Received: 8 July 2023 | Revised: 22 August 2023 | Accepted: 22 August 2023

DOI: 10.1002/elps.202300152

ELECTROPHORESIS

REVIEW

Recent developments in capillary and microchip electroseparations of peptides (2021–mid-2023)

Václav Kašička

Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czechia

Correspondence
Václav Kašička, Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Flemingovo n. 542/2 2, 160 00 Prague 6, Czechia.
Email: kasicka@uochb.cas.cz

Color online: See the article online to view Figures 1–4 and 7–9 in color.

Funding information
Czech Science Foundation, Grant/Award Number: 20-03899S; Czech Academy of Sciences, Grant/Award Number: RVO 61388963

Abstract

This review article brings a comprehensive survey of developments and applications of high-performance capillary and microchip electromigration methods (zone electrophoresis in a free solution or in sieving media, isotachophoresis, isoelectric focusing, affinity electrophoresis, electrokinetic chromatography, and electrochromatography) for analysis, micropreparation, and physicochemical characterization of peptides in the period from 2021 up to ca. the middle of 2023. Progress in the study of electromigration properties of peptides and various aspects of their analysis, such as sample preparation, adsorption suppression, electroosmotic flow regulation, and detection, are presented. New developments in the particular capillary electromigration methods are demonstrated, and several types of their applications are reported. They cover qualitative and quantitative analysis of synthetic or isolated peptides and determination of peptides in complex biomatrices, peptide profiling of biofluids and tissues, and monitoring of chemical and enzymatic reactions and physicochemical changes of peptides. They include also amino acid and sequence analysis of peptides, peptide mapping of proteins, separation of stereoisomers of peptides, and their chiral analyses. In addition, micropreparative separations and physicochemical characterization of peptides and their interactions with other (bio)molecules by the above CE methods are described.

KEYWORDS
capillary electrochromatography, capillary electrophoresis, peptides, proteins, review

1 | INTRODUCTION

Peptides are extremely numerous and extremely important class of biologically active compounds. They function as hormones, neurotransmitters, enzyme substrates and inhibitors, coenzymes, immunomodulators, ligands, receptors, ionophores, antibiotics, and toxins and play a crucial role in control and regulation of many vital processes in all living organisms. Moreover, many peptides

Abbreviations: 2-HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; AA, amino acid; AcOH, acetic acid; BFS, bare fused silica; CITP, capillary ITP; FA, formic acid; FESI, field-enhanced sample injection; FS, fused silica; GSH, glutathione; HPC, hydroxypropylcellulose; IMS, ion mobility spectrometry; IPA, isopropyl alcohol; LPA, linear polyacrylamide; LVSS, large-volume sample stacking; MCE, microchip CE; MeOH, methanol; MP, mobile phase; OT-CEC, open tubular CEC; PDADMAC, poly(diallyldimethylammonium chloride); PEI, polyethylenimine; SL, sheath liquid; SMIL, successive multiple ionic polymer layer; SP, stationary phase; t-ITP, transient-ITP.

or peptidomimetics are used as pharmaceuticals, or they are being developed as potential new drugs [1, 2] or peptide-based nanostructures for the drug delivery [3]. Antimicrobial peptides are considered potential new antibiotics [2, 4]. Peptides serve as biomarkers [5, 6], and the combinatorial peptide libraries are employed for finding and identification of low-abundant proteins [7]. In the current era of proteomics, peptidomics, and metabolomics, the importance of peptides is even increasing as these methodologies are the major analytical tools for a study of molecular bases of biological processes, as well as for finding new biomarkers and drug targets [8, 9]. These—omics technologies—are complex and interconnected disciplines. Both, structure and function of proteins are often investigated by means of their enzymatically generated peptide fragments in the so called bottom-up or shotgun proteomics [10–13]. These peptide fragments are separated and identified by liquid chromatography (LC), 2-DE, and capillary electrophoresis (CE) methods online coupled with MS detection [14–19]. In addition, for understanding of both normal and pathological physiological processes, a comprehensive investigation of the whole peptide set (peptidome) of a cell, organ, or organism at given time frame is necessary as well. This is the subject of peptidomics—a bridge between proteomics and metabolomics [12, 13, 20–23]. Thus, separation, analysis, isolation, purification, and characterization of peptides by capillary and microchip electromigration methods are one of the most challenging tasks of these high-performance separation techniques.

This review brings a comprehensive overview of the recent advances and developments in CE and microchip CE (MCE) methods for analysis and characterization of peptides in the period 2021–ca. mid-2023. It is an update of the previous reviews on CE and capillary electrochromatography (CEC) of peptides [24–35] covering the years 1997–2020. All modes of capillary and microchip electromigration techniques, both the electrophoretic modes (zone electrophoresis [ZE], isotachophoresis (ITP), isoelectric focusing (IEF), and affinity capillary electrophoresis (ACE)) and the mixed electrokinetic and electrochromatographic modes (EKC and CEC), have been intensively developed also in the last two and half years. Applications of these methods for analysis, micropreparation, and characterization of peptides have been further extended. Nowadays, CE methods are appreciated as recognized counterparts and/or complements of the (U)HPLC (high-performance liquid chromatography) and nano-LC methods [36–38] that are mostly used for both analytical and preparative peptide separations. In addition to the last one of the previous reviews [35], the recent developments of CE and CEC separations of peptides were reported in some other reviews [39–42], books [43, 44], and book chapters [22, 45, 46] dealing with particular aspects of CE and MCE of peptides, proteins, amino acids (AAs), and other biomolecules.

2 | ELECTROMIGRATION PROPERTIES OF PEPTIDES AND SELECTION OF SEPARATION CONDITIONS

In the period covered by this review (2021–mid-2023), the electromigration properties and behavior of polypeptides and proteins were investigated using machine learning models for the prediction of their migration times in CE and retention times in RP-HPLC [47]. For the migration time prediction in the CE–ESI–MS experiments performed in strongly acidic background electrolyte (BGE) (5% (833 mM) acetic acid [AcOH], pH 2.3), the semiempirical model of the structure-electrophoretic mobility relationship of proteoforms presented in the previous study [48] was employed. In this model, the size of the protein or polypeptide is derived from its relative molecular mass, M_r, and the charge, Z, at above pH 2.3 is estimated from the number of basic AA residues (His, Lys, Arg) and N-terminal amino groups in the protein/polypeptide molecule, that is, neglecting the partial dissociation of carboxylic groups of Asp and Glu AA residues. The electrophoretic mobility of a protein/polypeptide, μ, is calculated using the following equation:

$$\mu = a\frac{\ln(1 + 0.35 \times Z)}{M_r^{0.411}} + b \quad (1)$$

where a and b are two parameters related to the capillary zone electrop (CZE) settings [48]. From the calculated mobilities, the predicted migration time, t_m, in the CZE–MS experiment can be estimated by the following equation:

$$t_m = \frac{L^2}{(V_1 - V_2)\mu} \quad (2)$$

where L is the total capillary length, V_1 is the separation voltage, and V_2 is the electrospray voltage.

This model is in a qualitative agreement with the earlier published semiempirical relationships between the electrophoretic mobility, μ, and the charge/mass ratio (Z/M_r^{α}) or $\ln(1 + kZ)/M_r^{\alpha}$ with different exponents α of M_r [49, 50] that are used for estimation effective mobilities of peptides. Comparison of the predicted and experimental migration times has shown that a gated recurrent unit based model and a fully connected neural network model provided a high accuracy with the Pearson correlation coefficient of linearity close to 1 for proteoform migration time prediction, see Figure 1.

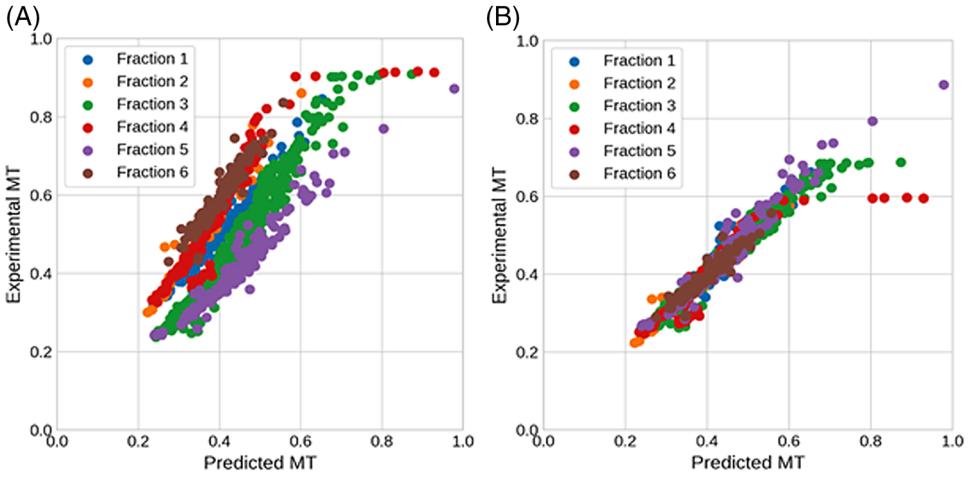

FIGURE 1 Correlation between predicted and experimental migration time (MT) for the capillary zone electrophoresis (CZE)-ONE data set (obtained from CZE/mass spectrometry (MS)/MS data of 1230 proteoforms of 430 proteins) with prefractionation into a training set of 878 proteoforms from 329 proteins and a test set of 352 proteoforms from 141 proteins. (A) MTs predicted by the semiempirical model are plotted against experimental MT in six CZE–MS runs. The Pearson correlation coefficient of linearity between predicted and experimental MTs is 0.956 on average for single runs and 0.792 for the combined data of the six runs. (B) The Pearson correlation coefficient of linearity between predicted and experimental MTs is improved to 0.954 for the combined data after calibration. *Source*: Reprinted with permission from Ref. [47].

The database of free solution effective electrophoretic mobilities of 276 metabolites, including peptides created in the CE–MS using 5% v/v formic acid (FA) as BGE in the mixed hydro-organic solvent (95/5 v/v water/methanol (MeOH) [51], can be advantageously utilized for the identification of peptides and other metabolites in the peptidomic and metabolomic studies [52].

In the selection of experimental conditions for CE and CEC separations of peptides, first, the general rules for BGE selection should be followed [53, 54]. Second, the electromigration and other specific properties of peptides, that is, their experimentally determined or theoretically calculated/estimated effective charge, effective mobility, isoelectric point (p*I*), hydrodynamic radius, relative molecular mass (M_r), steric structure, solubility, amphiphilicity, amphotericity, binding capabilities, chemical and thermal stability, and biological activity, should be taken into account. Various aspects of the BGE selection for CE separations of peptides and proteins, such as type and concentration of the BGE constituents with respect to their buffering capacity, mobility and electric conductivity, pH and ionic strength, additives suppressing sorption of peptides/proteins to the inner surface of the most often used fused silica (FS) capillaries or influencing separation selectivity, electroosmotic flow (EOF) direction and velocity, organic modifiers, temperature, Joule's heating effects, and compatibility with MS detection, were in detail described and discussed in the recent review [55]. Useful information and practical advices for CZE, capillary ITP (CITP), and CIEF analysis of peptides and proteins can be found also in the earlier published but still valid review and tutorial articles [56–59]. Important parameter for the selection of cationic or anionic mode of CZE or CITP separations of peptides is their p *I*, which can be found in some databases [60], or it can be calculated [61] or experimentally determined by IEF in capillary or slab gel format [59, 62] or by CZE at pH below and above p*I* [59, 63, 64].

If the effective mobilities of peptides are known or can be at least approximately estimated, then for optimization of their CZE and ITP separations, the freely available powerful simulation programs, PeakMaster and Simul, can be employed [65]. Another new open-source software for simulation of CITP and other CE separation process of various analytes, including peptides both in a classical plateau mode and in a more sensitive peak mode, was developed by the Santiago group [66]. The basic parameters for the selection of CZE experimental conditions, such as viscosity and electric conductivity of the BGE, injected and total capillary volumes, proportion and amount of injected sample, electric field strength, and others, can be obtained by the CE calculator freely available as an android application [67].

3 | SAMPLE PREPARATION

3.1 | Preconcentration and preseparation

Preconcentration and/or preseparation (sample cleanup) procedures have to be used for peptide analyses by CE

methods if the sensitivity of the detectors used and/or the separation power of these techniques are not sufficient for the direct analysis of peptides present at low concentrations and/or in complex (bio)matrices, such as body fluids, cell lysates, tissue extracts, foods, and feeds.

Sample preparation methods applied for preseparation and preconcentration of (bio)molecules including peptides in complex (bio)matrices before their CE analyses are in detail presented and evaluated in general recent reviews [68–75]. In these reviews, the applications of various procedures for sample cleanup are described, such as (ultra)filtration, solid-phase extraction (SPE), solid-phase microextraction (SPME), liquid–liquid extraction, and several electro-driven techniques. Several papers describe the particular sample cleanup techniques for the analysis of peptides and proteins, such as comparison of sample preparation workflows for proteomics [76], selective offline extraction of low-molecular-mass thiol peptides and AAs in saliva by Tween 20-capped gold nanoparticles [77] or offline selective enrichment of endogenous phosphopeptides with an exclusion of proteins (albumin) before their CE–MS analysis in tryptic digestion of beta-casein using TiO_2-modified fibrous core–shell mesoporous material [78]. SPE based on C-18 microcartridge online coupled with CE–MS and CLC–MS substantially (1000-times) increased the sensitivity of analysis of three opioid peptide biomarkers (dynorphin A, endomorphin 1, and methionine-enkephalin) in human blood plasma samples as compared to direct CE–MS and CLC–MS analysis [79]. The achieved limit of detections (LODs) were in the range 0.1–0.5 ng/mL. Glycopeptide enrichment in the digest of human plasma and brain (glyco)proteins using polyethylenimine (PEI)-benzoboroxole beads was improved 10 times when the amine-free buffers were employed [80].

Online phenylboronic acid-based extraction of glycopeptides from enzymatic digestion of recombinant human erythropoietin increased sensitivity of CE–MS analysis of this protein 500-times as compared to direct CE–MS and 200-times as compared to TiO_2–SPE–CE–MS [81]. A double-frit particle packed FS microcartridge (250/360 μm id/od × 7 mm) filled with phenylboronic acid sorbent was inserted at 75 mm from the injection end of the CE separation capillary.

For a particular peptide or specific peptide groups (e.g., phospho-, glyco-, or lipopeptides) preconcentration, the binding affinities of aptamers, lectins, or molecularly imprinted polymers [82, 83] or immunoaffinity capabilities of polyclonal and monoclonal antibodies [84] are advantageously utilized. Current status of proteolytic peptide capturing in bottom-up protein analysis and in peptide biomarker analysis using antibodies and molecularly imprinted polymers is comprehensively and critically evaluated in a recent review [85]. Online coupling of aptamer affinity SPE and immobilized enzyme microreactor (IMER) (see Figure 2) with CE–MS analysis of generated peptide fragments (shown in Figure 3) enabled highly sensitive targeted bottom-up analysis of α-synuclein—protein biomarker of various neurodegenerative disorders, including Parkinson's disease [86]. The online capturing and preconcentration of α-synuclein, its digestion by trypsin, and CE separation and MS identification of peptides and the whole protein were accomplished within 35 min with an LOD of 20 ng/mL of the digested protein with 66.7% coverage of peptide identification. This LOD was 125 that is 10 times lower than LOD of the IMER-CE–MS and aptamer affinity SPE–CE–MS alone systems, respectively.

In addition to the above chromatographic sorption-based principles, peptide preseparation and preconcentration can be achieved by online electro-driven sample-stacking techniques [87, 88]. They include field amplified sample stacking [89], field-enhanced or field-amplified sample injection (FESI or FASI) [77, 90, 91], large volume sample stacking (LVSS) [92–94], transient-ITP (t-ITP) [95, 96], electrokinetic supercharging [97, 98], and dynamic pH barrage junction and pH-mediated stacking [93, 99].

Online LVSS was used for peptide preconcentration in CE–UV analysis of four growth hormone-releasing hormone analogs (misused by athletes) in urine samples, including two diastereomeric peptides differing by the chirality of only one AA [94]. LVSS with polarity switching with injected sample volume equal to 80% of the total capillary volume was found to be more suitable than the sweeping concentration and allowed the LODs in the range of 75–200 ng/mL. In combination with C-18 SPE, the LOD was decreased 640 times as compared to the direct CZE.

Combination of the selective extraction of glutathione (GSH) and other low-molecular-mass thiols (cysteine, homocysteine, and methionine) by Tween-20-capped gold nanoparticles from saliva and FASI resulted in 1119–2067 values of sample enrichment factor and allowed the detection of unlabeled GSH and thiols with LODs in 0.15–1.5 ng/mL range [77]. The CE analyses were conducted in bare fused silica (BFS) capillary (25/360 μm id/od, 90/85 cm total/effective length), in BGE composed of 4.0 M AcOH, pH 2.08, at 24 kV separation voltage.

Combination of LVSS and dynamic pH junction made possible ultrasensitive CE separation of brain angiotensin peptides and their identification by online coupled nanoflow ESI trapped ion mobility time-of-flight (timsTOF) tandem high-resolution MS detection [93]. The LODs reached sub-picomolar or ca. 30 zmol (ca. 18 000 copies) level.

LVSS with EOF pump was employed for peptide preconcentration in CE and MCE analysis of

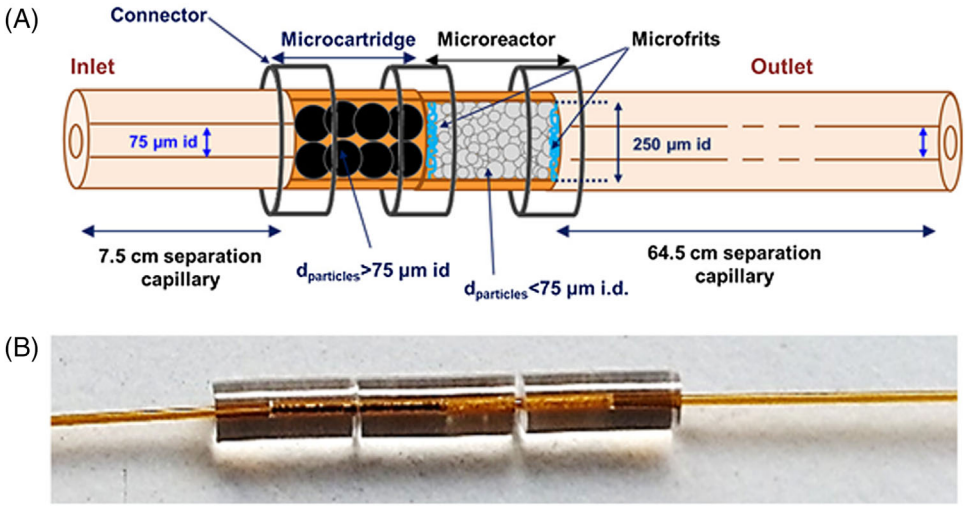

FIGURE 2 (A) Schematic representation and (B) picture under the optical microscope (100× magnified) of a modified capillary with a microcartridge and a microreactor for amino acid (AA)-solid-phase extraction (SPE)–immobilized enzyme microreactor (IMER)–CE–MS. *Source*: Reprinted with permission from Ref. [86].

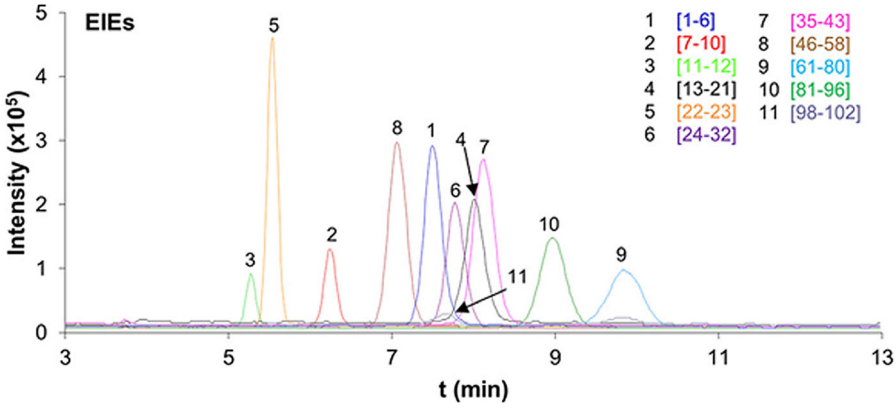

FIGURE 3 Extracted ion electropherograms (EIEs) of the tryptic peptides for the analysis by immobilized enzyme microreactor (IMER)–capillary electrophoresis (CE)–mass spectrometry (MS) of a 10-µg/mL recombinant α-syn standard. Conditions: microreactor (7 mm × 0.250 mm id), separation capillary (720 mm × 0.075 mm id) and BGE (50 mM acetic acid (AcOH), 50 mM formic acid (FA), and pH = 2.3). Sample dissolved in digestion buffer (10 mM NH_4HCO_3, pH 7.9), injected at 50 mbar for 15 s between two plugs of digestion buffer (50 mbar, 8 s), and pushed with BGE at 5 mbar for 600 s. Digestion/separation temperature 37°C and separation voltage +25 kV. Data extraction at the *m*/*z* of the most abundant molecular ions and a window of ±20 ppm. *Source*: Reprinted with permission from Ref. [86].

monophosphopeptide from bovine beta-casein in dynamically poly(vinyl pyrrolidone)-coated FS capillary or PDMS microchannel using 20 mM *N*-(2-hydroxyethyl)piperazine-2′-(2-ethanesulfonic acid) (HEPES) buffer, pH 9.0, as BGE [92]. High-sensitive enrichment factors of 260 and 240 were achieved in CE and MCE, respectively.

In-capillary FESI and multisegment injection were applied for high-sensitivity and high-throughput quantitative CE–MS analysis of therapeutic decapeptide triptorelin in complex pharmaceutical and biological matrices (commercial drug Diphereline and blood plasma, respectively), using 1 M FA, pH 1.88 as BGE. The FESI improved the LOD 50-fold to the 5–25 ng/mL level, and the multiple injection increased the throughput 3-fold.

A special approach, a combination of voltage polarity switching and the transient-ITP, t-ITP, the t-ITP, was applied for the analysis of five synthetic peptides (angiotensins I and II, bradykinin, neurotensin, and kemptide) diluted to 2 µM concentration [95]. Principle of this preconcentration procedure is shown in Figure 4A. Using 25 mM ammonium acetate, pH 4, as leading electrolyte (LE), and 100 mM AcOH as BGE, the method significantly enhanced sample loading capacity, up to 100% of the capillary volume, increased the peak capacity by 48%,

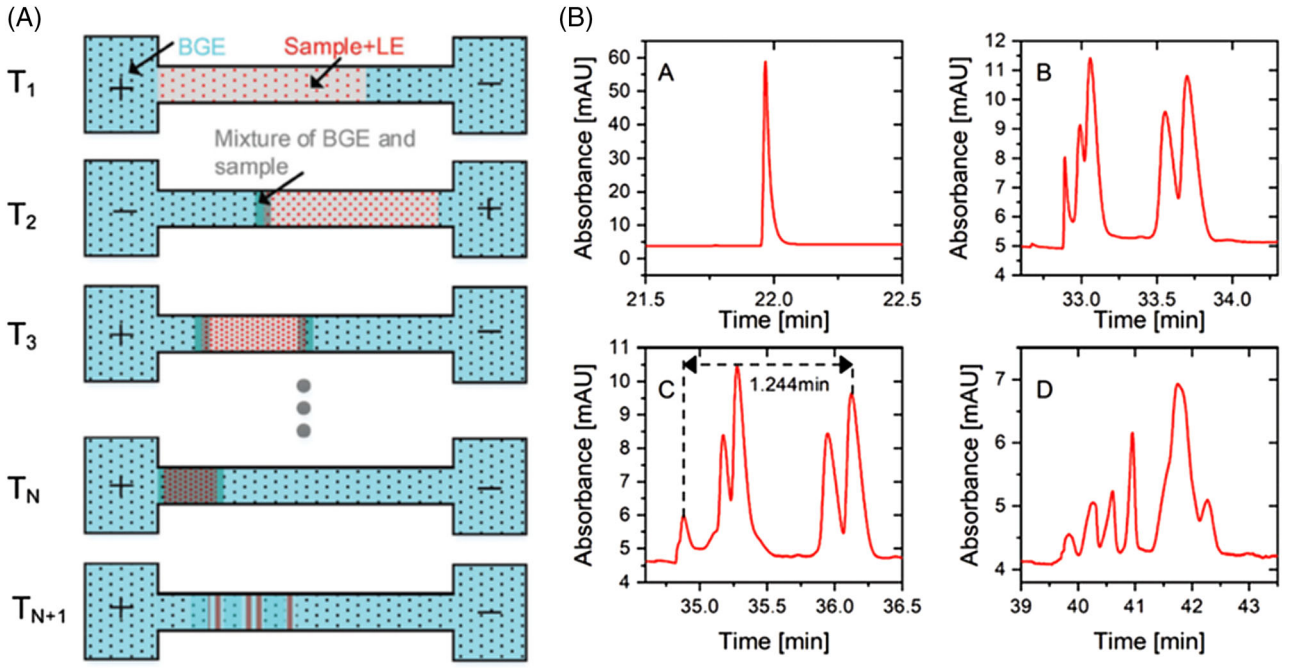

FIGURE 4 (A) Preconcentration procedure of polarity switching t-capillary ITP (CITP). (B) Single polarity switching t-CITP separation of five model peptides (kemptide, bradykinin, angiotensin I, neurotensin, and angiotensin II) at 100% sample loading with different polarity switching times at (A) 0 min, (B) 6 min, (C) 10 min, and (D) 16 min. *Source*: Reprinted with permission from Ref. [95].

and improved the resolution of separated peptides, see Figure 4B.

Combination of liquid–liquid extraction and pH-mediated stacking was employed for CE–UV determination of reduced and oxidized forms of GSH in human blood samples [99]. The LOQ for oxidized GSH enhanced 8-times to the 1.5 μM level. The pH-mediated stacking was implemented by injecting the sample zone between the zones of acidic citric acid and alkaline solution of sodium citrate. The BGE was composed of 75 mM sodium citrate, 200 μM cetyltrimethylammonium bromide (CTAB), 5 μM sodium dodecyl sulfate (SDS), pH 5.8.

Deep eutectic solvents (mixtures of coumarin, camphor, DL-menthol, and thymol) were used as a supported liquid membrane components in electromembrane extraction of 16 model oligopeptides containing 3–13 AA residues from 100 μL of 50 mM phosphate buffer, pH 3.0, into 100 μL of acceptor solution of 50 mM phosphoric acid, pH 1.8 [100].

In SPE and SPME, the new advanced nanoporous materials [101], metal-organic frameworks (MOFs) [102, 103], and metal nanoparticles [77, 104] are becoming useful sorbents for capture of endogenous peptides, especially glycopeptides and phosphopeptides, from complex biological samples. The classical offline formats of SPE are nowadays being substituted by their online modes. Both preconcentration and preseparation are achieved by a solid-phase packed or monolithic sorbents at the inlet capillary end.

3.2 | Derivatization

Sample preparation procedures include also the derivatization of analytes of interest, in this context, peptides. Derivatization is carried out to increase the sensitivity of their detection and/or selectivity of their separation. Peptides are mostly derivatized with fluorescent labels, such as FP-488-maleinimide, Alexa-488-maleinimide, CF-488-maleinimide, or Fluorescein-X5-maleinimide, to become detectable with (laser induced) fluorescence (LIF) detectors. The sensitivity of these detectors is about two to three orders higher than that of the most frequently used UV–vis spectrophotometric absorption detectors that do not need any derivatization for peptides.

Derivatization can be carried out both off- and online. In the latter mode, the derivatizing reagents can be components of the BGE or the sample solution, or they can be introduced into the capillary in their independent zone in front of or behind the sample zone [105]. The in-capillary simultaneous fluorescent and chiral derivatization of AAS by ortho-phthalaldehyde (fluorescent label) and *N*-acetyl-L-cysteine (chiral agent) [106] can be applied also for peptides. For other examples of precapillary, in-line, in-capillary, and postcapillary derivatization for the separation and detection of variable compounds, including peptides in CE with UV, LIF, and MS detection, see the recent review [107].

4 | SUPPRESSION OF PEPTIDE ADSORPTION AND REGULATION OF EOF

Adsorption of peptides, especially the hydrophobic and basic polypeptides to the inner surface of the FS capillaries and glass, quartz, or plastic chips, is one of the most serious issues in their analyses by CE methods. It significantly decreases separation efficiency and may cause partial or even complete loss of analyzed peptides. Thus, the suppression of adsorption is still a challenge in CE of peptides, proteins, and other analytes. In addition to the separations in strongly acidic or alkaline BGEs or in BGEs with high ionic strength, various types of capillary and microchip coatings are used. Dynamic coatings are based on reversible (dynamic) adsorption of small ions, for example, oligoamines and detergents (SDS, CTAB), or hydrophilic polymers and cellulose derivatives, for example, methylcellulose [108] or hydroxypropylcellulose (HPC) [109] added to the BGE to the capillary or microchannel wall. Static (permanent) modifications of the inner capillary or microchannel wall include covalent bonding or strong physical adsorption of the synthetic and natural polymers, for example, neutral linear polyacrylamide (LPA) [110], PVA [111], poly(vinyl pyrrolidone) [92], poly(ethylene oxide) (PEO) [112], PEG [113], fluorocarbon [114], and linear and grafted cellulose derivatives [108, 114], or charged polymers, such as cationic polybrene (PB) (hexadimethrine bromide) [94], poly(diallyldimethylammonium chloride) (PDADMAC) [113], and PEI [115], and anionic poly(sodium styrene sulfonate) (PSS) [113], and dextran sulfate [110].

Latest developments in column coatings for CE of proteins applicable also for peptides are presented in the recent reviews on CE analysis of proteins [41, 116]. Among them, the electrostatically bound bilayer or multilayered coatings, the so-called successive multiple ionic polymer layer (SMIL), relatively efficiently suppress peptide and protein sorption to capillary wall. However, their performance is strongly dependent on the character of the polyelectrolyte partners building the SMIL coating. Various aspects of their application, such as polyelectrolyte nature, molar mass and concentration, effect of the construction buffer pH and ionic strength, and the influence of the BGE pH and composition on the coating performance are in detail discussed in the recent review [117]. The effect of these parameters on the separation efficiency, EOF mobility, and coating stability and a survey of their recent applications are presented there. The influence of the chemical nature and the PEGylation of the last polycationic layer in the SMIL coatings on the previously adsorbed four layers PDADMAC/PSS_2 on the separation performance of a model mixture of four proteins (myoglobin, trypsin inhibitor, ribonuclease A, and lysozyme) was studied by the Cottet group [113]. They found out that from the five polycationic electrolytes tested, PDADMAC, PEI, ε-poly(L-lysine), α-poly(L-lysine), and poly(allylamine hydrochloride) (PAH), the last one was the best candidate to test the impact of PEGylation in this fifth layer. The SMIL coating with PEGylated last PAH layer generated the resulting electroneutral layer and allowed high-efficient separation of the above proteins. These findings can be applied also for CE of peptides.

In addition to the suppression of peptide sorption to the inner capillary wall, dynamic and/or permanent capillary coatings are utilized also for regulation of the EOF velocity/mobility. It is also a relevant parameter in CE separations because the EOF velocity/mobility also influences the separation efficiency, resolution, and speed of CE analyses of peptides and other analytes. New types of covalent cationic [118] and anionic [119] coatings of FS capillary are based on various ratio of charged and noncharged monomers covalently attached to silanized inner wall of the FS capillary. They allow us to tune EOF velocity/mobility for the optimization of CE separations of biomolecules including peptides.

For measurement of the EOF velocity and mobility, the selection of a suitable electroneutral marker is important. Martinkova et al. [120] have shown that some of the commonly used EOF markers may interact with the BGE constituents and may move with nonzero electrophoretic mobility. This may result in the biased EOF mobility of the markers and effective mobility of the analytes.

Peptide and protein sorption to the inner surface of the separation compartment and EOF regulation represent serious problems also in their CE analyses in glass/quartz or plastic microchips. Dynamic coating of cyclic olefin copolymer microchannels with SDS and static coating of these microchannels with BSA suppressed the sorption of dipeptide carnosine (β-Ala-L-His) and enabled the determination of this peptide and niacinamide in cosmetic preparations [121].

5 | SEPARATIONS BY THE PARTICULAR CE METHODS

5.1 | Zone electrophoresis

CZE is the simplest, universal, and major method in the family of CE techniques. It is used for the separation and analysis of majority of analytes, including peptides. ZE mode is usually considered when CE is presented without any further specification of the separation mode

[122]. In this section, only a few special topics of peptide CZE separations are presented; the majority of the CZE analyses are described in Section 7.

CZE is mostly performed in a free solution, where the separation of analytes is governed by a ratio of their effective charge and the hydrodynamic radius. Alternatively, CZE method is carried out in sieving media of classical agarose and cross-linked polyacrylamide gels or in physical gels of entangled networks of high-molecular mass linear polymers, for example, LPA and various polysaccharides. This mode of CZE is called capillary gel electrophoresis (CGE) or capillary sieving electrophoresis (CSE), and the analytes are separated according to hydrodynamic size of their molecules.

The separation principles, basic methodologies, a short historical overview, advantages, and disadvantages of CGE as well as its new developments and applications for separation of proteins and polypeptides in biopharmaceutical, biomedical, and agricultural areas were summarized in three recent reviews [123–125] and one book [126]. Native CGE and SDS-CGE in agarose gels with UV and ESI–MS detection were applied for the separation of peptides and proteins [127]. Three peptides (bradykinin, angiotensin II, and neurotensin) and three proteins (lysozyme, ribonuclease A, and insulin) were separated by native CGE in BFS capillary (80/70 cm total/effective length, 50/365 μm id/od) using 4% FA, 20% glycerol, pH 2.3, as BGE, and 0.6% agarose as sieving medium, see Figure 5. SDS–CGE under denaturizing conditions was employed for the characterization of a commercially available protein size standard (10 kDa) in the same capillary using 428 mM Tris-acetate-EDTA, 10% v/v glycerol, 0.1% SDS, pH 7.0, as BGE, and 0.6% agarose as sieving gel.

A special mode of CZE is a nonaqueous capillary electrophoresis. Recently, it was applied for the determination of emerging mycotoxins [128] and therapeutic mAb [129]. A mixed hydro-organic solvent water/acetonitrile (can) (95/5 v/v) was employed to suppress the sorption of polypeptides (parathyroid hormone and its fragments) to the BFS capillary in their CZE analyses in a strongly acidic BGE composed of 1 M FA [130].

Throughput of CZE analyses can be significantly increased when samples are injected in the sequential injection mode [131]. In this mode, the analyses of the preceding sample are stopped after some time (separation voltage is switched off), a new sample is introduced, separation voltage is switched on, and the analysis of both samples continues. Using this approach, the analyses of peptide in pharmaceutical and biological matrices [91] and qualitative analysis of biopharmaceuticals [132] were increased three times, and the time analysis of one sample was reduced the same time.

5.2 | Isotachophoresis

CITP is in fact displacement electrophoresis in discontinuous electrolyte system composed of LE possessing leading ion with highest effective mobility of the system and terminating electrolyte (TE) containing terminating ion with lowest effective mobility of the system. The analytes (introduced between LE and TE) are separated according to their effective mobilities, that is, charge to size ratio, in sharply separated neighboring zones between LE and TE. With the exception of bidirection CITP, conventional CITP operates in cationic or anionic mode, and within one experiment, it can separate either cations or anions. Recently, a wide review on the history and developments of ITP in the last 50 years was published [96], and the latest developments and applications of CITP were reported in another review [133]. Theory and microfluidic applications of ITP are presented in another comprehensive review [134].

In the case of peptide analysis, CITP is more often used for the determination of low molecular mass peptide counterions, see, for example, Refs. [135, 136], than for the analysis of peptides themselves. However, ITP or transient-ITP, t-ITP, is frequently used for the preconcentration of peptides at low concentration level prior to their separation and analysis by CZE method. As shown above in Section 3.1 and Figure 3, combination of t-ITP and voltage polarity switching made possible full capillary sample loading for CZE separation of five synthetic peptides (angiotensins I and II, bradykinin, neurotensin, and kemptide) at low 2 μM concentration [95].

5.3 | Isoelectric focusing

CIEF is a high-performance CE method separating peptides, proteins, and other amphoteric compounds according to charge distribution in their molecules manifested by differences in their p*I*s. It is appreciated especially for its high-resolution power and concentrating and self-sharpening effect. Recent progresses and applications of IEF of peptides and proteins both in the capillary and slab and narrow tube gel formats are presented and discussed in the recent review [137]. It describes the principles and various modes and platforms of IEF as well as usual issues in IEF and its application to separation and analysis of amphoteric compounds, especially proteins and peptides. Valuable information on CIEF of peptides and proteins, such as choice of carrier ampholytes, p*I* markers, sample application, and mobilization, avoiding of isoelectric precipitation, immobilized pH gradient, and selected applications can be found also in the earlier published tutorial and review articles [59, 62].

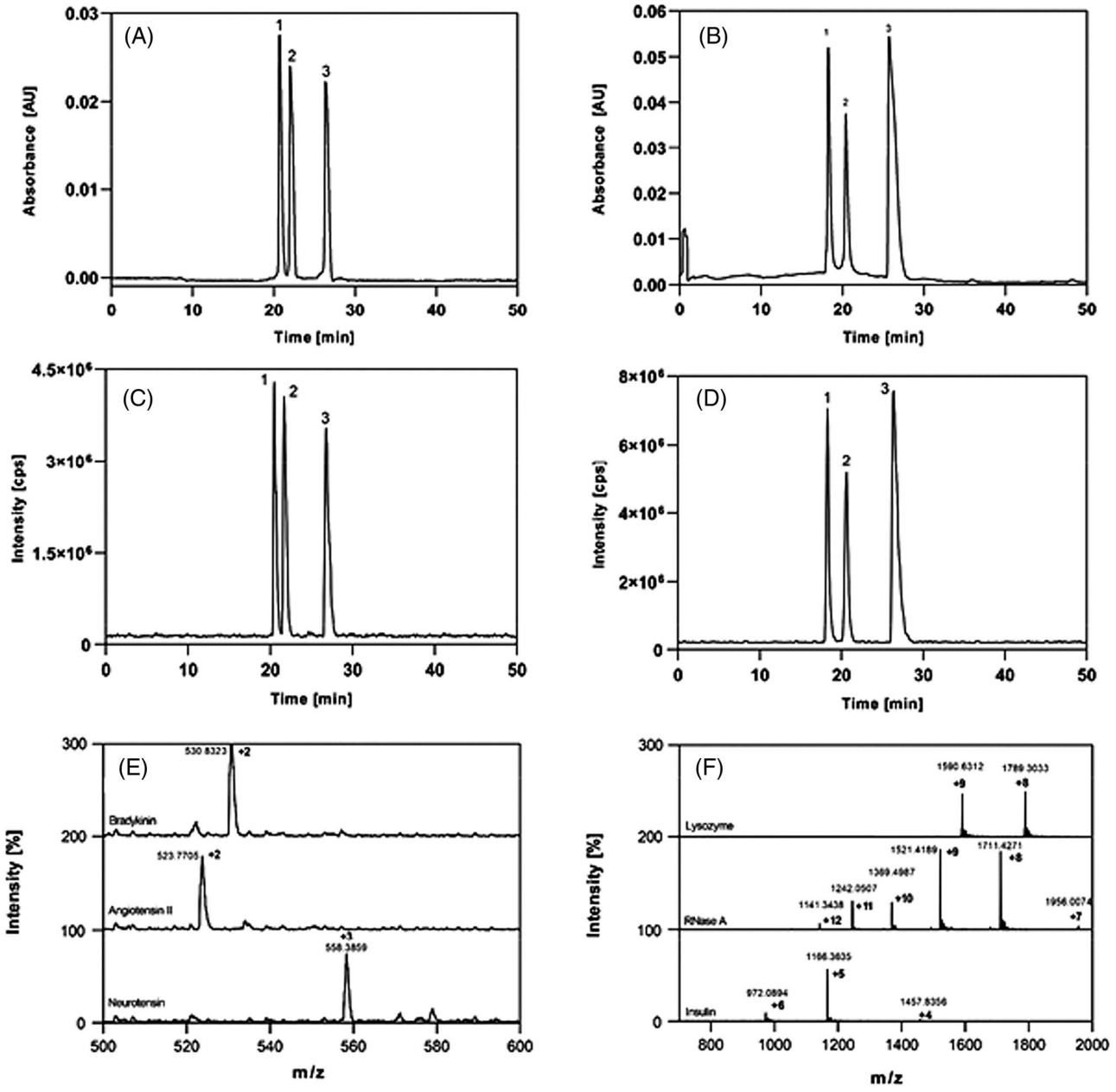

FIGURE 5 Native agarose capillary gel electrophoresis (CGE) analysis of peptide (panels A and C) and protein (panels B and D) mixtures with UV and mass spectrometry (MS) detection, respectively. Panels E and F show the resulting MS spectra. Peaks: (A and C) (1) bradykinin, (2) angiotensin II, and (3) neurotensin; (B and D) (1) lysozyme, (2) RNase A, and (3) insulin. Experimental conditions: CGE-UV: bare fused silica (BFS) capillary (50/365 µm id/od, 80/70 cm total/effective length); BGE 4% formic acid (FA), 0.6% agarose, 20% v/v glycerol, pH 2.3; electric field strength 285 V/cm; 214 nm detection wavelength. CGE–ESI–MS: capillary, BGEs at inlet vial and electric field strength are the same as in CGE-UV, BGE at outlet vial is 4% FA, 20% methanol (MeOH); spray voltage +2300 V; cone voltage: +30 V; desolvation gas flow rate 600 L/h; desolvation temperature 250°C; MS2 (full scan) between 400 and 1000 m/z for peptides and between 700 and 2000 m/z for proteins. *Source*: Reprinted with permission from Ref. [127].

No mobilization is necessary if the imaged CIEF (iCIEF) is performed with a whole-column imaging detection system, that is, with a continuous detection over a wide central section of the capillary with the diode array spectrophotometric or LIF detector. Latest innovation of the iCIEF and its applications for the analysis of proteins and peptides in pharmaceutical area are presented in Ref. [138].

iCIEF was applied for the fingerprinting of the trimeric receptor binding domain (RBD) of the spike (S) protein of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that is together with the whole S-protein the target to be genetically engineered for designing the wide-spectrum vaccine against SARS-CoV-2 [108]. The separation was performed in the fluorocarbon-coated FS

capillary column (50 mm long × 100/200 μm id/od) using 200 μL of the aqueous amphoteric electrolyte system composed of mixed ampholytes of 3 μL of Pharmalytes pH 3–10 and 1 μL of Pharmalytes pH 8–10.5, 10 μL of 200 mM iminodiacetic acid, 5 μL of arginine and 35 μL of 1% methylcellulose, and 1 μL of two p*I* markers (4.05 and 10.45). The finger prints of three batches of trimeric RBD were very well reproducible with relative standard deviations (RSDs) of p*I*s of the RBD and p*I* markers less than 1%. The method can be applied for the identification, batch-to-batch evaluation and stability study of the trimeric SARS-CoV-2 RBD protein, as a part of a quality control strategy during the vaccine production. The iCIEF is suitable for the separation of charge variants of intact proteins and their polypeptide fragments, especially in the development of therapeutic mAbs and other protein drugs [139–141].

For the successful CIEF analysis of peptides and proteins, especially for determination of their p*I*s, it is important to know the course of the pH gradient inside the capillary. Because it is impossible to directly measure pH inside the capillary, the profile of the pH gradient is evaluated indirectly using the compounds with well-defined p*I*s. Their role in CIEF is discussed, and the available p*I* markers and their synthesis are described in the recent review [142].

Due to its concentrating and self-sharpening focusing effects, CIEF is often used as the first concentrating step in two- or multidimensional separations of complex mixtures of peptides and proteins [143].

5.4 | Affinity electrophoresis

ACE is a combined method advantageously utilizing a high selectivity of (bio)affinity recognition/interactions and a high separation efficiency of CE. As shown in a recent reviews [144–147], ACE is widely used for investigations of non-covalent interactions of (bio)molecules including peptides under mild free-solution conditions and for the determination of the binding (association, stability, complexation, formation) constant or dissociation constant of the (bio)molecular complexes. For data processing and statistical evaluation of ACE experiments, the freely available program CEval is recommended [148].

ACE includes several modes that can be divided into two classes. The first one is based on the separation of the interacting species and the determination of their equilibrium concentration. It includes the Hummel–Dreyer method [149], the vacancy peak method [150], frontal analysis [151, 152], continuous frontal analysis [153, 154], and kinetic CE [155, 156]. The second class is based on the detection of a specific physicochemical property of the complexed analyte or its binding partner; mostly, it is the change of analyte effective mobility in the mobility shift ACE [151, 157] or change of migration times of analyte in the partial-filling ACE [158–161]. All these methods utilize the differences in the migration velocities of the interacting species. Both theoretical and experimental aspects of these methods and their applications for estimation of the binding constants have been outlined in the above reviews [144, 145, 147, 154]. The particular recent applications of ACE to quantitative characterizations of biopeptide interactions are presented in Section 7.3.

A special ACE methodology, indirect ACE, known also as competitive ACE was developed for the detection of protein binders at mM level, the determination of dissociation constants of peptide and protein complexes, and the characterization of the compound fragment binding site [162]. In this configuration, the effect of the analyte is evaluated indirectly via measuring the displacement of a known binder called reporter. Taking into account the multiple equilibrium theory, the dissociation constants of formed complexes on the micro- to millimolar range were determined. The method was applied on coagulation factor XIIa by evaluating pairs of fragments with expected behavior using pentamidine as a suitable reporter molecule.

5.5 | Electrokinetic chromatography

The recent developments and applications of capillary EKC including those dealing with EKC of peptides are described in general reviews on CE methods [39] and in a special review on chiral EKC presenting application of this method for enantioseparations of chiral compounds [163]. MEKC, the most popular mode of EKC with micellar pseudostationary phase constituted by ionogenic detergents, typically anionic SDS or cationic CTAB or zwitterionic CHAPS, is especially suitable for the separation of electroneutral peptides, that is, peptides with blocked or derivatized *N*- and *C*-terminus and other ionogenic groups of peptides, and/or for the separation of peptides with identical or very close charge/mass ratios but differing in their hydrophobicity. One of the latter examples is the MEKC separation of two octadecapeptides containing proline or hydroxyproline residue in the middle of peptide chain, thus differing only by one oxygen atom, using 65 mM SDS as micellar pseudostationary phase and 65 mM sodium tetraborate with 10% ACN organic modifier, apparent pH 9.4, as liquid phase [164]. The separation was performed in BFS capillary (50 μm id × 60/53 cm total/effective length) at 25 kV separation voltage, 25°C, and 216 nm detection wavelength, with LOD and LOQ equal to 10 μm and 50 μM, respectively.

5.6 | Electrochromatography

CEC is a hybrid separation technique combining the electrokinetic and chromatographic separation principles. It benefits both from the high selectivity of variable stationary phases (SPs) developed for peptide separations by (U)HPLC methods and from the flat (piston-like) profile and low dispersion of electroosmotically driven flow of mobile phase (MP). Despite these advantages, CEC in general, as well as in separation and analysis of peptides, did not fulfill the hopes connected with this method in the 1990s and in the beginning of this century. CEC did not become a widely used method for peptide separations and analyses.

CEC is implemented in three modes using packed, monolithic, and open tubular (OT) columns. Among them, in the last 2 and half years, mostly the OT-CEC (open tubular CEC) mode was preferred for further developments as the synthetic coating compounds and materials can be relatively easily physically or chemically immobilized on the inner FS capillary wall without the back-pressure problems or bubble generation in the sorbent or in the frits. Numerous coating materials, including organic and inorganic compounds and materials, hybrid materials, and polymeric materials, have been introduced as physically adsorbed of chemically covalently attached SPs as shown in a previous review [165]. The recent reviews mostly deal with new SPs, such as microporous materials (MOFs [166, 167], covalent organic frameworks [168], porous organic cages, and knitted aromatic polymers, nanoparticles, polymer materials, silica-based materials, and biomaterials). Various aspects of chiral CEC, including its history, advantages, bottlenecks, developments, and applications in the last 30 years and the future perspectives, were in detail discussed in a comprehensive review by Fanali and Chankvetadze [169]. They concluded that CEC still has a potential for further methodological development and widening of the application field.

CEC separations of peptides and proteins have been performed in different separation modes, with reverse-phase [170], affinity [166], mixed-mode [171], and cationic or anionic ion-exchange SPs [172], in classical FS capillary columns as well as in microfluidic chips.

Advances in stimuli-responsive polymeric coatings in OT-CEC were summarized in the recent review [173]. It describes latest developments in the design and synthesis of smart stimuli-responsive polymers, the procedures for their preparation on the inner capillary walls, strategies for improving the separation efficiency, and examples of its real-world application including separation and analysis of peptides. Compared with common polymeric coatings, the stimuli-responsive polymeric coatings exhibit stimuli-dependent reversible phase-changing properties in response to the external stimuli, such as light irradiation, temperature, electric or magnetic field, pH, organic solvent, and ionic strength, and they can (i) protect the capillary inner wall from the unfavorable/undesirable interactions, (ii) tune the coating properties, and (iii) adjust the EOF velocity by changing the coating structures, hydrophobicities, hydrophilicities, and surface charge. Thus, they have a potential to become powerful SPs for separation of both low-molecular-mass organic compounds as well as small and large biomolecules including peptides and proteins.

Two-layered copolymer coating containing vinylbenzyl chloride and divinylbenzene as components of the first thin monolithic monolayer and the second layer formed by reversible addition-fragmentation transfer polymerization of *N*-phenylacrylamide and styrene-generated SP that provided high separation efficiency in OT-CEC separation of complex mixture of synthetic peptides and peptide fragments of tryptic digestion of cytochrome *c* [174]. The separation efficiency of 2.4 million of theoretical places was achieved in separation of six synthetic peptides (Trp–Gly, Thr–Tyr–Ser, Pro–Phe–Gly–Lys, angiotensin 1, isotocin, and bradykinin), and more than 18 tryptic peptides of cytochrome *c* were resolved with this SP. The separation was performed in 50 μm id capillary (120/111.2 cm total/effective length) using the MP composed of 20 mM ammonium formate, pH 6.0, and ACN in 40:60 v/v ratio, at separation voltage of 20 kV, 25°C temperature and 214 nm detection wavelength. These results show that OT-CEC still possesses a potential for high-efficient separation of peptides and proteins.

Mixed mode SP prepared by copolymerization of three monomers with various polarity and hydrophobicity (*N*-[2-(acryloylamino)phenyl]acrylamide, methacrylic acid, and styrene) was employed for OT-CEC separation of six synthetic oligopeptides (dipeptides—hexapeptides) and tryptic peptide fragments of cytochrome *c* [171], see Figure 6. High efficient separation of all synthetic peptides and 27 tryptic peptides with high average theoretical plate number of 1.5 million per meter and high resolution, enhanced peak capacity and a wider retention time window, was achieved in 1 m long FS capillary (50 μm id × 92 cm effective length) using MP containing 50 mM ammonium formate, pH 6.5, and ACN in 30/70% v/v ratio. The separation voltage was 30 kV, and capillary was thermostated at 25°C.

OT-CEC column with covalently attached polystyrene sulfonate as SP was prepared by *in situ* polymerization using 4,4′-azobis(4-cyanopentanoyl chloride) as polymerization initiator [172]. It was successfully applied for the separation of different types of compounds, three peptides, five alkaloids, and five sulfonamides. A new covalent organic material composed of 4,4′-diaminodibenzo-18-crown-6-ether and 2,4,6-triformylphloroglucinol was

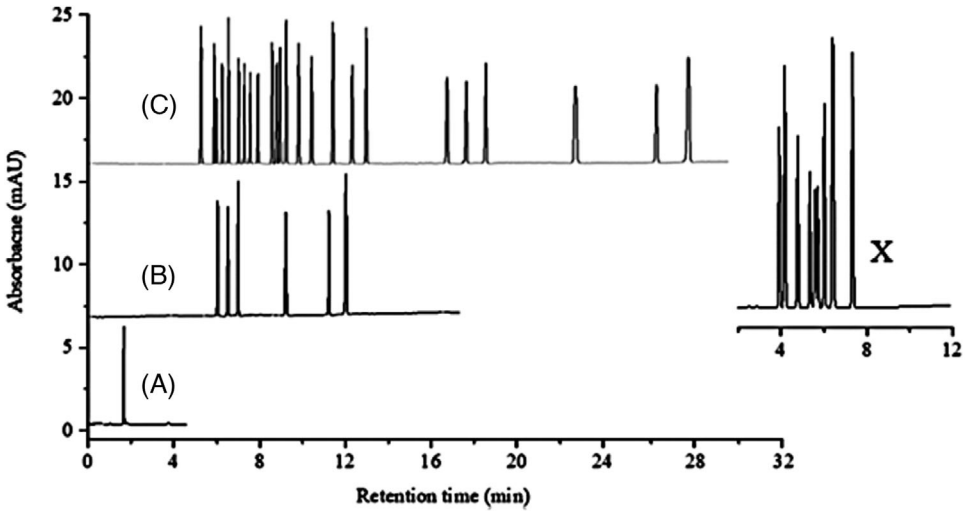

FIGURE 6 Records of capillary electrochromatography (CEC) separations of tryptic peptide fragments of cytochrome *c* on the column coated with stationary phase in the (C) presence and (X) absence of *N*-[2-(acryloylamino)phenyl]acrylamide, (B) six commercial peptides (eluted in order of 1: Trp–Gly, 2: Thr–Tyr–Ser, 3: Pro–Phe–Gly–Lys, 4: Tyr–Ile–Gly–Ser–Arg, 5: Phe–Leu–Glu–Glu–Ile, 6: Val–Glu–Pro–Ile–Pro–Tyr), and (A) the electroosmotic flow (EOF) marker acetone, using the elution conditions of acetonitrile (ACN)/20 mM ammonium formate (70/30% v/v, pH 6.5). Sample injection: 10 kV for 7 s; separation voltage 30 kV; current: 10–15 μA. *Source*: Reprinted with permission from Ref. [171].

developed and applied as SP in OT-CEC separation of compounds containing imidazole moiety [170]. The OT column provided specific selectivity for protonated imidazole structures and made possible separation of various other compounds, including three short peptides, Phe–Gly, Phe–Gly–Gly, and Ala–Trp using MP composed of 5–10 mM phosphate buffer and 10% of ACN.

Attractive alternatives to the classical CEC columns packed with particulate materials are the monolithic SPs prepared in the form of polymeric columns, silica columns, nanomaterials-based columns, and hybrid columns [175–177]. Their advantages are simplicity of their *in situ* preparation and variety of readily available chemistries for synthesis of monolithic materials.

The advantage of CEC as compared to MEKC is that the compositions of its MPs and the immobilized SPs are more compatible with online coupled ESI MS detection.

5.7 | Multidimensional separations

In spite of high separation efficiency of the particular CE methods, their resolution power is usually insufficient for a complete separation of complex mixtures of peptides/proteins in various biomatrices (body fluids, tissue extracts, cell lysates) and in (multi)enzymatic and chemical digests of large proteins. In these cases, a combination of two or more complementary separation principles, generating 2D or multidimensional separation systems, such as IEF and SDS–PAGE in the classical 2D gel electrophoresis (2D-GE) [178, 179] or in the differential or comparative 2D-GE [180] is needed.

The important role of multidimensional separations of biopeptides, peptide fragments of proteins and proteins in proteomic, peptidomic, and metabolomic studies was highlighted in the recent reviews [13, 39, 41]. Two- or multidimensional peptide separations are based on the combination of two or more separation methods with different separation principles, for example, LC and electrophoresis [181, 182]. If these principles are independent, the methods are classified as "orthogonal," and the peak capacity of this 2D separation is approximately equal to the product of the individual peak capacities of each dimension [183].

There are many combinations of separation methods applicable for 2D separations of peptides and proteins, such as LC–CE, CE–CE, CE–LC, where LC and CE represent different modes, for their particular combination see the earlier review [184]. When these 2D systems are online coupled with a single or tandem MS detection, these supra-powerful multidimensional separation systems can resolve and identify hundreds up to few thousands of peptides/proteins in the above complex biomatrices.

2D format implemented by online coupling of CIEF with ESI–MS detection [143] can be considered a current equivalent of the classical 2D-GE separating according to p*I* in the first dimension and according to size in the second dimension. CIEF–ESI–MS also provides both p*I* and M_r, but the obtained M_r values are much more precise

than those estimated from the second step of the 2D-GE, SDS–PAGE.

A powerful 2D-CZE-CZE setup composed of two BFS capillaries connected via customized 8-port valve and coupled with tandem ESI–MS detection was applied for the separation of charge variants of intact mAb in the first CZE dimension (in BGE composed of 380 mM e-aminocaproic acid, 2 mM triethylenetetramine, 0.05% HPC, pH 5.7) and for separation complex mixture of peptide generated by online in capillary reduction of mAb with tris(2-carboxyethyl)phosphine and digestion by pepsin in the second CZE dimension (in BGE consisted of 200 mM FA) [185]. Several common modifications as deamidation and oxidation were detected and localized in four different mAb molecules.

2D and 3D microfluidic chip electrophoresis system comprising CIEF in the first dimension and CGE in the second or ITP/CGE in the second and third dimensions was applied for the separation of complex mixture of peptides and proteins of the whole bacterial cell lysates [186]. Under the conditions partially specified only for IEF step (4% carrier ampholytes p*I* 3–10, 20 mM phosphoric acid as anolyte, 20 mM ammonium hydroxide as catholyte, 500 V/cm electric field strength in 23 mm long, 150 μm wide microchannel) and using an inverted fluorescence stereomicroscope equipped CCD camera for the visualization of the separation process, a high-resolution peptide fingerprinting with high peak capacity was obtained within 1 min.

6 | DETECTION

6.1 | UV-absorption

Thanks to relatively strong absorption of low UV radiation (185–220 nm) by the peptide bond, peptides can be advantageously detected by the spectrophotometric UV-absorption detection. This detection still belongs to the most often used detection schemes in CE analyses of peptides. Due to the low injected volume of analyzed samples and the short optical path of the on-column UV detector, the concentration sensitivity of UV detection is not too high. The typical LODs of the UV-detectors in 50 μm id capillary are at the micromolar level within a ca. nanoliter detection cell volume with 10–20 Hz data acquisition. This sensitivity can be enhanced by the earlier developed special detection cells with increased optical path, such as bubble, Z-shaped, or sleeve cells. For more details on miniaturized UV–vis spectrophotometric detectors in CE, see the earlier review [187] and in the recent review dealing with the current state of CE instruments including their detection systems [188]. Increased sensitivity of peptide detection is achieved at ultralow UV region below 200 nm, for example, at 195 nm in the CZE analysis of antimicrobial dipeptide *β*-Ala-Tyr [189].

6.2 | Fluorescence

Classical fluorescence detection with Xenon–Mercury lamp as a polychromatic light source and especially the LIF with the particular wavelength light sources belong to the most sensitive detection modes in separations of peptides by CE methods. In commercial CE devices with on-capillary LIF detection, the concentrations of LODs are usually in low-nM range; in the homemade special LIF detectors, the concentrations of LODs can be diminished to subnanomolar level, and the substance amount of LODs is in the zeptomole range [190].

Different aspects of LIF detection in CE (light sources, detection elements, optimization of excitation conditions, stability of the fluorophores, spectral filtering, and fluorescent labels) including the possibilities to further decrease the LODs of on-capillary LIF detectors were in detail analyzed in the previously published tutorial [191]. Applications of the standard and advanced fluorescence spectroscopy techniques (fluorescence microscopy, fluorescence recovery after photobleaching, fluorescence correlation spectroscopy, and fluorescence anisotropy) for detection as well as for investigation of transport, interfacial phenomena, and intermolecular interactions in CE separations of peptides and other biomolecules are discussed in recent review [192].

The inconvenience of the LIF detection of peptides is that they must mostly be derivatized by fluorogenic labels. The native fluorescence can be utilized only for the detection of peptides containing aromatic AA residues of Trp, Tyr, and Phe. However, for their excitation, deep UV-laser systems, such as Nd:YAG laser operating at 266 nm, or multiphoton excitation are necessary. With a native 280 nm light emitting diode as an excitation force and photomultiplier tube as a detector of emitted light, the LOD of 8.3 ng/mL was achieved for CE analysis of mAbs and their polypeptide light and heavy chains fragments [193]. The detector designs, performance, and applications of native fluorescence for the detection of biomolecules, including peptides and proteins, can be found in the review by Couderc et al. [194].

More often, the fluorescence detection of peptides is based on their precolumn or postcolumn derivatization with a fluorescent label (see Section 3.2). The disadvantage of this approach is that due to usual presence of multiple derivatization sites in peptides and proteins, several derivatives with different mobilities are generated resulting in

multiple peaks in electropherograms for originally single peptide or protein species.

6.3 | Mass spectrometry

Being both universal and selective and providing high-sensitivity and structurally rich information on the analyzed compounds, MS represents the most powerful detection mode for CE, LC, and other separation methods. The recent developments in the area of MS detection in CE methods have been described in several reviews dealing with CE–MS in general [195–198] and with its application to peptide, protein, peptidomic, proteomic, and metabolomic analyses especially [8, 16, 44, 199–201]. Importance of MS as analytical and structure elucidation method has significantly increased in the last years due to its crucial role in the previous omics technologies [44, 199, 202]. Hence, the relevance of MS hyphenation with CE separations of peptides and proteins is growing as well.

On- and offline coupling of CE methods with ESI–MS [203] and matrix-assisted laser desorption/ionization (MALDI)–MS [204] introduced more than two decades ago [205, 206] has become a breakthrough in the analysis and characterization of peptides and proteins. Hyphenation of CE separation with ESI–MS and MALDI–MS detection made possible not only a high-accuracy determination of M_r of CE separated peptides and proteins but also obtaining structural data on AA sequences [207], the sites of posttranslational modifications (PTMs), peptide mapping, and non-covalent interactions of peptides and proteins with other (bio)molecules. ESI and nanoESI are the most popular modes for online CE coupling with MS, especially thanks to their ability to generate multiple charged ions. It ensures that the ion m/z (mass/charge) ratios of even large polypeptides and proteins fit within the limited m/z detection range of most MS detectors.

In the beginning of their development, the CE–ESI–MS setups utilized for the ionization of the peptides and other analytes the adapted commercial sheath-flow interfaces employed for MS coupling with LC methods. However, in the sheath-flow interfaces, the eluting analytes are diluted with the sheath liquid (SL) at the exit of the FS capillary. This decreases the sensitivity of the MS detection. Consequently, in the next years, new types of low-flow (nanoflow) or sheathless interfaces were developed.

Increased ESI stability and detection sensitivity were achieved in a new flow-through microvial interface coupling CE with MS detection [115]. It was tested in combination with three commercial MS detectors, and LODs of five oligopeptides were in the range of 1.9–190 nM. The analyses were performed in PEI-coated capillary with reversed EOF in BGE composed 130 mM FA in 50% v/v water/MeOH mixed solvent at separation and ESI voltages of −25.5 and +4.5 kV, respectively.

Recently a special, easy, flexible, and robust nanoflow SL CE–ESI–MS interface "nanoCEasy" has been developed for CE hyphenation with high-resolution Orbitrap MS detector [208], see Figure 7. It is based on the previously introduced two capillary systems [209] and contains some 3D-printed components. The plug-and-play design allows introduction of capillaries and an emitter without any fittings in less than one minute. The transparent polymer components make possible visual observation of the liquid flow inside the interface. Robust function of the interface was demonstrated via the variable ESI voltage, the distance between the separation capillary and emitter tip, the distance between the emitter and the MS orifice, and different emitters of the same type. In optimized CE operational mode, the separation capillary tip was placed 1.8 mm from the emitter tip, and the position of the emitter tip was 3 mm in front of MS orifice. Reliable function of the interface was verified by CE–ESI–MS separation and detection of tryptic peptides of BSA using FS capillary (50 μm id × 65–70 cm total length) and BGE composed of 200 mM FA. SL consisting of 50% isopropyl alcohol (IPA) and 0.5% FA was delivered with 4–8 μL/min flow rate, and the dry gas flow rate was 3 L/min with a dry gas temperature of 150°C. The separation voltage was +30 kV, the ESI voltage was 1500–1600 V in positive ion mode, and the scan range was set to 250–1500 m/z with a target mass of 650 m/z.

Another robust hyphenation of CZE/CGE with ESI–MS detection was achieved by a closed-circuit coaxial sheath-flow reactor interface [127]. The post-column reactor placed in front of MS orifice made possible the usage of non-MS friendly components (SDS) in the BGE without significant sample ion suppression and supported stable electrospray. In SDS agarose CGE, the addition of γ-CD to the SL efficiently removed SDS from the sample and BGE in the flow reaction space by inclusion complexation and preserved the size-based separations of peptides and proteins with high separation efficiency. The BGE was composed of 428 mM Tris-acetate-EDTA buffer, pH 7.0, 10% v/v glycerol, 0.1% SDS, and contained 0.6% agarose. The SL consisted of 4% FA, 20% MeOH, and 0.8% γ-CD. The electric field strength was 215 V/cm with cathode at the injection capillary end. Spray voltage with positive ion detection mode was +2 300 V, cone voltage +30 V, desolvation gas flow rate 600 L/h, and desolvation temperatures of 250°C were the other operational conditions.

CE coupled with ion mobility spectrometry-tandem MS (IMS–MS/MS) allowed discrete detection of sequence isomeric peptides—three peptide isomers of 12 AA residues with direct and reverse regions of Ala–Val–Pro–Ile motif [210]. The separation took place in the BFS capillary (90 cm

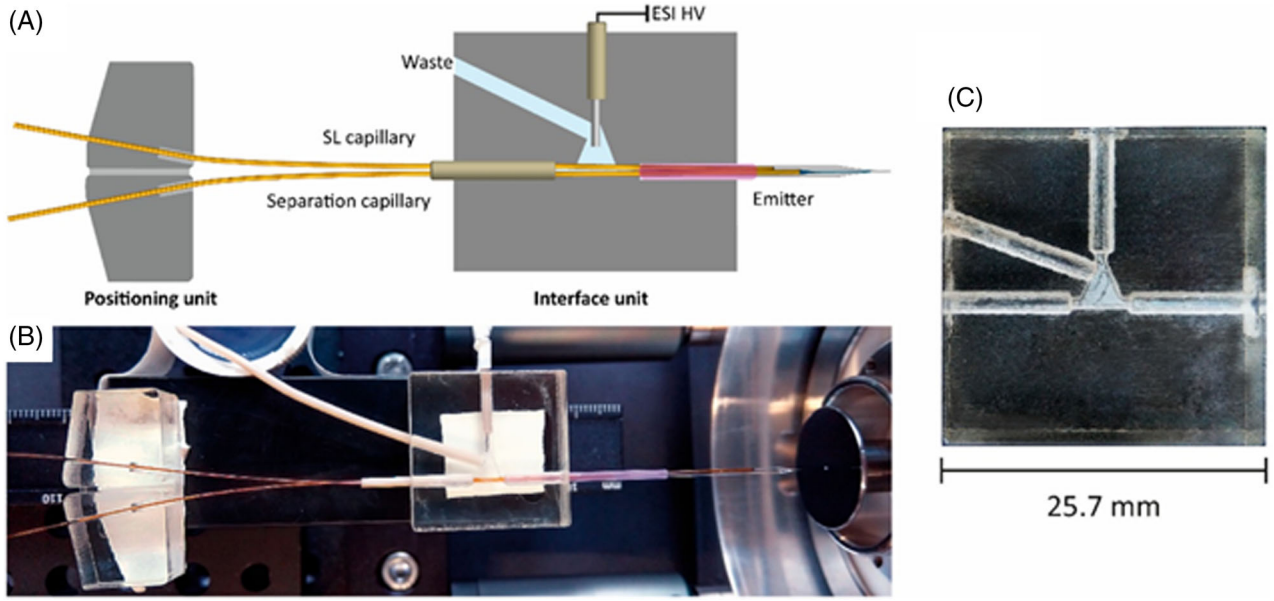

FIGURE 7 Setup of the nanoCEasy interface: (A) schematic view of the interface with attached capillaries and electrodes (not to scale); (B) image of the nanoCEasy interface mounted to a Bruker quadruple time of flight (QTOF) MS instrument; (c) close-up image of the 3D-printed interface part. *Source*: Reprinted with permission from Ref. [208].

long × 50 µm id) using 10 mM ammonium acetate adjusted with AcOH to pH 3.45 as BGE and electric field strength of 290 V/cm with normal polarity at 20°C. The SL consisted of 50% MeOH and 1% FA was injected into the electrospray at a 1.2 µL/min flow rate. The ESI voltage at positive ion mode was +4.0 kV, and the capillary tip was heated up to 80°C. The trap collision energy was 40 V in the MS/MS regime without the IMS, and the transfer collision energy was 50 V with the IMS involvement. In ion-mobility cell, the gas flow was 90 mL/min, and the IMS wave velocity was 650 m/s.

The second soft ionization mode, MALDI, is usually coupled with CE offline. Its advantage is that it generates preferentially single charged molecular ions of even large polypeptides and proteins with M_r up to 300 000. It allows the exact determination of M_r with an accuracy of ± 0.1% and identification of peptides and proteins isolated by micropreparative CZE, see Section 7.2.

6.4 | Other detection modes

Among the other detection modes, the universal capacitively coupled contactless conductivity detection (C^4D) is relatively frequently used in CE and MCE methods [211]. However, much more for small ions, such as acids, AAs [212, 213], and other small biomolecules [214, 215] than for peptides. One of a few examples is application of C^4D with 750 kHz excitation frequency and 90 Vpp (peak to peak) for the CE determination of GSH and other low-molecular-mass thiols (cysteine, homocysteine, and methionine) in saliva [77].

Electrochemical, NMR and some other detection modes are less suitable and hence much less employed for detection of peptides separated by CE methods than the above UV-absorption, fluorescence, MS, and C^4D detection schemes. For that reason, only a few recent reviews and papers are cited here, which are dealing with general developments and applications of these detectors in separation methods. They include electrochemical detection alone [216, 217] or in combination with MS [218], direct or indirect chemiluminescence [219], and electrochemiluminescence [220]. NMR spectroscopy is mostly offline combined with CE, especially for the structural analysis of analyte-selector complexes the strength of which was studied by CE [221–223].

7 | APPLICATIONS

7.1 | Analysis

7.1.1 | Quality control and determination of purity

Quality control and determination of peptide purity are necessary in all areas where peptides are isolated, synthesized, used, or studied. These areas include biological, biochemical, biomedical, physiological, and clinical research, for example, in the investigation and modeling of

variable biomolecular interactions, such as hormone-receptor, drug-receptor, enzyme-substrate, enzyme-inhibitor, ion-ionophore, and antigen–antibody, and in the identification of antigenic determinants (epitopes) of proteins. In addition, peptide and peptidomimetic drugs, and food and feed additives belong to the relevant compounds also in biotechnology, pharmaceutical, food, and feed industry [116, 224–226]. Hence, there is a wide need for qualitative and quantitative analysis of peptides by CE and other analytical methods. In most of the above applications of (bio)synthetic or naturally isolated peptides, CE methods can be used as sensitive control methods providing fast and accurate qualitative and quantitative information about purity of peptide preparations. Further, some recent applications of CE methods to peptide analyses will be presented, in addition to those shown in the previous sections.

Purity degree control and separation of human insulin and its six therapeutic analogs were performed CZE-UV and CZE–ESI–MS methods using three types of FS capillaries: untreated, SMIL coated, and LPA coated, and three types of acidic, neutral, and alkaline BGEs [110]. For uncoated capillaries, the BGEs were 1 M FA, pH 1.8, and 50 mM ammonium acetate, pH 7.0 and pH 10.0). For SMIL and LPA-coated capillaries, BGEs were 0.3 M FA, pH 2.3, and 50 mM FA, pH 2.6. A three layered SMIL coating was prepared by subsequent capillary flushing with 1 M NaOH for 30 min, 10% m/v PB for 20 min, 10 min water, 20 min 3% m/v dextran sulfate, 10 min water, 20 min PB, and 10 min water. The LPA coating was performed according to the original procedure developed by Hjerten [227]. The CE separations were performed in the capillaries with 50/375 μm id/od and 65/58 cm total/effective length at +25 kV for BFS capillaries, +30 kV for LPA-coated capillaries, and −30 kV for SMIL-coated capillaries. UV detection was set at 200 nm. For MS detection, the SL was IPA/water mixture at 1/1 ratio with 0.1% v/v FA, and the other parameters were in the following: ESI voltage at positive ionization mode was 3.5 kV, nebulizer pressure 0.3 bar, dry gas temperature 200°C, and dry gas flow rate 4.0 L min. MS detection was optimized for 600–2500 *m/z* mass range, and for MS/MS spectra, the collision energy was 45 eV. The coated capillaries exhibited a little bit better separation of human insulin and its analogs but some of them were migrating in wide distorted peak. The highest separation efficiency (192 000 theoretical plates per meter) was achieved in the SMIL-coated capillary. Surprisingly, slightly better LODs were obtained with UV-absorption detection (0.3–1.2 μg/mL) than with ESI–MS detection (1.0–3.4 μg/mL).

Purity of the insect antimicrobial dipeptide *β*-alanyl-tyrosine (*β*-Ala–Tyr) isolated from the extract of the hemolymph of larvae of the fleshfly *Neobellieria bullata* and purified by RP-HPLC and free-flow zone electrophoresis (FFZE) was tested by CZE in BFS capillary (50/375 μm id/od × 393/290 mm total/effective length) thermostated at 25°C using 500 mM AcOH, pH 2.50 as BGE, 20.0 kV as separation voltage, and 195 nm as detection wavelength [189].

CE–UV and CE–MS with sheathless ESI interface were employed for separation, identification, and quantification of impurities in cobratide peptide drug extracted from the venom of Chinese cobra that is widely used for the treatment of chronic intractable and persistent pain [228]. Under the optimized conditions (BGE composed of 50 mM ammonium acetate, pH 4.0, BFS capillary 90 cm × 30 μm id, separation voltage +10.0 kV, ESI voltage 1650 V, source heating temperature 50°C, collision cell energy 10 eV, data recorded at 800–2000 *m/z*, separation time 30 min), the LOQ of CE–UV method was 4.16 μg/mL.

The electrophoretic homogeneity of synthetic peptide containing 21 AA residues proposed as lactoferrin binding ligand by *in silico* modeling was confirmed by CE analysis in BFS capillary (75 μm id, 60/50 cm total/effective length) thermostated at 30°C using 10 mM Tris/HCl buffer, pH 7.2, as BGE, 20 kV as separation voltage, and 200 nm as detection wavelength [229]. The CE analysis of a mixture of this peptide with lactoferrin proved formation of peptide–lactoferrin complex.

Peptide drugs and peptides used in biological assays have to be characterized also by the content of their low-molecular-mass ionic admixtures and counterions [228]. Basic peptides frequently contain anionic counterions, for example, fluorides, chlorides, acetates, and trifluoroacetates (TFA) originating from synthesis and/or purification of these peptides. Peptides are often isolated as TFA salts by preparative HPLC or they may contain fluorides after their hydrogen fluoride cleavage in solid-state peptide synthesis. However, the toxic TFA and fluoride anions have to be completely exchanged for another counterion from the peptide preparations prior to their biological applications to avoid potential toxicity effect. Applications of CE methods in this field are presented in the previous reviews [135, 136].

7.1.2 | Determination in biomatrices

CE methods coupled with highly sensitive MS and LIF detectors and utilizing the online sample stacking effects possess great potential also for the determination of peptides at low concentration levels in complex biomatrices, such as biological fluids, cell lysates, tissue extracts, foods, and feeds. This fact and miniature injected sample volume (nL to pL) make CE methods powerful and highly appreciated tools in biomedical research and clinical analysis [230, 231], where often only minute volumes from

biopsy or dialysis samples are available, and in the single cell analysis [232–234].

Detection and identification of particular peptides in complex biomatrices are of great importance also in peptidomic and proteomic analyses of biological fluids (blood, serum, plasma, cerebrospinal fluid, urine, and saliva) [231]. It can result in the discovery of new specific peptide biomarkers applicable in diagnosis, therapy, and prognosis of some diseases, for example, various kidney diseases [235–237], Alzheimer's disease [5], prostate cancer [238], cardiovascular diseases [236], COVID-19 [239], and lupus nephritis (LN) disease [6].

CZE–ESI–MS setup has been applied for the determination of three peptidoglycan-derived peptides (tri-, tetra-, and pentapeptides) and their amidated derivatives in the extracts of bacterial cytoplasma [240]. These peptide fragments are generated by the bacterial autolysis of peptidoglycans—the relevant constituents of the bacterial cell wall. They are involved in the bacterial cell-wall stress response such as β-lactamase induction in the presence of β-lactam antibiotics. The absolute quantitation of one of these peptides (tripeptide) based on the [^{13}C, ^{15}N] isotopically labeled standard was carried out in the crude cytoplasmic extracts of bacteria grown in the presence or absence of a β-lactam antibiotic (cephalosporin C). The separation was performed in BFS capillary (50/365 μm id/od × 90 cm total length) using 100 mM FA as BGE and 10 mM FA, 50% IPA as SL delivered with 1.5 μL/min flow rate. The separation voltage was +30 kV at 25°C. The results have shown increased concentration of tripeptide in samples grown in the presence of antibiotics. In spite of high complexity of the sample, the repeatability of the CZE–ESI–MS quantitation was very good, with RSD of ca. 1%.

CE–ESI–MS in the mode of multiple segment injection and with in-capillary preconcentration via FESI was applied for high-throughput and high sensitive determination of therapeutic decapeptide triptorelin in complex pharmaceutical and biological matrices [91]. CE was run in 90 cm long × 50 μm id BFS capillary using BGE composed of 1 M FA, pH 1.88, and SL was a mixture of 5 mM ammonium acetate and MeOH (50/50, v/v). FESI increased the sensitivity 50 times to the LOD of 5 ng/mL in aqueous matrix of the commercial drug Diphereline and 25 ng/mL in complex blood plasma matrix spiked with triptorelin). The multisegment injection increased the throughput of the method three times as compared to conventional CE method with single injection per analysis.

3D printed, automated, pressure-driven injection microfluidic MCE device with 46 μm wide, 50 μm deep, and 9.8 mm long separation channel connected to two pneumatic valves (formed by membrane thickness of 5 or 10 μm) and reservoirs was developed using glycine and phenylalanine as model compounds and subsequently applied for the determination of preterm birth-related peptide and protein biomarkers, peptide 1, peptide 2, corticotropin releasing factor, ferritin, and lactoferrin, respectively [109]. The valves enabled control of fluid flow in microchannels during sample injection and MCE separation. The AlexaFluor fluorescently labeled peptides were injected within 0.5–1.5 s and separated at 460 V/cm electric field strength within less than 1 min in BGE composed of 50 mM HEPES, pH 8, and containing 1% HPC for dynamic microchannel coating. With the LIF detection with 532 nm excitation wavelength and 1.5 s injection time, the LODs for peptides 1 and 2 were 400 pM and 15 nM, respectively, and the linear detection range for peptide 2 was 50–400 nM.

Four biologically active peptides (Ala–Hyp, Pro–Hyp, Pro–Hyp–Gly, and Gly–Pro–Hyp) in simulated gastrointestinal digestion of two bovine collagen hydrolysates by pepsin (4% m/m in 100 mM HCl) were separated and quantified by CE with UV-absorption detection at 205 nm wavelength using BFS capillary (75 μm id × 60/53 cm total/effective length), BGE consisted of 100 mM phosphate buffer, pH 2.4, and separation voltage was 20 kV at 20°C [241].

Several CE methods have been developed for the determination of GSH, natural tripeptide γ-Glu–Cys–Gly, which plays an important role in several physiological processes in humans and animals. It maintains homeostasis and regulates the redox environment in the cells. Acting as antioxidant, it prevents and reduces oxidative damage of proteins in the living cells and participates in the reduction of disulfides and other molecules. The determination of GSH in body fluids is important for early diagnosis and monitoring of various diseases. CE analyses of GSH include, for example, the determination of GSH, cysteine, and homocysteine in human blood plasma and saliva [77], determination of reduced and oxidized forms of GSH in human blood samples [99], and quantification of naphthalenedicarboxaldehyde-labeled GSH in HepG2 cells with LOD and LOQ of 6.0 and 20.0 nmol/L, respectively, using Tris-borate buffer, pH 9.2, as BGE [242].

CE–MS analyses of urinary peptidomes (peptide profiling) have discovered large sets of urinary peptide biomarkers for various diseases. CE–MS study revealed a marker model of 83 urinary peptides that could be used for the diagnosis of periprosthetic joint infection with a sensitivity of 95%, a specificity of 90%, and an area under the curve of 0.96 [243]. On the other hand, CE–MS analyses of urinary peptidome of 55 patients with active LN and 38 patients with non-active LN have shown that urinary peptides cannot discriminate between active and non-active LN or predict early response to therapy [6].

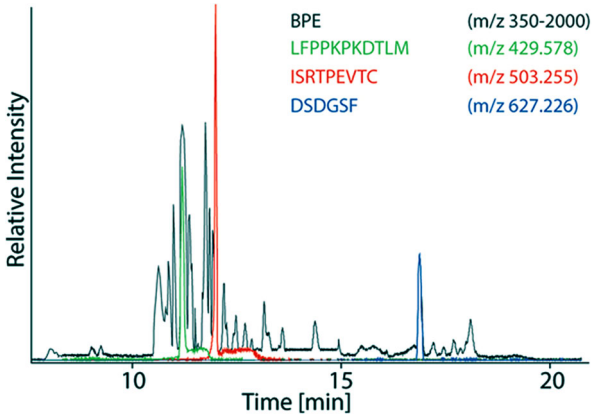

FIGURE 8 Base peak electropherogram of one-dimensional capillary zone electrophoresis (CZE) separation peptide fragments of in-capillary reduced and by pepsin digested mAb trastuzumab (1 mg/mL) using 40-mM tris(2-carboxyethyl)phosphine (TCEP) for reduction and 3.8 mg/mL pepsin for digestion, 70 cm poly(ethylene oxide) (PEO)-coated capillary, background electrolyte (BGE) 0.2 M formic acid (FA), 10 min digestion time, 30 kV, 350−2000 m/z (black trace). Extracted ion electropherograms of selected peptides: LFPPKPKDTLM (green trace, 429.578 m/z), ISRTPEVTC (red trace, 503.255 m/z), DSDGSF (blue trace, 627.226 m/z). *Source*: Reprinted with permission from Ref. [185].

In addition to the above peptide determinations in biological fluids, CE and CEC have been applied also to the analysis of peptides in complex mixtures of food and feed products [244, 245].

7.1.3 | Monitoring of chemical and enzymatic reactions

Besides analysis and characterization of "static" peptide preparations, CE methods are frequently applied also for monitoring and evaluation of chemical and enzymatic reactions and modifications of peptides and proteins, for example, hydrolysis, oxidation, reduction, deamidation, isomerization, and racemization. In addition to reactions performed in classical offline format, the course of reactions performed directly inside the separation capillary can be monitored by CE [246].

In-capillary digestion of charge variants of unstressed and thermally stressed mAb by pepsin was monitored by 2D-CE–ESI–MS setup described in Section 5.7. The peptide fragments were separated in 93 cm long 50 μm id capillary physically coated with PEO using 200 mM FA as BGE and 50% IPA, 0.2% FA as the SL with delivery flow rates of 3–8 μL/min [185], see Figure 8. The common PTMs of mAb, deamidation, oxidation, and others were detected and localized in the polypeptide chains.

A new concept of an IMER was introduced by Ryan and Bruening [247]. They placed the nylon membrane containing the immobilized pepsin between CE separation capillary and MS detector, that is, in a low-flow SL ESI source, which enabled protein digestion immediately before spray into the mass spectrometer. Thus, the proteolysis performed after separation limits the separation of digested peptides but allows the peptides from a given separated protein to enter the MS in a single plug. It may help in the identification of the given protein including utilizing its known migration time and effective mobility. Separation of six proteins (albumin, α-lactalbumin, insulin, carbonic anhydrase II, myoglobin, and ribonuclease B, each at 0.1 mg/mL concentration dissolved in 10 mM ammonium bicarbonate buffer) was carried out in 100 cm long LPA-coated FS capillary using 3% AcOH as BGE and aqueous 10% MeOH, 0.5% FA as the SL. Separation and spray voltages were set at +30 and +2 kV, respectively. Sequence coverages were greater than 75% for myoglobin and carbonic anhydrase II but much lower for proteins with disulfide bridges.

CE methods are frequently used for investigation of PTMs of peptides and proteins, such as phosphorylation, glycosylation, acetylation, methylation, glycation, and others [248–250]. PTM of an important protein, prostate-specific antigen (PSA), biomarker of increased risk of prostate cancer, was studied by CE–ESI–MS analysis of peptide fragments of PSA generated by tryptic digestion of PSA (isolated and immunopurified from urine by anti-PSA nanobodies and pretreated by reduction with dithiothreitol and alkylation with iodoacetamide) [251]. CE separation of peptides was performed in PEI coated FS capillary (30/150 μm id/od × 91 cm) with a porous tip at the outlet capillary end using 20% v/v (3.49 M) AcOH, pH 2.3, as BGE and −20 kV as separation voltage at 15°C. Sample volume was 41 nL, 6.3% of the total capillary length/volume. CE was hyphenated with ultrahigh resolution QQ–TOF–MS via sheathless CE–ESI–MS interface and nano-electrospray source. ESI voltage at positive ionization mode was +1350 V, nitrogen drying gas 1.2 L/min, quadrupole ion energy 5 eV, collision cell energy 5 eV, transfer time 120 μs; pre-pulse storage time 20 μs. The MS data were collected with a spectral acquisition frequency of 1 Hz within the m/z range 200–2200 for peptides and 600–3000 for the intact protein. Using this method, tri-, di-, mono-, and non-sialylated complex-type *N*-glycans were found on non-cleaved PSA as well as the non-glycosylated variant. The cleaved PSA showed that a similar relative abundance of glycoforms as non-cleaved PSA and the glycosylation profiles of intact protein was confirmed by more in-depth analysis of glycopeptides.

CE methods are widely used in monitoring of enzymatic digestions or conversions of peptides with the aim to study some details of these processes, such as drug metabolism, substrate or inhibition specificity, and/or

activity of enzymes and kinetics of their cleavage of peptides [246]. These studies include the determination of Michaelis–Menten kinetic data or the identification and characterization of inhibitors or substrates.

The CE-based enzyme assays are performed in two modes: (i) as precapillary assays with offline incubation of enzyme and substrate (and inhibitor) followed by CE analysis of substrate or product(s) [164, 252] and (ii) in-capillary assays, where the enzymatic reaction and analyte separation are performed in the same capillary [246, 253]. The latter mode is performed either as electrophoretically mediated microanalysis with enzyme, substrate and inhibitor mixing due to their different electromigration velocities [254, 255] or via their transverse diffusion of laminar flow profiles of their short in-series introduced closely neighboring zones [246, 256, 257].

Kinetic study of an important enzyme, prolyl hydroxylase 2, which is a key oxygen receptor regulating oxygen homeostasis and one of the targets for drug research for hypoxia related diseases, was based on MEKC separation and quantification of peptide substrate and product of enzymatic reaction [164]. A challenging separation of two peptides, octadecapeptides containing non-hydroxylated and hydroxylated proline residue in the middle of peptide chain, thus differing only by one oxygen atom, was achieved using 65 mM SDS as micellar pseudostationary phase and 65 mM sodium tetraborate with 10% ACN organic modifier, apparent pH 9.4, as MP. It enabled to determine Michaelis constant and maximum velocity of the enzymatic reaction, to screen the inhibitors of this enzyme, and to estimate their IC_{50} parameter (50% inhibition concentration). In several applications, the enzymatic reactions were carried out in IMERs that were directly connected with the CE separation [258].

A homemade CE device equipped with BFS capillary (75/365 μm id/od, 75/50 cm total/effective length) with semicircular bends and 25 mM sodium tetraborate BGE, pH 9.3, was applied to monitor hydrolysis of fluorescein labeled antimicrobial heptadecapeptide containing gelatinase cleavage sites by gelatinase secreted by *Staphylococcus aureus* [259]. The peptide substrate and one of the released peptide products were quantified by fluorescence detection (inverted fluorescence microscope) with excitation/emission wavelengths 492/516 nm. Linear dependence of the peak area of the peptide product on the concentration of *S. aureus* allowed application of this method for estimation of the content of *S. aureus* in the cell culture suspension.

The same BFS capillary, bent to 1, 2, or 4 semicircular-shaped turns with a 3-cm diameter and serving as a microreactor, was employed in a new assay for determination of PreScission protease activity in a microscale [260]. Due to different mobilities, the protease and the substrate (peptide labeled with ATTO 590 fluorescent dye and immobilized on GSH stabilized quantum dots) were mixed inside the bent capillary with sequential injections. The digestion of the peptide substrate was monitored by CE using 25 mM sodium tetraborate, pH 9.3, as BGE, 20 kV as separation voltage and Förster resonance energy transfer technology as the fluorescence detection.

In vitro simulated gastrointestinal digestion of two bovine collagen hydrolysates by pepsin (4% m/m in 100 mM HCl) was monitored by CE in BFS capillary (75 μm id × 60/53 cm total/effective length) using 100 mM phosphate buffer, pH 2.4, as BGE, at 20 kV separation voltage, 20°C, and 205 nm detection wavelength [241]. The two hydrolysates had distinct peptide profiles; 13 unidentified peptide peaks from each hydrolysate were not found in the other. No differences were found in AA content.

7.1.4 | Monitoring of molecular interactions and physicochemical changes

In addition to the previous chemical and enzymatic reactions of peptides, CE methods are often applied for studying of non-covalent molecular interactions of peptides with various ligands or receptors [261] and for monitoring of formation of covalent peptide adducts with other compounds, mostly antibodies or other carrier proteins [262]. Applications of different ACE methods for the investigation of the important interactions of drugs, including peptide drugs with serum proteins and related binding agents, can be found in a recent review [145].

CE–UV and CE–ICP–MS methods were applied to investigate non-covalent interactions of copper(II) ions with amyloid-β_{1-42} peptide [263]. CE–UV experiments were conducted in HPC coated FS capillaries (75 μm id × 64/54 cm total/effective length) using 10 mM Tris, 15 mM NaCl, pH 7.4 as BGE, at separation voltage of –20 kV, at 25°C, and detection wavelength of 200 nm. The analyses were assisted with pressure of 0.1 psi (689.5 Pa) to compensate the absence of EOF in the neutrally coated capillaries to speed up the CE analysis. Concentration of amyloid-β_{1-42} peptide species was quantified by their peak areas normalized by their migration times.

Hyphenation of CE with ICP–MS was implemented via a lab-made sheath-flow interface. The capillary goes through a microcross toward the nebulizer tip. The electrical connection is ensured by the ground electrode placed through the third inlet of the microcross. The SL (5 mM Tris, pH 7.4, containing 10 μM EDTA to prevent copper sorption on ICP-MS glass parts) was introduced by self-aspiration to the fourth inlet of the interface with flow rate about 450 μL/min. CE separations were carried out at 25°C, at –7 kV separation voltage assisted by 0.3 psi

(2068.5 Pa) pressure. Copper(II) was detected by ICP–MS at $m/z = 65$ with dwell time 1 s. ICP operating conditions were as follows: carrier gas flow rate 1 L/min, makeup gas flow rate 0.1 L/min, plasma gas flow rate 15 L/min, auxiliary gas flow rate 0.9 L/min, radio-frequency power 1550 W for the plasma. Quantification of copper was done by the integration of the Cu containing peaks. The results confirmed formation of several negatively charged copper complexes with aggregating beta-amyloid species and showed the enhancing aggregation rate with increasing copper concentration.

Degradation of α-amidated peptides, in particular 13 heptapeptides with C-terminal modification often used to improve half-life of therapeutic peptides, was monitored by CE–UV, LC–MS, and LC–MS/MS methods [112]. The CE–UV analyses were performed in PEO coated FS capillaries (50 μm id × 30/20 cm total/effective length) using 25 mM ionic strength sodium citrate buffer, pH 3.0, as BGE, detection wavelength of 214 nm and additional pressure 0.1 psi (689.5 Pa) to speed up the analysis. At separation voltage of 20 kV, current of ca. 27 μA, 25°C, the amidated and degradated (carboxylated) peptides were separated within a short time of ca. 5 min and provided complementary data for those obtained by LC–MS and LC–MS/MS methods.

Interactions of peptides with chiral selectors are described in Section 7.1.7.

CE methods are employed also for monitoring and evaluation of physicochemical processes and physical changes of peptides and proteins—aggregation, fragmentation, denaturation, and conformation changes during unfolding and folding processes [263].

7.1.5 | Amino acid and sequence analysis

CE methods can be employed also for the characterization and identification of peptides and proteins by their AA composition and AA sequence. A special comprehensive review summarizes the application of CE methods for the analysis of AAs in the last 20 years [264]. Various aspects of this topic, such as CE analysis of native and derivatized AAs with different types of detection (UV-absorption, fluorescence, contactless conductivity, MS) and the determination of AAs in different matrices including complete peptide and protein hydrolysates, are reported there. Another review article deals with the determination of branched-chain AAs by LC and CE methods [265].

AA sequences of CE separated peptides are usually obtained by their tandem MS detection [14, 207]. In fact, peptide and protein identifications in both bottom-up and top-down proteomic and peptidomic analyses are based on the determination of their AA sequences [16, 36, 266].

A special attention is paid to the determination of the less prevalent D-AAs in complex biological matrices, such as body fluids and tissue extracts [267]. They can serve as biomarkers of various human diseases, for example, some neurological pathologies, various cancers and kidney disorders. Chiral separations play a major role in their determination.

7.1.6 | Peptide mapping

High separation efficiency and resolving power make the CE methods suitable tools for peptide mapping (also called peptide finger printing) of proteins and polypeptides, that is, separation of peptide fragments created by the combination of specific chemical and/or enzymatic cleavage of proteins and polypeptides. The CE or LC record of such separation represents a characteristic map (finger print) of the particular protein or polypeptide. Peptide mapping is one of crucial techniques for protein/polypeptide identification, sequence determination of internal parts of proteins and polypeptides, monitoring of their PTMs and microheterogeneity, as well as for elucidation of protein and polypeptide structure [268]. Various aspects of peptide mapping of proteins by CE methods are presented and in detail discussed in the recent comprehensive review [42].

Due to the high complexity of peptide maps, namely of large proteins and biofluids, 1D- or 2D-CE hyphenated with MS detection are necessary for a complete resolution of peptides present in these complex mixtures (see Section 5.7). CE–ESI–MS/MS or CE–MALDI–MS/MS peptide mapping of proteins is now often used also for characterization and analysis of therapeutic mAbs [256]. Dadouch et al. elaborated a fast in-line methodology for the routine quality control of mAbs [257]. First, in the pretreatment step, the simultaneous denaturation and reduction with RapiGest surfactant and dithiothreitol, respectively, were carried out. Then, the tryptic digestion of mAb was performed in-line inside the capillary. Mixing of mAbs and trypsin resistant to autolysis in 100 mM NH_4HCO_3 as proteolysis buffer was realized by the transverse diffusion of laminar flow profiles under controlled temperature of 37.0°C. The released peptides were separated by CZE in a PEO coated FS capillary (30 μm id, 60/50 cm total/effective length) using aqueous 10% v/v AcOH as BGE, 15 kV separation voltage, and 214 nm detection wavelength. The whole methodology of CE-fingerprints was very fast (less than 2 h) and with good repeatability of migration times (RSD = 0.91% $N = 5$) and corrected peak areas (RSD = 9.6% $N = 5$). It was applied for the quality control of different commercial therapeutic mAbs and a mAb-drug conjugate.

Other types of peptide and protein maps are those (fingerprints) of particular biological fluid (blood serum and

plasma, urine, cerebrospinal fluid) or tissues. They are obtained as records of CE, LC, or MS separation and detection of peptides and proteins present in these complex biomatrices and can provide specific pattern (fingerprint) for the various states (standard vs. pathological) of people, animals, or plants, whose samples were analyzed. For example, urinary peptides were studied as potential noninvasive biomarkers for Lupus Nephritis using standard CE–MS method performed in commercially available Beckman Coulter Proteome Lab PA800 CE system online connected with Bruker Daltonic micrOTOF II MS detector [6].

Suitability of CE–MS methodology for the investigation of urinary peptide maps was confirmed in a special study that evaluated analytical performance of a large series of the CE–MS analyses of urine peptides with respect to the intra- and inter-day repeatability/reproducibility, variability and sensitivity of peptide detection, and characterization of the urinary peptidome [269].

7.1.7 | Chiral analysis and separation of stereoisomers

As peptides are composed of chiral building blocks, L- and D-AAs, the sequentially identical peptides may exist in different enantiomeric and diastereomeric configurations. This may result in their different biological activity and binding capability to chiral ligands or receptors. Hence, the chiral analysis of peptides and separation of their stereoisomers, especially in the case of peptide drugs, are very important. Thanks to their high separation efficiency and resolution power, short analysis times, and low consumption of not only sample but also chiral selectors and other BGE components, CE methods represent powerful tools for chiral analysis and separation of stereoisomers in general [163, 270–274] including chiral and stereoselective separations of peptides [232, 275]. Among the chiral selectors, native noncharged and especially the derivatized charged cyclodextrins still play the dominant role [276–278].

Single isomer CD derivative, heptakis(2,6-di-*O*-methyl)-*β*-CD, added at 15 mM concentration as a chiral selector into the BGE consisted of 100 mM phosphate buffer, pH 3.5, enabled enantioseparation of two polypeptide analogs of growth hormone-releasing hormone (sermorelin and CJC-1293) that differ by L- and D-configuration of only one AA, second L- or D-alanine of altogether 29 AA residues [94]. Separation was performed in an unusually long PB-coated FS capillary (110/100 cm total/effective length × 75 μm id) at separation voltage of –30 kV (cathode at the injection capillary end) and electric current of 70 μA. For a good repeatability of migration times and peak areas, the capillary was regenerated after each experiment by subsequent rinsing with 50 mM SDS for 7 min, water for 11 min, 100 mM NaOH for 5 min, 0.2% m/v PB in water for 5 min, and BGE for 7 min.

2-Hydroxypropyl-*β*-cyclodextrin (2-HP-*β*-CD) was found as a suitable stereoselector for chiral CE analysis of antimicrobial dipeptide *β*-Ala-Tyr isolated from the hemolymph of the fleshfly *Neobellieria bullata* and its synthetic amidated (*β*-Ala-D,L-Tyr-NH_2) and acetylated (N-Ac-*β*-Ala-D,L-Tyr) derivatives [279]. The baseline enantioseparation was obtained for all three pairs of enantiomers, see Figure 9. The best enantioseparation of *β*-Ala-D,L-Tyr was achieved in acidic BGE composed of 32 mM Tris, 50 mM H_3PO_4, pH 2.5, and containing 20 mg/mL of 2-HP-*β*-CD. The enantiomers of the amidated derivative *β*-Ala-D,L-Tyr-NH_2 were best separated in less acidic BGE consisting of 48 mM Tris, 50 mM H_3PO_4, pH 3.5, and containing 30 mg/mL of 2-HP-*β*-CD. The best enantioseparation of acetylated derivative (N-Ac-*β*-Ala-D,L-Tyr) was attained in alkaline BGE composed of 50 mM $Na_2B_4O_7$, 49 mM NaOH, pH 10.5, and 60 mg/mL 2-HP-*β*-CD. Chiral CE analyses of mixed samples of the isolated *β*-Ala–Tyr and synthetic standards of *β*-Ala-D-Tyr and *β*-Ala-L-Tyr have shown that the isolated *β*-Ala-Tyr was a pure *β*-Ala-L-Tyr enantiomer, see Figure 10.

The effect of chiral ionic liquids composed of tetraalkylammonium ions and the AAs (Asn, Asp, and Pro) on the CD-mediated CE enantioseparations of dipeptides Ala–Phe, Ala–Tyr, and Phe was studied by Konjaria and Scriba [280]. The separations were performed in BFS capillary (50/365 μm id/od × 50.2/40 cm total/effective length) at 25°C in BGE consisted of 50 mM sodium phosphate, pH 2.5 or 3.5, at 25 kV separation voltage, and 215 nm detection wavelength. The addition of a chiral ionic liquid to the BGE resulted in a higher, concentration-dependent enantioresolution of peptides than in the case of the BGE containing only alone *β*-CD or 2-HP-*β*-CD chiral selectors. The increase of resolution varied with both the tetraalkylammonium cation (tetramethylammonium and tetrabutylammonium) and the previous AA component of the ionic liquid. The presence ionic liquids in the BGE did not interfere with the pH-dependent reversal of the enantiomer migration order of the dipeptides when changing pH of BGE from 2.5 to 3.5.

In addition to separation of peptide enantiomers and diastereomers, CE with UV or MS detection can separate also positional and linkage isomers of glycopeptides and glycans generated by enzymatic cleavage of glycosylated proteins [281]. The separation and characterization of all the isomeric forms of glycans and glycopeptides is very important for the monitoring of glycoprotein- and glycosylation-related diseases, for example, prostate cancer [251].

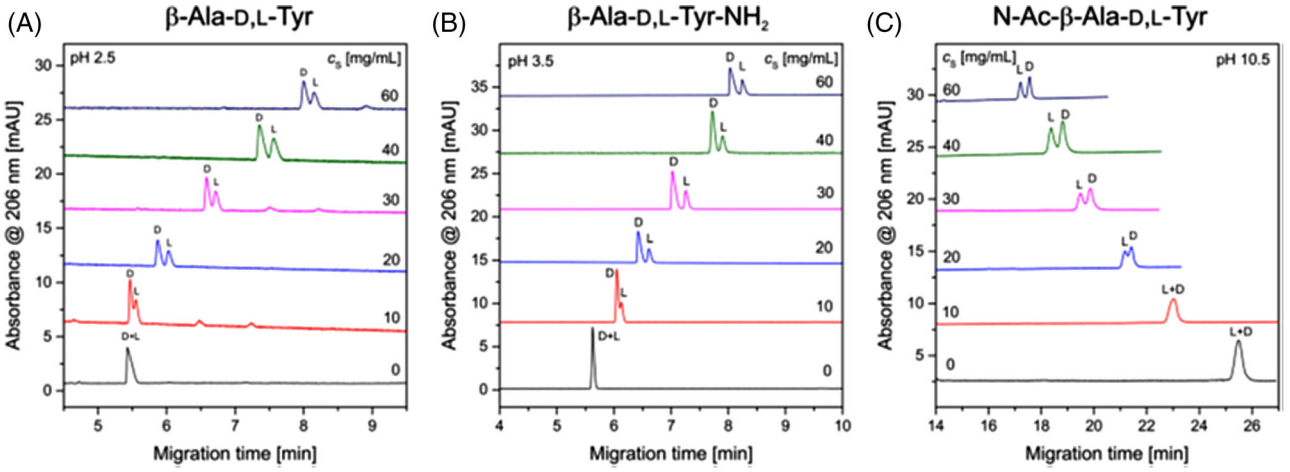

FIGURE 9 Records of capillary electrophoresis (CE) analyses of (A) β-Ala-D,L-Tyr in BGE composed of 32 mM Tris, 50 mM H_3PO_4, pH 2.5; (B) β-Ala-D,L-Tyr-NH_2 in BGE consisted of 48 mM Tris, 50 mM H_3PO_4, pH 3.5; and (C) *N*-Ac-β-Ala-D,L-Tyr in BGE containing 50 mM $Na_2B_4O_7$, 49 mM NaOH, pH 10.5, at increasing concentration of the chiral selector 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD) (0–60 mg/mL). *Source*: Reprinted with permission from Ref. [279].

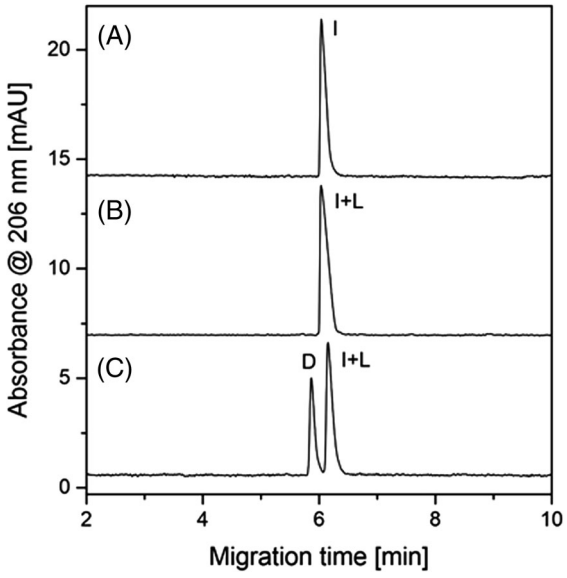

FIGURE 10 Capillary electrophoresis (CE) analysis of (A) isolated β-Ala–Tyr (peak I), (B) mixed sample of isolated β-Ala–Tyr and standard synthetic L-enantiomer (β-Ala-L-Tyr) (peak I + L), and (C) mixed sample of isolated β-Ala–Tyr and the standards of synthetic L- and D-enantiomers (β-Ala-L-Tyr and β-Ala-D-Tyr) in the background electrolyte (BGE) II, 32 mM Tris, 50 mM H_3PO_4, pH 2.5, 20 mg/mL 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD); U_{sep} = 25 kV, I_{sep} = 39.5 μA, injection 6.9 mbar, 5 s. *Source*: Reprinted with permission from Ref. [279].

Stereochemical configuration of three neuropeptides derived from the pleurin precursor in individual neurons isolated from the central nervous system of *Aplysia californica* was determined by CE coupled to trapped IMS MS (CE–TIMS–MS) [232]. Analysis of the mobility profiles has shown that more than 98% of the pleurin-derived peptides occur in non-isomerized, all-L forms in the individual neuron cells but 44% of the Plrn2 peptide was found to be present in the isomerized, D-residue-containing form in the nerve tissue. The CE separations were conducted in BFS capillary (75–80 cm long, 40/105 μm id/od), in the BGE containing 0.75% FA and 25% ACN, at 25 kV separation voltage. The lab-made CE–ESI interface was connected to a timsTOF Pro mass spectrometer. The emitter was grounded, and the ion guide capillary voltage of the MS detector was set 2000 V. The SL was aqueous solution of 50% MeOH and 0.1% FA. This setup proved to be a powerful tool capable to investigate peptide stereochemistry at the single cell level. Chiral separation is important especially in the determination of D-AAs in total hydrolysates of peptides and proteins because D-AAs can serve as specific metabolic biomarkers for detection of various pathological states and for evaluation of their recovery or progress [267].

Peptides are not only the subject of chiral CE separations, and they are used also as chiral selectors for enantioseparation of other chiral compounds. The most important peptide chiral selectors are macrocyclic glycopeptide antibiotics, vancomycin, ristocetin, teicoplanin, eromycin, and their fragments and analogs. They are used for a broad class of chiral separations both in electromigration and chromatographic techniques [282]. For example, vancomycin non-covalently or covalently attached to the inner FS capillary wall via photosensitive diazo resin as the coupling agent was applied for CE separation of enantiomers of chiral drug promethazine and three other chiral compounds (chlorpheniramine, benzoin, and mandelic acid) [283].

7.2 | Micropreparative separations

The application of CE techniques for preparative separations of peptides as well as of other (bio)molecules is much less than for their analyses and characterizations. This is caused by: (i) inherently low preparative capacity of the narrow bore capillaries with id less than 100 μm and (ii) more complicated adaptation of CE devices for preparative purposes ones than in LC systems. The latter problem, resulting from dipping of both capillary ends and electrodes into the BGE in the electrode vessels, by means of which the separation voltage is introduced in the capillary, was solved by special modifications of the CE devices. These modifications and some protocols elaborated for fraction collections from the capillary were reported in the earlier reviews [284, 285].

For peptide preparation and purification, CE methods are used mostly indirectly. They are utilized for the analysis of peptides separated and purified by other methods, mostly preparative LC, sometimes also by preparative free-flow electrophoresis. Direct application of CE methods allows only microscale isolation of a peptide or other compound of interest. In commercial apparatuses, the autosamplers are adapted to fraction collectors, and the electro- or pressure-driven flows are employed utilized for the elution of the particular compounds from the capillary.

The problem of limited preparative capacity of CE (mostly less than 1 μg per run) can be partially solved by (i) enlarging the capillary id (but compromising separation efficiency), (ii) repetitive fraction collection, and (iii) using the multicapillary systems. Multiple separations in a single narrow bore capillary and pooling of the fractions with the same mobility are used for the isolation of peptides prior to their subsequent AA or MS analysis for M_r determination and identification. Such approach was applied for micropreparative separation of peptide fragments generated by tryptic digestion of human serum proteins or proteins of the *Escherichia coli* cell lysate or conditioned cell media [286]. The CE separations were performed in BFS capillary with a wider id of 100 μm (375 μm od) and relatively short total/effective length of 30/20 cm thermostated at 25°C using 1 M AcOH as BGE, 7 kV as separation voltage, and 214 nm as detection wavelength. Fraction collection windows were selected by the calculation of migration velocity of peptides and extrapolating the migration time at detector to the migration time at the capillary outlet end. The fractions were collected by positioning the capillary outlet in the particular collection microvials throughout the experiment. The volume in the inlet and outlet vials during fraction collection was 125 μL. In the 8-times repeated experiments, a total amount of 2 μg of peptides was obtained with average mass of 400 ng of peptide per fraction. This peptide fractionation significantly improved identification of peptides and proteins in bottom-up proteomics of *E. coli* lysate, with ca. 185% more identified peptides and ca. 130% more identified proteins.

Microscale isolation of peptides is performed in the offline coupling of CE with MALDI–MS detection. Nanoliter volume fractions are collected on the MALDI compatible plates or membranes and subsequently analyzed by MALDI–MS [287, 288].

Substantial increase of preparative capacity can be achieved by the conversion of analytical CE separations into the preparative FFZE mode following the earlier developed procedures [289, 290]. This approach was applied for the final purification of the antimicrobial dipeptide β-Ala-Tyr isolated from the extract of the hemolymph of larvae of the fleshfly *Neobellieria bullata* and separated from the other extract components by RP-HPLC [189]. In FFZE, the two remaining admixtures of this dipeptide were separated, and pure dipeptide β-Ala-Tyr was obtained. Its electrophoretic homogeneity was confirmed by a single peak in the CZE analysis of the final product.

Other recent applications of various electromigration methods in the free-flow instrumental format for middle scale isolation of biomolecules including peptides are reported in recent reviews [291, 292]. FFZE implemented in microfluidic devices can also be used for microscale isolation of peptides and other biomolecules [293]. However, due to their limited preparative capacity, they are more suitable for continuous monitoring of selected analytes than for real preparative purposes.

7.3 | Physicochemical characterization

Besides many analytical applications, CE methods are frequently utilized also for the determination of several important physicochemical characteristics of (bio)molecules, including peptides, such as effective electrophoretic mobilities, actual and limiting (absolute) ionic mobilities, effective charges, p*I*s, M_rs, Stokes radii, diffusion coefficients, partition constants/coefficients (log $P_{o/w}$), distribution constants/coefficients (log $D_{o/w}$), acidity (ionization) constants (pK_a) of peptide ionogenic groups, binding (association, stability, formation, and complexation) or dissociation constants of peptide complexes, and Gibbs energy, enthalpy, entropy, rate constants, and activation energy of their chemical and enzymatic reactions and interactions [294].

CZE in sieving media (CSE or CGE) [123–126] provides data on the size (hydrodynamic radius) or mass (M_r) of the separated analytes. The M_r of polypeptides and proteins can be estimated from CGE of their complexes with SDS (capillary version of SDS–PAGE) [127]. Stokes radii

and effective (net) charges of peptides can be assessed from the effective mobilities measured by CZE and from the models relating peptide mobility and their charge/size ratio [49, 50]. Nevertheless, due to the approximate character of these models, only approximate values of charge, size (Stokes radius), and mass (M_r) are obtained from these measurements. Another important peptide/protein parameter related to the charge distribution in the amphoteric molecules, p*I*, can be determined by CIEF [59, 62] or by CZE [59, 63, 64]. However, also in this case, one should take into account that the obtained p*I* values are dependent on the composition of carrier ampholytes in CIEF or BGEs in CZE, and other experimental conditions.

Effective mobilities of Aβ_{1-42} peptide preparations prepared under self-aggregation conditions (incubated 48 h in pH 7.4 Tris buffer at room temperature) and under conditions limiting the aggregation (freshly dissolved in 0.1% NH_4OH) were determined by pressure assisted CZE-UV method in HPC-coated FS capillary (75 μm id × 64/54 cm total/effective length) using BGE composed of 10 mM Tris, 15 mM NaCl, pH 7.4, and separation voltage of –20 kV at 25°C [263]. Two values of mobilities were found to be equal to -20×10^{-9} m^2/V/s and -10×10^{-9} m^2/V/s. These values and the diameters of these populations determined by Taylor dispersion analysis (6.6 and 1.3 nm, respectively) helped to distinguish two populations in this peptide preparation. The first one, faster migration population with mobility of -20×10^{-9} m^2/V/s and larger diameter of 6.6 nm, was attributed to large oligomers, and the second one, slower migrating population with mobility of -10×10^{-9} m^2/V/s, was attributed to monomers and small oligomers.

Various ACE modes are widely applied for determination of the association or dissociation constants of biomolecular complexes with different types of both low- and high-molecular-mass ligands including peptides, for a survey, see the recent [144, 145, 147] or the earlier reviews [261, 295].

CE–ICP–MS method was applied for the determination of the affinity (binding) constant of the complex of a short beta-amyloid peptide 1–16 (Aβ_{1-16}) with copper(II) cation [296]. The affinity constant was determined by competition assays using nitrilotriacetic acid (NTA) as a suitable competitive chelator that forms with Cu(II) ion complex of approximately the same strength as the Aβ_{1-16} peptide but has a different mobility than the peptide-Cu(II) complex making possible to separate and detect both complexes by CE–ICP–MS. The mobilities of both complexes were determined by ACE and were found to be equal to -10×10^{-9} m^2/V/s for (Aβ_{1-16})-Cu(II) complex and -25×10^{-9} m^2/V/s for NTA–Cu(II) complex, being well different between themselves as well as being different from the mobilities of the free ligands, -8×10^{-9} m^2/V/s for Aβ_{1-16} and -50×10^{-9} m^2/V/s for NTA.

The determination of the binding constant of (Aβ_{1-16})-Cu(II) complex was based on the comparison of the affinity of Aβ_{1-16} and NTA for Cu^{2+} cations (added in the form of $CuCl_2$). Various solutions containing 10 μM $CuCl_2$, 100 μM Aβ_{1-16} and increasing concentration of NTA were analyzed by CE–ICP–MS after 2 h incubation at room temperature. Experiments were done in two BGEs (10 mM Tris, 15 mM NaCl, or 10 mM HEPES, 15 mM NaCl, both at pH 7.4) and in two types of FS capillaries (neutrally HPC coated or positively PB coated, both 64 cm long with 75/375 μm id/od), at –7 kV and pressure 0.3 psi (2068.5 Pa) in the former and at –15 kV in the latter capillary. For all experimental conditions, the distribution of Cu(II) between the two complex species was determined by ICP-MS where copper was detected at $m/z = 65$. Tris buffer was evaluated as unsuitable for the binding constant (K_b) determination as Tris increased dissociation of the copper complexes. Using the HEPES BGE and the PB- and HPC-coated capillaries, the Aβ_{1-16}–Cu complex was found to be very strong, with log K_b equal to ca. 9.9 in both types of capillaries.

The average apparent binding constants and the actual ionic mobilities of the complexes of the antimicrobial dipeptide β-Ala–Tyr and its amidated (β-Ala–Tyr–NH_2) and acetylated (*N*-Ac-β-Ala-Tyr) derivatives with chiral selector, 2-HP-β-CD, were determined by the mobility shift mode of the ACE [279]. The binding constants and the ionic mobilities of the complexes were obtained by nonlinear regression analysis of the dependence of effective mobilities of the dipeptide β-Ala–Tyr and its derivatives on the concentration of 2-HP-β-CD in the BGE. The complexes were rather weak, with the binding constants in the range 11.0–79.1 L/mol.

In addition to the determination of thermodynamic parameters, also kinetic characteristics of reactions and interactions can be estimated by CE methods [155, 156, 297, 298].

8 | CONCLUDING REMARKS AND FUTURE PROSPECTS

As shown in above sections, CE and MCE methods represent powerful techniques for analytical and micropreparative separations and characterizations of peptides. Nowadays, these methods are recognized/appreciated as valuable complements and/or counterparts of the other high-performance separation methods, especially (U)HPLC and nano-LC. Wide applicability of CE and MCE methods in chemistry and biochemistry of peptides is confirmed by their usage not only as high-efficient and high-sensitive analytical techniques, able to determine femtomole to zeptomole amounts of peptides in nano- to picoliter injected sample volume of complex biological matrices but also

as highly beneficial physicochemical methods providing important physicochemical and biochemical characteristics of peptides.

Peptides belong to the most important biologically active compounds with essential role in many vitally relevant physiological processes. Many of them and their functions have been identified but even more have to be revealed and elucidated. For a more detailed understanding of biological processes and diseases, a comprehensive investigation of a whole peptide set of a cell, organ or organism (peptidome) have to be performed. Moreover, the structure and function of proteins in proteomic studies are often explored by means of their peptide fragments. Hence, the analysis, purification and characterization of peptides will undoubtedly remain one of the most challenging issues of CE and MCE methods also in the next years. Concerning these methods themselves, their development will continue in the direction of further miniaturization, that is, to implementation in microfluidic devices and integration into micro-total analytical systems, online coupled with high-sensitive and high-resolution tandem MS or LIF detection. 2D and multidimensional separations based on combination of orthogonal principles of CE and LC methods, and their hyphenation with tandem MS detection will be necessary for analysis and identification of peptides and proteins in their complex mixtures in comprehensive peptidomic and proteomic studies.

ACKNOWLEDGMENTS

Some of the presented results were obtained with the support of the Czech Science Foundation, Grant no. 20-03899S, and by the Czech Academy of Sciences, research project RVO 61388963. The author thanks his coworkers, P. Sázelová, V. Šolínová, S. Štěpánová, R. Konášová, and D. Koval, for their help in preparation of this manuscript and for their cooperation in some of the presented results.

Open access funding provided by CzechELib.

CONFLICT OF INTEREST STATEMENT

The author has declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Václav Kašička https://orcid.org/0000-0003-1719-1432

REFERENCES

1. Muttenthaler M, King GE, Adams DJ, Alewood PE. Trends in peptide drug discovery. Nat Rev Drug Discov. 2021;20:309–25.
2. Manniello MD, Moretta A, Salvia R, Scieuzo C, Lucchetti D, Vogel H, et al. Insect antimicrobial peptides: potential weapons to counteract the antibiotic resistance. Cell Mol Life Sci. 2021;78:4259–82.
3. d'Orlye F, Trapiella-Alfonso L, Lescot C, Pinvidic M, Doan BT, Varenne A. Synthesis, characterization and evaluation of peptide nanostructures for biomedical applications. Molecules. 2021;26:4587.
4. Datta S, Roy A. Antimicrobial peptides as potential therapeutic agents: a review. Int J Peptide Res Therapeut. 2021;27:555–77.
5. Nguyen NV, Taverna M, Smadja C, Mai TD. Recent electrokinetic and microfluidic strategies for detection of amyloid beta peptide biomarkers: towards molecular diagnosis of Alzheimer's disease. Chem Rec. 2021;21:149–61.
6. Tailliar M, Schanstra JP, Dierckx T, Breuil B, Hanouna G, Charles N, et al. Urinary peptides as potential non-invasive biomarkers for lupus nephritis: results of the peptidu-LUP study. J Clin Med. 2021;10:1690.
7. Boschetti E, Zilberstein G, Righetti PG. Combinatorial peptides: a library that continuously probes low-abundance proteins. Electrophoresis. 2022;43:355–69.
8. Zhang W, Ramautar R. CE–MS for metabolomics: developments and applications in the period 2018–2020. Electrophoresis. 2021;42:381–401.
9. Mamani-Huanca M, Villasenor A, Gonzalez-Riano C, Lopez-Lopez A, Lopez-Gonzalvez A, Barbas C. Capillary electrophoresis mass spectrometry-based untargeted metabolomics to approach disease diagnosis. TrAC Trends Anal Chem. 2023;162:117049.
10. Zhang YY, Fonslow BR, Shan B, Baek MC, Yates JR. Protein analysis by shotgun/bottom-up proteomics. Chem Rev. 2013;113:2343–94.
11. Miller RM, Smith LM. Overview and considerations in bottom-up proteomics. Analyst. 2022;148:475.
12. Vitorino R, Guedes S, da Costa JP, Kasicka V. Microfluidics for peptidomics, proteomics, and cell analysis. Nanomaterials. 2021;11:1118.
13. Stepanova S, Kasicka V. Recent developments and applications of capillary and microchip electrophoresis in proteomics and peptidomics (mid–2018–2022). J Sep Sci. 2023;46:e2300043.
14. Miksik I. Coupling of CE–MS for protein and peptide analysis. J Sep Sci. 2019;42:385–97.
15. Lian ZR, Wang N, Tian YW, Huang LH. Characterization of synthetic peptide therapeutics using liquid chromatography–mass spectrometry: challenges, solutions, pitfalls, and future perspectives. J Am Soc Mass Spectrom. 2021;32:1852–60.
16. Chen DY, McCool EN, Yang ZC, Shen XJ, Lubeckyj RA, Xu T, et al. Recent advances (2019–2021) of capillary electrophoresis–mass spectrometry for multilevel proteomics. Mass Spectrom Rev. 2021;42:617–42.
17. Hynek R, Michalus I, Cejnar P, Santrucek J, Seidlova S, Kuckova S, et al. In-bone protein digestion followed by LC–MS/MS peptide analysis as a new way towards the routine proteomic characterization of human maxillary and mandibular bone tissue in oral surgery. Electrophoresis. 2021;42:2552–62.
18. Michalus I, Nguyen TV, Viktorova J, Cejnar P, Santrucek J, Kuckova S, et al. Liquid chromatography–tandem mass spectrometry analysis of peptides separated from the insoluble matrix by in-sample tryptic protein digestion for rapid

discrimination of various induced pathological states of human bone models in oral surgery. J Sep Sci. 2022;45:4388–96.
19. Stoll DR, Sylvester M, Euerby MR, Buckenmaier SMC, Petersson P. A strategy for assessing peak purity of pharmaceutical peptides in reversed-phase chromatography methods using two-dimensional liquid chromatography coupled to mass spectrometry. Part II: Development of second-dimension gradient conditions. J Chromatogr A. 2023;1693:463873.
20. Schrader M. Origins, technological development, and applications of peptidomics. Methods Mol Biol. 2018;1719:3–39.
21. Vitorino R, Choudhury M, Guedes S, Ferreira R, Thongboonkerd V, Sharma L, et al. Peptidomics and proteogenomics: background, challenges and future needs. Exp Rev Proteomics. 2021;18:643–59.
22. Stepanova S, Kasicka V. Peptidomics and capillary electrophoresis. In: Colnaghi Simionato AV, editor. Separation techniques applied to omics sciences: from principles to relevant applications. Cham: Springer; 2021. p. 87–104.
23. Foreman RE, George AL, Reimann F, Gribble FM, Kay RG. Peptidomics: a review of clinical applications and methodologies. J Proteome Res. 2021;20:3782–97.
24. Kasicka V. Capillary electrophoresis of peptides. Electrophoresis. 1999;20:3084–105.
25. Kasicka V. Recent advances in capillary electrophoresis of peptides. Electrophoresis. 2001;22:4139–62.
26. Kasicka V. Recent advances in capillary electrophoresis and capillary electrochromatography of peptides. Electrophoresis. 2003;24:4013–46.
27. Kasicka V. Recent developments in capillary electrophoresis and capillary electrochromatography of peptides. Electrophoresis. 2006;27:142–75.
28. Kasicka V. Recent developments in CE and CEC of peptides. Electrophoresis. 2008;29:179–206.
29. Kasicka V. Recent advances in CE and CEC of peptides (2007–2009). Electrophoresis. 2010;31:122–46.
30. Kasicka V. Recent developments in CE and CEC of peptides (2009–2011). Electrophoresis. 2012;33:48–73.
31. Kasicka V. Recent developments in capillary and microchip electroseparations of peptides (2011–2013). Electrophoresis. 2014;35:69–95.
32. Kasicka V. Recent developments in capillary and microchip electroseparations of peptides (2013–middle 2015). Electrophoresis. 2016;37:162–88.
33. Kasicka V. Recent developments in capillary and microchip electroseparations of peptides (2015–mid 2017). Electrophoresis. 2018;39:209–34.
34. Kasicka V. Recent developments in capillary and microchip electroseparations of peptides (2017–mid 2019). Electrophoresis. 2020;41:10–35.
35. Kasicka V. Recent developments in capillary and microchip electroseparations of peptides (2019–mid 2021). Electrophoresis. 2022;43:82–108.
36. Chen DY, Yang ZC, Shen XJ, Sun LL. Capillary zone electrophoresis–tandem mass spectrometry as an alternative to liquid chromatography–tandem mass spectrometry for top-down proteomics of histones. Anal Chem. 2021;93:4417–24.
37. Lenco J, Jadeja S, Naplekov DK, Krokhin OV, Khalikova MA, Chocholous P, et al. Reversed-phase liquid chromatography of peptides for bottom-up proteomics: a tutorial. J Proteome Res. 2022;21:2846–92.
38. Liu ZL, Xu MM, Zhang WQ, Miao XY, Wang PG, Li SW, et al. Recent development in hydrophilic interaction liquid chromatography stationary materials for glycopeptide analysis. Anal Methods. 2022;14:4437–48.
39. Gao ZT, Zhong WW. Recent (2018–2020) development in capillary electrophoresis. Anal Bioanal Chem. 2022;414:115–30.
40. Battat S, Weitz DA, Whitesides GM. An outlook on microfluidics: the promise and the challenge. Lab Chip. 2022;22:530–6.
41. Stepanova S, Kasicka V. Applications of capillary electromigration methods for separation and analysis of proteins (2017–mid 2021): a review. Anal Chim Acta. 2022;1209:339447.
42. Kasicka V. Peptide mapping of proteins by capillary electromigration methods. J Sep Sci. 2022;45:4245–79.
43. Colnaghi Simionato AV. (editor). Separation techniques applied to omics sciences: from principles to relevant applications. Cham: Springer; 2021.
44. Ramautar R, Chen DDY. Capillary electrophoresis–mass spectrometry (CE–MS) for proteomics and metabolomics: principles and applications. Weinheim: Wiley-VCH; 2022.
45. Stepanova S, Kasicka V. Analysis of peptides by capillary electromigration methods. In: Castro-Puyana M, Herrero M, Marina ML, editors. Capillary electrophoresis in food analysis. Singapore: Bentham Science Publishers; 2022. p. 109–46.
46. Stepanova S, Kasicka, V. CE-MS approaches for peptidomics. In: Ramautar R, Chen DDY, editors. Capillary electrophoresis–mass spectrometry (CE-MS) for proteomics and metabolomics: principles and applications. Weinheim: Wiley-VCH; 2022. p. 235–59.
47. Chen WR, McCool EN, Sun LL, Zang Y, Ning X, Liu XW. Evaluation of machine learning models for proteoform retention and migration time prediction in top-down mass spectrometry. J Proteome Res. 2022;21:1736–47.
48. Chen DY, Lubeckyj RA, Yang ZC, McCool EN, Shen XJ, Wang QJ, et al. Predicting electrophoretic mobility of proteoforms for large-scale top-down proteomics. Anal Chem. 2020;92:3503–7.
49. Cifuentes A, Poppe H. Behvior of peptides in capillary electrophoresis: effect of peptide charge, mass and structure. Electrophoresis. 1997;18:2362–76.
50. Solinova V, Kasicka V, Koval D, Hlavacek J. Separation and investigation of structure-mobility relationships of insect oostatic peptides by capillary zone electrophoresis. Electrophoresis. 2004;25:2299–308.
51. Petrov AP, Sherman LM, Camden JP, Dovichi NJ. Database of free solution mobilities for 276 metabolites. Talanta. 2020;209:120545.
52. Mamani-Huanca M, de la Fuente AG, Otero A, Gradillas A, Godzien J, Barbas C, et al. Enhancing confidence of metabolite annotation in capillary electrophoresis-mass spectrometry untargeted metabolomics with relative migration time and in-source fragmentation. J Chromatogr A. 2021;1635:461758.
53. Persat A, Chambers RD, Santiago JG. Basic principles of electrolyte chemistry for microfluidic electrokinetics. Part I: Acid-base equilibria and pH buffers. Lab Chip. 2009;9:2437–53.
54. Persat A, Suss ME, Santiago JG. Basic principles of electrolyte chemistry for microfluidic electrokinetics. Part II: Coupling between ion mobility, electrolysis, and acid-base equilibria. Lab Chip. 2009;9:2454–69.
55. Corradini D. Buffering agents and additives for the background electrolyte solutions used for peptide and protein capillary zone electrophoresis. TrAC Trends Anal Chem. 2023;164:117080.

56. Kasicka V, Prusik Z. Application of capillary isotachophoresis in peptide analysis. J Chromatogr. 1991;569:123–74.
57. Janini GM, Issaq HJ. Selection of buffers in capillary zone electrophoresis: application to peptide and protein analysis. Chromatographia. 2001;53:S18–26.
58. Scriba GKE, Psurek A. Separation of peptides by capillary electrophoresis. In: Schmitt-Kopplin P. (editor). Capillary Electrophoresis. Totowa, NJ: Humana Press; 2008. p. 483–506.
59. Righetti PG, Sebastiano R, Citterio A. Capillary electrophoresis and isoelectric focusing in peptide and protein analysis. Proteomics. 2013;13:325–40.
60. Kozlowski LP. Proteome-pI 20: proteome isoelectric point database update. Nucleic Acids Res. 2022;50:D1535–40.
61. Kozlowski LP. IPC 20: prediction of isoelectric point and pK(a) dissociation constants. Nucleic Acids Res. 2021;49:W285–92.
62. Pergande MR, Cologna SM. Isoelectric point separations of peptides and proteins. Proteomes. 2017;5:4–14.
63. Solinova V, Poitevin M, Koval D, Busnel JM, Peltre G, Kasicka V. Capillary electrophoresis in classical and carrier ampholytes-based background electrolytes applied to separation and characterization of gonadotropin-releasing hormones. J Chromatogr A. 2012;1267:231–8.
64. Solinova V, Kasicka V. Determination of acidity constants and ionic mobilities of polyprotic peptide hormones by CZE. Electrophoresis. 2013;34:2655–65.
65. Gas B. PeakMaster and Simul—Software tools for mastering electrophoresis. TrAC Trends Anal Chem. 2023;165:117134.
66. Avaro AS, Sun YX, Jiang KY, Bahga SS, Santiago JG. Web-based open-source tool for isotachophoresis. Anal Chem. 2021;93:15768–74.
67. Francois YN, Biacchi M, Gahoual R, Vezin A, Pansanel J. CEToolbox: specialized calculator for capillary electrophoresis users as an android application. Electrophoresis. 2021;42:1431–5.
68. John AS, Sidek MM, Thang LY, Sami S, Tey HY, See HH. Online sample preconcentration techniques in nonaqueous capillary and microchip electrophoresis. J Chromatogr A. 2021;1638:461868.
69. Thomas SL, Thacker JB, Schug KA, Marakova K. Sample preparation and fractionation techniques for intact proteins for mass spectrometric analysis. J Sep Sci. 2021;44:211–46.
70. Varnavides G, Madern M, Anrather D, Hartl N, Reiter W, Hartl M. In Search of a universal method: a comparative survey of bottom-up proteomics sample preparation methods. J Proteome Res. 2022;21:2397–411.
71. Badawy MEI, El-Nouby MAM, Kimani PK, Lim LW, Rabea EI. A review of the modern principles and applications of solid-phase extraction techniques in chromatographic analysis. Anal Sci. 2022;38:1457–87.
72. Poole CF. Selectivity evaluation of extraction systems. J Chromatogr A. 2023;1695:463939.
73. Yu RB, Quirino JP. Pseudophase-aided in-line sample concentration for capillary electrophoresis. TrAC Trends Anal Chem. 2023;161:116914.
74. Jeong S, Hajba L, Guttman A, Seol J, Chung DS. In-line microextraction techniques to improve the sensitivity and selectivity of capillary electrophoresis using commercial instruments. TrAC Trends Anal Chem. 2023;163:117058.
75. Tuma P. Progress in on-line, at-line, and in-line coupling of sample treatment with capillary and microchip electrophoresis over the past 10 years: a review. Anal Chim Acta. 2023;1261:341249.
76. Zheng WM, Yang PY, Sun CY, Zhang Y. Comprehensive comparison of sample preparation workflows for proteomics. Mol Omics. 2022;18:555–67.
77. Wang MM, Chen ZY, Jing XF, Zhou H, Wang Y, Ye JN, et al. Tween 20-capped gold nanoparticles for selective extraction of free low-molecular-weight thiols in saliva followed by capillary electrophoresis with contactless conductivity detection. J Chromatogr B: Anal Technol Biomed Life Sci. 2021;1176:122756.
78. Irfan A, Feng WX, Liu KX, Habib K, Qu Q, Yang L. TiO_2-modified fibrous core-shell mesoporous material to selectively enrich endogenous phosphopeptides with proteins exclusion prior to CE-MS analysis. Talanta. 2021;235:122737.
79. El Ouahabi O, Mancera-Arteu M, Pont L, Gimenez E, Sanz-Nebot V, Benavente F. On-line solid-phase extraction to enhance sensitivity in peptide biomarker analysis by microseparation techniques coupled to mass spectrometry: capillary liquid chromatography versus capillary electrophoresis. Microchem J. 2022;183:108089.
80. Morgenstern D, Wolf-Levy H, Tickotsky-Moskovitz N, Cooper I, Buchman AS, Bennett DA, et al. Optimized glycopeptide enrichment method-it is all about the sauce. Anal Chem. 2022;94:10308–13.
81. Mancera-Arteu M, Benavente F, Sanz-Nebot V, Gimenez E. Sensitive analysis of recombinant human erythropoietin glycopeptides by on-line phenylboronic acid solid-phase extraction capillary electrophoresis mass spectrometry. J Proteome Res. 2023;22:826–36.
82. Thevendran R, Citartan M. Assays to estimate the binding affinity of aptamers. Talanta. 2022;238:122971.
83. Guzman NA, Guzman DE, Blanc T. Advancements in portable instruments based on affinity-capture-migration and affinity-capture-separation for use in clinical testing and life science applications. J Chromatogr A. 2023;1704:464109.
84. Guzman NA, Guzman DE. Immunoaffinity capillary electrophoresis in the era of proteoforms, liquid biopsy and preventive medicine: a potential impact in the diagnosis and monitoring of disease progression. Biomolecules. 2021;11:1443.
85. Halvorsen TG, McKitterick N, Kish M, Reubsaet L. Affinity capture in bottom-up protein analysis—overview of current status of proteolytic peptide capture using antibodies and molecularly imprinted polymers. Anal Chim Acta. 2021;1182:338714.
86. Salim H, Pero-Gascon R, Gimenez E, Benavente F. On-line coupling of aptamer affinity solid-phase extraction and immobilized enzyme microreactor capillary electrophoresis-mass spectrometry for the sensitive targeted bottom-up analysis of protein biomarkers. Anal Chem. 2022;94:6948–56.
87. Slampova A, Mala Z, Gebauer P. Recent progress of sample stacking in capillary electrophoresis (2016–2018). Electrophoresis. 2019;40:40–54.
88. Kitagawa F, Soma Y. Recent applications of dynamic on-line sample preconcentration techniques in capillary electrophoresis. Chromatography. 2022;43:93–99.
89. Malak M, Ebrahim H, Sonbol H, Ali A, Aboulella Y, Hadad G, et al. Highly sensitive in-capillary derivatization and

field amplified sample stacking to analyze narcotic drugs in human serum by capillary zone electrophoresis. Separations. 2023;10:58.
90. Purgat K, Koska I, Kubalczyk P. The use of single drop microextraction and field amplified sample injection for CZE determination of homocysteine thiolactone in urine. Molecules. 2021;26:5687.
91. Piestansky J, Cizmarova I, Stefanik O, Matuskova M, Horniakova A, Majerova P, et al. Capillary electrophoresis-mass spectrometry with multisegment injection and in-capillary preconcentration for high-throughput and sensitive determination of therapeutic decapeptide triptorelin in pharmaceutical and biological matrices. Biomedicines. 2021;9:1488.
92. Kitagawa F, Hayashi A, Nukatsuka I. LVSEP analysis of phosphopeptides in dynamically PVP-coated capillaries and microchannels. Chromatography. 2022;43:37–41.
93. DeLaney K, Jia DS, Iyer L, Yu Z, Choi SB, Marvar PJ, et al. Microanalysis of brain angiotensin peptides using ultrasensitive capillary electrophoresis trapped ion mobility mass spectrometry. Anal Chem. 2022;94:9018–25.
94. Otin J, Tran NT, Benoit A, Buisson C, Taverna M. Online large volume sample staking preconcentration and separation of enantiomeric GHRH analogs by capillary electrophoresis. Electrophoresis. 2023;44:807–17.
95. Tang J, Wu HM, Hu JJ, Yu JC, Zhang JL, Wang CL, et al. On a separation voltage polarity switching transient capillary isotachophoresis method for higher sample loading capacity and better separation performance. Analyst. 2021;146:124–31.
96. Mala Z, Gebauer P. Analytical isotachophoresis 1967–2022: from standard analytical technique to universal on-line concentration tool. TrAC Trends Anal Chem. 2023;158:116837.
97. Lian LM, Zang YP, Yang F, Liu EH, Yan JZ, Tong SQ, et al. Online preconcentration methodology that realizes over 2000-fold enhancement by integrating the free liquid membrane into electrokinetic supercharging in capillary electrophoresis for the determination of trace anionic analytes in complex samples. Microchem J. 2022;174:107033.
98. Chu C, Lian LM, Gao W, Zang YP, Zou YF, Lv YB, et al. An efficient matrix solid-phase dispersion extraction combined with online preconcentration by electrokinetic supercharging in capillary electrophoresis for the sensitive determination of two alkaloids in complicated samples matrix. Microchem J. 2022;178:107314.
99. Ivanov AV, Popov MA, Aleksandrin VV, Kozhevnikova LM, Moskovtsev AA, Kruglova MP, et al. Determination of glutathione in blood via capillary electrophoresis with pH-mediated stacking. Electrophoresis. 2022;43:1859–70.
100. Rye TK, Martinovic G, Eie LV, Hansen FA, Halvorsen TG, Pedersen-Bjergaard S. Electromembrane extraction of peptides using deep eutectic solvents as liquid membrane. Anal Chim Acta. 2021;1175:338717.
101. Li YL, Sun NR, Hu XF, Li Y, Deng CH. Recent advances in nanoporous materials as sample preparation techniques for peptidome research. TrAC Trends Anal Chem. 2019;120:115658.
102. Zhong HF, Li YM, Huang YY, Zhao R. Metal-organic frameworks as advanced materials for sample preparation of bioactive peptides. Anal Methods. 2021;13:862–73.
103. Martinez-Perez-Cejuela H, Benavente F, Simo-Alfonso EF, Herrero-Martinez JM. A hybrid nano-MOF/polymer material for trace analysis of fluoroquinolones in complex matrices at microscale by on-line solid-phase extraction capillary electrophoresis. Talanta. 2021;233:122529.
104. Itterheimova P, Dosedelova V, Kuban P. Use of metal nanoparticles for preconcentration and analysis of biological thiols. Electrophoresis. 2023;44:135–57.
105. Glatz Z. On-capillary derivatisation as an approach to enhancing sensitivity in capillary electrophoresis. Electrophoresis. 2015;36:744–63.
106. Kartsova LA., Moskvichev DO. In-capillary chiral derivatization of amino acids. J Anal Chem. 2022;77:618–24.
107. Gotti R, Pasquini B, Orlandini S, Furlanetto S. Recent applications of the derivatization techniques in capillary electrophoresis. J Pharm Biomed Anal Open. 2023;1:100003.
108. Du JL, Wu G, Chen QY, Yu CF, Xu GL, Liu AH, et al. Fingerprinting trimeric SARS-CoV-2 RBD by capillary isoelectric focusing with whole-column imaging detection. Anal Biochem. 2023;663:115034.
109. Esene JE, Boaks M, Bickham AV, Nordin GP, Woolley AT. 3D printed microfluidic device for automated, pressure-driven, valve-injected microchip electrophoresis of preterm birth biomarkers. Microchim Acta. 2022;189:204.
110. Hamidli N, Pajaziti B, Andrasi M, Nagy C, Gaspar A. Determination of human insulin and its six therapeutic analogues by capillary electrophoresis – mass spectrometry. J Chromatogr A. 2022;1678:463351.
111. Villemet L, Cuchet A, Desvignes C, Sanger-van de Griend C. Protein mapping of peanut extract with capillary electrophoresis. Electrophoresis. 2022;43:1027–34.
112. Logerot E, Perrin C, Ladner Y, Aubriet F, Carre V, Enjalbal C. Quantitating alfa-amidated peptide degradation by separative technologies and ultra-high resolution mass spectrometry. Talanta. 2023;253:124036.
113. Roca S, Leclercq L, Gonzalez P, Dhellemmes L, Boiteau L, Rydzek G, et al. Modifying last layer in polyelectrolyte multilayer coatings for capillary electrophoresis of proteins. J Chromatogr A. 2023;1692:463837.
114. Kwok T, Chan SL, Shi J, Zhou M, Schaefer A, Bo T, et al. Imaged capillary isoelectric focusing employing fluorocarbon and methylcellulose coated fused silica capillary for characterization of charge heterogeneity of protein biopharmaceuticals. Sep Sci Plus. 2023;6:2200160.
115. Wang LY, Li YY, Chen DZ, Chen DDY. Electrospray ionization stability and concentration sensitivity in capillary electrophoresis-mass spectrometry using a flow-through microvial interface. Electrophoresis. 2021;42:360–8.
116. Stutz H. Advances and applications of capillary electromigration methods in the analysis of therapeutic and diagnostic recombinant proteins – a review. J Pharm Biomed Anal. 2023;222:115089.
117. Roca S, Dhellemmes L, Leclercq L, Cottet H. Polyelectrolyte multilayers in capillary electrophoresis. ChemPlusChem. 2022;87:e202200028.
118. Konasova R, Butnariu M, Solinova V, Kasicka V, Koval D. Covalent cationic copolymer coatings allowing tunable electroosmotic flow for optimization of capillary electrophoretic separations. Anal Chim Acta. 2021;1178:338789.

119. Solinova V, Tuma P, Butnariu M, Kasicka V, Koval D. Covalent anionic copolymer coatings with tunable electroosmotic flow for optimization of capillary electrophoretic separations. Electrophoresis. 2022;43:1953–62.
120. Martinkova E, Krizek T, Kubickova A, Coufal P. Mobilization of electroosmotic flow markers in capillary zone electrophoresis. Electrophoresis. 2021;42:932–8.
121. Chen YL, Xia L, Xiao XH, Li GK. Enhanced capillary zone electrophoresis in cyclic olefin copolymer microchannels using the combination of dynamic and static coatings for rapid analysis of carnosine and niacinamide in cosmetics. J Sep Sci. 2022;45:2045–54.
122. Maryutina TA, Savonina EY, Fedotov PS, Smith RM, Siren H, Hibbert DB. Terminology of separation methods (IUPAC Recommendations 2017). Pure Appl Chem. 2018;90:181–231.
123. Bhimwal R, Rustandi RR, Payne A, Dawod M. Recent advances in capillary gel electrophoresis for the analysis of proteins. J Chromatogr A. 2022;1682:463453.
124. Hajba L, Jeong S, Chung DS, Guttman A. Capillary gel electrophoresis of proteins: historical overview and recent advances. TrAC Trends Anal Chem. 2023;162:117024.
125. Holland LA, Casto-Boggess LD. Gels in microscale electrophoresis. Annu Rev Anal Chem. 2023;16:161–79.
126. Guttman A, Hajba L. Capillary gel electrophoresis. Amsterdam: Elsevier; 2022.
127. Sarkozy D, Guttman A. Analysis of peptides and proteins by native and SDS capillary gel electrophoresis coupled to electrospray ionization mass spectrometry via a closed-circuit coaxial sheath flow reactor interface. Anal Chem. 2023;95:7082–6.
128. Delgado-Povedano MD, Lara FJ, Gamiz-Gracia L, Garcia-Campana AM. Non-aqueous capillary electrophoresis-time of flight mass spectrometry method to determine emerging mycotoxins. Talanta. 2023;253:123946.
129. de la Nieta LNL, Bernardo FJG, Penalvo GC, Flores JR. Solid phase extraction prior to non-aqueous capillary electrophoresis with ultraviolet detection as a valuable strategy for therapeutic drug monitoring of cabozantinib. Microchem J. 2022;181:107830.
130. Nyssen L, Fillet M, Cavalier E, Servais AC. Highly sensitive and selective separation of intact parathyroid hormone and variants by sheathless CE-ESI-MS/MS. Electrophoresis. 2019;40:1550–7.
131. Li HL, Guo C, Zhang QC, Bao LC, Zheng QF, Guo ZP, et al. A substantial increase of analytical throughput in capillary electrophoresis throughput by separation-interrupted sequential injections. Anal Methods. 2021;13:1995–2004.
132. Kumar R, Sarin D, Rathore AS. High-throughput capillary electrophoresis analysis of biopharmaceuticals utilizing sequential injections. Electrophoresis. 2023;44:767–74.
133. Mala Z, Gebauer P. Recent progress in analytical capillary isotachophoresis (2018–March 2022). J Chromatogr A. 2022;1677:463337.
134. Ramachandran A, Santiago JG. Isotachophoresis: theory and microfluidic applications. Chem Rev. 2022;122:12904–76.
135. Stepanova S, Kasicka V. Determination of impurities and counterions of pharmaceuticals by capillary electromigration methods. J Sep Sci. 2014;37:2039–55.
136. Zhu QF, Scriba GKE. Analysis of small molecule drugs, excipients and counter ions in pharmaceuticals by capillary electromigration methods – recent developments. J Pharm Biomed Anal. 2018;147:425–38.
137. Farmerie L, Rustandi RR, Loughney JW, Dawod M. Recent advances in isoelectric focusing of proteins and peptides. J Chromatogr A. 2021;1651:462274.
138. Wu JQ, McElroy W, Pawliszyn J, Heger CD. Imaged capillary isoelectric focusing: applications in the pharmaceutical industry and recent innovations of the technology. TrAC Trends Anal Chem. 2022;150:116567.
139. Zhang XX, Kwok T, Zhou MK, Du M, Li VC, Bo T, et al. Imaged capillary isoelectric focusing (icIEF) tandem high resolution mass spectrometry for charged heterogeneity of protein drugs in biopharmaceutical discovery. J Pharm Biomed Anal. 2023;224:115178.
140. Schlecht J, Moritz B, Kiessig S, Neususs C. Characterization of therapeutic mAb charge heterogeneity by iCIEF coupled to mass spectrometry (iCIEF-MS). Electrophoresis. 2023;44:540–8.
141. Kwok T, Zhou MK, Schaefer A, Bo T, Li V, Huang TM, et al. Fractionation and online mass spectrometry based on imaged capillary isoelectric focusing (icIEF) for characterizing charge heterogeneity of therapeutic antibody. Anal Methods. 2023;15:411–8.
142. Dusa F, Kubesova A, Salplachta J, Moravcova D. Capillary isoelectric focusing: the role of markers of isoelectric point and recent applications in the field. TrAC Trends Anal Chem. 2023;162:117018.
143. Xu T, Sun LL. A mini review on capillary isoelectric focusing-mass spectrometry for top-down proteomics. Front Chem. 2021;9:651757.
144. Wang Y, Adeoye DI, Ogunkunle EO, Wei IA, Filla RT, Roper MG. Affinity capillary electrophoresis: a critical review of the literature from 2018 to 2020. Anal Chem. 2021;93:295–310.
145. Sharmeen S, Kyei I, Hatch A, Hage DS. Analysis of drug interactions with serum proteins and related binding agents by affinity capillary electrophoresis: a review. Electrophoresis. 2022;43:2302–23.
146. Suh K, Kyei I, Hage DS. Approaches for the detection and analysis of antidrug antibodies to biopharmaceuticals: a review. J Sep Sci. 2022;45:2077–92.
147. Asmari M, Michalcova L, Ibrahim AE, Glatz Z, Wätzig H, El Deeb S. Studying molecular interactions via capillary electrophoresis and microscale thermophoresis: a review. Electrophoresis. 2023;44:1114–42.
148. Dubsky P, Ordogova M, Maly M, Riesova M. CEval: all-in-one software for data processing and statistical evaluations in affinity capillary electrophoresis. J Chromatogr A. 2016;1445:158–65.
149. Rudnev AV, Aleksenko SS, Semenova O, Hartinger CG, Timerbaev AR, Keppler BK. Determination of binding constants and stoichiometries for platinum anticancer drugs and serum transport proteins by capillary electrophoresis using the Hummel–Dreyer method. J Sep Sci. 2005;28:121–7.
150. Dvorak M, Svobodova J, Benes M, Gas B. Applicability and limitations of affinity capillary electrophoresis and vacancy affinity capillary electrophoresis methods for determination of complexation constants. Electrophoresis. 2013;34:761–7.
151. Mlcochova H, Ratih R, Michalcova L, Wätzig H, Glatz Z, Stein M. Comparison of mobility shift affinity capillary electrophoresis and capillary electrophoresis frontal analysis for binding

constant determination between human serum albumin and small drugs. Electrophoresis. 2022;43:1724–34.
152. Mine M, Mizuguchi H, Takayanagi T. Kinetic analysis of the transphosphorylation with creatine kinase by pressure-assisted capillary electrophoresis/dynamic frontal analysis. Anal Bioanal Chem. 2021;413:1453–60.
153. Lounis FM, Chamieh J, Leclercq L, Gonzalez P, Rossi JC, Cottet H. Effect of dendrigraft generation on the interaction between anionic polyelectrolytes and dendrigraft poly(L-lysine). Polymers. 2018;10:45.
154. Nevidalova H, Michalcova L, Glatz Z. Capillary electrophoresis-based approaches for the study of affinity interactions combined with various sensitive and nontraditional detection techniques. Electrophoresis. 2019;40:625–42.
155. Miyabe K. Moment theory of affinity capillary electrophoresis for analysis of reaction kinetics of intermolecular interactions. J Chromatogr A. 2022;1684:463557.
156. Le ATH, Krylova SM, Krylov SN. Kinetic capillary electrophoresis in screening oligonucleotide libraries for protein binders. TrAC Trends Anal Chem. 2023;162:117061.
157. Gstottner C, Hook M, Christopeit T, Knaupp A, Schlothauer T, Reusch D, et al. Affinity capillary electrophoresis-mass spectrometry as a tool to unravel proteoform-specific antibody-receptor interactions. Anal Chem. 2021;93:15133–41.
158. Ansorge M, Dubsky P, Uselova K. Into the theory of the partial-filling affinity capillary electrophoresis and the determination of apparent stability constants of analyte-ligand complexes. Electrophoresis. 2018;39:742–51.
159. Solinova V, Zakova L, Jiracek J, Kasicka V. Pressure assisted partial filling affinity capillary electrophoresis employed for determination of binding constants of human insulin hexamer complexes with serotonin, dopamine, arginine, and phenol. Anal Chim Acta. 2019;1052:170–8.
160. Mitsuno E, Endo T, Hisamoto H, Sueyoshi K. Evaluation of the interactions between oligonucleotides and small molecules by partial filling-nonequilibrium affinity capillary electrophoresis. Anal Sci. 2022;38:851–9.
161. Miyabe K. Moment equations for partial filling capillary electrophoresis. Electrophoresis. 2022;43:559–70.
162. Davoine C, Pardo A, Pochet L, Fillet M. Fragment hit discovery and binding site characterization by indirect affinity capillary electrophoresis: application to factor XIIa. Anal Chem. 2021;93:14802–9.
163. El Deeb S, Silva CF, Nascimento CS, Hanafi RS, Borges KB. Chiral capillary electrokinetic chromatography: principle and applications, detection and identification, design of experiment, and exploration of chiral recognition using molecular modeling. Molecules. 2021;26:2841.
164. Zhang Y, Nsanzamahoro S, Wang CB, Wang WF, Yang JL. Screening of prolyl hydroxylase 2 inhibitors based on quantitative strategy of peptides. J Chromatogr A. 2022;1679:463411.
165. Miksik I. Capillary electrochromatography of proteins and peptides (2006–2015). J Sep Sci. 2017;40:251–71.
166. Wang M, Liu Y, Liu Y, Xia ZN. MOFs and PDA-supported immobilization of BSA in open tubular affinity capillary electrochromatography: prediction and study on drug-protein interactions. Talanta. 2022;237:122959.
167. Gao LD, Hu XF, Qin SL, Chu HT, Tang YM, Li X, et al. One-pot synthesis of a novel chiral Zr-based metal-organic framework for capillary electrochromatographic enantioseparation. Electrophoresis. 2022;43:1161–73.
168. Wang FL, Zhang YL, Wang GX, Qi SD, Lv WJ, Liu JJ, et al. Synthesis of a covalent organic framework with hydrazine linkages and its application in open-tubular capillary electrochromatography. J Chromatogr A. 2022;1661:462681.
169. Fanali S, Chankvetadze B. History, advancement, bottlenecks, and future of chiral capillary electrochromatography. J Chromatogr A. 2021;1637:461832.
170. Hu CJ, Li ZT, Hu Z, Li QY, Fu YY, Chen ZL. Synthesis of multifunctional crown ether covalent organic nanospheres as stationary phase for capillary electrochromatography. J Chromatogr A. 2022;1677:463323.
171. Ali F, Alothman ZA, Al-Shaalan NH. Mixed-mode open tubular column for peptide separations by capillary electrochromatography. J Sep Sci. 2021;44:2602–11.
172. Naithani V, Cheddah S, Yang KG, Xia ZH, Wang WW, Wang Y, et al. Preparation of open tubular capillary column covalently coated with polystyrene sulfonate with 4,4′-azobis(4-cyanopentanoyl chloride) as polymerization initiator for electrochromatographic separation of alkaloids, sulfonamides, and peptides. J Sep Sci. 2023;46:2200711.
173. Qi L, Qiao J. Advances in stimuli-responsive polymeric coatings for open-tubular capillary electrochromatography. J Chromatogr A. 2022;1670:462957.
174. Sun GL, Tang WY, Lu Y, Row KH. Growth of two-layer copolymer as the stationary phase with very high separation efficiency for separating peptides in capillary electrochromatography. Electrophoresis. 2021;42:2087–93.
175. Qin SL, Cui HS, Chu HT, Gao LD, Li X, Tang YM, et al. Preparation of a zeolite imidazole skeleton-silica hybrid monolithic column for amino acid analysis via capillary electrochromatography. Electrophoresis. 2022;43:1710–23.
176. Rajendiran V, El Rassi Z. Reversed-phase capillary electrochromatography of pre-column derivatized mono- and oligosaccharides with three different ultraviolet absorbing tags. J Chromatogr A. 2022;1671:463025.
177. Neequaye T, El Rassi Z. Poly(carboxyethyl acrylate-*co*-ethylene glycol dimethacrylate) precursor monolith with bonded (*S*)-(−)–1-(2-naphthyl) ethylamine ligands for use in chiral and achiral separations by capillary electrochromatography. J Chromatogr A. 2023;1688:463713.
178. Westermeier R. Looking at proteins from two dimensions: a review on five decades of 2D electrophoresis. Arch Physiol Biochem. 2014;120:168–72.
179. Pomastowski P, Buszewski B. Two-dimensional gel electrophoresis in the light of new developments. TrAC Trends Anal Chem. 2014;53:167–77.
180. König S. Differential vs comparative gel electrophoresis: new technology drives standardisation and quantification in protein two-dimensional gel electrophoresis. TrAC Trends Anal Chem. 2020;122:115731.
181. Kumar R, Shah RL, Ahmad S, Rathore AS. Harnessing the power of electrophoresis and chromatography: offline coupling of reverse phase liquid chromatography-capillary zone electrophoresis-tandem mass spectrometry for analysis of host

cell proteins in monoclonal antibody producing CHO cell line. Electrophoresis. 2021;42:735–41.
182. Kitagawa S. Development of novel analytical methods based on liquid chromatography and electrophoresis. Chromatography. 2023;44:1–9.
183. Giddings JC. Concepts and comparisons in multidimensional separation. J High Res Chromatogr Chromatogr Commun. 1987;10:319–23.
184. Ranjbar L, Foley JP, Breadmore MC. Multidimensional liquid-phase separations combining both chromatography and electrophoresis—a review. Anal Chim Acta. 2017;950:7–31.
185. Schlecht J, Jooss K, Moritz B, Kiessig S, Neususs C. Two-dimensional capillary zone electrophoresis-mass spectrometry: intact mAb charge variant separation followed by peptide level analysis using in-capillary digestion. Anal Chem. 2023;95:4059–66.
186. Quan ZL, Chen Y, Zhao XC, Yu SY, Li YR, Xu YQ, et al. Development of high-resolution multidimensional native protein microfluidic chip electrophoresis fingerprinting and its application in the quick analysis of unknown microorganisms. J Chromatogr A. 2022;1665:462797.
187. Pena-Pereira F, Costas-Mora I, Romero V, Lavilla I, Bendicho C. Advances in miniaturized UV-Vis spectrometric systems. TrAC Trends Anal Chem. 2011;30:1637–48.
188. Hartung S, Minkner R, Olabi M, Wätzig H. Performance of capillary electrophoresis instruments—state of the art and outlook. TrAC Trends Anal Chem. 2023;163:117056.
189. Solinova V, Sazelova P, Masova A, Jiracek J, Kasicka V. Application of capillary and free-flow zone electrophoresis for analysis and purification of antimicrobial beta-alanyl-tyrosine from hemolymph of fleshfly *Neobellieria bullata*. Molecules. 2021;26:5636.
190. Wu SO, Dovichi NJ. Capillary zone electrophoresis separation and laser-induced fluorescence detection of zeptomole quantities of fluorescein thiohydantoin derivatives of amino acids. Talanta. 1992;39:173–8.
191. Galievsky VA, Stasheuski AS, Krylov SN. Getting the best sensitivity from on-capillary fluorescence detection in capillary electrophoresis—a tutorial. Anal Chim Acta. 2016;935:58–81.
192. Thompson S, Pappas D. A fluorescence toolbox: a review of investigation of electrophoretic separations, process, and interfaces. Electrophoresis. 2019;40:606–15.
193. Zhu ZF, Lies M, Silzel J. Sodium dodecyl sulfate-capillary gel electrophoresis with native fluorescence detection for analysis of therapeutic proteins. J Pharm Biomed Anal. 2022;213:114689.
194. Couderc F, Ong-Meang V, Poinsot V. Capillary electrophoresis hyphenated with UV-native-laser induced fluorescence detection (CE/UV-native-LIF). Electrophoresis. 2017;38:135–49.
195. Ceribeli C, de Arruda HF, Costa LD. How coupled are capillary electrophoresis and mass spectrometry? Scientometrics. 2021;126:3841–51.
196. Della Posta S, Fanali C, Gallo V, Fanali S. Recent advances in the hyphenation of electromigration techniques with mass spectrometry. TrAC Trends Anal Chem. 2022;157:116800.
197. Lapizco-Encinas BH, Zhang YV, Gqamana PP, Lavicka J, Foret F. Capillary electrophoresis as a sample separation step to mass spectrometry analysis: a primer. TrAC Trends Anal Chem. 2023;164:117093.
198. Gosset-Erard C, Aubriet F, Leize-Wagner E, Francois YN, Chaimbault P. Hyphenation of Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) with separation methods: the art of compromises and the possible—a review. Talanta. 2023;257:124324.
199. Shah U, Patel A, Patel R, Patel M, Patel A, Kavad M, et al. Overview on capillary electrophoresis with mass spectrometry: application in peptide analysis and proteomics. Int J Pharm Chem Anal. 2021;8:6–15.
200. Seyfinejad B, Jouyban A. Capillary electrophoresis-mass spectrometry in pharmaceutical and biomedical analyses. J Pharm Biomed Anal. 2022;221:115059.
201. Schwenzer AK, Kruse L, Jooss K, Neususs C. Capillary electrophoresis-mass spectrometry for protein analyses under native conditions: current progress and perspectives. Proteomics. 2023;2023:e2300135.
202. Wu YY, Zhang WM, Zhao YY, Wang XY, Guo GS. Technology development trend of electrospray ionization mass spectrometry for single-cell proteomics. TrAC Trends Anal Chem. 2023;159:116913.
203. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass-spectrometry of large biomolecules. Science. 1989;246:64–71.
204. Karas M, Bachmann D, Bahr U, Hillenkamp F. Matrix-assisted ultraviolet-laser desorption of nonvolatile compounds. Int J Mass Spectrom Ion Process. 1987;78:53–68.
205. Olivares JA, Nguyen NT, Yonker CR, Smith RD. On-line mass spectrometric detection for capillary zone electrophoresis. Anal Chem. 1987;59:1230–2.
206. Smith RD, Olivares JA, Nguyen NT, Udseth HR. Capillary zone electrophoresis-mass spectrometry using an electrospray ionization interface. Anal Chem. 1988;60:436–41.
207. Ng CCA, Zhou Y, Yao ZP. Algorithms for de-novo sequencing of peptides by tandem mass spectrometry: a review. Anal Chim Acta. 2023;1268:341330.
208. Schlecht J, Stolz A, Hofmann A, Gerstung L, Neususs C. nanoCEasy: an easy, flexible, and robust nanoflow sheath liquid capillary electrophoresis-mass spectrometry interface based on 3D printed parts. Anal Chem. 2021;93:14593–8.
209. Hocker O, Montealegre C, Neususs C. Characterization of a nanoflow sheath liquid interface and comparison to a sheath liquid and a sheathless porous-tip interface for CE-ESI-MS in positive and negative ionization. Anal Bioanal Chem. 2018;410:5265–75.
210. Glazyrin YE, Mironov GG, Kichkailo AS, Berezovski MV. Separation abilities of capillary electrophoresis coupled with ion mobility mass spectrometry for the discrete detection of sequence isomeric peptides. Separations. 2022;9:106.
211. Kuban P, Kuban P. Novel developments in capillary electrophoresis miniaturization, sampling, detection and portability: an overview of the last decade. TrAC Trends Anal Chem. 2023;159:116941.
212. Tuma P. Determination of amino acids by capillary and microchip electrophoresis with contactless conductivity detection—theory, instrumentation and applications. Talanta. 2021;224:121922.
213. Tuma P, Hlozek T, Kamisova J, Gojda J. Monitoring of circulating amino acids in patients with pancreatic cancer and

cancer cachexia using capillary electrophoresis and contactless conductivity detection. Electrophoresis. 2021;42:1885–91.
214. Tuma P, Opekar F, Dlouhy P. Capillary and microchip electrophoresis with contactless conductivity detection for analysis of foodstuffs and beverages. Food Chem. 2022;375:131858.
215. Tuma P. Monitoring of biologically active substances in clinical samples by capillary and microchip electrophoresis with contactless conductivity detection: a review. Anal Chim Acta. 2022;1225:340161.
216. Costa BMD, Griveau S, d'Orlye F, Bedioui F, da Silva JAF, Varenne A. Microchip electrophoresis and electrochemical detection: a review on a growing synergistic implementation. Electrochim Acta. 2021;391:138928.
217. Nan P. Detection of diuretic doping by capillary electrophoresis and electro-chemical technology: a mini-review. Curr Pharm Anal. 2022;18:34–42.
218. Bohm D, Koall M, Matysik FM. Combining amperometry and mass spectrometry as a dual detection approach for capillary electrophoresis. Electrophoresis. 2023;44:492–500.
219. Zhao TJ, Lin HP, Li N, Shi HM, Kang WJ, Xu XD. Determination of folic acid by capillary zone electrophoresis with indirect chemiluminescence detection. RSC Adv. 2021;11:20063–9.
220. Sun SJ, Wei YF, Wang H, Tang LF, Deng BY. Determination of verapamil hydrochloride and norverapamil hydrochloride in rat plasma by capillary electrophoresis with end-column electrochemiluminescence detection and their pharmacokinetics study. J Chromatogr Sci. 2021;59:289–96.
221. Krait S, Salgado A, Peluso P, Malanga M, Sohajda T, Benkovics G, et al. Complexation of daclatasvir by single isomer methylated beta-cyclodextrins studied by capillary electrophoresis, NMR spectroscopy and mass spectrometry. Carbohydr Polym. 2021;273:118486.
222. Krait S, Salgado A, Malanga M, Sohajda T, Benkovics G, Szakaly PS, et al. Structural characterization of methyl-beta-cyclodextrins by high-performance liquid chromatography and nuclear magnetic resonance spectroscopy and effect of their isomeric composition on the capillary electrophoresis enantioseparation of daclatasvir. J Chromatogr A. 2022;1661:462675.
223. Hruzikova A, Cechova LM, Stepanova S, Tuckova L, Tichotova M, Ruzicka A, et al. A study of azopyrimidine photoswitches and their interactions with cyclodextrins: when the guest governs the type of accommodation at the host. Dyes Pigments. 2023;212:111099.
224. Reminek R, Foret F. Capillary electrophoretic methods for quality control analyses of pharmaceuticals: a review. Electrophoresis. 2021;42:19–37.
225. Krait S, Konjaria ML, Scriba GKE. Advances of capillary electrophoresis enantioseparations in pharmaceutical analysis (2017–2020). Electrophoresis. 2021;42:1709–25.
226. van der Burg D, Josefsson L, Emmer A, Sänger-van de Griend CE. Recent capillary electrophoresis applications for upstream and downstream biopharmaceutical process monitoring. TrAC Trends Anal Chem. 2023;160:116975.
227. Hjerten S. High-performance electrophoresis elimination of electroendosmosis and solute adsorption. J Chromatogr. 1985;347:191–8.
228. Liu B, Wang WT, Gao T, Huang L, Fan HH, Chen HX. Separation, identification and quantification of associated impurities in cobratide using sheathless CE-MS and CE-UV. Anal Methods. 2021;13:3845–51.
229. Zimina T, Sitkov N, Karasev V, Skorik Y, Kolobov A, Kolobov A. Design of peptide ligand for lactoferrin and study of its binding specificity. Chemosensors. 2023;11:162.
230. Kawai T. Recent advances in trace bioanalysis by capillary electrophoresis. Anal Sci. 2021;37:27–36.
231. Hruskova H, Voracova I, Reminek R, Foret F. Current applications of capillary electrophoresis-mass spectrometry for the analysis of biologically important analytes in urine (2017 to mid-2021): a review. J Sep Sci. 2022;45:305–24.
232. Mast DH, Liao HW, Romanova EV, Sweedler JV. Analysis of peptide stereochemistry in single cells by capillary electrophoresis-trapped ion mobility spectrometry mass spectrometry. Anal Chem. 2021;93:6205–13.
233. Johnson KR, Gao YF, Gregus M, Ivanov AR. On-capillary cell lysis enables top-down proteomic analysis of single mammalian cells by CE-MS/MS. Anal Chem. 2022;94:14358–67.
234. Cupp-Sutton KA, Fang ML, Wu S. Separation methods in single-cell proteomics: RPLC or CE? Int J Mass Spectrom. 2022;481:116920.
235. Saito R, Hirayama A, Akiba A, Kamei Y, Kato Y, Ikeda S, et al. Urinary metabolome analyses of patients with acute kidney injury using capillary electrophoresis-mass spectrometry. Metabolites. 2021;11:671.
236. Latosinska A, Siwy J, Faguer S, Beige J, Mischak H, Schanstra JP. Value of urine peptides in assessing kidney and cardiovascular disease. Proteomics Clin Appl. 2021;15:2000027.
237. Wendt R, Siwy J, He TL, Latosinska A, Wiech T, Zipfel PF, et al. Molecular mapping of urinary complement peptides in kidney diseases. Proteomes. 2021;9:49.
238. Frantzi M, Heidegger I, Roesch MC, Gomez-Gomez E, Steiner E, Vlahou A, et al. Validation of diagnostic nomograms based on CE-MS urinary biomarkers to detect clinically significant prostate cancer. World J Urol. 2022;40:2195–203.
239. Wendt R, Thijs L, Kalbitz S, Mischak H, Siwy J, Raad J, et al. Aurinary peptidomic profile predicts outcome in SARS-CoV-2-infected patients. eClinicalMedicine. 2021;36:100883.
240. Delvaux C, Dauvin M, Boulanger M, Quinton L, Mengin-Lecreulx D, Joris B, et al. Use of capillary zone electrophoresis coupled to electrospray mass spectrometry for the detection and absolute quantitation of peptidoglycan-derived peptides in bacterial cytoplasmic extracts. Anal Chem. 2021;93:2342–50.
241. Larder CE, Iskandar MM, Sabally K, Kubow S. Complementary and efficient methods for di- and tri-peptide analysis and amino acid quantification from simulated gastrointestinal digestion of collagen hydrolysate. LWT Food Sci Technol. 2022;155:112880.
242. Men X, Wu CX, Chen ML, Wang JH. Determination of glutathione in cells by capillary electrophoresis laser induced fluorescence. Chin J Chromatogr. 2023;41:87–93.
243. Omar M, Windhagen H, Krettek C, Ettinger M. Noninvasive diagnostic of periprosthetic joint infection by urinary peptide markers: a preliminary study. J Orthopaed Res. 2021;39:339–47.
244. Abril AG, Pazos M, Villa TG, Calo-Mata P, Barros-Velazquez J, Carrera M. Proteomics characterization of food-derived bioactive peptides with anti-allergic and anti-inflammatory properties. Nutrients. 2022;14:4400.
245. Valdes A, Alvarez-Rivera G, Socas-Rodriguez B, Herrero M, Cifuentes A. Capillary electromigration methods for food

analysis and foodomics: advances and applications in the period February 2019–February 2021. Electrophoresis. 2022;43:37–56.
246. Rozenski J, Asfaw AA, Van Schepdael A. Overview of in-capillary enzymatic reactions using capillary electrophoresis. Electrophoresis. 2022;43:57–73.
247. Ryan KA, Bruening ML. Online protein digestion in membranes between capillary electrophoresis and mass spectrometry. Analyst. 2023;148:1611–9.
248. Virag D, Dalmadi-Kiss B, Vekey K, Drahos L, Klebovich I, Antal I, et al. Current trends in the analysis of post-translational modifications. Chromatographia. 2020;83:1–10.
249. Saade J, Biacchi M, Giorgetti J, Lechner A, Beck A, Leize-Wagner E, et al. Analysis of monoclonal antibody glycopeptides by capillary electrophoresis-mass spectrometry coupling (CE-MS). Methods Mol Biol. 2021;2271:97–106.
250. Shrivastava A, Joshi S, Guttman A, Rathore AS. *N*-Glycosylation of monoclonal antibody therapeutics: a comprehensive review on significance and characterization. Anal Chim Acta. 2022;1209:339828.
251. Moran AB, Dominguez-Vega E, Nouta J, Pongracz T, de Reijke TM, Wuhrer M, et al. Profiling the proteoforms of urinary prostate-specific antigen by capillary electrophoresis—mass spectrometry. J Proteomics. 2021;238:104148.
252. Zhang Y, Zhao M, Wang CB, Wang Y, Nsanzamahoro S, Zhu LL. Screening prolyl hydroxylase domain 2 inhibitory activity of traditional Chinese medicine by CZE-UV. Electrophoresis. 2022;43:1601–10.
253. Liu ZT, Wang CL, Wang X, Van Schepdael A. On-line screening of indoleamine 2,3-dioxygenase 1 inhibitors by partial filling capillary electrophoresis combined with rapid polarity switching. J Chromatogr A. 2021;1651:462305.
254. Li W, Cui XY, Chen ZL. Screening of lactate dehydrogenase inhibitor from bioactive compounds in natural products by electrophoretically mediated microanalysis. J Chromatogr A. 2021;1656:462554.
255. Li W, Zhang BF, Chen ZL. Screening carbonic anhydrase IX inhibitors in traditional Chinese medicine based on electrophoretically mediated microanalysis. Talanta. 2021;232:122444.
256. Dadouch M, Ladner Y, Bich C, Montels JM, Morel J, Bechara C. In-capillary (electrophoretic) digestion-reduction-separation: a smart tool for middle-up analysis of mAb. J Chromatogr A. 2021;1648:462213.
257. Dadouch M, Ladner Y, Bich C, Montels J, Morel J, Perrin C. Fast in-line bottom-up analysis of monoclonal antibodies: toward an electrophoretic fingerprinting approach. Electrophoresis. 2021;42:1229–37.
258. Nagy C, Szabo R, Gaspar A. Microfluidic immobilized enzymatic reactors for proteomic analyses-recent developments and trends (2017–2021). Micromachines. 2022;13:311.
259. Hong TT, Qiu L, Rui W, Li Y, Zheng RH, Guo QQ, et al. Capillary electrophoretic method for *Staphylococcus aureus* detection by using a novel antimicrobial peptide. Electrophoresis. 2021;42:1217–20.
260. Zhou SW, Cui PF, Sheng JY, Zhang XL, Jiang PJ, Ni XY, et al. A novel assay for the determination of PreScission protease by capillary electrophoresis. Biophys Chem. 2022;281:106696.
261. Stepanova S, Kasicka V. Capillary electrophoretic methods applied to the investigation of peptide complexes. J Sep Sci. 2015;38:2708–21.
262. Piestansky J, Barath P, Majerova P, Galba J, Mikus P, Kovacech B, et al. A simple and rapid LC-MS/MS and CE-MS/MS analytical strategy for the determination of therapeutic peptides in modern immunotherapeutics and biopharmaceutics. J Pharm Biomed Anal. 2020;189:113449.
263. Duroux C, Hagege A. CE-ICP-MS to probe A beta(1–42)/copper(II) interactions: a complementary tool to study amyloid aggregation in Alzheimer's disease. Metallomics. 2022;14:mfab075.
264. Ta HY, Collin F, Perquis L, Poinsot V, Ong-Meang V, Couderc F. Twenty years of amino acid determination using capillary electrophoresis: a review. Anal Chim Acta. 2021;1174:338233.
265. Yin XW, Adams E, Van Schepdael A. Overview of chromatographic and electrophoretic methods for the determination of branched-chain amino acids. J Sep Sci. 2023;46:2300213.
266. Shen XJ, Xu T, Hakkila B, Hare M, Wang QJ, Wang QY, et al. Capillary zone electrophoresis-electron-capture collision-induced dissociation on a quadrupole time-of-flight mass spectrometer for top-down characterization of intact proteins. J Am Soc Mass Spectrom. 2021;32:1361–9.
267. Armstrong DW. Analysis of D-amino acids: relevance in human disease. LCGC North America. 2022;40:356–60.
268. Toole EN, Dufresne C, Ray S, Schwann A, Cook K, Ivanov AR. Rapid highly-efficient digestion and peptide mapping of adeno-associated viruses. Anal Chem. 2021;93:10403–10.
269. Mavrogeorgis E, Mischak H, Latosinska A, Siwy J, Jankowski V, Jankowski J. Reproducibility evaluation of urinary peptide detection using CE-MS. Molecules. 2021;26:7260.
270. de Koster N, Clark CP, Kohler I. Past, present, and future developments in enantioselective analysis using capillary electromigration techniques. Electrophoresis. 2021;42:38–57.
271. Chankvetadze B. Application of enantioselective separation techniques to bioanalysis of chiral drugs and their metabolites. TrAC Trends Anal Chem. 2021;143:116332.
272. Shamsi SA, Akter F. Capillary electrophoresis mass spectrometry: developments and applications for enantioselective analysis from 2011–2020. Molecules. 2022;27:4126.
273. Qian HL, Xu ST, Yan XP. Recent advances in separation and analysis of chiral compounds. Anal Chem. 2023;95:304–18.
274. Zhang L, Tan QG, Fan JQ, Sun C, Luo YT, Liang RP, et al. Microfluidics for chiral separation of biomolecules. TrAC Trends Anal Chem. 2023;158:116842.
275. Morvan M, Miksik I. Recent advances in chiral analysis of proteins and peptides. Separations. 2021;8:112.
276. Peluso P, Chankvetadze B. Native and substituted cyclodextrins as chiral selectors for capillary electrophoresis enantioseparations: structures, features, application, and molecular modeling. Electrophoresis. 2021;42:1676–708.
277. Chankvetadze B, Scriba GKE. Cyclodextrins as chiral selectors in capillary electrophoresis: recent trends in mechanistic studies. TrAC Trends Anal Chem. 2023;160:116987.
278. Fejos I, Kalydi E, Kukk EL, Seggio M, Malanga M, Beni S. Single isomer *N*-heterocyclic cyclodextrin derivatives as chiral selectors in capillary electrophoresis. Molecules. 2021;26:5271.

279. Sazelova P, Solinova V, Schimperkova T, Jiracek J, Kasicka V. Chiral analysis of beta-alanyl-D,L-tyrosine and its derivatives and estimation of binding constants of their complexes with 2-hydroxypropyl-beta-cyclodextrin by capillary electrophoresis. J Sep Sci. 2022;45:3328–38.
280. Konjaria ML, Scriba GKE. Effects of amino acid-derived chiral ionic liquids on cyclodextrin-mediated capillary electrophoresis enantioseparations of dipeptides. J Chromatogr A. 2021;1652:462342.
281. Reyes CDG, Jiang PL, Donohoo K, Atashi M, Mechref YS. Glycomics and glycoproteomics: approaches to address isomeric separation of glycans and glycopeptides. J Sep Sci. 2021;44:403–25.
282. Scriba GKE. Chiral recognition in separation sciences Part II: Macrocyclic glycopeptide, donor-acceptor, ion-exchange, ligand-exchange and micellar selectors. TrAC Trends Anal Chem. 2019;119:115628.
283. Xing J, Wang F, Cong HL, Wang S, Shen YQ, Yu B. Analysis of proteins and chiral drugs based on vancomycin covalent capillary electrophoretic coating. Analyst. 2021;146:1320–5.
284. Kasicka V. From micro to macro: conversion of capillary electrophoretic separations of biomolecules and bioparticles to preparative free-flow electrophoresis scale. Electrophoresis. 2009;30:S40–52.
285. Pes O, Preisler J. Off-line coupling of microcolumn separations to desorption mass spectrometry. J Chromatogr A. 2010;1217:3966–77.
286. Weaver SD, Schuster-Little N, Whelan RJ. Preparative capillary electrophoresis (CE) fractionation of protein digests improves protein and peptide identification in bottom-up proteomics. Anal Methods. 2022;14:1103–10.
287. DeLaney K, Li LJ. Capillary electrophoresis coupled to MALDI mass spectrometry imaging with large volume sample stacking injection for improved coverage of C borealis neuropeptidome. Analyst. 2020;145:61–69.
288. Zhou XM, Song WR, Novotny MV, Jacobson SC. Fractionation and characterization of sialyl linkage isomers of serum *N*-glycans by CE-MS. J Sep Sci. 2022;45:3348–61.
289. Kasicka V, Prusik Z, Sazelova P, Jiracek J, Barth T. Theory of the correlation between capillary and free-flow zone electrophoresis and its use for the conversion of analytical capillary separations to continuous free-flow preparative processes—application to analysis and preparation of fragments of insulin. J Chromatogr A. 1998;796:211–20.
290. Kasicka V, Prusik Z, Pospisek J. Conversion of capillary zone electrophoresis to free-flow zone electrophoresis using a simple model of their correlation—application to synthetic enkephalin-type peptide analysis and preparation. J Chromatogr. 1992;608:13–22.
291. Stastna M. Continuous flow electrophoretic separation—recent developments and applications to biological sample analysis. Electrophoresis. 2020;41:36–55.
292. Doria S, Yost J, Gagnon Z. Free-flow biomolecular concentration and separation of proteins and nucleic acids using teichophoresis. Talanta. 2023;255:124198.
293. Lee Y, Kwon JS. Microfluidic free-flow electrophoresis: a promising tool for protein purification and analysis in proteomics. J Ind Eng Chem. 2022;109:79–99.
294. Stepanova S, Kasicka V. Application of capillary electromigration methods for physicochemical measurements. In: CF Poole. editor. Capillary electromigration separation methods. Amsterdam: Elsevier; 2018; p. 547–91.
295. Dubsky P, Dvorak M, Ansorge M. Affinity capillary electrophoresis: the theory of electromigration. Anal Bioanal Chem. 2016;408:8623–41.
296. Duroux C, Hagege A. Interactions between copper(II) and beta-amyloid peptide using capillary electrophoresis-ICP-MS: Kd measurements at the nanogram scale. Anal Bioanal Chem. 2022;414:5347–55.
297. Miyabe K, Inaba S, Umeda M. A study on attempt for determination of permeation kinetics of coumarin at lipid bilayer of liposomes by using capillary electrophoresis with moment analysis theory. J Chromatogr A. 2023;1687:463691.
298. Mine M, Mizuguchi H, Takayanagi T. Kinetic analyses of two-steps oxidation from L-tyrosine to L-dopaquinone with tyrosinase by capillary electrophoresis/dynamic frontal analysis. Anal Biochem. 2022;655:114856.

How to cite this article: Kašička V. Recent developments in capillary and microchip electroseparations of peptides (2021–mid-2023). Electrophoresis. 2024;45:165–98.
https://doi.org/10.1002/elps.202300152