Review

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On-line capillary electrophoresis-mass spectrometry for the analysis of biomolecules

Mass spectrometry (MS) has become a key tool for the characterization of biologically relevant molecules in the last decade. Due to the complexity of most biological samples an upstream separation is essential. Capillary electrophoresis (CE) has gained much interest due to its high separation efficiency, speed, and often complementary selectivity to liquid chromatography. We describe the state-of-the-art of on-line CE-MS for the analysis of molecules of biological origin. The characterization of peptides, including the study of post-translational modifications, intact proteins, oligonucleotides, and related interaction studies are reviewed. Relevant publications are summarized in tables, including some important method parameters. Key applications are discussed with respect to the advantages and limitations of CE-MS. Coupling interfaces, preconcentration techniques, capillary coatings, and the different CE techniques, *e.g.*, capillary zone electrophoresis, capillary isoelectric focusing, capillary gel electrophoresis, *etc.* are briefly discussed against the background of their bioanalytical applications.

Keywords: Capillary electrophoresis-mass spectrometry / Oligonucleotides / Peptide / Protein / Review
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Abbreviations: ACE, affinity capillary electrophoresis; **APCI**, atmospheric pressure chemical ionization; **APS**, (3-aminopropyl)-trimethoxysilane; **BCQ**, [(acryloylamino)propyl]trimethylammonium chloride; **CGE**, capillary gel electrophoresis; **FTICR**, Fourier transform-ion cyclotron resonance; **Hb**, hemoglobin; **ICP**, inductively coupled plasma; **IMAC**, immobilized metal-ion affinity chromatography; **MAPTAC**, [3-(methacryloylamino)-propyl]trimethylammonium chloride; **PTM**, post-translational modifications; **PVA**, poly(vinyl alcohol); **RPLC**, reversed-phase liquid chromatography; **SQ**, single quadrupole; **TQ**, triple quadrupole

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1 Introduction

Genomics and, even more important, the wide field of proteomics and related clinical applications like biomarker screening dramatically increase the demand of sensitive and selective analytical tools for the analysis of biological samples. In this context, mass spectrometry (MS) plays a key role due to its specificity and power with respect to the identification of unknown compounds. Due to the complexity of most biological samples an upstream separation is essential. Capillary electrophoresis (CE) has gained much interest due to its high separation efficiency, speed, and often complementary selectivity to liquid chromatography [1].

The small amounts of solute analyzed by CE normally require a highly sensitive detection method. In this sense, sensitivity is of particular interest when dealing with limited sample amounts as, for instance, biomedical applications. In the last decade, MS detection for CE has been increasingly used and developed, especially in the field of proteomics proving its adequate sensitivity through the detection of peptides and protein digests at the attomole and femtomole level. MS is a more universal detector than UV-Vis, laser-induced fluorescence (LIF) or electrochemical detectors, because of its selectivity and specificity, which also compensates the variation in migration times that frequently occurs in CE. To achieve this sensitivity, a right selection of several parameters involved in the separation (*e.g.*, BGE composition, preconcentration steps), in the ionization technique, and with respect to the mass analyzer has to be carried out. MS provides a second dimension (*i.e.*, molecular weight) and, thus, enables the separation of comigrating biomolecules. Furthermore, structural information and unequivocal identification is possible by tandem-MS (MS/MS or MS^n). Relevant reviews summarizing either a field of CE-MS or the application of CE to the analysis of biomolecules are summarized in Table 1.

This paper describes the state-of-the-art of on-line CE-MS for the analysis of molecules of biological origin (all off-line or combined approaches as CE-MALDI, usually performed in an off-line mode, are skipped). The characterization of peptides, including the study of post-translational modifications (PTMs), intact proteins, oligonucleotides, and interaction studies are reviewed. In order to keep this paper to a reasonable size the applications of CE-MS on metabolite and drug determination are excluded as well as most applications on amino acid or nucleoside analysis. Readers interested on those small molecules can take resort of other reviews covering at least part of this subject [2, 4, 34]. Key applications are discussed with respect to the advantages and limitations of CE-MS, covering the different separation techniques coupled on-line to MS, *i.e.*, CZE, CIEF, CEC, capillary gel electrophoresis (CGE), MEKC, and ITP. All relevant publications are summarized in tables, including some important method parameters. In this review, we do not pretend to compile all the literature previously published about the subject but rather to show the actual situation of the analysis of oligonucleotides as well as proteins and their main fragments by CE-MS.

Table 1. Reviews concerning CE-MS and related areas in bioanalysis

Subject	Ref.
CE-MS, focus on coupling	[2]
CE-MS	[3]
CE-MS	[4]
CE-MS	[5]
CE-MS	[6]
Interfaces (a.o. CE-MS)	[7]
CE-MS and biological applications	[8]
Separations (including CE) coupled to MS	[9]
Proteins and peptides by CE-MS	[10]
Peptides and phosphorylation (IMAC) by LC-MS and CE-MS	[11]
Selective adsorbents CE-MS	[12]
CE-MS for chiral separation	[13]
CEC-MS	[14]
CE-ICP-MS	[15]
Alkaloids by LC-MS,CE, and CE-MS	[16]
Anticancer drug monitoring by LC-MS and CE-MS	[17]
Intact proteins by CE	[18]
CE of proteins 1999–2001	[19]
CE applied to biotechnology-derived therapeutic drugs	[20]
CE of peptides	[21]
CE of peptides	[22, 23]
CE of amino acids	[24]
CE of biopolymers	[25]
2-D electrophoresis compared to alternatives like CIEF-MS	[26]
DNA-adducts	[27]
CE for interaction studies	[28] [29]
Antigen-antibody studies by CE	[30]
Proteomics based on capillay separations	[1]
LC-MS in proteomics	[31]
Proteomics 2002	[32]
MS in proteomics	[33]

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2 Coupling

2.1 CE-ESI-MS

Electrospray ionization (ESI) is by far the most frequently applied coupling technique for the bioanalysis by CE-MS [3, 9], primarily because it allows molecules in the liquid phase to be transferred directly into ions in the gas phase and, also, because it allows the determination of high-molecular-weight molecules by bringing about the multiple charging of these biomolecules (most mass spectrometers have a limited mass range). Moreover, CE separates mostly charged analytes (at least in the mostly applied CZE mode) which makes ESI as the ionization technique for polar and ionic compounds (ions are formed in the liquid phase) the self-evident method of choice. Compared to LC-ESI-MS two additional key requirements have to be considered: the electric CE circuit needs to be closed at or close to the sprayer and very low and BGE-dependent flows need to be handled.

A general problem with respect to the robustness in the CE-ESI-MS coupling is to prevent the CE current from having an influence on the electrospray current. Currents in CE are typically three orders of magnitudes higher than those found in the electrospray. Therefore, a separation of the electrical circuit of CE from the circuit of the ESI source is required, considering the fact that electrochemical reactions are involved in both processes, potentially influencing the ionization efficiency of the electrospray. This is of importance especially in the case of negative separation potential combined with positive ESI detection. Moreover, the application of high CE currents is desired, as long as heating does not influence the separation, since a high electric field strength and a concentrated BGE enable fast and efficient separation. Grounding the ESI needle and applying the ESI voltage on the MS inlet is the most robust way to circumvent any problems with respect to CE-current-ESI interaction. However, only a few manufacturers of ESI-MS instruments have this hardware configuration [35].

2.1.1 Sheath-flow interface

Firstly developed by Smith *et al.* [36], this robust design offers more flexibility for BGE selection and, therefore, it can be used in a wide range of separations. For these reasons, this is the most commercialized and applied ESI interface at the moment [2]. The supply of a coaxial liquid enhances the robustness and delivers a stable spray. Thus, this coupling is also useful for separations with a low EOF. However, by diluting the analytes, sheath-flow interfaces reduce sensitivity compared to sheathless interfaces. Apart from the electrophoretic parameters that should be optimized in order to achieve a suitable separation of the analytes, there are several parameters involving the ESI itself that should also be taken into account and carefully optimized. The correct optimization of these parameters (*i.e.*, sheath-liquid composition, sheath-liquid flow, position of the capillary tip, dry gas temperature, and nebulizing gas flow and pressure) will yield a high MS signal and, therefore, low limits of detection. After optimizing these parameters, analysis can typically show detection limits in the low fmol-range for peptides, depending on the MS analyzer applied.

The use of sheath liquid greatly enhances the possibility of optimizing the electrospray process. Thereby it is possible to reduce the influence of nonvolatile components [37], or to improve the selectivity. This is clearly visible in the so called “wrong-way-around” detection. For example, Lewis *et al.* [38] separated peptides in a basic BGE, where separation was optimal, and detected the peptides by positive ESI-MS applying a low flow of sheath liquid consisting of 10% acetic acid.

2.1.2 Sheathless interface

First this nanospray interface was proposed by Olivares *et al.* [39]. In principle, it is the optimal design for coupling CE with ESI-MS due to its compatibility in terms of liquid flow and the high sensitivity that can be achieved. However, the main difficulty here is to close the electrical circuit required for any CE separation. Several strategies have been developed to close the CE separation circuit in a sheathless interface. Among these, coating the capillary outlet with a conductive metal or polymer or inserting a conductive wire into the outlet of the capillary are the most commonly used. However, the fabrication of this interface is delicated and time-consuming, while their robustness and stability have to be improved. Kelly *et al.* [40] described different procedures to produce durable gold coatings for combined CE-MS using a sheathless interface. Furthermore, they tested different coatings of the inner capillary surface to control the EOF. The sheathless interface was also compared to the coaxial sheath flow coupling with respect to performance and sensitivity. While the sheathless interface provided limits of detection for a set of standard peptides between 0.1–5 fmol, the coaxial sheath flow interface showed reduced sensitivity (3.1–21 fmol). However, in the sheathless interface an EOF close to zero as produced from neutral coatings or plastic capillaries can not be used. To avoid potential corona discharge, SF_6 can be applied as a sheath gas [41].

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2.1.3 Liquid-junction interface

Firstly developed by Henion and co-workers [42], the liquid-junction interface provides the electrical connection for closing the CE circuit *via* a liquid reservoir. An ESI emitter capillary is positioned opposite the end of the separation capillary with a gap of *ca.* 10–25 μm between both in such a way that the liquid and analytes from the separation capillary pass to the emitter tube and are sprayed afterwards. The main advantage of this setup is that CE and ESI can be operated independently due to the partial electrical and physical disconnection of the CE separation from the ESI emitter. The main disadvantages have been the difficulty of positioning the transfer capillary in a reproducible way, and the potential loss of separation efficiency in passage through the ESI emitter [43, 44]. Also, the ESI emitter can originate a counter hydrodynamic flow in the CE capillary, and, in some cases, a pressure (typically 20–40 mbar) has to be applied to the inlet capillary to counterbalance this effect. Although some new developments improving the original design have been reported [45–49], some of the original difficulties (*i.e.*, peak broadening and, thus, loss of separation efficiency) frequently remain, and the construction of the junction between the CE capillary and the ESI emitter continues being a difficult task.

2.2 Other ionization techniques

CE has frequently been coupled on-line to inductively coupled plasma-mass spectrometry (ICP-MS) [15]. We refer to this review with respect to the elemental speciation of metals in biological samples. Atmospheric pressure chemical ionization (APCI) and, more recently, atmospheric pressure photo ionization (APPI) are alternative ionization methods, which might be of interest to be coupled to CE, since they enable different ionization selectivity and they might be more tolerant than ESI with respect to the use of nonvolatile BGE. APPI has recently been applied to the use of nonvolatile BGE and compared to ESI for the analysis of pharmaceutical bases [50]. The results indicate that APPI is less affected by the used phosphate BGE leading to a cluster-free background and, thus, providing a better signal-to-noise ratio. Muijselaar *et al.* [51] applied APCI for the coupling of partial-filling micellar electrokinetic chromatography to mass spectrometry (MEKC-MS). The same group recently developed a low-flow triple tube sheath-liquid design for CE-APCI-MS [52]. Nevertheless, this technique is so far not systematically applied for the analysis of biological relevant samples.

3 Separation techniques

3.1 CZE-MS

CZE, the separation based on different migration velocities (*i.e.*, charge-to-size), is by far the most frequent CE-mode applied in combination with MS detection. This is mainly due to the fact that volatile electrolytes, as required for sensitive MS detection, are easily available for a broad pH-range (< 2 up to > 12), as long as no special BGE additives like cyclodextrins (for enantiomeric separation), micelles (for MEKC), gels (CGE) or ampholytes (CIEF) are used. Beside water a broad range of solvents (alcohols, acetonitrile, *etc.*) is meant for the combination with MS, which can be seen in a large number of nonaqueous CE-MS (NACE-MS) studies. However, the use of NACE to biological samples is focused on special applications since biological matrices are water-based.

3.2 CEC-MS

CEC is a hybrid of chromatography with an electrically driven system (EOF-driven). Thereby it tries to combine the advantage of the flat flow profile of the EOF (*i.e.*, high separation efficiency) with the versatility and loading capacity of chromatography. Packed columns are mostly used for separation but open-tubular CEC gained attraction due to its simplicity and the possibility to run it in commercial CE instrumentation. For details of CEC-MS see the review by Choudhary *et al.* [53].

3.3 CIEF-MS

CIEF provides the highest possible separation efficiency for separation of proteins. In a gel-free approach it is possible to couple CIEF on-line to ESI-MS offering a powerful tool for the analysis of very complex samples. The limitations for coupling of CIEF to MS are the volatility of the ampholytes and the transfer of the very narrow-focused zones into the MS without peak-broadening. For the latter, the cathodic combined with gravimetric mobilization seems to be best suitable [54, 55]. To solve the problem concerning the influence of the ampholyte on the ionization efficiency of the ESI, a dialysis interface prior to the ESI has been developed [56]. Furthermore, the introduction of a second dimension, like CZE or reversed-phase liquid chromatography (RPLC) provides a tool to separate the ampholytes from the analytes (compare Section 11). The majority of published data using CIEF-MS are focused on separation of very complex protein mixtures (cell lysates) in combination with high-resolution Fourier transform-ion cyclotron resonance-mass spectrometry (FTICR). Applications are discussed in Section 10.

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3.4 CGE-MS

The combination of CGE with MS is highly desired for, *e.g.*, size-sieving purposes as routinely applied in CE-LIF for sequencing of DNA or for the analysis of intact proteins. However, gels or monomeric impurities strongly decrease the ionization efficiency of ESI and cause contamination of the mass spectrometer. Nevertheless, the analysis of oligonucleotides could be achieved using the BGE additive poly(*N*-vinylpyrrolidine) [57, 58] or a size-sieving gel [59].

3.5 MEKC-MS

The application of micelles to the electrophoretic separation is intended to alter the separation selectivity due to the pseudochromatographic system of the micelles. However, micelle-forming molecules like sodium dodecyl sulfate are among the strongest inhibitors of ESI efficiency. Therefore, there is a strong need to prevent the micelles (and their monomers) to enter the MS by either applying APCI, counter-migrating micelles or by partial-filling techniques. However, few routine applications in the bioanalysis have been reported, especially where sensitive MS detection is required. The group around Riekkola [60] applied MEKC-MS a.o. to the analysis of iridoid glycosides. Some of the compounds could be detected in plants down to around 50 mg/L. Frommberger *et al.* [61] applied partial-filling MEKC-MS to the analysis of *N*-acyl-L-homoserins in bacteria. Partial-filling MEKC was used for the separation of closely related peptides by Koezuka *et al.* [62]. The on-line ESI-MS detection was not as sensitive as the UV detection, however, allowed the identification of the peptides. Petersson *et al.* [63] recently described the use of perfluorooctanoic acid up to a concentration of 100 mM without significant impact on the ESI process and with a detection limit down to the 25 ppb level for drug impurities. The use of such volatile micelles might significantly expand the application of MEKC-MS.

3.6 ITP-MS

From the early work by Smith *et al.* [64, 65] the on-line coupling of ITP with MS has gained attention because of the higher loading capacity compared to CZE. However, the low separation efficiency and difficulties in finding appropriate spacer limit its applicability. Thus, ITP is mainly used as an on-line preconcentration step prior to CZE (transient-ITP). This is clearly shown in a recent publication by Peterson *et al.* [66]. For low concentration of peptides ITP caused discrimination in the ESI-MS, whereas the comprehensive ITP-CZE-MS approach offered a loadability of 10 μL in conjunction with separation in the CZE and on-line MS detection. Zhao *et al.* [67] applied ITP-MS successfully to the analysis of radiation-induced damage of deoxynucleotides.

4 On-line preconcentration

Sensitivity is one of the most important issues when dealing with any analytical technique. The absolute sensitivity of a separation technique coupled to MS is almost independent of the separation method (despite the question of peak concentration/peak width). The absolute sensitivity mainly depends on the ESI process and the applied MS instrument and mode. On the other hand the concentration sensitivity is proportional to the amount of solution loaded on the separation device. One of the main disadvantages of CZE (and as a consequence of CZE-MS) is the limited amount of sample that can be injected into the capillary without ruining the separation resolution, which reduces considerably the concentration sensitivity of this technique (compared to, *e.g.*, HPLC). Moreover, when dealing with complex biological samples, it might also be necessary to perform a purification step of these samples prior to the separation. Both facts have brought about the need of on-line and off-line preconcentration procedures. Several on-line approaches have been developed in order to increase CZE-MS sensitivity. They are briefly described below.

4.1 Stacking

Stacking techniques developed for CZE-UV, as sample stacking, *i.e.*, the injection of the sample with low conductivity, are easily applicable for CZE-MS as well. Thus, the loadability of the capillary can often be increased by one order of magnitude, leading to the injection of roughly 10% of the capillary length (few hundreds of nL). The only limitation is the missing outlet vial, resulting in difficulties to use for instance sample stacking with large volumes or reversed polarity. The application of a switchable sheath liquid overcomes this problem, however, complicates the analysis procedure and automation.

4.2 Transient ITP

Transient ITP can be used to increase the injected sample volume without significant loss of separation efficiency and resolution. Firstly the analytes are concentrated in discrete sample zones between leading and terminating electrolyte. When the zones disintegrate, CZE separation takes place. Zhang *et al.* [68] reported the transient ITP-CZE-MS analysis of an angiotensin peptide mixture per-

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formed on a microdevice coupled to MS. Resolution and sensitivity were enhanced up to 10 times with the transient ITP preconcentration step. Larsson and Lutz [69] also used this approach for sensitivity enhancement in CZE-ESI-MS of peptides. The technique was applied to the qualitative analysis of a tryptic digest of cytochrome *c* and to the quantitative analysis of melagatran and two endogenous peptides. LODs were in the range of 6 nM corresponding to an absolute detection limit of 3 fmol. Amphoteric analytes, like peptides, can be preconcentrated by pH-mediated stacking, which also comprises transient ITP. This general method enhances the loadability at least 10-fold [35].

4.3 SPE on-line preconcentration

On-line preconcentration procedures based on solid-phase extraction promises the best improvement in loadability, since it allows injecting similar amounts as in LC. On-line SPE-CZE-MS has been developed with membranes or stationary phases packings near the capillary injection end [70–83]. As a general protocol, samples are passed through these devices. Then they are washed with BGE and cleaned in this way. Afterwards an organic solvent is usually applied to elute analytes before voltage application. Generally a transient ITP step is used to further concentrate the zone of eluted analytes before starting the CZE separation. For instance, Settlage *et al.* [46] reported a membrane preconcentration transient ITP-CZE-MS method for the sequence determination of tumor peptides using a porous glass junction μ-ESI interface. A polystyrene divinyl benzene (PSDVB) membrane preconcentration cartridge was installed at the head of the column facilitating the concentration of the sample. HPLC fractions from a human melanoma tumor cell extract were analyzed by mPC-transient ITP-CZE-MS. Detection limits of less than 50 amol in full-scan and MS/MS modes were observed.

Waterval *et al.* [70] have reported on preconcentration prior to the CZE separation of therapeutically active peptides in conjunction with UV and MS detection. An (3-aminopropyl)-trimethoxysilane (APS)-coated capillary and an acidic BGE were used after on-line preconcentration using silica columns. Mixtures of gonadorelin and angiotensin II in plasma could be separated. This preconcentration procedure allowed the loading of larger sample volumes and an improvement in sensitivity of 3–4 orders of magnitude could be obtained. Herring and Qin [71] have also made use of these preconcentrating devices. In this case, the preconcentrator is built with quartz wool and Poros beads and can be operated over a wide range of pH. It has been applied previously to CZE-MS to the analysis of a tryptic digest of the myosin I heavy chain kinase with concentration detection limits in the mid-picomolar range. Barroso and de Jong [72] have used an on-line preconcentrating column based on RP-C_{18} particles for the concentration and cleaning of proteolytic digests and major histocompatibility complex class I bound peptides. A 10-fold sensititivity improvement and a 100-fold increase in sample volume introduced in the capillary could be achieved.

5 Capillary coatings for CE-MS of peptides and proteins

It is already well known that separation of biomolecules (especially basic peptides and proteins) by CE using fused-silica capillaries is strongly hampered by solute adsorption onto the capillary wall [84]. Thus, adsorption of proteins onto the capillary wall seems to be the main reason for an observed efficiency loss [85], poor reproducibility in migration times, and low protein recovery rates [86]. Electrostatic interactions between positively charged residues of the proteins and negatively charged silanol groups are responsible for such adsorption effects [87]. Analysis by CE-MS suffers from the same adsorption problems than CE using other detection systems. Moreover, frequently a constant and high EOF is desirable in CZE-MS in order to maintain a stable electrical circuit. As a consequence, different strategies have been applied in CE-MS to overcome this limitation [4, 6]. In general, the main approach consists of using coatings covalently bonded to the capillary wall that can bear positive [88–90], neutral [91–93], or negative charges [94]. However, their main limitation is the time required to prepare the coating and its low stability at extreme pH. Also, the high price of some commercially available coated capillaries is an important drawback that needs to be considered. Other procedures used in CE-MS to reduce the protein adsorption consist of using noncovalent coatings onto the capillary wall as single [95] or multiple layers [96].

Different coatings have been developed for peptide and protein separation. Cationic coatings like APS provide stable electrospray currents by generating high EOF of the BGE. However, these types of coatings require long derivatization steps and they have relatively short lifetimes (1–2 days). Furthermore, for the analysis of complex samples like protein digests the EOF is often too high to obtain a good separation. Cao and Moini [97] studied the analysis of peptide mixtures and protein digests using three different types of capillary columns: untreated columns, freshly derivatized APS columns, and degraded APS-treated columns. The main reason for studying degraded APS columns is its lower EOF. Pressure assis-

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tance together with the use of APS-degraded columns gave best results for the separation of hemoglobin (Hb) S tryptic digest.

In a previous work, Li *et al.* [98] reported the use of an APS coating to separate tryptic digests of Hb in order to identify mutation sites in Hb chains (Hb A, S, and E). The total digestion of Hb normally results in a large number of digest products which are difficult to separate, especially when nonexpected digestion products are considered and the time for separation is short due to the strong EOF provided by the APS coating (*i.e.*, less than 5 min). In a latter work [99], they reported the analysis of Hb and its variants using a Polybrene coating, slowering down the EOF (compared to APS), and, thus, improving the resolution. The Polybrene-based coating was found to be highly reproducible, durable, and easy to prepare. 10–15 fmol of Hb variants A, S, C and D were analyzed separately in 10–15 min each. The use of a dynamic capillary derivatization with Polybrene for peptide separation was also reported by other authors like Tomlison *et al.* [81, 100].

Bateman *et al.* [101] studied several new disposable microsprayers for CE-MS coupling. Together with the optimization of the microsprayer configuration, they compared different capillary coatings ([3-(methacryloylamino)-propyl]trimethylammonium chloride (MAPTAC); [(acryloylamino)propyl]trimethylammonium chloride (BCQ); Polybrene, *etc.*) for the separation of peptides. Figure 1 shows the effect of the capillary coating on the separation of a selected group of peptides using different microsprayers. In this particular case, the capillaries were covalently modified with BCQ and MAPTAC, minimizing peptide adsorption when using an acidic BGE. BCQ and MAPTAC coatings provide separation efficiencies similar to those obtained using a Polybrene coating. However, the use of Polybrene required a recoating between analysis and other inconvenients and, therefore, BCQ coating was selected for further analysis. These conditions were applied to the analysis of glycopeptides in glycoprotein digests. Other coatings like polyacrylamide or poly(vinyl alcohol) (PVA) have also been used in CE-MS in order to reduce the strong adsorption biopolymer-capillary wall that can take place with acidic BGEs [47, 102–105].

Recently, a new physically adsorbed coating for CE-MS has been presented using an ethylpyrrolidine methacrylate-*N,N*-dimethylacrylamide polymer (EPyM-DMA). The capillary coating is simple and easy to obtain and only requires flushing the capillary with the aqueous polymer solution for 2 min. It is demonstrated that the use of this coating significantly reduces the electrostatic adsorption of proteins to the capillary wall. The physically adsorbed coating protocol is demonstrated to be compatible with

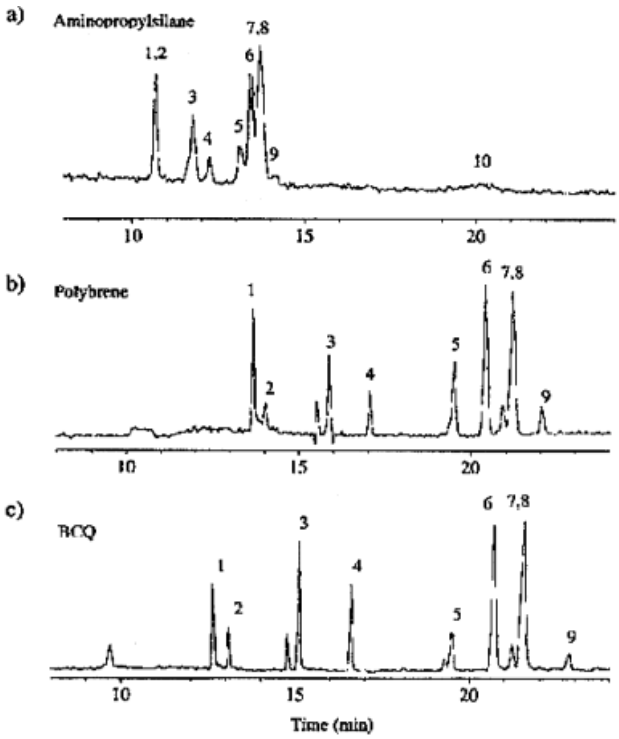


Figure 1. Effect of capillary coating and interface arrangement on separation efficiency for nanospray CZE-ESI-MS analysis of a peptide mixture. (a) One-piece column with aminopropylsilane coating; (b) butted-tip with Polybrene coating; (c) one-piece column with BCQ coating (MAPTAC gave the same result). Peak 1, Leu-enkephalin; 2, Glu-fibrinogen; 3, substance $P_{3\ 11}$; 4, cholecystokinin$_{10\ 20}$; 5, subsance $P_{1\ 9}$; 6, angiotensin I; 7, Val_4 Ile_7 angiotensin III; 8, $ACTH_{4\ 10}$; 9, $pGlu_4$-myelin basic protein $(MBP)_{4\ 14}$; 10, $ACTH_{11\ 24}$. Reprinted from [101], with permission.

ESI-MS *via* the reproducible separation of analytes (basic proteins), obtaining RSD (n = 5) lower than 1% for the analysis time reproducibility and lower than 5% for the peak height. The method was applied to address different problems in food analysis, based on CZE-MS analysis demonstrating the great possibilities of this technique to face real life problems [106].

6 MS instrumentation

CE has been coupled to many different types of mass analyzers, *i.e.*, ion traps (ITs), quadrupole (Q), FTICR, sector field (E,B) and time-of-flight (TOF)-type instruments. Quadrupoles have been the most commonly used mass analyzers for CE-MS, probably because of there availability in many labs. However, slow scanning speed and low resolution together with the relative low sensitivity in full-scan mode constitute important limitations of quadrupole

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analyzers. TOF mass analyzers, however, have a high duty cycle, good sensitivity, and high resolution. Concerning future CZE applications using high field strength (*e.g.*, chipCE-MS), where the expected peak width is in the range of milliseconds, only modern TOF analyzers with the possibility to acquire more than 20 spectra/s seem to be the alternative for MS detection. ITs are frequently used for the CE-MS analysis of peptides since they provide adequate scanning speed and sensitivity and, more interestingly, they are able to carry out fast MS^n experiments. The latter performance characteristic makes the IT-MS also interesting for structure elucidation.

The combinations of CE with other analyzers as, *e.g.*, FTICR, hybrid MS like Q-TOF, or triple quadrupole mass spectrometers (QqQ), have been reported [9]. FTICR-MS offers an excellent sensitivity, a high resolution ($>$ 100 000 with low ppm mass measurement error), and the possibility to carry out MS^n experiments, as has been demonstrated by the separation of tryptically digested cerebrospinal fluid [107]. Moreover, the new generation of FTICR-MS with magnetic fields higher than 9 Tesla is capable of sub-attomole sensitivity at nearly 1 s acquisition times [108, 109]. Recently, the principle usability of Hadamard transform TOF-MS (HT-TOF-MS) has been shown as a promising detector for any capillary-format separation that can be coupled to MS by electrospray ionization. HT-TOF-MS was able to sample peaks having widths in the millisecond range [110]. However, due to the high cost, sophisticated MS equipment (*e.g.*, Q-TOF, FTICR) can hardly be considered as routine detector for CE today.

7 Chip-based CE-MS

Over the past few years, microfabricated devices (microdevices, chips, microchips, lab-on-a-chip) have been developed to perform CE (and HPLC or CEC) on chips rather than in capillaries. The advantages to be expected are widespread: (i) the speed can substantially be increased due to shorter separation channels; (ii) the throughput can be increased furthermore due to parallelization; (iii) different injection schemes and miniaturization steps enable the handling of very small sample volumes; (iv) the two spatial dimensions and variable manufacturing possibilities enable the integration of CE separation on-chip combined with enzyme reactions, sample dilution, derivatization, concentration, and purification, *etc.*

An instrumental description of the different procedures to fabricate these devices and/or the different ways in which they can be coupled with MS is out of the scope of this review, an updated description of these points can be found in a recent review [7]. However, it is worth mentioning that most of these approaches suffer from difficulties in fabrication and they still require improvements in the LODs and separation power. Thus, this technique is still away to be routinely applied and, therefore, most papers deal with the possibility of chip-MS coupling itself, rather than the application. Furthermore, it is noticeable that most publications do not show an electrophoretic separation combined with on-line ESI-MS detection.

8 Applications to peptides

8.1 General aspects

Nowadays, the importance of peptides is evident in many fields. These biomolecules play an important role as hormones and neurotransmitters, in the design of new drugs, in clinical diagnosis, in proteomic studies, *etc.* In recent years, the separation of peptides by CE has become one of the most exciting application fields of this technique. The high efficiency (typically in the range of hundreds of thousands of plates) together with the fast analysis ($<$ 30 min) and the compatibility with small sample volumes have made CE-MS to a major laboratory tool and a complementary tool to widely used LC-MS for the separation, analysis, and characterization of such biomolecules. Tables 2, 3, and 4 summarize some of the main applications of CE-MS in peptide analysis, classified according to the type of interface used. Moreover, some other interesting information as the type of peptidic sample studied, the MS analyzer employed, the BGE used for the CE separation, the nature of the sheath liquid, the coating of the capillary (if any) are provided (when available), giving in this way an overview about the typical experimental parameters along with the achieved sensitivity of these type of analysis.

Table 2 shows a compilation of different peptide analyses that have been carried out using CE-MS with a sheath flow interface. Table 3 summarizes examples regarding the use of a sheathless interface for the CE-MS analysis of peptides or protein digests. As it can be seen, very low LODs have been achieved. In general, these LODs are lower than those obtained by a sheath-liquid interface, mainly due to the dilution of the sample by the sheath liquid and the dependence of the ESI on the concentration of the analyte. In the work by Cao and Moini [142] an in-capillary electrode sheathless interface is used for the analysis of a mixture of peptides, proteins, and tryptic digests. This in-capillary electrode consists of a platinum wire placed inside the capillary outlet. Interestingly, changing the wire length from 0.1–3.5 cm or varying the insertion of the Pt wire between 1 and 4 cm had no significant effect on the separation or ESI. Also, the possible background noise that the epoxy used to seal the hole

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Table 2. Peptide analysis by CE-MS using a sheath-flow interface

Sample	MS	BGE	Coating	Sheath liquid	Enrichment	Sensitivity	Ref.
Peptide std.[a] +digest	Sec.[b]	1% HOAc + 2 mM NH_4OAc	Dynamic: Polybrene		SPE	Shown: 40 fmol (dig) LOD: 8 fmol	[81, 100]
Peptide std.	TOF	0.1% HOAC	(Amino)	MeOH, 4 μL/min	–	LOD: 8 fmol	[95]
Peptide std.	TQ[c]	0.1 M formic acid	MAPTAC	25% w/v MeOH, 0.2% w/v formic acid, 2 μL/min	–	LOD: 3–21 fmol (SIM) shown: 1 pmol (dig.)	[40]
Digest	IT	1% HOAc (pH 2.2)	–	Ethanol-water-formic acid 60:39:1	–	Shown: 1.5 pmol	[111]
Peptide std. + digest	SQ[d]	155 mM HOAc + 55 mM formic acid or 20 mM ε-aminocaproic acid/HOAc (pH 4)	Polyacryl-amide	50% v/v isopropanol, 0.5% v/v acetic acid/formic acid, 5 μL/min	ITP	3 fmol/6 nM M (SIM, S/N = 3)	[69]
Peptide std. + digest	IT	0.2–0.5 M formic acid, 10–25 mM NH_3, 10% ACN	–	Isopropanol:water (50:50), 1–2 μL/min	pH-mediated stacking	500 amol/1 nM (full scan, S/N = 3)	[35]
Peptides in hemo-dialysis fluids	TOF	30% MeOH, 0.5% formic acid in water (pH 2.3–2.5)	–	30% MeOH, 0.5% formic acid, <10 μL/min	–	Shown: 100 fmol	[112]
Peptide std.	TOF	25 mM NH_4OAc/HOAc in water:methanol (1:1)(pH 5) or 100 mM NH_4OAc/HOAc (pH 4)	Untreated and APS	25 mM NH_4OAc in water:methanol (1:1) 0.1% HOAc, 4 μL/min	–	Shown: ~ 500 μM	[113]
Peptide std. + digest	IT	0.9 M amonium formate/formic acid (pH 2.0)	–	Methanol:water (50:50 v/v), 0.05% v/v formic acid 4 μL/min	–	fmol	[114]
Peptide std. + digest	IT	Ammonium formate/formic acid, NH_4OAc/HOAc	–	Methanol:water (50:50 v/v), 0.05% v/v formic acid 4 μL/min	–	Shown: 300 fmol	[115]
Peptide std.		0.1% HOAc or 10 mM NH_4OAc or 100 mM ammonium bicarbonate	–	MeOH 4 μL/min	–	Shown: 480 fmol	[116]
Peptides std.	TQ	20 mM Tris-HOAc (pH 8.1)	–	5 mM Tris-acetate (pH 8.1) H_2O:MeOH (25:75 v/v), 1.5 μL/min	–	–	[117]
Peptides in human urine	TOF	30% v/v MeOH + 0.5% v/v formic acid (pH 2.4)	–	30% v/v MeOH, 0.5% v/v formic acid (pH 2.4), 5 μL/min	–	–	[118]
Digest	TQ	Several BGEs (pH 3.1–8)	PVA	1:3 v/v mixture BGE/MeOH (1.5–2.5 μL/min)	–	Shown: 2.9–46 pmol/μL	[103]
Peptide std.	Sec.	0.01 M NH_4OAc (pH 8) or 0.01 M HOAc (pH 3.5)	Uncoated and APS	50:50 MeOH/3% HOAc	–	Shown: 700 fmol	[119]
Peptide std.	TQ	25 mM NH_4OAc (pH 3.5)	–	80% ACN, 20% 5 mM NH_4OAc pH 3.5, 2 μL/min	Hydro-dynamic or elec-trokinetic injection	–	[120]
Peptide std.	TQ	20 mM Tris-acetate (pH 8.1) 70–120 μM vancomycin	Polyacryl-amide, PVA	5–10 mM Tris-acetate, pH 8.1, in H_2O:MeOH (25:75 v/v), 1.5 μL/min	–	–	[102]
Digest	TQ	2 M formic acid	Polybrene	25% MeOH, 0.2% formic acid, 5 μL/min	–	Shown: 1–10 pmol	[121]

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Table 2. Continued

Sample	MS	BGE	Coating	Sheath liquid	Enrichment	Sensitivity	Ref.
Peptide std.	IT	Eluent: 50% ACN v/v + 3.2 mM NH_4OAc	CEC C_{18} columns	MeOH/H_2O/HOAc 80/20/0.1 v/v/v 1–1.5 μL/min	Electrokinetic injection	2 μM (full scan), 500 nM (SRM)	[122]
Peptide std. digest	SQ	0.01 M HOAc	–	MeOH, 4 μL/min	–	200 fmol (full scan) 1.5 pmol (SIM)	[123]
Peptide std.	SQ	1–2.5 mM CTAC (pH 4.3)	–	MeOH-H_2O-HOAc 80:10:10, pH 2.8	–	Shown: 4–64 pmol	[124]
Peptide std.	TOF	50 mM HOAc + 50 mM formic acid (pH 2.85)	–	isopropanol-H_2O, 0.05% formic acid, 60:40 v/v 2 μL/min	–	1–4 μg/mL	[125]
Peptide degradation products	IT	200 mM formic acid (pH 2.9, ammonia) +10% ACN	–	Isopropanol:H_2O 1:1, 2 μL/min	–	–	[126]
Peptide std.	TOF	50 mM NH_4OAc (pH 7.5)	–	MeOH:H_2O, 0.2% HOAc, 50:50 v/v, 1 μL/min	–	Shown: 100 fmol	[127]

a) std., standard; b) Sec., sector field type instrument; c) TQ, triple quadrupole; d) SQ, single quadrupole

Table 3. Peptide analysis by CE-MS using a sheathless interface

Sample	MS	BGE	Coating	Enrichment and sensitivity comments		Ref.
Peptide std. digest	TQ	15–20 mM NH_4OAc/HOAc (pH 5.0), 50% ACN	–	–	Shown: 10 pmol/154 μM (digest, full scan)	[128]
Digest	TQ	10 mM NH_4OAc/HOAc (pH 4.4)	APS	Electrokinetic injection	Shown: 30 fmol (full scan)	[129]
Peptide std.	TOF	50 mM HOAc, 50% MeOH	–	–	40–80 fmol/1–2 μM	[130]
Peptide std.	TQ	10.5 mM HOAc, 10% MeOH (pH 3.2)	APS	–	Pep. 300 amol/200 nM (S/N = 5) dig. 38–50 fmol (full scan, MS/MS)	[131]
Peptide std. digest	TQ	0.1 M formic acid	MAPTAC	–	0.1–5 fmol (SIM) shown 1 pmol (dig.)	[40]
Peptide std. digest	SQ	0.01 M HOAc	APS	–	0.6–4 fmol (full scan)	[90]
Peptide std. digest	TQ	0.1 M formic acid	BCQ	On-line SPE	Shown: 30 nM (dig.) (full scan/SIM)	[73]
Peptide std digest	IT	0.1 M HOAc 40% ACN	–	On-line SPE	1 fmol/1 nM (S/N $\geq$ 50:1) shown: 100 nM BSA dig.	[72]
Digest	IT	0.01 mM HOAc, 10% MeOH	–	On-line SPE	Shown: 150 fmol/ 20 nM	[71]
Peptide std. digest	TQ	1–5% HOAc	–	–	3.5 pmol/0.1 μM (S/N = 190) dig.:300 fmol (SIM, MS/MS)	[132]
Peptides in plasma	TQ	25 mM formic acid + 5% MeOH (pH 2.3)	APS	On-line SPE	Shown (≈LOD) 9 fmol/ 5 nM (full scan)	[70]
Peptide std. Digest	TOF	0.01 M HOAc (pH 3.4)	APS	–	Shown: 170–440 fmol (pep.)	[97]
Peptide std.	TOF	5% HOAc	–	–	Shown: 230 fmol	[74]
Digest	TOF	0.01 M HOAc (pH 3.4)	APS	–	Shown: 120 fmol	[133]
Peptide std. + digest	IT	0.1% HOAc	Monolithic polymeric columns	Electrokinetic injection	Shown: 50 fmol	[134]
Hemoglobin digest	IT/reTOF	10 mM NH_4OAc	APS		–	[98]

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Table 3. Continued

Sample	MS	BGE	Coating	Enrichment and sensitivity comments		Ref.
Hemoglobin digest	IT/reTOF	100 mM formic acid + 5 mM NH_4OAc (pH ~3)	Polybrene	Electrokinetic injection	Shown: 10–50 fmol	[99]
Peptide std. + digest	IT	95% 1.0 M HOAc/5% ACN	–	On-line SPE	500 amol (full scan)	[75]
Peptide std.	TOF	ACN/H_2O/HOAc (50:50:0.3 v/v/v)	–	–	Shown: 3.3–4.6 fmol (S/N ~35)	[135]
Peptide std.	FTICR	10 mM HOAc (pH 3.4)	APS	Electrokinetic injection	Shown: 500 amol	[136]
Digest	IT/reTOF	100 mM formic acid + 5 mM NH_4OAc (pH ~3)	Polybrene	Electrokinetic injection	Shown: < 10 fmol	[137]
Digest	TQ	1 M formic acid	BCQ	–	Shown: 2–15 pmol (full scan)	[138]
Peptide std. digest	IT/reTOF	10 mM HOAc/NH_4OAc (pH 3–4.9)	APS	–	Shown: 3–50 fmol (full scan)	[139]
Peptide std. digest	IT	1 M HOAc (pH 2.4)	–	–	Shown: 25 fmol, LOD ≈900 amol (S/N = 5) (peptides)	[140]
Peptide std.	TQ or IT	25 mM formic acid, 5% MeOH	–	On-line	Shown: 1.6–1.4 fmol (SIM)	[76]
Peptide std. + digest	TQ	0.1–2 M formic acid	BCQ	–	Shown: 6.25–18 fmol LOD 2–10 fmol (full scan). Shown: 1.5 pmol (dig.)	[101]

Table 4. Peptide analysis by CE-MS using a liquid-junction interface

Sample	MS	BGE	Coating	Enrichment	Sensitivity	Ref.
Digest	TQ	10 mM HOAc (pH 3.3) 10% MeOH	APS	On-line SPE	660 amol (full scan), 6 fmol (MS/MS)	[78]
Peptide std. digest	TQ	10 mM HOAc (pH 3.3) 10% MeOH	APS	Electrokinetic injection	Peptide: 300 amol (full scan), 600 amol (MS/MS). Shown: 38–50 fmol (MS/MS) digest	[141]
Peptide std. (CE) digest (CLC)	IT	20 mM 6-aminocaproic acid/HOAc (pH 4.4)	PVA	–	fmol range (full scan), amol range (SIM)	[47]

where the wire is inserted into the capillary was investigated. LODs in the low fmol range were achieved. Although the liquid-junction interface has been rarely applied to peptide analysis (see Table 4), several applications of this interface have been described by Figeys *et al.* [77, 78, 141] using a liquid junction μESI coupled to a triple-quadrupole as mass analyzer. Thus, Figeys *et al.* [141] reported a method for the identification of proteins by their amino acid sequence using APS-coated capillaries and acidic BGE. An LOD in the attomole range for peptides was reported. 50 and 38 fmol of β-lactoglobulin and BSA digest, respectively, were separated and determined by CZE-MS/MS. However, not all of the expected tryptic peptides were observed, which was attributed to matrix effects. In a more recent work [78], on-line SPE was introduced as a sample clean-up procedure in order to reduce matrix effects. Foret *et al.* [47] reported a new ESI interface (liquid junction interface with the ESI needle enclosed in a subsatmospheric chamber) for coupling the MS with microcolumn separations. CE separation of an angiotensin mixture using an acidid buffer in a PVA-coated capillary and capillary LC of cytochrome *c* tryptic digest were shown using this interface. The LODs for the analyzed peptides were in the low femtomole range (total ion monitoring) or in the high attomole range (SIM).

As can be deduced from Tables 2, 3, and 4, the main applications have been devoted to study mixtures of peptide standards. This is due to the fact that the main subject of most researchers has been the instrumental devel-

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opment of CE-MS required to carry out such analyses. However, some new and more application related studies have been proposed and are described in the following part.

Hb first variant was discovered in 1956 and, up to now, more than 700 structural variants of Hb are known. Hb abnormalities can occur as a result of a defense mechanism or even of random mutations, motivating the analysis of Hb variants. The structural characterization of Hb has been carried out by CZE-MS based upon the analysis of its tryptic digest [97–99] probing that CZE-MS constitutes an alternative and rapid method for the determination of Hb variants.

Lyubarskaya *et al.* [103] reported a new alternative for the continuous determination of inmunoreactive fragments in tryptic digests (epitope mapping). The method involves the use of on-line affinity capillary electrophoresis (ACE)-ESI-MS with a sheath-liquid interface and was applied to the determination of the inmunoreactive fragments of human β-endorphin tryptic digest. Different BGEs and PVA-coated capillaries were tested by separate injections of β-endorphin's tryptic digest, its antibody, and an incubated mixture of both. The antibody has a faster mobility than the tryptic digest fragments. Injected the monoclonal antibody immediately after the tryptic digest, inmmunoreactive peptides present in the digest bind (during voltage application) to the antibody and disappear from the electropherogram. This constitutes a different and fast method for the epitope mapping.

Kaiser *et al.* [112] reported the CZE-MS establishment of polypeptide patterns in hemodialysis fluids. An ESI sheath flow liquid interface was used in conjunction with a TOF analyzer. The composition of the sheath liquid was the same as the BGE (30% methanol and 0.5% of formic acid). Samples of hemodialysis of two different dialyzer membranes (low and high-flux) were passed through an anion exchange chromatography column and lyophilized in order to remove interfering salts and to concentrate the final sample, respectively. In high flux dialysates, more than 600 polypeptides were characterized in a single run of 45 min. The obtained polypeptide pattern of normal urine samples and urine after hemodialysis are compared. In a second work, the same group analyzed urine from patients suffering from renal diseases and impaired renal function [118]. Urine of unhealthy individuals contains different pattern and amounts of proteins compared to urine from a healthy control group. Therefore, CE-MS profiling of polypeptides from human urine provides suitable information of possible renal diseases. APS- and PVA-coated capillaries were tested but the best results were achieved with uncoated capillaries. More than 200 polypeptides could be determined in more than 50% of the individuals.

In any characterization of protein digests by CZE-MS, separation and detection depend (i) on the BGE composition and (ii) on the occurance of "unexpected" peptidic fragments during the enzymatic protein hydrolysis. In a work by Simó and Cifuentes [114], a new approach was used to optimize this problem, namely a theoretical model was developed that relates the electrophoretic behavior of peptides to their sequence. The effectiveness of this procedure was demonstrated by the fast attainment of good CZE-MS conditions for analyzing the peptides obtained from an enzymatic protein hydrolysate in a single run, including information about "unexpected" fragments from protein digests. An example of the usefulness of this procedure is given in Fig. 2 in which the theoreti-

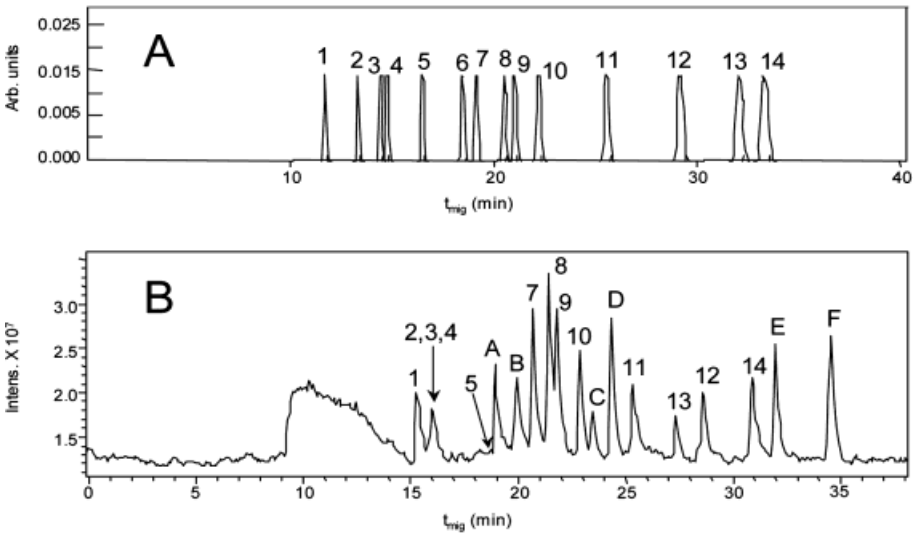


Figure 2. (A) CZE-ESI-MS predicted electropherogram, (B) CZE-ESI-MS total ion electropherogram of cytochrome *c* digest. BGE, 0.9 M ammonium formate/formic acid, pH 2. For more details see text. Redrawn from [114].

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cal and experimental CZE-MS electropherograms are depicted for a group of peptides obtained from a digested protein. The peaks indicated with numbers correspond to the expected fragments from the digestion of cytochrome *c* with trypsin, while the peaks marked with letters indicate the appearance of "unexpected" fragments. As can be seen, using the simulation program it was possible to find an adequate BGE providing a good separation of this mixture. Moreover, the simulation program also gave a good estimation of the expected order of migration of the different peptides simplifying the assignment of the compounds.

Although CZE is by far the most used CE mode, CEC, a hybrid technique between CE and HPLC, has also been used for peptide analysis providing adequate sensitivity. Gaspari *et al.* [122] reported the separation of seven peptides using 25 cm column packed with C_{18}-bonded silica particles as stationary phase and 50% acetonitrile in water with 3.2 mM ammonium acetate as eluent. This column allowed a fast separation of the compounds (*i.e.*, less than 6 min). LODs of 500 nM could be achieved. A different approach was developed by Ivanov *et al.* [134] for the separation of model peptide mixtures and BSA tryptic digest consisting of pressure-assisted CEC-CZE. Different mobile phases in conjunction with varying voltages and pressures were studied both for gradient and isocratic elution. Fast peptide separations were achieved.

8.2 Chip-MS applications to peptides

Among the compounds analyzed by microdevices coupled with ESI-MS, peptides and proteins account for the majority of studies. Karger and co-workers reported several microdevice-MS systems and their application to peptide analysis, listed in Table 5. In Fig. 3 the chip-CZE configuration used by Li *et al.* [146] is shown. A nanoelectrospray interface is formed by attaching a short capillary to the chip. Both chip channel and nanoelectrospray emitter were coated with BCQ. This device enabled the identification of peptides and in-gel digests of proteins with low-nanomolar detection limits. It was applied to the analysis of glycoproteins and identification of a subset of membrane proteins of extracts of different *Haemophilus influenzae* strains. Separations were achieved in less than 90 s. Zhang *et al.* [143] reported a microdevice with integrated liquid junction and attached electrospray capillary for the analysis of peptides, proteins, and protein digests. A subatmospheric electrospray interface was used and LODs in the attomole region were achieved. Figure 4 shows the separation of a mixture of angiotensins achieved with this microdevice [143].

Table 5. Application of microfabricated devices for CE-MS analysis of peptides and protein digests

Sample	MS	BGE	Capillary coating	Enrichment	Sensitivity	Ref.
Peptide std. + digest	IT	20 mM ε-aminocaproic acid/HOAc to pH 4	Polyacrylamide, PVA	Electrokinetic or pressure injection	LOD: 0.1–1 μM (peptides)	[143]
Digest	TOF	Eluent: 15 mM NH_4OAc (pH 4.6)/MeOH 70:30 v/v	Monolithic polymeric columns	Electrokinectic injection	Shown: 800 amol	[144]
Peptide std. + digest	IT	20 mM ε-aminocaproic acid/HOAc pH 4.4 or 1% v/v formic acid	Polyacrylamide	Electrokinetic injection. t-ITP	Low fmol to high amol	[68]
Digest	TQ	2 M formic acid	APTES[a)]	On-line microreactors	–	[145]
Peptide std. + digest	TQ, QqTOF	0.1 M formic acid	BCQ	Electrokinetic injection	LOD: 3.2–43.5 nM (SIM)	[146]
Peptide std.	TOF	MeOH/H_2O/HOAc (50:50:0.3 v/v/v)	–	–	Shown: 10 μM. Sub-amol to low-amol	[147]
Peptide std. + digest	TQ	30 mM NH_4OAc/NH_4OH, pH 8.8, 0.1 M formic acid	BCQ	Electrokinetic injection	LOD: 45–440 nM (SIM, peptides)	[148]

a) APTES, (3-aminopropyl)triethoxysilane

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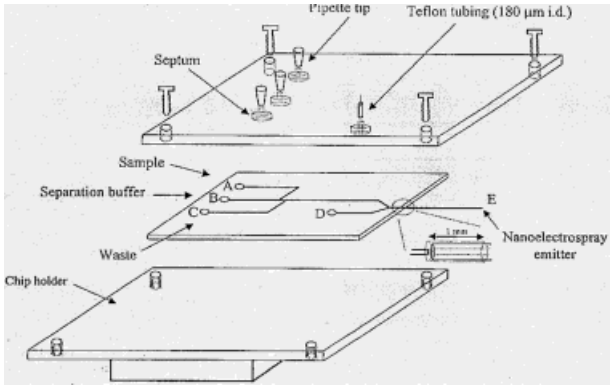


Figure 3. Schematic representation of the chip-CE configuration using a disposable nanoelectrospray emitter interface to either a triple-quadrupole or a hybrid Q-TOF mass spectrometer. Reprinted from [146], with permission.

9 Applications to the characterization of PTMs

Post-translational modifications (PTM) like phosphorylation and glycosylation play an important role in the functionality of biological systems. However, their analysis remains a major task, since the degree of modification is often low (*i.e.*, a few % of the respective protein). Furthermore, the separation often needs special conditions since, *e.g.*, glycopeptides with a large sugar tree are difficult to separate in standard C_{18}-RPLC due to their hydrophilicity. Thus, the use of CE-MS has gained attention in this field. Important CE-MS applications in this area are listed in Table 6. The characterization of PTMs based on intact proteins is included in Section 10.

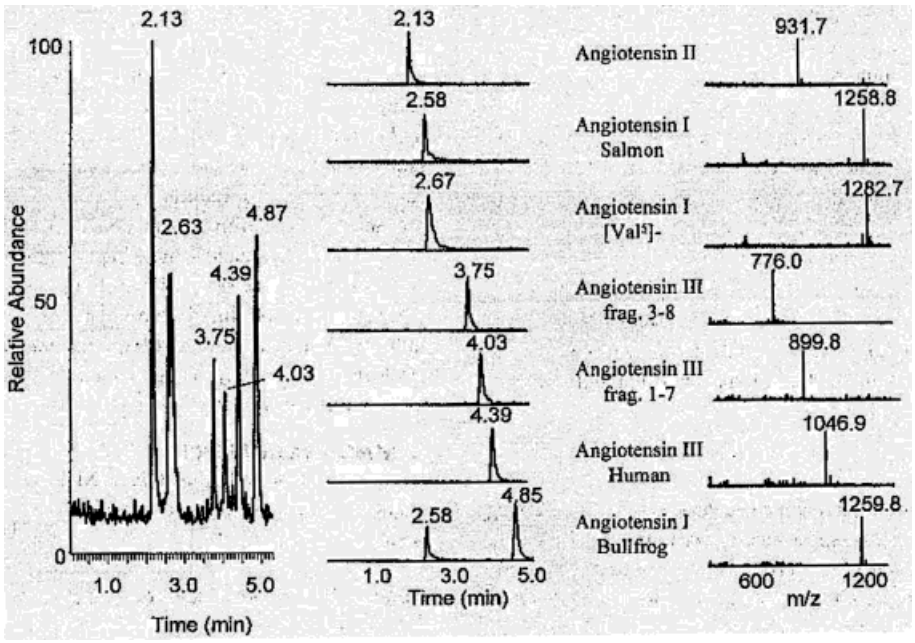


Figure 4. CZE-ESI-MS separation of a mixture of angiotensin peptides using a microdevice. Sample, 10 μg/mL of each peptide; BGE, 20 mM 6-aminocaproic acid/acetic acid, pH 4.4; electric field strength, 400 V/cm; liquid junction solution, 0.8% v/v acetic acid in 50% v/v methanol:water. Reprinted from [143], with permission.

9.1 Glycans

Bateman *et al.* [138] applied an acidic BGE in a BCQ-coated capillary coupled *via* a sheathless nanospray interface, which is operated in the positive mode to a triple-quadrupole mass spectrometer for the analysis of *O*- and *N*-linked glycopeptides. Glycopeptides were identified based on the detection of typical fragment ions like *m/z* 204 (GlcNAc oxonium ion) from CID in the skimmer/nozzle region. Further characterization has been performed by MS/MS experiments. In a similar setup, Zamfir and Peter-Katalinic [151] describe the analysis of the glycosylation by CZE-MS. They applied basic BGE, negative sheathless ESI, and QTOF-MS to the characterization of carbohydrates and *O*-glycosylated peptides. Several glycopeptides from urine samples of patients suffering from *N*-acetylhexosaminidase deficiency could be identified based on data-dependent MS/MS experiments.

Ultrafiltration was used to separate glycosylated from nonglycosylated peptides in phospholipase A of indivdual honeybees prior to the analysis by CZE-MS [150]. Several glycopeptides could be characterized by IT-MS. Different enzymatic carbohydrate releases were used to identify

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Table 6. Analysis of PTMs of proteins by CE-MS

Approach	Application	Interface	MS	Enrich-ment	Coating	BGE (comments)	Ref.
CZE-MS/MS	Glycoforms in tryptic digest of standard proteins	Sheathless	TQ	–	BCQ	0.1 or 1 M formic acid (in-source fragmentation)	[138]
SPE-CZE-MS/MS	Standard peptides and lectin digest	Sheathless/ sheath-liquid	TQ	On-line SPE	BCQ	(sensitivity: 30 nM (dig.))	[73]
CZE-MS	Digest of human erythropoietin (EPO)	Sheath-liquid	TQ	–	PB[a)]	0.67 M formic acid (analysis of RPLC fractions)	[149]
CZE-MS/MS	Glycoforms in tryptic digest of phospholipase of honey bees	Sheath-liquid	IT	–	SMIL[b)]/ PB		[150]
CZE-MS/MS	Mixture of glycopeptides	Sheathless	QTOF	–	–	Acetate, ammonia, 40% MeOH (pH 12)	[151]
CZE-MS/MS	Glcycopeptides in ribonuclease and lectin	Sheath-liquid	IT	–	PB	2 M formic acid	[152]
CZE-MS, MS/MS	(Modified) glycans from endo-toxins or lipopolysaccharides	Sheath-liquid	TQ	–	PB	50 mM ammonium formate (pH 8, NH_3)	[153, 154]
CZE-MS	Oligosaccharides and modified oligosaccharides from lipopolysaccharides	Sheath-liquid	TQ	Tran-sient ITP	–	50 mM triethylamine/ formate (pH 4.5)	[155]
CZE-MS/MS	Oligosaccharides and modified oligosaccharides from lipopolysaccharides	Sheath-liquid	TQ	On-line SPE	–	50 mM morpholine/ formate (pH 9) 2% MeOH	[156]
CZE-MS^n	Carbohydrate standards and glycans from fetuin digest	Sheath-liquid	IT	–	PVA	20 mM caproic acid (pH 4) (ANTS-labeled analytes)	[157]
CZE-MS	Carbohydrates (dextran hydrosylates)	Sheath-liquid	IT	–	–	1% HOAc (pH 3.4, NH_3) (ANTS-labeled analytes)	[158]
CZE-MS	Carbohydrates (standards and in wine)	Sheath-liquid	SQ	–	–	300 mM diethylamine	[159]
CZE-MS/MS	Heparin depolymerized disaccharides	Sheath-liquid	IT	–	–	30 mM formic acid (pH 3.2, NH_3)	[160]
CZE-MS	Phosphorylation (and deamidation) found in myelin basic protein (digest)	Sheathless	ITreTOF	–		100 mM formic acid, 5 mM NH_4OAc (pH 3)	[161]
ACE-MS^n	Phosphopeptide standards and from α- and β-casein digest	Sheath-liquid	IT	IMAC	APS	0.1% HOAc, 10% MeOH	[162, 163]

a) PB, Polybrene
b) SMIL, successive multiple ionic layers

high- and low-mannose-type *N*-glycosylation in ribonuclease B by CZE-MS [152]. The glycans of a lectin from *Bauhinia purpurea* could be characterized by on-line SPE-CZE-MS based on a 30 nM solution [73]. Boss *et al.* [149] achieved almost 100% sequence coverage for the digest of recombinant human erythroprotein applying CZE-MS after prefractionation by HPLC. Glycosylation sites and the carbohydrate tree could be studied in detail.

The characterization of endotoxins and lipopolysaccharides and their mild hydrolysis products by CZE-MS have been studied by Thibault and co-workers [153–155]. Modification/ heterogeneities like phosphates, phosphoethanolamine, and acyl groups could be determined. Both basic and acidic conditions for separating the nonderivatized polysaccharides have been applied allowing the separation of closely related oligosaccharides differing only in the number of carbohy-

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drates. Up to 1 μL (50% of the capillary) could be injected, enabling the characterization of 240 ng of glycans from *Pseudomonas aeruginosa* [155]. Up to 5 μL could be injected with good linear response using on-line SPE-CZE-MS [156].

The separation of most nonmodified carbohydrates, however, requires either the use of strong basic conditions (pH $\geq$ 12) [159] or they need to be derivatized. Derivatization with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) enables a good separation under acidic conditions and sensitive detection both in fluorescence and negative ESI-MS [158]. Gennaro *et al.* [157] used this method to study the glycans released from the model protein fetuin. The ANTS-labeled method was superior to the analysis of the underivatized sample, not that much with respect to separation of (in this case) sialylated glycans, but rather concerning the ability to run extensive MS/MS experiments, since the ANTS label (and thus a fixed negative charge) remains on the fragments and allows detailed structure analyses. Up to MS^5 in a CZE-peak could be performed based on predefined masses. Ruiz-Calero *et al.* [160] applied CZE-MS to the analysis of heparin-derived disaccharides. Due to the carboxyl and partly sulfate groups the separation can be performed without derivatization under acidic conditions (pH 3.2). LODs in the low-μM level allowed the characterization of enzymatically depolymerized mucosal heparins.

9.2 Phosphorylation

CZE-MS is a promising tool for the analysis of phosphorylated peptides, because the phosphatation bears a fixed negative charge and, thus, shifts the migration away from most non-phosphorylated peptides. Phosphorylation and deamidation sites of the various charge isomers of bovine myelin basic protein have been identified by analyzing the digest of each protein by CZE coupled on-line to an IT-storage reflectron-TOF mass spectrometer [161]. Cao and Stults [162, 163] developed a CZE-MS approach for the determination of phosphopeptides based on on-line preconcentration by immobilized metal-ion affinity chromatography (IMAC). This elegant method allows the selective preconcentration of phosphopeptides, washing of matrix components and results in LODs in the low-nM range (or below).

10 Applications to intact proteins

CE is frequently applied to separation of intact proteins. The usability of CE in protein and proteomic studies in the last years is reviewed by Corradini [164], Manabe [18], and Dolník and Hutterer [19]. A special review for CE separation of biotechnology-derived therapeutic proteins is published by Patrick and Lagu 2001 [20]. Publications reporting the on-line coupling of CE-MS for studies of intact proteins are summarized in Table 7. The type of separation method is given along with the interface, capillary coating, BGE, and MS system.

Valaskovic *et al.* [176] showed attomole sensitivity for proteins of 8–29 kDa isolated from human blood using very narrow capillaries (5 μm ID) with gold-coated ESI tips (2–5 μm ID) in a sheathless design that is coupled to an FT-MS. The detection of 7 amol carbonic anhydrase (CA) in a crude isolate from blood is shown which corresponds to the concentration of CA in a single blood cell. Using the resolving power of the FT-MS, the estimated errors were less than 1 Da in molecular mass determination. However, the sheathless design has still some drawbacks regarding the robustness. The documented life time of the capillary before failure (tip clogging) was, on average, more than 1 h.

Several research groups analyzed the protein components in single blood cells utilizing CZE-MS methods, however, the detection of CA in a single blood cell seems to be challenging [41, 142]. Cao and Moini [173] reported the analysis of α- and β-chains of Hb of a single intact red blood cell. This application clearly illustrates the advantage of the low sample requirement of CE and strong detection of on-line ESI-MS. The total amount of ≈ 450 amol Hb was sufficient for detection by the applied ESI-TOF-MS. In 2002, Moini *et al.* [174] reported LODs of 20 and 44 amol for CA I and CA II, respectively, using a split-flow interface and narrow capillaries (15 μm ID) for coupling of the CZE with an IT-MS. With this method, the research group was able to detect all four major proteins (α- and β-Hb, CA I and CA II) of the red blood cell in one analysis with minimal sample preparation.

Different BGEs were tested for CZE-MS of small proteins by Foret *et al.* [37]. Moving boundaries from differences in the ionic composition of the BGE and the sheath liquid have been observed due to an EOF close to zero. However, this setup enables the use of nonvolatile BGE at low electrolyte concentration. A similar approach has been shown for the separation of isoforms of ribonuclease B and recombinant human bone morphogenetic protein-2 using β-alanin as BGE in combination with on-line MS detection [170]. In a very recent study, different nonvolatile BGEs were tested in comparison to the formate-based system [182]. The expected improvement with respect to separation efficiency of the borate buffer remains to be shown, if so compensating the observed slightly reduced ionization efficiency of the borate buffer (≈ factor of 3).

A variety of BGE additives for CZE separation of specific protein mixtures has been reported by Dolník [183]. The optimization of a volatile BGE based on formic acid and

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Table 7. Characterization of intact proteins by CE-MS

Approach	Application	Interface	MS	Enrich-ment	Coating	BGE (comments)	Ref.
CZE-MS	Peptide and protein standard	Sheath-liquid	TQ		Microcoat™	0.01 M HOAc	[165]
CZE-MS	Ribonuclease B	Sheath-liquid	TQ	–	PB	3 M formic acid	[166]
CZE-MS	Proteins in aqueous humor	Sheath-liquid	Sector MS	SPE	PB	5% HOAc, 2 mM NH_4OAc	[167]
CZE-MS	Interleukin-6 dimer formation	Sheath-liquid	SQ			20 mM NH_4OAc, pH 4.2	[168]
CZE-MS	Histones	Sheath-liquid	TQ	–	Hydroxypro-pylmethyl-cellulose	0.1 M fomic acid	[169]
CZE-MS	α- and β-chains of hemoglobin of a single red blood cell	Sheathless (gold-coated)	FTICR		APS	10 mM HOAc (SF_6 as sheath gas)	[41]
CZE-MS	Glycoforms of intact proteins (15 kDa)	Sheath-liquid	SQ		Polyacryl-amide	50 mM β-alanine (pH 3.5) (in-source fragmentation)	[170]
CZE-MS/MS	β-Lactoglobulines and α-lactalbumin in whey drink	Sheath-liquid	TQ		Deriv. PS-EDA-diol nanoparticle monolayer	125 mM formic acid (pH 4, NH_3) (different HOAc and formic acid-based BGE)	[171]
CZE-MS	Reduced and oxidized forms of cytochrome *c*	Sheath-liquid	IT	–	CEP[a)] coating	20 mM Tris buffer (pH 6, HOAc)	[172]
CZE-MS	Intact and digested ribonuclease B with diffe-rent enzymatic cleavages	Sheath-liquid	IT	–	PB	2 M formic acid	[152]
CZE-MS	α- and β-chains of hemoglobin of a single red blood cell	Sheathless (in-capillary elec-trode)	TOF	–	APS	10 mM HOAc	[173]
CZE-MS	Red blood cell proteins hemoglobin (a,b) CA	Sheathless ("split-flow")	IT		APS	0.1% HOAc / ACN (50/50 v/v) for lysed blood	[174]
CZE-MS	Separation of oxidized forms of apolipoproteins (Δ = 16 Da)	Sheath-liquid	Q-TOF			50 mM ammonium bicarbonate with ACN (varying percentages)	[175]
CZE-MS	8–29 kDa proteins in human blood	Sheathless (gold-coated)	FTICR	Elec-trokinetic injection	APS	(sensitivity: 7 amol of carbonic anhydrase in blood)	[176]
CZE-MS	Standards of small proteins ($<$ 30 kDa)	Sheath-liquid	TQ	Transient ITP	Polyacryl-amide	20 mM 6-aminohexanoic acid (pH 4.4, HOAc)	[92]
CZE-MS	Metallothionein isoforms (proteins 5.5–7 kDa)	Sheath-liquid	IT			40 mM ammonium phosphate (pH 2.2), 5% i-propanol	[177]
CZE-MS	Metallothionein isoforms (proteins 5.5–7 kDa)	Sheathless	TQ			0.2 M formic acid (pH 2.2)	[178]
CIEF-MS	*E. coli* proteins	Sheath-liquid	FTICR		Polyacryl-amide	Pharmalyte 3–10 from Amersham Pharmacia Biotech	[179]
	E. coli proteins *D. radiodurans*	Sheath-liquid	FTICR		Polyacryl-amide	Pharmalyte 3–10 from Amersham Pharmacia Biotech	[180]

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Table 7. Continued

Approach	Application	Interface	MS	Enrich-ment	Coating	BGE (comments)	Ref.
CIEF-MS	On-line desalting of physiological samples	Sheath-liquid	Sector MS		PVA	Isolyte (pH 3–10) from ICN (Cleveland, OH, USA)	[181]
CIEF-MS	*E. coli* proteins model proteins	Sheath-liquid	FTICR		Polyacryl-amide	Pharmalyte 3–10 and 5–8 from Pharmacia	[55]
CIEF-MS	*E. coli* proteins	Sheath-liquid	TQ		Polyacryl-amide	Pharmalyte 3–10 and 5–8 from Pharmacia	[54]

a) CEP, permanently bonded polymer (Agilent Technologies, Waldbronn, Germany)

ammonia for separation of basic proteins has been reported by Huber *et al.* [171]. Using fused-silica capillaries coated with a monolayer of derivatized polystyrene nanoparticles the major whey proteins (different lactoglobulins) were identified in a whey drink by CZE-ESI-MS. In less complex biological samples like aqueous humor [167] and urine samples [112, 118, 184] peptides and small proteines separated by CZE can be characterized by CZE-MS after simple off-line preconcentration. The latter application shows the clear advantage of the CZE in combination with the high resolution of TOF-MS for biomarker screening in comparison to direct MS measurements with surface-enhanced laser desorption (SELDI)-MS.

A key application of CE-MS in the area of intact proteins is the differentiation of modifications like oxidation or glycosylation. In an early work, Bonfichi *et al.* [166] achieved mass characterization of eight isoforms of ribonuclease B after the separation of six isomeric forms. The results indicate less adduct formation in comparison to the analysis by MALDI-TOF-MS. Similar results were obtained for the characterization of histones in a hydroxymethylcellulose coated capillary by CZE-MS and MALDI-TOF-MS [169]. The separation, identification and quantification of the reduced and oxidized forms of cytochrome *c* using CZE-MS is shown by He *et al.* [172]. For the characterization of complex samples like apolipoproteins in plasma, CZE-ESI-MS is utilized [175]. With the addition of 10–20% acetonitrile to the BGE, different oxidized forms of human apolipoproteins are separated and on-line characterized by ESI-MS. An MS-compatible CZE BGE for the separation of recombinant human erythropoietin glycoforms was recently reported by López-Soto *et al.* [185] and Sanz-Nebot *et al.* [186], however, no CE-MS characterization of this important protein was achieved.

The on-line coupling of CIEF with MS detection was mainly driven by the high separation power of CIEF combined with the high resolution power of an FTICR-type mass spectrometer. Main applications in this field are the study of complex biological samples like physiological fluids containing a complex mix of proteins and the analysis of whole cell lysates for proteomic studies. Clarke *et al.* [181] developed a one-step-on-line desalting CIEF-MS method for analyzing physiologically derived samples containing high amounts of salts. Applying a linear voltage gradient across the separation capillary containing the sample mixed with ampholytes, the research group was able to separate and detect a mix of standard proteins containing 200 mM NaCl.

Similar to 2-D gel electrophoresis, the combination of CIEF-FTICR-MS provides detailed information for complex protein mixtures, *i.e.*, the p*I* and the mass of the proteins in excellent resolution. This technique was evaluated and developed by different groups using the cell lysates from *Escherichia coli* as a model [54, 55, 179, 180]. By optimizing the CIEF conditions and the coupling to the MS, the group of Smith [180] was able to detect 400–1000 putative proteins in mass range of 2–100 kDa from a total injection of 300 ng protein in a single CIEF-FTICR-MS analysis of cell lysates of *E. coli* and *Deinococcus radiodurans* [180]. In Fig. 5a 2-D display of the CIEF-FTICR-MS analysis is shown. Multidimensional separation techniques using on-line CIEF-MS are discussed below.

11 Applications to multidimensional and integrated approaches combining electrophoretic separation and MS

The complexity of biological samples brought up the idea to combine two separation techniques with independent selectivity (orthogonal) [187–189]. Different electrophoretic and chromatographic modes have been coupled to achieve good sensitivity (*i.e.*, application of a system cap-

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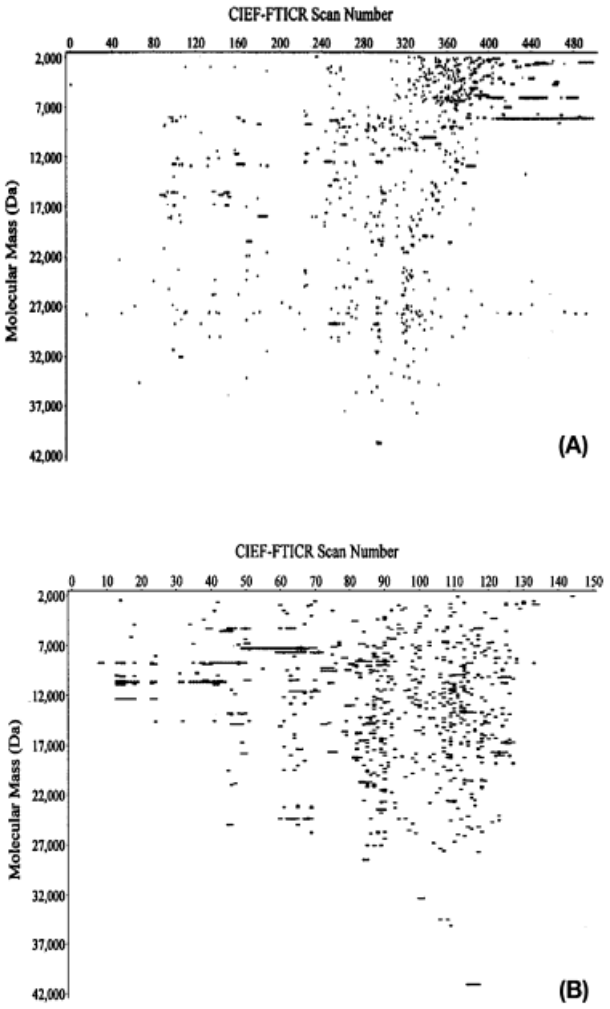


Figure 5. 2-D display of the CIEF-FTICR analysis of lysates from (A) *D. radiodurans* and (B) *E. coli* grown in normal media. Reprinted from [180], with permission.

able for high loadability as a first dimension) and high separation efficiency and speed as the second dimension. Thus, CZE is attractive as second dimension because of its speed and separation efficiency.

In an early work, Tomlinson *et al.* [190] fractionated immunoaffinity-enriched peptides by RPLC and analyzed these fractions by SPE-CZE-MS. In this way, they were able to determine peptides binding to the major histocompatibility complex in the low-fmol range. On-line multidimensional separation including CE-MS was pioneered by the group of Jorgenson [38]. They describe the comprehensive RPLC-CZE-MS, where LC is coupled to CZE by a flow gating interface. Electrokinetic injection into the CE capillary was performed by stopping the crossing BGE flow, which in-between prevents the outflow of the LC column to introduce into the capillary. Overlapping injections allowed a CZE separation every 15 s (the separation window of the peptides is about 15 s). A microsheath-liquid interface enables sensitive detection in the positive mode, while the separation proceeds under basic conditions. Recently, the on-line coupling of RPLC and CZE-MS was performed by a microfabricated device made from poly(dimethylsiloxane) [191]. Repeatability of the injection for the flow gating LC-CE interface was shown. The use of a TOF-MS enables fast detection of CZE-separated peptides ($<$ 60 s total CZE analysis time).

Mohan and Lee [192] developed a microdialysis junction to couple CIEF with transient ITP-CZE. Based on this interface an integrated multidimensional approach enabled the identification of 1174 unique proteins (26.5% of total) from *Shewanella oneidensis* [193]. The CIEF-CZE-FT-MS approach requires $<$ 500 ng and has a peak capacity of about 1600 in less than 3 h. The capacity of the separation and the resolution power of FT-MS is clearly illustrated in the 2-D plot of a CZE-FT-MS from one CIEF-fraction as shown in Fig. 6.

The on-line coupling of CIEF and LC using a microinjector interface was developed by Chen *et al.* [194]. Recently, a similar approach for protein and peptide mixtures has been reported, involving the on-line combination of CIEF with RPLC and MS [195]. Earlier, Gao *et al.* [196] have demonstrated the analysis of the proteome of a yeast cell lysate by off-line combination of CIEF and LC-FTICR-MS. In this approach, the collected fractions of the CIEF-separated proteins have been digested prior to LC-MS analysis. Stable isotope labeling with N^{15} and the accurate mass of the applied FTICR-MS enables the identification and relative quantitation of the proteins.

Both approaches, CIEF-CZE and CIEF-RPLC, take advantage of several benefits of isoelectrical focusing as the first dimension: upon completion of analyte focusing, the self-sharpening effect greatly restricts analyte diffusion and contributes to analyte stacking in narrowly focused bands with a concentration factor of more than two orders of magnitude. Moreover, CIEF as the first separation dimension resolves proteins/peptides on the basis of their differences in p*I*, thereby offering greater resolving power than that achieved in strong cation exchange chromatography. Furthermore, CIEF can be applied both to acidic and very basic proteins/peptides in broad or narrow pH-bands, due to the flexibility in the choice of ampholyte systems.

The combination of two highly resolving and orthogonal separation techniques of CIEF and RPLC or CZE, together with analyte focusing and concentration, significantly enhances the dynamic range and sensitivity of conventional MS towards the identification of low-abundance

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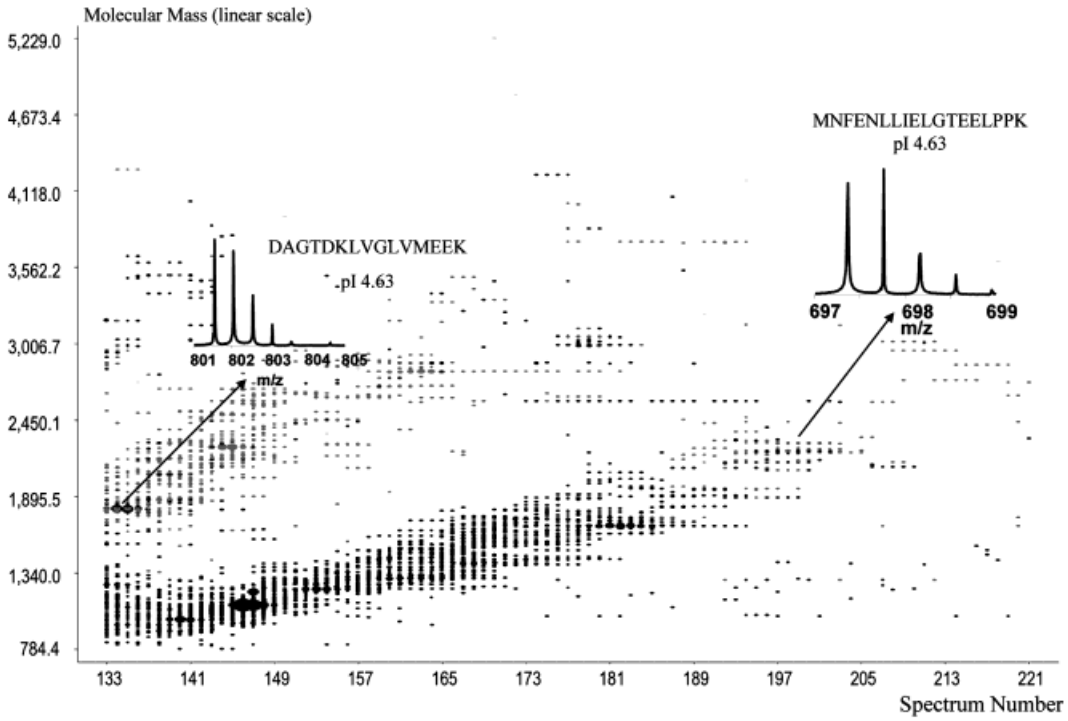


Figure 6. CZE-ESI-MS of fraction 3 of an on-line CIEF-CZE-FTICR-MS analysis of the tryptic digest from a lysate of *Shewanella oneidensis*. The different graying represent different charge states (see original publication for a colored figure). Reprinted from [193], with permission.

proteins. The CIEF-based multidimensional separation/concentration platform enables the identification of a greater number of soluble proteins and requiring 2–3 orders of magnitude lower protein loadings compared to classical methods like 2-D gel electrophoresis.

ITP-CZE not only enables the increase of loadability but can be used in a comprehensive way, as shown in an ITP-CZE-MS approach by Peterson *et al.* [66]. However, the ITP as the first dimension does not show the resolution as, *e.g.*, the CIEF-approach.

On-line SPE-CZE-MS with multistep elution from the chromatographic enrichment device [73, 197] is a 2-D approach, which significantly improves the power of the SPE-CZE-MS. Tong *et al.* [197] report a doubling of the found proteins for an 11-stage elution compared to the single stage elution. However, the difference is partly attributed to the gain of time for MS/MS experiments. The volume of the SPE-device has to be small in order to obtain a reasonable CZE separation, which restricts the use of such an approach compared to the 2-D LC setup (MudPIT) developed by the same group [198].

The integration of sample preparation into separation devices is highly attractive, since the idea of "lab-on-a-chip" has been developed in the mind of researchers. Cooper and co-workers [199] developed a miniaturized trypsin membrane reactor housed inside a commonly used capillary fitting, enabling rapid and sensitive protein identification by on-line proteolytic digestion and analysis of protein digests using nano-ESI-MS and MALDI-MS. According to the authors, the design and assembly of the capillary fitting-based trypsin membrane reactor were straightforward and highly robust, without the need for expensive fabrication technology and procedures. The resultant protein digests could also be further concentrated and resolved using capillary reversed-phase LC or transient ITP-CZE prior to the mass spectrometric analysis in an integrated platform. This nanoscale reaction system enables rapid proteolytic digestion (seconds) for a protein concentration of less than 10^{-8} M, it consumes little sample (about 5 fmol), and offers capillary interfaces with various separation and MS techniques.

12 Applications to oligonucleotides

The analysis of oligonucleotides has been studied by a couple of groups (Table 8). All of the studies used a BGE based on acetate or carbonate adjusted with NH_3 to a pH between 9.3 and 9.7, and ESI in the negative mode. In the early work by Linscheid and co-workers [200–202], sty-

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Table 8. CE-MS for the analysis of oligonucleotides and related molecules

Approach	Application	Interface	MS	Enrichment	Coating	BGE	Ref.
CZE-MS	Styrene oxide DNA-adducts (2–8bp)	Sheath-liquid	Q, Sector MS		–	30 mM NH_4HCO_3 (pH 6.5–6.7)	[200–202]
CZE-MS/MS	DNA and DNA-adducts	Sheath-liquid	TQ	Sample stacking with sheath-liquid-switching[a)]	–	100 mM $(NH_4)_2CO_3$ (pH 9.7)	[203]
CZE-MS/MS	Oligonucleotides (20–120 bp)	Sheath-liquid	Q-TOF	Sample stacking with compensating pressure	–	100 mM $(NH_4)_2CO_3$ (pH 9.7)	[204]
CZE-MS	Reaction product of phenylglycidyl ether with DNA	Sheath-liquid	TQ	Sample stacking	–	100 mM $(NH_4)_2CO_3$ (pH 9.7)	[205, 206]
CZE-MS/MS	Benzo[*a*]pyrene diol epoxide-DNA adducts (mononucleotides)	Sheath-liquid	Q-TOF	Sample stacking	–	20 mM NH_4OAc (pH 9.5)	[207]
CZE-MS	Anti-HIV nucleosides and their metabolites	Sheath-liquid	TQ	–	–	10 mM formic acid (pH 2.5, NH_3)	[208]
CZE-MS	Pt-adducts with DNA-nucleotides	Sheath-liquid	TQ	–	–	32 mM NH_4OAc (pH 9.6)	[209]
CZE-MS	Tetrahydrobenzo-[*a*]pyrenediolepoxide-DNA adducts	Sheath-liquid	TQ	Sample stacking	–	20 mM NH_4OAc (pH 9.3)	[210]
CE-MS	Oligonucleotides (4–6bp)	Sheath-liquid	TQ	–	PVA	14% polyvinyl-pyrrolidone in 20 mM NH_4OAc (pH 9.8)	[57]
CE-MS	Short isomeric oligonucleotides and amino acid derivatives	Sheath-liquid	IT	–	PVA	5% polyvinylpyrro-lidone in NH_4OAc (pH 9.5)	[58]
CGE-MS	Oligonucleotides (12–15 + 17–20 bps; phosphorothioate analogues	Sheath-liquid	TQ	–	PVA	20% PEG35000, 20 mM borate, 20 mM Bis-Tris, 20% ACN	[59]
CGE-MS	Synthetic oligonucleotides (5–20 bp)	Sheath-liquid	TQ	–	PVA	20% PEG35000, 20 mM borate, 20 mM Bis-Tris, 20% ACN	[211]

rene adducts of small oligonucleotides have been characterized by CZE-MS. In a more recent application, CZE-MS was used for the analysis of Pt-adducts with DNA-nucleotides [209]. Deforce and co-workers [203–207] developed a sensitive CZE-MS approach and applied this system on DNA and DNA-adducts, oligonucleotides up to 120 bp and reaction products of phenylglycidyl ether with DNA. The sensitivity is based on triple quadrupole and Q-TOF detection and, more important, on sample stacking with the EOF compensated by pressure. LODs down to 30 fmol were achieved. Similar sensitivity was achieved by Barry *et al.* [210] for the characterization of adducts formed between the benzo[*a*]pyrene metabolite tetrahydrobenzo[*a*]pyrenediolepoxide and DNA. Cahours *et al.* [208] applied CZE-MS to the analysis of anti-HIV nucleosides and their metabolites and achieved detection limits down to 2 μg/L using a triple quadrupole MS and acidic BGE. The analysis of DNA-adducts has been summarized by Andrews *et al.* [27].

Vouros and co-workers [57, 58] developed a CE-MS method based on polyvinylpyrrolidone as pseudophase to separate small oligonucleotides. The covalent PVA-coated capillary is needed to suppress the EOF. Despite

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the use of the polymer, a sensitive (100 fmol/ μL) characterization of nucleotides was achieved. A complete gel-filled capillary in conjunction with on-line ESI-MS detection was described by Freudemann *et al.* [55] and von Brocke *et al.* [211]. They applied a BGE containing 20% PEG 35000 to separate oligonucleotides up to 20 bp according to size. To prevent an EOF, polyvinylacetate-coated capillaries were used. However, the sensitivity is limited (data from 0.1 mM solutions shown).

13 Applications to molecular interactions

Several studies on the binding of various biological relevant species have been performed by CE [28, 30]. Some of these studies applied CE-MS. Strickmann *et al.* [212] studied the binding of the anti-tumor drug oxaliplatin to 5'-GMP. Various forms of Pt-complexes could be identified and the competition with L-methionine was proved. Affinity CE-MS for the description of the interaction between oligopeptides and vancomycin was applied by Chu *et al.* [102]. They screened a combinatorial library containing 361 tri- and tetrapeptides against the interaction with vancomycin. They found that D-alanine at the *C*-terminus and an aromatic amino acid in the penultimate position gave strongest interaction. In a follow-up approach, Dunayevsky *et al.* [117] determined the binding constants of 19 peptides. They obtained similar results for the ACE-UV experiment of a single compound compared to the results of the multiple ACE-MS experiments. Similar results were found by Lynen *et al.* [213]. They evaluated a combinatorial library containing 36 tetrapeptides and determined dissociation constants for a fraction of these and compared the results to those previously obtained by Chu *et al.* [102] for the stereoisomeric forms. For an overview of ACE and MS see Guzman and Stubbs [12].

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