



Universiteit
Leiden
The Netherlands

Exploring the proteome by CE-ESI-MS

Heemskerk, Anthonius Adrianus Maria

Citation

Heemskerk, A. A. M. (2016, April 28). *Exploring the proteome by CE-ESI-MS*. Retrieved from <https://hdl.handle.net/1887/38868>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/38868>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/38868> holds various files of this Leiden University dissertation

Author: Heemskerk, A.A.M.

Title: Exploring the proteome by CE-ESI-MS

Issue Date: 2016-04-28

Chapter 4

CE-MS for proteomics: advances in interface development and application (2007-2011)

Anthonius A.M. Heemskerk*, Rawi Ramautar*, Paul J. Hensbergen, André M. Deelder, Jean-Marc Busnel, Oleg A. Mayboroda

Journal of Proteomics, 2012, 75 (13), 3814–3828

* Authors have equal contribution

Abstract

Capillary electrophoresis-mass spectrometry (CE-MS) has emerged as a powerful technique for the analysis of proteins and peptides. Over the past few years, significant progress has been made in the development of novel and more effective interfaces for hyphenating CE to MS. This review provides an overview of these new interfacing techniques for coupling CE to MS, covering the scientific literature from January 2007 to December 2011. The potential of these new CE-MS interfacing techniques is demonstrated within the field of (clinical) proteomics, more specifically “bottom-up” proteomics, by showing examples of the analysis of various biological samples. The relevant papers on CE-MS for proteomics are comprehensively summarized in tables, including, e.g. information on sample type and pretreatment, interfacing and MS detection mode. Finally, general conclusions and future perspectives are provided.

1 Introduction

One of the main goals of proteomic studies is to achieve the highest possible proteome coverage. To this end, a multitude of multidimensional strategies have been evaluated during the last decade [1], with the main goal of introducing extra selectivity at various stages of the proteomic workflows so that the highest peak capacity and ultimately the most comprehensive analysis can be performed. In this context, miscellaneous separation/fractionation techniques, either chromatographic (ion exchange, size exclusion chromatography, reversed-phase liquid chromatography (RPLC) at different pH conditions, etc.) or electrophoretic (SDS-PAGE, iso-electric focusing (IEF), etc.) have been implemented as a first separation or fractionation step. However, reversed-phase liquid chromatography-mass spectrometry (RPLC-MS) is still the core technology for peptide and protein identification and it certainly will remain so in the foreseeable future [2-7]. For an update of the state-of-the-art of LC-MS for proteomics we refer to the review of Di Palma et al. in this issue of the Journal of Proteomics.

As the preferred final separation step in proteomic workflows RPLC offers numerous advantages, but as any analytical technology it has its limits. The most important ones are related to its separation mechanism – RPLC is a clearly suboptimal technique for small but also for rather large peptides, as these peptides are either not retained or irreversibly retained on conventional reversed-phase columns. Furthermore, at the flow rates at which RPLC is commonly operated, acidic and/or hydrophilic peptides are most vulnerable to ion suppression phenomena [8-10]. Finally, miniaturization of the LC-MS proteomics workflow is not an easy task and even the miniaturized workflows require at least 104 cells[11], which can be considered as a limitation when the analysis of really volume/material restricted samples is required [5].

In this review, we will concentrate on a different technology – namely, capillary electrophoresis–mass spectrometry (CE-MS). In CE, charged compounds in a fluid are separated from each other on the basis of their charge-to-size ratio under the influence of an electrical field. The first CE experiment in a fused-silica capillary with an internal diameter of 75 μm and a length of 100 cm was performed by Jorgenson and co-workers in 1981[12]. Since then, CE has emerged as a highly efficient separation technique and has, for example, played a central role in the deciphering of the Human Genome [13].

Nowadays, CE is considered as a powerful technique for the analysis of peptides as it exhibits a selectivity that is based on charge-to-size ratio differences and, therefore, in

comparison to RPLC, CE can provide complementary information on the composition of a biological sample. Furthermore, CE separations are highly efficient, relatively inexpensive (simple fused-silica capillaries vs. LC columns) and requires only small amounts of sample and reagents. As a result, the coupling of CE and MS is very attractive as it combines high-resolution separations with high detection selectivity and sensitivity. Still, the use of CE-MS in proteomics has lagged behind LC-MS. This might be due to the reduced migration time reproducibility which is occasionally observed as a result of sample-induced variations in electro-osmotic flow (EOF) often in combination with the use of non-appropriate washing conditions between runs and/or the poor concentration sensitivity compared to RPLC-MS.

However, since January 2007, a considerable number of papers have been published which report on the development and application of CE-MS for proteomics studies using electrospray ionization (ESI) and matrix assisted laser-desorption ionization (MALDI) ionization. In this review, we will summarize these studies and pay specific attention to emerging technological developments mainly related to the use of new interfaces for coupling CE to MS, enabling new possibilities for proteomics studies. We will focus on CE-MS-based bottom-up proteomics, i.e. the analysis of (complex) peptide mixtures obtained after enzymatic digestion of the protein sample. CE-MS for top-down proteomics, which concerns the analysis of intact proteins, is not covered in this review. Special attention is devoted to the advantages of CE-MS over RPLC-MS, e.g., operation at ultra-low flow-rates, capacity for miniaturization, and separation of polar and charged peptides, which often are poorly retained on RPLC columns. Selected illustrative examples are discussed in detail and some general conclusions and future perspectives are provided.

2 CE-MS methodology and applications

2.1 General aspects

CE has emerged as a highly efficient separation technique and, in effect, is used in a number of separation modes, such as capillary zone electrophoresis (CZE), normally referred to as “CE”, capillary electrochromatography (CEC), micellar electrokinetic chromatography (MEKC) and capillary iso-electric focusing (cIEF). In MEKC micelles are used as pseudo-stationary phases in the BGE allowing the simultaneous separation of neutral and charged compounds. However, the coupling of MEKC to MS is not straightforward and sensitivity of this combination remains limited. In cIEF, amphoteric

compounds, such as peptides, are separated according to their iso-electric point (pI) in a pH gradient formed by a mixture of carrier ampholytes (CAs) under the influence of an electric field[14]. After this so-called focusing step, the mobilization step is used to transfer the focused analytes toward the detector. Various strategies can be employed here but the most popular are the hydrodynamic and the chemical mobilizations, each providing various benefits. Major advantages of cIEF are related to its very high resolution capabilities and to the fact that this is a very efficient pre-concentration technique where the full separation capillary is initially filled with the sample to separate. In the scope of using it in conjunction with MS, the main limitation is the low volatility of the CAs (see section 2.2.2 for more details).

Detection in CE is most often based on on-column UV absorbance as it is easy to implement and it is widely applicable. However, it lacks both sensitivity, caused by the small optical path length as a result of the capillary diameter, and selectivity, as UV detection hardly provides any structural information of the analytes. For that reason, mass spectrometry has become increasingly popular as detection technique for CE. However, the coupling of CE to MS is not straightforward as a closed electrical circuit is required to maintain the high voltage across the capillary necessary for CE separation. Consequently, a CE–MS interface should provide a means to apply a voltage to the capillary outlet while insuring the independence of each of the electrical circuits (CE and ESI). The hyphenation of CE with MS started circa two decades ago, when Smith and co-workers introduced the first working interface[15]. Since then, research on this topic has been very active resulting in the development of various interfaces for several types of ionization sources[16-18].

For MS-based proteomics, ESI and MALDI are the two most widely used ionization methods. As different as they are, both ionization methods share one common trait, namely the ability to generate ions from large nonvolatile species without significant fragmentation. The hyphenation of CE and ESI-MS, however, appears to be more straightforward than CE and MALDI [19, 20]. With regard to CE-MS coupling, the composition of the BGE is the main constraint. The combination of CE and ESI-MS is compatible as both techniques are very well suited to compounds that can form ions in solution; yet if high buffer concentrations, nonvolatile constituents and/or surfactants are used as BGE, ion suppression effects are non-avoidable. Moreover, nonvolatile constituents may cause source contamination and high background signals. Therefore, only volatile BGEs like formic acid or acetic acid at low-pH (<3) conditions (most peptides migrate then toward the MS) using ESI in positive ionization mode are typically used for

CE--MS-based proteomics studies [21-24].

Method stability is of pivotal importance for a proteomics workflow. In CE adsorption of matrix components and/or analytes to the capillary wall may cause changes of the EOF which, consequently, leads to negative effects on the migration time reproducibility. Especially, when bare fused-silica (BFS) capillaries are used, separation efficiencies may be compromised as a result of adverse analyte–capillary wall interactions [25, 26]. Under these circumstances, it is important to implement systematic and rather extensive/harsh washing conditions between consecutive runs to allow the analysis of biological samples with minimal sample pretreatment. Another way to address this problem is to modify the inner capillary walls of BFS capillaries with polymers, either neutral or positive ones. This approach has been successfully applied in various CE-MS-based proteomics studies [25-27]. Particular attention has to be given to the stability of the coating under the used experimental conditions as any leakage of the used polymer toward the MS can usually not be tolerated. For this reason, covalent/permanent, rather than non-covalent/dynamic coating is preferred[28].

In the following sections, recent developments in CE-MS interfacing are discussed and exemplified for proteomics studies. As stated in the Introduction, only applications will be covered which concentrate on the use of CE-MS methodologies for bottom-up proteomics studies. This definition implies the analysis of (complex) peptide mixtures obtained after enzymatic digestion of the protein sample. Therefore, this section will not include the analysis of intact proteins which has been comprehensively reviewed by Haselberg et al. [29].

2.2 Sheath-liquid CE-MS

2.2.1 Coaxial interfacing

CE can be coupled to MS via a sheath-liquid or a sheathless interface, but for a variety of reasons the sheath-liquid interface has been so far the most widely used for CE–ESI-MS in proteomics studies [30-32]. Sheath-liquid interfaces use a coaxial sheath-liquid that mixes with the CE effluent. The aim of the sheath-liquid is to provide electrical contact between the CE separation and the ESI source. The sheath-liquid interface was first developed by the group of Smith[15]. In this configuration, the separation capillary is inserted in a tube of larger diameter in a coaxial setting (Figure 4-4). The conductive sheath-liquid, to which the CE terminating voltage is applied, is administered via this

outer tube and merges with the CE effluent at the capillary outlet. In most configurations, a gas flow is applied via a third coaxial capillary in order to facilitate the desolvation process. The sheath-liquid can be used to optimize the ESI process and, therefore, the composition and flow rate of the sheath liquid are critical parameters in optimizing the overall performance of the sheath-liquid CE-MS method. The sheath-liquid is often composed of a mixture of water and organic modifier, such as methanol or isopropanol, containing a volatile acid, such as formic or acetic acid. The operating flow rates in CE are usually between 20 and 100 nL/min, while the sheath-liquid flow rate typically ranges from 1 to 10 μ L/min. Thus, the flow rates of the sheath-liquid are at least one order of magnitude higher than the intrinsic CE flow rates which results in compromised ionization efficiencies and sensitivities of the analytes [24, 33, 34]. Additionally, the sheath-liquid can, to some extent, influence the peak efficiency. It has been shown that while the EOF presents a flat profile, the addition of both sheath-liquid and sheath-gas introduces a parabolic flow component which decreases the achievable peak efficiency [25, 35, 36]. The application of a counter pressure at the inlet of the separation capillary has been proposed to reduce this effect [25, 35, 36]. In general, the sheath-liquid interface can be considered as a fairly robust platform. This and the fact that sprayers are commercially available together might explain why the coaxial sheath-liquid design is so commonly used in CE-MS-based proteomics [37, 38]. An overview of CE-MS-based proteomics studies using a sheath-liquid interface over the period from January 2007 to December 2011 is given in Table 4-1. Selected examples are discussed below.

Catai et al. demonstrated that the use of a sheath-liquid interface in CE-MS for peptide analysis may cause some extra band broadening when compared to the results obtained with CE-UV [25]. The observed band broadening for the peptides was caused by both the hydrodynamic capillary flow induced by the nebulizer gas and the data acquisition rate of the ion trap mass spectrometer used. High separation efficiencies for peptide analysis could be obtained by cancellation of the hydrodynamic flow caused by the nebulizer gas and by using a sufficiently high-data acquisition rate.

CE coupled to TOF-MS via a sheath-liquid interface was used for the analysis of O- and N-glycopeptides of a tryptic digest of recombinant erythropoietin by Gimenez et al.[39]. The adsorption of glycopeptides to the inner surface of a bare fused-silica capillary wall was prevented by rinsing the capillary with 1 M acetic acid between runs. By using this washing procedure, RSDs (%) of migration times improved from ~3% to ~0.5%. It was demonstrated that the presence of reference compounds in the sheath-liquid had a detrimental effect on the detection of the peptides and glycopeptides of the protein digest

and as such the authors suggested that depending on the complexity of the sample to be analyzed, the use of references in the sheath-liquid can be inconvenient for the detection of the analytes of interest by CE-TOF-MS.

Elhamili et al. evaluated monoquaternarized piperazine, 1-(4-iodobutyl) 4-aza-1-azoniabicyclo[2,2,2] octane iodide (M7C4I) as a surface derivatization reagent for CE in combination with TOF-MS for the analysis of protein digests [26]. The M7C4I piperazine, at alkaline pH, forms a covalent bond via alkylation of the ionized silanols producing a cationic surface with a highly stable and reversed EOF. The obtained surface yielded fast separations (<5 min) of peptides at acidic pH with high separation efficiencies (up to 1.1×10^6 plates/m) while no bleeding of the coating reagent was observed into the MS instrument. The potential of CE-TOF-MS using M7C4I-coated capillaries was demonstrated for the analysis of protein digests.

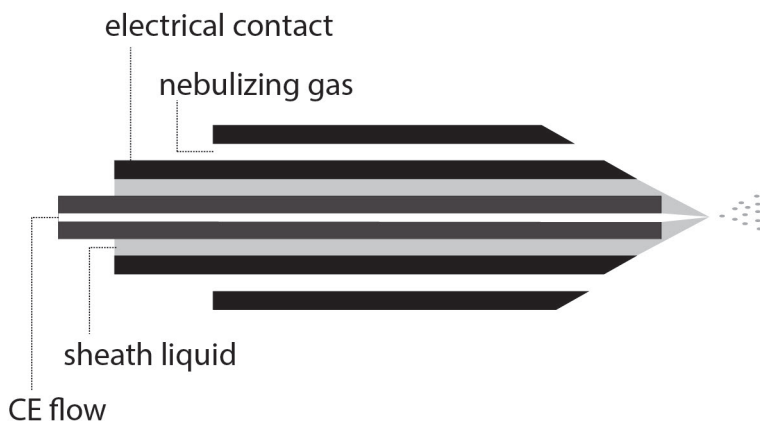


Figure 4-4: Schematic depiction of the coaxial sheath-liquid design as published by Smith and coworkers[15]

Tempels et al. developed an on-line SPE-CE-MS system for the analysis of peptides. Analytes were preconcentrated on a C18 microcolumn (5 x 0.5 mm id), which were then introduced into the CE system via a valve interface [40]. The CE system with a Polybrene-poly(vinylsulfonate) bilayer coated capillary was combined with an ion-trap MS via ESI using a co-axial sheath-liquid sprayer. The on-line coupling of the SPE and CE step by the valve interface allowed an independent functioning of the system parts. LODs for selected peptides were in the range of 1.5-3 ng/mL. The potential of the on-line SPE-CE-MS system was demonstrated by the analysis of a cytochrome C digest. However, some hydrophilic peptides did not show sufficient retention on the SPE column, and were lost during pre-concentration.

2.2.2 Liquid-junction interfacing

Another approach to couple CE to MS is via a liquid-junction interface, which was first reported by Henion and co-workers [41]. In a liquid-junction interface, the CE capillary and ESI emitter are separated by a small gap in which the CE effluent mixes with a sheath-liquid before entering the ESI emitter [34, 41, 42]. The mixing of the sheath-liquid with the CE effluent serves two purposes. The first is modifying the mixture of the BGE to make it more compatible with ESI-MS analysis. Secondly, and most importantly, it is used to create the closed electrical circuit required for CE separation. There are different solutions for the placement of the electrode required for a closed CE circuit and application of the ESI voltage. For example, the group of Dovichi used coated separation capillary ends [42] and sheath-liquid application [43-45] from a grounded vial (Figure 5-1 on page 96), while the group of Chen used a stainless steel ESI emitter as both ESI and CE electrode. The flow-rates of the sheath-liquid used in liquid-junction designs are typically in the range of 60 to 200 nl/min, which is significantly lower than the flow-rate used in coaxial sheath-liquid interfaces. The group of Dovichi recently described an interface based on electrokinetic pumping of the sheath-liquid and capable to operate in the nano-electrospray regime [44]. As a result, the system provides great flexibility in separation buffers, and allows the use of uncoated and inexpensive capillaries for separation. Low attomole range (high picomolar concentration range) LODs for selected peptides were reported.

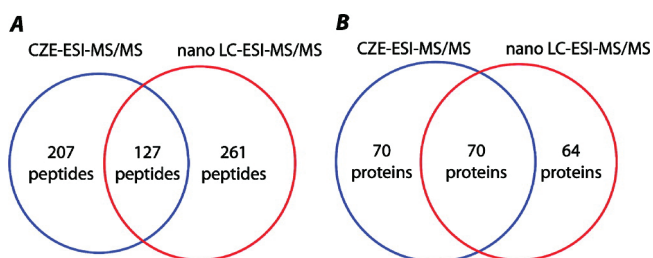


Figure 4-5: Venn diagram illustrating the overlap of peptides and proteins identified by both CZE-ESI-MS/MS and UPLC-ESI-MS/MS at 95% confidence (FDR < 0.15%): (A) peptides, (B) proteins. Taken from [45].

Recently, Li et al. evaluated the potential of CE coupled via an electrokinetically pumped sheath-liquid interface to ESI-MS for the analysis of a tryptic digest of a sample of intermediate protein complexity, the secreted protein fraction of *Mycobacterium marinum* [45]. For CE analysis, 11 fractions were generated from the sample using RPLC; each fraction was analyzed by CE-ESI-MS/MS, and 334 peptides corresponding to 140 proteins were identified in 165 min. In comparison, 388 peptides corresponding to 134

proteins were identified in 180 min by triplicate UPLC-ESI-MS/MS analyses, each using 250 ng of the unfractionated peptide mixture. Overall, 62% of the peptides identified with CE-ESI-MS/MS and 67% of those identified with UPLC-ESI-MS/MS were unique (Figure 4-5). CE-ESI-MS/MS favored basic and hydrophilic peptides with low molecular masses. Combining the two data sets increased the number of unique peptides by 53%.

Until now, only a few proteomic studies have been carried out with CE-MS using liquid-junction interfacing. However, the results obtained so far are promising and certainly will stimulate further development.

2.3 Sheathless interfacing

Sheathless interfacing is, in principle, the most straightforward way to couple CE to ESI-MS as the fundamental property of CE, i.e. the intrinsically low flow-rate, is used in its most optimal way. Therefore, various sheathless interfaces have been developed to enhance the concentration sensitivity of CE-MS [24, 46-51]. In the sheathless interface configuration, the CE voltage is directly applied to the CE buffer at the capillary outlet. This can be achieved by applying a metal coating to the end of a tapered separation capillary or by connecting a metal-coated, full metal or conductive polymeric sprayer tip to the CE outlet. Another way to make a closed circuit is by insertion of a metal micro-electrode through the capillary wall into the CE buffer end or by direct introduction of a micro-electrode into the end of a CE capillary [47]. While most of these interfaces have been proven to provide very high performances, mainly in terms of sensitivity, the main bottleneck has been the manufacturing complexity or the long-term stability of the sprayers. Concerning the latter parameter, the main limitation of, for example, metal coated tips is related to the stability of the metal coating which, upon operation, may degrade significantly till a point where the electrical contact is lost. A clogging is another frequent issue of these approaches, especially if a tapered tip used.

Moini developed a sheathless interface design consisting of a CE capillary with a porous tip as nanospray emitter[24]. The porous tip, being the outlet of the separation capillary, approach is very straightforward and does not require any fluidic connection. In this configuration, the properties of the glass portion of the capillary which has been etched with hydrofluoric acid provide electrical contact between the lumen of the CE capillary and the outside without the need for any liquid flow through the pores of the porous tip. To operate this interface, part of the porous portion of the tip is inserted in a buffer reservoir on which the electrical connections are being made. Besides the relative simplicity of the

approach, the major attributes of this strategy are the use of non-tapered tips (30 μm ID) and the displacement of the electrolysis reactions outside from the separation capillary, which are significant advantages, as compared to previous sheathless interfaces. This interface was used for the identification of proteins in complex mixtures and the separation of protein–protein and protein–metal complexes of erythrocytes, showing its potential for proteomic studies [24, 52, 53].

Based on the sheathless interfacing concept of Moini, a prototype high-sensitivity porous sprayer (HSPS) sheathless interface for CE–ESI–MS was recently developed by Beckman Coulter (Figure 5-2 on page 99). Busnel et al. used this novel approach for the analysis of tryptic digests of bovine serum albumin (BSA) and *E. coli*. [54]. It was found that the interface is capable of generating a stable spray with flow-rates ranging from below 10 nL/min to >340 nL/min, enabling its use in either the mass or concentration-sensitive

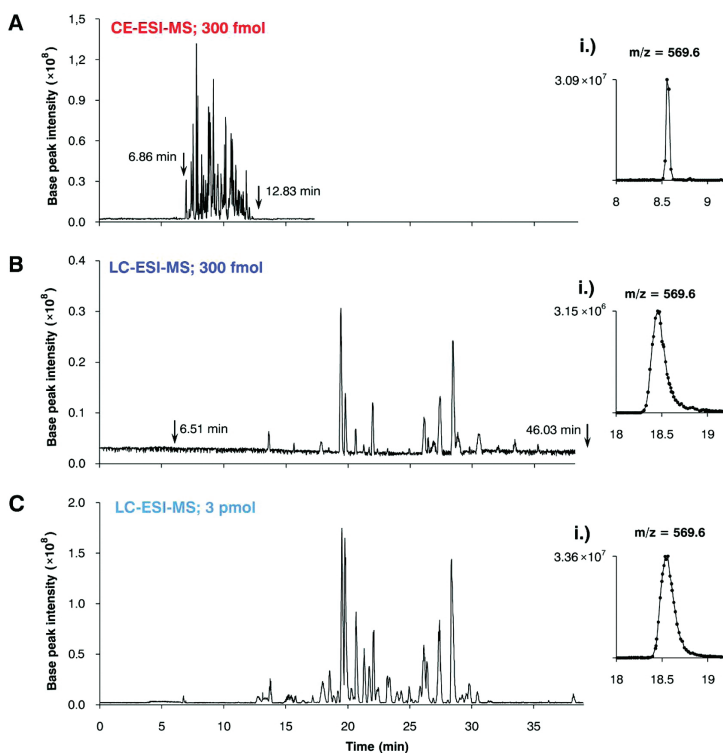


Figure 4-6: Base peak electropherogram and base peak chromatograms of rat testis H1 histones digested with endoproteinase Arg-C using (A) CE–ESI–MS, sample amount 6.15 ng (300 fmol); (B) LC–ESI–MS, 6.15 ng (300 fmol); (C) LC–ESI–MS, 61.5 ng (3.0 pmol). CE conditions were as described in Figure 5. LC–ESI–MS was performed using a homemade fritless column: packed 10 cm with 3 μm reversed-phase C18 (Reposil). The gradient (solvent A, 0.1% formic acid; solvent B, 0.1% formic acid in 85% acetonitrile) started at 4% B. The concentration of solvent B was increased linearly from 4% to 50% during 50 min and from 50% to 100% during 5 min. A flow rate of 250 nL/min was applied. (i.) Extracted mass trace of the ion $m/z = 569.6$ corresponding to the triply charged peptide KALAAAGYDVEKNNSR. Each dot represents a single full scan. Taken from [56].

region of the ESI process. The incorporation of transient isotachopheresis (t-ITP) in this approach increased the mass loading of the system resulting in concentration limits of detection in the sub-nanomolar range for selected peptides. The possibility to operate this interface at very low flow rates allowed the use of non-charged neutral capillary coatings at a flow rate below 10 nL/min, which resulted in a peak capacity above 320 for the separation of an *E. coli* tryptic digest. In addition to providing a very high sensitivity, the operation of this interface at such low flow rates reduces ion suppression effects normally observed when separation techniques (LC or CE) are hyphenated to ESI-MS using higher flow rates(> 200 nL/min)[55].

The group of Lindner has recently also evaluated the suitability of this sheathless CE-MS approach for peptide analysis [56]. Three different cationic capillary coatings were investigated for stability, resolution, and EOF and were found to enable reproducible separations by CE-ESI-MS. The sheathless CE-ESI-MS method was compared with nano-RPLC-ESI-MS by analyzing Arg-C-digested rat testis linker histones. With comparable amounts of sample injected, the number of identified peptides increased by more than 60% with sheathless CE-ESI-MS (Figure 4-6). Lindner et al. demonstrated that low-molecular mass peptides (<1400 Da) were preferentially identified by CE-ESI-MS, as this group of peptides poorly interacts with the reversed-phase material in the nano-LC system. This selectivity difference remained valid even with 10 times more sample loaded in the LC system than in the CE system.

The nanoliter to microliter per minute flow rate used in microchip CE shows a strong potential for interfacing such devices with nanoflow ESI-MS sources. Microfluidic chips can be coupled directly to an ESI interface using pressure-driven or EOF to direct the liquid into the spray [57, 58]. Although the use of microfluidic devices has been under investigation for some time now, their use in proteomic studies has been very limited so far. The potential of microchip CE-ESI-MS for proteomics has recently been investigated by Mellors et al. [59]. They developed a microchip CE system with an incorporated ESI sprayer, which required no junctions and therefore created no additional dead volume. As the developed spray tip required a significant EOF, a positively charged covalent capillary coating was used to create a reversed and strong EOF under which a stable spray could be achieved for the analysis of a BSA digest. With this system, plate numbers of 200,000 could be generated with peak widths around a second at half height. Overall, the developed approach shows a strong potential for proteomic studies as high peak capacities could be achieved in a narrow separation window.

2.4 Hyphenation of CE and MALDI-MS

In a nutshell the difference between the CE-ESI-MS and CE-MALDI-MS comes to a simple fact: the ionization process in MALDI is physically decoupled from the separation process. As a result, the influence of the BGE composition on the ionization is lower in MALDI and, if a correct ionization matrix is chosen, a higher ionic strength BGE can be used [60-62]. Since MALDI-MS generally operates under high vacuum conditions, offline approaches have so far been preferred. Although these approaches introduce a certain discontinuity to the workflow, they also permit to envisage the integration of on-plate reactions, which can be as diverse as enzymatic digestion, chemical modification or enrichment of subclasses of compounds.

While commercial CE instruments enable fraction collection, in practice this often leads to breakdown of the CE current occurring at each movement of the outlet reservoir, which induces a loss of peak efficiency. As a result, the potential applicability of these approaches is very limited. Several research groups have been developing home-made interfaces to couple CE and MALDI-MS, with one of the prerequisites being that these interfaces do not provoke any current interruption so that the high efficiency capabilities of CE can be preserved for the MALDI-MS detection. Interfaces based on the use of a T-junction, allowing the addition of a flow of sheath-liquid at the outlet of the separation capillary have for example been proposed [62]. T-junction based interfaces are suitable to most CE conditions with or without EOF; however, it has been demonstrated that, as in ESI-MS, the creation of a parabolic flow profile in the separation capillary using the addition of a sheath-liquid at the outlet of the separation capillary can compromise the peak efficiencies. As of today, T-junction interfaces are certainly the most common ones as they can be combined in a rather straightforward manner to commercial MALDI spotters. An alternative to T-junction based interfaces is the liquid-junction interface, however, a significant challenge with this strategy is to optimize the configuration to minimize dead volumes, which can, when they are too large, dramatically deteriorate the resolution. In an effort to provide a simple and powerful coupling of CE with MALDI-MS, an iontophoretic fraction collection approach has recently been proposed [60]. It is based on electromigration and molecular diffusion and does not rely on the use of any sheath-liquid, liquid-junction nor superimposed hydrodynamic pressure. The main advantage of this approach is that it is compatible with both bare fused-silica and neutrally coated capillaries and that the in-capillary resolution is totally independent from the spotting process. As a result, as many fractions as required can be collected without deteriorating the achievable resolution.

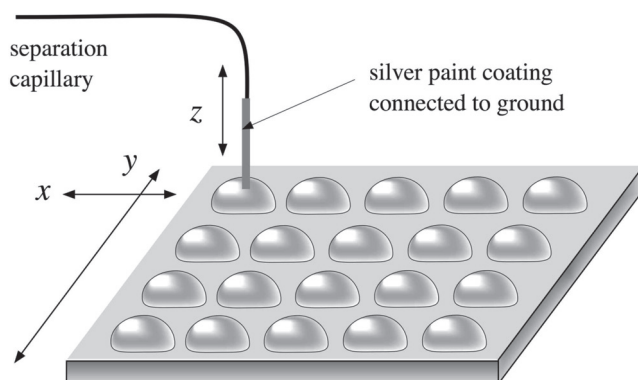


Figure 4-7: Schematic of the robotized fraction collection interface including a MALDI plate mounted on an x,y-stage and a separation capillary silver-coated at the tip for a connection to ground and mounted on a z-axis holder. Taken from [60].

In all the approaches described above, the fraction collection process is discontinuous in the sense that analytes separated by CE are collected in discrete droplets. As a result any discrete spotting strategy will induce a certain loss of resolution as compared to the in-capillary resolution. In this context, some groups have been proposing continuous offline deposition approaches. For example, Reijtar et al. have proposed an approach where the CE effluent was collected on a stainless steel MALDI plate through offline vacuum deposition [63]. In this configuration, the CE separated analytes were deposited on 100 micrometers wide streaks and the electrical contact at the interface was provided by the use of a liquid-junction. More recently, Wang et al. proposed a similar continuous deposition approach [64], but here the electrical contact was not made through the use of large liquid-junction but through the use of an open fracture [65] at the outlet of the separation capillary. Although the fabrication of the open fracture is not straightforward, the continuous aspect of the deposition presents a lot of capabilities, especially in view of the significant hardware and software advances which have recently been made in the field of MALDI imaging.

A number of CE-MALDI-MS applications have been developed and used for proteomics over the past few years, which are summarized in Table 4-4. Although most of them are mainly of academic nature, a few relevant cases will be discussed in detail to illustrate the potential of CE-MALDI-MS for proteomics. Zuberovic et al. performed an analysis of human CSF using a PolyE-323 positively charged coated capillary coupled to a sheath-liquid MALDI spotter [66]. The described strategy was also applied for quantitative proteomics in CSF after incorporating an iTRAQ labeling step in the sample preparation [67]. Absolute quantification could not be performed, but with the used strategy 43 proteins in CSF could be identified which varied in concentration after a traumatic brain injury.

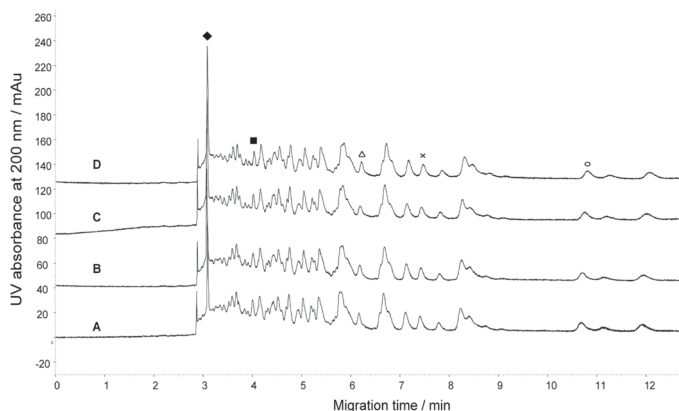


Figure 4-8: Impact of spotting interval time on separation resolution. Experimental conditions: HPC-coated capillary, total/effective length 80/21.5 cm \times 50 μ m i.d.; voltage, 30 kV; current, 12 μ A; UV absorbance at 200 nm; BGE, 10% acetic acid; sample, tryptic digest of α -lactalbumin (35 μ M) and β -lactoglobulin (27 μ M); sample injection, 30 mbar 30 s. (A) No spotting. (B) Spotting every 60 s. (C) Spotting every 30 s. (D) Spotting every 15 s. Taken from [60].

Busnel et al. developed a novel CE-MALDI spotting interface, shown in Figure 4-7, to achieve efficient fractionation at very low flow-rates using separation in a neutrally coated capillary [60]. The use of a coated capillary tip instead of a liquid-junction of sheath-liquid interface to achieve a closed circuit allowed fractionation onto a pre-structured MALDI plate enabling concentration into a small matrix spot (Figure 4-8). This application was tested on an 8 protein digest and compared to direct MALDI analysis of the same sample mixture, resulting in 87 peptides detected after MALDI fractionation compared to only 36 with direct analysis. Although an increase of almost 150% in the number of detected peptides was found the average sequence coverage only increased from 24% to 38%. A downside of the developed strategy was the need for sampling/spotting into larger volume droplets of MALDI matrix resulting in analyte dilution.

2.5 Multidimensional systems

The use of a single separation technique for the analysis of a complex sample such as protein digests of whole cell lysates or tissue lysates is often not sufficient. The larger is the number of peptides obtained after enzymatic digestion of a complex biological sample, the higher is the risk of co-migration if a single dimension separation approach is used. This, in turn, may lead to the difficulties in the interpretation of the MS/MS spectra of co-migrating peptides. The use of two complementary techniques generally results in an improved peak capacity thereby reducing co-migration of compounds. In this section, relevant applications of on-line and off-line multidimensional separation approaches are discussed. An overview is given in Table 4-2.

Chen et al. [68] coupled cIEF on-line to nano-RPLC-ESI-MS for the analysis of tryptic digests of Formalin-fixed and paraffin embedded (FFPE) tissue and yeast membrane [69-71]. The aim was to achieve maximum peptide coverage by coupling two complementary separation methods (Figure 4-9). A total of 14,478 distinct peptides were identified from micro-dissected FFPE glioblastoma tissue, leading to the identification of 2733 non-redundant SwissProt protein entries. For yeast tryptic digest, a total of 33,772 peptides and 3433 proteins could be determined after using a number of different sample preparation strategies. Although it was claimed that the sample consumption for this method was low, sample consumption for such a 2D technique will obviously be higher than for a single dimension separation technique.

A similar strategy was used by Fang et al. for the analysis of human saliva and mouse brain mitochondrial proteome [72, 73]. Their strategy consisted of an off-line coupling of an ITP/CZE to nano-RPLC-ESI-MS via a fraction collector. Like cIEF, ITP/CZE is enabling in-capillary sample concentration for the use of larger volume injections, but it provides better separation of the peptide bands reducing the appearance of peptides in multiple CE fractions. In a comparison with the previously described cIEF-LC method it was found that the developed ITP/CZE-LC-MS method was superior in the detection of both

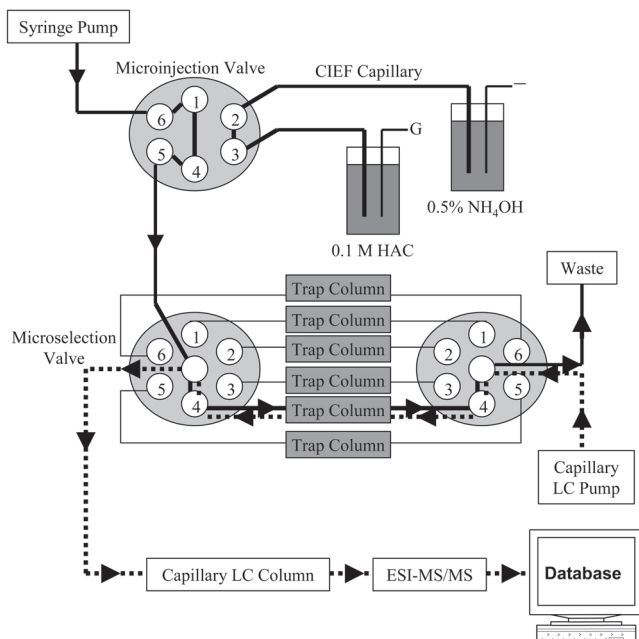


Figure 4-9: Schematic of on-line integration of CIEF with CRPLC as a concentrating and multidimensional separation platform. Solid and dashed lines represent the flow paths for the loading of CIEF fractions and the injection of fractions into a CRPLC column, respectively. Taken from [68]

peptides and proteins. Moreover, the use of both methods resulted in complementary data and resulted in an even higher number of detected mouse mitochondrial peptides and proteins.

An off-line CIEF-LC-MS method for the analysis of ovarian endometrioid adenocarcinoma was developed by Dai et al. [74] The developed strategy was used to compare the signaling pathways in two closely related carcinoma cell lines. A total of 2612 proteins could be identified of which only 1092 could be determined in both cell lines. For one of the cell lines 1749 proteins could be identified after merging the data from three analyses, but only 311 of these proteins could be identified in all three runs while 561 could be identified in only two runs leaving almost half of the proteins identified in just one run.

In principle, cIEF and CZE are not orthogonal separation techniques and, therefore, the coupling of the two methods will not yield much additional information for whole digests. Nevertheless, when separation of intact proteins is performed by cIEF followed by a digestion step and subsequent analysis by CZE the increase in information could

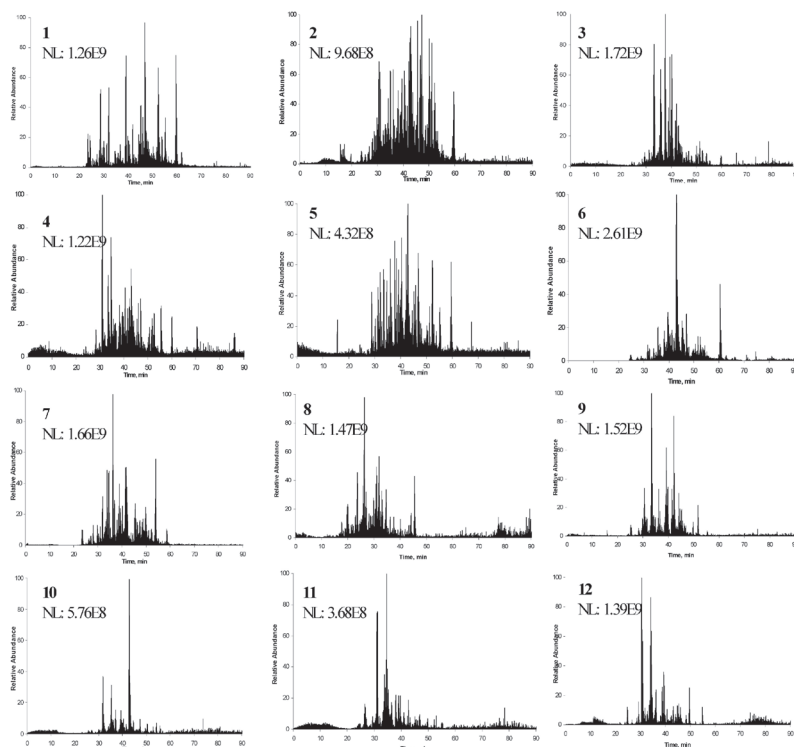


Figure 4-10: Base peak chromatograms of a representative CIEF/CRPLC multidimensional separation of 9.6 μ g of yeast tryptic peptides obtained from the soluble fraction of cell lysates. Each number represents the sequence of CIEF fractions further analyzed by CRPLC from acid to basic pHs. Taken from [68].

be significant. Using this strategy the most abundant proteins can (at least partially) be isolated from the less abundant compounds. This coupling of CIEF-CZE was used by Hanrieder et al. for the proteomic analysis of human follicular fluid [75]. In their method the intact protein content was first fractionated by cIEF into 10 fractions ranging from pH 3 to 10, which were then digested, and separated by CZE using a positively charged coated capillary, and this was finally followed by spotting on a MALDI target plate for subsequent analysis.

A method using iso-electric trapping (IET) in membrane separated wells and histidine as buffering electrolyte was developed by Cologna et al. [76] The investigation showed that histidine can serve as a very suitable buffer over a wide pH range and that the coupling of IET and CZE can have some benefits. So far, the method was only applied to BSA digested before the IET phase of the separation. As previously discussed, IET and CZE are not orthogonal methods and their coupling in this fashion will most likely not improve the sequence coverage when compared to an efficient single dimension separation strategy. If it could be proven that the IET strategy could also be used for the separation of whole proteins, then the coupling of these methods in a more orthogonal strategy comparable to the method developed by Hanrieder et al. [75] should be possible.

2.6 Relevant work in adjacent fields

Although the analysis of native peptides is a highly specialized area and is in many ways different from mainstream proteomics work it is difficult to ignore this important fraction of the literature. The group of Mischak et al. have used a CE-ESI-MS method that has been extensively characterized [23] and applied to the study of human urine [77-80], CSF [81] and bile [82]. The aim of their studies was to identify native peptide biomarkers for a large variety of diseases. In their strategy, CZE separation is coupled to TOF-MS through a co-axial sheath-liquid interface. Their CE-TOF-MS approach has been used for the analysis of thousands of biological samples, demonstrating the robustness of their approach for large-scale clinical studies. A comprehensive review of the use of CE-MS in peptidomics/proteomics for the discovery of biomarkers has recently been published by Mischak et al. [83].

3 Conclusions and outlook

Over the last 5 years, the hyphenation of CE with MS has significantly benefitted from technological developments in the field: today the coupling of CE to a mass spectrometer may be considered as simple and straightforward as coupling of a LC instrument to MS. The operation at ultra-low flow rates, capacity for miniaturization, and the ability to separate efficiently (highly) polar and charged peptides are the three main distinctive features of CE-MS. Although the loadability has always been a topic of discussion with regard to the general applicability of CE, novel in-capillary concentration methods and the gain of sensitivity due to the ultra-low flow-effects have reduced the relevance of this issue.

The next question is whether proteomics researchers are ready to use CE-MS for their applications and whether modern proteomics offers research questions that will be typically well-suited for a CE-MS approach. In that respect, we foresee that an exploration of the above mentioned ultra-low flow rate effects and the analysis of volume restricted samples will be a main area of CE-MS applications in proteomics. As discussed in this review, ultra-low flow conditions not only provide sensitivity but also has the advantage that under these circumstances ESI behaves “as a detector with almost equimolar responses for samples prepared from a complex serum matrix” [55]. Miniaturization is another strong aspect of CE-MS. Scaling down the LC-MS routines is an expensive and challenging process, CE, on the other hand, is a “nano-technique” by its nature.

Although CE-MS has until now been used primarily with relatively non-complex samples, we hope that this review will contribute to a more general appreciation of the possibilities of this technique and challenge the proteomics community to implement it in their workflows, and we hope to see the results of these experiments in the near future. Although single cell analysis might be considered the Holy Grail in proteomics, even hundreds of cells, which will still not be amenable to more conventional proteomic workflows, in principle provide enough material for a comprehensive CE-MS based proteomic analyses.

Sample	BGE/ampholytes	Coating	MS interface	Mass analyzer	Remarks	Ref.
Cytochrome C, β -lactoglobulin A and ribonuclease B tryptic digests	1 M Formic acid adjusted to pH 2.0 with NH_4OH	-	Sheath-liquid ESI 3 $\mu\text{L}/\text{min}$ 50/50 (v/v) MeOH/H ₂ O	IT	large volume injection with pH junction	[84]
Cytochrome c tryptic digest	20/80 ACN/20 mM Ammoniumformate (v/v)	Open Tubular n-octadecyl- and cholesterolbonded surfaces	Sheath-liquid ESI 50/50 (v/v) 1 mM Ammonium Acetate/MeOH	IT		[85]
Cytochrome C tryptic digest	200 mM Formic acid adjusted to pH 2.5 with NH_4OH	Polybrene and poly(vinyl sulfonate)	Sheath-liquid ESI 75/25 (v/v/v) ACN/water with 0.1% Formic Acid	IT	On-line SPE using a column switching system	[40]
BSA tryptic digest	10 mM Ammonium Acetate pH 3.0	monoquaternarized piperazine (positive coating)	Sheath-liquid ESI 2 $\mu\text{L}/\text{min}$ 80/20 (v/v) MeOH/H ₂ O with 10mM Acetic Acid	TOF		[26]
Erythropoietin and biosimilars tryptic digest	20 mM Ammonium Acetate pH 7.0	-	Sheath-liquid ESI 3.3 $\mu\text{L}/\text{min}$ 60/40 (v/v) iPrOH/H ₂ O with 0.5% Formic Acid		In-line immuno affinity SPE	[86]
BSA tryptic digest	30 mM Ammonium Acetate pH 3.0	N-Methylpolypyrrolidone (positive coating)	Sheath-liquid ESI 2 $\mu\text{L}/\text{min}$ 80/20 (v/v) MeOH/H ₂ O with 10mM Acetic Acid	TOF		[87]
Soy bean extract tryptic digest	0.5 M Formic Acid	-	Sheath-liquid ESI 3 $\mu\text{L}/\text{min}$ 50/50 (v/v) iPrOH/H ₂ O	TOF		[88]
Recombinant human erythropoietin (rhEPO) tryptic digest	50 mM Formic Acid and 50 mM Acetic Acid	-	Sheath-liquid ESI 3.3 $\mu\text{L}/\text{min}$ 50/50 (v/v) iPrOH/H ₂ O	IT		[39]
Four bovine protein tryptic digest	10 mM ammonium acetate pH 7.0	-	Electrokinetically junction at the tip ESI 50/50 MeOH/ H ₂ O 10 mM acetic acid	Orbitrap		[44]

Table 4-1: Overview of CZE-ESI-MS proteomics applications reported between 2007 and 2011.

Sample	First dimension	Second dimension	Remarks	Ref.
Human Saliva	Transient-Isotachopheresis using neutral hydroxypropyl cellulose coating. 2% Sigma Aldrich Pharmalyte as leading electrolyte and 100 mM Acetic acid as BGE	Nano-RPLC-ESI-MS linear gradient acetonitrile/water	tITP/CZE-LC coupling performed off-line using CE fraction collector equipped with sheath-liquid tip to obtain a closed circuit.	[73]
Formalin-fixed and paraffin-embedded (FFPE) tissues	cIEF using neutral hydroxypropyl cellulose coating and 2% Sigma Aldrich 3-10 ampholyte	Nano-RPLC-ESI-MS linear gradient acetonitrile/water 0.02% formic acid	cIEF-LC coupling performed by on-line column switching system. Protein digestion performed before cIEF	[69]
Yeast membrane tryptic digest	cIEF using neutral hydroxypropyl cellulose coating and 2% Sigma Aldrich 3-10 ampholyte	Nano-RPLC-ESI-MS linear gradient acetonitrile/water 0.02% formic acid	cIEF-LC coupling performed by on-line column switching system. Protein digestion performed before cIEF	[70]
Yeast membrane tryptic digest	cIEF using neutral hydroxypropyl cellulose coating and 2% Sigma Aldrich 3-10 ampholyte	Nano-RPLC-ESI-MS linear gradient acetonitrile/water 0.02% formic acid	cIEF-LC coupling performed by on-line column switching system. Protein digestion performed before cIEF	[71]
Mouse brain mitochondria	Transient-Isotachopheresis using neutral hydroxypropyl cellulose coating. 2% Sigma Aldrich Pharmalyte as leading electrolyte and 100 mM Acetic acid as BGE	Nano-RPLC-ESI-MS linear gradient acetonitrile/water	tITP/CZE-LC coupling performed off-line using CE fraction collector equipped with sheath-liquid tip to obtain a closed circuit	[72]
Ovarian endometrioid adenocarcinoma cell tryptic digest	cIEF using neutral hydroxypropyl cellulose coating and 2% Sigma Aldrich 3-10 ampholyte	Nano-RPLC-ESI-MS multi phase linear gradients acetonitrile/water 0.02% formic acid	cIEF-LC coupling performed off-line using fraction collector equipped with T-piece make-up flow and stainless steel needle to obtain a closed circuit. Protein digestion performed before cIEF	[74]
Human follicular fluid	cIEF in 1% RioRad 3-11 ampholyte	CZE-MS in unmodified capillary and 10 mM acetic acid BGE. Fraction collection followed by MALDI-TOF MS/MS	cIEF-CZE coupling performed off-line using hydrodynamic fraction collection. Protein digestion performed after cIEF fractionation	[75]
BSA tryptic digest	Iso Electric Trapping (EIT) using Poly(vinyl-alcohol) based buffering membranes	CZE-MS with neutral polyacrylamide coating and 250 mM Formic Acid BGE. Stacking performed with histidine as leading electrolyte. Detection by MALDI-TOF-MS	EIT-CZE coupling performed off-line. Protein digestion performed before EIT. MALDI spotting performed using sheath-liquid interface to provide closed circuit and MALDI matrix	[76]
<i>Mycobacterium marinum</i> secreted protein tryptic digest	RPLC using water:acetonitrile gradient followed by fractionation	CZE-ESI-MS using unmodified capillary and sheath-liquid interface	RPLC-CZE coupling performed off-line. Protein digestion performed before RPLC fractionation. MS analysis performed with Orbitrap	[45]

Table 4-2: Overview of multidimensional proteomics applications reported between 2007 and 2011.

Sample	BGE/ampholytes	Coating	MS interface	Mass analyzer	Remarks	Ref.
Six protein tryptic digest	0.1% Polybrene in 0.1% acetic acid	-	Sheathless porous tip interface	IT		[24]
BSA and <i>E.coli</i> tryptic digest	10 % acetic acid pH 2.2	polyacrylamide (neutral coating)	Sheathless porous tip interface	TOF	Large volume injection with tITP	[54]
Histone H1 endoproteinase Arg-C digest	0.1 % Formic Acid pH 2.7	M7C41, PolyE-323 and PEI (Positive coatings)	Sheathless porous tip interface	Orbitrap		[56]
BSA tryptic digest	50/50 (v/v) MeOH/0.4% Acetic Acid	PolyE-323 (positive coating)	Sheathless on-chip ESI sprayer	QTOF		[59]

Table 4-3: Overview of CZE-ESI-MS proteomics applications reported between 2007 and 2011.

Sample	BGE/ampholytes	Coating	MS interface	MALDI Matrix	Remarks	Ref.
E-coli tryptic digest	250 mM formic acid with 5 mM ammonium phosphate pH 2.2	-	MALDI spotter using make-up flow to provide matrix and electric circuit	CHCA	Large volume injection through stacking	[89]
BSA Myoglobin tryptic digest	10 mM acetate pH 4.25	1,2-dilauroyl-sn-phosphatidylcholine (neutral coating)	MALDI spotter with hydrodynamic spotting and no make-up flow	CHCA	Large volume injection using pH junction	[90]
Cerebrospinal fluid tryptic digest	10 mM Acetic Acid	PolyE-323 (positive coating)	MALDI spotter using make-up flow to provide matrix and electric circuit	HCCA		[66]
8 Protein tryptic digest	10% Acetic Acid pH 2.2	Hydroxypropylcellulose (neutral coating)	MALDI spotter with conductive coating on spotter tip for continuous separation voltage	CHCA	Large volume injection using ITP	[60]
a-lactalbumin tryptic digest	10 mM Ammonium Bicarbonate pH 9.0	-	MALDI spotter using make-up flow to provide matrix and electric circuit	CHCA		[91]
Cerebrospinal fluid tryptic digest	10 mM Acetic Acid	PolyE-323 (positive coating)	MALDI spotter using make-up flow to provide matrix and electric circuit	HCCA	Quantification with iTraQ labeling	[67]
6 protein tryptic digest	40mM Triethylamine to pH 2.3 with phosphoric acid and 20% v/v ACN	Fullerenol and poly(late) diol (neutral coating)	MALDI spotter using make-up flow to provide matrix and electric circuit	CHCA		[92]
3 protein tryptic digest	80mM phosphoric acid, 40mM TEA (pH 2.3) and 20% v/v ACN	Poly(late) diol (neutral coating)	MALDI with hydrodynamic spotting and voltage assisted spotting and no make-up flow	DHB	Large volume injection using ITP	[93]

Table 4-4: Overview of CZE-MALDI-MS proteomics applications reported between 2007 and 2011

References

- [1] Horvatovich P, Hoekman B, Govorukhina N, Bischoff R. Multidimensional chromatography coupled to mass spectrometry in analysing complex proteomics samples. *Journal of Separation Science*. 2010;33:1421-37.
- [2] Motoyama A, Yates JR. Multidimensional LC Separations in Shotgun Proteomics. *Analytical Chemistry*. 2008;80:7187-93.
- [3] Yates JR, Ruse CI, Nakorchevsky A. Proteomics by Mass Spectrometry: Approaches, Advances, and Applications. *Annual Review of Biomedical Engineering*. 2009;11:49-79.
- [4] Beck M, Claassen M, Aebersold R. Comprehensive proteomics. *Current Opinion in Biotechnology*. 2011;22:3-8.
- [5] Xie F, Liu T, Qian WJ, Petyuk VA, Smith RD. Liquid chromatography-mass spectrometry-based quantitative proteomics. *Journal of Biological Chemistry*. 2011;286:25443-9.
- [6] Ong S-E, Mann M. Mass spectrometry-based proteomics turns quantitative. *Natural Chemical Biology*. 2005;1:252-62.
- [7] Selvaraju S, El Rassi Z. Liquid-phase-based separation systems for depletion, prefractionation and enrichment of proteins in biological fluids and matrices for in-depth proteomics analysis – An update covering the period 2008–2011. *ELECTROPHORESIS*. 2012;33:74-88.
- [8] Wilm M, Mann M. Analytical properties of the nanoelectrospray ion source. *Analytical Chemistry*. 1996;68:1-8.
- [9] Schmidt A, Karas M, Dulcks T. Effect of different solution flow rates on analyte ion signals in nano-ESI MS, or: when does ESI turn into nano-ESI? *Journal of American Society of Mass Spectrometry*. 2003;14:492-500.
- [10] Gangl ET, Annan M, Spooner N, Vouros P. Reduction of signal suppression effects in electrospray MS using a nanosplitting device. *Analytical Chemistry*. 2001;73:5635-44.
- [11] Masuda T, Sugiyama N, Tomita M, Ishihama Y. Microscale Phosphoproteome Analysis of 10000 Cells from Human Cancer Cell Lines. *Analytical Chemistry*. 2011;83:7698-703.
- [12] Jorgenson JW, Lukacs KD. Free-zone electrophoresis in glass capillaries. *Clinical Chemistry*. 1981;27:1551-3.
- [13] Karger BL, Guttman A. DNA sequencing by CE. *ELECTROPHORESIS*. 2009;30 Suppl 1:S196-202.
- [14] Silvertand LH, Torano JS, van Bennekom WP, de Jong GJ. Recent developments in capillary isoelectric focusing. *Journal of Chromatography A*. 2008;1204:157-70.
- [15] Smith RD, Udseth HR. Capillary zone electrophoresis-MS. *Nature*. 1988;331:639-40.
- [16] Maxwell EJ, Chen DD. Twenty years of interface development for capillary electrophoresis-electrospray ionization-mass spectrometry. *Analytica Chimica Acta*. 2008;627:25-33.
- [17] Hommerson P, Khan AM, de Jong GJ, Somsen GW. Ionization techniques in capillary electrophoresis-mass spectrometry: principles, design, and application. *Mass Spectrometry Reviews*. 2011;30:1096-120.
- [18] Klampfl CW, Buchberger W. Coupling of capillary electroseparation techniques with mass spectrometric detection. *Analytical and Bioanalytical Chemistry*. 2007;388:533-6.
- [19] Stutz H. Advances in the analysis of proteins and peptides by capillary electrophoresis with matrix-assisted laser desorption/ionization and electrospray-mass spectrometry detection. *ELECTROPHORESIS*. 2005;26:1254-90.
- [20] Aebersold R, Goodlett DR. Mass Spectrometry in Proteomics. *Chemical Reviews*. 2001;101:269-96.
- [21] Pelzing M, Neususs C. Separation techniques hyphenated to electrospray-tandem mass spectrometry in proteomics: capillary electrophoresis versus nanoliquid chromatography. *ELECTROPHORESIS*. 2005;26:2717-28.
- [22] Busnel JM, Lion N, Girault HH. Electrokinetic supercharging for highly efficient peptide preconcentration in capillary zone electrophoresis. *ELECTROPHORESIS*. 2008;29:1565-72.
- [23] Wittke S, Mischak H, Walden M, Kolch W, Rädler T, Wiedemann K. Discovery of biomarkers in human urine and cerebrospinal fluid by capillary electrophoresis coupled to mass spectrometry: Towards new diagnostic and therapeutic approaches. *ELECTROPHORESIS*. 2005;26:1476-87.
- [24] Moini M. Simplifying CE-MS Operation. 2. Interfacing Low-Flow Separation Techniques to Mass Spectrometry Using a Porous Tip. *Analytical Chemistry*. 2007;79:4241-6.
- [25] Catai JR, Torano JS, de Jong GJ, Somsen GW. Efficient and highly reproducible capillary electrophoresis-mass spectrometry of peptides using Polybrene-poly(vinyl sulfonate)-coated capillaries. *ELECTROPHORESIS*. 2006;27:2091-9.

- [26] Elhamili A, Wetterhall M, Arvidsson B, Sebastiano R, Righetti PG, Bergquist J. Rapid capillary electrophoresis time-of-flight mass spectrometry separations of peptides and proteins using a monoquaternarized piperazine compound (M7C4I) for capillary coatings. *ELECTROPHORESIS*. 2008;29:1619-25.
- [27] Sebastiano R, Mendieta ME, Contiello N, Citterio A, Righetti PG. An N-methylpolyvinylpyridinium cationic polymer for capillary coating in electrophoresis of proteins and peptides. *ELECTROPHORESIS*. 2009;30:2313-20.
- [28] Huhn C, Ramautar R, Wuhler M, Somsen GW. Relevance and use of capillary coatings in capillary electrophoresis-mass spectrometry. *Analytical and Bioanalytical Chemistry*. 2010;396:297-314.
- [29] Haselberg R, de Jong GJ, Somsen GW. Capillary electrophoresis-mass spectrometry for the analysis of intact proteins 2007-2010. *ELECTROPHORESIS*. 2011;32:66-82.
- [30] Schmitt-Kopplin P, Englmann M. Capillary electrophoresis - mass spectrometry: survey on developments and applications 2003-2004. *ELECTROPHORESIS*. 2005;26:1209-20.
- [31] Kašička V. Recent developments in CE and CEC of peptides (2009-2011). *ELECTROPHORESIS*. 2012;33:48-73.
- [32] Herrero M, Ibanez E, Cifuentes A. Capillary electrophoresis-electrospray-mass spectrometry in peptide analysis and peptidomics. *ELECTROPHORESIS*. 2008;29:2148-60.
- [33] Marginean I, Kelly RT, Prior DC, LaMarche BL, Tang K, Smith RD. Analytical characterization of the electrospray ion source in the nanoflow regime. *Analytical Chemistry*. 2008;80:6573-9.
- [34] Zhong X, Maxwell EJ, Chen DD. Mass transport in a micro flow-through vial of a junction-at-the-tip capillary electrophoresis-mass spectrometry interface. *Analytical Chemistry*. 2011;83:4916-23.
- [35] Mokaddem M, Gareil P, Belgaied JE, Varenne A. A new insight into suction and dilution effects in capillary electrophoresis coupled to mass spectrometry via an electrospray ionization interface. Part I-Suction effect. *ELECTROPHORESIS*. 2008;29:1957-64.
- [36] Mokaddem M, Gareil P, Belgaied J-E, Varenne A. New insight into suction and dilution effects in CE coupled to MS via an ESI interface. II – Dilution effect. *ELECTROPHORESIS*. 2009;30:1692-7.
- [37] Simpson DC, Smith RD. Combining capillary electrophoresis with mass spectrometry for applications in proteomics. *ELECTROPHORESIS*. 2005;26:1291-305.
- [38] Fonslow BR, Yates JR, 3rd. Capillary electrophoresis applied to proteomic analysis. *Journal of Separation Sciences*. 2009;32:1175-88.
- [39] Giménez E, Ramos-Hernan R, Benavente F, Barbosa J, Sanz-Nebot V. Capillary electrophoresis time-of-flight mass spectrometry for a confident elucidation of a glycopeptide map of recombinant human erythropoietin. *Rapid Communications in Mass Spectrometry*. 2011;25:2307-16.
- [40] Tempels FWA, Underberg WJM, Somsen GW, de Jong GJ. On-line coupling of SPE and CE-MS for peptide analysis. *ELECTROPHORESIS*. 2007;28:1319-26.
- [41] Wachs T, Sheppard RL, Henion J. Design and applications of a self-aligning liquid junction-electrospray interface for capillary electrophoresis-mass spectrometry. *Journal of Chromatography B*. 1996;685:335-42.
- [42] Liu CC, Zhang J, Dovichi NJ. A sheath-flow nanospray interface for capillary electrophoresis/mass spectrometry. *Rapid Communications in Mass Spectrometry*. 2005;19:187-92.
- [43] Wojcik R, Dada OO, Sadilek M, Dovichi NJ. Simplified capillary electrophoresis nanospray sheath-flow interface for high efficiency and sensitive peptide analysis. *Rapid Commun Mass Spectrom*. 2010;24:2554-60.
- [44] Wojcik R, Li Y, Maccoss MJ, Dovichi NJ. Capillary electrophoresis with Orbitrap-Velos mass spectrometry detection. *Talanta*. 2012;88:324-9.
- [45] Li Y, Champion MM, Sun L, Champion PA, Wojcik R, Dovichi NJ. Capillary Zone Electrophoresis-Electrospray Ionization-Tandem Mass Spectrometry as an Alternative Proteomics Platform to Ultraperformance Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry for Samples of Intermediate Complexity. *Anal Chem*. 2012.
- [46] Klampfl CW. Review coupling of capillary electrochromatography to mass spectrometry. *Journal of Chromatography A*. 2004;1044:131-44.
- [47] Zamfir AD. Recent advances in sheathless interfacing of capillary electrophoresis and electrospray ionization mass spectrometry. *Journal of Chromatography A*. 2007;1159:2-13.
- [48] Sanz-Nebot V, Balaguer E, Benavente F, Barbosa J. Comparison of sheathless and sheath-flow electrospray interfaces for the capillary electrophoresis-electrospray ionization-mass spectrometry analysis of peptides. *ELECTROPHORESIS*. 2005;26:1457-65.
- [49] Li J, Thibault P, Bings NH, Skinner CD, Wang C, Colyer C, Harrison J. Integration of microfabricated devices to capillary electrophoresis-electrospray mass spectrometry using a low dead volume

- connection: application to rapid analyses of proteolytic digests. *Analytical Chemistry*. 1999;71:3036-45.
- [50] Janini GM, Conrads TP, Wilkens KL, Issaq HJ, Veenstra TD. A sheathless nanoflow electrospray interface for on-line capillary electrophoresis mass spectrometry. *Analytical Chemistry*. 2003;75:1615-9.
- [51] Issaq HJ, Janini GM, Chan KC, Veenstra TD. Sheathless electrospray ionization interfaces for capillary electrophoresis-mass spectrometric detection advantages and limitations. *Journal of Chromatography A*. 2004;1053:37-42.
- [52] Nguyen A, Moini M. Analysis of major protein-protein and protein-metal complexes of erythrocytes directly from cell lysate utilizing capillary electrophoresis mass spectrometry. *Analytical Chemistry*. 2008;80:7169-73.
- [53] Garza S, Thomas PW, Fast W, Moini M. Metal displacement and stoichiometry of protein-metal complexes under native conditions using capillary electrophoresis/mass spectrometry. *Rapid Communications in Mass Spectrometry*. 2010;24:2730-4.
- [54] Busnel J-M, Schoenmaker B, Ramautar R, Carrasco-Pancorbo A, Ratnayake C, Feitelson JS, Chapman JD, Deelder AM, Mayboroda OA. High Capacity CE-ESI-MS: Coupling a Porous Sheathless Interface with Transient-Isotachopheresis. *Analytical Chemistry*. 2010;82:9476-83.
- [55] Valaskovic GA, Utley L, Lee MS, Wu J-T. Ultra-low flow nanospray for the normalization of conventional liquid chromatography/mass spectrometry through equimolar response: standard-free quantitative estimation of metabolite levels in drug discovery. *Rapid Communications in Mass Spectrometry*. 2006;20:1087-96.
- [56] Faserl K, Sarg B, Kremser L, Lindner H. Optimization and Evaluation of a Sheathless CE-ESI-MS Platform for Peptide Analysis: Comparison to LC-ESI-MS. *Analytical Chemistry*. 2011;83:7297-305.
- [57] Lee J, Soper SA, Murray KK. Microfluidic chips for mass spectrometry-based proteomics. *Journal of Mass Spectrometry*. 2009;44:579-93.
- [58] Michael C B. Capillary and microchip electrophoresis: Challenging the common conceptions. *Journal of Chromatography A*. 2012;1221:42-55.
- [59] Mellors JS, Gorbounov V, Ramsey RS, Ramsey JM. Fully Integrated Glass Microfluidic Device for Performing High-Efficiency Capillary Electrophoresis and Electrospray Ionization Mass Spectrometry. *Analytical Chemistry*. 2008;80:6881-7.
- [60] Busnel J-M, Josserand J, Lion N, Girault HH. Ionophoretic Fraction Collection for Coupling Capillary Zone Electrophoresis with Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *Analytical Chemistry*. 2009;81:3867-72.
- [61] Silvertand LH, Torano JS, de Jong GJ, van Bennekom WP. Improved repeatability and matrix-assisted desorption/ionization - time of flight mass spectrometry compatibility in capillary isoelectric focusing. *ELECTROPHORESIS*. 2008;29:1985-96.
- [62] Huck CW, Bakry R, Huber LA, Bonn GK. Progress in capillary electrophoresis coupled to matrix-assisted laser desorption/ionization – time of flight mass spectrometry. *ELECTROPHORESIS*. 2006;27:2063-74.
- [63] Rejtar T, Hu P, Juhasz P, Campbell JM, Vestal ML, Preisler J, Karger BL. Off-line coupling of high-resolution CE to MALDI-TOF and TOF/TOF MS. *Journal of Proteome Research*. 2002;1:171-9.
- [64] Wang J, Ye H, Zhang Z, Xiang F, Girdaukas G, Li L. Advancing Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometric Imaging for Capillary Electrophoresis Analysis of Peptides. *Analytical Chemistry*. 2011;83:3462-9.
- [65] Wang J, Ma M, Chen R, Li L. Enhanced Neuropeptide Profiling via Capillary Electrophoresis Off-Line Coupled with MALDI FTMS. *Analytical Chemistry*. 2008;80:6168-77.
- [66] Zuberovic A, Hanrieder J, Hellman U, Bergquist J, Wetterhall M. Proteome profiling of human cerebrospinal fluid: exploring the potential of capillary electrophoresis with surface modified capillaries for analysis of complex biological samples. *European journal of mass spectrometry*. 2008;14:249-60.
- [67] Zuberovic A, Wetterhall M, Hanrieder J, Bergquist J. CE MALDI-TOF/TOF MS for multiplexed quantification of proteins in human ventricular cerebrospinal fluid. *ELECTROPHORESIS*. 2009;30:1836-43.
- [68] Chen J, Balgley BM, DeVoe DL, Lee CS. Capillary Isoelectric Focusing-Based Multidimensional Concentration/Separation Platform for Proteome Analysis. *Analytical Chemistry*. 2003;75:3145-52.
- [69] Guo T, Wang W, Rudnick PA, Song T, Li J, Zhuang Z, Weil RJ, DeVoe DL, Lee CS, Balgley BM. Proteome Analysis of Microdissected Formalin-fixed and Paraffin-embedded Tissue Specimens. *Journal of Histochemistry & Cytochemistry*. 2007;55:763-72.

- [70] Wang W, Guo T, Song T, Lee CS, Balgley BM. Comprehensive yeast proteome analysis using a capillary isoelectric focusing-based multidimensional separation platform coupled with ESI-MS/MS. *PROTEOMICS*. 2007;7:1178-87.
- [71] Balgley BM, Wang W, Song T, Fang X, Yang L, Lee CS. Evaluation of confidence and reproducibility in quantitative proteomics performed by a capillary isoelectric focusing-based proteomic platform coupled with a spectral counting approach. *ELECTROPHORESIS*. 2008;29:3047-54.
- [72] Fang X, Wang W, Yang L, Chandrasekaran K, Kristian T, Balgley BM, Lee CS. Application of capillary isotachopheresis-based multidimensional separations coupled with electrospray ionization-tandem mass spectrometry for characterization of mouse brain mitochondrial proteome. *ELECTROPHORESIS*. 2008;29:2215-23.
- [73] Fang X, Yang L, Wang W, Song T, Lee CS, DeVoe DL, Balgley BM. Comparison of Electrokinetics-Based Multidimensional Separations Coupled with Electrospray Ionization-Tandem Mass Spectrometry for Characterization of Human Salivary Proteins. *Analytical Chemistry*. 2007;79:5785-92.
- [74] Dai L, Li C, Shedden KA, Misek DE, Lubman DM. Comparative proteomic study of two closely related ovarian endometrioid adenocarcinoma cell lines using cIEF fractionation and pathway analysis. *ELECTROPHORESIS*. 2009;30:1119-31.
- [75] Hanrieder J, Zuberovic A, Bergquist J. Surface modified capillary electrophoresis combined with in solution isoelectric focusing and MALDI-TOF/TOF MS: A gel-free multidimensional electrophoresis approach for proteomic profiling—Exemplified on human follicular fluid. *Journal of Chromatography A*. 2009;1216:3621-8.
- [76] Cologna SM, Williams BJ, Russell WK, Pai P-J, Vigh G, Russell DH. Studies of Histidine As a Suitable Isoelectric Buffer for Tryptic Digestion and Isoelectric Trapping Fractionation Followed by Capillary Electrophoresis–Mass Spectrometry for Proteomic Analysis. *Analytical Chemistry*. 2011.
- [77] Mullen W, Delles C, Mischak H, action moEC. Urinary proteomics in the assessment of chronic kidney disease. *Current Opinion in Nephrology and Hypertension*. 2011;20:654-61 10.1097/MNH.0b013e32834b7ffa.
- [78] Metzger J, Chatzikyrkou C, Broecker V, Schiffer E, Jaensch L, Ipchofer A, Mengel M, Mullen W, Mischak H, Haller H, Gwinner W. Diagnosis of subclinical and clinical acute T-cell-mediated rejection in renal transplant patients by urinary proteome analysis. *PROTEOMICS – Clinical Applications*. 2011;5:322-33.
- [79] Mullen W, Gonzalez J, Siwy J, Franke J, Sattar N, Mullan A, Roberts S, Delles C, Mischak H, Albalat A. A Pilot Study on the Effect of Short-Term Consumption of a Polyphenol Rich Drink on Biomarkers of Coronary Artery Disease Defined by Urinary Proteomics. *Journal of Agricultural and Food Chemistry*. 2011;59:12850-7.
- [80] Foucher C, Schiffer E, Mischak H, Ansquer J-C, Wilbraham D. Effect of fenofibrate treatment on the low molecular weight urinary proteome of healthy volunteers. *PROTEOMICS – Clinical Applications*. 2011;5:159-66.
- [81] Jahn H, Wittke S, Zürgb P, Raedler TJ, Arlt S, Kellmann M, Mullen W, Eichenlaub M, Mischak H, Wiedemann K. Peptide Fingerprinting of Alzheimer's Disease in Cerebrospinal Fluid: Identification and Prospective Evaluation of New Synaptic Biomarkers. *PLoS ONE*. 2011;6:e26540.
- [82] Lankisch TO, Metzger J, Negm AA, Voßkuhl K, Schiffer E, Siwy J, Weismüller TJ, Schneider AS, Thedieck K, Baumeister R, Zürgb P, Weissinger EM, Manns MP, Mischak H, Wedemeyer J. Bile proteomic profiles differentiate cholangiocarcinoma from primary sclerosing cholangitis and choledocholithiasis. *Hepatology*. 2011;53:875-84.
- [83] Mischak H, Schanstra JP. CE-MS in biomarker discovery, validation, and clinical application. *Proteom Clin Appl*. 2011;5:9–23.
- [84] Imami K, Monton MRN, Ishihama Y, Terabe S. Simple on-line sample preconcentration technique for peptides based on dynamic pH junction in capillary electrophoresis–mass spectrometry. *Journal of Chromatography A*. 2007;1148:250-5.
- [85] Yang Y, Boysen RI, Matyska MT, Pesek JJ, Hearn MTW. Open-Tubular Capillary Electrochromatography Coupled with Electrospray Ionization Mass Spectrometry for Peptide Analysis. *Analytical Chemistry*. 2007;79:4942-9.
- [86] Giménez E, Benavente F, de Bolós C, Nicolás E, Barbosa J, Sanz-Nebot V. Analysis of recombinant human erythropoietin and novel erythropoiesis stimulating protein digests by immunoaffinity capillary electrophoresis–mass spectrometry. *Journal of Chromatography A*. 2009;1216:2574-82.
- [87] Elhamili A, Wetterhall M, Sjödin M, Sebastiano R, Bergquist J. Analysis of peptides using N-methylpolyvinylpyridium as silica surface modifier for CE-ESI-MS. *ELECTROPHORESIS*.

- 2010;31:1151-6.
- [88] Simó C, Domínguez-Vega E, Marina ML, García MC, Dinelli G, Cifuentes A. CE-TOF MS analysis of complex protein hydrolyzates from genetically modified soybeans – A tool for foodomics. *ELECTROPHORESIS*. 2010;31:1175-83.
 - [89] Williams BJ, Russell WK, Russell DH. Utility of CE-MS Data in Protein Identification. *Analytical Chemistry*. 2007;79:3850-5.
 - [90] Nesbitt CA, Jurcic K, Yeung KKC. Nanoliter-volume protein enrichment, tryptic digestion, and partial separation based on isoelectric points by CE for MALDI mass spectral analysis. *ELECTROPHORESIS*. 2008;29:466-74.
 - [91] Vannatta MW, Whitmore CD, Dovichi NJ. CE-MALDI interface based on inkjet technology. *ELECTROPHORESIS*. 2009;30:4071-4.
 - [92] Bachmann S, Vallant R, Bakry R, Huck CW, Corradini D, Bonn GK. CE coupled to MALDI with novel covalently coated capillaries. *ELECTROPHORESIS*. 2010;31:618-29.
 - [93] Bachmann S, Bakry R, Huck CW, Polato F, Corradini D, Bonn GK. Peptide mapping using capillary electrophoresis offline coupled to matrix-assisted laser desorption ionization time of flight mass spectrometry. *ELECTROPHORESIS*. 2011;32:2830-9.

