Received: 2 August 2018 | Revised: 12 September 2018 | Accepted: 13 September 2018

DOI: 10.1002/jssc.201800817

REVIEW ARTICLE

JOURNAL OF SEPARATION SCIENCE

Coupling of CE-MS for protein and peptide analysis

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Funding information
Grantová Agentura České Republiky, Grant/Award Number: 17-10832S

The review is focused on the latest developments in the analysis of proteins and peptides by capillary electrophoresis techniques coupled to mass spectrometry. First, the methodology and instrumentation are overviewed. In this section, recent progress in capillary electrophoresis with mass spectrometry interfaces and capillary electrophoresis with matrix-assisted laser desorption/ionization is mentioned, as well as separation tasks. The second part is devoted to applications—mainly bottom-up and top-down proteomics. It is obvious that capillary electrophoresis with mass spectrometry methods are well suited for peptide and protein analysis (proteomic research) and it is described how these techniques are complementary and not competitive with the often used liquid chromatography with mass spectrometry methods.

KEYWORDS
capillary electrophoresis, mass spectrometry, peptides, proteins

1 | INTRODUCTION

Electrophoretic methods were closely linked to biopolymer analysis in the early years, mainly in the analysis of protein. In this context, we have to mention that electrophoresis as an analytical method was introduced by Arne Tiselius in the 1930s [1]. His pioneering work was rewarded by a Nobel prize in 1948 "for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins" [2].

Limitation of separation in free solution, as was established by Tiselius [1], is based on molecular diffusion and convection. For this reason, traditionally used media are anticonvective, such as polyacrylamide or agarose gels. These gels are traditionally used for the size-dependent separation of biological macromolecules, such as proteins and nucleic acids. The alternative to the slab ("planar") or tube format is separation in capillaries. In principle, capillaries have a low conductance. They generate only small amounts of heat and are in principle anticonvective. Therefore, the use of gel media in capillaries for electrophoresis is not necessary—free solution electrophoresis, as well as the use of gel media in the capillary—can be applied.

If we are talking about capillary electrophoresis, we have to mention the initial work of Hjertén in 1967 [3]; however, only millimetre-bore capillaries were available. In 1981, Jorgenson and Lukacs [4] showed the potential of capillaries smaller than 100 μm, and this date can be assumed as the "birthday" of capillary electrophoresis.

In the field of proteomic and peptidomic analysis, it is necessary to obtain not only reasonable separation but also the exact determination of structure. It means not only determination of molecular masses of compounds, their sequence (amino acid composition) but also various post-translational modification(s) (PTMs), interaction with other compounds (low as well as high molecular weight), changing of their tertiary or quaternary structure, etc. For this analysis, a mass spectrometer and its coupling to the separation technique is crucial equipment. The traditional (and routine) technique used for this purpose is HPLC, but capillary electrophoresis (or capillary electromigration techniques) can give some new information regarding another separation mechanism—these techniques are not competitive but complementary.

First attempts to connect capillary electrophoresis with MS are dated to the end of the 80s [5]. Of course, in the beginning, all instrumentation and interfaces were experimentally developed. Nowadays, there are commercially available instrumentations broadly used for a wide spectrum of analysis and compounds. From this instrumentation, it is possible to mention commercially available interfaces: coaxial sheath-flow interface G1607A (Agilent Technologies, USA), porous tip interface, CESI 8000 (AB SCIEX, USA), electrospray emitter with EOF driven sheath liquid, EMASSII (Prince Technologies,

Article Related Abbreviations: PTM, post-translational modification; SCX, strong cation exchanger; tITP, transient isotachophoresis

Netherlands) and interface CEi SP20 (Reeko Instrument, Xiamen, China) developed for combination of ICP with MS. To date, there is a plethora of books, book chapters, and review articles about the use of coupling capillary electrophoretic methods with MS in various fields of application and separation science, e.g. [6–25]. The use of CE and MS is still of great interest, as evidenced by the statistic from the Web of Science (Clarivate Analytics). When you choose as search topics CE and MS you obtain 7336 hits during last 10 years (2008–2017) with maximum at 2011 (796 hits) and minimum at 2017 (672 hits) when numbers are fluctuated without tendency.

This review attempts to summarize recently introduced methods (i.e. mainly during last five years) in the field of analysis of proteins/peptides (proteomic or peptidomic approaches) by capillary electromigration methods coupled to MS.

2 | METHODOLOGY AND INSTRUMENTATION

The most useful ionization techniques for CE-MS coupling are ESI and MALDI. Both are soft ionization techniques and so well usable for the analysis of proteins/peptides. Both can be used as online as well as offline coupling. Online coupling by ESI is the preferred method; however, offline coupling of CE to MALDI has a lot of interest due to some advantages, such as higher tolerance of salts, greater sensitivity to some compounds, versatility of separation modes and buffer choice.

2.1 | CE-ESI-MS

ESI is the most frequently used technique for CE-MS coupling and, of course, for analysis of proteins/peptides. The closing of the electrical circuit of CE at the electrospray tip can be materialized by the implementation of some conductive components (such as a conductive coating of the capillary tip or attached emitter) or by addition of auxiliary liquid, so-called sheath liquid. So, in principle, we can divide interfaces to two categories: sheath liquid (when the total liquid flow is in the range of μL/min), which is the most frequently used), and sheathless liquid (flow rate at range tens of nL/min). Online coupling of capillary electrophoresis to MS via ESI has several fundamental issues [18]: (i) consolidation of the CE and ESI circuits, (ii) stable electric contact, (iii) proper emitter geometry for stable ESI and (iv) suitable electrolyte for separation by CE as well as for ESI.

2.1.1 | ESI interfaces with additional liquid (sheath liquid)

There are three main reasons to add liquid to the electrolyte (BGE) of CE: (i) electrical connection of the CE circuit, (ii) adjustment of spray voltage, and (iii) stabilization of electrospray, its flow rate [17].

Coaxial sheath-liquid interface

The coaxial sheath-liquid interface is a "traditional" interface for coupling CE-MS. This technique was developed in 1988 [26]. In this arrangement, the capillary from CE is inserted inside the metal needle and sheath liquid is coaxially delivered inside this needle (and washing out of the capillary). This approach provides electrical contact and a constant flow mixing with the capillary effluent (BGE) at the sprayer tip. Of course, for a stable sprayer, we need additional nebulizer gas. The separation capillary usually extrudes out of the sheath-liquid tubing by 1–2 mm to produce a stable spray. This instrument is commercially available. Sheath-liquid flow rates are around 1–10 μL/min (typically 4 μL/min).

Although this device is relatively simple and is universally used, its use is limited. First, the high flow rate of sheath liquid makes a significant dilution of CE effluent and so causes a decrease of sensitivity. Second, nebulizer gas makes a suction effect at the capillary outlet that causes a parabolic flow inside the capillary, resulting in a decrease in the separation efficiency.

Nanoflow sheath-liquid interface (liquid junction interface)

A liquid junction interface is another choice to use for the liquid flow adding to CE effluent. This arrangement can overcome the high dilution of effluent by coaxial sheath-liquid interface. In this case, flow liquid is added to CE effluent via a 20–200 μm gap between the capillary end and the spray emitter.

For proteomic applications, Dovichi's group developed three variants of the sheath-flow nanospray interface (for a review, see [24]). In principle, the capillary was threaded through a plastic cross into a glass emitter. For example, Sun et al. [27] demonstrated instrumentation that enables picogram to femtogram amounts of *E. coli* digests; for example, over 100 proteins were identified from 16 pg digests by MS/MS (see Figure 1). Schiavone et al. [28] demonstrated the application of this instrumentation for peptide analysis (bovine serum albumin digest). They used 28 cm, uncoated capillary (10 μm id/150 μm od) when its 5 mm was etched with hydrofluoric acid to a resulting od ∼60 μm. The applied separation voltage and electrospray voltage were 29.5 kV and 1.5 kV, respectively. The separation buffer was 0.5% v/v formic acid. The sheath liquid was 10% v/v methanol containing 0.1% v/v formic acid. The spray emitter opening size was 7–9 μm. CZE-MS/MS analysis of the bovine serum albumin digest identified 31 peptides, produced 52% sequence coverage, and generated a peak capacity of ∼40 across the 1 min separation window (CE coupled to Q-Exactive mass spectrometer).

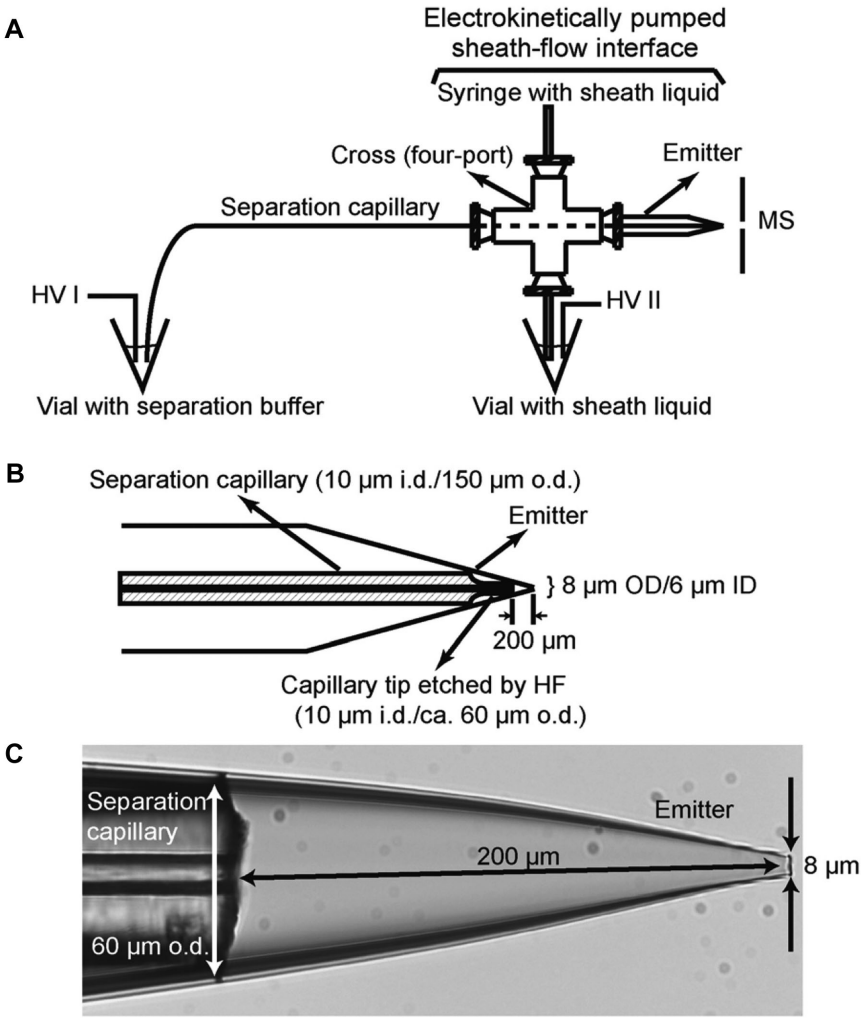

FIGURE 1 (A) CZE-ESI-MS/MS system; (B) schematic representation of the etched capillary in the electrospray emitter; (C) micrograph of the etched capillary in the emitter. i.d. = inner diameter, o.d. = outer diameter. Reprinted with permission from [27]. Copyright 2018 John Wiley and Sons

2.1.2 | ESI interfaces without additional liquid (sheathless liquid)

Unlike the sheath-liquid interface, the sheathless liquid interface utilizes only the BGE for spray generation. Therefore, for all separation conditions, electrolyte composition has a direct influence on the spray performance. The main advantage of this technique is that it does not dilute eluent/sample and therefore should increase sensitivity. For the sheath-liquid arrangement, grounding of the system is made through this sheath-liquid. At the sheathless grounding, the interface electrical contact can be provided by some other ways. An electrical conducting coating is usable and well described. In this case, the main problem is the lifetime of this coating. There are described coatings by electroplating of metals or fabrication of emitter from conductive polymers or carbon. Inserting metal wires through small holes close to the end of the capillary is also described.

Probably only one commercially available (CESI 8000 by Sciex) sheathless interface using a porous tip interface was introduced by Moini in 2007 [29]. This approach is based on etching the capillary wall at the outlet over a length of several cm to a wall thickness of 5 µm, where the glass becomes porous and conductive. This porous tip allows electrical contact with the surrounding metal cylinder filled with electrolyte and serves as an emitter. In the commercially available instrument, 30 µm id capillaries are used. The flow rate should be 2–20 nL/min allowing low ion suppression and high sensitivity when the usability for intact protein analysis was demonstrated [30]. Guo at al. [31] improved previous porous tip interfaces by replacing the porous glass wall by the metal-coated surface of the ESI emitter. Applicability of this method was proved by separation of peptide standards.

Tycova and Foret [32] published an "interface-free" approach. In this case, the CE-MS analysis was performed in a narrow bore (< 20 µm id) electrospray capillary. The separation capillary and electrospray tip formed one entity, so the applied high voltage served for both the separation and electrospray ionization. In this case, optimal current conditions must be adjusted to obtain a stable electrospray signal. The applicability of this method was demonstrated by the analysis of cytochrome c tryptic digest [32].

2.2 | CE-MALDI-MS

The combination of CE and MALDI is realized in an offline regime [18]. This offline arrangement has two main advantages: (i) better tolerance to salts and (ii) possibility to store samples/analytes. The offline combination also means that it is possible to make independent optimization of CE separation and MS detection. Of course, these advantages are not only in the case of CE-MS combination but are also properties of other separation methods, such as the HPLC-MS combination.

The most important problem with CE-MALDI-MS is to maintain electrical continuity when collecting the CE effluent at the capillary outlet. In this case, methods used in CE-ESI-MS coupling can be adapted for CE-MALDI-MS coupling, such as the coaxial sheath-flow liquid interfaces, liquid-junction, or sheathless interface using a metal-coated capillary. It is also a challenge to collect CE effluent with minimum perturbation to the separation process and maintaining maximum separation efficiency. In principle, commercially available MALDI spotters for nanoLC can be used, however, a speed 4 s/fraction is a limitation and usable for systems with sheath liquid flow only.

If we are talking about coupling CE with MALDI-MS by a sheath-flow liquid interface, we have to mention two advantages: (i) integrity of the CE circuit by grounding the sheath liquid and (ii) creation of droplets for better sample deposition. Several papers have described this. For example, Biacchi et al. [33] constructed a fully automated offline CE-MALDI interface with additional UV detection for characterization of monoclonal antibodies. The separation capillary is connected to the voltage via a liquid junction inside the cross-connection needle (that is manipulated by a robotic x-y-z axis motion system). The matrix solution is supplied by a second pressurized capillary attached to the same robotic motion system at a distance of two neighboring spots on a MALDI target. The performance of the method was evaluated with separation of five intact proteins and a tryptic digest mixture of nine proteins. Comparison with Nano-LC-MALDI-MS/MS showed complementarity at the peptide level with an increase of 42% when using CE-MALDI-MS coupling.

A sheathless interface can lead to better sensitivity; however, this approach is complicated by current breakdown during sample collection. Wang et al. [34,35] developed a "porous polymer joint" sheathless interface. This porous polymer joint was immersed in a buffer vial with a grounded electrode, and the capillary column end went through the vial for sample deposition. Subsequent fraction collection was performed on a series of predeposited nanoliter volume 2,5-dihydroxybenzoic acid spots on a Parafilm-coated MALDI sample plate. This instrumentation was applied, e.g. for neuropeptide analysis in individual neuronal organs [34].

A sheathless interface using silver-coated capillary column outlet for iontophoretic sample deposition on MALDI target was also described [36,37]. Individual droplets of several microliters were predeposited on the MALDI target to receive CE zones exiting the capillary end by electromigration and diffusion when the capillary tip dipped into these droplets. For separation, a neutral-coated capillary was used that eliminates EOF, and current breakdown during the separation process could be avoided. This method was used, for example, for analysis of a tryptic digest of eight proteins [36].

Another interesting approach is using offline CE and MALDI-MSI. The problem in CE-MALDI-MS coupling is in the time interval among sample depositions that influence peak resolution/column efficiency. This problem can be minimized by a faster sample deposition; however, loss of on-column resolution always occurs. Collecting continuous CE traces on a moving target followed by MALDI-MS detection along the whole trace could alleviate this problem. This approach was introduced in 1993 [38] using a sheath-liquid interface and later realized by a sheathless interface [39]. In some other instruments in which the interface involves sliding the CE capillary distal end within a machined groove on a MALDI sample plate are described. This plate is precoated with a thin layer of the matrix for continuous sample deposition [40]. It was demonstrated that this method is robust and suitable for quantitative analysis of peptide mixtures with a wide dynamic range.

Recently, an approach was described by Jiang et al. for profiling neurotransmitters and metabolites from the hemolymph of a Jonah crab [41]. The inlet of the capillary was positioned 15 cm higher than the outlet to maintain syphoning flow and the outlet was positioned right above the matrix plate. The CE eluate was continually deposited in the form of sample strip directly onto a MALDI plate. After that, the strip was covered with matrix by a sprayer and analyzed.

2.3 | Separation tasks

As in any analytical case, the success in the analysis of proteins/peptides by CE-MS is a multifactorial task. Some problems are mentioned at Table 1.

The main problem in CE separation of proteins is protein adsorption on the capillary wall due to the electrostatic interaction between positively charged proteins and dissociated silanols on the capillary wall. One of the most used approaches to overcome this problem is to use low pH buffer ($pH < 3.0$) that suppress dissociation of silanols (and minimizing also EOF). At this pH, adsorption of the positively charged proteins is reduced, when good resolution and reproducibility can be achieved. However, this approach is not suitable for native proteomics protein complex studies. So, another possibility is to use static or dynamic coating (both for negative or positive charging of the inner capillary wall).

TABLE 1 Factors influencing success of protein/peptide identifications in CE-MS

Factor	Solving problem
Sample amount	Bigger injection volume
	Sample stacking
	On-line SPE
	Sample prefractionation
	Larger internal diameter of capillary
MS sensitivity	ESI interface (nano)
	Modify sheath liquid or sheath-less interface
	Prevent protein/peptide absorption on capillary wall
	Electrolyte compatible with MS (low ion suppression)
	Adjust electrolyte (compostion, pH)
	Adjust sheat-liquid (in case of sheat liquid interface)
Unadequate separation	Prolonging separation by using lower voltage or longer capillary
	Neutral coated capillaries: use nonacidic buffer
	Fused silica capillaries: use acidic (or basic) buffers
	Use of static/dynamic coated capillaries

It is necessary to mention that positive charging leads to the reverse of the EOF and so reverse polarity is then required. In the case of neutral coating, absence of constant flow rate can lead to problems with ESI stability and separation time is longer. This problem can be overcome by positive pressure at the capillary inlet, which gives a stable ESI and reduces separation time. If we use dynamic coating (replaced after every analysis) we have to use compounds compatible with MS detection and this compound can influence separation repeatability and ion suppression.

It is necessary to mention also the problem with sensitivity in protein/peptide analysis by CE-MS. The most frequently used methods for improved sensitivity are various online or offline concentration techniques such as SPE, liquid–liquid extraction, pH-mediated stacking, field-amplified stacking, and transient ITP (tITP) (for reviews see [42–48]). Really popular is using dynamic pH junction methods for stacking large volume injections of proteins [46]. Normally used is injection of only a few nanoliters (0.5–5% of the capillary volume). In the case of dynamic pH junction method, we use acid background electrolyte (e.g. 10% acetic acid) and inject sample diluted in a basic buffer (by pressure). The analytes (proteins/peptides) are focused at the basic/acidic interface with the application of a voltage. This method can increase the sample loading volume to sub-μL volumes (up to 25% of the capillary volume) without significant loss of separation capacity for bottom-up proteomic analysis [46].

Some interesting new ideas are fritless SPE microcartridges when the diameter is large enough to prevent their entrance into the separation capillary [17]. Dovichi's group described another possibility for improved sensitivity in the analysis of proteins/peptides (at the bottom-up analysis of proteins)—detachable SCX monolith, integrated with CZE and coupled with pH gradient elution [49,50]. In this case, the protein mixture was captured, reduced, alkylated, and tryptically digested by sequential introduction of reagents (at optimized conditions). After the last step, the microreactor was flushed by acidic solution washing out all the undesirable substances. At the next step, the microreactor was connected to a coated capillary (by linear polyacrylamide) (see Figure 2). Peptides (tryptic digest) from the reactor were flushed by a zone of basic buffer followed again by the acidic electrolyte. The sharp increase of pH also served as the pH-mediated stacking at capillary electrophoretic separation. The use of this approach was demonstrated on bottom-up analysis of Xenopus laevis zygote homogenate giving a signal of 1274 peptides within 40 min [51].

Sample enrichment manipulation can also be made offline, i.e. to prepare sample outside CE instrument. These approaches are well described in the preparation for protein and/or peptide analysis by HPLC (and HPLC-MS) and do not significantly differ for separation by CE-MS. It is only necessary to highlight the significance of the desalting step, electrically driven separations are sensitive to salt concentration. Because these offline sample preparations are commonly used for all proteomic analyses, this review is not focused on this problem and readers are encouraged to read some specific review(s) (see e.g. [52,53]).

3 | APPLICATIONS

Proteomic analysis is a diverse task involving analysis of a broad spectrum of compounds, from relatively low molecular peptides to high molecular proteins. So, it involves analysis of protein digests, peptides (bottom-up, shotgun, and middle-down proteomics) as well as analysis of intact/native protein forms (top-down proteomics). So, if we are talking about definitions, we can use three main approaches for protein analysis:

(i) Proteins are enzymatically digested into smaller peptides using proteases such as trypsin or pepsin. The collection of peptide products is then introduced to the mass analyser. When the characteristic pattern of peptides is used for the identification of the protein, the method is called peptide mass fingerprinting if the identification is performed using the sequence data determined in

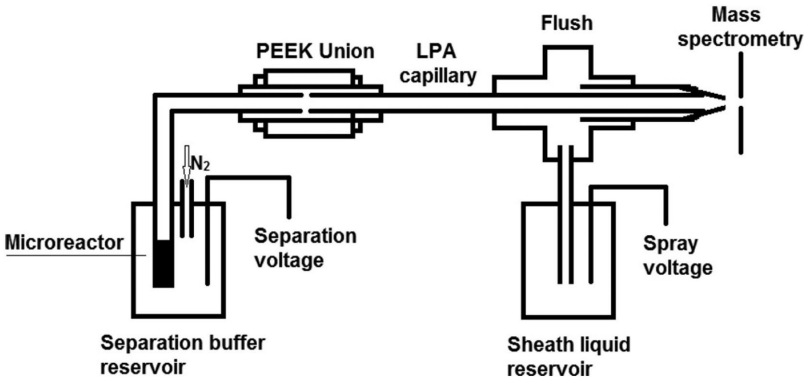

FIGURE 2 A schematic diagram of the SCX-microreactor-CZE-MS/MS system. Reprinted with permission from [51]. Copyright 2016 American Chemical Society

tandem MS analysis it is called de novo sequencing. These procedures of protein analysis are also referred to as the "bottom-up" approach.

(ii) Identification of proteins (peptides) in complex mixtures using a combination of CE (or HPLC) and MS—so-called "shotgun proteomics."

(iii) Analysis of intact proteins; this approach is referred to as the "top-down" strategy of protein analysis.

The "bottom-up procedure is "classical" and it is the most often used procedure in proteomic analysis. The main enzyme for protein cleavage is trypsin; however, some other enzymes can be used. These enzymes can be highly specific (trypsin, AspN, GluC, ArgC, LysC), less specific (chymotrypsin, pepsin), or nonspecific (proteinase K). In addition, chemical cleavage can be used (CNBr). These enzymes can also be used in combination. This procedure allows a large number of identifications in a wide molecular weight range and it involves biologically active peptides or naturally occurring protein fragments. Investigation of undigested proteins allows analysing of whole protein modifications, some "snapshot profile" of the in vivo proteome, exploring some disease pathogenesis at the molecular level.

There is also the "middle-down proteomics" that is applied to the analysis of big protein fragments. It is a method between "bottom-up" and "top-down" approaches. This technique can give us some new information about the localization of post-translational modifications.

Crucial parameters about protein/peptide analysis by CE-MS were mentioned in previous chapters (interfaces, capillaries, online sample preparation). In the next section, only some new important applications will be described.

3.1 | Bottom-up protemic and peptide analysis

As was mentioned previously, the bottom-up technique is the most common approach in proteomics. In this technique, a mixture of peptides arising after cleavage (enzymatic and/or chemical) is separated by separation methods and analyzed by tandem MS. For many years the "gold" standard for separation techniques was HPLC, namely reversed-phase. However, a good alternative can be separation by capillary electrophoresis. As is often mentioned, CE and HPLC are not competitive but are complementary techniques. Frankly, we have to mention some certain CE restrictions—lower sample loading, shorter separation windows—leading to a smaller number of MS spectra and therefore of protein identification. However, the CE, as well as new electrospray interfaces, were developed (see previous chapters) and in combination with high-speed mass spectrometers (such as TOF and Orbitrap), it allows improved CE-MS protein/peptides analysis.

Generally speaking, we can attribute that slower separation (longer separation window) can be achieved using neutral-coated capillaries and BGE at low pH and high concentration (such as 10% acetic acid or 1 M formic acid). Under these conditions, EOF is significantly reduced and separation of positively charged peptides is slowed. Another benefit of this approach is that the sticking of peptides on the capillary is minimized. In the case of using a sheath-liquid interface, sheath liquid differs from BGE and consists mainly of acidic water–organic solvent system (e.g. 10% methanol with 0.5% acetic acid).

As was mentioned previously, coated capillaries are suggested for analysis of proteins and peptides. However, many studies also used uncoated capillaries (fused-silica capillaries) for these separations. For example, it was described using uncoated capillary (bare fused-silica capillaries (total length 91 cm; 30 μm id; BGE was 10% acetic acid)) for analysis of a limited quantity (100 ng) of yeast mitochondria [54]. In this analysis, a sheathless interface with capillary porous tip was used. Run time was 60 min. Total of 300 proteins were identified by CE-MS (1765 unique peptides) and 271 proteins (976 unique peptides) by nanoLC-MS. Overall, 349 proteins (78 only by CE-MS, 49 only by nanoLC-MS, 222 by both techniques) were identified, and 2177 peptides (1201 only by CE-MS, 412 only by nanoLC-MS, 564 by both techniques). So, this approach demonstrates the complementary of both techniques. CE-MS enabled identification of larger peptides and detected those having extreme p*I*.

CE-MS and HPLC-MS methods were also used for analysis of insoluble proteins in an avian eggshell matrix. Eggshell was divided into four distinct layers (cuticle, two palisades, and a mammillary layer), chemically and/or enzymatically split with CNBr/trypsin and proteinase K and analyzed by CZE and HPLC online coupled to MS detection. The CZE analysis was done with a fused-silica capillary of 100 cm total length and 75 µm id with 0.25 M formic acid (pH 2) as BGE. The CE-MS interface was the sheath-liquid type (sheath liquid was 5 mM ammonium acetate/2-propanol 1:1, v/v, at a flow rate of 3 µL/min). Five main proteins were identified (ovocleidin-116, ovocalyxin-32, ovocalyxin-36, ovocleidin-17, and ovalbumin). The CE-MS peptide maps of eggshell proteins were compared with the HPLC-MS ones, and it was demonstrated that both methods have a different mechanism of separation (migration/elution order) [55].

As was mentioned previously, sample enrichment is also a frequently mentioned task mainly in the analysis of a mixture of peptides. Often used methods are various stacking approaches, such as dynamic pH junction (see the chapter about Separation tasks). An interesting method uses a SPE cartridge at the inlet tip of the separation capillary [48]. For example, a detachable SCX monolith, integrated with capillary zone electrophoresis and coupled with pH gradient elution, is also described. This approach was successfully applied for analysis of tryptic digest of Xenopus laevis zygote homogenate giving a signal of 1274 peptides within 40 min [51]. The separation was done at linear polyacrylamide-coated capillary (60 cm long, 50 µm id) with BGE consisting of 50 mM formic acid. The interface CE-MS was sheath-liquid with 10% methanol (with 0.1% formic acid).

To improve protein coverage it is, besides analysis by LC and CE separately, a good choice to couple techniques with different (orthogonal) mechanisms. From the view of peptide analysis, it is coupling LC (mainly reversed-phase) and CE. This approach is useful mainly for the analysis of complex proteomes. Of course, the simplest way to make offline combination (online coupling could be a more effective technique but technically it is not simple to develop the necessary apparatus. Chen et al. [56] analyzed mouse brain proteome digest after a prefractionation by RPLC (60 fractions collected and combined into 15 or 30 fractions). Each fraction was analysed by CE-MS using linear polyacrylamide-coated capillary (96 cm long, 50 µm id) when samples were stacked by dynamic pH junction and separated at 5% v/v acetic acid. MS was coupled by the sheath-liquid interface (0.2% formic acid in 10% methanol, both v/v) when the run time was 140 min (i.e. separation window). The benefit of a dynamic pH junction-based CZE–MS system was demonstrated by analysis of 500 nL and 50 nL samples when the weight of the injected sample was the same (see Figure 3). In this case, the analysis of the 0.4 mg/mL sample with 500 nL injection volume generated significantly higher numbers of protein and

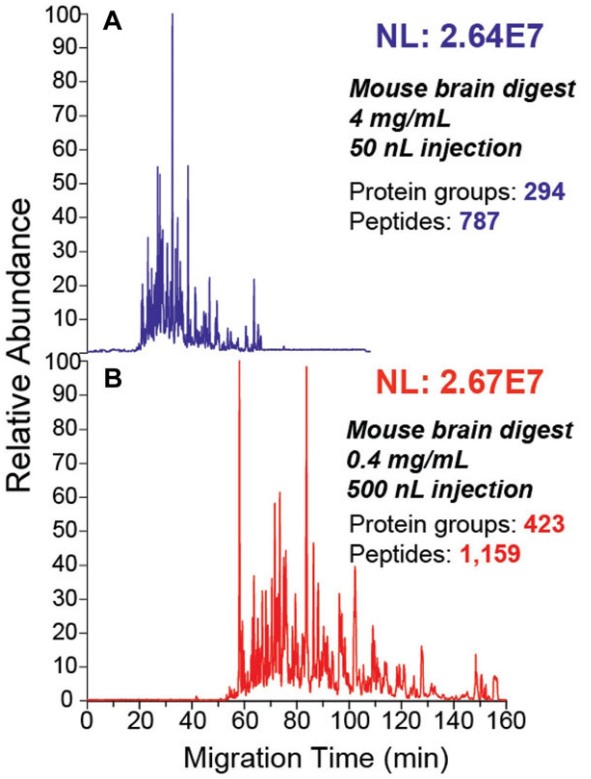

FIGURE 3 Electropherograms of the mouse brain proteome digests in 10 mM NH_4HCO_3 (pH 8) after CZE–MS analysis. (A) 4 mg/mL of the mouse brain proteome digest with 50 nL injection volume; (B) 0.4 mg/mL of the mouse brain proteome digest with 500 nL injection volume. Separation conditions: linear polyacrylamide-coated capillary (length 96 cm, 50 µm id), BGE 5% acetic acid, sheath liquid 10% methanol with 0.2% formic acid v/v in methanol. Republished with permission of Royal Society of Chemistry, from [56] (2017); permission conveyed through Copyright Clearance Center

peptide IDs than the analysis of the 4 mg/mL sample with 50 nL injection volume (423 versus 294 protein groups and 1159 versus 797 peptides). This CE-MS method was suitable for identification of 1600 and 3000 protein groups identified from 50 and 500 µg of protein digests, respectively. The same group extended this work more deeply in a combination of LC and CE methods [57]. They developed an SCX-reversed-phase LC-CZE-MS platform for deep bottom-up proteomics. This technique enables identification of around 8200 protein groups and 65 000 unique peptides from a mouse brain proteome digest in 70 h when the peak capacity of this orthogonal SCX-RPLC-CZE platform was around 7000. The result was in principle the same as was obtained in combination 2D-LC-MS/MS (8200 versus 8900 protein groups, 65 000 versus 70 000 unique peptides). Peptides were separated by SCX-RPLC into 60 fractions (after a 3-salt step experiment) or 40 fractions (after a two-salt step experiment). Fractions were analyzed at 92-cm long linear polyacrylamide-coated capillary (50 µm id) by BGE consisting of 5% acetic acid and using

a sheath-liquid interface for coupling to MS (sheath-liquid buffer was 0.2% formic acid containing 10% methanol, v/v).

Capillary electrophoresis also has a high possibility for study and analysis of posttranslational modifications of proteins/peptide. These modifications can alter the electrophoretic mobilities of peptides. The identification (characterization) and quantification of individual PTMs is fundamental for understanding the role and function of proteins within intracellular and extracellular environments. Nowadays, the most studied PTMs are glycosylation (enzymatic and nonenzymatic—glycation) and phosphorylation. These modifications are connected with various stages of ageing, as well as with different pathological states.

Detection and characterization of glycoproteins and glycopeptides is not only a challenge for analytical scientists but their biomedicine and biotechnology (incl. biomarkers) aspects are really important. The sugars are rather voluminous and significantly influence the ion radius; moreover, the presence of sialic acids (with a pK_a of 2.6) in the glycan structure further modifies the electrophoretic mobility of the peptides. The CE-MS technique allowed the baseline separation of α2,3- and α2,6-sialylated IgG Fc glycopeptides without previous sample derivatization [58]. These separations were done at bare fused-silica capillary (90 cm long) using 10% acetic acid as BGE when the interface to MS was sheathless (separation window was 60 min). The method was applied to the analysis of glycopeptides of prostate-specific antigen and identified 75 glycopeptides. The same group of authors also developed a method for CE-MS analysis based on enhancing ionization efficiency and spray stability in electrospray ionization by enrichment of the gas with an organic dopant. They used the CE-MS system with a sheathless interface for glycopeptide analysis [59]. The obtained up to 25-fold higher sensitivities for model glycopeptides.

There was also described analysis of a glycation reaction, when the exact determination of arising monotopic product, Nε-(carboxymethyl)-lysine, at bovine serum albumin and collagen type I was described [60,61]. In this approach, CZE separation was used when coupling with ion-trap MS was done by the sheath-liquid interface. It is necessary to mention that analysis of collagens is often complicated due to their rigid structure and cleavage resistance (for review see [62], when a common procedure for analysis of glycated proteins is also reviewed [63,64]).

Phosphorylation is another important and common PTM of proteins/peptides. This modification affects their electrophoretic mobilities and results in either one or two extra negative charges depending on the pH of the BGE. Capillary electrophoresis is an excellent method for investigation of many modifications, such as asparagine deamidation, aspartate isomerization, arginine citrullination, phosphopeptide isomers [65] as well as analysis of phosphopeptides, and acetylated, methylated and nitrated peptides [66]. The presence of deiminated Arg (at positions 3 and 17 of histone H4) was possible to detect by MS/MS analysis with electron transfer dissociation fragmentation [65]. Moreover, based on CE-MS, isobaric mono-phosphorylated peptides obtained in the course of a kinase activity study were separated and individual positional isomers quantified. There were also investigated variously modified separation capillaries as a factor significantly affecting the analysis of the PTM of peptides [66]. Using neutrally coated capillary led to the highest overall signal intensity of singly modified peptides and separation selectivity. Bare fused silica capillary was the best choice for identification of multiply phosphorylated peptides (see Figure 4). However, the combination of results obtained by CE-MS and nanoLC-MS are complementary and the combination of both methods together gives a higher level of information—from identified 8143 phosphopeptides (PC-12 pheochromocytoma cells) 38.5% were determined by CE-MS only, 30.2% by nanoLC-MS only and only 31% were identified by both methods [66].

The diagonal CE coupled to MS was described as a suitable method for accurate determination of peptide phosphorylation stoichiometry [67]. Diagonal capillary electrophoresis is a 2D separation method when an immobilized alkaline phosphatase microreactor is present at the end of the first capillary. At the first dimension, a mixture of the phosphorylated and unphosphorylated forms of a peptide is separated. The resulting fractions were dephosphorylated in the reactor. The products were transferred to the second capillary and analyzed by MS. Both capillaries were bare fused silica 25 cm length. The phosphorylated and unphosphorylated peptides differ in charge so they were separated in the first-dimension separation. After the reaction, both peptides (unphosphorylated and dephosphorylated) peptides were identical with the same mobility, and phosphorylation stoichiometry can be determined by the ratio of the signals of the two forms [67].

Highly hydrophobic proteins are traditionally analysed by reversed-phase HPLC. If we are talking about capillary electromigration methods, in principle we can use the micellar electrokinetic chromatographic technique, however, the combination of this method with MS has some limitations because the surfactants used are not volatile. Cheng and Chen described another approach—nonaqueous CE coupled with MS [68]. The BGE consists of 20% acetonitrile, 78% methanol and 2% formic acid, with 20 mM ammonium formate. Separation was done with a fused-silica capillary (72 cm length, 50 μm id). This method makes it possible to separate (and identify) a mixture of highly hydrophobic temporin peptides, which was separated in 12 min (see Figure 5).

3.2 | Top-down proteomics

Capillary electrophoresis in combination with MS has a great potential for protein analysis of entire proteins. In

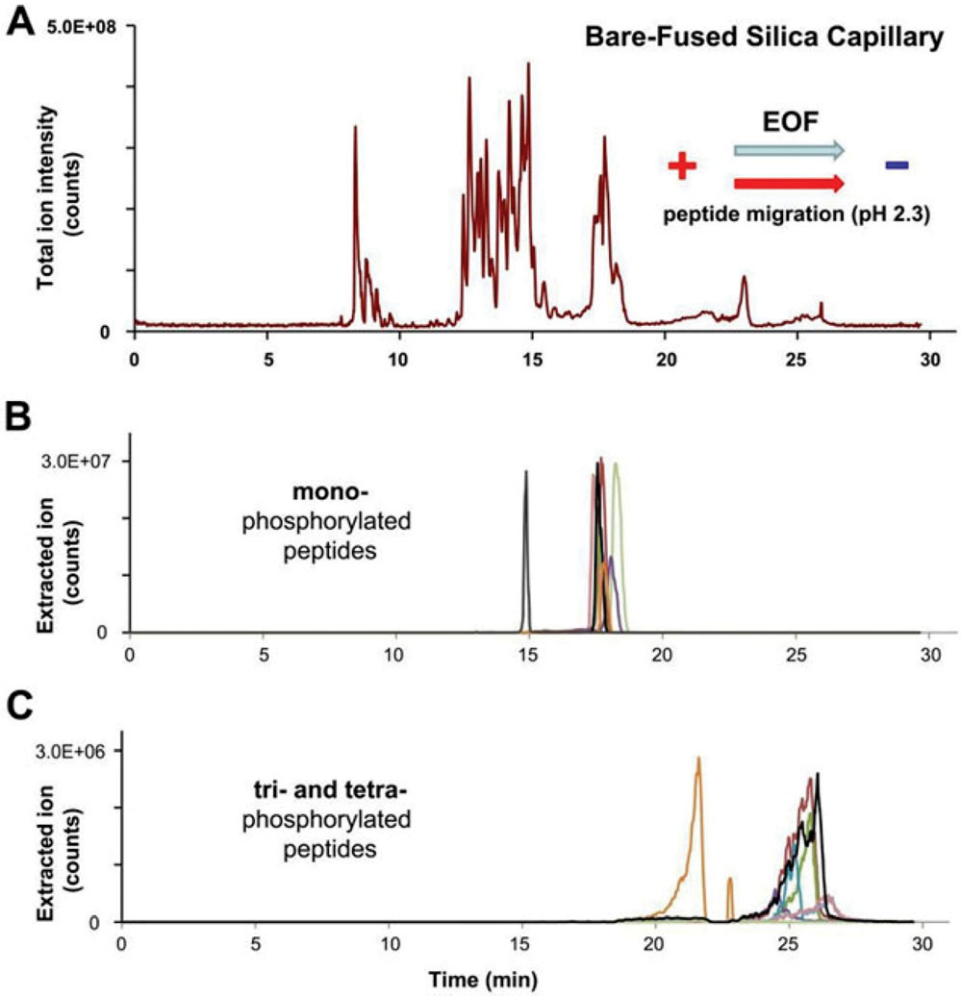

FIGURE 4 CE-MS separation of posttranslational modified synthetic peptides using a bare fused silica capillary. (A) Total ion current electropherogram and extracted ion electropherograms of (B) monophosphorylated and (C) tri- and tetra-phosphorylated peptides. CE conditions: Separation capillary length: 100 cm with porous tip, id: 30 µm; BGE: 10% v/v acetic acid; separation voltage: +30 kV. Reprinted with permission from [66] Copyright 2018 John Wiley and Sons

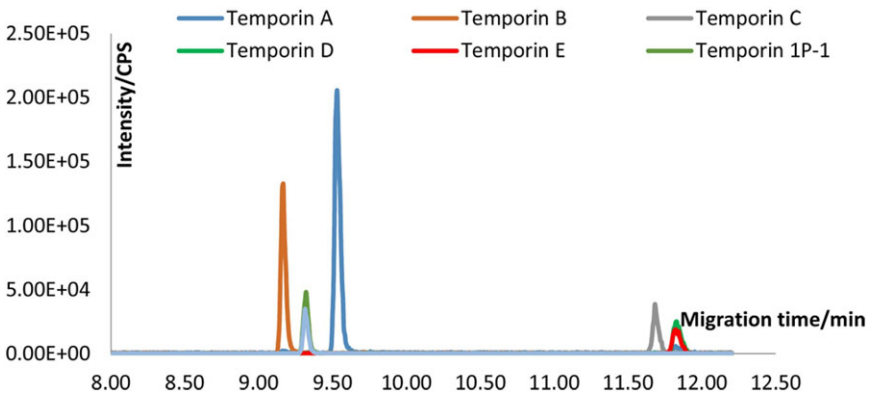

FIGURE 5 Nonaqueous CE-MS separation of highly hydrophobic peptides. Bare fused silica capillary (72 cm length, 50 µm id); separation voltage: 30 kV; BGE: 78% methanol, 20% acetonitrile, and 2% formic acid with 20 mM ammonium formate; modifier: ethanol with 2 mM ammonium formate, flow rate at 2.0 µL/min. Temporins D and E are not separated. Reprinted with permission from [68]. Copyright 2018 John Wiley and Sons

comparison to the widely used LC-MS, CE-MS has an advantage in its high resolution. The most important feature is to couple CE with high scanning rate (for narrow CE peaks) of high-resolution MS (such as Orbitrap). For coupling of these techniques, either sheath-liquid or sheathless interfaces can be used. In the analysis of intact proteins, the problem of sticking/absorption to the capillary wall is really important. For this reason, static or dynamic-coated capillaries are used when separation is done at acidic BGE, usually containing 5–10% of organic solvent. Good information about separation

and analysis optimization (such as capillary coating, background electrolyte, sheath liquid, and nebulizer gas pressure) for analysis of small- to medium-sized proteins are described in the paper by Taichrib et al. [69].

The identification of 580 proteoforms from yeast was described [70]. Of course, it was impossible to make this analysis by a single analysis. First, the yeast proteome was separated into 23 fractions by RP-LC. These fractions were analyzed by CE-MS using an electrokinetically pumped sheath-flow interface (sheath liquid composed of 10% methanol, 0.5% formic acid, v/v). The capillary was coated by linear polyacrylamide and the BGE was 5% acetic acid. This approach enabled identification of 580 proteoforms and 180 protein groups, and an additional 3243 protein species were detected.

The comparison of separation methods is really interesting. Klein et al. [71] compared CE-MS with reversed-phase nanoLC-MS. They identified 905 unique peptide sequences with high confidence, 50% of those were identified only with LC, 20% only with CE and 30% with both techniques. The advantage of the CE technique was in the identification of small and highly charged peptides, likely unable to bind to the reversed-phase LC column. LC provides better identification of hydrophobic peptides. So, this analysis can serve as a well-documented example that both methods are complementary and using both methods can serve to obtain better results with increased coverage.

A top-down CE-MS to discover disease biomarkers in biofluids has also been described [72–74]. The arrangement of the equipment is simple. For CE separation, uncoated capillaries are used, the BGE is formic acid containing 20% acetonitrile v/v. At the review of the clinical application of CE-MS proteomic/peptidomic [73] the importance of sample preparation when desalting is highlighted as being really important for correct analysis.

4 | CONCLUSIONS

Electrophoretic methods are historically connected to the analysis of proteins. So, it is not surprising that capillary electromigration methods as well as connection to MS are well suited for peptide and protein analysis (proteomic research). CE methods have a big advantage with their high efficiency (resolution) separation; however, the disadvantage can be in the small amount of introduced sample. The main future tasks in the analysis of peptides/proteins can be sample preparation (online and/or offline) and developing a highly sensitive routine and robust CE-MS interfaces. Two-dimensional approaches will also be greatly welcome. However, we can conclude that CE-MS for protein/peptide analysis is a well-described technique that is complementary (not competitive) to the often used LC-MS methods.

ACKNOWLEDGMENTS

This work was supported by the Czech Science Foundation (Grant No. 17–10832S), and support for the long-term conceptual development of research organization RVO: 67985823.

CONFLICT OF INTEREST

The author has declared no conflict of interest.

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How to cite this article: Mikšík I. Coupling of CE-MS for protein and peptide analysis. *J Sep Sci* 2019;42:385–397. https://doi.org/10.1002/jssc.201800817