as shown for tricine-based separation of anions in Fig. 1. In addition, low electrolyte concentration limits the possible injection volume. The most common strategy for coupling CIEF with ESI-MS is the direct hyphenation using low ampholyte concentrations. Tang et al. [16] described the first direct hyphenation of CIEF with MS using an ampholyte concentration of 0.5% for a compromise between CIEF resolution and ESI-MS detection. Further approaches of direct CIEF-MS are described in detail in a recent review [17]. Another alternative is the use of partial filling techniques, which have been applied especially for the coupling of chiral CZE and MEKC to ESI-MS [18, 19]. In these cases, the EOF and the migration direction/order play a crucial role and need to be optimized during method development.

Most CE-MS applications have been performed using a sheath liquid interface, where a sheath liquid at a flow rate in the low microliter-per-minute range provides the contact

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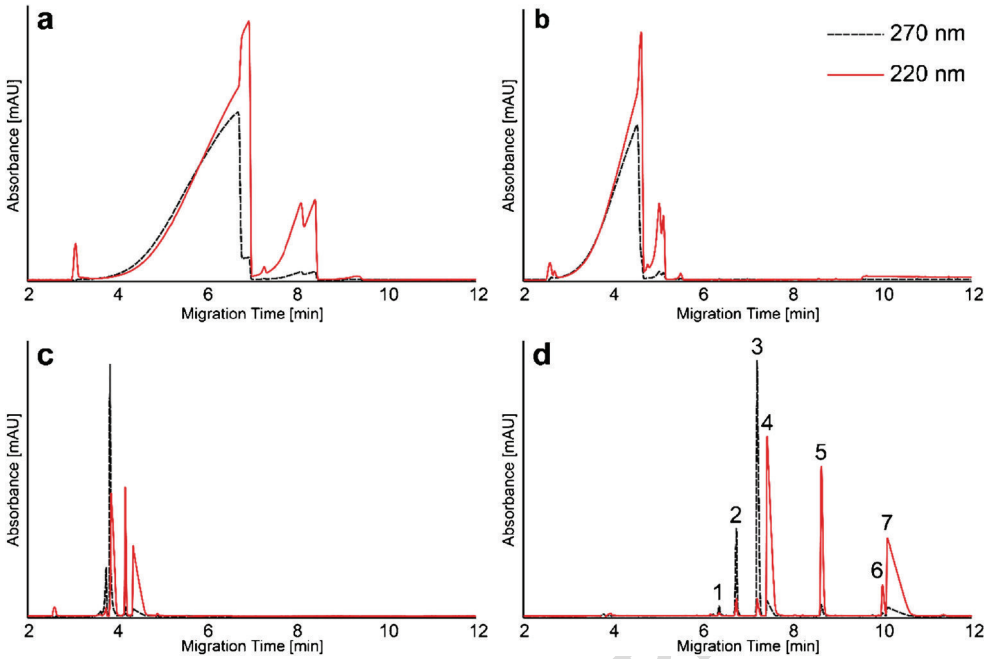

Q3 **Fig. 1** Comparison of different BGEs for the separation of ascorbic acid (AA), acetylsalicylic acid (ASA), and related degradation products in degraded effervescent tablets (RH 75%, $T = 30$ °C, $t = 72$ h). The CZE-UV separation was performed applying + 20 kV in a 50-cm fused silica capillary. Two different wavelengths were used: 220 nm (red, solid) and 270 nm (black, dashed). Four different BGEs are compared: 25 mM ammonium formate, pH = 6 (**a**); 25 mM ammonium acetate, pH = 9 (**b**); 25 mM tricine, pH = 8.8 (**c**); 100 mM tricine, pH = 8.8 (**d**). The peaks in the 100 mM tricine BGE were assigned to diacetylated AA (1), monoacetylated AA (2), AA (3), ASA (4), naphatalenesulfonic acid as internal standard (5), saccharin (6), and salicylic acid (7)

between the capillary outlet and a surrounding metal needle. In the last years, nanospray interfaces have become more popular, especially since the commercialization of the porous tip and a nanosheath liquid interface. For details of coupling, we refer to recent reviews [20, 21]. NanoESI processes (flow rates < 1000 nL/min) are generally more tolerant to ESI-interfering compounds and offer higher sensitivity. Thus, these interfaces are expected to be more useful for coupling ESI-interfering CE methods with MS. The field of application regarding nanoESI in combination with non-volatile BGEs is relatively new. Still, the direct hyphenation of CIEF with MS was recently introduced by Dai et al. utilizing a nanosheath liquid interface [22]. Still, there is need for further systematic investigations regarding the degree of compromise regarding concentration of interfering substances, if nanoESI interfaces are used.

A different approach for coupling CE to MS and reduce BGE-related MS interference is the use of alternative ion sources to ESI. Examples are matrix-assisted laser desorption/ionization (MALDI) [23], atmospheric pressure chemical ionization (APCI) [24], atmospheric pressure photo ionization (APPI) [25], and inductive-coupled plasma (ICP) [26]. However, ICP is limited to elemental analysis, especially metals, whereas APCI and APPI are more suited for small, less polar analytes, which are typically not accessible by electromigration separation techniques. The use of MALDI for CE separations is limited to spotted fractions.

Still, in order to maintain the original separation performance in combination with a sensitive mass spectrometric detection, further approaches such as multidimensional separation methods need to be considered.

CE-CE-MS for coupling MS-interfering capillary electromigration methods to mass spectrometry

Two-dimensional (2D) separation techniques are commonly used to increase peak capacity by combining two separation techniques/modes with different selectivities (e.g., combination of LC and CE methods). Hence, superior separation performance can be achieved compared with the respective individual separation dimensions [27]. Here, we discuss an alternative purpose to allow interference-free mass spectrometric detection of analytes separated in highly ESI-interfering electrolyte systems applied as the 1st dimension [28]. Remaining interfering electrolyte compounds of the 1st dimension are either completely removed from the sample or separated from the analytes of interest in the 2nd dimension prior to MS detection. Considering any CE mode as 1st dimension, there are generally two different approaches classified as offline and online.

Applying offline 2D coupling methods, fractions of a 1st separation dimension are collected and subsequently

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transferred to a 2nd separation dimension. This approach is rather simple and often preferred, especially if additional sample preparation steps in between the two separation methods are required (e.g., solvent exchange, derivatization, or digestion). Nevertheless, fraction collection in CE is associated with high dilution of the sample, as typical fractions in CE are in the lower nanoliter range which have to be collected in at least a few microliters of solvent [29]. This challenge can be tackled by performing an additional concentration step prior to injection in the 2nd dimension; however, this makes the entire 2D system more complex and sensitive against disturbances. In general, offline methods are time consuming, labor intensive, and also automation is limited. Thus, in contrast with LC, fraction collection is rarely applied in CE.

In contrast, online coupling of two CE dimensions potentially enables a direct sample transfer with minor dilution. For online coupling a dedicated sample transfer in the nanoliter range from the 1st to a 2nd dimension is required. A variety of 2D concepts have been developed, including dialysis, flow gating interfaces, and microfluidic chips [30, 31]. Still, most of this work has been performed with optical detection and ESI-interfering electrolyte systems in the 2nd dimension. Especially, flow-gating interfaces and interface-free microfluidic chips are challenging to couple to MS, due to the open nature of the system (absence of outlet vial).

Mechanical-valve-based interfaces are frequently used in LC-LC coupling. Regarding the use of such a valve for CE-CE, certain other requirements need to be fulfilled. Due to the high voltages applied in CE, a fully isolated valve is required and thus, metal components in direct contact or near proximity of the electrolyte solutions must be avoided. Furthermore, based on the miniature nature of CE, the transfer of very small volumes (low nanoliter range) from the 1st to the 2nd separation dimension needs to be achieved. In recent years, our group has developed a CE-CE-MS system using a four-port mechanical valve as interface. A scheme of the general CE-CE-MS setup is depicted in Fig. 2. The valve consists of three major parts: the stator, the rotor comprising the sample loop (4–20 nL), and the motor. A mixture of polyether ether ketone and polytetrafluoroethylene was chosen as rotor and stator material to provide sufficient resistivity and tightness of the valve. Due to the material properties and the close distance of the rotor channels, up to ± 15 kV can be applied in both dimensions in order to avoid potential current breakthroughs [28]. The inlet and outlet capillaries of the 1st and 2nd separation dimension are connected to the four-port valve, respectively. In general, the 1st dimension can be operated in any CE mode (CZE, CIEF, CE(SDS), etc.) and usually UV detection is applied provided by an external detector placed right in front of the valve through the inlet. During the CE separation of the 1st dimension, the mechanical valve is kept in loading position, where the sample loop is connected to the 1st dimension, until the analyte of interest is positioned in the sample

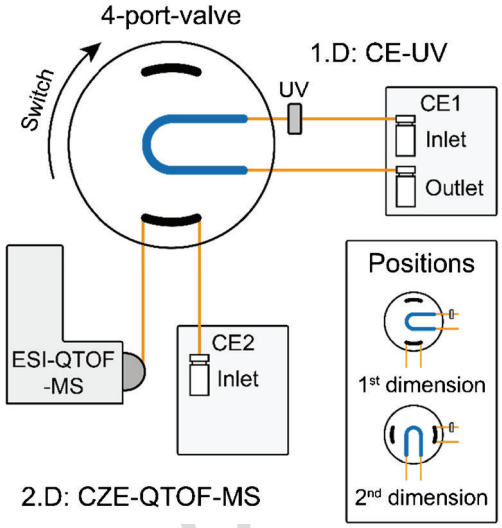

Fig. 2 General setup of the CE-CE-MS system developed in our group. The inlet and outlet capillary of the 1st dimension CE-UV method (CE1 instrument) are connected to the four-port valve (upper, right side). An external UV detector cell is positioned in front of the valve through the inlet of the 1st dimension. Various CE separation modes can be applied as 1st dimension including CZE, CIEF, and CE(SDS). The inlet and outlet capillaries of the 2nd-dimension CZE-QTOF-MS (CE2 and MS instrument) are connected to the remaining channels of the four-port valve (lower part). During the CE(UV) separation, the mechanical valve is kept in loading position where the sample loop is connected to the 1st dimension. When the desired analyte is located in the sample loop, the valve is switched to inject position transferring the analyte from the 1st to the 2nd dimension. Subsequently, high voltage (10 to 15 kV) is applied for separation. The insert contains the position of the valve for the separation in the 1st dimension and the position of the valve for the separation in the 2nd dimension

loop. The external UV detector and the known distance between detector cell and center of the sample loop is used to determine the correct time to cut desired peaks. Subsequently, the valve is switched to inject position transferring the analyte from the 1st to the 2nd dimension and high voltage (± 10 to 15 kV) is applied for separation. Typically, CZE is performed in the 2nd dimension due to the availability of volatile BGEs (e.g., formate or acetate). A major characteristic of this 2D setup is, that both dimension are operated completely independent (e.g., coating and equilibration procedures).

Several applications have been developed utilizing the above-mentioned CE-CE-MS system. In our group, a CZE-UV for the simultaneous determination of ascorbic acid (AA), acetylsalicylic acid (ASA), and their related degradation products in effervescent tablets has been developed. Since the BGE contains 100 mM tricine, direct coupling of this method to ESI-MS was not possible. Thus, this method was the first showcase for a highly ESI-interfering CZE method applied as 1st dimension in the CE-CE-MS setup. In this way, it was possible, for the first time, to identify mono- and diacetylated AA as major degradation products of AA in the presence of ASA [32]. Another example of an MS-incompatible CZE method as 1st dimension was the

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characterization of intact monoclonal antibody (mAb) charge variants using a generic ε-aminocaproic acid (EACA)-based BGE [33]. These electrolyte systems are routinely applied as pharmaceutical application and cannot be coupled directly to MS [6]. The CZE-UV electropherogram of the deglycosylated model mAb Trastuzumab is shown in Fig. 3a. Three peaks, including the main form (1) and two acidic variants (2 + 3), were cut in a heart-cut approach, transferred, and analyzed via CZE-MS in the 2nd dimension. It was possible to separate the co-transferred ESI-interfering EACA in the 2nd separation dimension prior to MS detection, as indicated in Fig. 3b. In this way, interference-free, highly precise mass data (deviation 0.4–0.8 Da) of intact charge variants of Trastuzumab were achieved (Fig. 3c–e). In combination with the electrophoretic separation, the acidic variant peaks 2 and 3 were identified as deamidation products. Another application was the separation and characterization of hemoglobin and its glycated form ($\Delta pI = 0.036$) by CIEF-CZE-MS setup [34]. In addition, Trastuzumab was analyzed with the same CIEF-CZE-MS setup, and the results were in accordance with the findings of the CZE-CZE-MS measurements [35]. In this work, the possibility to perform multiple heart-cuts in the same CIEF analysis was evaluated and confirmed. In addition, the 2D system was extended to imaging (i)CIEF as 1st dimension, which is a powerful technique commonly applied for the analysis of biopharmaceuticals [36]. In this work, the larger injection volume enabled the characterization of a basic variant of Trastuzumab. The observed mass shift could be explained by either succinimide formation (−17 Da) or partial cyclisation of N-terminal glutamic acid (−18 Da). Another interesting field of high ESI interference are CE methods utilizing SDS for mAb impurity analysis. We have developed a CZE method for the characterization of SDS-complexed samples based on the co-injection of positively charged surfactants and methanol as organic solvent to remove SDS from proteins. This method can be applied as 2nd dimension enabling the mass spectrometric characterization of mAb fragments and impurities (manuscript in preparation).

All these examples demonstrate the versatility of this CE-CE-MS approach using a mechanical valve. This is of special interest for the MS coupling of generic and validated methods, utilizing ESI-interfering electrolytes as frequently applied in the pharmaceutical context.

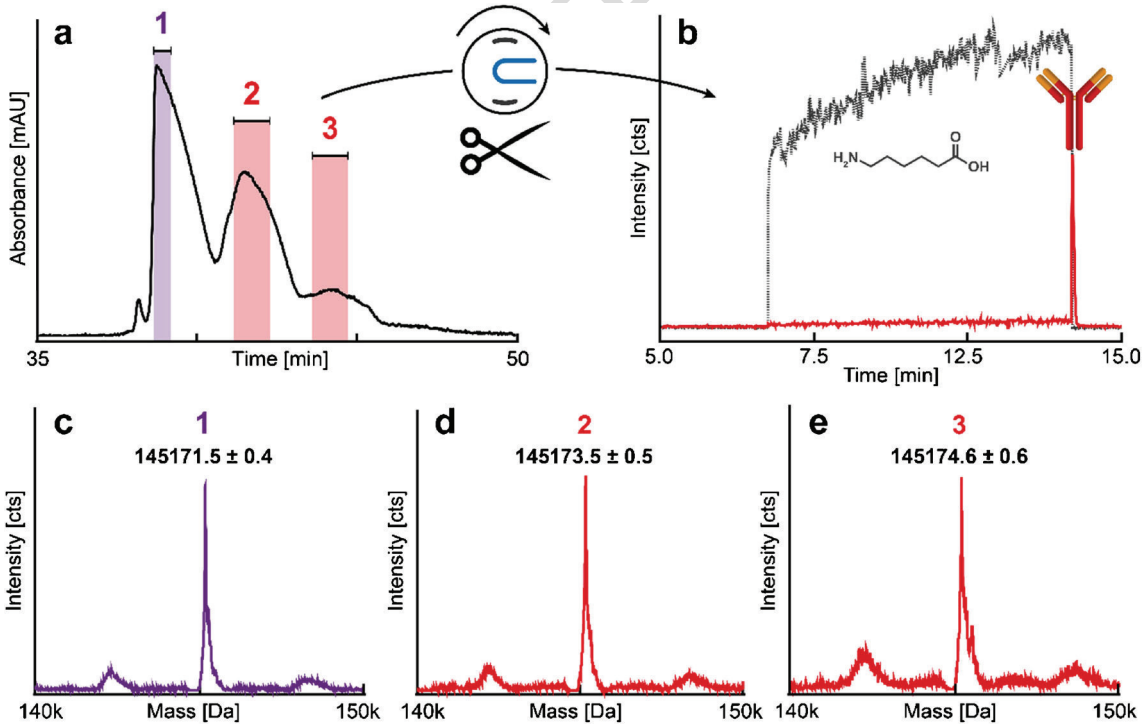

Fig. 3 CZE-CZE-MS for the characterization of intact monoclonal antibody (mAb) charge variants. 1st-dimension CZE-UV electropherogram of deglycosylated model mAb Trastuzumab (6 mg/mL) (**a**) at 380 mM EACA, 1.9 mM TETA, and 0.05% HPMC (pH = 5.7) was used as BGE, commonly used as pharmaceutical application [6]. A separation voltage of + 10 kV was applied. Analyte peaks (10–20 nL) were transferred in a heart-cut approach from the CZE-UV to the CZE-MS dimension. CZE-MS (2nd dimension) electropherogram of highly ESI-interfering EACA (gray, dashed) and mAb variant (red, solid) (**b**): 2 M HAc was used as BGE and in-house PVA-coated capillaries were applied. A separation voltage of + 10 kV was applied. The co-transferred ESI-interfering EACA was successfully separated from the mAb signal in the 2nd CZE dimension prior to MS detection. Deconvoluted mass spectra of the main form M (peak 1, 10 nL cut) (**c**) and acidic variant A1 (peak 2, 20 nL cut) (**d**) and A2 (peak 3, 20 nL cut) (**e**). The minor mass difference of + 2.0 and + 3.1 Da is an indication for the presence of deamidation products. For the cut of acidic variant 3, a higher concentrated sample was applied (30 mg/mL). Modified from ref. [33]

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Outlook

Despite the ongoing development of new CE methods, many applications are still not compatible with MS detection due to the nature of the electrolytes used. Thus, there is a need for techniques to enable MS detection of analytes separated in such ESI-interfering BGEs. Nanospray interfacing will certainly make CE-MS more powerful in both, existing and new fields of application. Still, to what extent this will also enable direct coupling of ESI-interfering CE methods with MS remains open.

The introduced CE-CE-MS setup based on a mechanical valve interface is a promising approach to face the above-mentioned challenges, which is substantiated by the presented applications. Despite the already achieved results, there is still room for improvement of this design. So far, the maximum applicable voltage is limited (± 15 kV) which influences the total method run time. A complete automation of the mechanical valve-based CE-CE-MS setup is aspired, being supported also by a detection closer to or even in the loop. In addition, interfacing for 2D coupling can be improved potentially by the use of different materials and larger distances of the channels. These characteristics will be tackled in future studies. Such improvements will contribute to expand the application of CE-MS toward classical 2D approaches for the analysis of complex samples. In this context, the combination of chromatographic and electromigration techniques is of major interest. Furthermore, the role of microfluidic chips in one and two-dimensional electromigration techniques will certainly grow in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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