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Review

Advances in capillary electrophoresis for the life sciences

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ABSTRACT

Capillary electrophoresis (CE) played an important role in developments in the life sciences. The technique is nowadays used for the analysis of both large and small molecules in applications where it performs better than or is complementary to liquid chromatographic techniques. In this review, principles of different electromigration techniques, especially capillary isoelectric focusing (CIEF), capillary gel (CGE) and capillary zone electrophoresis (CZE), are described and recent developments in instrumentation, with an emphasis on mass spectrometry (MS) coupling and microchip CE, are discussed. The role of CE in the life sciences is shown with applications in which it had a high impact over the past few decades. In this context, current practice for the characterization of biopharmaceuticals (therapeutic proteins) is shown with CIEF, CGE and CZE using different detection techniques, including MS. Subsequently, the application of CGE and CZE, in combination with laser induced fluorescence detection and CZE-MS are demonstrated for the analysis of protein-released glycans in the characterization of biopharmaceuticals and glycan biomarker discovery in biological samples. Special attention is paid to developments in capillary coatings and derivatization strategies for glycans. Finally, routine CE analysis in clinical chemistry and latest developments in metabolomics approaches for the profiling of small molecules in biological samples are discussed. The large number of CE applications published for these topics in recent years clearly demonstrates the established role of CE in life sciences.

1. Introduction

After a long development, capillary electromigration techniques are now very strong in various fields [1]. Separation is mainly based on charge-to-size ratio and high efficiencies can be obtained with short separation times. Next to the use of capillary zone electrophoresis (CZE) for different types of application, capillary isoelectric focusing (CIEF) and capillary gel electrophoresis (CGE) are powerful for the analysis of biopolymers. The reproducibility and robustness of capillary electrophoresis (CE) was often less than that of liquid chromatography (LC) and gas chromatography (GC) but during the last decades this has been improved by reliable injection and stable electroosmotic flows (EOF) in the capillaries. The development and commercialization of suitable automated instruments played an important role in the progress of CE.

Electrophoresis has been known for a long time and different principles have been developed [2]. In 1937 Tiselius described protein separations using an U-tube [3] but only a separation of two components was obtained and the applicability was still limited. Separation efficiency was rather low due to thermal diffusion and convection. It should be noted that the thesis of Tiselius on electrophoresis as an analytical technique already appeared in 1930. For this work in separation science Tiselius was awarded a Nobel Prize. In the next years a few new developments and applications were published. A main contribution was offered by Martin who obtained an efficient separation of chloride, acetate, aspartate and glutamate using displacement electrophoresis (isotachophoresis, ITP) [4].

Convection was considered a major drawback and its suppression was obtained by carrier materials in the separation tube and plates containing gels. A successful approach was the use of capillaries of 0.1–0.5 mm i.d. which were useful for gel electrophoresis. Initial work in open-tube electrophoresis was presented by Hjerten [5] using capillaries rotated along their longitudinal axis to minimize the effects of convection. Everaerts et al. [6] applied capillaries with similar diameters made from glass and Teflon for ITP. Subsequently, the diameter was further reduced for the optimization of heat dissipation. In 1981, Jorgenson and Lukacs [7] showed the potential of fused silica capillaries smaller than 100 µm. The use of bare fused-silica (BFS) capillaries is considered a real breakthrough in the history of capillary electrophoresis. The role of the EOF as driving force was stressed and a stable EOF could be obtained. Just as in capillary GC, these capillaries

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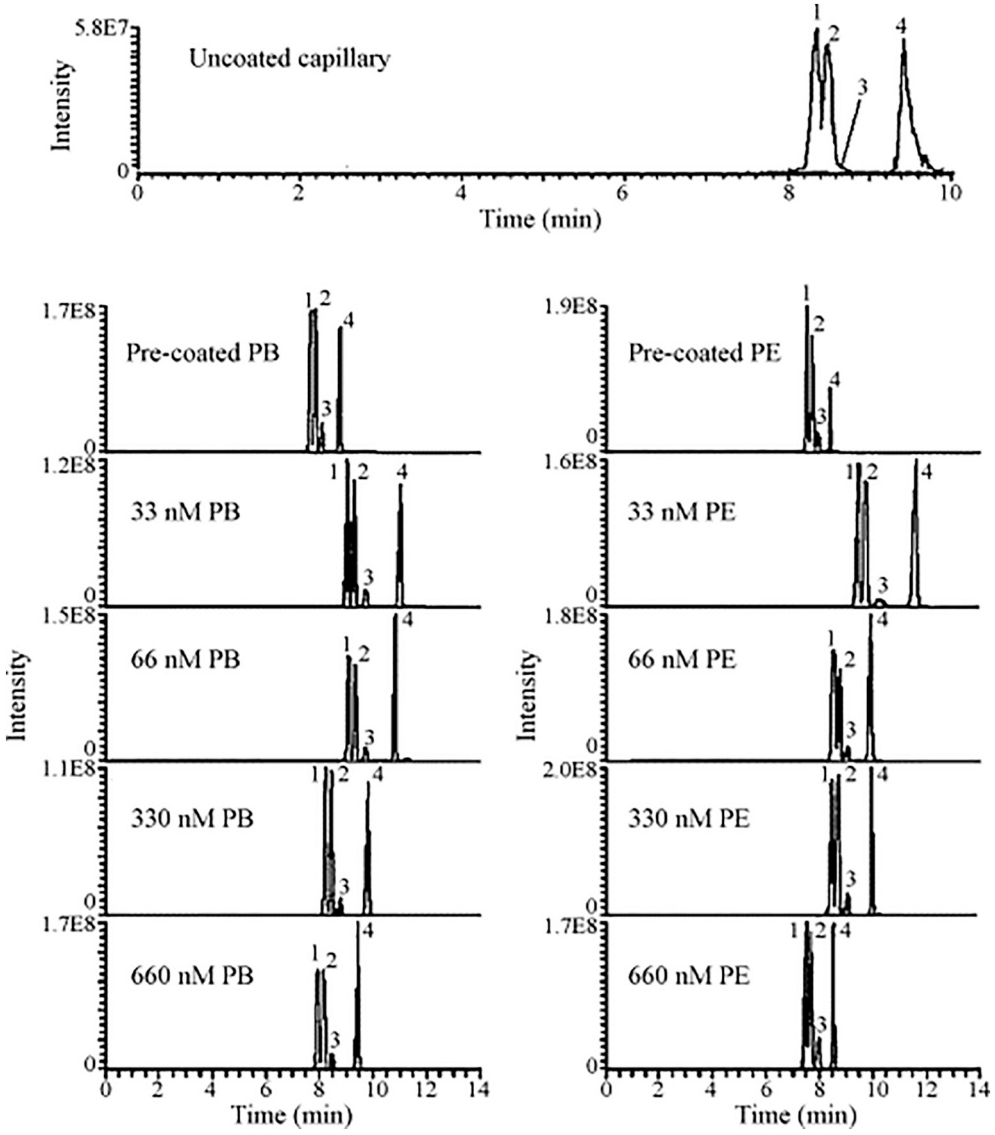

Fig. 1. Comparison of CE-MS analyses of peptide standard using an uncoated capillary (top panel), polybrene (PB) and PolyE-323 (PE) pre-coated capillary, and self-coated capillary using 33–660 nM PB and PE (from top to bottom).
Reproduced from reference [19] with permission.

increased the possibilities of CE for routine analysis. On the end of the last century much attention was paid to CE and promising results were shown. However, the competition with existing high-performance separation techniques such as LC was hard. In the last decades the real value of CE has been demonstrated, especially for the life sciences.

Based on developments in the past, the state of the art and advances of CE in life sciences are presented. In the technology section the different principles are described and attention is paid to instrumentation. The combination of CE and mass spectrometry (MS) is now mature [8], contributing to the complementarity of CE to LC and LC-MS and is therefore discussed in a separate section. CE is very suitable for the separation of polar and ionogenic compounds and the applicability in main fields of the life sciences is shown. The potential of CE for the analysis of biopharmaceuticals (therapeutic proteins) and glycans is high. Also in clinical chemistry and metabolomics various applications demonstrate the possibilities of capillary electromigration separation techniques for the analysis of complex samples, especially for profiling and biomarker discovery. Concerning the utility of CE for chiral analysis, we refer to more dedicated reviews [9,10]. Advanced systems and perspectives of CE are also discussed and some figures illustrate that CE is now a strong technique in life sciences.

2. Technology

2.1. CE modes

In this section different CE modes are shortly discussed and important developments are described that stress the versatility of CE. In the CZE part related capillary electromigration techniques are also mentioned. Special attention is paid to the progress in microfluidics CE.

2.1.1. CZE

Separations in CZE are obtained by differences in electrophoretic migration of analytes in an electric field, based on their charge-to-size ratio. The application of high voltages leads to fast analysis, as the migration of analytes is inversely proportional to the voltage. Generally, the mobility of analytes also depends on the EOF, which is caused by the charged silanol groups of the BFS capillary. The high separation efficiency of CE is due to the flat flow EOF profile and low diffusion. Basically, only longitudinal diffusion, i.e. the B-term of the

Van Deemter curve, is contributing to band broadening making this technique well-suited for the highly efficient and selective analysis of polar and charged compounds. Since the separation efficiency is inversely proportional to the diffusion coefficient of the solute, large biomolecules will have small diffusion coefficients, and will be particularly suited to analysis by CZE, leading to highly efficient separations with large plate numbers.

Neutral compounds can also be separated by using micelles in the buffer solution (micellar electrokinetic chromatography, MEKC). This important extension of the possibilities was developed by Terabe et al. [11]. The micellar solution is called a pseudostationary phase as compounds can partition between the micelles and the solution outside the micelles. The use of different surfactants (also volatile) and other methodological innovations have made MEKC a powerful technique [12]. Other additives that can offer special selectivity through molecular interaction in CZE are e.g. cyclodextrins for chiral separations [13]. The chiral separation of drugs and amino acids is one of the main application fields of CE. Solvent consumption in CE is small as flowrates are very low (nL/min range) and aqueous buffers are mostly used as background electrolyte (BGE). This latter aspect means that the technique is biocompatible and suitable for the analysis of intact proteins. Capillary electrochromatography (CEC) with packed and open-tubular columns has also been developed for the analysis of charged and neutral compounds [14–16]. Here, separation is based on differences in electrophoretic mobility and the partition between stationary and mobile phase. The mobile phase flows through the column by the EOF, generated by the silanol groups of the stationary phase. Neutral analytes move through the packed column by the EOF and are separated by partition between the stationary and mobile phase, while charged analytes progress through the packed column with the additional contribution from the electrophoretic mobility of the analytes.

For protein analysis a special aspect plays an important role: Electrostatic interactions induce protein adsorption on the capillary inner wall due to the presence of negatively charged silanols. This can result in the total collapse of the EOF as well as peak broadening or even peak deformation. Modification of the surface of the capillary wall with a dynamic or permanent coating is a successful strategy to prevent these phenomena [17,18]. Dynamic coating of the capillary with a buffer additive, such as hydroxypropylmethylcellulose (HPMC) or polybrene, are commonly used for the characterization of biopharmaceuticals. Fig. 1 shows the effect of dynamic coatings on the separation of peptides and the correspondence between precoated and self-coated capillaries [19]. Polymers adsorb on the capillary wall minimizing the interaction between proteins and silanols, but the coating can be released, requiring regeneration steps in-between runs. As an alternative, modification of the capillary wall by covalent bonding of neutral or charged polymers is a good option. Although modified capillaries are still commercially available, many coating procedures are described in the literature. A coating can also increase the repeatability of the EOF. It should be noted that positively-charged coatings change the direction of the EOF and the CE polarity should be reversed to move analytes towards the detector. Various coatings have been applied for the separation of proteins but also for the analysis of complex samples containing small cationic and anionic compounds such as in metabolomics [20].

Microchip capillary electrophoresis (MCE) is an important development to miniaturize CE methods [21,22]. The properties of microchips (e.g. rectangular channel of about 10–100 µm in width and height and 10–100 mm in length) make MCE a fast separation system with separation times < 1 min. Joule heating and radial dispersion of the analytes are reduced with respect to capillaries with larger diameters. Decrease of separation time can make MCE to a new approach for very fast and efficient analysis. Several commercial systems are available with ultraviolet (UV) detection. Dynamic coating with HPMC is used for monoclonal antibodies (mAbs) charge heterogeneity analysis [23,24]. More recently, Ramsey et al. introduced an MCE-MS device based on a surface coating procedure with chemical deposition of an aminopropylsilane layer and modification with polyethylene glycol [25,26]. The design is shown in Section 2 and the separation and detection of intact mAbs variants are demonstrated in the section biopharmaceuticals.

2.1.2. CGE

The principle of CGE is the separation of biomacromolecules in a sieving medium. For proteins, sodium dodecyl sulfate (SDS) is also added to the BGE to denature them. The charge homogeneity of SDS-protein complexes allows separation based on their hydrodynamic radius. CGE adapts SDS-polyacrylamide gel electrophoresis (PAGE) to a miniaturized design that reduces the drawbacks of SDS-PAGE, such as a long separation time, limited reproducibility and low resolution. The traditional slab gel has been replaced by soluble polymers employed as replaceable gels allowing the efficient separation of deoxyribonucleic acid (DNA) fragments and proteins [27,28]. Highly automated capillary array electrophoresis was developed and the sample throughput increased considerably [29]. The technique is very suitable for the separation of DNA fragments and commercial apparatus for DNA sequencing are available. The use of a sheath-flow fluorescence detector with intercalating dyes was also an important step in this field [30]. The rapid progress in the Human Genome Project was enabled by this technique and the elucidation of the complete genetic blueprint was an important step in the history of biology [31]. In CGE, EOF often must be suppressed to ensure that the separation is based only on differences in the hydrodynamic radius. Several procedures for neutral coatings have been described to eliminate EOF during CGE analysis, using e.g. polyacrylamide (PAA) hydroxypropyl cellulose, polyvinyl alcohol (PVA) and some other (commercial) coating agents [32]. Since the end of the 80s of the previous century, CGE methods have been used for protein separations and characterization. With the development of commercial sieving kits, CGE has become a high-throughput method for quality control of biopharmaceuticals and more specifically for glycan analysis.

2.1.3. CIEF

CIEF is a CE separation mode that provides a high resolution separation of proteins according to their isoelectric point (pI) and has mainly been applied for the analysis of charge heterogeneity of biopharmaceuticals [33,34]. In CIEF, a mixture of ampholytes and sample fills the capillary. A basic catholyte, generally sodium hydroxide, is placed at the cathode and an acidic anolyte, e.g. phosphoric acid, is placed at the anode. Due to the ampholyte properties, a pH gradient is established in the capillary under influence of the electric field. Proteins are focused until the region where the pH and the pI of the protein are equal. At this point, the net charge of the protein is zero and the migration stops. In the next step, the individual components are transported to the detector by electrophoretic or hydrodynamic mobilization. The position of the detector at the capillary end makes the mobilization step necessary, which may cause negative effects on the resolution due to possible peak broadening. To prevent this effect, whole-column imaging CIEF (iCIEF) was developed to provide faster separations with higher resolution, better reproducibility, and reduced sample volume consumption [35]. iCIEF uses short capillaries (5 cm) with CCD camera detection and enables monitoring of the focusing process. No mobilization step is necessary in iCIEF for detection of the proteins. Concerning detection modes, UV detection at 280 nm represents almost all applications described to date. For structural information, MS detection coupled with CIEF allows identification based on m/z value. However, the potential of CIEF-MS coupling is limited by the presence of salts and ampholytes. A recent paper shows the possibilities of CIEF-MS using special conditions for CIEF and MS interfacing [36]. Fig. 2 shows a promising application including the comparison with iCIEF-UV. Two-dimensional CE consisting of CIEF and CE or CGE was developed for complex protein samples. Ampholytes were removed after the CIEF separation and different interfaces were optimized [37]. Presently, CIEF is a reference method for the characterization of

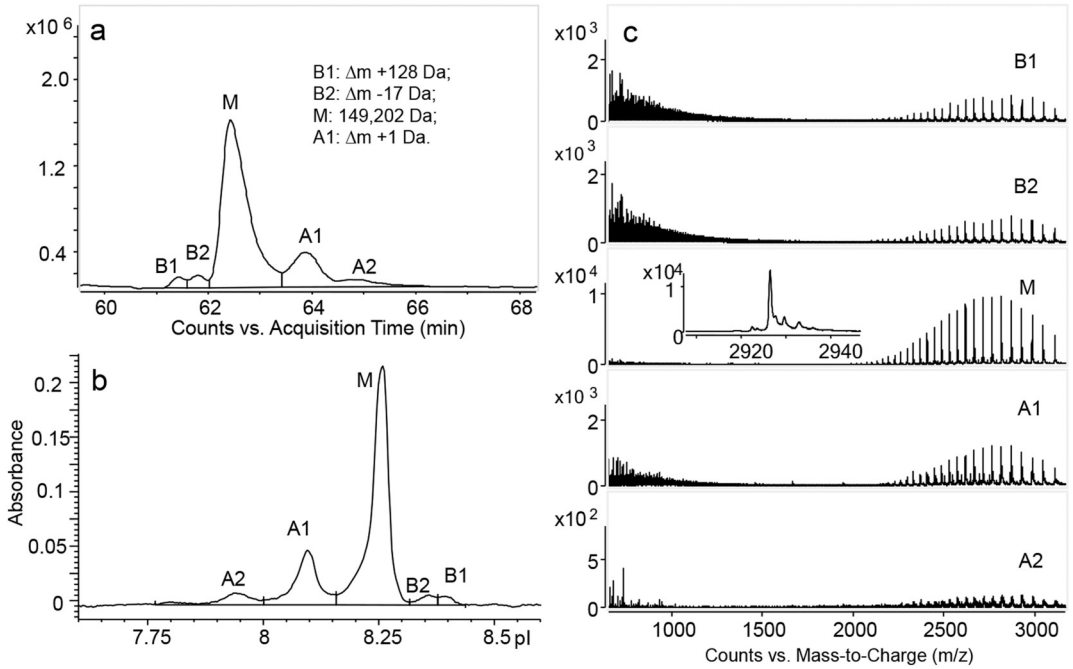

Fig. 2. Bevacizumab CIEF-MS analysis in comparison with iCIEF-UV: (a) CIEF-MS extracted ion electropherogram showing basic variants B1 and B2, main peak M, and acidic variants A1 and A2; (b) iCIEF-UV electropherogram; (c) CIEF-MS mass spectra of major variants. The insert in part c is the expanded view of the main peak in the mass spectrum.
Reproduced from reference [37] with permission.

biotherapeutics. Similar to CGE, with the development of commercial kits and instruments, CIEF became an important technique in the biopharmaceutical industry.

2.2. CE-MS

The coupling of CE and MS is indispensable to obtain a complementary technique to LC-MS. CE-MS provides high resolution separation under more physiological conditions, detailed structural information and lower limits of detection compared to UV. To fully use the combined potential, it is important to have access to robust CE-MS interfaces. Electrospray ionization (ESI) [38] is most often used to interface liquid-based separation techniques to MS [39]. It effectively ensures that dissolved molecules can enter the MS analyzer as gas-phase ions. For CE however, coupling is not that straightforward as there is no outlet vial nor an electrode at the capillary outlet to close the electrical circuit. Furthermore, the separate electrical circuits of CE and MS are not easily combined as currents used in CE are much higher than those encountered in ESI. In addition, the low liquid flow in CE, ranging from zero to sub-μL/min, hampers stable electrospray formation.

2.2.1. Sheath liquid interface

CE and MS were successfully coupled by connecting the outlet of the capillary with a stainless steel ESI needle, using it as both a capillary outlet and ESI emitter electrode [40]. In this way direct electrical contact was accomplished with the CE effluent. This approach was further developed by increasing the flow rate of the sprayed liquid with a co-axial sheath-liquid (SL) interface, thereby ensuring a more effective electrospray (Fig. 3A) [41]. In this setup a regular CE capillary was encompassed by a conductive SL establishing electrical contact between the metal sprayer and BGE at the sprayer tip [42]. The SL acted as outlet vial and provided electrical contact at the capillary outlet to ensure a stable and independent CE flow. In this way the electrospray was created directly at the capillary outlet.

The material of the spray needle has a significant effect on the measurement, especially on the signal of anions. Stainless steel needles can oxidize and corrode when using reverse CE polarity due to electrolysis and anionic compounds can complexate with the iron oxides, reducing detection intensity [43]. The application of platinum ESI needles however, prevented oxidation and needle corrosion. This resulted in limit of detection improvements up to 63 fold for anions, better reproducibility and improved capillary lifetime, compared to a stainless steel needle [43]. Another way to circumvent the issue with oxidation and corrosion of the stainless steel ESI needle is to analyze anionic compounds in normal polarity CE mode at high pH separation conditions.

The SL interface is often used with MS instruments that use a grounded sprayer needle, delivering the ESI voltage from the MS inlet and ensuring two separate and adjustable current flows for CE and MS. This allows for independent optimization of the BGE and the SL composition. The liquid used in the co-axial SL interface typically consists of a mixture of volatile organic solvents and water with volatile acidic or alkaline additives to facilitate ion formation. The additives in the SL however, can also be used to facilitate chemical reactions upon mixing capillary effluent. This concept was used to obtain additional structural information in the analysis of phenolic compounds using stable free radicals, and in the determination of the number of exchangeable hydrogens in analytes by adding deuterated solvents to the SL [44]. SL flows are typically several μL/min to ensure a stable spray. This results in dilution of the CE effluent, depending on the EOF rate, and thus a lower signal intensity. Furthermore, suction effects caused by the sheath-liquid flow can cause a laminar flow inside the CE capillary which results in band broadening if the pressure is not compensated through the inlet vial [45]. On the other hand, non-volatile BGE additives that provide effective separation but are in principle not compatible with MS can be used in low amounts without causing too much ion-suppression. Non-volatile buffers were applied in the BGE for protein analysis with CE-ESI-MS [46]. Sodium phosphate strongly suppressed ionization, but 25 mM ammonium borate could be used without compromising signal intensities. Phosphate could be used for the analysis of antihistamines, applying it in the BGE and SL in low concentrations. Minimal ion-suppression was observed by using an

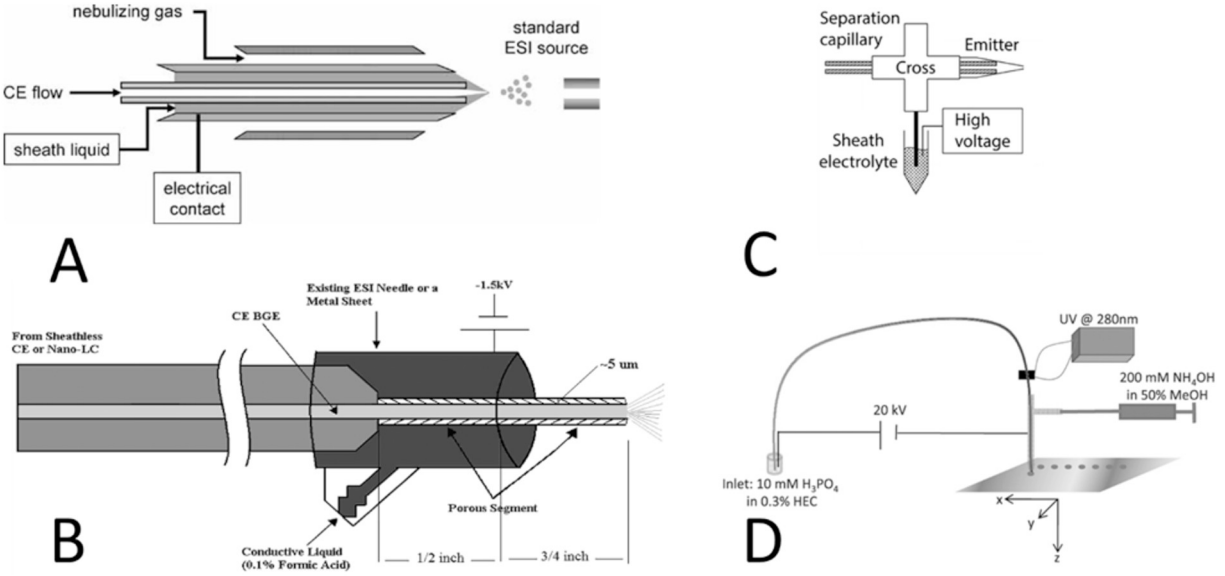

Fig. 3. Common interfaces for CE-MS. (A) co-axial sheath liquid ESI interface, (B) sheathless ESI interface, (C) miniaturized liquid junction ESI interface and (D) MALDI spotter sheath liquid interface.
Reproduced from references [42],[51],[60] and [63] with permission.

interface with low SL flown and performing the analysis under acidic conditions to ensure mobility of phosphate ions towards the capillary inlet [47]. For the analysis of drugs of abuse, the application of ammonium phosphate in the BGE also showed high ionization performances and good resolution. In contrast, tris(hydroxymethyl)aminomethane and borate as BGE constituents, could not be used in this case due to severe ion suppression [48]. Apparently, low concentrations of non-volatile buffers can be used with SL interfaces, but applicability may be dependent on analyte characteristics and SL composition.

The SL interface has been widely applied and proven to be very robust and is therefore considered the standard for CE-MS. Recent adjustments to the SL sprayer tip, in combination with ESI source and sprayer positioning modifications, even improved spray quality, ionization efficiency and signal intensity. It is therefore expected that this interface will continue to be used extensively.

The SL interface was recently miniaturized for working in the nanoflow range to further improve ionization efficiency, while retaining the benefits of the SL [49]. In this setup, the capillary end was encompassed by a thin grounded metal needle. The SL was pumped through the needle at a flow rate of 1 μL/min using a T-connection and contacted the CE effluent at the capillary end. Due to the lower SL flow rate in this setup, no nebulizing gas was required for effective ESI, which eliminated the suction effect. To improve the stability of this interface, the outer diameter of the separation capillary and the inner diameter of the needle were further reduced, allowing SL flow rates of 300 nL/min. This resulted in a smaller Taylor cone and minimized peak broadening by the ESI process. Better separation efficiency was obtained with this setup, while maintaining comparable limits of detection as with the conventional SL interface [50].

2.2.2. Sheathless interface

To completely avoid dilution of the CE effluent a porous tip sheathless interface was used (Fig. 3B). The end of the CE capillary was etched with hydrofluoric acid to obtain a porous capillary end to provide a passage for ions and electrons [51]. The capillary end was encompassed by a metal cylinder that acted as outlet vial and electrode. In this way the BGE inside the capillary was in contact with the BGE surrounding the outside of the capillary. The end of the capillary protruded slightly from the metal cylinder and acted as sprayer tip.

Relative standard deviations of 1–3% were achieved for migration time repeatability [52] and a stable and more effective ESI was obtained [53], which resulted in a 5 to 140 fold improvement of limit of detection for proteins [54] and 8 to 30 fold for selected metabolites [55], compared to the SL interface.

The sheathless interface is universal and can be used with all types of ESI-MS instruments, independent of the electrical circuit of the MS. Although, specially treated and relatively expensive capillaries are required for this commercialized interface, which can only be obtained from the manufacturer. Furthermore, a CE device from the same manufacturer is needed and, therefore, it is difficult to predict whether this interface will receive widespread attention.

2.2.3. Liquid junction interface

Liquid junction is a third, common type of interface that uses, like the SL interface, the principle of mixing the CE effluent with an outlet electrolyte that is connected to a ground electrode. The electrolyte is added through a T-connection to the CE effluent before ESI and usually has the same composition as liquids used in SL interfaces [56]. The CE effluent and the outlet electrolyte are combined between the MS emitter and the CE capillary end. This causes peak broadening and hence lower resolution. Furthermore, analytes are still diluted, just like with the SL interface, and therefore compromising intensity. On the other hand, the liquid junction interface is universally applicable and can also be used with MS instruments with ESI potential on the sprayer tip.

The liquid-junction approach has been used in a miniaturized device using a flow-through microvial to combine CE effluent and outlet electrolyte. This nanoflow ESI interface [57] used a stainless steel hollow needle with asymmetrical geometry [58]. The needle enclosed the CE capillary and acted as outlet electrode and vial, through which the outlet electrolyte was pumped. The outside tip of the needle acted as electrospray emitter. An electrolyte flow rate of ≥100 nL/min through the needle ensured minimal CE effluent dilution using normal size capillaries. This approach was also applied for interfacing cIEF-ESI-MS by supplying catholyte and mobilization solutions through the microvial [59]. Recently, a similar miniaturized liquid-junction interface, but using an electrokinetically pumped outlet electrolyte and a borosilicate glass emitter, was developed (Fig. 3C). The emitter had a 15–35-μm diameter tip and a 20-μm spacing between the separation capillary and the emitter tip [60]. This geometry resulted in an EOF of approximately 50 nL/min in the emitter, while the EOF in the CE

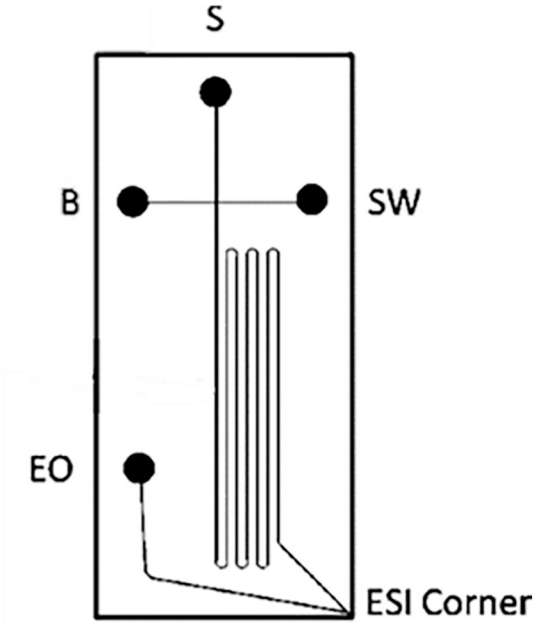

Fig. 4. MCE-ESI device. S = sample reservoir; B = BGE reservoir; SW: sample waste reservoir; EO: electroosmotic pump reservoir.
Adjusted from reference [61].

capillary was around 20 nL/min, resulting in minimal dilution of the CE effluent before MS analysis, compared to SL and regular liquid-junction interfaces [60]. With this nanoflow ESI liquid junction interface an approximately 20-fold improvement for limits of detection was obtained, compared to the regular SL interfaces. The interface was recently commercialized, and it is therefore expected that its application will increase significantly in the future. In view of the wide applicability, regarding the choice of CE capillaries and CE-MS instruments, in combination with the improvement in signal intensity, the miniaturized liquid-junction interface could well become a potential new standard in CE-MS interfacing.

MCE devices have been used to miniaturize CE systems. It allows for high speed analyses, the possibility to combine different CE separation modes and the integration of ESI in one device. An integrated microfluidic CE-ESI device, combined with MS detection, was recently demonstrated (Fig. 4) [61]. The device incorporated, beside the CE part, an integrated ESI emitter and electroosmotic pump to assist ESI. Junctions between individual components were absent and therefore no dead volumes were present. This CE-ESI was commercialized and applied successfully to the analysis of biomolecules [25,62].

2.2.4. CE-MALDI MS

CE can also be coupled indirectly to matrix assisted laser desorption/ionization (MALDI)-MS, which is less prone to ion-suppression by non-volatile BGE additives compared to ESI. Both CE separation and MS detection can be optimized without compromising each other, as the instruments are not directly coupled. SL interfacing is most commonly used for CE-MALDI-MS [63] in combination with a spotting device to deposit analytes on a MALDI target plate (Fig. 3D). The MALDI matrix can be added through the SL before or after sample spotting. In another approach, liquid free interfacing was used with a silver-coated tip for connection to ground. Droplets of solution were predeposited on the MALDI plate to receive the analytes upon contact with the capillary [64]. A sheathless interface was obtained by coupling the CE and a separate spotting capillary through a porous polymer. This provided a passage for ions and electrons [65]. The porous joint was encompassed by a reservoir containing the outlet BGE to ensure a closed electrical circuit. MALDI is more tolerant to non-volatile additives than ESI and can therefore be used with CE modes that are less compatible with ESI-MS. The analysis of proteins with cIEF-MALDI-MS for example, could be performed in the presence of ampholytes, detergents and viscosity increasers, using a SL interface which provided the catholyte solution [63]. A big disadvantage of CE-MALDI however, is the loss of resolution after deposition. Therefore, this combination is mainly used for identification of peaks. Given the many developments in ESI-MS instrumentation and CE-ESI-MS interfaces and the few new developments in CE-MALDI-MS, the use of the latter is likely to decrease rapidly.

Recent developments in CE-MS coupling focus on miniaturization using universally applicable and nanoflow ESI interfaces that provide the lowest possible dilution of CE effluent and most efficient ionization [66,67]. The application of non-volatile BGE constituents however, is more limited with these interfaces and CE separation often has to be performed at lower currents [60], reducing the freedom in the choice of constituents and concentrations of the BGE. On the other hand, it is the expectation that miniaturized liquid junction interfaces will become more important in the future, as they allow the measurement of lower concentration levels and the use of regular separation capillaries.

3. Selected applications in the life sciences

3.1. Biopharmaceuticals

Biopharmaceuticals are biomolecules (nucleic acids, peptides, proteins) used for therapeutic purpose. These products have emerged at an exceptional speed and several important products are now on the market. They are mainly produced using recombinant technologies, which enable the production of proteins on a large scale. Biopharmaceutical products include different types of biomolecules, e.g. hormones such as insulin and growth factors (erythropoietin, EPO), interferons, interleukins (IL-2), vaccines, mAbs and related products (bispecific antibodies and antibody-drug conjugates). Characterization of biopharmaceuticals requires a combination of several techniques in order to investigate the different structural properties of biomolecules. Analytical techniques used are separation methods such as CE and LC. These allow separation of the main isoform from variants and impurities. Since the introduction of modern CE, capillary electromigration techniques have been shown to be very suitable for the separation of biomolecules. The advantages of CE are its high separation efficiency and the use of aqueous buffers which is favorable to preserve the higher order structure of proteins. Therapeutic peptides and proteins are typically charged molecules. Their modification often induces a change in the net charge and/or hydrodynamic radius. Therefore, electrokinetic separations using CE-based techniques are well suited for the separation of biopharmaceutical variants [68]. CE separation can be coupled to sensitive detection techniques including laser-induced fluorescence (LIF) and MS. MS is very useful for the structural characterization of biopharmaceutical products. The characterization of biopharmaceuticals has been described using different CE separation modes from CZE to CGE and CIEF. This chapter describes some applications of CE-based methods for the characterization of biopharmaceutical products. The possibilities of each separation mode for routine analysis are stressed and a few relevant figures are shown. Many biopharmaceuticals, such as mAbs, are glycosylated and therefore characterization of glycans is important. This part is described in a separate section on glycan analysis.

3.1.1. CGE

The size heterogeneity of proteins is influenced by modifications such as post translational modifications (PTMs) and fragmentation. A shift of the profile towards acidic forms (deamidation, sialylation, glycation) or basic forms (succinimide formation, C-terminal lysine heterogeneity) [69] can be observed and this may influence the safety and efficacy of the product [70]. PTMs can be effectively mapped with CGE and therefore Salas-Solano et al. optimized a generic CGE-LIF

method for quality control (QC) and stability monitoring of mAbs. The validation of this method under the guidelines of the International Committee on Harmonization demonstrates that the method determines the consistency of mAb manufacturing [71]. Han et al. also proposed a CGE-LIF method for mAb separation and their method was transferred to the QC laboratory for lot release testing of therapeutic antibodies [72]. As CGE proved to be very suitable for mAbs characterization, the United States Pharmacopeia (USP) released a general protocol for the CGE-UV characterization of mAbs. However, it was shown that the USP method induced fragmentation for three mAbs under non-reducing conditions. This extent of fragmentation was not found using an in-house developed method [73], demonstrating that molecule-specific methods can be essential to minimize artifacts. The potential of this approach, using CGE separations in pharmaceutical laboratories, was reported in several publications [74].

Further improvements in the performance of CGE have been made by Szekely et al., proposing a generally applicable multicapillary SDS-CGE-LIF method for high-throughput quality control analysis of mAbs with reducing conditions [75] and Kubota et al. [76], demonstrating the analysis of a mAb and cleaved fragments using orthogonal analytical methods, including CGE-UV (Fig. 5). Progress in CGE instrumentation to reduce separation times have focused on MCE with SDS. While MCE-SDS is an attractive alternative to conventional CE-SDS for protein analysis, there are still opportunities for improvement, e.g.

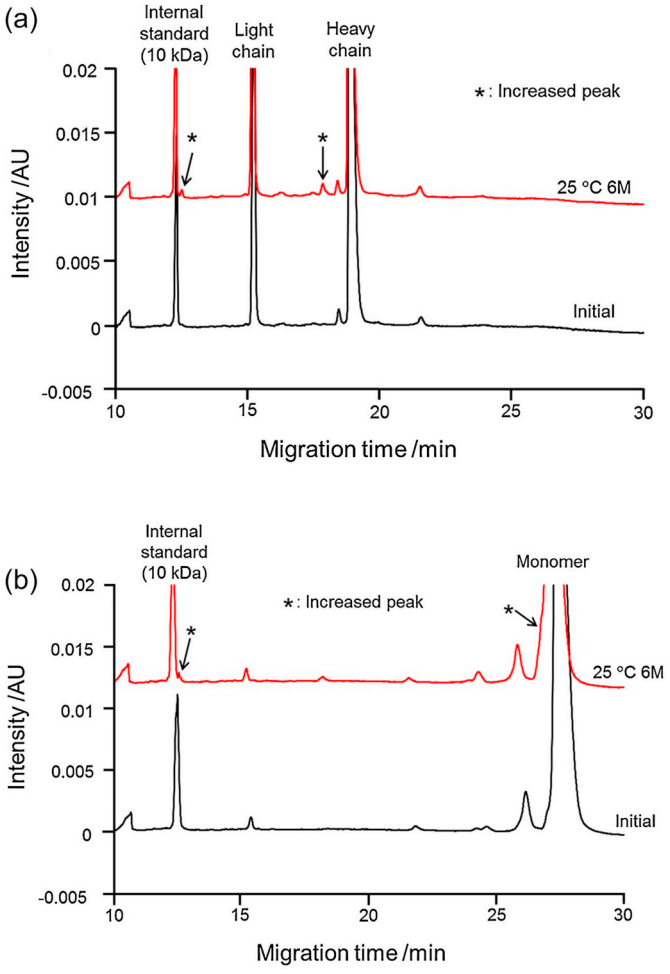

Fig. 5. Electropherograms of the mAb-A initial sample (lower trace) and degradation sample (upper trace) obtained by SDS-CGE (a) reduced and (b) non-reduced conditions. The internal standard peak, light chain peak, heavy chain peak, monomer peak, and increased peaks are indicated.
Reproduced from reference [76] with permission.

higher resolution to separate product variants of similar sizes. Smith et al. validated an MCE-SDS method with LIF detection for analysis of mAbs by separation and quantitation of size variants. The method is suitable for good manufacturing practice release and stability studies [77].

3.1.2. CIEF

The charge heterogeneity of biopharmaceuticals is an important quality parameter. Charge heterogeneity profiles can also be influenced by modifications such as PTMs and fragmentation. CIEF and iCIEF with UV detection are fully implemented in QC laboratories and have become reference methods for the characterization of charge heterogeneity. Identification of intact and reduced therapeutics and PTMs can be realized using this CE mode. Many papers describe the potential of CIEF- and iCIEF-UV as useful tools in the process development of biotherapeutics [78,79]. Suba et al. validated a CIEF-UV method for identification testing of mAb drug products with a pI between 7.0 and 9.0. The CIEF method provided a good pH gradient for internal calibration (R2 > 0.99) and good resolution between all isoforms. These authors also found CIEF-UV to be an accurate analytical method to confirm protein identity in QC and release tests in the biopharmaceutical industry, especially with the use of commercial standardized kits [80]. CIEF- and iCIEF-UV methods are also suitable for the characterization of mAbs-related products. An improved method for the characterization of acidic and basic variants of an antibody used a preparative immobilized pH gradient for fractionation and the fractions were further characterized by CGE-UV and LC-MS [81]. To demonstrate the accuracy of CIEF- and iCIEF-UV methods as references for charge variants characterization, Salas-Solano et al. organized interlaboratory studies [82]. An international group of 12 laboratories from biopharmaceutical companies was formed to evaluate the precision and robustness of CIEF- and iCIEF-UV to determine the charge heterogeneity of mAbs. This has stimulated the application of CIEF- and iCIEF-UV methodologies both in process development and QC of biopharmaceutical companies. Efficient coupling of CIEF with MS is not easy but will further increase the possibilities of (i)CIEF (see Section 2).

Further improvement of separation time was obtained by the development of microchip CIEF. Kinoshita et al. reported a comparison between microchip CIEF-UV and conventional CIEF-UV methods for the evaluation of mAbs charge heterogeneity [83]. A 10-fold decrease in separation time and an excellent correlation of calculated pI values and the relative amounts of the charge variants were observed, but automation of the microchip method is still limited. However, this strategy is promising and improvements will be realized in the future.

3.1.3. CZE-MS

CZE can easily be coupled with MS and structure information of products can be obtained. Middle-up analysis is the characterization of therapeutic proteins after limited proteolysis, which generates peptides usually larger than 10 kDa. Proteolytic treatment is performed with enzymes such as pepsin, papain and streptococcal cysteine proteinase. The use of a limited digestion is interesting as it reduces the complexity of the sample in order to focus the analysis on specific parts of the investigated protein. As a consequence, it is mainly employed for the analysis of highly complex proteins like mAbs, especially because specific enzymes for Immunoglobulin (IgG)1 proteolysis are available [84]. The analysis of mAbs after reduction of disulfide bridges was developed using online CE-ESI-MS [85]. Results showed the separation of the mAbs heavy (H) chain and light (L) chain in a mixture of reduced mAbs. CE-ESI-MS was used for the characterization of biopharmaceuticals after different sample pretreatments. The complete separation of the mixture composed of mAbs H chain, L chain, and fragments produced by IdeS (igG-degrading enzyme of *Streptococcus pyogenes*) digestion was demonstrated. In addition, the different lysine variants for the fragment crystallizable region (Fc) fragments were identified. The various applications of CE-ESI-MS middle-up characterization demonstrate that

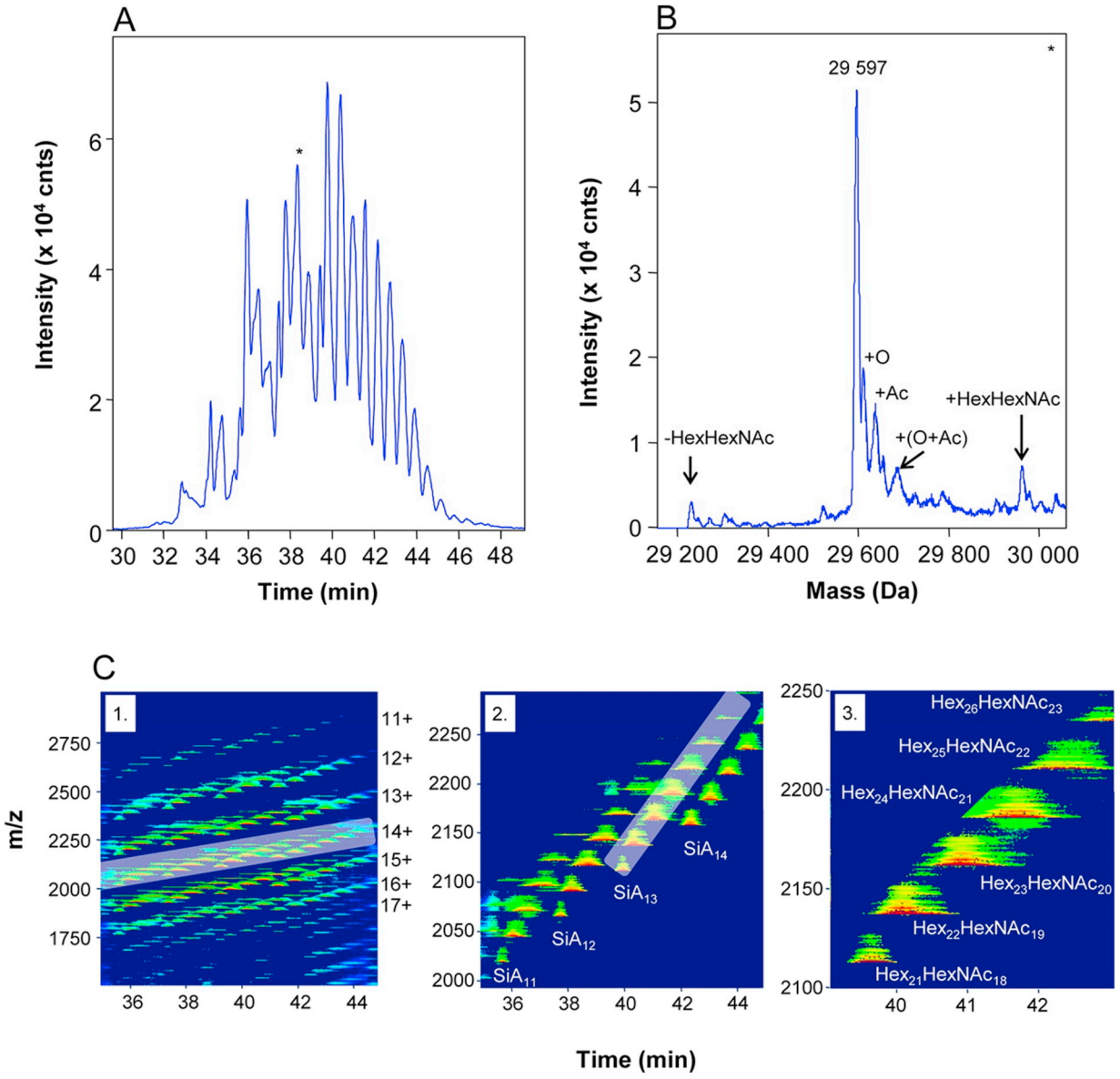

Fig. 6. Sheathless CE–MS of recombinant human EPO (200 µg/mL) employing a neutrally coated capillary. (A) BPE; (B) deconvoluted mass spectrum obtained at the apex of the peak migrating at 38.0 min; (C1) contour plot with zooms of (C2) the 14+ charge state of the glycoforms and (C3) the SiA13 sialoforms of the 14+ glycoforms.
Reproduced from reference [53] with permission.

CE is suitable for the separation of large protein fragments. Biacchi and coworkers developed an off-line CE-MALDI/ESI-MS method for the analysis of different fragments produced from the IdeS digestion of cetuximab. They demonstrated the baseline separation of Fc lysine variants and the independent characterization of the N-glycosylation sites present of the protein [86].

For characterization of biopharmaceutical products, the analysis of intact proteins is the main goal. A useful protocol for rapid CZE method development for top-down and middle-up analysis of mAbs was presented by Suba et al. [87]. MS offers a high level of structure information and an efficient separation in front of MS increases the information obtained from the spectra. CE-ESI-MS with a sheathless interface was used to develop a method for the characterization of human EPO (Fig. 6). This method provided fractionation and subsequent characterization of a large number of EPO glycoforms in a single analysis. The separation of isoforms prior to MS improved sensitivity and significantly increased the number of identified isoforms [53]. Characterization of intact interferon-β1 was realized using CE-ESI-MS/MS analysis. This approach allowed the identification of glycoforms and modified variants by MS/MS analysis. The use of electron transfer dissociation and high-energy collision dissociation fragmentation demonstrated the possibility of performing a characterization of different isoforms and the presence of PTMs [88]. The potential of CE-MALDI-MS was also demonstrated and applied for the separation and MS analysis of intact charge variants of trastuzumab [89]. CZE-MS has also been applied for stability studies and deamidation and oxidation products have been identified. Especially time-of-flight (TOF) MS was found to be very suitable for this type of analysis as small mass differences can be observed [90,91].

A CE-nano ESI-MS microchip was used for the analysis of intact infliximab [25]. Lysine variants were successfully separated and identified by MS (Fig. 7). The same system was used for the characterization of intact antibody drug conjugates (ADCs). The microchip MS analysis demonstrated the possibility to determine the drug-to-antibody ratio (DAR) [26]. ADCs are complex samples in the field of therapeutic protein products. Said and coworkers developed an analytical

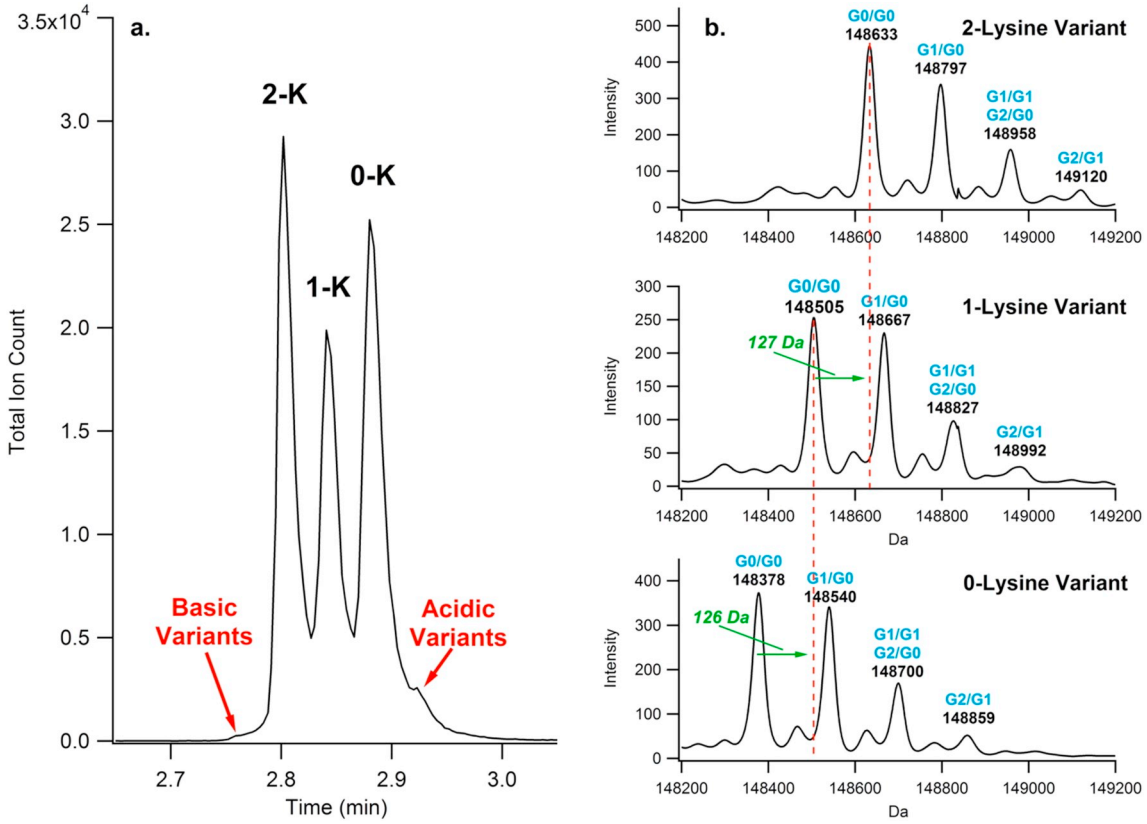

Fig. 7. (a) Separation of intact Infliximab charge variants by MCE-MS. Identified lysine variants are labeled as 2-K, 1-K and 0-K. (b) Deconvoluted mass spectra for each lysine variant.
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methodology for ADCs analysis on the peptide, middle-up, and intact level. This workflow is mainly based on sheathless CE-ESI-MS. It enables the characterization of the amino acid sequence, to localize the different conjugation sites to estimate the relative conjugation levels for each site and obtain the glycoprofile of the protein [92]. Because the ESI ionization yield is relatively low for intact proteins, CE-ESI-MS methods for intact proteins will profit from the development of a more sensitive CE-MS coupling. The characterization of intact therapeutic proteins using CE-MS remains a challenge.

3.2. Glycans derived from proteins

Carbohydrates are among the most abundant group of organic molecules on the planet and play essential roles in different biological processes [93]. Glycans conjugated to proteins consist of monosaccharide building blocks that are linked together in different sequences and linkages. The chains can be linear or branched, yielding an enormous number of possible glycan structures and isomeric forms. In most glycoproteins, the glycans are linked at the anomeric center to the protein by *N*- or *O*-linkages of amino acids (Fig. 8).

Since the establishment of the configuration of carbohydrates at the end of the 19th century, a steady progress has been made in glycan research, usually associated with important technological developments [93]. Recently, the glycomics field is strongly emerging with the growing demand for glycan biomarkers for disease diagnosis and the vast growing market of protein pharmaceuticals and vaccines. Furthermore, innovation in separation science and MS, together with novel synthesis approaches for complex carbohydrates provide new opportunities in this area. Currently, glycan analysis is mainly performed with hydrophilic interaction chromatography (HILIC)-MS and porous graphitized carbon (PGC) LC-MS [94], but CE also plays a major role. This section highlights important developments in the analysis of protein-derived glycans by means of CE with special attention to glycan derivatization.

3.2.1. Sample preparation

Glycans can be released from proteins enzymatically or chemically. *N*-glycans are usually released intact from isolated glycoproteins by denaturation of the protein and cleavage of the *N*-acetylglucosamine-asparagine bond by endoglycosidases such as peptide-N-glycosidase F (PNGase F) [95]. For *O*-linked glycans there is no universal enzyme-based cleavage procedure available and chemical cleavage is used instead. β-elimination at high alkaline pH is commonly used to release *O*-linked glycans, converting them thereby to the reduced form which makes the derivatization with a tag more complicated [96]. Release with anhydrous hydrazine is also possible, but the peeling degradation of glycans increases compared to the reducing β-elimination approach and more modified carbohydrate residues are obtained [97].

Since most glycans do not contain an UV-active chromophore, labeling of released glycans is often required. Furthermore, if no *N*-acetylneuraminic acid (Neu5Ac) or other chargeable groups are present, it is difficult to charge glycans for electrophoretic separation or during ESI and therefore an ionizable group is attached. A negative chargeable tag is often applied for CE of glycans to charge neutrals and increase the charge on negatively charged compounds. A positive chargeable tag on the other hand decreases the net charge and electrophoretic mobility of negatively charged glycans and is therefore only applied when Neu5Ac residues are neutralized. Initially 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) labeling was used for the analysis of carbohydrates with CE coupled LIF detection, allowing for low attomole detection limits of small carbohydrates and mapping of *N*-glycans derived from proteins [98]. CBQCA was gradually replaced by reagents that gave more sensitive and stable derivatives such as: anthranilic acid (2-aminobenzoic acid; 2-AA) [99] and sulphated

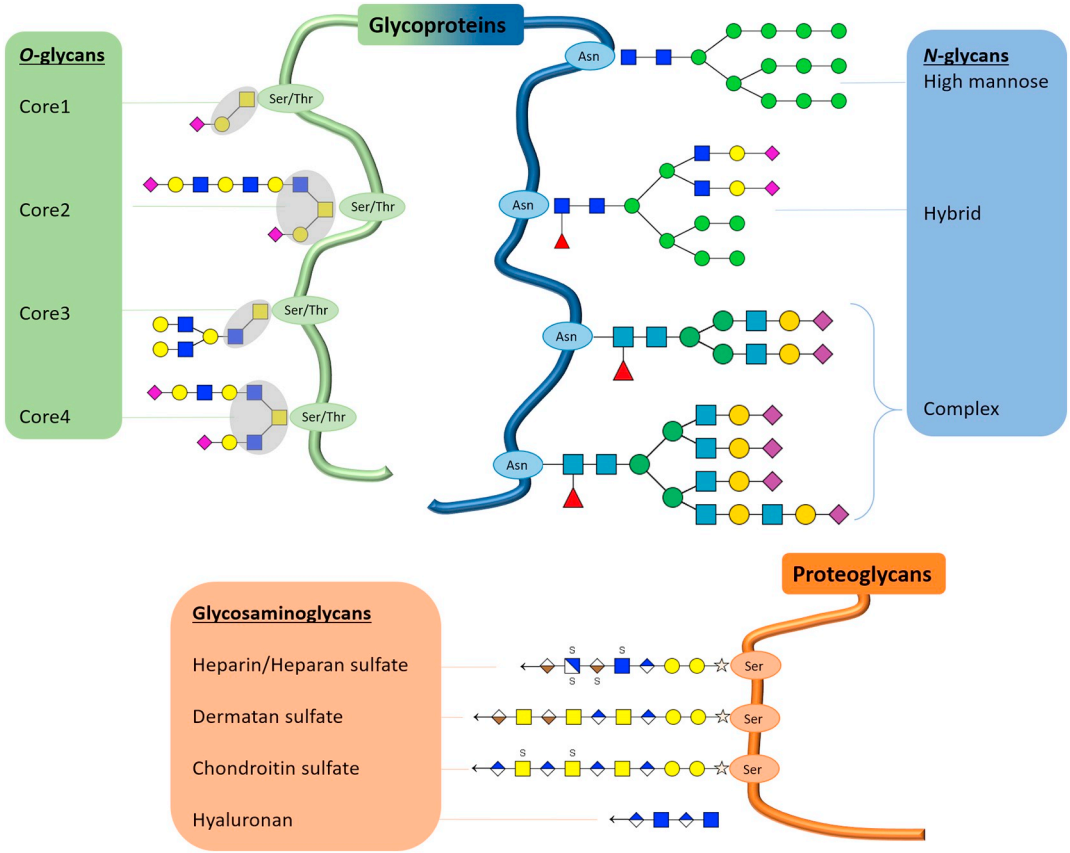

Fig. 8. Structures of glycans linked to proteins. Yellow circle = galactose (Gal); yellow square = *N*-acetylgalactosamine (GalNAc); blue square = *N*-acetylglucosamine (GlcNAc); green circle = mannose; red triangle = fucose, purple diamond = *N*-acetylneuraminic acid (Neu5Ac). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reagents like aminonaphthalene trisulfonic acids (ANTS) [100], 9-aminopyrene-1,4,6-trisulfonic acid and 8-aminopyrene 1,3,6-trisulfonic acid (APTS) [101]. The latter two offer a higher molar absorbance at the excitation wavelength for derivatized carbohydrates and a different emission wavelength for reacted and unreacted label which ensures a lower background signal [102]. Nowadays, the derivatization reagents 2-AA and APTS are commonly used to introduce negative charges to glycans allowing also for the CE separation of neutrals and detection with both LIF and MS. An improvement of the APTS derivatization method was recently introduced by Khan et al. with the availability of a new derivatization reagent Teal™ [103]. This compound consists of three sulfonic acid moieties, like APTS, but has a higher reactivity towards glycans. Therefore, less amount is needed for the derivatization. Furthermore, signal intensities were slightly increased with respect to APTS derivatives.

Reagents for glycan release and derivatization are often added in large excess for quantitative labeling. Since these additives and matrix components can interfere with the further analysis, they are removed after the respective reactions. Native and derivatized glycans can be purified and concentrated using solid-phase extraction modes such as reversed-phase [104], gel filtration, size exclusion, PGC [105] and HILIC [106].

3.2.2. CE-LIF of glycans

The combination of CZE or CGE with very sensitive LIF detection has been used for the analysis of glycans in clinical samples since many years and has become an important tool in the biopharmaceutical industry [32]. By using multicapillary CE-LIF analyzers, multiplexing can be performed for high-throughput analysis of glycans [107]. CGE-LIF makes use of replaceable sieving matrices like PAA and cellulose derivatives [108]. Furthermore, neutral capillary coatings like PAA [109] or the more hydrophilic PVA [110] are used to reduce the EOF while preserving the liquid sieving matrix in the capillary. These systems can be used to obtain separation of polydisperse intact polysaccharides, with a resolution of 1 carbohydrate unit [111], and oligosaccharide hydrolysates derivatized with ANTS [112].

By partially hydrolyzing an oligosaccharide derived from glucose monomers, a mixture of chains consisting of different numbers of glucose units is obtained. The high resolution CGE analysis allows the baseline separation of this oligosaccharide ladder that can be used for the identification of unknown glycans using glucose unit (GU) calculation. GU values can be obtained from the migration times of separated homooligomers (oligomer consisting of identical monomers) and calculated for the unknown glycan using the closest migrating homooligomers as follows [113]:

$$GU_A = dp_1 + \frac{t_A - t_1}{t_2 - t_1}$$

where dp_1 is the degree of polymerization of the preceding homooligomer peak, t_A is the migration time of the unknown glycan and t_1 and t_2 are the migration times of the homooligomers preceding and following the peak of the unknown glycan, respectively [113]. Structural elucidation, including linkage and position information of carbohydrates is possible by removing carbohydrates stepwise from the non-reducing end with exoglycosidase enzymes, subsequential analysis with CGE and identification using GU values [114].

CE-LIF was recently used for *N*-glycan profiling of human serum proteins to discover and evaluate biomarkers for several diseases: in hepatocellular carcinoma abundance alterations of two fucosylated bi- and triantennary glycans were found [115], gastric cancer could be diagnosed by using one specific triantennary glycan and observing an

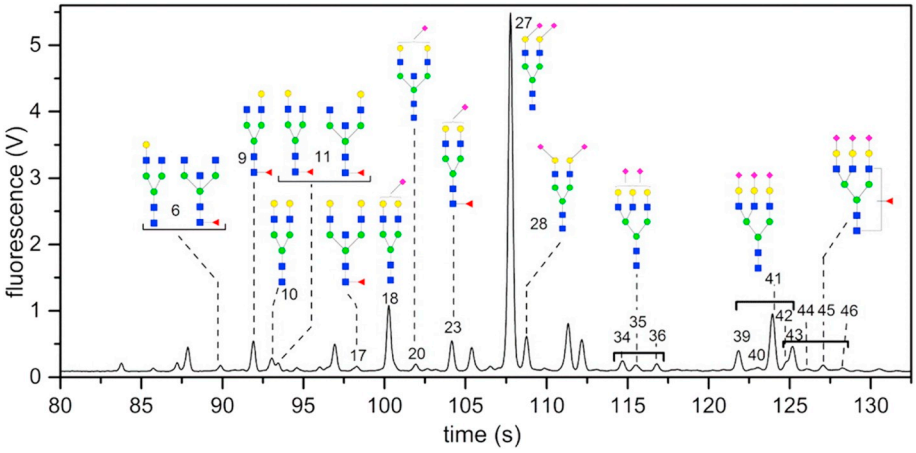

Fig. 9. MCE of methylamidated serum *N*-glycans from a colorectal cancer patient. Separation efficiencies were up to 700,000 theoretical plates. Annotated peaks correspond to statistically different glycans between control and pathological samples.
Reproduced from reference[120].

overall decrease in core-fucosylation [116] and a decrease in core-fucosylation could be related to all known subtypes of congenital disorders [117]. Additionally, *N*- and *O*-glycans originating from skin cells were analyzed to screen for basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) biomarker. An increase in high-Man *N*-glycans and core 2 *O*-glycans was observed on carcinogenic cells, while α2-3-Neu5Ac levels were decreased in SCC but not in BCC [118].

Microchip CE-LIF was used in the analysis of serum *N*-glycans [119], where Neu5Ac residues were derivatized with methylamine to neutralize the charge. All *N*-glycans were then labeled with APTS, separated using the microchip device and detected with LIF. In combination with separate MALDI-TOF analysis, a total of 52 different glycans were identified in serum samples, including linkage and positional isomers (Fig. 9). The same approach was used for the analysis of serum samples from patients with colorectal cancer, revealing an increase in the abundance of several fucosylated tri- and tetra-antennary glycans [120]. As described above, a change in fucosylation is characteristic for several different forms of cancer, but by applying high-resolution MCE separation it was possible to assign alterations in different fucosyl isomers specifically to colorectal cancer [120]. CE-LIF has not only made its way in clinical research as described above, but has also become a widely used technique for glycan analysis in the biopharmaceutical industry [121]. The technique has been used for the characterization of glycans on mAbs [122] (Fig. 10A) and on influenza A virus glycoproteins used in vaccine manufacturing [123].

CE became an established technique for glycan analysis after it was coupled to LIF as it offers high resolution with good reproducible migration times and peak areas [32] together with unrivaled detection sensitivity for the analysis of glycans. The technique is nowadays applied in clinical biomarker analysis and pharmaceutical quality control. However, for identification purposes, well-defined glycan standards or the application of several exoglycosidase digestion steps are necessary. Correspondingly, multiple CE analyses are required to elucidate single glycan structures using exoglycosidases and although high-throughput analyzers can be used, this approach is more difficult for the identification in biological samples containing high number of different and co-migrating (isomeric) glycans [32]. Mass spectrometry can be a suitable complement for identification.

3.2.3. CE-MS of glycans

Although limits of detection measured with CE-LIF are lower than with CE-MS, the latter offers the possibility to easily determine glycan composition using accurate mass measurements and to obtain linkage information by multi-stage fragmentation experiments [124]. SL interfacing is most often used with CE-MS and to obtain high detection sensitivity in negative MS mode, an alkaline SL is commonly applied. This can influence glycan separation as was shown by the application of triethylamine as a SL additive; In the reverse CE polarity mode, positively charged ions from the SL enter the CE capillary and improve the separation efficiency by creating moving ion boundaries and ion interactions [125].

The separation of negatively chargeable glycans in CE-MS is almost always performed in reverse CE polarity with a neutrally coated capillary to suppress the EOF and ensure a larger separation window [110]. Both permanent (e.g. PVA or PAA) and dynamic coatings (e.g. based on soluble PAA) can be used to suppress the EOF and their application results in similar CE glycan profiles [125]. A PVA coating was employed for the study of *O*-acetylation on Neu5Ac residues of *N*-glycans obtained from fish serum. Separation was performed in reverse CE polarity mode at low pH and a flow-through microvial was used for interfacing with MS. The analysis revealed up to three different *O*-acetylated isomers for disialylated bi- and triantennary glycans [126]. The same method was used for the analysis of unlabeled *N*-glycans released from human IgG and recombinant human EPO expressed in Chinese hamster ovary cells [127]. Neutral glycans migrated with the low EOF and were not separated, while several sialylated glycans were separated in the window before the EOF [127]. To obtain a more robust coating and repeatable migration times, a multilayer PVA coating was applied for the analysis of *N*-glycans, which was reported to be superior in both separation efficiency and detection sensitivity over single-layer coated PVA capillaries. Several *N*-glycans released from human serum were separated and identified using MS with a SL interface [104].

In a different approach, phospholipids were applied as semi-permanent inner wall coatings, using 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine in a 1:2 concentration ratio. These quaternary ammonium components with long alkyl tails self-assemble on the inner surface of the capillary, thereby forming a stable zwitterionic bilayer with a zero net charge while reducing the EOF [128]. Two APTS-labeled, isomeric triantennary *N*-glycans consisting of the same monosaccharide units and containing three galactose (Gal) residues were released from asialofetuin, treated with the exoglycosidase enzyme β1–4 galactosidase and analyzed using a co-axial SL sprayer as interface. The analyzed glycans differed in the linkage of one terminal Gal residue (β1–3 instead of β1–4 linkage) in the middle antenna of one glycan. All terminal Gal residues were removed by the enzyme, except for the β1–3 linked Gal, yielding two different compounds that could be baseline separated [128].

New labeling strategies have been applied for glycan analysis with CE-MS to further improve limits of detection and gain structural

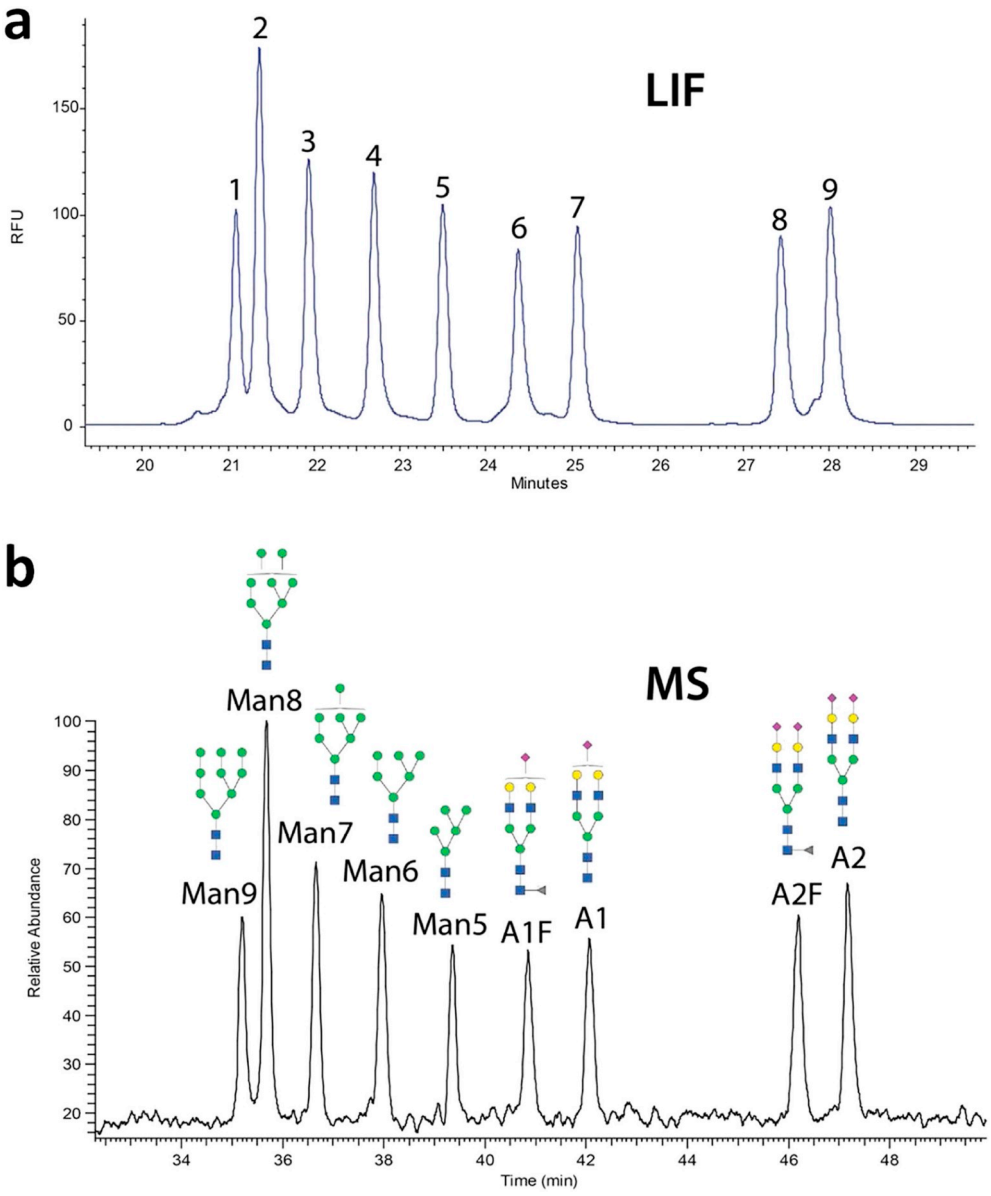

Fig. 10. Electropherograms obtained with CZE-LIF (a) and CZE-MS (b). The extracted ion electropherogram and LIF trace show the analysis results of common *N*-glycans (1.25 nmol/mL) observed on mAbs, labeled with Teal™ and separated using a high methanol content in the BGE. The signal to noise ratio of the LIF trace is clearly superior to the ratio of the MS trace.
Reproduced from reference [103] with permission.

information. *N*-glycans derivatized with Teal™ were analyzed using an electrokinetic pump-based nanospray SL ion source with high methanol content in the BGE for separation and ESI support (Fig. 10B) [103]. Several glycan structures were separated using the Teal™ reagent and similar signal intensities were obtained, compared to ATPS labeling [103]. In addition to derivatizing at the reducing end, Neu5Ac residues can be labeled to prevent their loss during analysis, to neutralize their charge or to obtain detailed structural information during MS analysis. Therefore, Neu5Ac residues on *N*-glycans were derivatized with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM) to amidate α2,6-linked Neu5Ac and form lactones with α2,3-linked Neu5Ac residues. This reaction labeled each type of Neu5Ac with a unique mass to determine specific linkages on sialylated *N*-glycans [124]. *N*-glycans were derivatized with DMTMM and ATPS and analyzed with both CE-MS, using a sheathless interface, and a microfluidic device using a linear PAA coating to minimize the EOF and prevent sample adsorption. A number of 77 *N*-glycan structures derived from human serum were detected, differentiating between specific linkages on sialylated isomers [124].

Due to the small injection volumes used in CE (typically ~5–20 nL) the sensitivity of CE-MS is often not sufficient to analyze low abundant glycans in biological samples. For the analysis of ATPS-labeled *N*-glycans anion-exchange sorbent beads were used in a solid-phase extraction (SPE)-CE set-up consisting of an inlet, an SPE and separation capillary joined with a polymeric sleeve [129]. The inner diameter of the inlet and separation capillaries was smaller than the SPE beads diameter and the SPE capillary, effectively trapping the beads between inlet and separation capillary. By introducing 90 µm particles into a 100 µm capillary, a single bead strain was created that better prevented the SPE column from clogging than with the application of a multi-layer bead packing. A 10-µL injection volume of the glycan sample led to an 800-fold increase in detection sensitivity compared to a CE-MS analysis using a regular injection volume. However, the flow through the capillary for loading and eluting of analytes needed to be adjusted

continuously to the gradually changing backpressure of the SPE construct. Unfortunately, the robustness of SPE-CE is still low and therefore this approach cannot yet be applied routinely.

3.2.4. Quantitative CE-MS for glycans

Till now, most CE-MS analyses methods for glycans are focused on identification, since the quantitative determination is complicated by the lack of reliable standards. Therefore, other approaches that are more generally applicable for relative quantification have been developed. One of those methods is stable isotope labeling of glycans to compare concentrations of glycans between different samples [130]. For one sample a normal label is used and for the other sample a heavier label containing a stable isotope. Glycans existing in one sample are detected as single ions, whereas common glycans are detected as paired ions with a normal and heavy label. Stable isotope twoplex labeling with ^{12}C and ^{13}C 2-AA isotopes was used for the analysis of *N*-glycans released from a mAb [131]. With CE-MS using a PVA coating and an SL interface it was possible to quantify several glycans in two different drug product lots of a commercial mAb [131]. Quantitative analysis has also been reported using tandem mass tags (TMTs) for multiplexed semiquantitative MS/MS of identical analytes in different samples. The TMTs have a modular structure consisting of a reactive group to attach the tag to the analyte, a reporter group that can be modified with different stable isotopes for multiplexing and a balance group to counterbalance the different masses of the reporter ion to ensure that all tags have the same mass prior to fragmentation. Analytes are labeled with TMT's with different reporter ion masses for each different sample. The samples are mixed and analyzed with a separation method and tandem MS. During separation single peaks are obtained for each analyte in the mixed sample. These peaks consist of analytes with isobaric tags and therefore have the same mass in MS. Upon fragmentation in the second MS step unique known reporter ion masses are obtained for each sample that can be used to distinguish the different samples and to determine the relative concentration of analytes in each. Recently, TMTs were modified with a carbonyl-reactive aminooxy group to make the tag suitable for glycan analysis [132]. The applicability of this TMT was demonstrated by the characterization of *N*-linked glycosylation profiles of human colon carcinoma cells with MS, revealing down-regulation of high-mannose glycans in the metastatic cell line [132]. The TMT contains a tertiary amine that provides electrophoretic migration and ESI under acidic conditions and therefore it could also successfully be applied in CE-MS quantification of *N*-glycans released from bovine fetuin [133]. In another application, a microfluidic system with integrated liquid-junction MS interface was used for the quantification of *N*-glycans in positive CE and MS mode [62]. Capillary surfaces were coated with an aminopropyl silane reagent to minimize EOF and glycans were derivatized with aminooxy-TMT to introduce a positive charge for electrophoretic migration and to enable multiplexing in multi-stage MS experiments. Neu5Ac residues were derivatized to prevent interaction with the positively charged capillary surface coating and to obtain linkage-specific mass-shifts for sialylated glycans. The authors claimed that the application of this chip-based CE method improved glycan separation compared to frequently used chromatographic methods [62].

CE-MS is an attractive technique that has developed into an alternative to HILIC- and PGC-MS for glycan separation and identification. Different developments in CE-MS interfacing have resulted in improved signal intensities, however detection limits in CE-MS of glycans remain an issue to be dealt with. In recent years, progress in the CE field has been made regarding injectable volumes to increase sensitivity and stable, MS compatible capillary coatings to obtain better resolution and migration time repeatability. Furthermore, several new derivatization strategies were developed to increase identification and quantification possibilities. It is expected that these new technologies will contribute to further developments in the glycomics field that will expand the application of CE-MS and make it an even more indispensable technique in the field next to LC-MS.

3.3. Clinical chemistry

In the field of clinical chemistry, CE has been used as an effective tool for the reliable, fast and selective analysis of various small molecules and proteins in a wide range of biological samples. CE especially gained interest in clinical laboratories after the introduction of commercially available CE instruments, often supported by reagents kits designed for the analysis of specific compounds. Nowadays, enzymatic assays, immunoassays, and chromatographic separation techniques are generally used in a clinical setting for the analysis of specific small molecules, peptides and proteins in biological samples. In this section, attention is paid to a few CE and CE-MS applications that have been and still are used for routine analysis in clinical laboratories. The CGE system for the analysis of DNA fragments has been described in Section 2.1.2.

The use of CE-UV has shown to be an effective analytical tool for the characterization of haemoglobin (Hb) in humans, which is of high clinical interest due to various disorders associated with abnormal blood Hb composition [134]. These diseases are grouped into anaemia resulting from the presence of deleterious genes, coding for defective sequence variances of Hbs, and thalassaemia, which is characterized by abnormal levels of normal globulin chains. Given the prevalence of genetically derived anaemia in many populations, routine clinical screening of heamoglobulin variants in Hb disorders by CE has been implemented in clinical laboratories around the world. Analyses is often performed fully automated, with dynamic coatings and on-column UV detection [135,136], requiring relatively small amount (circa 20 μL) of samples, It has been often used for the assessment of HbA1c combined with a HbA(1c) kit specifically designed for this purpose. Currently, CE is still used in clinical laboratories for the screening of Hb variants in haemoglobin disorders, however, LC methods are increasingly employed for this purpose, in some cases as a complementary technique whereas in other cases as a replacement for CE [137]. CE is used for the profiling of proteins in human serum, with a main focus on the globulins as changes in each of these proteins can be correlated with a patient's health status [138]. A typical serum protein profile obtained by CZE for a healthy subject is shown in Fig. 11, in which the characteristic globulin bands are easily observed [139].

The group of Harald Mischak has developed CE-MS methods for the reproducible profiling of native peptides in > 20,000 human urine samples at different laboratories with an acceptable inter-laboratory reproducibility over the past decade [140,141]. For the discovery of peptide markers for the prediction of chronic kidney disease (CKD), Good et al. developed a CE-MS-based proteomics analytical workflow for the profiling of urinary peptides in 230 patients with CKD and in 379 healthy subjects [142]. CE–MS analysis was performed at low pH conditions using a BFS capillary, which was coupled to ESI-MS via an SL interface. In total 273 peptides were found as potential biomarkers for CKD using a Support Vector Machine model. To evaluate whether this set of peptides could be used as a classifier for CKD, a validation study was performed using an independent cohort of 144 human urine samples, revealing a sensitivity of 85% (95% CI, 77.5–91.4) and a specificity of 100% (95% confidence interval, 89.6–100.0). Overall, this work clearly indicates the value of CE-MS for clinical and biomarker discover studies.

3.4. Metabolomics

In this section, the utility of CE and CE-MS is highlighted for the analysis of (endogenous) metabolites in biological samples. CE can be used for both non-targeted and targeted profiling in biological samples. In the first approach, the aim is to analyze as many metabolites as possible without having a priori knowledge on the nature and identity. In the second approach, the focus is on the quantitative analysis of pre-

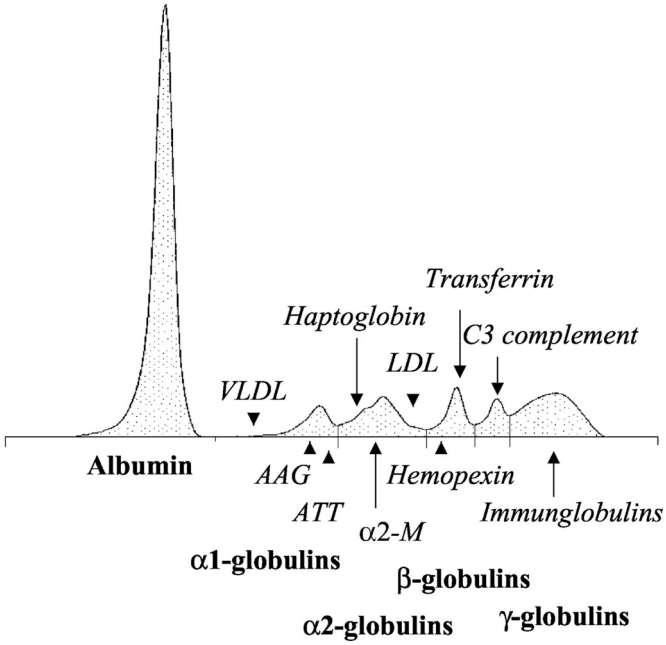

Fig. 11. CE of serum proteins from a healthy subject. The automated instrument (β1-β2+ reagent set) separates human serum proteins into six main fractions detected at the cathodic end of the capillary in the following order: γ-globulins, β2-globulins, βl-globulins, α2-globulins, αl-globulins, and albumin. Electrophoretic mobilities of individual proteins were determined by immunosubtraction or overload, depending on the protein. AAG, α1-acid glycoprotein; ATT, α1-antitrypsin; α2-M, α2-macroglobulin.
Reproduced from reference [139] with permission.

selected (target) metabolites. CE has a long history for the analysis of (endogenous) metabolites in a wide range of biological samples [143], as it is very suited for the selective analysis of polar and charged compounds [144]. As only small sample volumes are required for the injection, CE is very suitable for the analysis of volume-limited biological samples. However, depending on the detection technique (and obviously on the goal of the study), small injection volumes may result in compromised concentration sensitivities. This issue can be tackled by using electrokinetic- or chromatographic-based preconcentration techniques [145,146].

On-line sample preconcentration in CE-MS is relatively easily performed by in-capillary stacking based on analyte mobility differences within a discontinuous BGE system. Therefore, direct injection of long sample plug lengths (> 10% capillary length) can be performed as electrokinetic focusing of analytes into narrow zones occurs prior to ESI-MS analysis. In general, transient-ITP and/or dynamic pH junction has been often used for enrichment of mainly cationic metabolites in biological samples obtaining detection limits in the low nanomolar range, while using conventional CE-MS approaches [147]. The use of chromatographic-based preconcentration by employing coupled SPE-CE systems is another way to improve concentration sensitivity of CE-MS. For example, Pont et al. recently demonstrated the utility of SPE coupled in-line to CE-MS for the profiling of metabolites in deproteinized plasma samples from mice [148].

Nowadays, CE-MS can be considered a useful analytical technique for addressing biological and clinical questions using a metabolomics approach [149,150]. In the first part of this section, emphasis is on works which clearly exemplified the (early) potential of various CE systems for profiling metabolites in biological samples. This is followed by a section which provides an overview of the initial CE-MS approaches developed for global or non-targeted metabolic profiling studies. Special attention is paid to CE-MS methods with new interfacing techniques in order to increase the coverage. Finally, some thoughts on the direction of CE-MS in metabolomics are provided.

3.4.1. CE systems for metabolic profiling of biological samples

The actual potential of CE (referring here to CZE) for the profiling of endogenous metabolites in body fluids has been demonstrated circa three decades ago by Jellum et al. [151–153]. In this work, human urine samples were analyzed by CE without the use of any sample pretreatment [152]. Circa 50 urinary metabolites were analyzed in 15 min and the identification was based on migration times and characteristic UV spectra. This approach was applied to the screening of urine from patients with metabolic disorders, such as pyroglutamic aciduria. Authors already anticipated at that time the potential of CE for profiling of small-volume samples in the clinical field. Another pioneering work in the development of CE approaches for screening metabolic disorders in body fluids emerged from Barbas and co-workers [154]. This group developed a relatively fast and automated CE-UV method for organic acids profiling in urine [155]. Samples were analyzed directly after centrifugation and dilution and separation was in reverse polarity mode employing a PAA-coated capillary and phosphate buffer (pH 6.0) containing a small amount of methanol as BGE. Various other groups have also designed CE approaches for the profiling of organic acids [156,157]. For example, Dolnik et al. developed a CE method using a capillary coated with linear PAA and indirect UV detection [156]. Serum could be analyzed directly, without using a deproteinization step. The CE method was used for the analysis of organic acids in serum from a pediatric patient with respiratory insufficiency (Fig. 12).

In general, mostly CZE has been used for the analysis of endogenous metabolites in biological samples. This CE mode is fully compatible with ESI-MS as volatile buffers can be employed for the analysis. In order to improve selectivity, MEKC was employed for the profiling of biological samples [158]. For example, both CZE and MEKC were used for profiling of urine samples from diabetic rats [158]. CZE, employing a PAA-coated capillary and reverse CE polarity, was used for the analysis of anions, whereas MEKC was used for the profiling of cations and neutral compounds. Overall, an extended metabolic profile of the urine sample was obtained by combining the data obtained with both methods. A sulfated β-cyclodextrin-modified MEKC approach has also been used for human urine, allowing the separation of 80 compounds within 25 min [159]. Charged cyclodextrins have different shapes, size and binding properties, compared to underivatized cyclodextrins, and can offer different selectivity for analytes and improve resolution [160]. Although an improved separation and additional selectivity can be obtained by MEKC, the hyphenation of MEKC to ESI-MS is problematic and often provides limited sensitivity. CEC has also been evaluated for the profiling of endogenous metabolites. For example, a pressure-assisted CEC approach was developed for rat urine and the performance of this method was compared with chromatographic methods [161]. CEC can be used for the separation of a wide range of compounds and has therefore a strong potential for global profiling studies. However, the design of CEC columns in a consistent manner remains a challenging task.

Regarding detection, mainly UV, but also contactless conductivity, electrochemical and LIF detection have been used [162–166]. Notably, CE-LIF can be considered a very strong tool for the highly sensitive and selective analysis of numerous metabolites in small-volume biological samples. For example, Harstad et al. developed a high-speed microdialysis-CE method for assessing the uptake/release dynamics of branched chain amino acid in 3T3-L1 cells using LIF detection [167]. Currently, CE-LIF approaches are often used for the selective profiling of derivatized amino acids and small peptides in small-volume biological samples [168].

Many CE approaches developed a few decades ago, e.g. for organic acids, have not been implemented in actual clinical settings due to variability in EOF, and therefore migration times, when employing BFS capillaries in combination with biological samples. In addition to

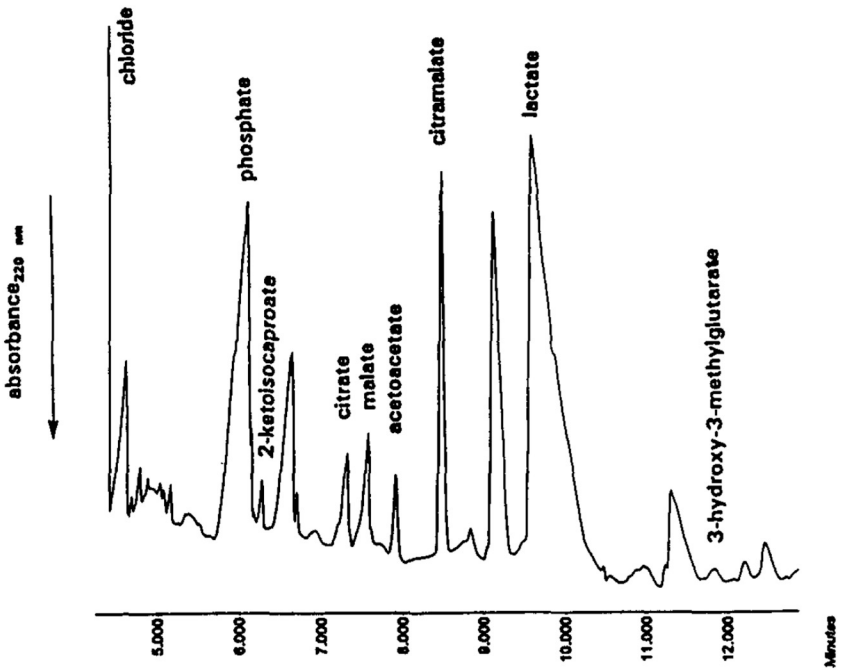

Fig. 12. Determination of organic acids in serum from a pediatric patient with respiratory insufficiency. Experimental conditions: linear polyacrylamide-coated capillary, 75 µm I.D.; BGE, 10 mM e-aminocaproic acid-10 mM mandelic acid; voltage, −20 kV; detection, absorption at 220 nm. Undiluted serum injected by pressure using100 mbar for 6 s. Reproduced from reference [156] with permission.

generating irreproducible EOF, adsorption of matrix components to the inner wall of BFS capillaries may also compromise separation efficiencies and peak area repeatability. Therefore, it is important to incorporate, in addition to an effective sample pretreatment procedure, an effective rinsing procedure between runs when employing BFS capillaries with (especially protein-rich) biological samples. Obviously, a rinsing procedure increases the total analysis time per sample. Another way to address this issue is to modify the inner capillary wall of BFS capillaries with polymers.

3.4.2. CE-MS systems for global metabolic profiling studies

MS has emerged as a key technology for the selective and sensitive analysis of metabolites in biological samples, providing the ability to quantify and identify analytes. Combination of MS with a front-end separation technique is often needed in order to reduce ion suppression, to allow the separation of isobars and isomers, and to have additional information on the physicochemical properties of the metabolites for identification. One of the first applications reporting CE-MS in a clinical context, emerged from the group of Jellum [169]. In this work, a CE-MS method was developed for the selective analysis of metabolites in dried blood spots from patients with the metabolic disorder propionic aciduria. CE was coupled to MS via a SL interface and electrophoretic separation was performed with a BFS capillary employing 20 mM ammonium acetate (pH 8.5) as BGE. Around the same period, Soga et al. developed a CE-MS method for the analysis of 19 naturally occurring amino acids without derivatization [170]. To enable the simultaneous and selective analysis of all amino acids by CE-MS, 1 M formic acid (pH 1.8) was used as BGE. The CE-MS method provided detection limits in the low µM-range for most amino acids and its utility was demonstrated by the analysis of soy sauce, beer and urine. CE-MS approaches for global or non-targeted profiling studies were developed by the same group using a first method for profiling cationic metabolites, employing 1 M formic acid (pH 1.8) as BGE and a BFS capillary, and a second method for profiling anionic metabolites using a cationic polymer-coated capillary with 50 mM ammonium acetate (pH 8.5) as BGE [171]. By using both CE-MS approaches, > 1600 metabolic features could be observed in an extract from *Bacillus subtilis*, of which 150 could be identified (Fig. 13). The developed CE-MS approaches allowed the selective and highly efficient analysis of a broad range of polar metabolite classes, including amino acids, amines, nucleosides, nucleotides, organic acids and sugar phosphates. The CE-MS approach for anionic profiling has been further improved by the same group over the last decade [172]. Based on these CE-MS methods, the authors initiated Human Metabolome Technologies (HMT), a Japan-based company which is providing procedures and workflows for CE-MS-based metabolomics. The CE-MS approaches of HMT were recently used for the profiling and absolute quantification of polar and charged metabolites in plasma samples from the Tsuruoka Metabolomics Cohort Study (> 10,000 human subjects) [173], thereby clearly illustrating the capability of CE-MS for large-scale studies.

Most CE-MS-based studies have been performed with the SL interface, however, the SL is generally provided at a flow-rate between 5 and 10 µL/min. Considering the fact that both CE and ESI-MS perform most optimal at low flow-rate conditions, CE-MS should preferably be carried out via an interface which effectively uses the intrinsically low flow separation property of CE and the improved ESI efficiency under these conditions. For example, CE-MS using a sheathless porous tip interface, has been evaluated for the profiling of cations in human urine at low-pH separation conditions resulting in an information-rich metabolic profile [55]. This approach allowed for an enhanced coverage of polar and charged metabolites of the urinary metabolome with nanomolar detection limits. Approximately 900 molecular features were detected with sheathless CE-MS, whereas 300 were observed by SL CE-MS. Hirayama and co-workers also assessed the performance of this approach and obtained a tenfold increase in the number of detected peaks compared to SL CE-MS methods [174]. The long-term performance of this approach still needs to be assessed in more extended studies analyzing larger numbers of clinical samples. The sheathless porous tip interface was also evaluated for anionic metabolites using the same separation conditions as for cations, but with a reversed MS detection and CE separation polarity [175]. A broad range of anionic metabolite classes could be profiled under these conditions, including sugar phosphates, nucleotides and organic acids. An injection volume of circa 20 nL resulted in nanomolar detection limits, which was a significant enhancement compared to the micromolar detection limits typically obtained with classical SL CE-MS methods.

Metabolic profiling approaches are now more widely used for large-scale phenotyping studies (e.g., clinical samples from Biobanks). Therefore, the ability to analyze thousands of biological samples with a reliable high-throughput precision becomes crucial. In this context, the

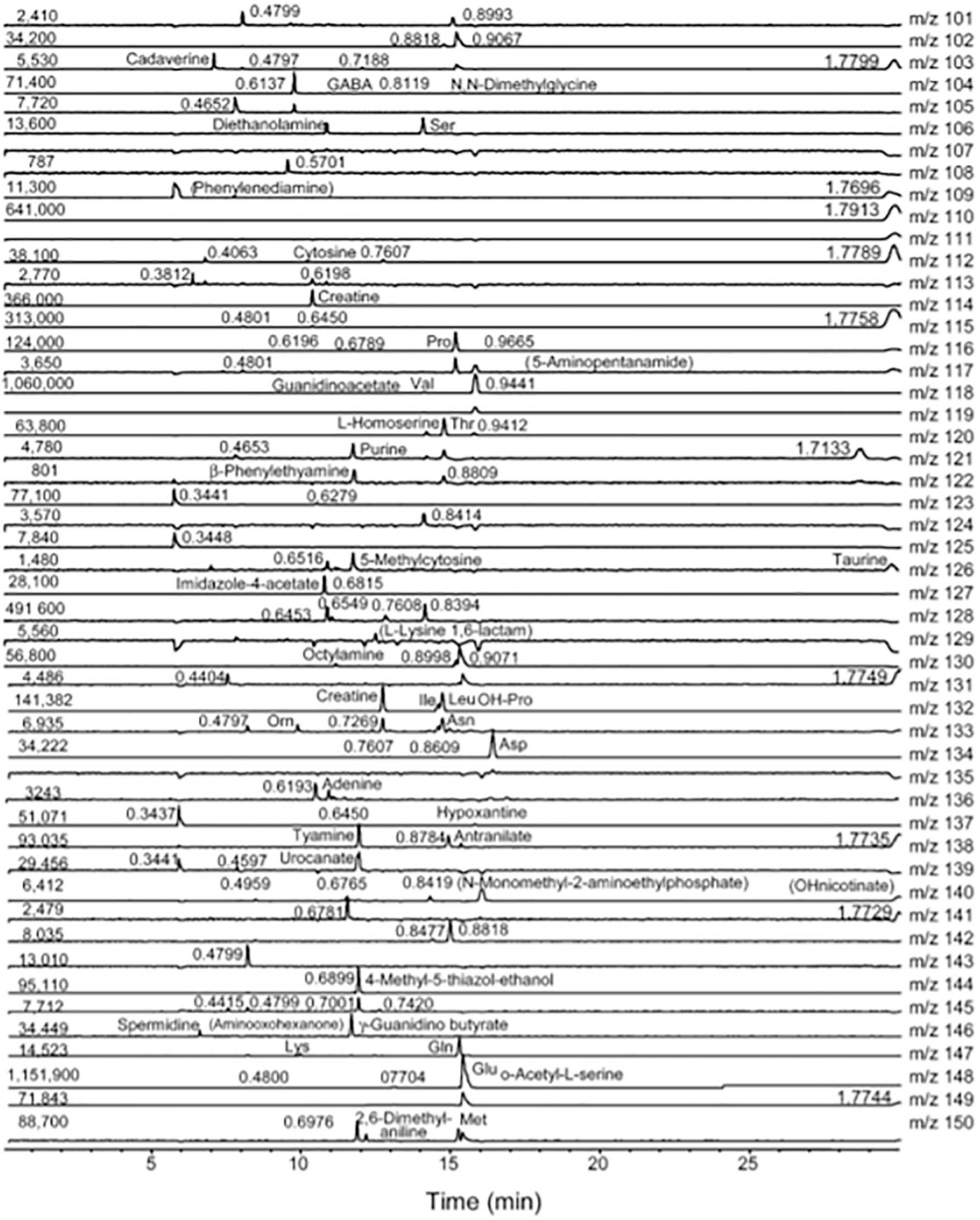

Fig. 13. Selected ion electropherograms for cationic metabolites observed in an extract from *Bacillus subtillis* by CE-MS in the *m/z* range of 101 to 150. The number provided in the upper left corner of each trace is the abundance associated with the highest peak in the electropherogram.
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recently developed multi-segment injection CE-MS approach can be considered a practical tool, as this approach allows serial hydrodynamic injection of seven or more discrete sample segments within a single capillary (Fig. 14). Sample throughput is increased up to one order of magnitude without ion suppression/enhancement, while maintaining separation performance [176].

In comparison to other analytical techniques, the use of CE-MS in metabolomics is still underrepresented. However, with recent developments in improved interfacing designs, it is anticipated that this approach will be considered more for the profiling in material-limited biological samples, such as single cell analyses. Therefore, a complete analytical workflow based on CE-MS employing a low-flow SL interface was recently developed [177,178]. Moreover, a recent study revealed that CE-MS could be used for the analysis of > 76% of the compounds present in a commercially available metabolite library mixture which comprised 596 compounds [179]. A comparison with HILIC-MS and reversed-phase LC-MS demonstrated that CE-MS is particularly useful for the profiling of amino acids, sulfated and phosphorylated compounds, thereby clearly illustrating the added value of CE-MS for metabolomics [179].

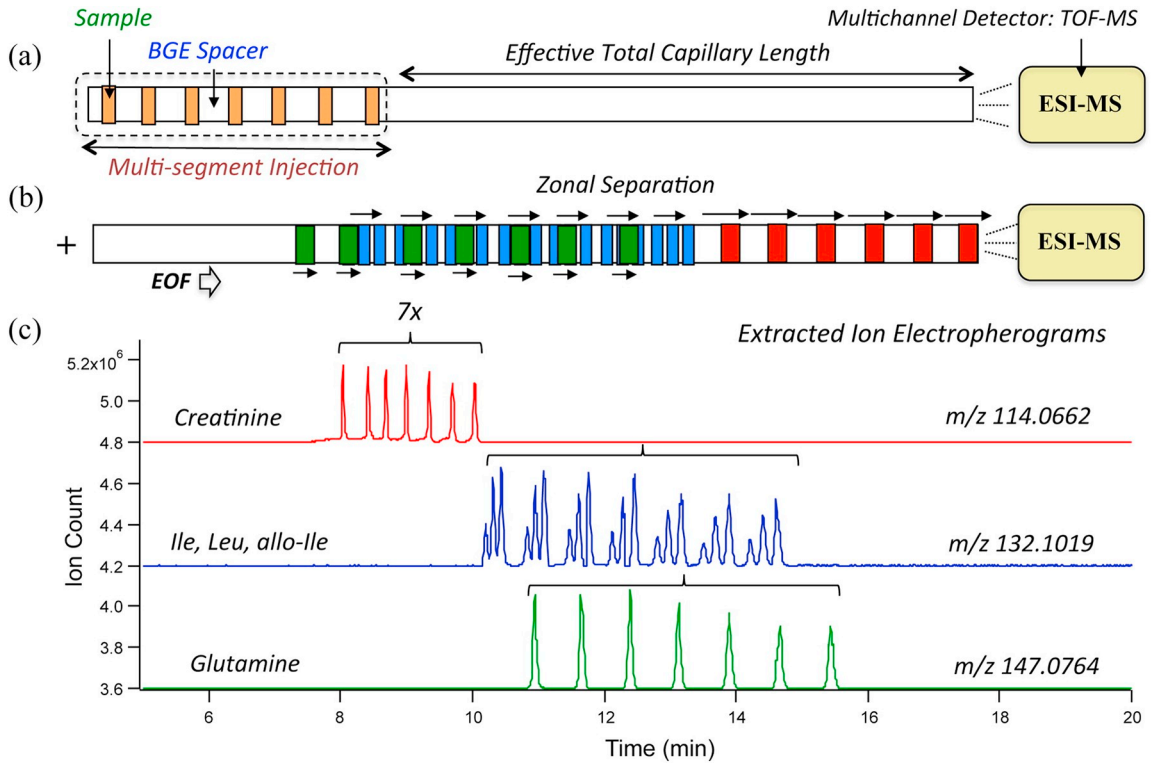

Fig. 14. Multi-segment injection in CE-MS. (A) Multiplexed separation based on serial injection of seven discrete sample segments within a single capillary by multi-segment injection CE-MS; (B) ions migrate as a series of zones in free solution prior to ionization; (C) the procedure enables reliable quantification of polar metabolites and their isomers in different samples as ionization occurs within a short-time interval (≈2–6 min) under steady-state conditions when using ESI-MS. Reproduced from reference [176] with permission.

4. Concluding remarks

CE became strong in life sciences with the rapid and efficient analysis of small highly polar compounds and large biomolecules such as DNA, peptides and proteins. Many of these compounds are often analyzed with more robust and sensitive systems, partly due to developments in other techniques such as LC. However, there are applications where CE performs better or gives complementary information, and in those fields the various CE modes have become indispensable techniques. Furthermore, several developments in instrumentation and applications have ensured that CE is no longer being regarded as a non-robust technique. In addition, the development of accessible miniaturized devices, which allows very fast analyses, contributes to the application of CE as an attractive alternative for routine high-throughput analysis.

CGE, CIEF and CZE are currently, in addition to many other techniques, routinely used in the pharmaceutical industry and other laboratories for the characterization of intact proteins. Combined with MS, CE is used to quickly resolve protein glycoforms with high resolution and to identify the compounds. The analysis of released glycans in contrast is usually performed with CZE- or CGE coupled to LIF detection, because detection limits with CE-MS are not sufficient due to the low injection volumes in CE. Recent developments in MS interfaces have led to lower detection limits with CE-MS, although LIF detection remains unrivaled. Despite the higher detection limits, it is anticipated that the application of CE-MS in this area will increase with the growing glycomics field and demand for glycan biomarkers. CE is attractive for the clinical and biomarker field, especially for the analysis of polar small molecules in studies were limited sample volume is available. Moreover, high resolution is important when profiling biological samples containing large numbers of metabolites and therefore nowadays CE(-MS) is routinely applied in the metabolomics field. CE is now an established technique in the fields described in this review. For further applicability, it is important that still more progress will be made in on-line preconcentration systems to inject larger volumes routinely in CE. In that case it is expected that CE could become of added value in more areas.

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References

[1] P. Schmitt-Kopplin, Capillary Electrophoresis: Methods and Protocols, Humana Press, New York, 2016.
[2] O. Vesterberg, History of electrophoretic methods, J. Chromatogr. 480 (1989) 3–19.
[3] A. Tiselius, A new apparatus for electrophoretic analysis of colloidal mixtures, Trans. Faraday Soc. 33 (1937) 0524–0530.
[4] A.J.P. Martin, 1942, (Unpublished results).
[5] S. Hjertén, Free zone electrophoresis, Chromatogr. Rev. 9 (1967) 122–219.
[6] F.M. Everaerts, J.L. Beckers, T.P.E.M. Verheggen, J.L. Beckers, Isotachophoresis: Theory, Instrumentation and Applications, Elsevier, Amsterdam, 1976.
[7] J.W. Jorgenson, K.D. Lukacs, Zone electrophoresis in open-tubular glass-capillaries, Anal. Chem. 53 (1981) 1298–1302.
[8] G.J. de Jong, Capillary Electrophoresis - Mass Spectrometry (CE-MS): Principles and Applications, Wiley-VCH, Weinheim, 2016.
[9] P. Rezanka, K. Navratilova, M. Rezanka, V. Kral, D. Sykora, Application of cyclodextrins in chiral capillary electrophoresis, Electrophoresis 35 (2014) 2701–2721.
[10] J.M. Saz, M.L. Marina, Recent advances on the use of cyclodextrins in the chiral analysis of drugs by capillary electrophoresis, J. Chromatogr. A 1467 (2016) 79–94.
[11] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Electronic separations with micellar solutions and open-tubular capillaries, Anal. Chem. 56 (1984) 111–113.

[12] M. Silva, Micellar electrokinetic chromatography: a review of methodological and instrumental innovations focusing on practical aspects, Electrophoresis 34 (2013) 141–158.
[13] C.E. Sänger – van de Griend, Y. Hedeland, C. Pettersson., Chromatography Today, vol. May/June (2013) 32–37.
[14] V. Pretorius, B.J. Hopkins, J.D. Schieke, Electro-osmosis - new concept for high-speed liquid-chromatography, J. Chromatogr. 99 (1974) 23–30.
[15] T. Tsuda, K. Nomura, G. Nakagawa, Open-tubular microcapillary liquid-chromatography with electroosmosis flow using a UV detector, J. Chromatogr. 248 (1982) 241–247.
[16] J.H. Knox, I.H. Grant, Miniaturizing in pressure and electroendosmotically driven liquid-chromatography - some theoretical considerations, Chromatographia 24 (1987) 135–143.
[17] J. Horvath, V. Dolnik, Polymer wall coatings for capillary electrophoresis, Electrophoresis 22 (2001) 644–655.
[18] L. Hajba, A. Guttman, Recent advances in column coatings for capillary electrophoresis of proteins, Trac-Trend Anal Chem 90 (2017) 38–44.
[19] S. Garza, S. Chang, M. Moini, Simplifying capillary electrophoresis-mass spectrometry operation: eliminating capillary derivatization by using self-coating background electrolytes, J. Chromatogr. A 1159 (2007) 14–21.
[20] C. Huhn, R. Ramautar, M. Wuhrer, G.W. Somsen, Relevance and use of capillary coatings in capillary electrophoresis-mass spectrometry, Anal. Bioanal. Chem. 396 (2010) 297–314.
[21] A. Manz, D.J. Harrison, E.M.J. Verpoorte, J.C. Fettinger, A. Paulus, H. Ludi, H.M. Widmer, Planer chips technology for miniaturization and integration of separation techniques into monitoring systems - capillary electrophoresis on a chip, J. Chromatogr. 593 (1992) 253–258.
[22] S.C. Jacobson, R. Hergenroder, L.B. Koutny, J.M. Ramsey, High-speed separations on a microchip, Anal. Chem. 66 (1994) 1114–1118.
[23] H.L. Han, E. Livingston, X.Y. Chen, High throughput profiling of charge heterogeneity in antibodies by microchip electrophoresis, Anal. Chem. 83 (2011) 8184–8191.
[24] M. Dawod, N.E. Arvin, R.T. Kennedy, Recent advances in protein analysis by capillary and microchip electrophoresis, Analyst 142 (2017) 1847–1866.
[25] E.A. Redman, N.G. Batz, J.S. Mellors, J.M. Ramsey, Integrated microfluidic capillary electrophoresis-electrospray ionization devices with online MS detection for the separation and characterization of intact monoclonal antibody variants, Anal. Chem. 87 (2015) 2264–2272.
[26] E.A. Redman, J.S. Mellors, J.A. Starkey, J.M. Ramsey, Characterization of intact antibody drug conjugate variants using microfluidic capillary electrophoresis-mass spectrometry, Anal. Chem. 88 (2016) 2220–2226.
[27] M.C. Ruizmartinez, J. Berka, A. Belenkii, F. Foret, A.W. Miller, B.L. Karger, DNA-sequencing by capillary electrophoresis with replaceable linear polyacrilamide and laser-induced fluorescence detection, Anal. Chem. 65 (1993) 2851–2858.
[28] H. Swerdlow, R. Gesteland, Capillary gel-electrophoresis for rapid, high-resolution DNA sequencing, Nucleic Acids Res. 18 (1990) 1415–1419.
[29] R.J. Zagursky, R.M. McCormick, DNA sequencing report - DNA sequencing separations in capillary gels on a modified commercial DNA sequencing instrument, Biotechniques 9 (1990) 74–79.
[30] H. Swerdlow, S. Wu, H. Harke, N.J. Dovichi, Capillary gel-electrophoresis for DNA sequencing - laser-induced fluorescence detection with the sheath flow cuvette, J. Chromatogr. 516 (1990) 61–67.
[31] N.J. Dovichi, J.Z. Zhang, How capillary electrophoresis sequenced the human genome, Angew. Chem.-Int. Edit. 39 (2000) 4463–4468.
[32] A. Szekrenyes, S.S. Park, M. Santos, C. Lew, A. Jones, T. Haxo, M. Kimzey, S. Pourkaveh, Z. Szabo, Z. Sosic, P. Feng, C. Varadi, F. de l'Escaille, J.B. Falmagne, P. Sejwal, T. Niedringhaus, D. Michels, G. Freckleton, M. Hamm, A. Manuilov, M. Schwartz, J.K. Luo, J. van Dyck, P.K. Leung, M. Olajos, Y.M. Gu, K. Gao, W.B. Wang, J. Wegstein, S. Tep, A. Guttman, Multi-site N-glycan mapping study 1: capillary electrophoresis - laser induced fluorescence, mAbs 8 (2016) 56–64.
[33] F. Kilar, Recent applications of capillary isoelectric focusing, Electrophoresis 24 (2003) 3908–3916.
[34] K. Shimura, Recent advances in IEF in capillary tubes and microchips, Electrophoresis 30 (2009) 11–28.
[35] Q.L. Mao, J. Pawliszyn, Capillary isoelectric focusing with whole column imaging detection for analysis of proteins and peptides, J. Biochem. Biophys. Methods 39 (1999) 93–110.
[36] H.C. Liu, L.H. Zhang, G.J. Zhu, W.B. Zhang, Y.K. Zhang, An etched porous interface for on-line capillary electrophoresis-based two-dimensional separation system, Anal. Chem. 76 (2004) 6506–6512.
[37] J. Dai, J. Lamp, Q.W. Xia, Y.R. Zhang, Capillary isoelectric focusing-mass spectrometry method for the separation and online characterization of intact monoclonal antibody charge variants, Anal. Chem. 90 (2018) 2246–2254.
[38] M. Dole, L.L. Mack, R.L. Hines, Molecular beams of macroions, J. Chem. Phys. 49 (1968) 2240–2249.
[39] M. Yamashita, J.B. Fenn, Electrospray ion-source - another variation on the free-jet theme, J Phys Chem-Us 88 (1984) 4451–4459.
[40] J.A. Olivares, N.T. Nguyen, C.R. Yonker, R.D. Smith, Online mass-spectrometric detection for capillary zone electrophoresis, Anal. Chem. 59 (1987) 1230–1232.
[41] R.D. Smith, C.J. Barinaga, H.R. Udseth, Improved electrospray ionization interface for capillary zone electrophoresis - mass spectrometry, Anal. Chem. 60 (1988) 1948–1952.
[42] P. Hommerson, A.M. Khan, G.J. de Jong, G.W. Somsen, Ionization techniques in capillary electrophoresis-mass spectrometry: principles, design, and application, Mass Spectrom. Rev. 30 (2011) 1096–1120.
[43] T. Soga, K. Igarashi, C. Ito, K. Mizobuchi, H.P. Zimmermann, M. Tomita, Metabolomic profiling of anionic metabolites by capillary electrophoresis mass spectrometry, Anal. Chem. 81 (2009) 6165–6174.
[44] T.J. Causon, L. Maringer, W. Buchberger, C.W. Klampfl, Addition of reagents to the sheath liquid: a novel concept in capillary electrophoresis-mass spectrometry, J. Chromatogr. A 1343 (2014) 182–187.
[45] C.L. do Lago, D.T. Vidal, K.J. Francisco, V.B. dos Santos, A simple approach to compensate the suction caused by the electrospray ionization source in capillary electrophoresis-mass spectrometry systems, Electrophoresis 35 (2014) 2412–2416.
[46] J.H. Eriksson, R. Mol, G.W. Somsen, W.L. Hinrichs, H.W. Frijlink, G.J. de Jong, Feasibility of nonvolatile buffers in capillary electrophoresis-electrospray ionization-mass spectrometry of proteins, Electrophoresis 25 (2004) 43–49.
[47] C.T. Chien, F.A. Li, J.L. Huang, G.R. Her, CE-MS of antihistamines using non-volatile phosphate buffer, Electrophoresis 28 (2007) 1454–1460.
[48] R. Gottardo, I. Miksik, Z. Aturki, D. Sorio, C. Seri, S. Fanali, F. Tagliaro, Analysis of drugs of forensic interest with capillary zone electrophoresis/time-of-flight mass spectrometry based on the use of non-volatile buffers, Electrophoresis 33 (2012) 599–606.
[49] V. Gonzalez-Ruiz, S. Codesido, S. Rudaz, J. Schappler, Evolution in the design of a low sheath-flow interface for CE-MS and application to biological samples, Electrophoresis 39 (2018) 853–861.
[50] V. Gonzalez-Ruiz, S. Codesido, J. Far, S. Rudaz, J. Schappler, Evaluation of a new low sheath-flow interface for CE-MS, Electrophoresis 37 (2016) 936–946.
[51] M. Moini, Simplifying CE-MS operation. 2. Interfacing low-flow separation techniques to mass spectrometry using a porous tip, Anal. Chem. 79 (2007) 4241–4246.
[52] Y. Yin, G. Li, Y. Guan, G. Huang, Sheathless interface to match flow rate of capillary electrophoresis with electrospray mass spectrometry using regular-sized capillary, Rapid Commun. Mass Spectrom. 30 (Suppl. 1) (2016) 68–72.
[53] R. Haselberg, G.J. de Jong, G.W. Somsen, Low-flow sheathless capillary electrophoresis-mass spectrometry for sensitive glycoform profiling of intact pharmaceutical proteins, Anal. Chem. 85 (2013) 2289–2296.
[54] R. Haselberg, C.K. Ratnayake, G.J. de Jong, G.W. Somsen, Performance of a sheathless porous tip sprayer for capillary electrophoresis-electrospray ionization-mass spectrometry of intact proteins, J. Chromatogr. A 1217 (2010) 7605–7611.
[55] R. Ramautar, J.M. Busnel, A.M. Deelder, O.A. Mayboroda, Enhancing the coverage of the urinary metabolome by sheathless capillary electrophoresis-mass spectrometry, Anal. Chem. 84 (2012) 885–892.
[56] E.D. Lee, W. Muck, J.D. Henion, T.R. Covey, Liquid junction coupling for capillary zone electrophoresis/ion spray mass spectrometry, Biomed. Environ. Mass Spectrom. 18 (1989) 844–850.
[57] E.J. Maxwell, X. Zhong, H. Zhang, N. van Zeijl, D.D. Chen, Decoupling CE and ESI for a more robust interface with MS, Electrophoresis 31 (2010) 1130–1137.
[58] E.J. Maxwell, X.F. Zhong, D.D.Y. Chen, Asymmetrical emitter geometries for increased range of stable electrospray flow rates, Anal. Chem. 82 (2010) 8377–8381.
[59] X. Zhong, E.J. Maxwell, C. Ratnayake, S. Mack, D.D. Chen, Flow-through microvial facilitating interface of capillary isoelectric focusing and electrospray ionization mass spectrometry, Anal. Chem. 83 (2011) 8748–8755.
[60] L. Sun, G. Zhu, Z. Zhang, S. Mou, N.J. Dovichi, Third-generation electrokinetically pumped sheath-flow nanospray interface with improved stability and sensitivity for automated capillary zone electrophoresis-mass spectrometry analysis of complex proteome digests, J. Proteome Res. 14 (2015) 2312–2321.
[61] N.G. Batz, J.S. Mellors, J.P. Alarie, J.M. Ramsey, Chemical vapor deposition of aminopropyl silanes in microfluidic channels for highly efficient microchip capillary electrophoresis-electrospray ionization-mass spectrometry, Anal. Chem. 86 (2014) 3493–3500.
[62] K. Khatri, J.A. Klein, J.R. Haserick, D.R. Leon, C.E. Costello, M.E. McComb, J. Zaia, Microfluidic capillary electrophoresis-mass spectrometry for analysis of monosaccharides, oligosaccharides, and glycopeptides, Anal. Chem. 89 (2017) 6645–6655.
[63] L.H. Silvertand, J.S. Torano, G.J. de Jong, W.P. van Bennekom, Development and characterization of cIEF-MALDI-TOF MS for protein analysis, Electrophoresis 30 (2009) 1828–1835.
[64] J.M. Busnel, J. Josserand, N. Lion, H.H. Girault, Iontophoretic fraction collection for coupling capillary zone electrophoresis with matrix-assisted laser desorption/ionization mass spectrometry, Anal. Chem. 81 (2009) 3867–3872.
[65] J. Wang, M. Ma, R. Chen, L. Li, Enhanced neuropeptide profiling via capillary electrophoresis off-line coupled with MALDI FTMS, Anal. Chem. 80 (2008) 6168–6177.
[66] A. Tycova, M. Vido, P. Kovarikova, F. Foret, Interface-free capillary electrophoresis-mass spectrometry system with nanospray ionization-analysis of dexrazoxane in blood plasma, J. Chromatogr. A 1466 (2016) 173–179.
[67] J. Krenkova, K. Kleparnik, J. Grym, J. Luksch, F. Foret, Self-aligning subatmospheric hybrid liquid junction electrospray interface for capillary electrophoresis, Electrophoresis 37 (2016) 414–417.
[68] S. Fekete, D. Guillarme, P. Sandra, K. Sandra, Chromatographic, electrophoretic, and mass spectrometric methods for the analytical characterization of protein biopharmaceuticals, Anal. Chem. 88 (2016) 480–507.
[69] B. Moritz, V. Schnaible, S. Kiessig, A. Heyne, M. Wild, C. Finkler, S. Christians, K. Mueller, L. Zhang, K. Furuya, M. Hassel, M. Hamm, R. Rustandi, Y. He, O.S. Solano, C. Whitmore, S.A. Park, D. Hansen, M. Santos, M. Lies, Evaluation of capillary zone electrophoresis for charge heterogeneity testing of monoclonal antibodies, J Chromatogr B Analyt Technol Biomed Life Sci 983-984 (2015) 101–110.
[70] A. Beck, E. Wagner-Rousset, D. Ayoub, A. Van Dorsselaer, S. Sanglier-Cianferani, Characterization of therapeutic antibodies and related products, Anal. Chem. 85 (2013) 715–736.

[71] O. Salas-Solano, B. Tomlinson, S. Du, M. Parker, A. Strahan, S. Ma, Optimization and validation of a quantitative capillary electrophoresis sodium dodecyl sulfate method for quality control and stability monitoring of monoclonal antibodies, Anal. Chem. 78 (2006) 6583–6594.
[72] M. Han, D. Phan, N. Nightlinger, L. Taylor, S. Jankhah, B. Woodruff, Z. Yates, S. Freeman, A. Guo, A. Balland, D. Pettit, Optimization of CE-SDS method for antibody separation based on multi-users experimental practices, Chromatographia 64 (2006) 335–342.
[73] A.L. Esterman, A. Katiyar, G. Krishnamurthy, Implementation of USP antibody standard for system suitability in capillary electrophoresis sodium dodecyl sulfate (CE-SDS) for release and stability methods, J. Pharm. Biomed. Anal. 128 (2016) 447–454.
[74] S. Yin, C.V. Pastuskovas, L.A. Khawli, J.T. Stults, Characterization of therapeutic monoclonal antibodies reveals differences between in vitro and in vivo time-course studies, Pharm. Res. 30 (2013) 167–178.
[75] A. Szekely, A. Szekrenyes, M. Kerekgyarto, A. Balogh, J. Kadas, J. Lazar, A. Guttman, I. Kurucz, L. Takacs, Multicapillary SDS-gel electrophoresis for the analysis of fluorescently labeled mAb preparations: a high throughput quality control process for the production of QuantiPlasma and PlasmaScan mAb libraries, Electrophoresis 35 (2014) 2155–2162.
[76] K. Kubota, N. Kobayashi, M. Yabuta, M. Ohara, T. Naito, T. Kubo, K. Otsuka, Identification and characterization of a thermally cleaved fragment of monoclonal antibody-A detected by sodium dodecyl sulfate-capillary gel electrophoresis, J. Pharm. Biomed. Anal. 140 (2017) 98–104.
[77] M.T. Smith, S. Zhang, T. Adams, B. DiPaolo, J. Dally, Establishment and validation of a microfluidic capillary gel electrophoresis platform method for purity analysis of therapeutic monoclonal antibodies, Electrophoresis 38 (2017) 1353–1365.
[78] E. Tamizi, A. Jouyban, The potential of the capillary electrophoresis techniques for quality control of biopharmaceuticals-a review, Electrophoresis 36 (2015) 831–858.
[79] S. Fekete, A.L. Gassner, S. Rudaz, J. Schappler, D. Guillarme, Analytical strategies for the characterization of therapeutic monoclonal antibodies, Trac-Trend Anal Chem 42 (2013) 74–83.
[80] D. Suba, Z. Urbanyi, A. Salgo, Capillary isoelectric focusing method development and validation for investigation of recombinant therapeutic monoclonal antibody, J. Pharm. Biomed. Anal. 114 (2015) 53–61.
[81] Z.G. Wu, P. Zhou, X.X. Li, H. Wang, D.L. Luo, H.Y. Qiao, X. Ke, J. Huang, Structural characterization of a recombinant fusion protein by instrumental analysis and molecular modeling, PLoS One 8 (2013) 1–8.
[82] O. Salas-Solano, K. Babu, S.S. Park, X.F. Zhang, L. Zhang, Z. Sosic, B. Boumajny, M. Zeng, K.C. Cheng, A. Reed-Bogan, S. Cummins-Bitz, D.A. Michels, M. Parker, P. Bonasia, M.F. Hong, S. Cook, M. Ruesch, D. Lamb, D. Bolyan, S. Kiessig, D. Allender, B. Nunnally, Intercompany study to evaluate the robustness of capillary isoelectric focusing technology for the analysis of monoclonal antibodies, Chromatographia 73 (2011) 1137–1144.
[83] M. Kinoshita, Y. Nakatsuji, S. Suzuki, T. Hayakawa, K. Kakehi, Quality assurance of monoclonal antibody pharmaceuticals based on their charge variants using microchip isoelectric focusing method, J. Chromatogr. A 1309 (2013) 76–83.
[84] B. Vincents, U. von Pawel-Rammingen, L. Bjorck, M. Abrahamson, Enzymatic characterization of the streptococcal endopeptidase, IdeS, reveals that it is a cysteine protease with strict specificity for IgG cleavage due to exosite binding, Biochemistry 43 (2004) 15540–15549.
[85] M. Han, B.M. Rock, J.T. Pearson, D.A. Rock, Intact mass analysis of monoclonal antibodies by capillary electrophoresis-mass spectrometry, J. Chromatogr. B 1011 (2016) 24–32.
[86] M. Biacchi, R. Gahoual, N. Said, A. Beck, E. Leize-Wagner, Y.N. Francois, Glycoform separation and characterization of cetuximab variants by middle-up off-line capillary zone electrophoresis-UV/electrospray ionization-MS, Anal. Chem. 87 (2015) 6240–6250.
[87] D. Suba, Z. Urbanyi, A. Salgo, Method development and qualification of capillary zone electrophoresis for investigation of therapeutic monoclonal antibody quality, J. Chromatogr. B 1032 (2016) 224–229.
[88] D.R. Bush, L. Zang, A.M. Belov, A.R. Ivanov, B.L. Kargert, High resolution CZE-MS quantitative characterization of intact biopharmaceutical proteins: proteoforms of interferon-beta 1, Anal. Chem. 88 (2016) 1138–1146.
[89] M. Biacchi, R. Bhajun, N. Said, A. Beck, Y.N. Francois, E. Leize-Wagner, Analysis of monoclonal antibody by a novel CE-UV/MALDI-MS interface, Electrophoresis 35 (2014) 2986–2995.
[90] A. Taichrib, M. Pelzing, C. Pellegrino, M. Rossi, C. Neususs, High resolution TOF MS coupled to CE for the analysis of isotopically resolved intact proteins, J. Proteome 74 (2011) 958–966.
[91] R. Haselberg, V. Brinks, A. Hawe, G.J. de Jong, G.W. Somsen, Capillary electrophoresis-mass spectrometry using noncovalently coated capillaries for the analysis of biopharmaceuticals, Anal. Bioanal. Chem. 400 (2011) 295–303.
[92] N. Said, R. Gahoual, L. Kuhn, A. Beck, Y.N. Francois, E. Leize-Wagner, Structural characterization of antibody drug conjugate by a combination of intact, middle-up and bottom-up techniques using sheathless capillary electrophoresis - tandem mass spectrometry as nanoESI infusion platform and separation method, Anal. Chim. Acta 918 (2016) 50–59.
[93] A. Varki, N. Sharon, Historical background and overview, in: A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler (Eds.), Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY), 2009, pp. 1–22.
[94] L. Veillon, Y.F. Huang, W.J. Peng, X. Dong, B.G. Cho, Y. Mechref, Characterization of isomeric glycan structures by LC-MS/MS, Electrophoresis 38 (2017) 2100–2114.
[95] S. Gaunitz, G. Nagy, N.L. Pohl, M.V. Novotny, Recent advances in the analysis of complex glycoproteins, Anal. Chem. 89 (2017) 389–413.
[96] N.G. Karlsson, C. Jin, M.A. Rojas-Macias, B. Adamczyk, Next generation O-linked glycomics, Trends Glycosci. Glycotechnol. 29 (2017) E35–E46.
[97] M. Fukuda, T. Kondo, T. Osawa, Studies on hydrazinolysis of glycoproteins - cor structures of oligosaccharides obtained from porcine thyroglobulin and pineapple stem bromelain, J. Biochem. 80 (1976) 1223–1232.
[98] J.P. Liu, O. Shirota, D. Wiesler, M. Novotny, Ultrasensitive fluorometric detection of carbohydrates as derivatives in mixtures separated by capillary electrophoresis, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 2302–2306.
[99] K.R. Anumula, Quantitative-determination of monosaccharides in glycoproteins by high-performance liquid-chromatography with highly sensitive fluorescence detection, Anal. Biochem. 220 (1994) 275–283.
[100] P. Jackson, The use of polyacrylamide-gel electrophoresis for the high-resolution separation of reducing saccharides labeled with the fluotophore 8-amino-naphthalene-1,3,6-trisulfonic acid - detection of picomolar quantities by an imaging-system based on a cooled charge-coupled device, Biochem. J. 270 (1990) 705–713.
[101] R.A. Evangelista, A. Guttman, F.T.A. Chen, Acid-catalyzed reductive amination of aldoses with 8-aminopyrene-1,3,6-trisulfonate, Electrophoresis 17 (1996) 347–351.
[102] R.A. Evangelista, M.S. Liu, F.T.A. Chen, Characterization of 9-aminopyrene-1,4,6-trisulfonate-derivatized sugars by capillary electrophoresis with laser-induced fluorescence detection, Anal. Chem. 67 (1995) 2239–2245.
[103] S. Khan, J. Liu, Z. Szabo, B. Kunnummal, X. Han, Y. Ouyang, R.J. Linhardt, Q. Xia, On-line capillary electrophoresis/laser-induced fluorescence/mass spectrometry analysis of glycans labeled with Teal fluorescent dye using an electrokinetic sheath liquid pump-based nanospray ion source, Rapid Commun. Mass Spectrom. 32 (2018) 882–888.
[104] Y.W. Zhang, M.Z. Zhao, J.X. Liu, Y.L. Zhou, X.X. Zhang, Double-layer poly(vinyl alcohol)-coated capillary for highly sensitive and stable capillary electrophoresis and capillary electrophoresis with mass spectrometry glycan analysis, J. Sep. Sci. 38 (2015) 475–482.
[105] M.C. Hennion, Porous graphitic carbon: liquid chromatography, in: I.D. Wilson (Ed.), Encyclopedia of Separation Science, Academic Press, Oxford, 2000, pp. 3937–3946.
[106] M.H. Selman, M. Hemayatkar, A.M. Deelder, M. Wuhrer, Cotton HILIC SPE microtips for microscale purification and enrichment of glycans and glycopeptides, Anal. Chem. 83 (2011) 2492–2499.
[107] W. Laroy, R. Contreras, N. Callewaert, Glycome mapping on DNA sequencing equipment, Nat. Protoc. 1 (2006) 397–405.
[108] B.C. Durney, C.L. Crihfield, L.A. Holland, Capillary electrophoresis applied to DNA: determining and harnessing sequence and structure to advance bioanalyses (2009-2014), Anal. Bioanal. Chem. 407 (2015) 6923–6938.
[109] M. Beneito-Cambra, P. Anres, J. Vial, P. Gareil, N. Delaunay, Stability and effectiveness of linear polyacrylamide capillary coating to suppress EOF in acidic media in the presence of surfactants, ionic liquids and organic modifiers, Talanta 150 (2016) 546–552.
[110] D. Belder, A. Deege, H. Husmann, F. Kohler, M. Ludwig, Cross-linked poly(vinyl alcohol) as permanent hydrophilic column coating for capillary electrophoresis, Electrophoresis 22 (2001) 3813–3818.
[111] J.P. Liu, O. Shirota, M.V. Novotny, Sensitive, laser-assisted determination of complex oligosaccharide mixtures separated by capillary gel electrophoresis at high resolution, Anal. Chem. 64 (1992) 973–975.
[112] A. Klockow, H.M. Widmer, R. Amado, A. Paulus, Capillary electrophoresis of ANTS labeled oligosaccharide ladders and complex carbohydrates, Fresenius J. Anal. Chem. 350 (1994) 415–425.
[113] M. Kerekgyarto, A. Guttman, Toward the generation of an aminonaphthalene trisulfonate labeled N-glycan database for capillary gel electrophoresis analysis of carbohydrates, Electrophoresis 35 (2014) 2222–2228.
[114] N. Callewaert, S. Geysens, P. Molemans, R. Contreras, Ultrasensitive profiling and sequencing of N-linked oligosaccharides using standard DNA-sequencing equipment, Glycobiology 11 (2001) 275–281.
[115] M. Fang, Y.P. Zhao, F.G. Zhou, L.G. Lu, P. Qi, H. Wang, K. Zhou, S.H. Sun, C.Y. Chen, C.F. Gao, N-glycan based models improve diagnostic efficacies in hepatitis B virus-related hepatocellular carcinoma, Int. J. Cancer 127 (2010) 148–159.
[116] Y.P. Zhao, X.Y. Xu, M. Fang, H. Wang, Q. You, C.H. Yi, J. Ji, X. Gu, P.T. Zhou, C. Cheng, C.F. Gao, Decreased core-fucosylation contributes to malignancy in gastric cancer, PLoS One 9 (2014) e94536.
[117] Y.P. Zhao, C.P. Ruan, H. Wang, Z.Q. Hu, M. Fang, X. Gu, J. Ji, J.Y. Zhao, C.F. Gao, Identification and assessment of new biomarkers for colorectal cancer with serum N-glycan profiling, Cancer 118 (2012) 639–650.
[118] U. Moginger, S. Grunewald, R. Hennig, C.W. Kuo, F. Schirmeister, H. Voth, E. Rapp, K.H. Khoo, P.H. Seeberger, J.C. Simon, D. Kolarich, Alterations of the human skin N- and O-glycome in basal cell carcinoma and squamous cell carcinoma, Front. Oncol. 8 (2018) 70.
[119] I. Mitra, C.M. Snyder, X. Zhou, M.I. Campos, W.R. Alley Jr., M.V. Novotny, S.C. Jacobson, Structural characterization of serum N-glycans by methylamidation, fluorescent labeling, and analysis by microchip electrophoresis, Anal. Chem. 88 (2016) 8965–8971.
[120] C.M. Snyder, W.R. Alley Jr., M.I. Campos, M. Svoboda, J.A. Goetz, J.A. Vasseur, S.C. Jacobson, M.V. Novotny, Complementary glycomic analyses of sera derived from colorectal cancer patients by MALDI-TOF-MS and microchip electrophoresis, Anal. Chem. 88 (2016) 9597–9605.
[121] A. Guttman, Capillary electrophoresis in the N-glycosylation analysis of

biopharmaceuticals, TrAC Trends Anal. Chem. 48 (2013) 132–143.
[122] Z. Szabo, A. Guttman, J. Bones, B.L. Karger, Rapid high-resolution characterization of functionally important monoclonal antibody N-glycans by capillary electrophoresis, Anal. Chem. 83 (2011) 5329–5336.
[123] J. Schwarzer, E. Rapp, U. Reichl, N-glycan analysis by CGE-LIF: profiling influenza A virus hemagglutinin N-glycosylation during vaccine production, Electrophoresis 29 (2008) 4203–4214.
[124] C.M. Snyder, X. Zhou, J.A. Karty, B.R. Fonslow, M.V. Novotny, S.C. Jacobson, Capillary electrophoresis-mass spectrometry for direct structural identification of serum N-glycans, J. Chromatogr. A 1523 (2017) 127–139.
[125] S.C. Bunz, E. Rapp, C. Neususs, Capillary electrophoresis/mass spectrometry of APTS-labeled glycans for the identification of unknown glycan species in capillary electrophoresis/laser-induced fluorescence systems, Anal. Chem. 85 (2013) 10218–10224.
[126] R.G. Jayo, J. Li, D.D. Chen, Capillary electrophoresis mass spectrometry for the characterization of O-acetylated N-glycans from fish serum, Anal. Chem. 84 (2012) 8756–8762.
[127] R.G. Jayo, M. Thaysen-Andersen, P.W. Lindenburg, R. Haselberg, T. Hankemeier, R. Ramautar, D.D. Chen, Simple capillary electrophoresis-mass spectrometry method for complex glycan analysis using a flow-through microvial interface, Anal. Chem. 86 (2014) 6479–6486.
[128] T.J. Langan, L.A. Holland, Capillary electrophoresis coupled to electrospray mass spectrometry through a coaxial sheath flow interface and semi-permanent phospholipid coating for the determination of oligosaccharides labeled with 1-aminopyrene-3,6,8-trisulfonic acid, Electrophoresis 33 (2012) 607–613.
[129] K. Jooss, J. Sommer, S.C. Bunz, C. Neususs, In-line SPE-CE using a fritless bead string design—application for the analysis of organic sulfonates including inline SPE-CE-MS for APTS-labeled glycans, Electrophoresis 35 (2014) 1236–1243.
[130] J. Yuan, N. Hashii, N. Kawasaki, S. Itoh, T. Kawanishi, T. Hayakawa, Isotope tag method for quantitative analysis of carbohydrates by liquid chromatography–mass spectrometry, J. Chromatogr. A 1067 (2005) 145–152.
[131] C. Varadi, S. Mittermayr, S. Millan-Martin, J. Bones, Quantitative twoplex glycan analysis using (12)C6 and (13)C6 stable isotope 2-aminobenzoic acid labelling and capillary electrophoresis mass spectrometry, Anal. Bioanal. Chem. 408 (2016) 8691–8700.
[132] H. Hahne, P. Neubert, K. Kuhn, C. Etienne, R. Bomgarden, J.C. Rogers, B. Kuster, Carbonyl-reactive tandem mass tags for the proteome-wide quantification of N-linked glycans, Anal. Chem. 84 (2012) 3716–3724.
[133] X. Zhong, Z. Chen, S. Snovida, Y. Liu, J.C. Rogers, L. Li, Capillary electrophoresis-electrospray ionization-mass spectrometry for quantitative analysis of glycans labeled with multiplex carbonyl-reactive tandem mass tags, Anal. Chem. 87 (2015) 6527–6534.
[134] J. Gerritsma, D. Sinnige, C. Drieze, B. Sittrop, P. Houtsma, N. Hulshorst-Jansen, W. Huisman, Quantitative and qualitative analysis of haemoglobin variants using capillary zone electrophoresis, Ann. Clin. Biochem. 37 (2000) 380–389 Pt 3.
[135] A. Woodworth, N. Korpi-Steiner, J.J. Miller, L.V. Rao, J. Yundt-Pacheco, L. Kuchipudi, C.A. Parvin, J.M. Rhea, R. Molinaro, Utilization of assay performance characteristics to estimate hemoglobin A(1c) result reliability, Clin. Chem. 60 (2014) 1073–1079.
[136] O. Heylen, S. Van Neyghem, S. Exterbille, C. Wehlou, F. Gorus, I. Weets, Evaluation of the Sebia CAPILLARYS 2 Flex Piercing for the measurement of HbA1c on venous and capillary blood samples, Am. J. Clin. Pathol. 141 (2014) 867–877.
[137] H. Khashoggi, S. Pignalosa, C. Russo, M. Pieri, S. Bernardini, New HPLC instrument performance evaluation in HbA1c determination and comparison with capillary electrophoresis, Scand. J. Clin. Lab. Invest., DOI https://doi.org/10.1080/00365513.2018.1487072(2018).
[138] J. Bienvenu, M.S. Graziani, F. Arpin, H. Bernon, C. Blessum, C. Marchetti, G. Righetti, M. Somenzini, G. Verga, F. Aguzzi, Multicenter evaluation of the Paragon CZE 2000 capillary zone electrophoresis system for serum protein electrophoresis and monoclonal component typing, Clin. Chem. 44 (1998) 599–605.
[139] C. Gay-Bellile, D. Bengoufa, P. Houze, D. Le Carrer, M. Benlakehal, B. Bousquet, B. Gourmel, T. Le Bricon, Automated multicapillary electrophoresis for analysis of human serum proteins, Clin. Chem. 49 (2003) 1909–1915.
[140] M. Pejchinovski, D. Hrnjez, A. Ramirez-Torres, V. Bitsika, G. Mermelekas, A. Vlahou, P. Zurbig, H. Mischak, J. Metzger, T. Koeck, Capillary zone electrophoresis on-line coupled to mass spectrometry: a perspective application for clinical proteomics, Proteomics Clin. Appl. 9 (2015) 453–468.
[141] N. Drouin, J. Pezzatti, Y. Gagnebin, V. González-Ruiz, J. Schappler, S. Rudaz, Effective mobility as a robust criterion for compound annotation and identification in metabolomics: toward a mobility-based library, Anal. Chim. Acta, DOI https://doi.org/10.1016/j.aca.2018.05.063(2018).
[142] D.M. Good, P. Zurbig, A. Argiles, H.W. Bauer, G. Behrens, J.J. Coon, M. Dakna, S. Decramer, C. Delles, A.F. Dominiczak, J.H. Ehrich, F. Eitner, D. Fliser, M. Frommberger, A. Ganser, M.A. Girolami, I. Golovko, W. Gwinner, M. Haubitz, S. Herget-Rosenthal, J. Jankowski, H. Jahn, G. Jerums, B.A. Julian, M. Kellmann, V. Kliem, W. Kolch, A.S. Krolewski, M. Luppi, Z. Massy, M. Melter, C. Neususs, J. Novak, K. Peter, K. Rossing, H. Rupprecht, J.P. Schanstra, E. Schiffer, J.U. Stolzenburg, L. Tarnow, D. Theodorescu, V. Thongboonkerd, R. Vanholder, E.M. Weissinger, H. Mischak, P. Schmitt-Kopplin, Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease, Molecular & cellular proteomics: MCP 9 (2010) 2424–2437.
[143] R. Ramautar, CHAPTER 1 Capillary Electrophoresis–Mass Spectrometry for Metabolomics – From Metabolite Analysis to Metabolic Profiling, Capillary Electrophoresis–Mass Spectrometry for Metabolomics, The Royal Society of Chemistry, 2018, pp. 1–20.
[144] J. Godzien, A. Garcia, A. López-Gonzalvez, C. Barbas, Chapter 8 CE-MS for Metabolomics: A Comparison With Other Techniques, Capillary Electrophoresis–Mass Spectrometry for Metabolomics, The Royal Society of Chemistry, 2018, pp. 161–183.
[145] M.C. Breadmore, A. Wuethrich, F. Li, S.C. Phung, U. Kalsoom, J.M. Cabot, M. Tehranirokh, A.I. Shallan, A.S. Abdul Keyon, H.H. See, M. Dawod, J.P. Quirino, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2014–2016), Electrophoresis 38 (2017) 33–59.
[146] J.P. Quirino, On-line Electrophoretic, Electrochromatographic, and Chromatographic Sample Concentration in CE-MS, Wiley-VCH, Weinheim, 2016.
[147] E.E. Baidoo, P.I. Benke, C. Neususs, M. Pelzing, G. Kruppa, J.A. Leary, J.D. Keasling, Capillary electrophoresis-fourier transform ion cyclotron resonance mass spectrometry for the identification of cationic metabolites via a pH-mediated stacking-transient isotachophoretic method, Anal. Chem. 80 (2008) 3112–3122.
[148] L. Pont, F. Benavente, J. Jaumot, R. Tauler, J. Alberch, S. Gines, J. Barbosa, V. Sanz-Nebot, Metabolic profiling for the identification of Huntington biomarkers by on-line solid-phase extraction capillary electrophoresis mass spectrometry combined with advanced data analysis tools, Electrophoresis 37 (2016) 795–808.
[149] A. Hirayama, M. Wakayama, T. Soga, Metabolome analysis based on capillary electrophoresis-mass spectrometry, TrAC Trends Anal. Chem. 61 (2014) 215–222.
[150] R. Ramautar, Capillary electrophoresis-mass spectrometry for clinical metabolomics, Adv. Clin. Chem. 74 (2016) 1–34.
[151] E. Jellum, A.K. Thorsrud, E. Time, Capillary electrophoresis for diagnosis and studies of human disease, particularly metabolic disorders, J. Chromatogr. 559 (1991) 455–465.
[152] E. Jellum, H. Dollekamp, C. Blessum, Capillary electrophoresis for clinical problem solving: analysis of urinary diagnostic metabolites and serum proteins, J. Chromatogr. B Biomed. Appl. 683 (1996) 55–65.
[153] E. Jellum, H. Dollekamp, A. Brunsvig, R. Gislefoss, Diagnostic applications of chromatography and capillary electrophoresis, J Chromatogr B Biomed Sci Appl 689 (1997) 155–164.
[154] C. Barbas, N. Adeva, R. Aguilar, M. Rosillo, T. Rubio, M. Castro, Quantitative determination of short-chain organic acids in urine by capillary electrophoresis, Clin. Chem. 44 (1998) 1340–1342.
[155] A. Garcia, C. Barbas, Capillary electrophoresis for the determination of organic acidurias in body fluids: a review, Clin. Chem. Lab. Med. 41 (2003) 755–761.
[156] V. Dolnik, J. Dolnikova, Capillary zone electrophoresis of organic acids in serum of critically ill children, J. Chromatogr. A 716 (1995) 269–277.
[157] M. Shirao, R. Furuta, S. Suzuki, H. Nakazawa, S. Fujita, T. Maruyama, Determination of organic acids in urine by capillary zone electrophoresis, J. Chromatogr. A 680 (1994) 247–251.
[158] M. Vallejo, S. Angulo, D. Garcia-Martinez, A. Garcia, C. Barbas, New perspective of diabetes response to an antioxidant treatment through metabolic fingerprinting of urine by capillary electrophoresis, J. Chromatogr. A 1187 (2008) 267–274.
[159] C. Guillo, D. Barlow, D. Perrett, M. Hanna-Brown, Micellar electrokinetic capillary chromatography and data alignment analysis: a new tool in urine profiling, J. Chromatogr. A 1027 (2004) 203–212.
[160] Z. Juvancz, R.B. Kendrovics, R. Ivanyi, L. Szente, The role of cyclodextrins in chiral capillary electrophoresis, Electrophoresis 29 (2008) 1701–1712.
[161] G. Xie, M. Su, P. Li, X. Gu, C. Yan, Y. Qiu, H. Li, W. Jia, Analysis of urinary metabolites for metabolomic study by pressurized CEC, Electrophoresis 28 (2007) 4459–4468.
[162] J. Hong, R.P. Baldwin, Profiling clinically important metabolites in human urine by capillary electrophoresis and electrochemical detection, J Capillary Electrophor 4 (1997) 65–71.
[163] J. Schneede, P.M. Ueland, Application of capillary electrophoresis with laser-induced fluorescence detection for routine determination of methylmalonic acid in human serum, Anal. Chem. 67 (1995) 812–819.
[164] M.P. Lorenzo, A. Villasenor, A. Ramamoorthy, A. Garcia, Optimization and validation of a capillary electrophoresis laser-induced fluorescence method for amino acids determination in human plasma: application to bipolar disorder study, Electrophoresis 34 (2013) 1701–1709.
[165] M.P. Lorenzo, A. Navarrete, C. Balderas, A. Garcia, Optimization and validation of a CE-LIF method for amino acid determination in biological samples, J. Pharm. Biomed. Anal. 73 (2013) 116–124.
[166] T. Lapainis, C. Scanlan, S.S. Rubakhin, J.V. Sweedler, A multichannel native fluorescence detection system for capillary electrophoretic analysis of neurotransmitters in single neurons, Anal. Bioanal. Chem. 387 (2007) 97–105.
[167] R.K. Harstad, M.T. Bowser, High-speed microdialysis-capillary electrophoresis assays for measuring branched chain amino acid uptake in 3T3-L1 cells, Anal. Chem. 88 (2016) 8115–8122.
[168] T. Jako, E. Szabo, T. Tabi, G. Zachar, A. Csillag, E. Szoko, Chiral analysis of amino acid neurotransmitters and neuromodulators in mouse brain by CE-LIF, Electrophoresis 35 (2014) 2870–2876.
[169] K.B. Elgstoen, J.Y. Zhao, J.F. Anacleto, E. Jellum, Potential of capillary electrophoresis, tandem mass spectrometry and coupled capillary electrophoresis-tandem mass spectrometry as diagnostic tools, J. Chromatogr. A 914 (2001) 265–275.
[170] T. Soga, D.N. Heiger, Amino acid analysis by capillary electrophoresis electrospray ionization mass spectrometry, Anal. Chem. 72 (2000) 1236–1241.
[171] T. Soga, Y. Ohashi, Y. Ueno, H. Naraoka, M. Tomita, T. Nishioka, Quantitative metabolome analysis using capillary electrophoresis mass spectrometry, J. Proteome Res. 2 (2003) 488–494.
[172] A. Hirayama, T. Soga, CE-MS for anionic and cationic metabolic profiling: system optimization and applications, Capillary Electrophoresis–Mass Spectrometry for Metabolomics, The Royal Society of Chemistry, 2018, pp. 134–160.

[173] S. Harada, A. Hirayama, Q. Chan, A. Kurihara, K. Fukai, M. Iida, S. Kato, D. Sugiyama, K. Kuwabara, A. Takeuchi, M. Akiyama, T. Okamura, T.M.D. Ebbels, P. Elliott, M. Tomita, A. Sato, C. Suzuki, M. Sugimoto, T. Soga, T. Takebayashi, Reliability of plasma polar metabolite concentrations in a large-scale cohort study using capillary electrophoresis-mass spectrometry, PLoS One 13 (2018) e0191230.
[174] A. Hirayama, M. Tomita, T. Soga, Sheathless capillary electrophoresis-mass spectrometry with a high-sensitivity porous sprayer for cationic metabolome analysis, Analyst 137 (2012) 5026–5033.
[175] M.C. Gulersonmez, S. Lock, T. Hankemeier, R. Ramautar, Sheathless capillary electrophoresis-mass spectrometry for anionic metabolic profiling, Electrophoresis 37 (2016) 1007–1014.
[176] N.L. Kuehnbaum, A. Kormendi, P. Britz-McKibbin, Multisegment injection-capillary electrophoresis-mass spectrometry: a high-throughput platform for metabolomics with high data fidelity, Anal. Chem. 85 (2013) 10664–10669.
[177] R.M. Onjiko, S.E. Morris, S.A. Moody, P. Nemes, Single-cell mass spectrometry with multi-solvent extraction identifies metabolic differences between left and right blastomeres in the 8-cell frog (Xenopus) embryo, Analyst 141 (2016) 3648–3656.
[178] R.M. Onjiko, D.O. Plotnick, S.A. Moody, P. Nemes, Metabolic comparison of dorsal versus ventral cells directly in the live 8-cell frog embryo by microprobe single-cell CE-ESI-MS, Analytical Methods: Advancing Methods and Applications, vol. 9, 2017, pp. 4964–4970.
[179] N. Drouin, J. Pezzatti, Y. Gagnebin, V. Gonzalez-Ruiz, J. Schappler, S. Rudaz, Effective mobility as a robust criterion for compound annotation and identification in metabolomics: toward a mobility-based library, Anal. Chim. Acta 1032 (2018) 178–187.