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ELECTROPHORESIS

REVIEW

Strategies for capillary electrophoresis: Method development and validation for pharmaceutical and biological applications—Updated and completely revised edition

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Abstract

This review is in support of the development of selective, precise, fast, and validated capillary electrophoresis (CE) methods. It follows up a similar article from 1998, Wätzig H, Degenhardt M, Kunkel A. "Strategies for capillary electrophoresis: method development and validation for pharmaceutical and biological applications," pointing out which fundamentals are still valid and at the same time showing the enormous achievements in the last 25 years.

Abbreviations: ADC, antibody–drug conjugates; AIQ, analytical instrument qualification; APTS, 3-(aminopropyl)trimethoxysilane; AQbD, analytical quality by design; ATRP, atom transfer radical polymerization; BLA, biological license application; CE-FA, capillary electrophoresis frontal analysis; CMC, critical micellar concentration; COC, cyclic olefin copolymers; cosmo, cationic polymer–coated capillary; CS, chiral selector; DoE, design of experiments; DQ, design qualification; DS, dextran sulfate; eACA, ε-aminocaproic acid; EK, electrokinetic; EKS, electrokinetic supercharging; EME, electromembrane extraction; FASS, field-amplified sample stacking; FC, fluorocarbon; FESI, field-enhanced sample injection; FITC, fluorescein isothiocyanate; GO, graphene oxide; HD, hydrodynamic; HPMC, hydroxypropyl methylcellulose; HR, high reverse coatings; iCIEF, imaged capillary isoelectric focusing; iEK, insulator-based electrokinetics; IQ, installation qualification; LE, leading electrolyte; LN, low normal; LPA, linear polyacrylamide; MAPTAC, [3-(methacryloylamino)propyl]trimethylammonium; MCE, microchip electrophoresis; ms-ACE, mobility shift affinity capillary electrophoresis; μTAS, micro-total analysis systems; OQ, operational qualification; PB, polybrene; PDMS, polydimethylsiloxane; PMMA, poly(methyl methacrylate); PSP, pseudostationary phases; PQ, performance qualification; PVA, polyvinyl alcohol; PVS, poly(vinyl sulfonate); QC, quality control; QCC, QC checks; RAFT, reversible addition-fragmentation chain transfer; SCARAFT, surface-confined aqueous reversible addition-fragmentation chain transfer; SMIL, successive multiple polymer layers; SST, system suitability test; TE, terminating electrolyte; TEA, triethylamine; TETA, triethylenetetramine; T-EthA, triethanolamine.

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The structures of both reviews are widely similar, in order to facilitate their simultaneous use. Focusing on pharmaceutical and biological applications, the successful use of CE is now demonstrated by more than 600 carefully selected references. Many of those are recent reviews; therefore, a significant overview about the field is provided. There are extra sections about sample pretreatment related to CE and microchip CE, and a completely revised section about method development for protein analytes and biomolecules in general. The general strategies for method development are summed up with regard to selectivity, efficiency, precision, analysis time, limit of detection, sample pretreatment requirements, and validation.

KEYWORDS
biopharmaceuticals, capillary electrophoresis, method development, troubleshooting, validation

1 | INTRODUCTION AND INSTRUCTIONS FOR USE

Capillary electrophoresis (CE) is an analytical technique used to separate charged molecules in solution based on their electrophoretic mobility. Due to its high efficiency, selectivity, resolution, and sensitivity, CE has become increasingly popular in recent years as a method for analyzing complex mixtures of molecules, leading to a number of developments that have significantly impacted the field. Intending to summarize such advances, this review aims to provide a general update on various topics related to CE and CE method development, from a previous review presented in 1998, which has been cited over 200 times and is well known among all CE scientists [1]. Now, almost 25 years later, the CE world has seen multiple innovations, countless new devices, techniques, methods, and practices, pushing CE into an established and frequently used analytical technique. As an example, it is worth mentioning that a Google Scholar search for "capillary electrophoresis" would render over a million hits in the last 25 years. There are almost 50 thousand papers about capillary zone electrophoresis (CZE), about 20 thousand capillary isoelectric focusing (CIEF) and almost 400 thousand hits about CE with sodium dodecyl sulfate (CE–SDS). In contrast, a search for the same terms (but dating before 1998) would render only 130,000 hits on CE in general. This trend is further illustrated by a presentation of Marjorie Shapiro from the Office of Biotechnology Products at the Food and Drug Administration on state-of-the-art analytical methods for the development of therapeutic proteins [2]. Most of the biological license applications (BLAs) nowadays use one or more CE methods for characterization and release that are implemented at the quality control (QC) level.

Although much of the information on the original review [1] is still valid and the fundamentals have not changed over the years, herein additional new examples, focusing on the most significant updates, are provided. Considering that it is not possible to provide a comprehensive coverage of the recent literature, Table 1 shows the sections that did not need an update. Here a brief visit to the original review is worthwhile. The original and this present review are strongly related, which is underlined by the widely maintained structure.

The previous Section 3 on "Peptide and protein separations" by CE has been restructured and now refers not only to peptides and proteins but also to all biopharmaceuticals. Section 3.2.4 of micellar electrokinetic chromatography (MEKC), which has become less relevant in recent years and is still dealt with in Section 2.4 in relation to selectivity, has been replaced by the promising method of CE coupled to mass spectrometry (CE–MS). In addition, there is a section on miniaturization in this update (Section 11), which has long been desired in order to shorten analysis times and has now been realized through the development of microchip instruments. A detailed new section on sample pretreatment (Section 6) has been written, which is why the former sections on sample stacking do not appear in the update. Section 9, Validation, was only briefly touched upon; an update of that would be promising as a standalone paper in the future. The chapter on Troubleshooting

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TABLE 1 Overview of the sections of the original review [1] that have not been updated, with information which remained interesting and valid.

Non-updated chapters from Ref. [1]
2.1.2 Aims of method development
2.1.4 Initial considerations (method development)
2.2.3 Complexing reagents for nonstereoselective separations
2.2.5 Capillary electrophoresis with polymeric separation media
2.5.1 General considerations (EOF)
2.5.2 Determination of the EOF and mobility μ
2.6 EOF
3.2.4 MEKC of proteins
5 Indirect detection
5.1 Performance
5.2 Method development
7.1 General considerations (quantitation)
7.3 Peak height or peak area?
7.4 Migration time precision and peak area precision
7.5 The role of the sample amount
7.6 The detection wavelength
7.8 Measuring precision
7.9 Miscellaneous
8.1 General considerations (LOD)
8.2 Instrumental aspects
8.2.1 Installation of more sensitive detector cells
8.3 Derivatization
8.4 Column overloading
8.5 Stacking
8.5.1 Principles
8.5.2 Field amplification without discontinuous electrolytes
8.5.2.1 In-column FASS
8.5.2.2 Head-column FASS (FASI)
8.5.2.3 FASS for Uncharged or Weakly Charged Analytes
8.5.3 Field amplification with discontinuous electrolytes
9 Validation

(Section 10) has been completely restructured and filled with very interesting and practical information.

2 | METHOD DEVELOPMENT FOR OPTIMAL SEPARATIONS

2.1 | Starting method development

2.1.1 | Selection of the appropriate separation technique

The first questions to ask when developing a method have not fundamentally changed since the original review: *What do I want to analyze, with what goal in mind, and what is the best technique for doing the analysis? If one of the appropriate techniques is CE, then which CE mode is most appropriate for my task?*

The first thing to consider is the requirements for the analysis. Of course, the basic requirements for analytical methods come to mind immediately. What is desired is a high sensitivity, separation efficiency, precision, speed, and ease of use. Also, the signal-to-noise (S/N) ratio should be appropriate. A high sample throughput should be possible and only a low sample concentration minimally necessary. The analytes should be stable and easily detectable in the chosen method. A design of experiments (DoE) approach is great for identifying a suitable analytical method, as later described in Ref. [3].

2.1.2 | Search for references

Compared to the original review, the amount of published materials has changed immensely. When searching for literature and interesting methods, one therefore encounters a flood of information and should be trained to select papers with regard to their quality.

2.2 | Selectivity

The term "selectivity" can be confusing. On the one hand, most analysts have a clear internalized concept of that word. However, not many people are able to clarify the exact meaning in one sentence. The major reason for the confusion might be the existence of the term "specificity," which can roughly be translated as the *absolute* absence of any interference [4]. Unfortunately, that clear distinction has not always been used very thoroughly in the past. Even in the R1 version of the ICH Q2 (still in use)—guidelines for specificity and selectivity, those terms were not properly used (https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf) [5]. Fortunately, the committee clarified both terms in the upcoming version (R2 (https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q2r2-validation-analytical-procedures-step-2b_en.pdf)) [6]. In order to avoid any confusion, the term "selectivity" is used in accordance to the IUPAC recommendations of 2001 [4].

In that definition, selectivity indicates how suitable a method is to determine analytes in matrices or mixtures without interfering with similarly behaving molecules [4].

From an ICH Q2 perspective, specificity or selectivity, in addition to potential impurities, degradation products, related substances or matrix, implies the absence of interference from other components present in the operating

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environment. This includes other pharmaceutical drug substances and products generated or analyzed in the same facility.

For any separation method, it is important to realize that selectivity is not the end goal of a method, but the means to achieve the ultimate goal that can be the quantification of a certain analyte, purity testing, and so on with certain precision and accuracy. Selectivity is, however, a very important prerequisite to reach these goals as a method cannot be accurate and precise without selectivity. For separation techniques, such as HPLC or CE, two fundamental types of parameters related to selectivity have to be distinguished. First, if the selectivity is related to the detection, it would be, for example, possible to selectively analyze one of more co-eluting substances using an MS detector. The other type of selectivity is directly related to the separative efficacy of the technique or method [4]. As the application of selectivity enhancing detectors is not unique to electrophoresis, the following focuses on separation selectivity.

Selectivity in CE is mostly related to differences in apparent mobility of two or more compounds of a mixture [7–9]. Thus, the most efficient way to influence selectivity in CE would be to alter the experimental conditions, to maximize the differences in apparent mobility. It can mainly be achieved by influencing the state of ionization, introducing complexing argents, or adding (pseudo)stationary phases [7–9]. However, other factors such as peak broadening due to longitudinal diffusion, Joule heating, length of the sample plug, and slow mass transfer (e.g., packed capillaries or wall adsorption) should be considered as they affect efficiency (see Section 2.6) and thus resolution [7, 9]. The authors of the review article "selectivity in capillary electrokinetic separations" [7] suggested to classify selectivity in CE by the separation mechanism. They divided the methods into two main areas: (1) CZE, CZE with organic modifiers, and nonaqueous capillary zone separations (NACE); and (2) all other mechanisms that include interactions with added selectors or stationary phases [7].

2.3 | Selectivity in CZE

As the effective charge and thus the apparent mobility of many important substances is dependent on the pH, the selection of an appropriate background electrolyte (BGE) is the most effective way to influence the selectivity in CZE [1, 7–10]. The most important factors of that selection are the type of buffer (pH range, buffer substances, and co-ions) and the ionic strength [7]. Depending on the analytes' physicochemical properties, a standard BGE should be considered the starting point for method development. It is recommended to begin method development using a BGE that (1) provides sufficient buffer capacity at a pH value where all relevant analytes are ionized and (2) allows the ionic strength to be clearly defined and reproducibly prepared. The latter can be achieved by mixing well-defined quantities of buffer compounds [7], (https://blog.sepscience.com/pharmascience/method-development-in-capillary-electrophoresis-ce-selecting-your-background-electrolyte), (https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf) [11]. Two recommended standard BGEs are 20 mmol/L sodium tetraborate (pH ≈ 9.3) or a mixture of 100 mmol/L phosphoric acid and 70 mmol/L Tris (pH ≈ 2.5) for acidic or basic analytes, respectively [8–10]. The tables with additional standard BGE compositions can be found in Refs. [8–10] (https://blog.sepscience.com/pharmascience/method-development-in-capillary-electrophoresis-ce-selecting-your-background-electrolyte). Using a pH, where all analytes are fully ionized, could be inappropriate for chemically similar substances. If such substances cannot be separated, the pH must be adjusted. Consider, for example, two positional isomers with different pK_a values. The maximal difference of the effective mobilities, and thus, the maximal selectivity will be achieved at a pH of $(pK_{a1} + pK_{a2})/2$ [8]. For the same reason, it is frequently argued that an optimal separation of comparable compounds can be expected to be obtained with pH values close to their pK_a-values [12]. However, it must be noted that in these pH ranges, the effective mobilities are very sensitive to small pH changes, which makes the method less robust [8, 10]. An alternative option to influence the selectivity without directly modifying the pH value would be the addition of organic modifiers or complexing agents [7].

An often-underestimated factor is the role of the co-ions in the BGE. Appropriate selection of the co-ion reduces electromigration dispersion and can also support in-capillary concentration injection possibilities such as sample stacking or transient ITP [7, 13]. In addition, BGE components can influence or control the electroosmotic flow (EOF) or prevent the adsorption of sample components to the capillary wall [7].

Unfortunately, there are situations in which a simple CZE will inevitably lead to a dead end. Of these, the two main cases are pH-independent neutral molecules and racemic mixtures. For such situations, it is recommended to use alternative electrophoretic separation techniques such as MEKC [8, 9]. For chiral compounds, BGEs containing chiral selectors (CSs) should be applied [14–17]. Furthermore, the field of NACE provides a broad margin for optimizing separation methods, or selectivity [7]. Each of these topics will be discussed in more detail in the following sections.

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2.4 | MEKC

MEKC is an established separation method that combines some aspects of CE (electromigration) with some aspects of chromatography (partition). Here, a surfactant (SURFace ACTive AgeNT) is dissolved in the BGE at a concentration that exceeds its corresponding critical micellar concentration (CMC), leading to the aggregation of monomers into micelles. These micelles serve as a "pseudostationary phase (PSP)" that, albeit at much lower rate than typical analytes, can move in response to the electric field and that can provide a phase for analytes to partition and thus provide an additional mechanism to improve the separation process. An overview of fundamentals and developments related to MEKC is given in a review from 2011 [18], but recent years have witnessed multiple advances in this field, including the exceptional mechanistic work from Farah and Tavares [19–22]. As expected, micelles are the soul of this separation method and its efficiency is controlled by the formation and characteristics of these aggregates (density, charge, hydrophobicity, size, solubility, etc.), the structure of the selected surfactant [23] and the partition coefficient of the analytes. Along the same reasoning, readers are strongly encouraged to consult a report from Kenndler's group, describing different equilibria involving surfactant monomers and their effect on migration in MEKC [24].

As a first consideration to rationalize the separation process, the micelle's structure and charge should be considered. Although perhaps the most commonly used surfactant for MEKC is SDS, the use of other negatively charged surfactants (such as those terminated in sulfate or carboxylic acids), surfactants with different charges (e.g., the cationic cetyltrimethyl ammonium bromide [CTAB] or tetradecyl trimethyl ammonium bromide [TTAB]), a noncharged (such as Brij 35) or zwitterionic surfactants should be considered. Adding to the versatility of this approach, surfactants can also be combined forming mixed micelles. Although not specifically addressed in this section, it is important to note that the addition of surfactants to the BGE can not only affect the separation mechanism but also the EOF [25] and the detection step [26]. In addition, it is worth mentioning that MEKC not only provides a versatile separation mode but also a simple way to work with poorly soluble analytes, as it is the case of Sudan dyes [27], alkaloids [28], some pesticides [29], or natural products [30]. Moreover, and due to its paramount importance to CE, the use of MEKC has been extensively discussed and reviewed in the literature focusing on their use for chiral separations [18], analysis of drug purity, log *P* value estimation [31], or the separation conditions for several different kinds of pharmaceutical compounds [32]. Among those, readers should consider two sequential reviews by Silva that provided foundational information related to approaches to increase resolution and sensitivity [33, 34] as well as innovations focusing on practical aspects [35]. More recent reviews have also covered the use of MEKC toward the separation of antidepressants [36]. It is also important to note that various micellar phases have been applied to facilitate stacking (e.g., [36–40]) or sweeping, an aspect that is specifically addressed in Section 6.1 of this review.

Understandably, MEKC methods are reasonable starting points to address multiple analytical problems [41] and multiple groups have reported applications of MEKC using a common BGE composed of a buffer system capable of supporting high EOF values (phosphate or borate) at concentrations in the 5–50 mM range, a soluble, negatively charged surfactant (such as SDS) at concentrations in the 10–50 mM range, and an organic modifier (such as acetonitrile or alcohol) at concentrations in the 5%–20% range. Under these conditions, it is important to carefully monitor the separation current, as Joule heating could become problematic and favor the development of bubbles, especially if a volatile modifier (such as methanol) is used. Optimization of these variables can be accomplished by a number of strategies, including univariate modes [42], multivariate analysis [43, 44], a linear decision tree [45], DoE [3], or even DFT calculations [46].

Applications of MEKC are abundant in the literature [31, 36, 37, 47–50], and thus only a few examples will be specifically mentioned in this section. Among those, we would like to highlight the applicability of MEKC to develop a microchip CE (MCE) method to differentiate original and seized, adulterated whiskeys. Here, the optimum BGE was composed of 50 mM sodium phosphate, pH 9 and 30 mM SDS; allowing the analysis of ethanol, butanol, and pentanol with limit of detections (LODs) of 0.17%, 0.18%, and 0.50%, respectively [51]. Table 2 [28, 42, 44, 45, 49, 52–59] provides additional examples of combinations of BGEs optimized for the separation of specific analytes.

2.4.1 | Microemulsion electrokinetic chromatography (MEEKC)

The separation mechanism of microemulsion electrokinetic chromatography (MEEKC) is similar to that in MEKC, with the difference that MEEKC uses microemulsions instead of micelles. MEEKC seems to be more advantageous than MEKC when applied for the separation of more hydrophobic analytes [60–62], because hydrophobic analytes are more likely to enter the microemulsion droplets (compared to the micelles) and MEEKC has a larger and more controllable separation window. Harang studied the effect of the microemulsion composition using compounds with different charges

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TABLE 2 Combinations of optimized BGEs for separation challenges.

Target analyte	Notes	References
Phytocannabinoids	BGE: 130 mM CHOL, 100 mM Tris, 100 mM CHES	[44]
Cardiovascular drugs	BGE: 10 mM borate buffer of pH 10.5 containing 25 mM SDS and 11% 1-propanol	[45]
Coumarin	150 mM SDS and 25% MeOH in 25 mM H_3PO_4 (pH 2.5)	[52]
Triazoles	Pseudostationary phase (15 mM SDS, 17.5 mg/mL SγCD) and the BGE consisted of 100 mM Tris, 100 mM phosphoric acid in-mixed hydro-organic solvent (80/20 v/v water/methanol), apparent pH 4.8	[53]
Unsaturated C_{18} fatty acids	50 mM ammonium perfluorooctanoate pH 9.5/MeOH (90:10, v/v) and BGE: 50 mM trimethylammonium perfluorooctanoate pH 9.5 water/MeOH (90:10, v/v)	[42]
Intestinal bacteria	The BGE were 1 × TBE containing SDS (10–35 mM) and PEO (5.0×10^{-6}–5.0×10^{-5} g/mL)	[54]
Synthetic cathinones	75 mM borate buffer at pH 9.3 and 0.4 mM of C12E10 surfactant	[55]
Caffeine and its main metabolites	35 mM phosphate, pH of 10.5, and 25 mM SDS	[56]
Apolipoproteins on human VLDL	20 mM sodium phosphate, 40 mM bile salts (50% sodium cholate and 50% sodium deoxycholate), 25 mM carboxymethyl-β-cyclodextrin (CM-β-CD) (pH 7.0)	[57]
EKC Marker	Review	[58]
Proteins	Review	[49]
Chiral separations	Review	[59]
Plant alkaloids	Review	[28]

Abbreviation: PEO, poly(ethylene oxide); SDS, sodium dodecyl sulfate; TBE, Tris-borate-EDTA.

and different lipophilicities [63]. A recent review nicely summarizes studies that investigated the effects of different modifiers or their concentration changes on the microemulsion [61]. Interestingly, one study compared the separation of methyl derivatives of quinoline with different CE techniques. These include CZE, NACE as well as MEKC and MEEKC with negatively charged or neutral micelles/microemulsions under various pH ranges, organic phases, surfactant concentrations, and ionic strengths. Although all reported methods have their particular strengths, it was noted that the best overall methods for separating the selected analytes were NACE and MEEKC (SDS-based). This study also highlighted the importance of analysis time (NACE being twice as fast as MEEKC) as well as the effect of the BGE on the sensitivity (impaired for BGE containing formamide) and peak symmetry. More importantly, the study provides a detailed description of how the elution order can drastically change depending on the surfactant (type and concentration), core phase for MEEKC (e.g., *n*-heptane or *n*-octane), pH (between 6 and 9.5), ionic strength, and electrolyte selected [64]. Similar findings regards migration time, resolution, and peak shape were reported considering additions of NaCl (up to 200 mM) and methanol (up to 10%) [64, 65]. In addition to MEEKC, it is important to note the possibility to use liposome electrokinetic chromatography (LEKC), an especially interesting alternative to estimate the octanol–water partition coefficient (log $P_{O/W}$) that also allows accounting for interactions with lipid bilayers [60, 66].

2.4.2 | Surfactants, modifiers, and markers

Although a broad number of surfactants have been used for several analytical techniques [67], SDS, sodium di-(2-ethylhexyl)-sulfosuccinate (AOT), Brij 35, polyethylene glycol, PEG, and a few alcohols are typically used in MEKC or MEEKC (see Table 3). Additional examples are listed in Table 5 and Table 6. In any of these cases, it is important to note the effect of the surfactant type/concentration on the surface charge of the capillary [25] and on the ionic strength of the BGE, as both variables can affect the magnitude and/or direction of the EOF. Because MEKC is also often used with organic modifiers to increase the solubility for hydrophobic analytes, it should be considered that the CMC of surfactants (including that of SDS or sodium lauryl ether sulfate) in most BGE will be different (typically lower) than the corresponding value in a pure aqueous solutions [68, 69]. In this regard, the pH of the BGE should also be carefully monitored, because it may not only determine if the migration of the analyte will be affected by electrostatic interactions with the surface of the micelle but may also affect the aggregation of the surfactant monomers [70].

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TABLE 3 Surfactants and modifier used in MEKC and/or MEEKC with example literature [18, 31, 48, 50, 68, 70, 72–74].

Type	Surfactant/modifier	References
Anionic	SDS	[18, 31, 70]
	Lithium dodecyl sulfate, sodium octane sulfonate	[18]
	Bile salts	[50]
	Sodium cholate (bile salt)	[72]
	Sodium lauryl ether sulfate	[68]
	Sodium tetradecyl sulfate (STS), sodiumdecyl sulfate	[73]
Cationic	Cetyltrimethyl ammonium chloride, cetyltrimethyl ammonium bromide (CTAB), didodecyldimethylammonium bromide	[18]
	Tetradecyl trimethyl ammonium bromide (TTAB)	[18, 70]
	Dodecyl trimethyl ammonium bromide (DTAB)	[73]
Non-ionic	Polyoxyethylene dodecyl ether (Brij 35), polyoxyethylene sorbitan monolaurate (Tween-20), cocamide monoethanolamine	[18]
	Polyoxyethylene dodecanol	[73]
Others	(Hydrophobic) Ionic liquids	[48]
	Polymeric surfactants	[18]
Chiral	Review	[74]

Abbreviation: SDS, sodium dodecyl sulfate.

Bile salts, cholesterol-based surfactants, can also be used for MEKC. Commonly used bile salts include sodium cholate, sodium taurocholate, or sodium deoxycholate [71]. For more detailed information, we also refer to an overview covering the years 2000–2020 [50]. As a recent example, it is worth mentioning that sodium cholate was applied in nonaqueous MEKC to separate compounds from *Salviae miltiorrhizae*. As expected, authors noted that concentrations higher than 140 mM (CMC = 111 mM) were beneficial in terms of resolution, but such improvements were at the expense of separation time and joule heating [72].

As previously stated, it is possible to modify the BGE with different substances to adjust the interaction between analytes and micelles and therefore the overall separation. Although common modifiers include organic solvents and electrolytes, a few groups have explored the use of additional compounds such as hydrophobic ionic liquids, which seem to act as a cosurfactant and improve the separation [48]. Another type of modifiers are nanomaterials, which can be used as support for the PSP. For example, BGE modified with multiwalled carbon nanotubes coated with SDS were successfully applied for the separation of herbicides, barbiturates, dansyl-DL amino acids, dipeptides, and model proteins (ribonuclease A, lysozyme, human serum albumin, α-lactalbumin and myoglobin; not baseline separated) [47].

Wiedmer, Lokajová, and Riekkola noted that a quantitative description of MEKC and MEEKC often requires calculating the mass distribution (or retention) factor; which is expressed in terms of the times required for the analyte, EOF marker, and the pseudostationary phase marker to reach the detector [58]. Although researchers have a large selection of potential markers, it is worth noting that Sudan III and dodecylbenzene are the most commonly used pseudostationary phase marker for MEKC, whereupon dodecylbenzene can be used in most MEEKC applications. Additional examples or markers, including hydrophobic deep eutectic solvents, have been recently reported [21, 75–79]. It should be noted, however, that not every marker is ideal for every situation or BGE, but the variety available allows one to choose the most appropriate one [58].

2.4.3 | Chiral MEKC separations

Separation of chiral compounds is important for many industries, but essential for the pharmaceutical industry because different enantiomers can feature very different biological effects [80]. For the general use of MEKC for chiral separation, recently published reviews [50, 59, 81] should be specifically highlighted. Fortunately, it is also possible to couple MEKC with chiral separation (CMEKC) followed by MS detection. Among the surfactants that enable that possibility, it is worth mentioning polymeric glucopyranoside-based surfactants [82, 83]. In addition, it is important to mention that bile salts can not only be used for normal, but also for chiral MEKC separations, supporting the use of co-CSs such as β-cyclodextrins (CDs) [50, 84]. However, bile salts can generally cause chiral separation on their own [85], whereas achiral ionic-surfactants such as SDS need a co-CS [74]. As

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mentioned, chiral CE–MS is an important field and is frequently discussed [36], as recently by Shamsi and Akter [86] and by Zhang et al. [74], who gave a good overview of usable CSs. Chiral MEKC–MS is also a topic here, and the transition from capillary to MS detection is discussed in detail. Coupling must be considered, as the BGE and CSs can cause contamination of the ionization source, ionization suppression, or strong background noise in the MS signal. This can be overcome by varying techniques and/or the selection of appropriate reagents [86].

2.4.4 | MEKC–MS applications

As previously noted, one of the challenges when coupling MEKC with MS is that the reagents usually used in MEKC (including CSs [36, 81]) are not compatible with MS detection. These cases require replacing nonvolatile PSP by a volatile PSP or non-disturbing PSP as well as reducing the amount (or avoiding) of PSP which is brought into the MS by depletion or reversed migration direction [35, 49, 87, 88]. For example, Moreno-González used ammonium perfluorooctanoate as a volatile surfactant, to separate different Sudan dyes via MEKC and to directly analyze them with MS. This also allowed the quantification of these Sudan impurities in chili food products with a limit of quantitation (LOQ) of 22 µg/kg at an S/N of 10 [89]. It is important to note that the same surfactant was also used by Couderc's group to separate C_{18} fatty acids, keeping simple UV–Vis detection [42]. Mol used atmospheric pressure photoionization (APPI) instead of electrospray ionization (ESI) for coupling MEKC with MS and demonstrated that APPI is less sensitive than ESI for ion suppression, and the use of SDS did not affect the photoionization [90]. Another way to couple MEKC (with troublesome reagents) and MS is to introduce a second separation step, such as a two-dimensional (2D) CE approach. With a CE–CE–MS approach, the analytes are in the first dimension separated by the main method, such as CGE, MEKC, or MEEKC. The second dimension is then introduced via a valve system, the respective peaks are transferred, and then separation is performed by CZE that leads to the MS detection for which the reagents are more compatible with MS [91]. Other techniques to directly couple CE to MS are also briefly discussed [91].

2.4.5 | Separation of biomolecules, peptides, and proteins by MEKC

MEKC is already established as a versatile separation approach for small biomolecules such as amino acids [92], but it can be also used for the separation of peptides [93, 94] and proteins, also with chirality [74]. In this case, the same surfactants can be used for the separation of proteins. Again, depending on the p*I* of the protein (or compound), the surfactant may need to be changed to improve the separation efficiency and, for example, avoid ion pairing behavior and impair the separation. As discussed in a recent review, it is possible to increase resolution/separation efficiency of proteins by implementing 2D separation [28]. Peptide separation via MEKC and MEEKC was applied to separate six different pharmaceutical insulin drugs from each other. In both variants, the insulin could not be completely baseline separated, whereby the separation with MEEKC was inferior. However, the scope of this study was small and the MEEKC buffer was without an organic modifier compared to the MEKC method [95].

2.4.6 | Principles in enantioselectivity

Fundamentals

Enantiomers have almost identical physical and chemical properties. In true-electrophoretic mode, when a strong electric field is applied across a buffer solution, separation relies on different migration velocities resulting from different charge densities among the analytes. As the charge density (charge-to-size ratio) among enantiomers does not differ in the isotropic medium, enantioseparation is impossible to obtain in electrophoretic-only modes [96–98].

However, they can be distinguished by their interactions with other chiral molecules in anisotropic environments [99]. The differences in the spatial arrangement of atoms or groups around a chiral center result in different affinities toward these CSs. In the presence of a CS, either stable diastereoisomers or reversible diastereoisomeric complexes are formed. The preferential interaction between a CS and one enantiomer over its counterpart is defined as enantioselectivity. Thus, enantiomers can be distinguished [16, 100–102].

Direct and indirect CE methods have been established in chiral separation. The indirect approach involves an optically pure reagent to form diastereoisomeric derivatives. As a result, the chemical and physicochemical behaviors of both enantiomeric products, now diastereomers, are different. Therefore, separation in achiral (isotropic) environments becomes possible. The separation can be achieved with this approach, although it is not always simple due to the complexity of the derivatization process [99, 103, 104]. On the other hand, direct chiral separation relies on reversible diastereoisomers interactions in anisotropic environments. The chiral system is formed by employing a chiral species known as a CS. It can be used as a

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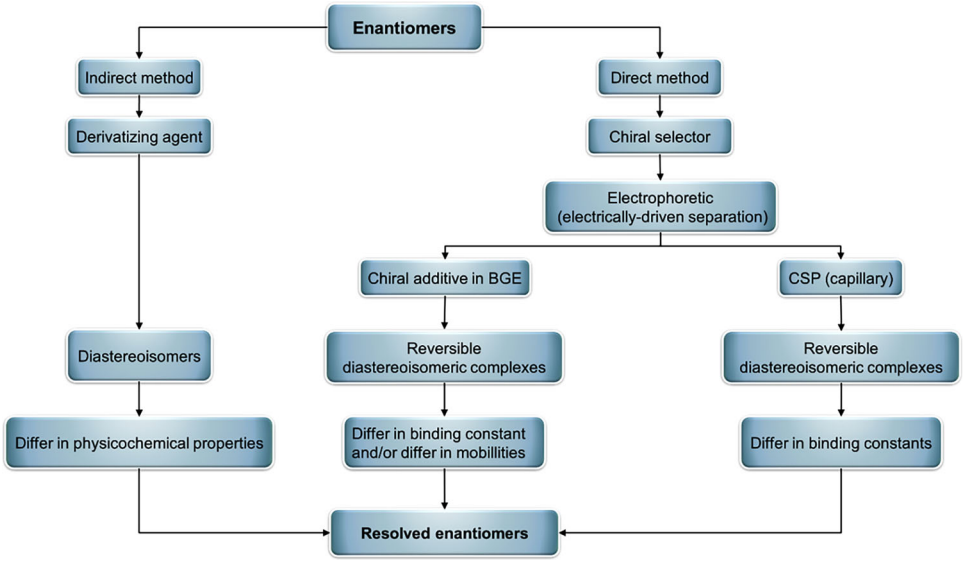

FIGURE 1 A scheme of the general concept of direct and indirect methods in chiral CE illustrating the formation of diastereoisomeric complexes in chiral CE. BGE, background electrolyte; CSP, chiral stationary phase. *Source*: Integrated from Refs. [99, 103, 104].

chiral additive in free solution or coated/immobilized into the capillaries as chiral stationary phases.

Chiral selectors in the background electrolyte

The simplest application is by adding the CS in the BGE. Thus, CSs containing BGE fill the entire capillary and form a dynamic phase due to their electrokinetic (EK) mobility as a pseudostationary phase. CE using a CS in the BGE is sometimes termed chiral electrokinetic chromatography (*c*EKC) ([105]; compare Section 2.4). This approach has been widely accepted in the chiral separations of pharmaceuticals [17, 106–108], biofluids such as drug metabolite in urine samples [109], and amino acids [110].

The CSs employed should fulfill several requirements, such as being stereoselective and being able to form a transient diastereomeric complex at least with one of the enantiomers. Moreover, it should be soluble, chemically stable, not interfere with the detection, and exhibit fast complexation kinetics ([16, 102, 104, 107]; see also Table 3 in Ref. [1]). A scheme of the general concept of direct and indirect methods has been established for chiral CE and is depicted in Figure 1.

Generally, for method development, an appropriate CS needs to be first identified. Selecting a CS is one of the most critical tasks in the molecular recognition of enantiomeric pairs [16, 102, 107, 111]. Numerous CSs have been widely used extensively over the years to improve the separations of various classes of analytes. In general, commonly used CSs are classified as macrocyclic/other antibiotics, chiral crown ethers, monomeric/polymeric surfactants, cyclic/linear polysaccharides, cyclic/linear oligosaccharides, and proteins [112]. Although computer modeling techniques can nowadays be used to predict the interaction of an enantiomer with a CS to some extent, it is still difficult to predict interaction *differences* of the enantiomers with a CS as the model uncertainties are generally larger than the difference needed of chiral CE separation. This is illustrated by the CD screening for the enantioseparation of the eight enantiomer pairs of a tetrapeptide [107, 113]. Some of the enantiopairs were not separated, while, under the same conditions, another enantiopair of the tetrapeptide showed a resolution as high as 7.5. Hence, the selection of an appropriate CS needs to be done experimentally by a screening experiment [17, 107, 113]. Method development strategies for direct methods using CS as BGE additives were outlined in Refs. [17, 102, 107, 114]. A simple method development starting strategy can be sketched as follows for basic, acidic, and neutral enantiomers.

Basic enantiomers are charged at low pH. A good generic low-pH buffer consists of 100 mM phosphoric acid and 70 mM Tris, resulting in a pH of 2.5. As a first step, screen for an appropriate CS in this buffer. Typically, neutral cyclodextrins are a good first choice. Inject the enantiomers in the buffer without CS and then screen the CDs for enantioseparation. A second option is the screening of charged cyclodextrins or other CSs.

Acidic analytes are negatively charged at a high pH. A good starting point is a 100 mM Tris–100 mM boric acid buffer, of which the pH is around 8.2. Although it is worthwhile to screen with neutral cyclodextrins also for acidic compounds, there is a risk that the separation window is too narrow [17], so charged cyclodextrins should also be taken along in the first screen. If the charged cyclodextrin–acidic enantiomer interaction is strong, it is possible that

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a reversed polarity is required. If a neutral cyclodextrin shows favorable, but indeed the separation window is too narrow, use a neutral coated capillary and reverse voltage. As a second choice for acidic enantiomers, cyclodextrin-modified MEKC can be tested (see Chapter 5 [16]).

For neutral enantiomers, charged CDs or chiral MEKC are the options of choice. When the CS is selected, optimize the concentration of the CS and other appropriate method parameters, see also Chapter 8 [16] and [17, 102, 107, 114]. For the optimization of the CS concentration, make a plot of the enantiomer migration difference against the CS concentration [102, 107, 115–117]. Under the right condition, this plot shows an optimum, although the optimum can be temperature dependent [118]. Please note that different plot shapes can be expected, if the complexation between enantiomer and CS is more complex than 1:1 stoichiometry [113].

It is important to point out that a simple separation of the two enantiomers is often not sufficient for the analytical task. For example, to screen one enantiomer as an impurity in the presence of the other enantiomer as the main compound, that is, to check the presence of the distomer in the presence of the eutomer, one needs to overload the sample to obtain a good S/N ratio for quantification [119]. Therefore, a resolution of at least 3 should be achieved here [120].

The previously reported separation approaches show that chiral compounds naturally possess unique molecular behaviors in chiral environments. Thus, bringing uniqueness to a chiral CE system requires certain conditions of CS concentration, pH, applied voltage, organic additive, and controlled or ambient temperature. All mentioned factors need to be optimized in the presence of CS as a buffer additive or coated/immobilized into a stationary phase and in a dynamic coating system to achieve selective and effective separation. Some new developments in chiral CE and applications are listed in Table 4 [96, 121–135].

Permanently and dynamically immobilized chiral selectors

In order to develop enantioselective methods, system optimization and modification involving novel (synthesized) selectors combined with column (capillary) pretreated techniques under certain CE conditions have been studied.

Xu et al. applied a combination approach of CS immobilization into a nanocoated capillary. The inner wall of the capillary was pretreated with a nanoparticle coating of poly(glycidyl methacrylate) with subsequent immobilization of glucosyl-β-cyclodextrin (selector). The separation method optimization was set up at 20 mM phosphate buffer, 30 kV, and ambient temperature. Under these constant CE conditions, increases in pH and selector concentration (40–60 mM) resulted in the increasing resolution (Rs) and selectivity factor (α) of racemic drugs (chlorpheniramine, CHL and nefopam, NEF) [135].

Another separation approach was conducted on similar racemic drugs using L-histidinium functionalized β-CD derivative as chiral ionic liquid buffer additive at different concentrations. Separation factors of buffer pH, applied voltage, and the organic additive were systematically reported. The CE conditions were set up at a constant concentration of 50 mM phosphate buffer and a capillary temperature of 25°C. The Rs increased with increasing selector concentration up to 20 and 25 mM for NEF and CHL, respectively. Rs for both drug models increased when the buffer pH was raised (up to 3.0). An increase in applied voltage (10–15 kV) led to an increase in Rs and separation efficiency and, on the other hand, a shorter migration time. Increasing the CS concentration led to a higher Rs because of stronger interactions. However, Rs decreased at a certain concentration of CS (25 mM). This indicates the interaction between CS and enantiomers reached maximum complexation. Meanwhile, a higher pH (3.6) caused a peak-broadening effect, thus reducing Rs. At an applied voltage of 25 kV, the effect of excessive Joule heating on decreased Rs was found. Furthermore, methanol at 20% (v/v) was found to be the best organic additive compared to ethanol and acetonitrile regarding the peak shape and Rs [136].

A dynamic coating technique has been applied using hydroxyethyl cellulose (HEC) as the coating agent and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) as the CS. Improvements in peak symmetry, migration time, and peak area precision were reported. The coating solution, for example, a polymer- or a low molecular mass-material, was simply flushed through the capillary. Under slightly alkaline conditions (pH 8.5), the adsorbed coating agent provided effective separation with excellent durability and reproducibility. Despite its simplicity, interference of the coating agent with the analyte might occur [134].

2.4.7 | Use of organic modifiers in CE

In order to achieve maximum efficiency, selectivity and resolution in CE analysis, organic modifiers are often used to modify the properties of the BGE [137]. They can be used to modulate the EOF. Organic modifiers can also enhance the selectivity by altering the solute–solvent interactions of the analytes. By using these organic modifiers, it is possible to optimize the separation of complex mixtures of molecules in CE. For the sake of simplicity and clarity, Table 5 [138–143], Table 6 [7, 144, 145], and Table 7 [7, 139, 141] contain only the additives that have been added in recent years. All other common additives can be found in the original version of this review from 1998 [1].

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TABLE 4 Method development- and applications-related techniques (compare [1], Section 2.2.4, Figures 10 and 11).

Chiral CE-system	Chiral selector (CS)	Coverage	Separation factor	Sample (category)	References
Buffer additive	*N*-Heterocyclic cyclodextrin derivates	Chiral recognition mechanism	pH and CS conc.	Organic acids, NSAIDs, antihistamines, fluoroquinolone antibiotics, and dansylated amino acids (negatively charged and zwitterionic model racemates)	[121]
	Heptakis-(2,3,6-tri-*O*-methyl)-*β*-cyclodextrin (TM-*β*-CD), native, derivitized, neutral, ionic CDs	Method optimization, validation, and application	pH, temperature, voltage, pressure, BGE, and CS conc.	Amlodipine (pharmaceutical)	[96]
	Heptakis-(2,3,6-tri-*O*-methyl)-*β*-cyclodextrin (TM-*β*-CD), native- and derivitized-CDs	Method optimization, validation, and application	pH, temperature, voltage, pressure, BGE, and CS conc.	Verapamil (pharmaceutical)	[122]
	Heptakis-(2,3,6-tri-*O*-methyl)-*β*-cyclodextrin (TM-*β*-CD), neutral- and charged-CDs	Method optimization, validation, and application	pH, temperature, voltage, pressure, BGE, and CS conc.	Mexiletine (pharmaceutical)	[123]
	Quaternary ammonium *β*-CD	Quality by design in chiral purity testing	pH, temperature, voltage, BGE, and CS conc.	*S*-Tenofovir (enantiopure antiretroviral drug)	[124]
	Maltodextrin (dextrose equivalent 4–7)	Method optimization, application, and robustness	pH, voltage, and CS conc.	Amlodipine (pharmaceutical)	[125]
	Sulfated cyclofructan-6 and sulfated cyclofructan-7	Method development and validation	pH, temperature, voltage, BGE, and CS conc.	Amphetamine and cathinone derivates (psychoactive substances)	[126]
	Human serum albumin	Method development and binding affinity determination	pH, voltage, and CS conc.	Amlodipine and verapamil (racemic and enantiopure substances)	[127]
	Gamithromycin	Method development and optimization	Voltage, organic solvent, BGE, and CS conc.	Amlodipine, primaquine (chiral primary amine substances)	[128]

(Continues)

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TABLE 4 (Continued)

Chiral CE-system	Chiral selector (CS)	Coverage	Separation factor	Sample (category)	References
	Kasugamycin	Method development and chiral recognition mechanism	pH, voltage BGE, and CS conc.	Ephedrine–pseudoephedrine, quinine–quinidine, cinchonine–cinchonidine, amlodipine, promethazine, and ofloxacin	[129]
Buffer additive (BGE containing CS with cationic additives)	Heptakis-(2,3,6-tri-*O*-methyl)-*β*-cyclodextrin (TM-*β*-CD)	Method validation	pH, BGE (buffering agent and cationic additives: Mg^{2+}, Ca^{2+}, Zn^{2+})	Dexibuprofen (pharmaceutical)	[130]
Buffer additive (BGE containing CS with chiral ionic liquids (CIL) additives)	Glucosyl-*β*-CD (with CIL additives amino acids: trifluoroacetate-L-hydroxyproline, nitricacid-L-hydroxyproline and trifluoroacetate-L-threonine)	Method development	pH, voltage, CIL conc., CS conc. Type, and proportion of organic modifier	Amlodipine, nefopam, econazole (pharmaceutical)	[131]
	Maltodextrin (dextrose equivalent 4–7) (with CIL additives: tetramethylammonium-D-gluconic acid and tetramethylammonium-shikimic acid)	Method development	pH, voltage, and CS conc.	analgesics, antidepressants, antiallergics, antifungal, antihypertensive, and antiparkinsonian (pharmaceutical)	[132]
Buffer additive (partial filling CE-FLD)	G-rich double-strand DNA, G-quadruplex, and DNA tetrahedron	Method development	pH, voltage, plug length of DNA selector, and injection interval	Ofloxacin (racemic substance)	[133]
Dynamic coating capillary	Hydroxyethyl cellulose (HEC), hydroxypropyl-*γ*-cyclodextrin (HP-*γ*-CD)	Method development and validation	pH, voltage, BGE conc., and CS conc. (HEC and HP-*γ*-CD)	Mitiglinide (pharmaceutical)	[134]
Coated/immobilized CSP	Glucosyl-*β*-CD	A combination of nanoparticle coating and immobilized selector	pH and CS conc.	Chlorpheniramine, nefopam (pharmaceutical)	[135]

Abbreviations: BGE, background electrolyte; CE-FLD, capillary electrophoresis- fluorescence detector; CSP, chiral stationary phase.

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TABLE 5 Buffer additives to enhance selectivity.

Additive	References
Ion pairing reagents	
Tetrabutylammonium bromide (TBA)	[138]
Hexamethonium bromide	[138]
Diammonium hydrogen phosphate	[138]
PDADMA	[138]
Polyethyleneimine (PEI)	[138]
Camphor-sulfonate	[139]
Surfactants	
Sodium tetradecyl sulfate (STS)	[139]
Sodium hexadecyl sulfate (SHS)	[139]
Dodecyltrimethylammonium bromide (DTAB)	[140]
Tetradecyltrimethylammonium bromide (TTAB)	[140]
Polyoxyethylene sulfate (Brij-S)	[141]
Complexing reagents	
PVP	[141]
MoO_4^{2-} or WO_4^{2-}	[142]
Proteins	
β-Lactoglobulin	[143]
Casein	[143]
Cellobiohydrolase	[143]
Human serum transferrin	[143]
Riboflavin binding protein	[143]
Miscellaneous	
2,10-Ionene	[141]

TABLE 6 Surfactants used in CE.

Additive	References
Anionic, SDS similarity	
Poly(sodium-10-undecylenate)	[7]
Poly(sodium-10-undecanylsulfate)	[7]
Anionic, Sodium cholate similarity	
Bile acids	[144]
Cationic, C_nTAB similarity	
Benzalkonium chloride (BAK)	[145]

TABLE 7 Chiral selectors.

Additive	References
Cyclodextrins	
ODMS-γ-CD	[139]
Noncyclic saccharides	
Dextran sulfate	[7]
Miscellaneous	
Camphorsulfonates	[7]
Calixarenes; (p-sulfonic calix[4]-arene)	[7, 141]
Ergot alkaloids	[7]
Quinidine, other cinchona alkaloids and derivates	[139]
Tert-butyl-carbamoylquinine	[139]
(−)-2,3:4,6-di-O-isopropylidene-2-keto-L-gulonic acid (DIKGA)	[139]

2.5 | CE with constant current or constant power

Most articles primarily mention using constant voltage for CE measurements, while occasional references are made to constant current or constant power methods. However, the actual utilization of these alternative methods is rare. Despite the potential drawbacks of constant voltage, such as excessive Joule heating, it remains the preferred measurement approach due to its prevalence in commercial instruments and the additional complexity associated with deviating from it. For example, the common equations for calculating effective mobilities include a voltage specification, which is usually the result of the setting in the software. Constant current mode is said to have more precise (intra-day) migration times because of smaller temperature changes [146, 147] whereas constant voltage mode might be the preferred choice when looking for a better inter-day repeatability [148].

Nonetheless, there are cases where conducting measurements under constant current or constant power conditions can be beneficial, particularly in mobility shift affinity capillary electrophoresis (ms-ACE) studies aiming to isolate analyte–ligand interactions while minimizing other electrophoretic variations. When performing ms-ACE measurements, one needs to make sure that the changes in electrophoretic mobility are solely due to analyte–ligand interactions. Therefore, there is a need to minimize all other changes in electrophoretic conditions [149]. Those changes might occur because of changes in temperature or changes in the BGE's properties.

Constant current or constant power modes offer advantages in terms of consistent and reproducible separations, reduce Joule heating, improve temperature control, and enable to optimize conditions for faster and more efficient separations with higher resolution and repeatability.

The constant current mode ensures that the current remains constant as the sample is loaded and run, which helps to avoid sample band broadening due to changes in the applied electric field. In 2022, da Costa et al. published a research article dealing with different approaches for controlling the electrophoretic migration [150]. They concluded that the current-controlled mode is superior to the voltage-controlled one. Furthermore, they give substantial theoretical background, including valuable equations,

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how to, for example, calculate mobility working under the current- or power-controlled mode.

Additionally, constant current as well as constant power mode helps to reduce Joule heating, which can cause sample degradation. The modes have a self-compensating effect as far as the correlation of temperature, conductivity, and applied voltage is concerned [151]. By maintaining a constant power, the separation can be faster and more efficient [152], resulting in better resolution and repeatability. Because of its better temperature control, this mode is recommended for BGEs with high conductivity, isoelectric focusing (IEF) and ACE [149, 152–154]. Regardless of the chosen mode, minimizing the impact of variable BGE composition is recommended by working with defined-concentration BGEs rather than pH-titrated ones (see Section 8).

2.6 | Separation efficiency

When an analyte is injected into a capillary and an electric field is applied, the sample zone does not remain focused but widens over time due to diffusion effects. The parameter for the dimension of peak broadening during electrophoresis is separation efficiency. Therefore, the dimensionless parameter N is often calculated, in order to compare the quality of separations by CE to chromatography, where N is the plate number, the plate height H, and the capillary length L are used to describe the processes mathematically as has already been described in chromatography (Equation 1) [1, 155]:

$$N = \frac{L}{H} \tag{1}$$

If diffusion effects occurring in an electric field and the analyte's mobility are respected, the following equation can be used for calculations:

$$N = \frac{\mu U l}{2DL} \tag{2}$$

where μ is the electrophoretic mobility of the analyte, D the molecular diffusion coefficient, l is the effective length, L the total capillary length, and U the applied voltage [155, 156].

The different reasons for peak broadening are longitudinal diffusion, Joule heating, the influence of sample plug length, the influence of the EOF, the effect of electromigration dispersion protein, and adsorption to the capillary wall [157].

Longitudinal diffusion has the greatest influence on band broadening as concentration gradients form at sample interfaces. These zones get broader with increasing time [155]. It seems reasonable to use a higher applied potential to shorten separation time and reduce the previously mentioned effect. On the other hand, using higher voltages increases the current and can lead to excessive Joule heating (see also Section 8 for verification with Ohm's plot) [158, 159]. Because the wall side of the capillary conducts heat faster to the outside, a temperature gradient of a parabolic profile inside of the capillary is created. This temperature gradient along the cross section is responsible for differences in viscosity of the BGE and therefore in different electrophoretic mobilities of analytes resulting in band broadening. This does not apply to EOF because its velocity depends only on the viscosity near the capillary wall [158, 160]:

$$J\ (t) = i^2Rt = \frac{U^2t}{R} = \frac{U^2At}{\rho L} \tag{3}$$

Equation (3) shows Joule heating (J) generation as a function of time (t), which is related to the current (i) and resistance (R). The effect can be reduced by a smaller cross-sectional area (A), given that the same conditions of voltage (U), capillary length (L), and resistivity of the solution (ρ) are valid [158]. It would also be beneficial to use longer capillaries and low conductivity buffers to increase the resistance and reduce heat generation [160].

Another possibility to diminish the effect of Joule heating is the dissipation of the heat that is dependent on the surface-to-volume ratio. Therefore, it is often advantageous to use capillaries of narrow inner diameter. Thus, other negative effects such as a limited loading capacity, poor detection sensitivity, and high flow-induced backpressure appear [158, 161]. To facilitate the dissipation of heat, a heat sink [162] or cooling system can be used. When strong currents are prevalent, those that cool with liquids are a bit more efficient than those that cool with a forced airflow [163], although the latter represent the simplest and most cost-efficient option, Rogers et al. suggest an approach to lower Joule heating by using microstructured fibers (bundled capillaries), hence increasing the surface while maintaining the volume. Using the same conditions, they determined an 82% better separation efficiency than for using standard capillaries of the same cross-sectional area [158].

Kenndler et al. compared the effect of Joule heating between two organic solvents and water (all with the same initial electrical conductivity). They found that the temperature gradients are larger in the organic solvents than in water, but that the effect has only a small influence because longitudinal diffusion is of much greater importance [157].

Another effect on separation efficiency is the influence of electromigration dispersion. It can occur when the

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sample is overloaded, low conductivity buffers are used, or when the mobility of the BGE co-ion does not match with the mobility of the analyte [8]. As the current in the capillary is constant and represents a product of conductivity and electric field, a variation in conductivity in the sample zone will change the local electric field and thus the migration velocity of the sample molecules. The varying conductivities of the analytes in the sample zone result in different migration velocities, leading to an asymmetric and triangular peak shape. Although a high sample concentration is good for detector sensitivity and a low buffer conductivity is useful to reduce Joule heating, those also contribute to electromigration dispersion as mentioned before [160]. The electromigration dispersion effect can also be reduced by better matching of the BGE co-ion mobility with the analyte's mobility [8].

To maintain a high separation efficiency, the maximum size of the sample plug should be generally <1%, or about <5% of the total capillary length when using stacking [163, 164]. Here, hydrodynamic (HD) injection is the most commonly used injection technique at the moment whereby the injected sample amount can be controlled by variation in the injection time or the applied pressure [163]. Furthermore, the effect of the EOF on separation efficiency is substantial. If the EOF is directed against the analyte's direction of migration, then there is more time for diffusion. Compared to a situation without EOF or with an EOF directed in the same direction as an analyte, the peak would be wider and the separation efficiency lower [157].

Other contributing factors to separation efficiency are analyte–wall interactions [165]. Leclercq et al. investigated their influences using coated capillaries. They developed a method to quantify protein adsorption based on the calculation of retention factors by determining separation efficiency. Finally, they suggest the consideration of optimum electric field strength, a sufficiently long capillary, and a low internal diameter to get the best possible separation efficiency [156]. Another investigation was done examining the correlation between separation efficiency and cationic bilayer coatings [166]. Didodecyldimethylammonium bromide as coating reagent and capillaries of 5, 10, and 25 μm inner diameter were used. It was shown that by using coated capillaries with a smaller inner diameter (5 or 10 μm instead of 25 μm), higher plate numbers could be achieved even after several runs without renewing the coating. For smaller inner diameters, the separation efficiency increased, whereas the S/N ratio decreased. Furthermore, the relative stability of coatings was demonstrated by calculating separation efficiencies for the separation of proteins. The more separations were conducted, the lower the separation efficiencies got [166].

Capillary coatings can be either dynamic or permanent. Hajba et al. gave a good overview of often-used coatings for protein analysis by CE [167]. There is also the possibility to use nanomaterials as coatings. Some examples are gold-, metal-oxide, or polymer nanoparticles. Their big advantage is a large surface-to-volume ratios [168]. Although many surface chemistry options are possible, care must be taken because of possible interference with detection systems, such as interference with ionization in mass spectrometric detection or light scattering in UV–VIS detection. Thus, they are mostly employed as permanent coatings [167].

As illustrated here, there are many different factors influencing separation efficiency. Many of them are interrelated or influence each other so compromises must be found to obtain the best possible results.

2.7 | Analysis time

As stated in the 1998 version of this article [1], the most used strategy for reducing analysis time is still reducing the length of the capillary. A reduction of 25% of the migration time is obtained when the capillary length is reduced by 50%, as shown in the following equation:

$$t_{M,2} = t_{M,1} \frac{l_2}{l_1} \frac{L_2}{L_1} \tag{4}$$

where $t_{M,1}$ and $t_{M,2}$ refer to the analyte migration time, l_1 and l_2 represent the capillary effective length, and L_1 and L_2 are the overall capillary length that depends on the specific experimental setup [153]. The use of short capillaries offers the potential to shorten analysis time down to seconds, which is quite comparable to miniaturized analysis (discussed in Section 11). However, short capillaries are suitable in cases where enough separation resolution can rapidly be achieved. There is a limit on how short the capillary length can be in commercial CE instruments [169, 170]. Analysis time can also be shortened by either increasing the overall EOF by increasing the applied separation voltage or modifying the characteristics of the BGE (pH and ionic strength), or by the addition of pressure-driven flow. However, these strategies can be employed only if they do not undermine the separation process and if the injection plug length is appropriate. Furthermore, changes in analysis time can also significantly influence the baseline noise of the separation, which in turn would affect the precision of the analyte quantification.

It is important to remember that migration times cannot be directly used in CE to identify unknown analytes, as it is not a robust parameter. Migration times depend on EOF and the presence of pressure-driven flow, perhaps added to assist EOF, during the separation. The effective electrophoretic mobility is a better parameter to be used

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for analyte identification [171]. Analysis time also depends on variations in migration time caused by analyte–wall interactions that, in turn, depend on the conditions of the capillary wall, which vary along the lifetime of the capillary. This can only be reduced by employing coated capillaries or the addition of surfactants to the BGE. A less than optimal repeatability of migration times has been considered a weakness of CE and must be taken into account during method development [171, 172]; however; with analytical quality by design (AQbD) method development, excellent precision can be achieved (see Section 8). A precise determination of migration time is one of the main characteristics to consider when evaluating CE instrumentation and equipment [173]. In summary, there are several strategies that can be employed to modify analysis time, depending on if the priority is separation precision or separation speed [1].

3 | PROTEINS AND BIOPHARMACEUTICALS

CE offers a vast range of possibilities for the analysis and characterization of proteins and biopharmaceuticals. CE is not only the method of choice for high-resolution DNA sequencing, but it is also very useful for the analysis and QC of therapeutic proteins, particles such as viral vaccines or lipid nanoparticles (LNPs), intact cells and much more from small inorganic ions to high-molecular-weight biomolecules [174]. Indeed, one can argue that the progress in the field has been driven by the need for minute characterization and precise analysis of biotherapeutics.

The characterization of biopharmaceuticals continues to be a challenge, as they are very large and structurally complex, and generally display a multitude of microheterogeneities [175]. A year ago, Kumar et al. comprehensively reviewed the applications of CE for the characterization of biopharmaceutical products [176]. In their review, they outline the analytics of biopharmaceuticals such as monoclonal antibodies (mAbs), antibody–drug conjugates (ADCs), fusion proteins, growth factors, cytokines, nucleic acids, and viral vectors, particularly related to the characterization of quality attributes. The review by Lechner et al. discusses state-of-the-art analysis of mAbs and ADCs [177] and additional reports [178–183] are discussed in other sections of this review. The application of CE in the pharmaceutical industry, related to the analysis and characterization of therapeutic proteins, is well described by Kaur et al. [184]. In the review, the advantages and disadvantages of the individual capillary electrophoretic techniques are explained and the latest developments of CE-based methods for therapeutic proteins are described.

The review by Fekete et al. provides insight regarding various chromatographic, electrophoretic, and mass spectrometric approaches for the analysis of protein biopharmaceuticals, with particular emphasis on mAbs [185]. Many different techniques are compared there. Among them are chromatographic techniques, such as ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography, hydrophilic interaction liquid chromatography, and reversed phase liquid chromatography (RPLC), as well as electrophoretic techniques, such as CZE, CIEF, capillary gel electrophoresis (CGE), and also mass spectrometric techniques. Mass spectrometric approaches include intact protein and middle-up measurements, top-down and middle-down measurements, bottom-up measurements, and glycan MS measurements [185].

3.1 | Benefits of capillary electrophoresis

CE has long been criticized for the lack of robustness of the methodology; however, this aspect has been significantly improved in recent years by developments in instrumentation [169], reliable injection, and stable EOFs in capillaries [173]. In addition, best practices for CE have been identified, especially within the (bio)pharmaceutical industry, further improving precision and robustness. Process tools such as AQbD also improved focus on developing for the intended use of a method, influencing choices made during development. Extensive examples are described in the work of van Tricht [186] and Geurink [187]. There are applications, such as biopharmaceutical analysis, where CE performs better or provides complementary information, and in these areas, the various CE modes have become indispensable techniques. Furthermore, the development of accessible miniaturized instruments that allow very rapid analysis is helping to make CE an attractive alternative for routine high-throughput analysis. The advantages of CE over other separation techniques also include its high separation efficiency and the use of aqueous buffers, which favor the preservation of the higher order structure of proteins [182]. One notable advantage of CE over most LC modes is its environmental friendliness, as it requires significantly smaller volumes of solvents, resulting in a considerably smaller amount of waste produced [188]. Compared to HPLC, CE consumes significantly less reagents, buffers and sample, and has a higher separation efficiency with often shorter analysis times. Furthermore, many different separation modes can be used with CE and different detection schemes introduced [189]. CE is an established analytical technique for biopharmaceuticals, due to the benefic characteristics

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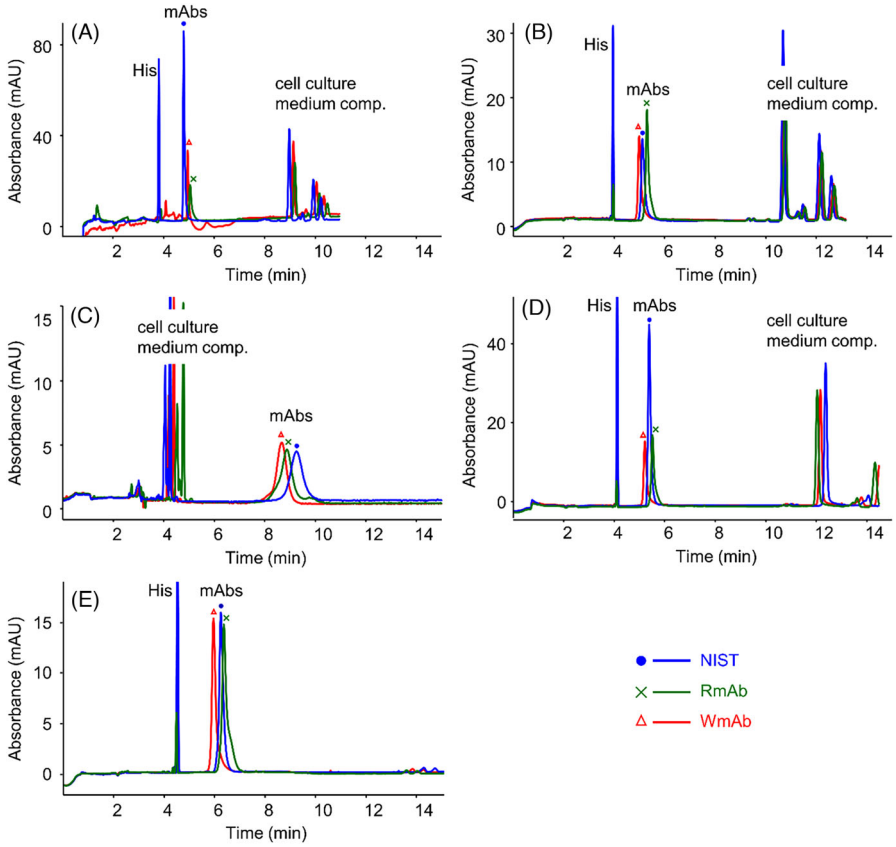

FIGURE 2 Rituximab, Waters monoclonal antibodies (mAb), and NISTmAb analyzed on (A) an uncoated capillary, (B) a polyvinyl alcohol (PVA)-coated capillary, (C) a successive multiple polymer layer (SMIL)-3 coated capillary with BGE: 100 mM phosphate, 70 mM Tris, and 0.1% poloxamer, (D) a dynamic triethanolamine (T-EthA)-coated capillary with BGE: 100 mM phosphate, 70 mM T-EthA, 0.1% poloxamer, or (E) a dynamic triethylamine (TEA)-coated capillary with BGE: 100 mM phosphate, 70 mM TEA, 0.1% poloxamer. Effective separation length was 24.5 cm. *Source*: From Ref. [193].

such as high resolution separation and the possible time and resource saving miniaturization [176, 177, 190].

3.2 | Separation strategies for proteins and biopharmaceuticals

3.2.1 | CZE

CZE is also very popular for the analysis of proteins because it is independent of the use of commercial kits (although kits with known compositions are highly appreciated) and often offers fast analysis times. This is important for QC, but particularly attractive for process analysis. CZE and (i)CIEF show comparable separation efficiencies [191, 192].

In the meantime, two methods have become particularly established for the analysis of mAbs. One uses triethanolamine (T-EthA) and coated capillaries [193] and focuses on the mAb concentration determination, including complex matrices such as upstream process samples (see Figure 2). The other, often called the He–CZE-method [194] or eACA–CZE method, uses ε-aminocaproic acid (eACA) [170] and determines charge heterogeneity (see Figure 3). Both methods are shown in Table 8. Their performance is well discussed in the given publications. These two methods are also given high priority in recent research papers [170, 193].

There are a number of variants of both methods [170, 191, 193, 195]. Those may be optimized for a specific mAb (see Section 9.1.1) [196]. However, these methods are characterized in less detail than the methods described in Table 7, for example, with respect to precision and robustness. Based on similar starting conditions as the T-EthA method, Villemet et al. mapped complex samples of peanut allergen proteins [197]. In order to increase buffering capacity and influence resolution between proteins, some of the BGE base was replaced with glycine, which buffers in the same pH-range as the first pK_a of phosphoric acid.

Besides the T-EthA and the eACA method, there are several alternatives, which again are less well characterized but offer a wide range of alternative conditions,

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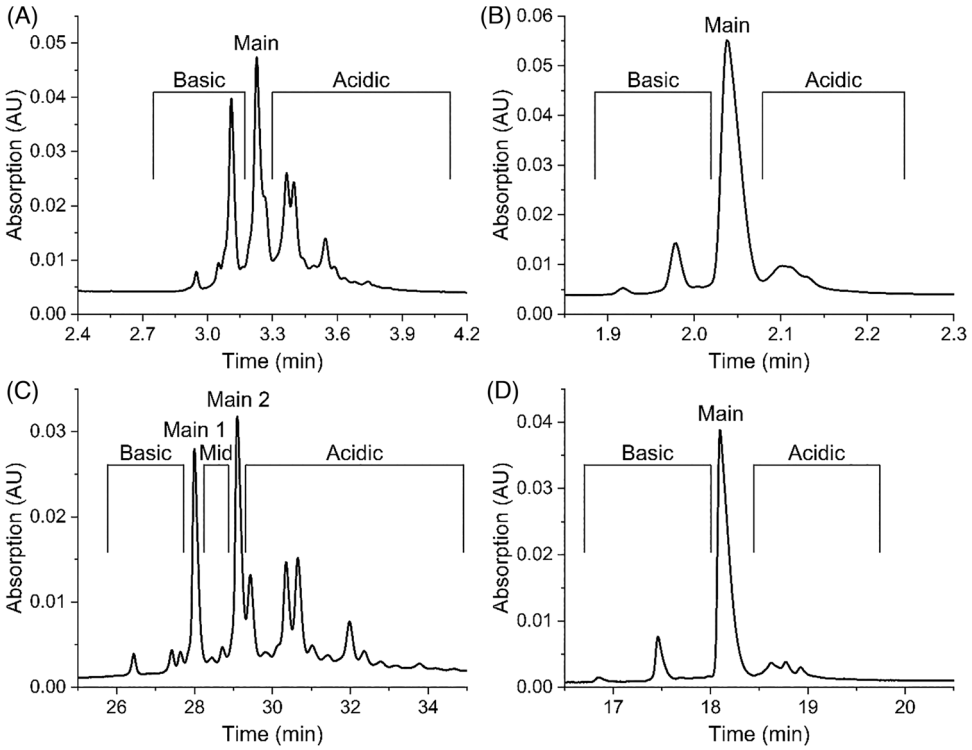

FIGURE 3 Peak profiles of Waters Intact monoclonal antibody (mAb) Mass Check Standard (A and C) and NISTmAb (B and D), high-speed (HS) ε-aminocaproic acid (eACA) capillary zone electrophoresis (CZE)–UV method used in (A) and (B); HR eACA CZE–UV method used in (C) and (D). *Source*: From Ref. [170].

for example, buffer pH values ranging from below 3 up to 10, various coatings and alternative reagents such as diaminobutane, tetraethylene pentamine, as most recently reviewed by Stutz in 2023 [183]. These methods can be a good starting point for the development and validation of own CZE methods, if neither the T-EthA nor the eACA methods can be successfully applied for one's own analytical task. However, keep in mind that these methods were initially developed for mAbs with pI ranges from 7 to 9. An example of an AQbD-developed method with significant impact on the production process was described by van Tricht and Geurink [187, 198–200]. This method's purpose was to determine adenovirus type 26 (Ad26) particles in all process intermediates as well as in drug substance and drug product. The tris–tricine pH 7.6 BGE for this method was carefully designed. Tris has been used in Ad26 formulation buffers, and tricine was previously used in AEX. The pK_as of tricine and tris are in the same range, so both tris and tricine buffer the BGE. In addition, tris and tricine are both low-conducting components, so relatively high concentrations were used, resulting in high buffering capacities (214 mM, according to calculations with PeakMaster [201]). The BGE could be precisely and robustly prepared by fixed concentrations, where the pH was measured to control the preparation. Polysorbate-20 was added to the BGE to prevent adsorption by matrix components of crude process samples. The resulting method was highly robust and precise, so that the 3-day-hold time previously required for the analysis of the in-process control measurement could be reduced to less than 2 h. The effect of this cannot be overestimated. In the COVID-19 pandemic, this meant that the production time of every batch of COVID-19 vaccine (ca. 10–50 $\times 10^6$ doses) was reduced by 3 days each, improving the quality with less risk for degradation and more precise Ad26 particle concentration measurements. The inherent robustness of the method facilitated transfer to multiple production sites [187].

3.2.2 | CIEF and iCIEF

CIEF and its closely related mode imaged capillary isoelectric focusing (iCIEF) are treated together within this section. CIEF was introduced by Hjertén in 1985 [202]; however, iCIEF was not mentioned in our original publication and is one of the important developments in this field. An in-depth summary about iCIEF has been provided in a recent review by Wu et al. [203], and some key points are incorporated in the following. Its development started in the 1990s [204] and it is now (2022) commercialized by two companies, namely ProteinSimple (Bio-Techne Corp.) and Advanced Electrophoresis Solutions Ltd, in

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TABLE 8 Two established methods for capillary zone electrophoresis (CZE) of monoclonal antibodies (mAbs): method and performance parameters.

(I) T-EthA method for the concentration determination of mAb (in example process samples) [193]

Polyvinyl alcohol (PVA)-coated capillaries with 50-µm id, a total length of 33 cm with an effective length of 24.5 or 8.5 cm (e.g., Agilent Technologies)

Separation voltage: 16 kV, ramped over 0.5 min (approx. 45 µA)

Sample injection at 10 mbar for 5 s, followed by the injection of a BGE plug using the same conditions

$T = 20°C$

$\lambda = 210$ nm

Conditioning: PVA capillary successively flushed with 10 mM phosphoric acid, water, and BGE at 1 bar for 20 min each before first used, and for 10 min each at beginning of each working day. Before injection, capillary flushed with 10 mM phosphoric acid for 1 min and BGE for 2 min, each at 1 bar

BGE: 100 mM phosphoric acid, 70 mM T-EthA, 0.1% poloxamer. pH after preparation: 2.5

The intra-day precision and accuracy were 2%–12% and 88%–107%, respectively, and inter-day precision and accuracy were 4%–9% and 93%–104%, respectively

t_{ana} = 6–11 min

(II) eACA method for the charge heterogeneity determination in drug substance and drug product [170]

The BGE comprising 400 mM eACA and 2.0 mM. The different reasons for peak broadening are longitudinal diffusion, Joule heating, the influence of sample plug length, the influence of the EOF, the effect of electromigration dispersion protein, and adsorption to the capillary wall TETA adjusted to pH 5.7 with glacial acetic acid, and 0.05% HPMC was used. First, 420 mM eACA, and 2.1 mM TETA at pH 5.7 was prepared, which can be stored at 2–8°C with an expiration date of 6 months. Add 13.8 g of eACA and approximately 170.0 mL of deionized water to a 250-mL glass beaker and stir until dissolved. Add 76.8 µL of TETA and stir to mix the solution. Adjust pH to 5.7 ± 0.05 with glacial acetic acid. Transfer the solution to a 250-mL glass volumetric flask and fill to 250.0-mL volume with deionized water. Afterward, the 1% (w/v) HPMC was prepared which can be stored at ambient temperature with an expiry date of 3 months. A volume of 30.0-mL deionized water was added to a 50-mL glass volumetric flask followed by 0.3 g of HPMC slowly added to help disperse HPMC. Stir covered overnight or until dissolved at ambient temperature. Both solutions (19.0 mL of 420 mM eACA 2.1 mM TETA buffer [pH 5.7] and 1.0 mL of 1% HPMC into a 20-mL glass volumetric flask) were combined to reach the final concentration of the BGE. The BGE can be used for 2 weeks and needs to be stored at 5°C in the refrigerator. For capillary storage, 10 mM phosphoric acid, for the rinsing procedure, 0.1 M hydrochloric acid was used.

There are *two versions* of the method that differ in capillary lengths and separation voltages:

High speed with a capillary of 10 cm (Sciex)/8.5 cm (Agilent) effective length, and of 30 cm (Sciex)/33 cm (Agilent) total length, and a separation voltage of 29 kV (Sciex)/30 kV (Agilent), as well as, *high resolution* with a capillary of 40 cm (Sciex, and Agilent) effective length, and of 50 cm (Sciex)/48.5 cm (Agilent) total length, and 20 kV (Sciex)/19.4 kV (Agilent)

$\Lambda = 214$ nm, 8 Hz (Sciex)/10 Hz (Agilent)

$T = 25°C$ (separation); $T = 15°C$ (sample storage)

Sample injection by applying 35 mbar (0.5 psi) for 5 s for high speed and for 10 s for high resolution.

More method details can be found in Wiesner et al. (in press) and the corresponding supplementary material (Tables S2–S4).

%Corrected peak area: 0.2%–2% RSD

t_{ana} = 2.5–4 min (high speed), 20–35 min (high resolution)

Abbreviations: BGE, background electrolyte; eACA, ε-aminocaproic acid; HPMC, hydroxypropyl methylcellulose; PVA, polyvinyl alcohol; T-EthA, triethanolamine.

dedicated iCIEF systems [205, 206]. Recently, an integrated chip-based iCIEF-MS system was introduced by Intabio (now SCIEX) [207]. In comparison to CIEF, iCIEF has several advantages. Through the nature of the employed so-called whole column imaging detection [208], it is possible to monitor the focusing process in real time, thus helping with method development and troubleshooting. Furthermore, as the mobilization step is not needed, time is saved and possible challenges during mobilization, for example, peak broadening or distortion, are avoided [203]. Generally, shorter capillaries with a larger internal diameter than in CIEF are used. This reduces the focusing time and improves the LOQ/LOD. Despite these impressive advantages, it should not go unmentioned that the available systems are proprietary and one is locked into a vendor-based solution, including reagents (kits) and capillary cartridges.

CIEF and iCIEF have matured into techniques that are routinely applied in analytical laboratories, especially in the biopharmaceutical industry. Exemplary applications include, but are not limited to, monoclonal antibodies [192, 209–215], bispecific antibodies [216–218], ADCs [219, 220], PEGylated proteins [221, 222], glycoproteins, in particular erythropoietin [213, 223, 224], and fusion proteins [213, 225]. Because this subsection is part of the former "proteins" section, which was extended to all biopharmaceuticals, it should not go unmentioned that the range of applications has been expanded beyond

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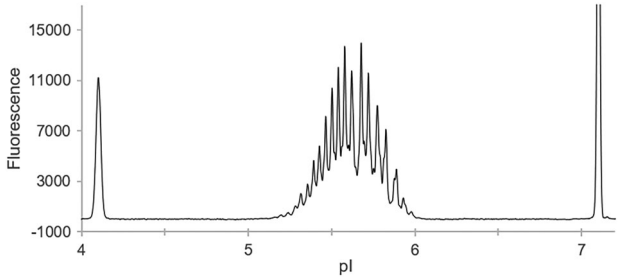

FIGURE 4 Imaged capillary isoelectric focusing (iCIEF) electropherogram of the hACE2/His receptor. *Source*: From Ref. [239].

proteins and includes oligosaccharides [226], LNPs and related mRNA vaccines [227–230], virus-like particles [231, 232], poliovirus [230, 233], adeno-associated virus [230, 234–237], papillomavirus [238], and bacteria and yeast cells [230]. Figure 4 shows a very exemplary iCIEF electropherogram of the hACE2/His receptor, which provides a clear representation of the signals of the individual isoforms of a protein.

In comparison to the preceding paper [1], the separation principle and general setup remain unchanged, whereas practical implementation and method development evolved. For the CIEF method development, the work of Mack et al. [240] provided a platform method. Many publications on CIEF after this influential paper incorporate some of the concepts applied therein. For iCIEF, the vendors provide initial method parameters for method development. As CIEF and iCIEF share most concepts, the parameters described later are applicable to both modes if not otherwise indicated.

Normally, method development starts with a platform method and parameters are subsequently optimized. Usually a so-called master mix, consisting of water, ampholytes, p*I* markers, spacers or sacrificial agents, solubilizing agents and a dynamic coating, and viscosity enhancing agent, is prepared. Aliquots thereof are mixed with the samples in the required ratio. A "master mix" preparation is commonly used for several samples, ensuring a consistent composition of the contained components. Optimization of its composition is one of the main objectives during method development. Beforehand, the sample's salt content should be minimized and potentially interfering substances removed [241–243]. This may be achieved through a buffer exchange. At the same time, it should be kept in mind that this may adversely affect the stability of the contained protein(s).

The lower and upper limits of the pH range are usually considered to be around 2.5 and 10–12, respectively. They result from the specifications/properties of the carrier ampholytes, whose useful range ends there. Individual differences between the proprietary ampholytes ranges and properties exist. Ampholytes were characterized in-depth in a series of six articles by Righetti and collaborators. The investigated ampholytes were all well suited for acidic to neutral pH ranges but became worse in the basic regions (above pH 8) [244–249]. Ampholytes are available as broad range and narrow range ampholytes and their selection plays an important role for the $\Delta pH/dx$ of the pH gradient and thus resolution [250, 251]. Common brands include Pharmalyte, Bio-Lyte, Servalyte, and AESlyte. The latter ones are relatively new and first reports about their use are published [252]. In an application note, the background noise of different ampholytes was compared, indicating differences between brands [253]. During CIEF–MS, Wu et al. described a significantly higher background noise in the high mass range for Servalytes compared to Pharmalytes [218]. Lot-to-lot (in)consistency has been an issue and a recent patent suggest an LC–MS approach to ampholyte analysis before use in (i)CIEF [254]. The use of immobilized pH-gradients is reported in the literature [255–257], but they are currently not commercially available.

As marker for the p*I*, short peptide sequences with a defined amino acid sequence are commonly used [258, 259]. These peptide markers are available through different manufacturers. Alternatively, low-molecular-mass markers were suggested [260, 261]. The p*I* of the analyte(s) is then determined through linear regression using adjacent p*I* markers [242, 262]. It is important to realize that the pH gradient is intrinsically not linear, due to a bidirectional isotachophoretic process [242, 263, 264]. The nonlinearity is exacerbated if mixtures of ampholytes are used, which is common practice in (i)CIEF. Then, the pH gradient shows two or more distinct slopes. However, as shown by Wu and Huang, the assignment of an (apparent) p*I* value with an SD of 0.1 pH units is still possible using linear fit and two p*I* markers [242]. The mobilization step in CIEF may introduce additional variability of the slope [265]. Additionally, the (apparent) p*I* may be influenced by many other factors [241]. Hence, it should be treated as a method specific value.

Spacers or sacrificial agents are mostly either L-arginine (basic side) or iminodiacetic acid (acidic side) [240]. As they decrease the available capillary focusing volume, their use increases the $\Delta pH/dx$ of the pH gradient and impairs resolution. In CIEF, L-arginine is necessary to block the part of the capillary after the detection window. In iCIEF, they often can be omitted unless the protein is very basic or acidic and if ampholyte loss is not an issue. At the same, time beneficial effects have been described [266].

Urea is still one of the most used additives to prevent precipitation [192, 209–211, 216, 219, 223, 224, 267, 268]. However, several other substances have proven their usefulness. The list includes (without claim to completeness):

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ethylurea [229, 269, 270], formamide [271], dimethyl sulfoxide [272], sulfobetaines [273, 274], glycerol [275], taurine [273, 274], sucrose [273, 276], sorbitol [273], and polysorbate 20 [267, 270, 277].

Most "master mixes" contain a portion of a neutral dynamic coating agent, a proprietary CIEF gel or aqueous methylcellulose solutions that are frequently used. Additionally, the gel doubles as a viscosity-enhancing agent. The increased viscosity reduces diffusion and associated peak broadening and further reduces residual EOF. The choice depends on the capillary used and one should follow the recommendations of the respective capillary or capillary cartridge vendor.

In iCIEF, the capillary cartridge is sold by the manufacturers. Neutral coated capillaries are used to suppress the EOF and allow for focusing in specified positions. Available coatings are fluorocarbon (FC)-based [278] or contain immobilized methylcellulose [279]. For CIEF, in many cases, the neutral coated capillary from SCIEX is used in conjunction with the aforementioned CIEF gel [240, 272, 280]. Alternatives were used in selected publications, for example, an FC-coated capillary from Agilent [281], linear polyacrylamide (LPA) [282], or PVA [268, 275]. The use of homemade coatings has fallen out of popularity and is only seldomly reported.

For both CIEF and iCIEF, phosphoric acid and sodium hydroxide are the commonly used anolyte and catholyte, respectively, albeit in different concentrations. Mack et al. introduced the use of 200 mM phosphoric acid and 300 mM sodium hydroxide to improve repeatability [207]. The capillary must of course tolerate those (for CE) quite harsh conditions. In iCIEF, the anolyte and catholyte are sold by the instrument vendors and again phosphoric acid and sodium hydroxide are used, albeit at lower concentrations.

Mobilization is either not needed (iCIEF) or performed through the replacement of the catholyte (chemical mobilization). Acetic acid, which was first described by Manabe et al. [265, 283] and adopted by Mack et al. [240], is frequently used, often at a concentration of 350 mM [240]. Depending on the investigated protein(s), other reagents might be better suited for chemical mobilization. For example, Kristl and Stutz found 50 mM glutamic acid pH 10.5 best suited for the mobilization of ovalbumin [284]. Ren et al. used 100 mM ammonium hydroxide as anolyte replacement for the mobilization of the acidic erythropoietin and under CIEF conditions with reversed polarity to mirror the pH gradient [224]. Mobilization through the addition of salt to the catholyte fell out of favor and is not used anymore. (Assisted) Pressure-based mobilization is mostly used in combination with MS.

The (online) coupling of (i)CIEF with MS has gained significant popularity in recent years. It allows unambiguous peak identification and provides additional structural information about the analyzed protein species. Different approaches have been described, for example, online CIEF–MS [218, 275, 282, 285], microchip CIEF–MS [207, 217, 286], CIEF–CZE–MS [287, 288], and online iCIEF–MS [289–292]. In the case of direct online CIEF–MS, the catholyte and anolyte are additionally replaced with volatile compounds, for example, ammonia (ammonium hydroxide) and formic acid, and glycerol is used as dynamic coating and viscosity enhancing agent. Detailed information has been collected in reviews [293, 294].

By far the most important detection mode is UV detection. It should be noted that a UV filter assembly is necessary due to absorbance of most ampholytes at low-UV wavelengths. Native fluorescence is an alternative [211], if the instrument supports the detection mode and offers improved sensitivity [192]. Capacitively-coupled contactless conductivity detection (C^4D) detection (Section 7.1) in combination with microchip CIEF has been recently reported. Besides the different detection mode, the analysis time was also significantly lower than with regular CIEF [295].

Another possibility to improve the throughput in CIEF is a recently introduced multi-capillary (array) electrophoresis system by SCIEX [296]. Publications concerning its performance, benefits, and drawbacks are anticipated.

The applied field-strength should be set as high as practically possible, due to its beneficial effect on resolution [251]. The maximum usually results from the limit of the instrument for the applied voltage. Commonly, the focusing starts with a lower field-strength, which is increased after a short time to the maximum. This prevents excessive heating, as the current decreases rapidly as the focusing process proceeds. The focusing time, and for CIEF methods the mobilization time, are optimized during method development [240].

Reproducibility or repeatability were assessed in several studies. Emphasis was placed on the determined apparent p*I* value and the percentage area of the individual isoforms. Mack et al. reported RSD between 0.04% and 0.09% for apparent p*I* values and between 0.62% and 3.04% for the percentage area [240]. In two intercompany studies, the performances of CIEF [210] and iCIEF [209] for the analysis of mAbs were investigated in-depth. In both cases, excellent values were obtained. With CIEF, RSD values ≤0.5% for the apparent p*I* values and ≤4.4% (outliers removed) for the percentage area were obtained [210]. For iCIEF, RSD values ≤0.8% for the apparent p*I* values and ≤11% (outliers removed) for the percentage area were reported [209]. A recent intercompany study using two iCIEF instruments reported RSDs values ≤0.28% for the

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apparent p*I* and RSDs values ≤6.25% for the percentage area of NISTmAb [215]. Kahle et al. used the NISTmAb and infliximab in a comparative study and achieved RSD values of 0.05% for the apparent p*I* value and 1.3% for the percentage area of the NISTmAb main peak, whereas for the infliximab main peak, the reported RSD values were 0.04% and 2.4% for p*I* and percentage area, respectively. For the infliximab main peak using UV detection, LOD was 2 µg/mL and LOQ 9 µg/mL. This value improved with fluorescence detection to 0.9 µg/mL (LOD) and 3 µg/mL (LOQ). All values were obtained with iCIEF [192]. CIEF was employed by Suba et al., who reported RSD values for the p*I* value between 0.05% and 0.17% and between 2.5% and 3.9% for the relative migration time [281]. An interlaboratory method validation was conducted by Wu et al. using iCIEF. Reported values for the LOQ range between 0.9 and 6.9 µg/mL [214]. The publication of Bonn et al. described the implementation of a system suitability standard in CIEF [268]. Based on this work, a follow-up study by Cruzado-Park investigated some issues with the method. The modified method was suitable for more than 100 runs on the same capillary [272].

Naturally, (i)CIEF has been discussed in numerous reviews, some of them were already referenced within this section. They may provide additional resources and information to the interested reader, for example, [183, 184, 190, 191, 203, 241, 261, 264, 293, 294, 297–302].

3.2.3 | CE–SDS

The basics of CE–SDS can be found in the original review. CE–SDS can be seen as a further development of the established SDS–PAGE and is nowadays a widely used analytical technique. The comparison between the conventional SDS–PAGE and the newer CE–SDS is the subject of some interesting papers, in which the possibilities for quantification and automation are emphasized for CE–SDS, whereas the general performance in size determination is not so much decided by the results, but only by the amount of work, which is significantly greater for SDS–PAGE [303–305].

In 2019, Sänger-van de Griend, one of the co-authors of this present article, wrote a review article on CE–SDS in terms of method development, validation, and best practice [306]. This comprehensive review excellently summarizes the developments of the last years. It contains all important chapters, such as the basic separation mechanism, the methodology, and good working practices, including the steps sample preparation, pipetting and mixing, incubation, up to detection, a chapter on troubleshooting and finally the validation of the CE–SDS. The review [306] can therefore be regarded as the basis for this article. Further method development for non-mAb proteins was presented in a 4-step approach by Geurink [307]. Guttman explained the separation of glycosylated protein from the non-glycosylated protein by interaction of the glycosylated group with borate–dextran complex in the gel buffer [308].

Rustandi et al. published an article in 2008 reporting on the applications of CE–SDS in the development of biopharmaceutical antibody-based products. CE–SDS is used both to determine product quality at early stages of development and to characterize the final product, as it is an automatable method that provides quantitative and robust results and can be used for a wide range of products [309]. CE–SDS finds a very wide range of applications. A few applications are protein mapping [197], analysis and characterization of ADCs [310], or the characterization and elimination of artifacts from mAbs [311]. Another interesting CE–SDS method was developed by Wagner et al. to determine the amount of size variants in drugs, such as therapeutic antibodies and ADCs, which can be used for batch release, batch consistency, determination of stability, and shelf life [312]. In the analysis of adeno-associated-virus-based gene therapy products, laser-induced fluorescence (LIF) detection was used for the first time in CE–SDS. Zhang et al. developed a method using an AQbD approach that has a simple and robust sample preparation workflow, followed by the separation and quantitation using CE–SDS–LIF [313].

The performance of CE–SDS instruments currently available on the market has been compared and published with respect to the analysis of proteins [173].

With both CE–SDS and CIEF, too high salt concentrations can quickly lead to problems. However, the high salt concentration is often caused by the samples or the buffer in which the analytes (mostly proteins) are dissolved. To remove this salt, which interferes with the electrophoresis, a buffer exchange is suitable, which can be done, for example, with the help of Amicon columns.

3.2.4 | CE–MS

The use of CE–MS can make the analysis of proteins, and in particular mAbs, much more efficient. The already excellent separations by CE-based approaches can be complemented by valuable structural information about the analytes. Differences in glycosylation patterns and several PTMs, including deamidation or amino acid composition, could be easily detected from the mass shifts [286, 314]. CE–MS is just getting really exciting due to recent commercial solutions from companies such as (in alphabetic order)

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Advanced Electrophoresis Solution, Agilent, Bio-Techne, and SCIEX [169].

The injection volume in CE is usually in the nanoliter range. Therefore, the sensitivity of CE–MS is generally lower than that of LC–MS for comparable analytical tasks. Typical analyte concentrations range from 0.25 to 2 mg/mL. However, this is not a major limitation as sufficient amounts of compounds are available, for example, in biopharmaceutical development and manufacturing. Repeatability for major constituents is reported in the range of 2% for relative peak areas [169].

The structural information available with these new commercial approaches is very valuable and much more accessible than with previous LC–MS approaches. CE–MS is often superior to LC–MS, where frequently the chromatographic separation is not completely satisfactory. For example, the analysis of very polar and ionic substances, such as those found in body fluids, is difficult using conventional RPLC–MS [315]. For such substances, CE–MS is then more advantageous than LC–MS, as possible other LC techniques, such as the use of ion-pair reagents, can cause significant ionization suppression of analytes in MS [316]. Furthermore, it is very valuable to have both CE–MS and LC–MS results for characterization. The fact that a combination of both techniques can provide more information than each technique on its own is shown, for example, by the application example of Klein et al. [317]. However, reliable instruments are the prerequisite for this high performance.

Usually CZE–MS is meant when writing CE–MS, and various volatile ammonium buffers, such as formate, acetate, and bicarbonate, have been used in combination with organic solvents such as acetonitrile, methanol, and isopropanol [183, 217, 286]. Acidic pH values are often preferred because they reduce protein adsorption (Section 3.3.2) Evaporation of volatile BGE components can cause shifting migration times; however, evaporation can greatly be reduced by the use of a mineral oil layer on top of the BGE (Ten Pierick in Ref. [318]). Surfactants such as SDS and ampholytes are often not compatible with MS applications, which complicates the use of CE–SDS–MS and CIEF–MS. However, there are also concepts for avoiding these interferences [91, 230, 286, 289]. In the case of CIEF–MS, there is also the need for highly sensitive and robust ESI interfaces for coupling CIEF with MS, as the analytes in ESI are often suppressed by the ampholytes. The review by Xu et al. described recent technical advances in this field and some application examples [294].

The limitations of MS detection, the equipment required, the methodological aspects to be considered, and the various CE applications are well summarized in recent reviews. The analysis of whole proteins, for example, regular biotherapeutics such as glycosylated mAbs, but also advanced variants such as bispecific proteins currently, plays a special role, as does the characterization of proteomes or glycosylation patterns [169, 178–183], and applications have been excellently tabulated [179, 183].

The efforts of the various equipment companies over the last few years are now bearing fruit and necessary conditions are now being met.

Due to the limited experience with new commercial systems, it is necessary to gain practical knowledge on which aspects require specific attention during the validation process.

Initially, one can be guided by the new Q2 guideline. It may be that the mass detector or interface requires further investigation beyond that for validation.

There are already a number of very interesting publications with prototypes. Recently, Mack et al. [207], He et al. [286], and Ostrowski et al. [314] successfully used a prototype consisting of a microfluidic chip-based iCIEF–MS technology and CE users are really looking forward to the moment when these instruments become available.

3.3 | Choice of the capillary

3.3.1 | Adsorption

Adsorption effects and associated altering of the capillary wall are major challenges in CE. Typical consequences are drifting of the EOF, a low separation efficiency, asymmetric peak shapes as well as a poor reproducibility of the migration times and peak areas. Adsorption effects occur due to several types of interactions, mainly electrostatic and hydrophobic. Electrostatic interactions highly depend on the pH and prominent when the analytes and the capillary wall have different charges. On the other hand, hydrophobic interactions are rarely dependent on the pH value and are influenced by the physiochemical properties of the analytes [1, 319]. Stutz wrote an excellent review on protein adsorption in CE [320].

3.3.2 | Strategies for minimizing adsorption

Analyzing small molecules, electrostatic interactions can be usually controlled depending on their p*I*. However, it is more challenging to minimize electrostatic interactions when analyzing proteins and other large molecules ($Mr > 5000$). As proteins typically have many charged sites, they have many options for electrostatic interactions. Even when proteins are near their p*I*, they can still contain regions with high charge density. These regions will be further able to interact with the capillary wall. Thereby, only moieties should be considered, which are directed

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to the outside. Toward the protein core directed moieties are usually not able to show interaction with the capillary wall [319].

The use of extreme pH values is an option to minimize (but not entirely prevent) electrostatic interactions. The use of low pH is widely common in CE–MS. Low pH values are favorable with the mostly used mode positive ESI–MS. Acetic or formic acid–based BGEs are often used. However, in many cases, applying extreme pH is limited. First, the separation efficiency is often insufficient at extreme pH values. Second, many analytes are not soluble or not stable at extreme pH values. Furthermore, some analysis must be performed in a defined pH range. For example, when studying binding properties in some CE modes, like ACE, a defined pH value of the BGE is often crucial and has to be near the physiological value, for example, for better reliability and biological significance.

High ionic strengths using ion pairing reagents or surfactants are other options to decrease electrostatic attractions and thus adsorption. Co-ions in the BGE compete with the surface binding sites. Ion-pairing reagents or surfactants decrease the net protein charge or prevent protein molecules from approaching the capillary surface [321]. However, higher ionic strengths are generally associated with higher buffer conductivities, which are often limited by higher Joule heating, leading to peak distortion and unstable baselines. Zwitterionic buffers are a proven alternative [322] and, for example, also used in the aforementioned CZE method for mAb analysis (Section 3.2.1) [170, 194]. In the case of CE–MS, nonvolatile salts can interfere with ESI and might contaminate the ion source [321]. In reality, often a multivariate approach is required to prevent adsorption from very complex matrices, such as cell culture samples, as demonstrated in Refs. [198, 323].

3.3.3 | Capillary coating

Capillary coatings are increasingly used for minimizing wall adsorption. Coatings allow working under the defined conditions without manipulating the properties of the BGE, like the pH or molarity. They can be specifically chosen or adapted for the particular analysis. For achieving reproducible results, coatings must show several requirements. They should enable a full coverage of the capillary surface and should be stable under the used conditions (pH, used [organic] solvents), allowing analyzing a high number of runs. Coatings with polymers are more common than coatings with small molecules; thus polymer coatings allow higher surface coverage than the surface modification by small molecules. Moreover, nanomaterials are being used as coatings due to their large surface area, enabling an effective separation of proteins and chiral compounds [319].

Coatings can be classified depending on their charge into neutral, cationic, and anionic. Most coatings strategies are based on inhibiting electrostatic interactions. Neutral coatings minimize adsorption through their surface inertness or by steric hindrances, whereby charged coatings are based on electrostatic repulsion interactions [321].

Currently, many coating strategies are being applied. The most popular paths are static, static adsorbed, and dynamic coatings. The nomenclature of coating methods is inconsistent, some publications consider static covalent coating as "permanent coating" and report about "dynamic coating" for "static adsorbed coatings." Therefore, it is sometimes challenging for the reader to figure out the applied coating type.

Static covalent coating

Static-covalent coatings are based on covalent binding (Si–O–Si–C or direct Si–C bonds) between the coating agent and the silanol capillary. Thereby, Si–C bonds are more stable against hydrolysis and more applicable at higher pH values. A general statement about the stability of static covalent coatings is difficult. However, static-covalent coatings often have an improved stability compared to static adsorbed coatings. They rarely show bleeding effects and associated problems with detectors. The simultaneous use of covalent coatings and additives may be generally recommended. The coating process is usually very laborious and often commercial products are used. Most static covalent coatings are neutral and suppress the EOF. Neutral coatings are usually stable in low pH values and have no limitation by the charge of the analytes. Additionally, other agents are ionic (positive or negative) and build an EOF. Hereby, capillary and analyte charges have to be alike to avoid adsorption. Therefore, cationic coatings are beneficial when working at low pH values and anionic coatings are suitable when working at high pH values [319].

The most used static covalent coatings are as follows: PVA, polyacrylamide, LPA, polydimethylsiloxane (PDMS), hydroxypropyl methylcellulose (HPMC), and graphene oxide (GO) [321].

PVA is compatible with the most common organic solvents and stable over a wide pH range. However, its stability is best under acidic conditions. PVA is highly hydrophilic; therefore, adsorption of most proteins is low. By this, PVA is very suitable for protein separations. When analyzing basic pharmaceuticals, a higher resolution and loadability can be achieved with PVA capillaries than with bare fused capillaries. In the review [321], a study of Fanali et al. was presented, in which a modification of PVA with glutaraldehyde as a cross-linker improved the stability of

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PVA in acidic milieus and enabled the measurement of 900 runs in one capillary.

Another option to enhance pH stability of PVA capillaries is to induce crystallization by heating the capillary [321].

Polyacrylamide is compatible with organic solvents and more hydrophobic than PVA, although polyacrylamide-coated capillaries are sensitive for contact with air. Polyacrylamide-coated capillaries are commercially available and can also be coated by self. This coating has been successfully used for analyzing peptides and glycoproteins [321].

LPA coatings are synthetized from N-substituted acrylamide copolymers. Several polymerization methods are available for synthetizing of LPA. Most used are for example: the free radical polymerization method developed by Hjertén, the thermally initiated free-radical polymerization method, the living radical polymerization techniques (atom transfer radical polymerization (ATRP), and reversible addition-fragmentation chain transfer (RAFT) polymerization) and the surface-confined aqueous RAFT (SCARAFT)-coating method [324]. The properties and the quality of the synthetized polymers vary widely depending on the applied polymerization type. Most polymerization methods have issues regarding homogeneity and reproducibility of the polymerization. For example, when applying free radical polymerization, oxygen in the air may quench the reaction and causes an irreproducibility of the coating. Further, acrylamide monomers, which react with the capillary surface, may also react together in the solution. The produced polymer may clog the capillary. When using the ATRP process, high concentrations of transition-metal ions are required for catalyzation. Residual amounts of these ions may build an anodic EOF and disturb the analyses, especially when working at low pH values. In the RAFT polymerization method, the structure of the resulted copolymer is not defined. Thus, the monomers were added to the polymerization mixture before polymerization. A better homogeneity and reproducibility have been reported for the SCARAFT-coating. Furthermore, it is possible to optimize the coating by simply choosing the suited functional vinyl monomers. SCARAFT-coating is additionally environmentally friendly, because avoiding the use of organic solvents. This coating was applicable for bottom-up proteomic analysis in CE–MS. A very low EOF allowed a 200-min separation window and thus single-shot CZE–MS analysis. The coating additionally showed a good stability with a migration time reproducibility between 2% and 3% for 200 h of operation [324].

The nanomaterial GO is used as covalent static coating for capillaries. After coating, different reagents can be coupled on GO, such as DR, poly(ethylene oxide) (PEO), dopamine, or enzymes, building a multilayered system. GP–DR and GO–PEO systems showed a great separation performance for proteins and excellent stability. GO–dopamine system is being used for protein conjugation by hydrophobic or π–π interactions. The immobilization of enzymes on GO provided a better stability and reproducibility [319].

Static adsorbed coatings

Static adsorbed coatings can be self-synthesized by simple rinsing steps with the needed polymer solutions. Some coatings are also commercially available. As the coating is semi-permanently adsorbed to the capillary, this coating type is usually compatible with MS. In the case of coupling with MS, the MS apparatus has to be decoupled during the coating process to avoid contamination. Multiple-layer coatings of alternating charge are usually produced to enhance stability and achieve the desired surface charge. In some cases, a recoating is not necessary and in others a regular regeneration between the runs is needed. The adsorption of cationic coatings is usually based on coulomb interactions between the silanol surface and a large number of cationic amine groups of the polymers or a polyacrylamide backbone. Cationic coatings minimize the electrostatic attraction and the hydrophobic interaction with the proteins, due to a large number of amine groups of the coating agents. When an anionic coating is required, the capillary must be first rinsed with the cationic polymer solution and afterwards with the anionic coating solution. The anionic polymer binds on the cationic polymer. Neutral and hydrophilic polymers are fixed to the wall by hydrogen bonding and other weak interactions. Advantages of this coating type are the uncomplicated adsorption and desorption by simple rinsing steps and the limited volume needed for the coating process. Disadvantages are the low stability of neutral and cationic polymers at high pH values. Coating with a multilayered generally shields the silanol groups more effectively and often provides better stability than one-layered coatings. Additionally, it minimizes the peak tailing [321].

3-(Aminopropyl)trimethoxysilane (APTS), [3-(methacryloylamino)propyl]trimethylammonium (MAPTAC), mono- and multilayers of polybrene (PB), polyamine-based coating (PolyE-323), poly-LA 313, monoquaternarized piperazine, cationic polymer–coated capillary, and *N*-methylpolyvinylpyridinium are examples for cationic static adsorbed coatings [325].

APTS shows a very fast EOF and often an insufficient resolution. However, the resolution can be improved using longer capillaries. In their review, Huhn et al. compared APTS capillaries to MAPTAC and PB-coated capillaries for the analysis of proteins, peptides, and lysed blood. On the one hand, PB and MAPTAC capillaries showed slower EOF and better separation efficiency than APTS capillaries. MAPTAC capillaries provided a better sensitivity and

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precision for migration times and peak areas of peptides with RSD% values of 0.7% and 3%–8%, respectively. On the other hand, APTS-coated capillaries showed a better stability than capillaries with PB coating.

MAPTAC shows a relatively strong EOF. The performance of MAPTAC capillaries for the separation of glyphosate and glufosinate at pH 6.5 was compared to LPA and bare fused silica (BFS) capillaries. The analytes have acidic functions and an amine group. The best results were achieved in LPA capillaries, followed by MAPTAC-coated capillaries [321].

The positively charged successive multiple polymer layers (SMIL) coating consists of layers of PB–DS–PB. SMIL induces a reversed EOF and was applied for the analysis of four anionic organic acids in a negative voltage mode by CE–MS. The SMIL coating produced a sufficient solvent for a stable (80 nL/min) EOF and MS signal in the pH range from 3 to 9. RSD values for migration times and MS signal intensities of less than 2% and 5% ($n = 3$) were obtained, respectively. In contrast to the one-layered coating of PB, the SMIL coating is compatible with organic solvents.

PolyE-323 has a similar structure to PB but with secondary amines instead of quaternary. It has a positive net charge under neutral to acidic conditions and can be used over a wide pH range between 2 and 10. This coating enabled a stable EOF and allows an efficient separation for tryptic peptides. Poly-LA 313 is hydrophobic and is used to analyze proteins [326].

Neutral low normal (LN) and high reverse coatings (HR) are neutral. LN coatings show a low stability at low pH values, wherein HR coatings provide good pH stability. PB- and LN-coated capillaries were compared for the separation of glycoforms. Hereby, PB-coated capillaries showed a faster separation and a lower resolution than LN-coated capillaries [321].

The bilayered coating of PB and poly(vinyl sulfonate) (PVS) (PB–PVS), the four-layered coating of PB and dextran sulfate (PB–DS–PB–DS) and CEofix are examples for anionic static adsorbed coatings.

The bilayer of PB–PVS provides a strong and pH-independent EOF in the pH range between 2 and 9. It showed a good stability and achieved good RSD values below 1% for the migration time of cationic alkaline compounds in low pH (500 mmol/L formic acid [pH 2.5]) [321]. The negatively charged multiple polyelectrolyte coating PB–DS–PB–DS produced a strong EOF, which was necessary for maintaining a stable MS signal for analyzing four positively charged analytes (alkaloids). The used BGE was ammonium formate in 50% v/v acetonitrile (pH 3.09). The migration time repeatability was at 0.8% and 2.7% for intraday (run-to-run, $n = 5$) and interday (day-to-day, $n = 3$). The RSD for batch-to-batch repeatability ($n = 5$) of the sheathless interfaces was not more than 3.0%. The variation of the mass signal intensity was less than 5% [327]. CEofix is a commercially available bilayered coating of a positively and a negatively charged polymer. This coating was applied for analyzing insulin, α-lactalbumin, and β-lactoglobulin B in CE–UV and achieved RSD values for migration time below 1%. The coating was also used for testing basic drugs in CE–DAD and CE–MS. The RSD values for migration time and peak area were below 0.25% and 2.40% for the CE–DAD system and below 0.85% and 14.3% for CE–MS [321].

Wall immobilization of biological units

The immobilization of proteins, enzymes, and cells onto the capillary wall can be used to produce online microreactors in CE. This approach can be conducted by adsorption, cross-linking, and other immobilization processes. Enzymatic reactions can be performed on immobilized-enzyme microreactors that enable analyte incubation, product separation, and detection in one capillary. Enzyme immobilization increases enzyme stability and reduces the experimental cost by avoiding the waste of enzyme. However, immobilization may be complicated and hard to control and can result in decreasing activity. Cells act as drug processors and are more suitable than enzymes for drug screening. Thus, cells show the natural conformation and bioactivity of proteins. The adsorption of cells to the capillary wall, similarly to proteins, does not need a covalent linkage [319].

Dynamic coatings

This coating method involves dissolving agents in the BGE, which interact with the silanol groups on the capillary surface, reducing the interaction between the analyte and the surface. The advantages of this coating process include its ease of use, flexibility, and prevention of coating bleeding by renewing the coating with each run. However, there are several disadvantages to consider. First, adding agents to the BGE increases its ionic strength, leading to Joule heating, and related issues. However, using a surface-active amine as the basic component of the BGE can mitigate this effect without altering the ionic strength [8, 117, 323].

Second, there is a possibility of interaction between the coating agent and the analyte, which can affect the properties of the analytes and the separation. Third, compatibility with MS can be problematic due to the presence of added agents in the BGE, as they may suppress ionization (especially with surfactants), contaminate the ion source, and produce background signals. Commonly used agents are cationic or neutral in nature. Cationic agents bind reversibly to the negatively charged surface and include polyamines, surfactants, and polysaccharides such as spermine, butanediamine, cetyl trimethyl ammonium bromide, or chitosan. Neutral agents, on the other hand,

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slow down the EOF by covering the capillary surface and can include polymeric substances like HPMC, hydroxyethyl cellulose, dextran, and PVA [319]. An example of this coating method is the eACA–CZE method described in Section 3.2.1, which uses a polyamine (TETA) and HPMC [170, 194]. Dynamic coatings are also used in combination with static coatings, for instance, in (i)CIEF (Section 3.2.2).

3.3.4 | EOF and coatings in MS

In a sheathless interfacing CE–MS system, the ion source replaces the outlet vial. A sufficient EOF toward the outlet site is often important in order to achieve a good reproducibility of the migration times and MS signals. An interruption of the fluid flow to the outlet may cause a discontinuation of the electrical circuit. When a reverse polarity is required, BFS capillaries are not the best option to conduct the separation. Thus, the EOF would be unfavorable directed toward the cathode (to the inlet). In this case, applying pressure is an option to provide a constant flow of fluid toward the outlet. However, HD flows may induce band broadening and loss of resolution. In contrast to that, the use of a cationic coated capillary may reverse the EOF and ensure a stable fluid streaming to the ion source at the outlet [321]. Thereby, the addition of coating agents or related substances increases the background noise, suppresses analyte signals, and/or contaminates the ion source and MS optics. For these reasons, when working in a CE–MS system, it is recommended to avoid dynamic coatings and to use other coating strategies with a good stability [321].

3.4 | Must-have chemicals

To perform CE, one should have a collection of reagents in the laboratory that are frequently needed for the application. In this chapter, we give a "shopping list" of substances that are frequently needed in CE analysis.

For example, CZE always requires buffer solutions, which must be varied in pH and ionic strength. Therefore, the necessary substances for a few common buffers should always be available, such as phosphoric acid and/or phosphate salts for phosphate buffer, tris base, and borate buffer. To regulate the pH value, only the mixing ratios of the two buffer substances should be changed. In the case of doubt, an acid such as HCl or a base such as NaOH can also be added, but it should be noted that these change the ionic strength. Sodium hydroxide should be present anyway when working with BFS capillaries, as it is often, but not always, needed to condition the capillary. If you want to change the ionic strength of the BGE, NaCl is very suitable and should therefore not be missing in any laboratory. For some samples, it is necessary to rinse the capillary with organic solvents, so a selection of such should also be available.

In addition to substances to regulate pH and ionic strength, additives are often put into the BGE to increase the selectivity of the method. Of course, this also includes the chiral separators for chiral separations. The frequently used cyclodextrins are also used in some cases for non-chiral separations [328, 329].

In the case of CE–SDS, completely different reagents are again required, first and foremost the surfactant SDS. Detergents such as SDS or polysorbate 20 should also be present in every CE laboratory. They are useful both as rinsing substances for some samples and essential for CGE methods, especially of course CE–SDS. Polysorbate 20, Poloxamer, or other neutral surfactants as BGE additive can prevent adsorption of matrix components [187, 193, 198, 323, 330]. As protein analysis often involves working under reduced conditions, reducing agents such as β-mercaptoethanol are also must-haves for the laboratory bench. CE–SDS also always requires a viscous separation matrix and molecular weight markers to determine apparent molecular weights. As with the other CE techniques, a sample buffer, washing, and conditioning solutions may be required.

The chemicals required depend, of course, on the method used. For example, completely different reagents are needed for CIEF. A selection of ampholytes and p*I* markers should always be available for this purpose. Furthermore, additives that prevent precipitation, such as urea or glycerol (see Section 3.2.2 for more details), should be present, as well as methyl cellulose to control viscosity and possibly cathodic spacers, such as arginine. Anolytes such as phosphoric acid and catholytes such as sodium hydroxide are also always needed and should not be missing in any CIEF laboratory.

Of course, there are countless other useful additives, which are often very individual and specific to a task. Here, only a shopping list with basic chemicals is provided, which should be in the laboratory at the beginning of the work with CE in order to start the first measurements. After that, depending on the problem, further additives can be researched and ordered individually. Those have been mentioned in the previous sections, including Sections 2.4, 3.2.1–3.3.3.

4 | ESTIMATION OF PHYSICOCHEMICAL PROPERTIES BY CE

In addition to being an excellent separation technique, CE can also be applied for the determination of a wide range

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of physicochemical properties [1, 331, 332]. For the determination of some of these parameters, (capillary) electrophoresis is in some cases even a standard technique. For example, the apparent molecular weight of proteins and their isoelectric points are conventionally assessed by (capillary) gel electrophoresis and IEF, respectively [176, 183]. As the determination of these two properties is directly related to specialized techniques, further information can be found in the respective Sections 3.2.3 and 3.2.2. Next to the mentioned prominent properties CE was successfully used for the determination of pK_a-values, effective charges, water–octanol partition coefficients, distribution coefficients, diffusion coefficients, and kinetic/thermodynamic parameters [1, 60, 331]. As the intention of this article is not to provide a complete description of the methodologies, only relevant reviews as well as highly outstanding articles are quoted in this section. For more detailed descriptions, we explicitly refer to the cited publications and the references therein.

4.1 | pK_a-values and effective charges

CE is an outstanding technique when it comes to determining pK_a values. Over the last several years, a number of articles have been published on this topic; see Refs. [60, 331–333] and the references within. There are two main variations in the literature. The first measures the effective mobility as a function of various different pH values and calculates the pK_a value(s) by nonlinear regression, whereas the second approach directly compares mobilities of marker substances with similar pK_a-values [331, 332, 334]. Two advantages of choosing CE for this type of measurement are that it does not require a high amount of sample material and that these materials do not necessarily have to be extremely pure or stable [331]. However, it should be mentioned that measurements of mobility are highly dependent on temperature and ionic strength [60, 331, 335]. Especially the mathematical correction of the latter factor can be very complex. But fortunately, an excellent computer program has been developed by Malý et al. for this task [335]. As it is possible to determine pK_a values, it should not be surprising that also the effective charge of particles/molecules can also be determined by using CE [331, 336]. Ibrahim et al. demonstrated various approaches for performing such analyses [336].

4.2 | Water–octanol partition coefficients/distribution coefficient

Lipophilicity can be characterized by partitioning a molecule between two immiscible phases. In the case of CE, MEKC, MEEKC, and VEKC/LEKC are valuable techniques for estimating that property [331, 337, 338]. A molecule's retention factor can be used as surrogate for its distribution between, for example, the BGE and the inner part of a micelle. With that data, the water–octanol partition coefficient or the distribution coefficient can be estimated using the retention factors of reference substances [331, 337, 338]. Štěpánová and Kašička provided a brilliant overview about different procedures and the benefits of different pseudostationary phases [331].

4.3 | Diffusion coefficients

Diffusion coefficients can be derived from the variance of CE peaks [331, 339, 340]. As the total variance of any peak is related to many influencing parameters, the fraction caused by longitudinal diffusion has to be extracted. Due to its effect over time, the variance can be explicitly determined by specialized CE techniques, such as the low field, the (multiple) stopped migration, or graphical, and HD velocity–related methods [331, 339–342].

4.4 | Binding parameter

In the past few decades, a number of different CE-based ligand binding assays have been introduced [331, 343, 344]. Among these, ms-ACE and CE frontal analysis (CE–FA) are the two most prominent techniques used [345].

In ms-ACE, the effective mobility is measured as a function of ligand concentration. First, the mobility of the pure analyte is determined. Subsequently, defined quantities of a ligand are added to the BGE and then the mobility is measured again. As the ligand is in dynamic equilibrium with the ligand–analyte complex within the BGE, the measured mobility shifts from the value of the pure analyte to that of the fully saturated complex, as the concentration of the ligand increases. The apparent binding constant can then be derived from these data using nonlinear regression [331, 345, 346]. As the determination of mobilities in discontinuous BGEs is not to be considered trivial, Dubský et al. developed a powerful computer program for the evaluation of ms-ACE experiments. That software can be used to analyze asymmetric peaks optimally, perform the nonlinear regression, and calculate the asymmetric confidence intervals [346].

The basic concept of CE frontal analysis (CE FA) is completely different. Here, the analyte and its ligand are mixed before injection and the equilibrium is allowed to stabilize. Thereafter, a relatively large volume is injected into the capillary. The high injection volumes result in plateau-like peaks. Under certain conditions, it is possible to separate the ligand and its analyte, or the complex, from each other. In this case, the plateau height of the ligand peak is

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proportional to its free concentration. Using the known total concentration and the determined free one, the concentration of the bound ligand and the quotient of bound ligand and analyte concentration can be calculated. In the end, the determined quotient and the free ligand concentration are fitted to the CE FA function. That procedure yields not only information regarding the binding constant, but also about the number of binding sites [331, 345].

As mentioned at the beginning of this section, there are many other techniques available. Galievsky et al. published a very well written comprehensive summary about those different techniques/methods in 2015 [343]. Furthermore, it is worth to be mentioned that in the last decade techniques for the determination of rate constants have been introduced. These include, for instance, nonequilibrium CE of equilibrium mixtures or moment analysis [331, 343, 347–349].

5 | INDIRECT DETECTION

Indirect detection is a reasonable option, if a substance is poorly detectable. For example, the majority of CE instruments are equipped with UV detectors. If a substance cannot be detected this way because it lacks a chromophore, a BGE with good (UV) light absorbing properties can be used (such as chromate, benzoate, phthalate, or pyrometallitate [350, 351]) resulting in negative substance signals. This is particularly a good option, if the analytes of interest are charged, which is usually the case working with electrophoresis.

However, as plenty of detection modes are available nowadays [169], there has been less interest in this field recently. Please refer to the original article for method development using indirect detection [1].

6 | DIRECT INJECTION, SAMPLE PRETREATMENT, AND PRECONCENTRATION

One of the most critical aspects of CE, as it relates to its ability to target real samples and applications is its, though limited, ability to inject and separate components from untreated samples, a topic that has raised interest for decades [352]. Typical (sample) problems that influence the selection of both, injection methods and the potential inclusion of sample pretreatment steps, are the presence of particles (that could simply clog the capillary), the abundance of proteins (resulting in non-specific adsorption), the concentration of salts (that may result in stacking or de-stacking), and the overall concentration of the analytes of interest. Fortunately, there are plenty of options to mitigate these shortcomings. Thus, this section aims to provide the readers with general guidance related to these topics, as discussed in the literature as the original review was published and considering that only a few representative examples are herein provided and discussed. Additional reviews describing various aspects of this section can be found elsewhere [353, 354].

6.1 | General considerations related to sample injection in CE

Regardless of the specific methodology, injection methods can be broadly classified as HD or EK. The former is based on the use of a pressure difference between both ends of the capillary and is implemented by pressurizing the sample vial (as recently implemented by Furter [355]), applying vacuum at the detector side, or changing the relative height of the sample versus the outlet vial. Considering its simplicity and the fact that both the pressure difference and injection time can be accurately controlled, leading to the injection of a plug with identical composition to the sample, HD methods are often the default option in commercial CE instruments. On the other side, EK injection methods take advantage of a combination of EOF and electrophoretic mobility of the ions present in the sample to introduce a sample plug and can be implemented in a variety of ways, as long as there is a potential difference between the sample and the outlet reservoir. As expected, the main advantage (or limitation) of this approach is that the plug introduced can be enriched (or depleted) of certain ions, leading to multiple approaches to perform sample preconcentration as well as matrix cleanup [356–358]. EK is often the preferred injection method in MCE [359, 360], as the approach can be implemented without additional hardware (pumps or valves [361, 362]). In either case, the volume of sample injected can be calculated using relatively simple functions (as described by Krivácsy [363]) or specific software packages (as reviewed by Thormann [364]). Android users can also take advantage of CEToolbox, a practical app that provides extensive injection information as injected volume, total capillary volume, proportion and amount of injected sample, among other options [365]. Please note that the actual amount injected in EK depends on the local field strength over the injection vial, not the overall field strength. This local field strength varies with the composition of the sample [8].

6.1.1 | Injection modes to improve the sensitivity

Perhaps one of the simplest options to increase the sensitivity of the analysis is by integrating sample stacking, a rather efficient way to compress the length of a sample plug

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and that can be broadly classified as either proportional or boundary stacking [366], depending on the mechanism involved. In general terms, the *stacking* effect results from a difference between the velocity of the analytes in the sample (low conductivity) and the BGE (high conductivity). Under these conditions, the analytes will experience a higher electric field strength and migrate faster until they encounter the BGE, where they slow down, causing an accumulation of the ions at the boundary. This approach is often referred to as field-amplified sample stacking (FASS, when coupled to HD injection) or field-enhanced sample injection (FESI, when coupled to EK injection). Although fundamental developments have been established in the field for several years [358, 367–370], it is worth mentioning a theoretical analysis presented by Dubey [371] and a carefully prepared electrical description by da Costa [150]. In addition, reviews by Grochocki [372] (focusing on stacking toward metabolomics), John [373] (preconcentration in nonaqueous CE), and Suntornsuk [374] (preconcentration of pharmaceutical and related substances) have also been recently published. As expected, most of the recent reports involving stacking are focused on the application of the approach to various samples, including drinking water [375, 376], brain tissue [377], fish [378], meat [379], plants [380], human urine and serum [381], noting that the injection of these samples often requires homogenization, filtration, evaporation, and/or dilution [382, 383]. It is also worth mentioning a recent approach by Perrin's group, describing the possibility to combine desalting, protein precipitation, automated liquid–liquid extraction, in-line CE stacking and electrophoretic separation [384], the possibility to combine surfactants and pressure to increase the concentration of analytes (×3000) [385], and the approach described by Graf coupling ITP–CE for the analysis of glyphosate at pM levels [386].

Hybrid modes (such as pressure-assisted EK injection, PAEKI) have been described and used to improve the sensitivity of the analysis of proteins [387], antibiotics [388, 389], biogenic amines [390], or inorganic contaminants [391]. Although the approach was applied to a nanofluidic device, electro-preconcentration diagrams were used to optimize molecular enrichment with low counter pressure using albumin as a model analyte, reaching preconcentration factors of 70 after only 2 min [387].

Besides the effects produced by differences in concentration of salts in the BGE, differences in pH have been also used to improve CE analysis. Among those approaches, dynamic pH barrage junction focusing is based on the fact that the velocity of the analyte can change under different pH conditions as determined by the pK_a values of its different functional groups [392, 393]. Also taking advantage of pH changes around the capillary inlet end in the sample vial (from 7.1 to <4, upon the application of +10 kV for 100 s), Hattori and Fukushi were able to charge analytes and control the behavior of L-histidine and creatinine during the EK injection [394] using a simple strategy called mobility boost. As a pH-based variation of EK supercharging (EKS), Koukalová [395] used an asymmetric boundary, where one side featured lower pH (for the focusing of strongly acidic ampholytes and the accumulation of weak acids) and the opposite side comprised a neutral/basic nonconductive zone of the ampholyte. Under optimized conditions, authors achieved a 14-fold accumulation (in 25 min) compared to that by classical ITP, reaching and LOD of 0.9 μM for glyphosate. With pertinent variations, other groups have also used pH-mediated stacking toward the analysis of glutathione in blood [396] or antibiotics in milk products [397].

Combining FESI with a sequence of a small plug of high ionic strength leading electrolyte (LE), a small plug of water, a long EK injection of the sample, and a final plug of low ionic strength terminating electrolyte (TE) leads to EKS. This sequence causes a gradient of the electric field across the sample zone, allowing the analytes to concentrate into distinct zones, whereas the LE and TE eventually dissipate and a CZE separation occurs under counter-flow or reduced EOF conditions. Applications of EKS to the determination of neurotransmitters were recently reviewed by Wells [398]. Since then, the approach has been applied, for example, to the analysis of amino acids in cerebrospinal fluid [399], alkaloids in rat fecal samples [400], and various anionic analytes in plant/feces [401] reaching 30–100, 1500, and 2000-fold improvements, respectively. The use of tITP in combination with a cleverly designed BGE resulted in an LOD of 0.8 pmol/L and LOQ of 2.5 pmol/L for adenovirus particles [200].

6.1.2 | Injection modes to improve the throughput

Besides manipulating the EOF [402], the (relative) low-throughput associated with individual runs can be addressed by injecting multiple segments of sample between BGE zones, allowing the components to migrate into those spacers [403]. This approach has been used not only to decrease the analysis time of creatinine (by approximately 70%) [404, 405] but also to inject multiple serum filtrate samples for metabolomic studies, including those of women in the last trimester of their pregnancy [406]. Other applications of this approach have been also recently described [407–410], noting that current analytical constraints of the approach include poor concentration sensitivity, potential loss in isobar/isomer resolution, sample carryover, its restriction to ionic metabolites [403], as well as the need to perform extensive method

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optimization to improve the compatibility with NACE protocols [411].

Another alternative to improve the throughput in CE is to perform sample stacking from a flow of solution, an approach first proposed by Kuldvee [412] but that has been used multiple times since then [413–415]. Here, the fluidic components (pumps, valves, manifolds, etc.) are used to deliver a stream of sample to the inlet of the capillary, enabling a fraction of the sample to enter the capillary through a combination of EK and/or HD mechanisms. Additional developments of this basic concept include sequential injection-CE [416–418], methods based on asymmetric flow field-flow fractionation [419], and flow-gated injection [420]. It is also pertinent to note that a number of groups have described the multiple capillaries [421–423] or presented custom instrumental approaches [361, 424–427] that not only address the throughput but also offer automation and open-source designs [428].

6.2 | General considerations related to sample treatment in CE

Although the previously described sample preconcentration approaches can be performed in-line (carried out directly in the separation capillary [416]), low analyte concentrations or matrix-related issues can also be addressed by implementing an extraction step, where analytes are transferred from the sample matrix to another phase, leading to an increase in the concentration. In general terms, and as described by Xia [429, 430], this can be accomplished via passive (adsorption, partition, sized-based recognition, etc.) or assisted modes (acoustic wave, microwave, electrical field [431], etc.). Considering the differences in the underlying mechanisms, sample pretreatment steps involving a chemical transformation of the analyte (derivatization) are presented separately. All things considered, and despite representing the bottleneck of rapid sample analysis [429], most authors implementing routine sample treatment steps choose the simplicity offered by off-line methods. Reasons for the selection of other methods [432] and pertinent examples are herein discussed, noting that many groups are now also considering green [433, 434] and/or QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) [435] metrics along with performance.

6.2.1 | Passive sample pretreatment steps

One of the simplest (and most widely used) sample pretreatment procedures (for clean-up and preconcentration) prior to CE is off-line solid-phase extraction (SPE).

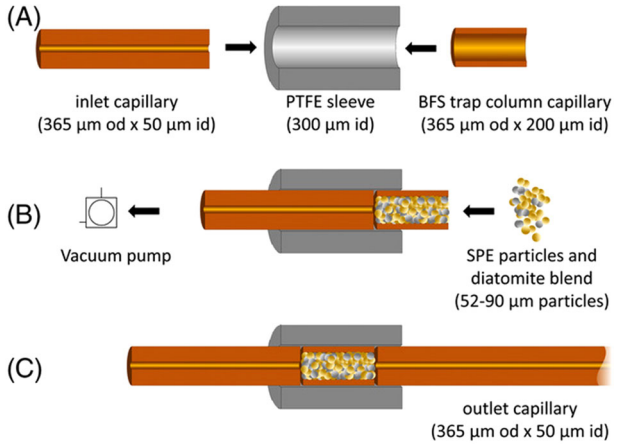

FIGURE 5 Schematic overview of the construction of a trap column used to incorporate solid-phase extraction (SPE): (A) cross sections of bare fused silica (BFS) capillaries in a polytetrafluoroethylene (PTFE) sleeve. The capillaries have an outer diameter (od) that matches the inner diameter (id) of the sleeve. (B) The capillaries are inserted into the sleeve and thereafter sorbent particles, with a larger diameter than the in- and outlet capillaries, are introduced into the SPE capillary by applying a vacuum through the inlet capillary. (C) The packed SPE capillary is pushed to the center of the sleeve with a BFS capillary, and the outlet capillary is connected. *Source*: From Ref. [461].

This concept has been extensively reviewed considering the material used [436–440] or the target analytes (i.e., parabens [441], peptides [442], natural products [443], drugs of abuse [444], pollutants [445], organic acids [435], or oligonucleotides [446]). Beyond the use of non-specific hydrophobic (C_{18} [447] or carbon-based materials [165, 448]) or electrostatic interactions [449, 450], perhaps the most significant advancements reported involve the use of much smaller volumes (typically just a few µL) of both sample and elution [451] and the application of custom materials [452] that can give some selectivity prior to the separation. In addition, many groups have invested significant efforts into the incorporation of novel phases into cartridges (for online [453, 454]) or in-tube [455–457] SPE. These phases can be synthetized *in situ* (monoliths) [432, 454, 455, 458] or simply assembled using capillaries of different dimensions able to trap large sorbent particles (60 µm Oasis HLB [459], a mixed polymeric phase with good water wettability and an ability to capture both apolar and polar organic molecules [460]). As shown in Figure 5, this is one of the simplest strategies to incorporate SPE and that can be adapted to combine multiple materials, as demonstrated in Ref. [461].

Although not specifically applied to CE, Ken Marcus' group has recently presented several examples illustrating the advantages of capillary-channeled fibers for the

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preconcentration of extracellular vesicles from various sources [462, 463].

In-line with these developments, it is also worth highlighting the implementation of affinity phases for the online enrichment of specific biomolecules [345]. Following the pioneering work by Guzman, who combined the use of antibodies and/or other affinity ligands as highly selective capture agents [464, 465], many other groups have applied similar approaches for the analysis of protein biomarkers [466, 467] and other molecules [468, 469]. Although the preparation of these stationary phases requires a careful selection of the experimental conditions (to control the orientation of the antibodies and maximize their efficiency), they also offer exquisite selectivity and the possibility (when integrated with side channels) of injecting and rinsing the phase before performing the separation step. Subsequent preconcentration strategies based on affinity have been presented for lysozyme [470] or biotherapeutics [471].

Another alternative to perform sample pretreatment is to incorporate a liquid–liquid extraction step [353]. Far away from traditional approaches [472], the main advantages of recently described methods coupled to CE are that they only require the use of a small volume of nonaqueous solvent (typically, placed at the end of the capillary) and that they allow their integration into the analytical process without significant hardware modifications. For instance, Figure 6 shows the modifications performed by Lindenburg's group to a Beckman Coulter CE apparatus (replacing the existing electrode with a longer platinum electrode of 4 cm, which was isolated with a PTFE sleeve) to enable the analysis of biogenic amines in urine samples, at low nM levels [473].

Additional details, including fundamentals and extensive classifications, can be found in recent reviews [474–476]. Among other clever approaches, it is worth mentioning the possibility to use the capillary to add solvents and mix the sample to determine tyrosine kinase inhibitors in plasma [384], additional uses of droplet extraction [477], including single bubble in-tube microextraction [478], and the use of alternative solvents [479] such as deep eutectic solvents [480] or ionic liquids [481].

Pavel Kuban's group recently reported the advantages of at-line coupling of hollow fiber liquid-phase microextraction to CE for trace determination of basic [482, 483] and acidic drugs [484] in complex samples. Although this approach is established, they considered a 3D-printed microextraction device (inexpensive and disposable, Figure 7) with a hollow fiber placed in the sample vial of a commercial CE instrument.

Also using a liquid extractant, Mora's group used a subcritical water extraction (185°C for 10 min [362]) to release amino acids from soil or small inorganic anions and a series of carboxylic acids from soil or cells (200°C for 30 min [485]), noting that pressure is needed to keep the water in its condensed phase [486]. Albeit used to perform hydrolysis, the addition of in-line heating (90°C) was used by Zhang [487] toward the analysis of carbamates. Another option is to analyze the glyphosate content of soil and also beer samples using CE–MS, which are alkaline solutions containing sodium phosphate (e.g., 50 mM) as sample pretreatment [488, 489].

The integration of dispersive (micro)extraction methods is another trend. These methods are quickly gaining popularity [490] and are based on the emulsification of an extractant (organic solvent, ionic liquid, and deep eutectic solvent [491]) and a dispersant (for instance, a surfactant [381]) in the sample, followed by its phase separation by solidification or centrifugation. This approach is somewhat similar to the one used in cloud-point extraction, where a surfactant above the CMC is used for the extraction [492–494]. These are both considered mature approaches, and most of the recent papers describe different modes to improve the extraction process using ultrasound (fluoxetine and norfluoxetine enantiomers [383]) or vortex (quinolone antibiotics in honey [491], free fatty-acids in biodiesel [495]) as well as the application of those methods toward the analysis of specific molecules (such as antipsychotic, phenothiazine drugs in urine reaching LODs in the 2–10 nM range [496]). Last but not least, several groups performing for *in vivo* sampling have reported microdialysis [497–500], an approach that can now be performed using air-assisted flow gating [501].

6.2.2 | Active sample pretreatment steps

As previously described, many extraction processes can be affected by the addition of energy, which accelerates the mass transfer but do not affect the driving force for the process. On the contrary, this section describes a series of pretreatment steps, where energy is absolutely needed to drive the process. Among those, perhaps the simplest methodology has been to heat samples (off-line) to denature thermolabile proteins that could otherwise interfere with the separation step. This can be accomplished at moderate temperatures (70°C for 20 min [502] or 90°C for 10 min [466]) followed by a filtration or centrifugation step. In addition, one of the most intriguing directions in sample pretreatment is the use of electric fields [503, 504] to accelerate the mass transfer of charged analytes into a liquid [505] or a supported liquid phase. Although the former typically uses drops (or droplets, where the analyte is transferred between immiscible phases) [473, 506, 507], the latter is typically known as electromembrane extraction (EME) and has become a very active area [508, 509].

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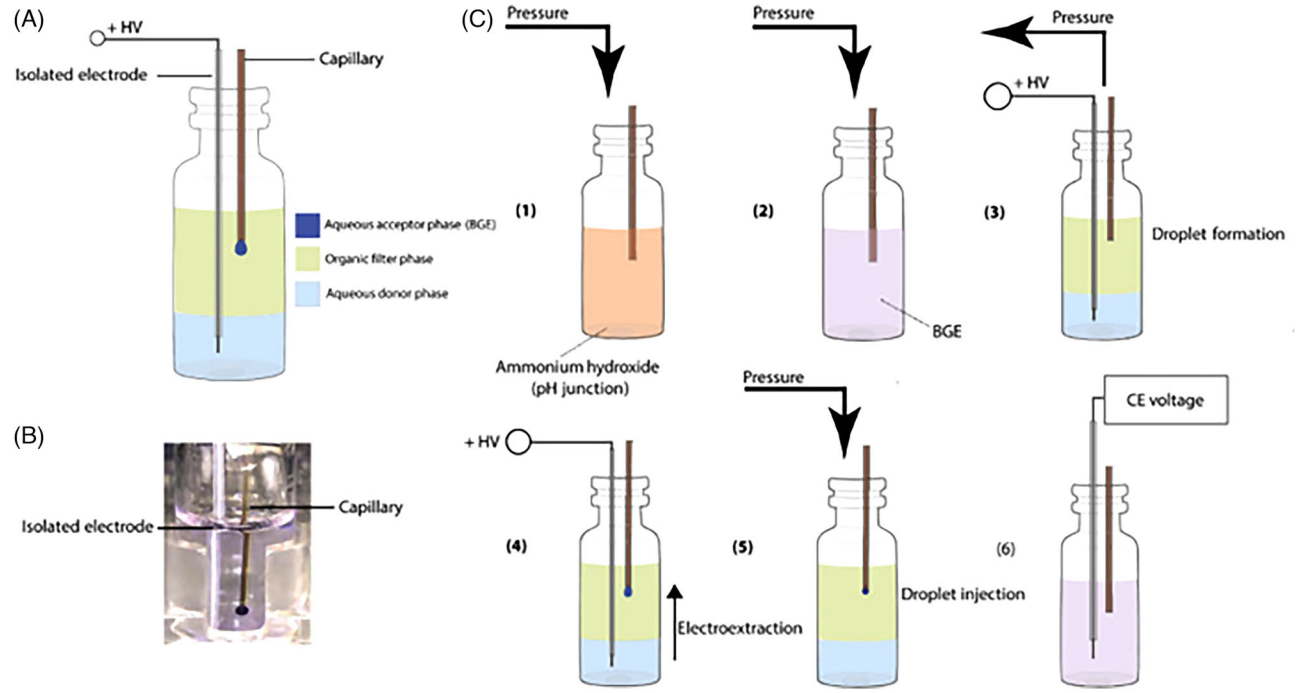

FIGURE 6 Illustration of the modifications performed by Lindenburg's group to a Beckman Coulter CE apparatus. (A) Schematic representation of the 3PEE setup, and (B) actual setup incorporating the modified electrode configuration used during experiments (bottom of vial not visible). (C) Schematic representation of the key steps in the extraction procedure in the CE–UV system: (1) injection of ammonium hydroxide, (2) injection of BGE, (3) application of negative pressure, (4) application of voltage, (5) retraction of droplet using pressure, (6) vial switch to BGE and start of CE separation. BGE, background electrolyte; CE, capillary electrophoresis. *Source*: From Ref. [473].

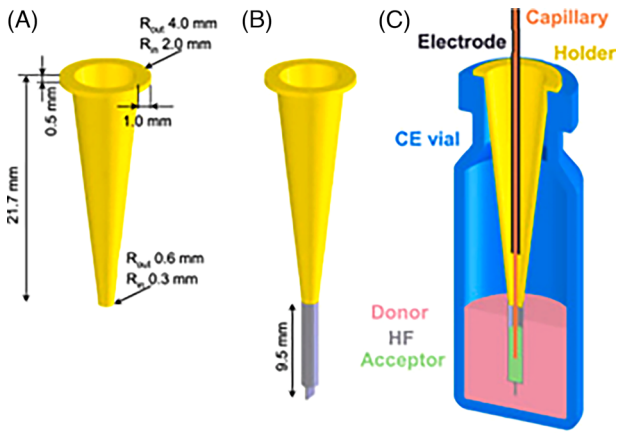

FIGURE 7 Illustration of a 3D-printed microextraction device. (A) Scheme and dimensions of the 3D-printed holder, (B) scheme of the 3D-printed holder with an attached hollow fiber, (C) scheme of the 3D-printed holder with the hollow fiber inside the CE vial depicting the tubular electrode and the separation capillary of the CE instrument. CE, capillary electrophoresis. *Source*: From Ref. [484].

This approach was recently used for the determination of phenytoin in plasma [510], experiments that only allowed the extraction of free forms of the compounds are typically extracted.

Among the most recent developments in this area, it is worth mentioning the work by Opekar and Tuma, who described and online approach to integrate EME with CE toward the determination of creatinine and basic amino acids in human urine (up to fivefold improvements in sensitivity after 300 s extraction at 150 V) [511] as well as Yuan et al. who proposed a nonaqueous miscible liquid–liquid electroextraction technique that enables fast (5 s) enrichment of amphetamine-type drugs from a vial containing mL of sample into a µL-level acceptor in a tube [512].

6.2.3 | Derivatization

Many groups have reported the possibility of including derivatization steps to aid in the detection step. Although those efforts are certainly relevant for the overall analysis, this section is focused on those reports using derivatization as a sample treatment step. Thus, perhaps a good starting point for this section are reviews from Underberg and Waterval (procedures to introduce charges in saccharides [513]) and Wuethrich and Quirino (derivatization for separation and detection in CE [514]). Other reviews have also described derivatization procedures applied to CE analyses [353, 515], including those targeting biogenic amines [516], amino acids [517, 518], cocaine [519], lipids [520],

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cells [521], or bacterial samples [522]. Perhaps one of the most important aspects of these procedures is establishing a reproducible procedure to control the yield (or density of the functionalization on large particles), so the extraction process is systematic.

As previously described [514], these reactions can be performed off-line (implemented before the separation), in-line (sequential injection and flow-gated CE), in-capillary (sample modified in the separation capillary), or post-capillary (after the separation). Generally speaking, and among different protocols that can be applied to improve the preconcentration or separation of target analytes, perhaps the most common ones are off-line (also described as pre-column). These methods are generally manually performed by allowing researchers to control a wide range of conditions [523], including heating the sample and have been applied toward the analysis of acrylamide [524], mono- and oligosaccharides [525], sialylated *N*-glycan linkage isomers [526], 3-hydroxyaspartate [527], or acidic metabolites [528]. Off-line approaches have also allowed taking some advantage of advanced oxidation methods (combining H_2O_2 and UV light) to follow the degradation of pharmaceuticals [529] or to convert alcohols into the corresponding acids, thus facilitating their separation by CE [350, 530]. In order to avoid evaporation of the samples during pretreatment, Romson et al. used an FC lid [531], a simple and clever approach that uses FC-770 Fluorinert (an inert, fully fluorinated liquid used in many instruments as coolant, $\delta = 1.79$ g/mL). This off-line approach was used to incorporate trypsin digestion (2 h at room temperature) and identify sequence homologies in spermatophore proteins from *Pieris napi*.

In-line options are more convenient, as they can be implemented by modifying the injection sequence, but require that the experimental conditions and reactions kinetics are compatible with the CE. This is, for instance, the approach recently selected by Dadouch et al. to incorporate digestion (with IdeS enzyme) prior to the analysis of infliximab [532] or adalimumab [533] (the latter, incorporating a reduction step with tris(2-carboxyethyl) phosphine hydrochloride). The implementation of this in-line methodology reduced reactant consumption by a factor of 1000 and doubled the efficiency of the separation, later noting that a careful selection of the trypsin grade was essential to avoid autolysis issues [534]. The same group also applied a similar approach for the analysis of a conjugate of poly-L-lysine and 5-hydroxytryptophan, demonstrating the versatility of the methodology toward pharmaceutical analysis [535]. Other groups have also used trypsin reactors toward the analysis of proteins in human tears [536] as well as *Escherichia coli* lysates [537] or discovering enzyme-inhibitor drug leads [538]. Other examples of the use of enzyme reactors include the immobilization of L-lactate dehydrogenase for the analysis of pyruvate [539], xanthine oxidase to determine the inhibitory capacity of flavonoids [540], or the screening of dual-target inhibitors against thrombin and factor Xa [541].

van der Burg recently developed an in-line approach for automated derivatization and sample handling for the analysis of mono- and disaccharides in cell culture media on either capillary or chip systems [193].

7 | QUANTITATION

Although quantification is a very important aspect in CE, there are not very many new aspects to this topic since the 1998 review [1]. This is perhaps because many aspects were already studied at that time because they are so important. Therefore, the original review is still valid in this regard.

Instrumental aspects of CE, including quantification and precision, have recently been reviewed [169], and the state of the art in CE precision has been reexamined in this context. Interestingly, this state of the art was described as about 1% in Ref. [1] but more recently has been assumed to be about 2%. This change for the worse was generally attributed to today's main application for biomolecules, whereas 25 years ago, very many small molecules were studied. More recently, however, CZE applications have again allowed percentage RSDs in the 1% range for mAb applications [169, 170, 187, 199].

7.1 | LODs and the influence of more sensitive detectors

Currently, UV/VIS detection is the most commonly used detector in CE because it is available in almost every instrument, inexpensive, and simple. However, with LODs in the range of 10^{-5}–10^{-6} M, the sensitivity is often insufficient, which is due to the short detection path length and the injected sample volumes in the nanoliter range [180, 542, 543].

Therefore, several strategies can be applied to improve the sensitivity. One option is to modify the detection cell of the capillary to increase the optical path length, for example, by using a bubble cell or a Z-shaped cell [180]. Adenoviruses could be analyzed down to picomolar concentrations combining a bubble cell with transient isotachophoretic stacking [187, 200]. Other possibilities are in-capillary sample preconcentration techniques such as sample stacking (see Section 6.1), sample treatments to remove interfering matrix compounds from the sample, and finally the use of alternative detection methods [542], which will be examined in this section.

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Today, highly sensitive detectors used in CE are fluorescence detectors and mass spectrometers. Fluorescence detectors can usually determine quantities in the nanomolar range [180, 542], although there are some articles reporting zeptomole or yoctomole range [544, 545]. Many aspects affect the sensitivity of fluorescence detection. These include the excitation light source and its arrangement, the use of optical filters, and the influence of light scattering on background noise [546]. Although lasers are powerful as an excitation source and achieve the best sensitivity, light-emitting diodes (LEDs) are increasingly used because they are small, inexpensive, are more flexible in choice of wavelengths, and have a longer lifetime [547, 548].

For fluorescence detection, the analytes must either be natively fluorescent, such as the amino acids tyrosine, tryptophan, or phenylalanine, or they must be labeled with a fluorescent dye [549]. A frequently used dye is fluorescein isothiocyanate (FITC), which shows strong fluorescence at an excitation wavelength of 488 nm. Labeling can also impair the sensitivity if analytes are not adequately labeled and therefore have a high fluorescent background [544]. In addition, the achievable LOD can be affected depending on the dye used.

Today, many CE instruments have a built-in fluorescence detector. However, commercial fluorescence detectors, which can be connected to already present instruments, are also available. For FITC, those instruments typically have an LOD in the nanomolar range [313, 549–551]. Research on home-built LIF and LED-induced fluorescence detectors is also an ongoing topic.

MS detection as a highly sensitive method has some advantages, such as no need for fluorophore or chromophore compounds. However, it is associated with other challenges, such as the need for a dedicated interface, a suitable buffer, and the incompatibility with highly saline samples or complex matrices of biological samples [543]. Seyfinejad et al. provided a tabular overview of MS detection in pharmaceutical and biomedical analyses. Therein, the conditions of the respective methods, including the determined LODs, can be found [543]. A nice comparison of some currently available commercial CE–MS interfaces and their sensitivities is given by Chen et al. [178]. Although ESI is the most widely used ionization method in combination with CE at the moment [552], inductively coupled plasma MS (ICP–MS) is an emerging field and can be used to overcome the lack of sensitivity of UV/VIS detection for nanoparticles [553].

Another detector is based on C^4D. It is also commonly used in microchip devices for the detection of organic and inorganic ions, as it is simpler to implement in terms of geometry. To obtain low noise and a stable baseline, it is necessary to use buffers of low conductivity. C^4D is not as sensitive as LIF or MS detection but achieves detection levels in the µM to nM range. In addition, it is a universal detector suitable for all analytes, with and without chromophores [554].

8 | SENSITIVITY AND THE LIMIT OF DETECTION

The content of this earlier chapter in the original article [1] was mainly moved to Section 6, some instrumental aspects are also discussed in Section 7.1.

9 | VALIDATION

9.1 | Method validation: a few adjustments

As before, ICH Guideline Q2* defines how validation should be performed in pharmaceutical analytics (https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf), [555]. The related concepts were already quite mature in the 20th century. Therefore, nothing fundamental has changed in the requirements since the publication of Ref. [1] but rather adjustments and modifications of existing concepts took place. However, an amendment to Q2 is already very far along and is about to be adopted. In this amendment ICH Q2(R2)**, the basic concept remains the same, but some definitions and specifications are brought up to the current state of scientific knowledge. After ICH Q2(R2)** has come into force, we will certainly write a separate article on the resulting consequences for CE in a similar place (https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q2r2-validation-analytical-procedures-step-2b_en.pdf).

*Q2: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. *Validation of Analytical Procedures: Text and Methodology Q2(R1)*: *ICH Q2(R1)*, Step 4; International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2005 (Q2(R1)). Available online: https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf (accessed on January 17, 2023).

**Q2(R2): International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. *Validation of Analytical Procedures: Text and Methodology Q2(R2)*: *ICH Q2(R2)*, Step 2b: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q2r2-validation-analytical-procedures-step-2b_en.pdf.

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What has changed: We do not longer talk about instrument validation (Section 9.2 in Ref. [1]), but about instrument qualification [556]. Extensive work has recently been published on the quality of various CE instruments [169, 173].

Moreover, DoE has become much more important in recent decades. This tool helps a lot to evaluate the robustness of analytical methods, as well as AQbD or the principle of Lifecycle Management.

A few best practice examples from vaccine and antibody analyses and the quantitation of an enzyme may round up this update [186, 187, 196, 198–200, 557].

Moreover, perhaps most importantly, our attitude toward method validation had changed. We no longer look at method validation as an exercise to press the best we can out of a method in the hands of the expert. Our current attitude toward method validation is that it should give a reliable prediction toward future use in the hands of QC analysts.

9.1.1 | Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters, and provides an indication of its reliability during normal usage [555]. It should be evaluated during the development stage of a method. The relevant parameters for robustness remained unchanged during the last decades [1], but DoE [558] became a tremendously helpful and powerful tool to investigate robustness and the proper range of method parameters. Once it is known which two or three parameters are most decisive for the robustness of a method, for example, through preliminary experiments or a (Plackett–Burman) screening design, a DoE approach can define the proper parameter range with an acceptably high number of experiments.

For example, 12 vitamins from a biotechnical process have been separated from each other by MEKC. The pH and the SDS concentration have been identified as most critical parameters. Using a central composite orthogonal design for BGE optimization in the range 50–100 mM SDS and pH 8–9, only 11 experiments were sufficient to create a Sweet Spot Plot (see Figure 8). This plot makes it clear that a separation of all 12 vitamins is possible in a range from approximately 60–90 mM SDS, but the pH needs to be kept in a range of 8.6 ± 0.05. This is a very important information. It is easy to keep the pH within that range when taking good care, but it is good to know that good care is actually required in this case [559].

Another excellent example for the use of DoE, using various models, was demonstrated to optimize an eACA method to characterize 16 mAbs and their side compounds [196]. Resolution, peak width, and the number of peaks have been chosen as response parameters, the concentrations of eACA, TETA, HPMC, butanolamine, and acetonitrile have been varied, as well as the pH, resulting in a *compromise method* (400 mM EACA, 4 mM of TETA, 0.05% HPMC, 24 mM of butanolamine, and 0.4% of acetonitrile at a final pH of 5.7) considering all response parameters, and a *highest number of peaks method* (400 mM EACA, 4.4 mM TETA, 0.05% HPMC, 47 mM of butanolamine, and 6% acetonitrile at a final pH of 6.0). For more information on the TETA and eACA method, please see Section 3.2.1.

9.2 | Instrument qualification

The qualification of instruments is of great importance for the validity of generated data. Especially in pharmaceutical QC, analytical instruments and procedures must guarantee that they can reliably work to ensure the safety and efficacy of drug products [556, 560]. Therefore, instrument qualification is not a single constant process but consists of several activities introduced by the United States Pharmacopoeia chapter ⟨1058⟩. The analytical instrument qualification (AIQ) is the fundament for quality management in laboratories. It consists of four activities which are design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) [556, 561]. DQ concerns the responsibility of the manufacturer for a robust design and the availability of specifications and requirements, whereas users should consider their need for support installation and services [556]. The next step is the IQ, which involves the setup of the instrument in its environment and must be done for new and pre-owned devices. The OQ consists of the testing of the basic instrument functions after the first implementation or significant modification to verify the declarations specified by the manufacturer and to test the operating limits [561]. The final step involves the PQ. This examines whether the instrument performs according to the user's specifications [556]. According to Pögel et al., a PQ should be done at least once a month and also in combination with system suitability tests (SSTs) and QC checks (QCC) [561]. An SST is a procedure that tests whether the method and instrument combined perform according to the requirements set based on knowledge of critical method parameters and handling, on the occasion of the actual analysis. In general, the frequency of OQ/PQ depends on the instrument's reliability and how critical the measurements are for the laboratory routine [556]. Today, in pharmaceutical companies, AIQ is

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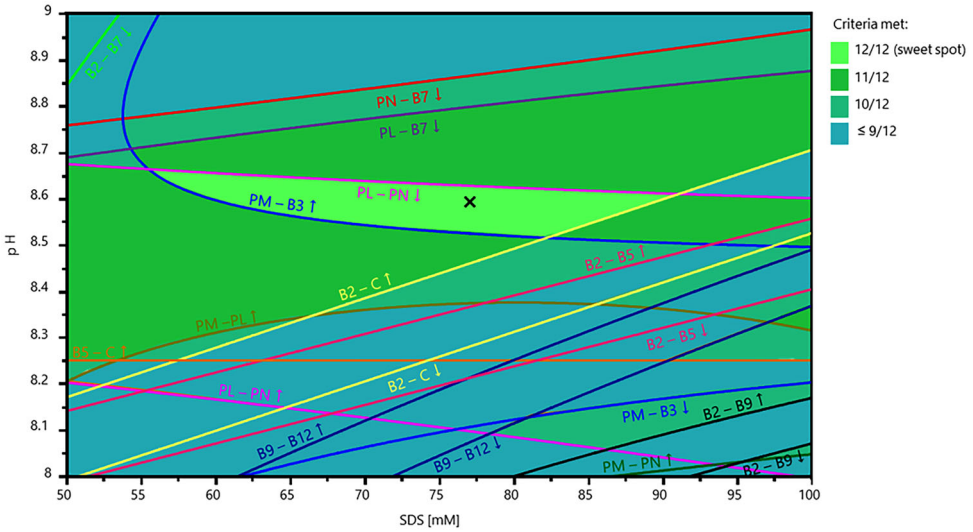

FIGURE 8 Sweet Spot Plot of resolutions of critical vitamin pairs, criterium is $R \geq 1.0$ for the resolution between PN and PL and $R \geq 1.5$ for all other critical vitamin pairs. The arrows indicate at which side of the border the criterion was met. The cross indicates the selected conditions. B1, thiamine; B2, riboflavin; B3, nicotinamide; B5, D-calcium pantothenate; B7, biotin; B9, folic acid; B12, cyanocobalamin; PL, pyridoxal; PM, pyridoxamine; PN, pyridoxine. *Source*: From Ref. [559].

implemented by the performance of standard operating procedures (SOPs) [562]. Cianciulli et al. defined parameters, test methods, and acceptance criteria for accurate AIQ for CE devices [556]. An important parameter is the consistency of temperature during analysis, which can be controlled by accurate thermostatting. The stability and accuracy of the applied voltage can be examined by reproducibility tests of migration times or calculation of effective mobilities. For the verification of UV/VIS detectors, wavelength accuracy and linearity as well as S/N have to be investigated. Finally, it has to be tested, if the injection is working appropriately. The authors provide these information including parameters, procedures, and acceptance criteria in a tabular overview [556].

9.3 | Method transfer

Once a CE method has been validated, it can then be formally transferred to the application laboratories. The method can be applied to the desired product after the method transfer and is therefore essential and actually the biggest challenge of the method. Method transfer is often the interface between the researching institution, such as a university or R&D lab, and the performing industrial institution or QC lab that produces a certain product. After a successful method transfer, both institutions should arrive at the same results independently of each other [328, 563].

The fundamental method transfer has not changed over the years, which is why the ideas from the original review have also been taken up in later works [564].

10 | TROUBLESHOOTING AND PREVENTION: GOOD CE PRACTICES

The principles of troubleshooting have not fundamentally changed since the original review [1]. The recommendations for troubleshooting described there, which are largely based on the studies by Altria [565] and Engelhardt et al. [566], can therefore still be adopted. In addition, as CE community we have collected best practice over the years, and this "tribal knowledge" often helps to prevent the need for troubleshooting [8] (https://blog.sepscience.com/pharmascience/topic/capillary-electrophoresis-ce) [567]. An illustrative example is the investigation of the role of eACA in the CZE method for charge heterogeneity determination mentioned in Section 3.2.1, where willingness to share method details and to perform an interlaboratory study lifted a lot of misunderstanding and clarified that most companies' methods deviated from the original method and that the eACA quality was not a contributing factor to the observed issues [170].

10.1 | Precision

Paramount to good method performance in CE, it is important to apply best practice and not to select conditions or settings that interfere with the method's precision.

For good repeatability in CE, it is generally advisable to change the inlet and outlet vial after each run (or so), to prevent shifts in the t_M due to buffer depletion. The easiest way to do this is to use a replenishment system. How

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frequent the buffer vials need to be replaced if there is no replenishment system, depends on the BGE buffering capacity and the volume of BGE in the vials. The liquid levels in inlet and outlet vials need to be the same to prevent syphoning. This also implies that capillary conditioning should be programmed such that the inlet and outlet BGE vial do not change in volume. Therefore, the actual conditioning steps should be performed from separate vials and a waste vial. Vials should not be filled for more than 75% of the height, as overfilling the vials might cause liquid to enter the tubing system of the instrument. The waste vial should contain sufficient liquid (e.g., water or BGE) so that the capillary end reaches the liquid to prevent the formation of large droplets of conditioning solutions that could then be carried over to a next vial. Always cap vials, as for most systems the cap is the soft material that can be compressed to make the system pressure-tight, required for good conditioning and precise injection.

Which capillary conditioning steps need to be performed for good method repeatability and reproducibility is different for each method and has to be investigated as part of method development. This includes initial conditioning of a new capillary and cleaning and storage conditions. Although literature gives the impression that a fused silica capillary should initially be rinsed with an NaOH solutions, this is not the case. For instance, Shi et al. demonstrated that rinsing with NaOH completely destroyed the charge-heterogeneity determination of mAbs [568]. The development of the conditioning of a PVA-coated capillary to prevent and reverse adsorption was an important aspect of developing a successful method for virus particle concentration determination [198].

The BGE needs to be prepared such that the composition is controlled. Titrating a BGE solution to the intended pH causes variability in the ionic strength that, in turn, results in fluctuating currents. It is better to determine what specific concentrations of each BGE component are required as this will result in a constant quality and reproducible current levels. Water of appropriate quality needs to be used. Usually, Milli*Q* filtered water works well. Not all bottled water types are suitable. If there is a risk for incomplete dissolution of the BGE components or precipitation, the BGE solutions need to be filtered over 0.22–0.45 µm pore filters. After preparation, the BGE needs to be degassed. This can be done in different ways, for example, filtering over 0.22–0.45 µm pore filters, sonication, under vacuum, and so on.

Generally speaking, CE instrument sample injections are precise and injection repeatabilities of ≤1% RSD are feasible. To prevent carryover from a sample droplet sticking to the capillary end, the capillary inlet is dipped in BGE or water after sample injection. In addition, the polyimide outer coating of fused silica capillaries should be removed, for example, using a gas flame, to prevent sample sticking into the pockets between the silica and polyimide, which can be the result of polyimide delamination after capillary cutting. Precision can be further improved by injection of a BGE plug after the sample plug. If the capillary hits the bottom of the vial, injection or conditioning artifacts can be expected. Assure that the capillary is cut straight. If not, resolution between close-migrating peaks can be lost [569]. Although some advice to polish the capillary end with the flat side of the ceramic capillary cutter if there are any cutting artifacts, we strongly advice against that as silica debris might enter the capillary.

When applying the voltage, voltage ramping is usually beneficial. Typically, the voltage is ramped over 0.5 min, although longer ramping times can be appropriate after large-volume injections of low-conducting sample.

The detection settings during analysis should fit the expected peak width, such that at least 20 data points are collected so that the integration software can detect the peak and integrate properly. For non-baseline-resolved peaks, the method SOP should include integration instructions so that every time the peaks are integrated in a similar way.

Last but not least for good precision is to keep the instrument clean. Any kind of spill might cause barriers against pressure-tightness and cause improper conditioning or injection. There is also a risk that dried conducting liquids create current hot spots, damaging the capillary or short-cutting the electric field. Some solutions such as gels are very sticky, and insufficient cleaning might cause vials or caps to stick in turn.

10.2 | The capillary

Different capillary batches often have different properties. This is commonly observed and can then have a significant impact on, for example, the stability of dynamic coatings and the duration of equilibration processes. For example, in some batches the coating may be stable and the analytes may show the same migration behavior over 100 repeat injections, but in other batches, the coating is unstable and shifts in migration time occur after a few injections. The differences between the distinct batches are probably the result from the varying densities of silanol groups on the BFS surface. A solution to this problem would be to choose a coating that is not so sensitive to the small changes in the capillary and is therefore more robust. In order to be able to monitor this problem, an SST for the capillaries should be developed in relevant cases, in form of a method that is easy to perform and sensitive to small differences in the capillaries, so that these become apparent.

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Another common trouble with any type of CE is a broken capillary. Most often the break happens at the detector window, as the outer coating has been removed there, weakening the capillary. A broken capillary can be detected in the UV trace as well as in the leak current and current trace. In the SI, all three traces are shown in the event of a capillary breakage to demonstrate how quickly to detect this problem (Figure S1). It can then be fixed by changing the capillary and cleaning the interface.

A clogged capillary would also lead to faulty separations. Capillary clogging can happen, for example, due to the presence of particles, use of solutions that are too viscous, adsorption on the inner capillary wall, or precipitation. An example of an electropherogram with a clogged capillary can be seen in Figure S2. To avoid clogging, the use of syringe filters and the adoption of extended rinsing procedures should be considered, along with the use of a different coating, less prone to adsorption.

To check whether a capillary is clogged, high pressure can be applied to an air-filled inlet vial, positioned in a vial filled with water. If no bubbles are observed, then the capillary is likely clogged or broken.

If the capillary is not conditioned properly despite all best practice as described before, it is possible that the pump is malfunctioning. To verify, first set vials filled with water at the capillary inlet and outlet and manually, through the system diagram, apply a pressure of ca. 1 bar. Verify if the pressure is built up and remains constant. If not, check all tubing and connections to assure that the system is pressure tight. If the pressure was built up and remained constant, next the inlet vial for a vial with a solution is changed that gives a strong response with the detector settings. Again apply a pressure of ca. 1 bar and verify if the detector signal changes when the liquid should have reached the detection window, typically after 20–30 s. If this is not the case, check whether the capillary is broken or clogged, whether the capillary touches the vial bottom, whether the capillary window is centered in the detector, whether debris obstructs detection, or whether the detection slit in the aperture/alignment interface is correct.

10.3 | Detection

Most CE instruments work with UV detection, that is, they use a lamp which, like any lamp, is only functional for a certain period. For example, UV lamps are typically guaranteed for 500–2000 h of operation. After this time, there is a risk that the intensity of the excitation source is reduced, which will, of course, also be reflected in the electropherograms. Examples of how this looks with CZE (Figure S3) and CE–SDS (Figure S4) can be seen in the SI. This problem occurs regularly with every CE with UV detection after a certain time, which is why it is good to recognize this quickly and then replace the lamp. Many instruments have a lamp intensity test integrated in the software, which allows to quickly test whether the lamp is still in order. For the same reason S/N tests of reference peaks are included into AIQ/PQ and SSTs [556].

Some instruments have a plastic aperture that will wear with time due to the constant exposure to UV light. Regularly check the aperture and replace when needed. When using filter UV detectors, the filters also need to be replaced regularly.

With CIEF, it is very important to use a UV filter to avoid destroying the baseline due to the absorption of the ampholytes. An example electropherogram, which was recorded without UV filter, can be found in Figure S5. The baseline is very wavy and the peaks are difficult to recognize, so a UV filter or suitable wavelengths should always be used.

Baseline disturbances can also affect, for example, the quantification of protein impurities in CE–SDS. One way to avoid these baseline effects is to shorten the injection sequence [570].

10.4 | Excessive Joule heating

High voltage can be applied in CE for fast and efficient separations, provided that there is no excessive Joule heating. Generally, the current for a specific BGE–capillary combination is linear with the applied voltage as long as the Joule heating is dissipated efficiently. The heat dissipation is supported with the capillary cooling system that most instruments have, albeit that different constructions show different cooling efficiencies. In order to verify whether the Joule heating is dissipated efficiently and excessive Joule heating is avoided, make an Ohm's plot. For this, fill the capillary with the BGE and stepwise increase the applied voltage while measuring the current. The plot of the current versus the applied voltage starts deviating from a linear correlation when there is excessive Joule heating. Cianciulli et al. developed a method for measuring the temperature during CE, which can also be used to calculate the maximum suitable electrical power per unit length [571].

If the CE does not cool properly, it is often worthwhile to disassemble the device and see if the cooler/heat exchanger is seated properly. If not, it should be adjusted, the screws tightened if necessary, and the problem is quickly solved. When using liquid cooling, regularly check the cooling liquid levels and adjust, as the cooling liquid is volatile.

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10.5 | Troubleshooting

Troubleshooting implies making a series of observations and tests that helps identify where the actual issue is. When the unexpected occurs and troubleshooting is required, do not take the instrument apart immediately. First, reconsider carefully whether all best practice was applied and whether all solutions were prepared as should. Then, without changing anything, observe the system. Are there any error messages in the system log? Are all vials and caps in place and at the proper positions? Are the right solutions in the vials, is there precipitation, or are there any air bubbles visible? Are the capillary ends at the appropriate length and are they undamaged? Is the capillary broken or visibly blocked? Are the electrodes straight and clean? Was the detector lamp switched on and did it have sufficient energy?

If all is as it should, perform the manual pressure test as described before. If the pressure test passes, fill the capillary with BGE and put BGE vials at the inlet and outlet. Apply the voltage and check whether the current is as usual. If an Ohm's plot was not made before, do so now. If all looks OK, run the actually programmed method stripped from conditioning and injection. If there are no issues observed, step-by-step build the programmed method back and verify when the issue occurs. In this way, most of the time the (root) cause of the issue can be identified and solved.

Generally, the current trace is a powerful tool to support troubleshooting, as the current trace is usually very specific for a certain method and sample. Consequently, show a typical current trace in the method SOP.

Mostly, when CE best practices are taken into account and the method is well developed, the majority of the issues during sample analysis are caused during sampling and sample preparation. Provided sensitivity is not limiting, injection repeatability for a CE method can be well within 1%–2% RSD. The added method uncertainty/variability can be assessed by uncertainty/error analysis of the sample preparation steps. Especially variabilities introduced by pipetting, inappropriate mixing, and serial dilution are frequently underestimated.

11 | MINIATURIZATION OF CAPILLARY ELECTROPHORESIS: MICROCHIP ELECTROPHORESIS (MCE)

The 1998 version of this review article [1] did not contain a section on MCE, but 25 years later, the miniaturization of CE is now a reality. Significant advances in the miniaturization of CE systems have been reported during the last two decades, making MCE systems a routine method for DNA and protein analysis. The first applications of microfluidic devices were in the field of analysis, working on the microscale allowed exploiting new mechanisms not available in traditional bench-scale analytical systems [572]. There are attractive benefits offered by miniaturized systems, such as reduced analysis time, reduced sample consumption, higher resolution and integration, and the potential of portable devices [573]. Challenges such as appropriate sample introduction schemes and analysis robustness have been successfully overcome, allowing MCE to become available in commercial instruments [169, 173, 574].

11.1 | First microchip electrophoresis devices

MCE systems were made possible thanks to the advances in miniaturization and microfabrication technologies. MCE is one of the earliest versions of micro-total analysis systems (μTAS) [169, 173, 574]. The very first miniaturized analytical device was reported by Terry et al. in 1979 with the development of a miniaturized gas chromatograph on a silicon wafer for the separation of a mixture of hydrocarbons in 10 s [575]. However, at that time, there was no significant interest in the development of miniaturized systems as microfabrication facilities were not popular. The next significant advancement in miniaturization was the concept of μTAS developed by Manz et al. in 1989 [574]. A μTAS integrates all required steps for a complete analysis within the same device, which includes sample injection, reagent mixing, separation, and detection of target analytes.

Some of the main contributions to the early stages of the field of MCE were made by Harrison and Manz [576–579] and Jacobson and Ramsey [580, 581]. These pioneer reports in MCE employed devices made from glass, as it was one of the first materials utilized in microfabrication technologies. The use of high electric fields and short electrophoretic channel lengths allowed for analysis times in the range of 1–2 min, reaching plate heights down to 0.3 μm/plate [579]. The development of integrated sample injection schemes made possible the design of complex separation strategies that permitted valveless fluid switching by means of EOF [577]. Traditional capillaries were replaced by short channels on microchips with lengths between 1 and 10 mm, which were employed to carry out electrophoretic separations with almost no dead volume [578]. The use of MCE systems made possible the analysis of small sample volumes, in the range of a few nanoliters to a few microliters [582]; along with low consumption

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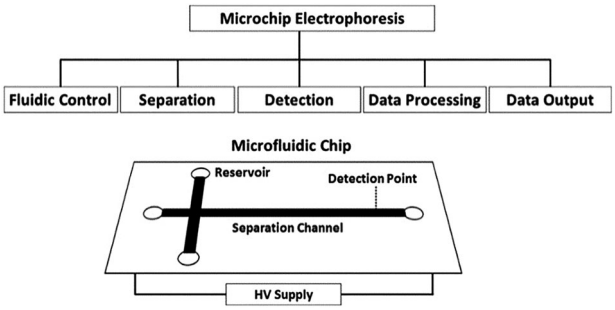

FIGURE 9 (Top) Schematic of the five stages of an electrophoretic separation, from fluidic control to data output. (Bottom) Illustration of a microfluidic device for electrophoretic separation. *Source*: Adapted with permission from Ref. [584], copyright (2019) Elsevier.

of reagents, low waste, short processing times, portability, and reduced cost when compared to traditional bench scale equipment [583].

The configuration of MCE systems is rather simple, as in many systems the EOF is used for liquid pumping and devices are made from transparent substrates with optical properties that facilitate fluorescence detection methods. LIF is commonly used in MCE systems as some target analytes possess native fluoresce and there is a large availability of suitable fluorescent labeling reagents for labeling target analytes [583]. Electrochemical detection is also popular in MCE systems in both amperometric and conductometric modes [584, 585]. MS is another major detection method used in MCE systems as it offers high sensitivity and resolving capabilities; MS detection is particularly advantageous in the detection of carbohydrates and proteomics analysis [582].

In terms of the method development aspect, in MCE systems the selection of the device material and separation channel design are essential components of the method development process, in contrast with traditional CE systems where commercially available equipment is employed. Perhaps this is the main dissection in method development between MCE and CE systems. The most common materials employed for the fabrication of MCE systems are glass, some ceramic materials, and polymers such as PDMS, poly(methyl methacrylate), and cyclic olefin copolymers. The use of polymers has received significant attention, as polymers allow for mass production and disposable devices [583]. The use of 3D printing methodologies has enabled the creation of complex structures that were not feasible employing traditional lithographic methods [586]. Figure 9 contains a schematic of the five distinct stages of a miniaturized electrophoretic separation and an illustration of a MCE device [584]. The simple T-shaped microchannel design is formed by the main separation channel that is perpendicularly connected to the sample channel, which is required to perform EK injection (discussed in Section 11.2).

11.2 | Sample introduction in microchip electrophoresis

Significant advances have also been made in the development of strategies for sample injection during the last two decades. The 1998 version of this review article correctly stated that at the time sample injection in MCE remained a major challenge [1]. Since then, numerous HD and EK injection schemes have been successfully developed [587, 588] and thus, sample injection is no longer a major hindrance. An accurate sample injection process is critical to the success of an electrophoretic separation (see Section 6.1), as both, the resolution and efficiency of the separation depend on the quality of the sample zone introduced into the MCE system [589]. Figure 10 contains an illustration of the three steps of an EK sample injection process in an MCE system [590].

The main objective of a sample injection process is to deliver a fixed sample volume to the main separation channel; for this purpose, a plethora of channel designs with T-shaped intersections, such as the one depicted in Figure 10, have been developed [591–593]. Many of these processes employ one of the two common schemes: a two-step process that includes injection and separation and a three-step process that includes loading, gating, and injection. These injection modes employed with MCE systems are classified as follows: pinched injection, floating injection, gated injection, and dynamic injection. Briefly, pinched injection is when the sample volume is determined by the channel intersection (such as the illustration in Figure 10), resulting in an accurate, well-defined, and reproducible sample volume that produces high separation efficiency. However, it can only inject small volumes of the sample solution into the channel, as the volume of the injected sample is limited by the volume of the channel intersection. Floating injection is similar to pinched injection, with the extra capability of being able to inject a slightly larger sample volume into the channel. Gated injection, on the other hand, offers high flexibility on the volume of the sample injected into the channel, as the sample volume is determined by the applied injection voltage and duration of the injection step. However, gated injection is significantly affected by injection bias. Dynamic injection also allows variability in the volume of the sample injected, and into the channel, but the sample is introduced as several plugs; and injection bias is also significant [594].

Although significant progress has been made, some challenges still remain, such as injection bias and dispensing bias [587, 594]. Injection bias is when a specific

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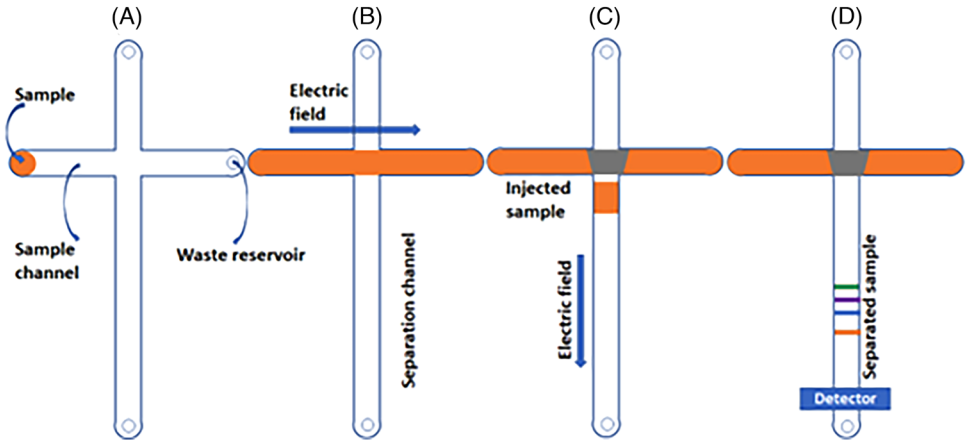

FIGURE 10 Illustration of an electrokinetic injection process in an MCE system. (A) Introduction of the sample into the sample reservoir. (B) Sample loading step across the sample channel, in which the sample is derived to the waste reservoir. (C) Sample gating step in which the sample volume is cut and introduced into the main separation channel. (D) Sample injection step, during which the sample migrates along the length of the main separation channel and is resolved into its distinct components. *Source*: Adapted with permission from [590], copyright (2022) John Wiley and Sons.

analyte is favored during the injection process, that is, more of one analyte is injected than of the others. Analytes with a higher overall EK mobility would enter the main separation channel more rapidly than analytes with a lower overall EK mobility (see Section 6.1), this produces a greater response in the detector for the higher mobility analyte [587]. Significant efforts have been made to reduce or eliminate injection bias, as it can be highly detrimental to quantitative applications of MCE systems. Injection bias is not a major problem in the measurement of migration times or qualitative electrophoresis analysis. To address injection bias issues, hybrid injection schemes that combine hydrostatic forces have been proposed, in which, for example, the sample injection is accomplished by employing both a syringe pump and a voltage sequence [594].

11.3 | Overview of method development advances and recent applications in microchip electrophoresis

Numerous novel applications of MCE have been recently developed, as new advances allow for new configurations. Similarly to CE systems (see Section 2.1), to initiate proper method development for an MCE application, it is essential to first assess if an electrophoretic-based approach is the best option for the analysis/separation in mind. If this is the case, one needs to ask: *What specific type of MCE system and technique are the best suited for my particular analysis?*

As with CE, the selectivity (see Section 2.3) of the selected method is paramount. Selectivity is defined as the degree to which the method is able to distinguish an analyte from a mixture without interference from other analytes or the matrix. However, the majority of desired MCE applications are separation processes where selectivity, while important, is not the only primary objective. The ultimate goal of method development is to develop a well-resolved, reproducible, and efficient separation process. After selecting a suitable method and operating conditions (BGE and pH) to achieve proper selectivity, the next steps in process development are selecting the detection mode and ensuring a successful separation in terms of analysis time, reproducibility, and others. The validation of MCE systems follows the same protocol followed with CE systems (see Section 9 and [1]), that is, validation is ensuring that the method selected is capable to achieve the desired separation process. Validation guidelines and criteria vary depending on the industry and/or institution (e.g., IUPAC and FDA). Recently van der Burg demonstrated that a method developed on a conventional CE instrument with a fused silica capillary could be transferred easily to a fused silica chip MCE system [193].

Examples of novel and recent applications of MCE systems include the use of AC sinusoidal waves, polarity switching, dual-channel systems, the use of *in situ* photopolymerized gels, and the addition of sponge-like materials to the channel liquid reservoirs to function as capillary force suction pumps [595]. In these recent applications, method development included the evaluation and selection of these additional operating conditions. For example, the selection of the characteristics of AC potential, frequency, and peak amplitude, which allowed for the simultaneous determination of cations and anions [596]; and the design of a polarity switching scheme to achieve simultaneous enrichment and separation of fluorescent analytes [597].

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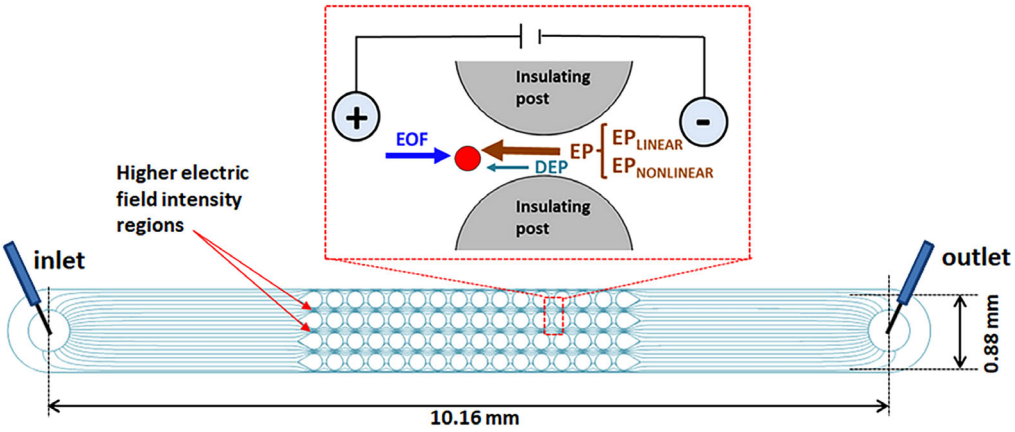

FIGURE 11 Illustration of an iEK microchannel, depicting the electric field as streamlines. The regions between the insulating cylindrical posts are the electric field reaches its highest intensity. Nonlinear electrokinetic effects, including nonlinear electrophoresis, arise in these regions of higher field intensity. The top panel depicts a negatively charged particle as a red circle, and all forces exerted on the particle/analyte are depicted as arrows, noting that the dominant forces are EO flow and electrophoresis (EP) linear and nonlinear with a small contribution from dielectrophoresis (DEP). This illustration corresponds to a channel with a negative zeta potential, where the magnitude of the negative particle zeta potential is greater than that of the channel ($|\zeta_P| > |\zeta_W|$) [605].

The fields of bioanalysis [583] and clinical applications [584] have benefited the most from the miniaturization of CE systems. In the field of bioanalysis, MCE systems have enabled rapid assessments that can replace time-consuming laboratory analysis. One example is the detection and quantification of intact pathogens. The first electrophoretic separation of intact microbes *in the manner of molecules* was reported by Armstrong et al. in 1999 [598]. Since then, numerous electrophoretic approaches, including a vast range of MCE systems, have been successfully employed for the detection and separation of intact virus, bacterial, and yeast cells [590, 599, 600]. A new approach in microfluidic devices that exploits nonlinear electrophoretic effects have also been recently proposed for the rapid electrophoretic-based separation of intact microorganisms, microparticles, and proteins [601–604]. This method, called insulator-based EK (iEK), employs microchannels with 3D insulating posts embedded into the channel, where the presence of the insulating posts distorts the electric field distribution along the microchannel, creating regions of higher electric field intensity. An illustration of an iEK system, depicting the forces acting on the target analyte, is included in Figure 11. It is precisely in these regions of higher field intensity where nonlinear EK effects arise and influence the overall migration of the target analyte. Method development in iEK systems includes the selection of linear or nonlinear effects to carry out the separation, as the elution order of a separation process can be switched by varying the magnitude of the applied electric potential [605]. This flexibility of simply varying the magnitude of the applied potential to control which EK mechanism is the dominant effect in the system is unique to MCE systems. It is possible to design and fabricate tailored MCE system to enable and enhance the switching between linear and nonlinear EK mechanisms, by designing devices that allow for the generation of high electric field zones within the device.

Some general rules can be identified in method development of MCE systems. First, similar to CE; target analytes can range from nano to larger biomolecules to micron-sized microorganisms, the latter more commonly analyzed in MCE systems due to their higher potential for integrating several processes within the device [606]. Second, the selection of the suitable electrophoretic process offers more alternatives than with traditional CE systems, as MCE devices allow for higher flexibility in their operation. For instance, the switching between linear and nonlinear EK effects can change the elution order, just by varying the magnitude of the applied electric potential [603, 605]. By considering both linear and nonlinear EK effects, it is possible to design highly discriminatory separations of target analytes with similar characteristics (similar size, shape, and electrical charge). Significant efforts are being devoted to the characterization of nonlinear electrophoretic migration of particles and cells [607, 608], in order to employ this data for the design of effective iEK separation systems [609].

In the field of clinical applications, the use of MCE systems (including also gel-based MCE systems) has the potential to expedite disease diagnosis and accelerate treatment. For example, cancer diagnosis with MCE relies on the detection and quantification of small molecules, peptides, and proteins as well as the detection of mutations of DNA and RNA samples extracted from bodily fluids

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and tissue. Immune disorders can also be diagnosed by employing MCE systems by detecting small molecules and proteins from bodily fluids and tissue samples. Cardiovascular diseases and organ diseases/dysfunctions (e.g., diabetes, pancreatic dysfunctions) have also been diagnosed with miniaturized electrophoretic systems by assessing proteins, enzymes, and small molecules in urine, blood, plasma, and serum samples. An excellent summary of the use of MCE systems in clinical diagnosis was published by Wuethrich and Quirino, the reader is referred to the original article for further information [584, 585]. Another recent review discusses the use of MCE for cell culture process samples and upstream and downstream processing [610].

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Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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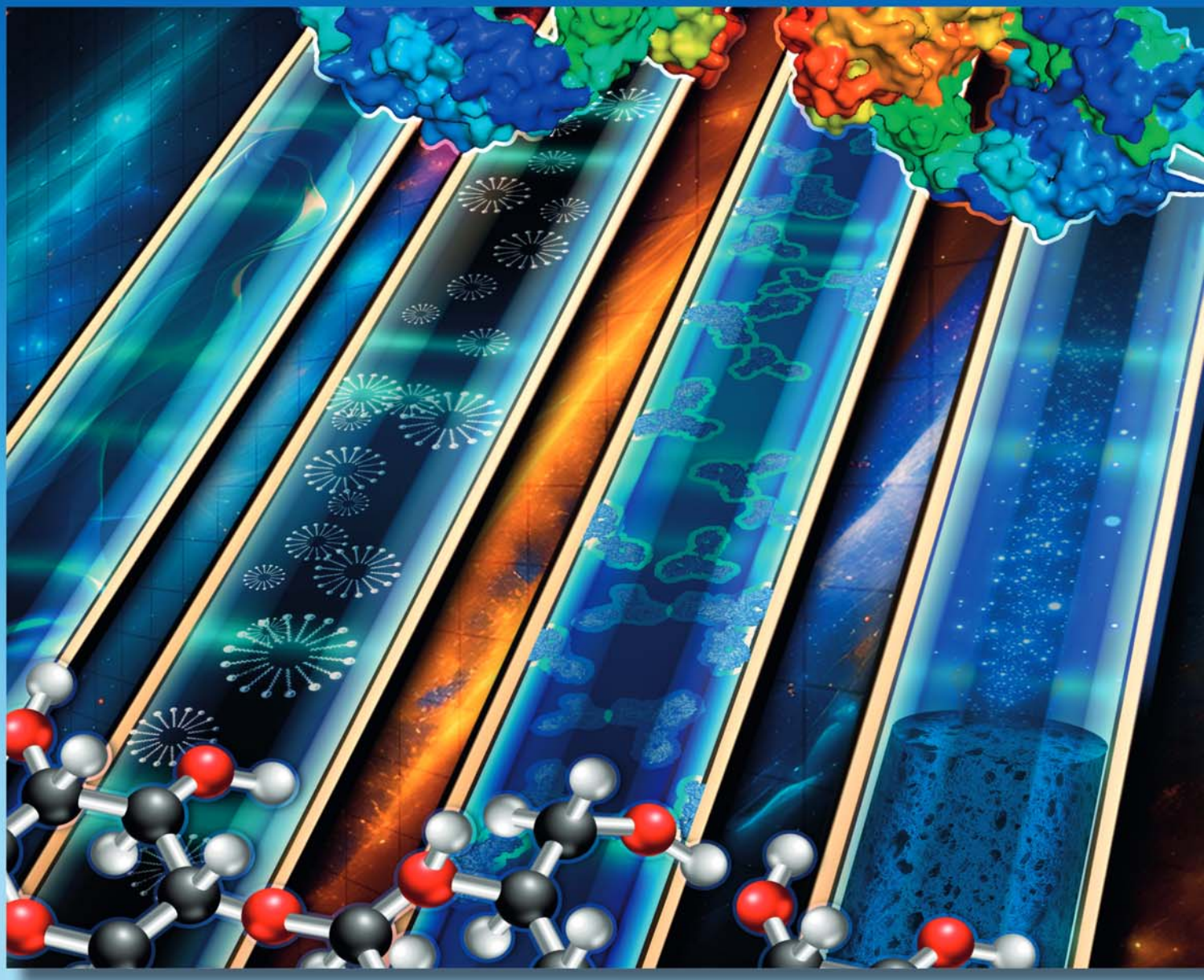

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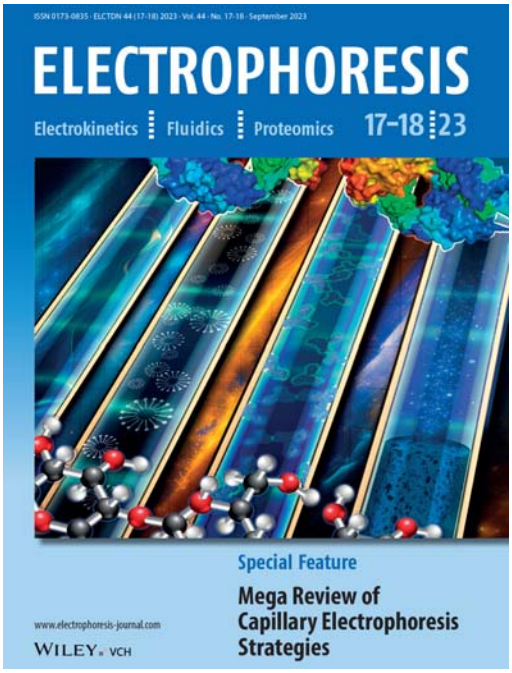

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The cover picture includes four capillaries running separations of different samples and using different modes, illustrating the various uses of capillary electrophoresis (CE) for pharmaceutical and biological analysis. With over 600 carefully chosen references, this review focuses on the major developments in methodological and validation strategies in CE over the past 25 years.

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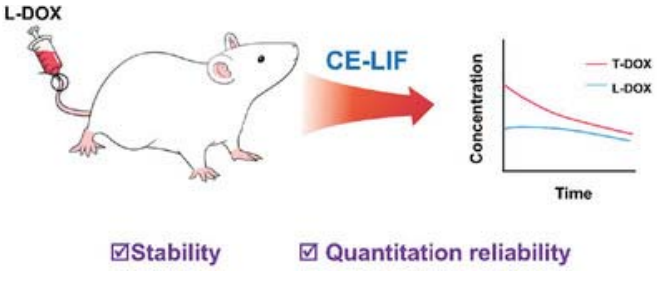

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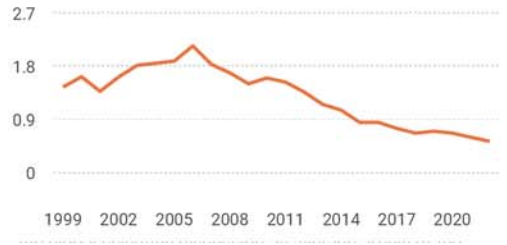

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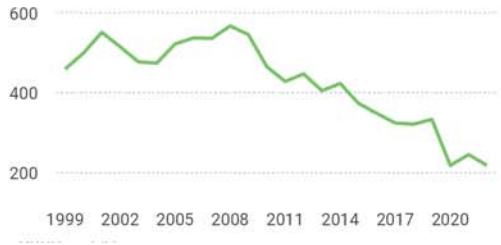

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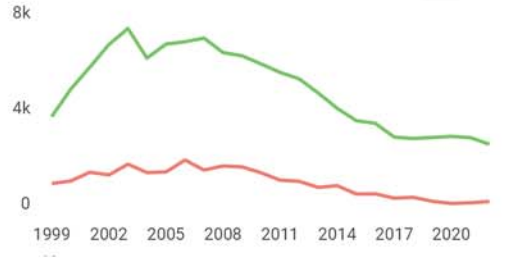

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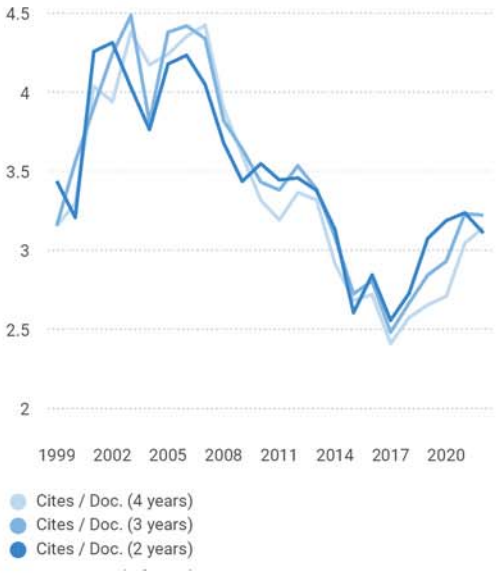

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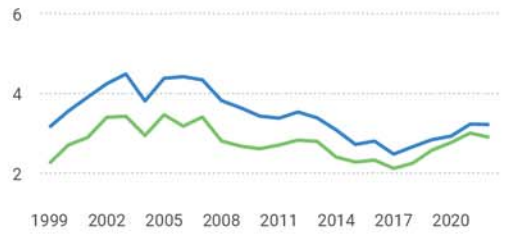

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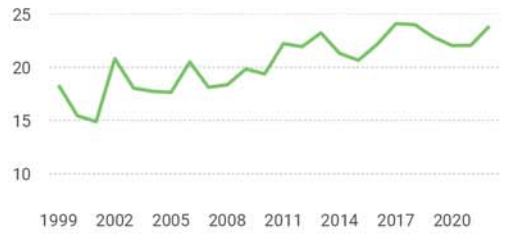

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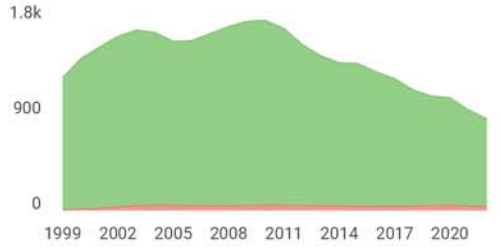

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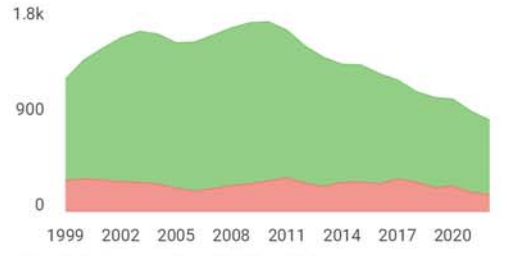

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