Rapid Capillary Zone Electrophoresis Along Short Separation Pathways and Its Use in Some Hyphenated Systems: A Critical Review

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Contents

1. Introduction

1.1. Present Position of Analytical Measurements in Scientific Research and Practice

The contemporary research in natural sciences and technologies and its applications to a wide range of human activities are characterized by two primary features:

(a) The immense volume of concrete findings is growing more and more rapidly.

(b) The greatest development takes place at the boundaries among scientific disciplines, e.g., at the boundaries between mathematics and physics, chemistry, and biology. Consequently, successful solutions of research problems require teamwork, with intense and continuous cooperation among the team members specialized in various fields. To attain this, the specialists in individual disciplines must possess a sufficient overview and understanding of the theoretical and experimental approaches used in the cooperating fields and of the characteristics of all the results contributed.

Analytical chemistry is a very good example of this situation. It is an applied discipline that widely uses the results of not only the general chemistry but also of other fields, primarily mathematics, informatics, and physics, and progressively more those of biology, imitating or directly utilizing the processes occurring in living organisms. On the other hand, most scientific and technological projects cannot be solved without analytical data on the identity and amounts of a great variety of substances present in various materials and on the distribution of these substances in space and time. Therefore, the analytical field is very wide and its boundaries with other disciplines are quite diffuse. Analytical chemists are, or should be, members of very many research teams and should continuously cooperate with the other team members who, on the other hand, should sufficiently understand the possibilities and limitations of analytical procedures, the character of the analytical data, and their reliability.

The requirements on the amount of analytical data and on the quality of analytical information are rapidly increasing. The most common analytical tasks involve:

(a) Identification and sufficiently selective, sensitive, and reliable simultaneous determinations of the components of large sets of very similar substances (e.g., in biochemistry, biology, and medicine);

(b) Monitoring of rapid changes in the presence and concentration of a component or several components in complex matrices (e.g., in biology, theoretical and practical medicine, or environmental monitoring, or in studies of reaction mechanisms and kinetics in many fields);

(c) Determinations of spatial distribution of substances in various matrices (e.g., in material sciences, solid-phase physics, geology, etc.);

(d) Identification and determination of ultratrace amounts of various analytes (e.g., in physics, electronics, or material sciences);

(e) Simple, rapid and inexpensive analyses of very large numbers of samples (e.g., in practical medicine, environmental monitoring, or food industry);

(f) Completion at a satisfactory technical level, possibly with extensive miniaturization and automation, and with acceptable financial costs.

Analytical chemistry employs a very wide range of experimental approaches and their combinations to meet all these requirements. Among the most important ones are the uses of high-performance separations (primarily gas and liquid chromatography or capillary electrophoresis), hyphenated with various measuring techniques (mostly mass spectrometry and other spectrometric measurements) and many more complex combinations. This review concentrates on the very young and highly promising area of capillary electrophoresis along short separation pathways, because it has so far been covered by the literature much less than the classical fields of high-performance separations, because it is developing rapidly and makes it possible to carry out rapid, reliable, relatively technically simple, and not very expensive analyses of very complex samples, primarily in the fields of biology, medicine, and environmental studies.

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1.2. Role of Rapid, Highly Efficient, and Selective Separations

Especially powerful and efficient analytical methods separate and subsequently determine analytes in fluid streams. Capillary electrophoresis (CE), which is at present rapidly developing, belongs in this group. The CE techniques have a number of important advantages: they are experimentally simple, the instruments are inexpensive and mostly do not contain moving mechanical components, and the analyses are rapid and employ very small volumes of both the samples and the reagents. Furthermore, the movement of the background electrolyte, if required at all, is provided by the electroosmotic flow (EOF), whose velocity and direction can readily be controlled by varying the magnitude of the separation voltage, by modifying the inner surface of the separation capillary, and by changing the electrolyte composition. Sharp profiles of the zones of the analytes separated and efficient removal of Joule's heat from the capillary contribute to high separation efficiencies of CE procedures.

The separation times attained in standard CE instruments mostly range from ca. 5 to 30 min, which is quite satisfactory for common analyses. However, substantially shorter separation times are required if CE is to be employed for, e.g., monitoring of the kinetics of (bio)chemical reactions or if it should be used as a follow-up separation procedure in multidimensional separation systems. The requirements on the rapidity also increase in analyses of large sets of samples where the number of samples analyzed within a time unit strongly affects the practical applicability of the procedure and its cost.

One way of increasing the number of samples analyzed within a time unit lies in the use of a bunch of separation capillaries; this approach has been applied to the investigation of the human genome (see the appropriate sections of the review in ref 1). Another approach utilizes the fact that, for the reasons given above, CE is readily miniaturized and the separation pathway is shortened. Electrophoresis on glass or plastic chips is at present very popular;[2,3] see section 4. Nevertheless, classical capillaries with common CE instruments also yield a number of possibilities for substantial shortening of the separation time without losing on the separation efficiency and selectivity, by means of

(a) increasing the intensity of the electric field in the capillary;

(b) accelerating the EOF by the covering of the capillary internal surface with a specific agent, or adding an EOF modifier to the background electrolyte;

(c) using a multiple sample injection; and

(d) shortening of the separation pathway.

These principles and their combinations have been evaluated and validated in detail, both for standard[4] and chiral[5] separations. The results obtained have shown that all these principles contribute significantly to the rapidity of analysis and, thus, to the sample throughput. It should be added that another strategy for acceleration of the separations employs an external increase in the pressure within the capillary during the separation.[6]

The present review concentrates on the methodology of rapid separations in systems involving short separation pathways, obtained either through a specific approach to the standard CE instruments with common capillaries, 50 to 80 cm long, or using specialized apparatuses with capillary lengths of a few centimeters. The results are evaluated in terms of the separation efficiency, expressed by the number of theoretical plates, N, and the migration time, t. These quantities are defined[7] by the following equations:

$$N = \frac{\mu U_{ef}}{2D} \quad (1)$$

and

$$t = \frac{L_{ef} L}{\mu U} \quad (2)$$

where μ is the electrophoretic mobility [$cm^2\ V^{-1}\ s^{-1}$], U and U_{ef} are the separation voltages [V] over the whole capillary length, L, and over its section between the injection and the detection sites, L_{ef}, respectively (lengths given in cm), and D is the diffusion coefficient [$cm^2\ s^{-1}$]. Therefore, see eq 1 and the work in ref 7. The CE separation efficiency expressed as the number of theoretical plates, N, increases with the increase of the applied effective voltage, U_{ef}, but, in contrast to liquid chromatography, is independent of the length of the separation pathway. At a constant separation voltage, the electric field intensity increases with shortening of the separation pathway, and thus the migration time and the time of analysis decrease. The separation efficiency should increase, but all the factors leading to the analyte zone broadening must remain constant under these conditions. The most important of these factors is the Joule heat generated by the passage of electric current through the capillary, and this heat increases with increasing electric field intensity. To suppress this effect, the separation capillary should have the lowest possible internal diameter and the background electrolyte should exhibit a low electric conductivity. On the other hand, if the time of analysis is not important and the migration times can be similar to those obtained in long capillaries, then lower separation voltages and more conductive background buffers can be used with short separation pathways, and this may be useful from the point of view of the separation process mechanism.

Note: It is evident that there is a great difference between the capillary overall length, L, and the distance between the sample injection and the analyte detection points, i.e., the length of the separation pathway, L_{ef}. The latter is decisive for the resultant separation, even if longer capillaries have often to be used for experimental reasons. As both these values are important, the "length of the capillary" will be expressed as the fraction, L/L_{ef}, wherever required.

2. Fast Separations in Commercial Electrophoretic Systems

From the experimental point of view, practical applications are easiest when using common, commercial electrophoretic systems, which, however, do not permit the use of capillaries whose overall length is below ca. 20 to 30 cm. Nevertheless, short migration times can sometimes be attained even with these capillaries. For example, the separation of derivatized amino acids[8] in a 10 μm i.d. capillary with length values of 35/20 cm, using fluorescence detection, provided migration times of Trp, Phe, Val, Ser, Ala, Gly, Glu, and Asp within 70 s, in a borate buffer containing 0.02 M KCl, at a pH of 9.5, and at the separation voltage of 30 kV. This work principally aims at demonstrating that the removal of the Joule heat is sufficient, even with considerably conductive buffers and at high electric field intensities, provided that the capillary internal diameter is sufficiently small and that high separation efficiency, of the order of 10^6 plates per meter, is preserved.

Analogously, proteins in human serum were determined[9] within 100 s, using a 25 μm i.d. capillary with lengths of 25/18.5 cm and a voltage of 20 kV. A similar capillary with lengths of 23/16 cm was used[10] for rapid indication of the presence of drugs, namely, morphine, caffeine, and amphetamine, in human serum and urine. A 27/20 cm, 50 μm i.d., capillary was employed[11] to separate and determine many impurities in pharmaceutically important substances within a time shorter than 100 s; the authors have coined the term high-speed capillary electrophoresis, HSCE, for this technique. Antipyrine was determined[12] in saliva within 70 s, using a 30/23 cm capillary under a voltage of 25 kV. The same time was required for the separation of fluorescein isothiocyanate (FITC) marked insulin during immunochemical tests, with a 30/12 cm, 10 μm i.d., column at a voltage of 30 kV.[13]

A much more frequent approach to the use of commercial electrophoretic systems for rapid separations of substances is based on the sample injection into the capillary outlet, i.e., into the site that is much closer to the detection cell than the regular sample injection point (short-end injection, SEI). The only important thing is to properly select the polarity of the voltage applied (the opposite to that used with normal sample injection); the other procedures (the sample size, washing of the capillary, etc.) remain unchanged. The basic properties of this technique are carefully reviewed in refs 14 and 15, where typical applications to substances of various types are also given. The separation efficiencies attained usually are in a range from 10^5 to 10^6 theoretical plates per meter. There are several tens of papers describing the use of this type of sample injection; examples of the systems separated and the principal separation and detection parameters are listed in Table 1. Procedures involving sample injection into the capillary outlet have also been tested and validated for chiral separations in aqueous[31−33] and nonaqueous[34] media.

Separations along short capillary sections are highly advantageous for rapid, orientative analyses of even rather complex samples, as demonstrated in ref 35 on a separation of a model mixture of 16 amines that are often abused as drugs, e.g., amphetamine and its derivatives, nicotine, cocaine, methadone, codeine and its derivatives, lidocaine, etc. This procedure employed an almost nonaqueous background electrolyte consisting of 25 mM ammonium acetate and 1 M acetic acid in a mixture of acetonitrile, methanol,

Table 1. Examples of CE Analyses with Sample Injection into the Capillary Outlet End (Short-End Injection) (SDS, Sodium Dodecylsulfate; TRIS, Tris(hydroxymethyl)aminomethane; HDB, Hexadimethrine Bromide (polybren); HIBA, Hydroxyisobutyric Acid; MC, Methylcellulose; TEA, Triethanolamine)

analyte	sample	LOD	capillary[a]	separation time, s	notes	ref.
Procaine Lidocaine	model sample		50 μm, 48/8	75	100 mM TRIS-phosphate buffer (pH 2.5), hydrodynamic injection 35 mbar/4 s, 30 kV, UV detection at 200 nm	4
amphetamine and its derivatives	model sample		50 μm, 64/8	165	100 mM TRIS-phosphate buffer (pH 2.5) + 25 mg/mL 2-hydroxypropyl-β-cyclodextrin, hydrodynamic injection 15 mbar/5 s, 30 kV, UV detection at 200 nm	5
$S_2O_3^{2-}$, Cl^-, SO_4^{2-}, oxalate, SO_3^{2-}, CO_3^{2-}	model samples		50 μm, 32/7	20	5 mM Na_2CrO_4 + 0.001% HDB + 20% acetonitrile (pH 11), hydrodynamic injection 10 mbar/10 s, 30 kV, indirect UV detection at 185 nm	15
3-, 4-, 2-methylphenol, 3,4-, 2,5-, 2,3- and 2,6-dimethylphenol				52	15 mM H_3PO_4 + 10 mM borate + 0.001% HDB + 20% methanol + 20% 2-propanol + 5% acetonitrile (pH 11.5), hydrodynamic injection 5 mbar/3 s, 30 kV, UV detection at 214 nm	
carboxylic acids pyruvic, citraconic, mesaconic, itakonic				39	5 mM potassium hydrogenphtalate + 0.001% HDB (pH 3.85), hydrodynamic injection 5 mbar/10 s, 30 kV, indirect UV detection at 185 nm	
creatinine	urine	0.5 mg L^{-1}	50 μm, 32/8	22 (migration time)	10 mM TRIS + 20 mM HIBA (pH 3.93), hydrodynamic injection 50 mbar/9 s, 30 kV, UV detection at 215 nm	16
creatinine	human serum		75 μm, 60/10	66 (migration time)	40 mM phosphate buffer (pH 2.35), hydrodynamic injection 35 mbar/5 s, 30 kV, UV detection at 190 nm	18
NO_3^- NO_2^-	model sample	6.2 ppb 46 ppb	50 μm, 27/7	12	20 mM phosphate buffer (pH 2.5), electrokinetic injection 10 kV/10 s, 25 kV, UV detection at 214 nm	17
glycerol	biodiesel	4.3 mg L^{-1}	50 mm, 32/?	28	determination of HIO_3 released at reaction of glycerol with HIO_4, 20 mM glycine + 10 mM trifluoroacetic acid (pH 2.6), hydrodynamic injection 50 mbar/5 s, 30 kV, UV detection at 210 nm	19
opiate alcaloids morphine codeine oripavine thebaine	processed liquors		50 μm, 50/8	120	100 mM TRIS-phosphate buffer + 30 mM hydroxy-propyl-β-cyklodextrin (pH 2.8), hydrodynamic injection 50 mbar/5 s, 25 kV, UV detection at 214 nm	20
Norfluoxetine Fluoxetine	plasma	4 μg L^{-1}	50 μm, 30/10	240	80 mM phosphate buffer + 60 mM TEA +5% methanol (pH 2.5), electrokinetic injection 5 kV/100 s, 10 kV, UV detection at 214 nm	21
cocaine	Erythroxylum coca leves		75 μm, 32/8	120 (migration time)	50 mM TRIS-phosphate buffer (pH 2.5), hydrodynamic injection 25 mbar/4 s, 15 kV, UV detection at 195 nm; see also ref 23	22
carbohydrates in heroin drugs glucose sucrose lactose manitol	heroin samples		50 μm, 50/9	300	65 mM borate (pH 12), 60 °C, hydrodynamic injection 50 mbar/1 s, 8 kV, UV detection at 195 nm	24
Citalopram and its metabolites	pharmaceuticals	0.15 mg L^{-1}	50 μm, 48/8	90	25 mM citrate buffer + 0.04% β-cyclodextrin (pH 5.5), 10 °C, hydrodynamic injection 50 mbar/10 s, 20 kV, UV detection at 205 nm	25
synthetic peptides	model sample		100 μm, 34/8	200	10 mM phosphate buffer (pH 2), hydrodynamic injection 5 mbar/2 s, 7 kV, UV detection at 214 nm	26
Heparin	model sample		50 μm, 60/11	110 (migration time)	50 mM phosphate buffer (pH 3), hydrodynamic injection 50 mbar/20 s, 20 kV, UV detection at 200 nm	27
cystein methylcystein	blood	40 nmol L^{-1}	50 μm, 60/10	90	100 mM TRIS-phosphate buffer (pH 3.75), hydrodynamic injection 35 mbar/10 s, 30 kV, UV detection at 280 nm	28
adenine nucleotides	red blood cells, spermatozoa	6 μmol L^{-1}	75 μm, 30/10	ATP: 81 ADP: 111 AMP: 280 (migration times)	60 mM acetate buffer + 0.01% MC (pH 3.8), hydrodynamic injection 30 mbar/5 s, 17 kV, UV detection at 254 nm	29
chondroitin sulfate	plasma	0.5 mg L^{-1}	75 μm, 30/10	120	30 mM acetate buffer (pH 3.8), hydrodynamic injection 30 mbar/5 s, 30 kV, UV detection at 254 nm	30
benzoate sorbate	beverages	0.9 mg L^{-1} 0.3 mg L^{-1}	50 μm, 32/9	28	25 mM TRIS + 12.5 mM HIBA (pH 8.1), hydrodynamic injection 50 mbar/3 s, 30 kV, UV detection at 200 nm (benzoate) and 245 nm (sorbate)	110

[a] Internal diameter, total length/length from the sample injection point to the detection site (cm, rounded).

glycerol, and water at a respective volume ratio of 75:15:8.5:1.5. The samples (0.1 or 0.2 mg L^{-1}) were dissolved in methanol; the 32.5/8.5 cm capillary, 25 μm i.d., was used; the samples were injected by applying negative pressure at capillary inlet; the separation voltage amounted to 30 kV; and the UV photometric detection employed a wavelength of 214 nm. All 16 amines were separated within 2 min, and the identification of the drugs separated was enhanced by diode array UV/vis detection.

Rapid separations permitted by this sample-injection technique have further been utilized to solve problems that are not purely analytical. Because the required sample volumes may be very small (of the order of nL), the kinetics of enzymatic reactions can be followed using the injection vial as the reaction vessel. The injection, separation, and detection of the substrate and the products can thus proceed *online*, repeatedly and in short time intervals, without changes in the volume and in the concentrations of the reactants. For example, during the monitoring of enzymatic hydrolysis of cyclic adenosine-3′,5′-monophosphate yielding adenosine-5′-monophosphate,[36] the separation of the two substances was attained within 25 s, using a 48.5/8.5, 50 μm i.d., capillary and a separation voltage of 30 kV. Analogous procedures have been employed to determine the enzymatic activity of rhodanase,[37] haloalkane dehalogenase,[38] and the angiotensine converting enzyme.[39] In the last work,[39] the substrate and the enzyme were injected directly into the separation capillary, within which the reaction proceeded and the products were separated and detected by switching-on the separation voltage. This method, the electrophoretically mediated microanalysis, EMMA, integrated with the substance injection into the capillary outlet, has been evaluated in detail and validated.[40]

EMMA has also been proposed as an alternative to the classical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the study of protein oligomeration induced by homocysteine thiolactone.[41] The separation required ca. 70−90 s, with a 31/10 cm, 75 μm i.d., capillary and a separation voltage of 6 kV. This technique has further been applied to the monitoring of products of polymerase chain reactions with similar molecular masses.[42] The 375 and 400 bp (base pairs) products were separated within ca. 80 s in a 27/7 cm, 50 μm i.d., capillary at a separation voltage of 15 kV.

Sample injection into the capillary outlet has also considerably accelerated a determination of the dissociation constants of 20 proteinogenic amino acids[43] and of many pharmaceuticals (ibuprophen, benzoic acid, lidocaine).[44] A further decrease in the migration times has been attained when applying a pressure on the solution inside the capillary during the whole separation time (pressure-assisted CE),[44] which is especially important for separations in acidic background buffers when the EOF is suppressed. Another application deals with a study of the rate of liberation of the Campral active substance (the calcium salt of acamprosate) from the pharmaceutical tablets.[45] An advantage over the standard liquid chromatographic procedure lies here not only in the saving of time but also in decreasing the cost of the analysis; for experimental and statistical evaluation of the robustness of this technique, see ref 46.

The separation process can further be accelerated by shifting the injected sample zone toward the detection site by a background electrolyte zone under a suitable pressure applied (extended short-end injection)[47]—the effective separation pathway has thus been shortened to 1.8 cm in a 33/8.5 cm, 25 μm i.d., capillary at a separation voltage of 30 kV. Under these conditions and in a phosphate buffer containing 2% of sulfonated β-cyclodextrine, the ormeloxiphene enantiomers have been separated within 10 s. Another, "non-mechanical" way to accelerate separations and improve their efficiency is based on the use of two background electrolytes with different conductivities.[48] The larger part of the separation capillary is filled with the more conductive electrolyte, and the less conductive one is only contained in the short capillary section from the injection site at the capillary outlet to the place close to the detection site. Because of the increased electric field intensity over the section containing the less conductive electrolyte, the zone of the injected sample is sharpened (the stacking effect) and the ions move more rapidly to the detection site. This approach has been termed field-amplified separation in CE, claiming that the time of analysis is triply shortened and that its sensitivity is triply increased compared to a single-electrolyte analyses; the separation efficiency is also improved. All this has been demonstrated on a separation of 2-hydroxy-3,5-dinitrobenzoic, salicylic, nicotinic, and 2-aminobenzoic acids, taking less than 40 s, in a phosphate buffer of pH 7.47 (ionic strength 150 and 30 mmol L^{-1}), in a 57/7 cm, 50 μm i.d., capillary and at a separation voltage of 10 kV.

The rapidity of separations can also be influenced by combining the sample injection into the capillary outlet with suitable modifications of the EOF. For example, in determining anions,[15] a polycationic surface active substance, polybren (HDB), was added to the background electrolyte, thus causing the reversal of the EOF direction to be the same as the direction of anion migration. This approach has also been employed in chiral separations of derivatized amino acids with vancomycine as the chiral selector, using the HDB[49] or polydimethylacrylamide[50] added to the background electrolyte for modification of the capillary internal surface.

Another possibility of using standard electrophoretic apparatuses for rapid separations is based on connecting a short separation capillary with a small internal diameter to a sufficiently long auxiliary capillary whose internal diameter is substantially larger. When the capillaries are filled with a background electrolyte, the electric resistance in the separation capillary is significantly higher than that in the auxiliary one. On applying a high separation voltage, the electric field intensity is thus much higher in the separation capillary and the migration velocity of the analytes is enhanced; moreover, the connection of a separation capillary with a long, wider auxiliary one (or with two auxiliary capillaries at both ends of the separation capillary) permits the placing of the high-voltage electrodes at a safe distance.[51] This arrangement was used for the determination of the dissociation constant of a monoclonal antibody−insulin complex,[52] with a 7.5/3.5 cm, 25 μm i.d., separation capillary and a 55 cm long, 150 μm i.d., auxiliary capillary. With a total voltage of 20 kV applied to both capillaries, 16.6 kV was across the separation one (2.2 kV cm^{-1}), whereas only 3.6 kV (0.065 kV cm^{-1}) remained on the auxiliary one. The separation required was attained within <6 s.

In the end of this section, it should be pointed out that the technique of sample injection into the capillary outlet is also often used in further capillary electromigraion methods, e.g., electrokinetic chromatography, micellar electrokinetic chromatography, or microemulsion electrokinetic chromatography.

Table 2. Examples of CE Analyses Employing Short Capillaries in Simple Laboratory-Assembled Apparatus (CAPS, *N*-Cyclohexyl-3-aminopropanesulfonic Acid; CTAB, Cetyltrimethylamonium Bromide)

analyte	sample	LOD	capillary[a]	separation time, s	notes	ref.
Cl^-, NO_2^-, SO_4^{2-}, NO_3^-, F^-, $H_2PO_4^-$, HCO_3^-	snow	0.03−0.2 mg L^{-1}	50 μm, 15/11	55	6 mM Na_2CrO_4 + 2.5 mM CTAB + 3.6% acetonitrile (pH 9.5), electrokinetic injection 500 V/8 s, 5 kV, indirect UV detection at 254 nm	53
Cl^-, NO_2^-, SO_4^{2-}, NO_3^-, F^-, $H_2PO_4^-$, HCO_3^-	model sample, plasma, milk	0.3−1.0 mg L^{-1}	50 μm, 15/11	50	6 mM Na_2CrO_4 + 2.5 mM CTAB + 3.6% acetonitrile (pH 9.3), hydrodynamic injection 3 cm/25 s, 5 kV, indirect UV detection; see similar determination of anions in water[55]	54
porfyrins	model sample	$\approx 10^{-8}$ mol L^{-1}	50 μm, 14/6	300	20 mM SDS + 40 mM CAPS + 8% dimethylformamide (pH 10.8), hydrodynamic injection 14 cm/10 s, 3 kV, fluorescence detection	56
Lidokaine Ofloxacine	urine	$3\cdot10^{-8}$ mol L^{-1} $5\cdot10^{-7}$ mol L^{-1}	50 μm, 10/10	60	10 mM phosphate buffer (pH 8.0), electrokinetic injection 3.5 kV/3 s, 3.5 kV, electroluminiscence detection	57
Enoxacin Ofloxacin Fleroxacin Pazufloxacin	pharmaceuticals	23−65 μg L^{-1}	75 μm, ?/9	170	30 mM TRIS + 4 mM H_3PO_4 (pH 8.9), hydrodynamic injection 1.6 cm/15 s, 3 kV, potential gradient detection.	109

[a] Internal diameter, total length/length from the sample injection point to the detection site (cm, rounded).

3. Specialized Electrophoretic Instruments with Short Capillaries

Specialized electrophoretic instrumentation has been designed for the use of short separation capillaries with lengths of a few centimeters. The main technical problems lie in the fact that such short capillaries cannot be manipulated very much, as far as changes in the shape and position are concerned. This places higher demands on the sample injection, which should be designed so that the separation capillary position is unchanged.

3.1. Laboratory-Assembled Apparatus

The simplest laboratory instruments are, in principle, miniaturized common electrophoretic sets. The two ends of a short capillary (mostly 50 μm i.d.) are fixed in small vessels that also contain the CE electrodes. These vessels are filled, in dependence on the actual step of the overall separation procedure, by the background separation buffer, the sample solution, or the washing liquid. The samples are usually injected electrokinetically. This arrangement has been used for separations and determinations of various substances—for typical examples, see Table 2.

Laboratory-assembled instruments for separations in short capillaries have further been successfully utilized for rapid separations of DNA fragments. The capillaries tested for this purpose were 10−15 cm long, with distances to the detection cell of 1−7 cm; laser-induced fluorescence (LIF) has been found especially advantageous for this type of separation, because it permits monitoring of very narrow zones in the capillary. Separations of 20−1000 bp fragments of DNA were completed within 60 s in a 7 cm long capillary (separation voltage intensity, 556 V cm^{-1}) or even in a 2 cm capillary (185 V cm^{-1}); a 1 cm long capillary was already too short for adequate resolution of the fragments separated.[58]

A series of publications[59−62] deals with separations of DNA fragments after their alkaline denaturing, using the LIF detection. These procedures represent a substantially faster alternative to the classical gel electrophoresis—the separation times are shorter by several orders of magnitude. In a 12/6 cm capillary with the internal surface covered by a linear polyacrylamide film and with an electric field strength of 256 V cm^{-1}, 116−210 bp fragments were separated within ca. 150 s, resolving those differing by at least 4 bp. The separation was carried out at a somewhat increased temperature of 30 °C, in a 0.1 M TRIS and 0.1 M TAPS background buffer containing 2% agarose.[59] The use of a shorter, 5/2.5 cm capillary, whose internal surface was coated with covalently bound polyvinyl alcohol, at an electric field intensity of 600 V cm^{-1} and a temperature of 60 °C, further accelerated the separation and improved its efficiency[61]—201−217 bp fragments were separated within 42 s. The increased temperature enhanced the denaturing strength of the background electrolyte and increased the electrophoretic mobility of the fragments. As pointed out above, the samples are injected electrokinetically in most laboratory instruments with short capillaries. However, diffusion injection has also been tested in ref 62 by immersing the capillary end into the sample solution for a defined time period. Utilizing the stacking effect, even fragments differing in size by a single nucleotide were partially resolved in a 12.5/7 cm capillary, with a background electrolyte containing 4% linear polyacrylamide in 0.04 M NaOH and at electric field strength of 240 V cm^{-1}; the separation efficiency amounted to ca. 10^6 theoretical plates per meter.

An original technique has been described[63] for sample injection into a short capillary; see Figure 1A. In the immediate vicinity of the injection end of a 7 cm long separation capillary, 25 μm i.d., fixed in the background buffer reservoir, the orifice is located of an auxiliary capillary, 100 μm i.d. A small volume (a few hundreds of nL) of the sample solution is forced by a microliter syringe into the stirred background buffer, close to the separation capillary inlet. The amount of the sample injected is determined by the combination of the volume ejected from the auxiliary capillary and the velocity of the background buffer convection. An advantage lies in the fact that the separation voltage need not be changed or switched off during the sample injection, the sample manipulation is very simple, and the separation capillary position need not be changed. This procedure has been termed capillary batch injection (CBI).

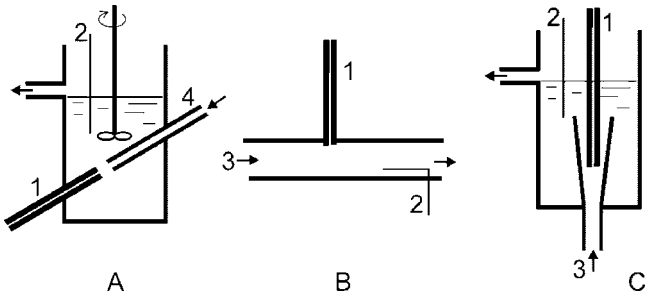

Figure 1. Principal schemes of some FI (SI)−CE interfaces applicable to short CE capillaries. The schemes are based on ref 63 (A), ref 73 (B), and ref 78 (C). 1, CE separation capillary; 2, electrophoretic electrode; 3, FI (SI) outlet; 4, sampling injection needle. For discussion, see the text.

The separation and determination parameters attained using a silica capillary and a glass chip have been compared[64] for a mixture of FITC and its complex with antihuman immunoglobuline G (FITC-IgG). A fluorescence detector, and a background buffer of 30 mM TRIS plus acetic acid (pH 8.6), were used. The sample was injected hydrodynamically (14 cm/10 s) into the capillary and electrokinetically (2.5 kV/2 s) into the channel on the chip. The results are summarized in Table 3. It can be seen that miniaturized systems are preferable to the standard CE procedures from the point of view of the separation efficiency and the time of analysis. On the other hand, the reproducibility of the migration times and the peak areas is somewhat poorer for chip and short capillary separation, apparently due to small, uncontrollable fluctuations of the experimental conditions during the sample injection and the analyte detection, which is quite understandable. The limits of detection given in Table 3 cannot have a general validity, because they strongly depend on the detection technique; the high value obtained for the chip separation is given by the small depth of the separation channel (8 μm), which is unfavorable for the fluorescence detection.

In an overwhelming majority of CE analyses, the analyte zones are detected at a single point close to the detection end of the separation capillary. However, short separation capillaries permit optical detection of the substances separated along the whole capillary length. The radiation from a source is incident on the capillary at right angles to its longitudinal axis, and the response, the intensity of the radiation passed in the absorption measurements, the intensity of the fluorescence generated, or the changes of the refractive index are monitored along the whole capillary, using a digital camera (whole-column imaging detection, WCID). This technique is often used in capillary isoelectric focusing, see, e.g., the review in ref 65 and the references therein. WCID has also been employed for a study of CE separation of proteins (hemoglobin, cytochrome c, myoglobin, β-galactosidase) in a glass capillary 4 cm long, 200 μm i.d., filled with an agarose gel[66] and using an absorption detection at 390−420 nm. This approach permitted direct visualization of the separation process and also the monitoring of the interactions of the proteins separated with various biologically active substances contained in the separation medium. An analogous procedure has been used to study the reactions of cytochrome c with SDS and the Congo red dye, and the interaction of proteins with the capillary walls.[67] The capillaries, 200 and 100 μm i.d., 3−6 cm long, had the polyimide outer film removed along the whole length, and the absorption of radiation was detected at 280 nm. The effect of the sample-injection techniques on the separation has also been studied in this work.

A Teflon separation capillary, 6 cm long, 42 μm i.d., has been used for separations with a highly sensitive LIF detection.[68] The exciting radiation with a wavelength of 480 nm was brought from a laser source through a light-conducting fiber placed axially to the end of the separation capillary. Because of the total reflection inside of the capillary, the radiation passed along the whole capillary length and the fluorescence response could be monitored, again at the whole capillary length, at right angles to the exciting radiation. This system was tested with FITC, 5-carboxyfluorescein, and FITC-marked human serum albumine. The method is also suitable for monitoring of photodegradation of fluorescing substances. The WCID visualization of the separation process has also been used to follow the dynamics of the analyte zones movement through various background buffers in a 5 cm long capillary, with a UV detection.[69]

3.2. Coupling of CE with Flow-Injection or Sequential-Injection Analysis

The flow-injection (FI) and sequential-injection (SI) analytical methods can readily be hyphenated with CE in short capillaries and bring many advantages. They permit sample injection into very short capillaries without necessity of any mechanical operations with the capillary and, even more important, they make possible efficient sample pretreatment procedures, such as preconcentration, preliminary separation, or derivatization. The sample introduction into the system and its pretreatment in the liquid flow can be automated, which favorably affects the number of samples analyzed within a time unit. The combination of these techniques exhibits synergistic favorable effects, impossible for these methods alone.[70] Therefore, the FI−CE and SI−CE couples, with the CE step carried out in a short capillary or on a chip, are analytically powerful, as illustrated in some recent reviews.[70−72]

Note: From the general point of view of coupling with CE, the FI and SI techniques are identical. Therefore, the FI symbol will primarily be used in the text below, because most of the experimental work has been done with flow-injection procedures, but it should be kept in mind that all the conclusions obtained hold for both FI and SI.

A suitable interface is required for the coupling of CE with FI, which should primarily secure that only a small fraction of the FI outlet is introduced into the CE system (split sampling) and that the FI system is separated from the high-voltage part of the CE apparatus. An example of such an interface[73] is depicted in Figure 1B. The sample injection end of the CE separation capillary is located in the FI outlet tubing, at right angles to the liquid flow; this tubing also contains the grounding electrode of the CE high-voltage source. During the passage of a sample zone along the CE injection end, a small sample portion is electrokinetically introduced into the separation capillary. This interface has been tested on determinations of anions in artificial samples and in samples of drinking and rain waters, after separations in a common length (80/45 cm) capillary, with indirect UV detection. The main advantage of this arrangement is in very rapid sample introduction, resulting in a high frequency of analyses, up to 150 samples per hour. This interface has been modified[74] by placing a valve on the FI outlet; when it is closed, the pressure in the system increases and the sample

Table 3. Comparison of Some Parameters of the CE Determination of FITC-IgG using a Glass Chip and Silica Capillaries of Different Lengths; Based on the Data Presented in Ref 64

parameter	glass chip[a]	A 14 cm capillary[b]	A 47 cm capillary[b]
total/effective lengths of the separation channel, cm	4.5/2.8	14/6	47/35
electric field, V cm^{-1}	526	268	383
migration time, s	11.2	44.7	152
theoretical plate count	49 580	41 820	27 750
migration time reproducibility, RSD, %	0.90	0.81	0.25
peak area reproducibility, RSD, %	5.78	4.17	2.51
limit of detection, ppm	250	10	100

[a] The width × depth of the separation channel, 50 μm × 8 μm. [b] Internal diameter, 50 μm

is introduced into the CE part of the apparatus hydrodynamically, the sample size being determined by the time for which the valve is closed.

This interface has mostly been used in combination with CE capillaries of common lengths; however, it has also been applied[75] to a CE system with a short capillary (15/8 cm, 10 μm i.d.), with the FI system being arranged to permit exact timing of hydrodynamic sample introduction into the separation capillary. Inorganic cations (NH_4^+, K^+, Ca^{2+}, Na^+, Mg^{2+}) and anions (Cl^-, NO_3^-, SO_4^{2-}) were completely separated within 11 s. To completely resolve organic anions (oxalate, tartrate, malate, succinate, gluconate, benzoate), the length of the capillary had to be increased to 25/15 cm, obtaining the separation time of 40 s. Using a longer, 30 cm capillary, this principle can be applied to simultaneous determination of both cations and anions.[76] The analytical system contains two FI−CE interfaces at both ends of the CE separation capillary. The sample is thus injected into both capillary ends, and a contactless conductivity detector is located in between, with the effective capillary length being 17.5 cm for the determination of cations and 12.5 cm for the determination of anions; at a voltage of 7.5 kV, the separation took 60 s.

A principle identical with that depicted in Figure 1B has been used[77] to design a much simpler interface consisting of an FI outlet plastic tube bent at right angles, whose wall is pierced with the end of a CE separation capillary. If this end is at right angles to the FI outlet flow, the sample injection into the CE capillary is electrokinetic; if it directed toward the flow, the sampling is combined, electrokinetic plus hydrodynamic.

Another type of the FI−CE interface[78] can be seen in Figure 1C. The sample injection end of the CE separation capillary is placed in the widened FI outlet; the sample is injected in a combined manner, electrokinetically and hydrodynamically. This interface was tested on separations of an artificial mixture of magnolol and benzoic acid in a 30 cm long capillary. It has been applied to various separations using common length CE capillaries (see the review in ref 71 and the references therein). The study of the rate of dissolution of a pill containing trimethoprim and sulfamethoxazole[79] employed, however, a short CE capillary (29.0/14.5 cm, 75 μm i.d.), with the advantage of automated and frequent sample introduction and noninterruped separation process.

Another type of FI(SI)−CE interface has solely been proposed for separations in short CE capillaries.[80] It has the so-called H-configuration (Figure 2A), and its dimensions are analogous to those used with electrophoretic chips; however, it consists of discrete components. It is sometimes called the microfluidic CE system. The separation occurs in a common CE silica capillary whose ends are placed in side arms functioning as the inlet and outlet vessels of the CE

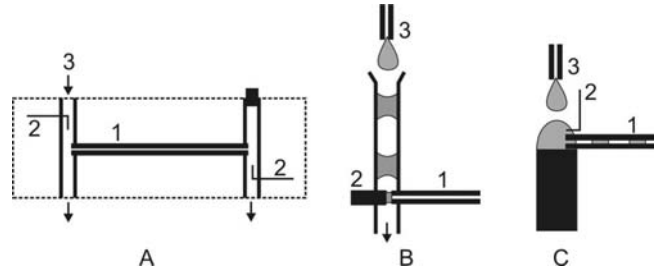

Figure 2. FI(SI)−CE interface in the H-configuration according to ref 80 (A), and the principal schemes of the falling-drop FI−CE interface according to ref 85 (B) and ref 88 (C). 1, CE separation capillary; 2, electrophoretic electrode; 3, solution inlet from the FI(SI) system. For discussion, see the text.

apparatus. This set is glued to a glass or plastic plate of the size of a microscopic slide. This interface was first used in combination with a SI system and fluorescence detection for the determination of FITC-marked amino acids.[81] The amino acids (Arg, Phe, Gly) were separated within 8 s in a 6.0/3.5 cm capillary, 75 μm i.d., with a voltage of 9 kV; 48 samples could be analyzed per hour. A similar interface permitted determination of Arg, Phe, Ser, and Gly after automated continuous online derivatization with *N*-acetyl-L-cysteine and *o*-phthaldialdehyde, permitting UV detection at 214 nm.[82] This separation took 240 s in a 6.5/3.5 cm capillary, 75 μm i.d., at a voltage of 1 kV. The same interface has further been applied to the separation of trimethoprime, sulfadiazine, and sulfamethoxazole contained in pharmaceuticals;[83] direct UV detection was possible, and the separation was complete within 150 s. Contactless conductivity detection was employed in the separation of inorganic cations in surface waters[84] using a 5.5 cm capillary, 25 μm i.d. At a separation voltage of 1.5 kV, NH_4^+, K^+, Ca^{2+}, Na^+, and Mg^{2+} were separated within 60 s.

As mentioned above, FI(SI)−CE hyphenated systems require an efficient electric separation of the high-voltage CE component from the FI(SI) one, for both functional and safety reasons. For example, air bubbles were used for this separation in a FI−CE determination of sucrose and glucose;[85] the air-segmented FI outlet liquid dropped freely into the suitably modified inlet arm of the interface, from which the solution was pumped out (falling-drop interface, FDI). The electrophoretic electrode was located close to the inlet of the CE separation capillary in such a way that a liquid film was preserved between the electrode and the capillary, even during the passage of an air bubble (see Figure 2B), thus avoiding any interruption of the separation voltage. The CE separation of the two analytes at a voltage of 1.7 kV over a 5 cm long, 25 μm i.d., capillary took ca. 60 s; amperometric detection at a Cu indication electrode was used. Similar arrangement[86] served for the separation and determination of Co^{2+} and Cu^{2+} ions; chemiluminescence detec-

tion was based on the reaction of luminol contained in the background buffer with H_2O_2 present in the outlet vessel, under catalysis by the analytes. The Pro, Val, and Phe amino acids were separated and determined analogously,[87] with the chemiluminescence detection. The luminescence reagent, $Ru(bpy)_3^{3+}$, was generated electrochemically at a Pt electrode placed in an outlet vessel. In both these works the luminescence radiation was led to the detector by a light-conducting fiber placed close to the capillary detection end.

A modification of the FDI for a miniaturized CE system[88] is depicted in Figure 2C. The FI outlet solution freely drops, in accordance with the FI system program, on the surface of a vertical glass rod, where the inlet end of the CE capillary is placed, together with one of the CE electrodes. When a drop of the sample solution is present on the rod, the sample is electrokinetically injected into the CE separation capillary; when a drop of the background buffer is on the rod, the CE separation takes place. This system permitted separation and determination of 48 samples of a pharmaceutical containing trimethoprim and sulfamethoxazole per hour, using a 6.5/5.0 cm, 75 μm i.d., capillary, at a voltage of 1 kV and with UV detection at 254 nm.

If the silica CE separation capillary is covered with a film of a material whose refractive index is lower than that of the solution within the capillary, then the capillary not only serves for the separation but also functions as a waveguide with a liquid core (Liquid-Core Waveguide, LCW). Such a material is, e.g., the 2,2-bistrifluoromethyl-4,5-difluoro-1,3-dioxol polymer, provided by the DuPont Company under the commercial name Teflon AF. Teflon AF-coated capillaries are usually used in miniaturized FI(SI)−CE systems with fluorescence detection and the H-configuration interfaces (FI(SI)−LCW−CE). The analyte contained in the solution within the CE separation capillary is excited by radiation brought through the capillary wall, at right angles to the separation channel, and the fluorescence radiation generated is brought by the liquid to the capillary ends. A classical waveguiding fiber is attached to the detection end of the capillary and led to a photomultiplier, or a photomultiplier is placed directly at the capillary detection end, possibly through a simple absorption filter. The fluorescence is excited by light-emitting diodes, LEDs; for details, see the reviews in refs 89 and 90 and the references therein.

A very simple FI−LCW−CE apparatus has been employed[91] in the separation of FITC-marked amino acids, Arg, Phe, Gly. Fluorescence was excited by a blue LED (478 nm) without any focusing optics; the radiation was led to a photomultiplier by an optical fiber, through a green plastic foil functioning as an optical filter. The separation was performed in a 14.0/12.8 cm, 48 μm i.d., capillary at a voltage of 7 kV, making possible up to 144 analyses per hour, with detection limits (LOD) of 1.3 μmol L^{-1} for Arg and of 1.9 μmol L^{-1} for Phe and Gly. The signal-to-noise ratio was substantially improved by using two LEDs and by modulation of their radiation;[92] the blue LED (470 nm) served as the excitation source, and the red one (645 nm) was the reference source for compensation of the background, with the two LEDs being switched on alternately, at a frequency of 1 kHz. Using the 4.5/4.0 cm, 48 μm i.d., capillary at a voltage of 0.9 kV, the above FITC-marked amino acids were determined with LOD values by about 2 orders of magnitude lower than those obtained in ref 91. A similar apparatus with an SI sample injection was used[93] to separate DNA fragments in a 5/4 cm, 75 μm i.d., capillary. To improve the signal/noise ratio, the exciting radiation produced by a LED (520 nm) was modulated by a frequency of 900 Hz. The photomultiplier signal, obtained through an optical filter at the detection end of the separation capillary, was treated by a lock-in amplifier. This arrangement permitted the obtaining of analytical results comparable to those provided by the much more complex LIF detection.

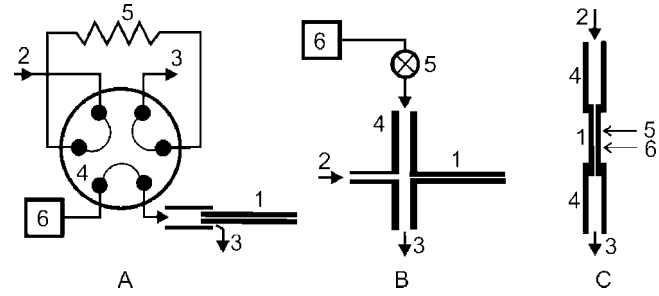

Figure 3. Principal schemes of basic interfaces for LC−CE separations (1, CE separation capillary; 2, inlet of the eluate from the LC column; 3, outlet to waste). A standard interface with a six-way sampling valve according to ref 94 depicted in the loop-filling position (A) (4, sampling valve; 5, sampling loop; 6, auxiliary pump). A liquid-flow gated (FG) interface according to ref 97 (B) (4, tube for the gating liquid inlet; 5, valve; 6, auxiliary pump). Optically gated (OG) interface according to ref 51 (C) (4, an auxiliary capillary; 5, photolyzing laser beam; 6, fluorescence-exciting beam).

3.3. Coupling of CE with Liquid Chromatography

Separations of complicated mixtures of substances can often be managed by combining several separation principles, one of the combinations involving the hyphenation of liquid chromatography (LC) with CE. The former technique, e.g., the reversed-phase LC, separates the analyte molecules primarily on the basis of their hydrophobicity, whereas the CE differentiates them in dependence on their charge and size. The CE procedures are rapid, especially when using short separation pathways, and thus are suitable for functioning as the follow-up procedure, permitting optimization of the first, chromatographic separation step. The applicability of LC−CE combinations is improved by automation of the sample introduction into the second separation step.

One of the first works[94] dealing with automated introduction of the eluent from an LC column into a CE separation capillary describes a separation of standard mixtures of peptides, involving angiotensine I, Met-Leu-Phe, methionine enkefalinamide, leucine-enkefaline, and ovalbumine hydrolyzate, marked by fluorescamine. The LC−CE interface consists of an electronically controlled six-way injection valve (see Figure 3A), which simultaneously functions as the grounding electrode of the CE system. The LC column eluent fills the injection loop (10 μL). The valve is turned at regular intervals and the injection loop content is brought to the sample injection end of the CE capillary, where the analyte is electrokinetically introduced. The background buffer, propelled by an auxiliary pump, passes around the injection end of the CE capillary when the injection loop is filled. The capillaries used in this work had a total length of 38 cm (41 and 50 μm i.d.,) and an effective length of 6.5 cm. Fluorescence detection was used, and the separation took 60 s at an electric field intensity of 579 V cm^{-1}. A similar instrumentation has been used for separations of peptides in hydrolyzates of bovine and equine cytochrome C[95] and of proteins in human, equine, and bovine serum.[96]

The valve with a sampling loop (Figure 3A) has a limited applicability; it can only be used in combination with conventional, packed LC columns which have sufficiently large mobile-phase flow rates capable of filling the sampling loop. This is generally rather wasteful, and the coupling of highly efficient CE separation with an LC separation exhibiting a substantially poorer separation efficiency is often not very convenient. Therefore, combinations of LC microcolumns or capillary columns with CE, where the consumption of the mobile phase and the sample is much smaller and the separation efficiencies of the two techniques are much more similar, should mostly be preferred. A special flow-gating (FG) interface has been developed[97] for the purpose (see Figure 3B), and its functioning has been tested on size-exclusion LC−CE separations of protein standards, using CE capillaries of various lengths.

As can be seen in Figure 3B, the outlet from the LC microcolumn is located at a short distance against the sample injection end of the CE capillary and the gating solution (the CE background electrolyte) is pumped at right angles to the LC eluate; the flow rate of the background electrolyte is controlled by a valve. During the gating solution flow, the LC eluate is led to the waste and thus cannot enter the CE capillary. The electrokinetic sample introduction into the CE system is only possible when the gating solution flow is stopped for a defined time. The character of the liquid flow and of the analyte zones in dependence on the operating conditions of the FG interface made of a transparent material have been studied in detail using a dye solution;[98] the transparent interface material makes also possible precise mutual positioning of the LC column outlet and the CE capillary inlet. The interface properties have been improved by filing the CE capillary end to a conical shape. The reproducibility of this sample injection has been determined from 400 separations of FITC-marked phenylalanine and glutamic acid, obtaining RSD values of 2.5, 3.0, and 0.07% for the peak height, the peak area, and the migration time, respectively. The test substances were separated within 35 s, when using a 25/15 cm, 17 μm i.d., CE capillary, the electrokinetic sample injection at 1 kV/3 s, and a separation voltage of 30 kV; the separation efficiency obtained, nearly 500 000 theoretical plates, indicated that the interface had virtually no effect on the analyte zone width.

The FG interface has been found to be an efficient way of sample injection into short CE capillaries even in the absence of the preceding LC step; the sample must then be introduced into the interface by an auxiliary pump. In this way, ascorbic acid was determined in the nucleus caudatus of a rat brain,[99] by in-line coupling microdialysis with CE. The ascorbic acid migration time was 40 s with a 50/15 cm, 25 μm i.d., capillary, at a voltage of 30 kV. Another advantage of the FG interface lies in the possibility of rapid, repeated sample injections into the CE capillary, by electronically controlled interruptions of the gating liquid flow. This was utilized for an online, competitive immunochemical determination of insulin from the ratio of FITC-marked insulin free and bound in the complex with anti-insulin monoclonal antibody.[100] The samples were repeatedly, electrokinetically injected from the reaction space, where the complex was competitively formed, into a 8/2 cm, 25 μm i.d., capillary; the sampling time was several tens of milliseconds, with a 10 s sampling interval. The separation took 3 s, and the LIF detection limit was 0.3 nmol L^{-1}. This analysis was further shortened when using a 5.0/0.8 cm, 5 μm i.d., capillary at an electric field intensity of 4 kV cm^{-1}; the migration times amounted to mere 0.7 and 0.97 s for the complexed and free FITC-insulin, respectively. Under these conditions, it was possible to inject samples by interrupting the gating liquid flow for 200 ms, with a 3 s interval between injections.

This system was used to carry out an automated online competitive determination of insulin in a single Langerhans islet, on the islet stimulation by glucose and tolbutamide.[101] An analogous technique was used to immunochemically determine glucagon and insulin.[102] By placing a short LC capillary column in front of the CE step,[103] the glucagon limit of detection was decreased to 20 pmol L^{-1}, compared to the value, 760 pmol L^{-1}, attained in ref 102, and further substances reacting with the antibody could be detected. The same apparatus, combining reversed-phase capillary LC with CE, was used for competitive immunochemical determination of neuropeptide Y, even when mixed with glucagon.[104]

In addition to optical detection, primarily LIF, contactless conductivity detection has also been used[105] for CE separations with rapid, automated sample injection by means of the FG interface. The authors consider the possibility of continuously varying the capillary effective length by shifting the detecting electrodes along the capillary to be the main advantage of this detection technique; this, of course, is impossible with optical detection in polyimide-coated capillaries. The system was tested on separations of inorganic ions, saccharides, amino acids, and pharmaceuticals in capillaries with effective lengths from 5 to 20 cm and internal diameters of 15, 25, 50, and 75 μm, at electric field intensities from 570 to 850 V cm^{-1}.

Both the traditional CE and all the so far mentioned types of CE with short separation pathways employ sample injection into the capillary in the form of a zone, whose length is controlled by a device operating on some mechanical principle. This is a factor that prevents injection of very short sample zones and the attainment of high sample injection rates, which, however, is very desirable for multidimensional separation methods. For these purposes and analytes that can be provided with a suitable fluorescence mark, the optically gated (OG) interface has been developed, permitting a defined sample injection for times of mere tens of milliseconds, into capillaries with effective lengths of units to tens of millimeters.[51] The analytes can then be separated within subsecond time periods. The principal scheme of this interface can be seen in Figure 3C. The solution components, including a fluorescence-marked (e.g., by FITC) analyte pass continuously through the separation capillary, due to electromigration and the EOF. The sample size is defined by the radiation from a continuous laser source. This radiation is divided into two beams that are focused at the capillary at a certain mutual distance and have different energies. The beam with more than 90% of the overall energy is focused at a site closer to the inlet end of the capillary and causes photolysis of the marked analyte with the formation of a nonfluorescing product. The other beam, focused at a point closer to the detection end of the capillary, is a part of the LIF detector and excites the fluorescence of marked analytes. When the photolyzing beam is not shaded, then only nonfluorescing compounds enter the LIF detector. A short-time shading of the photolyzing beam (for a period of a ms order) produces the sample zone containing the marked

analyte, which can then be detected. The separation pathway is thus defined by the capillary section between the two beams.

The OG interface was tested[51] on the separation of FITC-marked amino acids (Arg, Phe, Glu). The separation was attained within ca. 1.4 s, in a capillary with an internal diameter of 10 μm and an effective length (the distance between the laser beams) of 1.2 cm, a sampling time of 40 ms, and an electric field intensity of 3.3 kV cm^{-1}. To make the instrument operation easier and to better separate the high-voltage electrodes, the short separation capillary was placed between two auxiliary capillaries with a substantially larger internal volume. The effect of the OG interface operational parameters on the separation characteristics of the FITC-marked amino acids was investigated in detail,[106] using a 6 μm i.d. capillary and in the absence of auxiliary capillaries. Under extreme conditions, with a capillary effective length of 5 mm and a sampling time of 5 ms, the baseline separation of an Arg, Phe, Gly, and Glu mixture was attained within 0.7 s. With a capillary effective length of 1 mm, this mixture was separated within a mere 140 ms; however, Phe and Gly were not completely resolved. These data indicate potential possibilities of the OG interface, especially in the monitoring of the rates of various (bio)chemical reactions and in rapid multidimensional separations; it has been applied to LC−CE separations of various peptide hydrolyzates.[107,108] Its wider application to analytical practice may apparently be hindered by the complexity of the instrumentation required.

4. Fast Separations by Chip-Based CE

A short separation channel with a small internal diameter created on a chip permits rapid and efficient CE separations within units to tens of seconds. In addition to the basic structure, i.e., the separation and injection channels, further structures can readily be created on chips for sample treatment and modification of the separation/detection (microfluidic or lab-on-chip systems). Chips with sets of separation channels are also being tested. The most important advantage of chip-separation devices lies in the fact that it is possible to tailor specialized structures for jobs in hand; however, a certain drawback is often caused by higher financial costs compared to the classical CE (which, of course, is dependent on the instrumental setup, primarily on the detection technique used) and by the need for a special instrumentation.

In contrast to separations along short capillaries or short sections of classical CE capillaries, which are discussed in detail in the present review, rapid separations on CE chips have been dealt with in dozens of reviews of both the instrumentation aspects and the application fields. Therefore, some recent works can be cited, involving general descriptions of microfluidic system structures, the instrumentation required, and the procedures employed,[1,2] and specialized publications on sample injection, the detection techniques, coupling of chip CE with other analytical procedures, and applications to various analyte and sample types.

Electrokinetic sample introduction into the separation channel is most common in chip separations, owing to its experimental simplicity; for various approaches, see ref 111. The detection techniques are reviewed much more frequently. Among the electrochemical methods, amperometry is common,[112−115] including electrochemiluminescence technique.[114] The classical potentiometric technique and conductometry with the electrodes in direct contact with the test solution are encountered much less often.[116] At present, an important detection technique for electromigration separations is the contactless conductivity detection (often abbreviated as C^{4}D, capacitively coupled contactless conductivity detection), in which the electrodes are separated from the test solution by an insulant layer—for reviews of the principles and applications to chip CE, see, e.g., refs 117−120.

Optical detection methods for chip CE are described and evaluated in ref 121. The LIF and the LED excited fluorescence are considered to be the most important detection techniques. Chemiluminescence has a poorer sensitivity, but it is instrumentally simpler because it does not require an excitation source and complex optical components for focusing and collimation. The standard method for capillary separations, the UV/vis detection, is less common in chip CE; diode array detectors are used to advantage. Specialized detection techniques for microfluidic devices, based on IR and Raman spectroscopy, NMR, surface plasmon resonance, thermal lens effect, chemi- and electrochemiluminescence, and other principles are reviewed in ref 122. The authors of the last paper consider thermal lens method and the surface plasmon resonance as a prospective approach to microfluidic systems. A laser is the source of radiation in both the systems, but it is also commonly used in the established LIF detection.

In addition to standard CE performed on a chip with a single channel, multichannel chips are being developed and tested (with several hundred channels on a chip), especially for demanding bioanalytical assays. The methods of multiplex detection, based on various principles (in addition to standard ones, such as LIF or electrochemical detection, less common ones are tested, e.g., acousto-optical deflection, scanning mirror, WCID, etc.) and applications of these systems to separations of biomolecules are discussed in ref 123.

Similar to classical CE, the chip CE can be combined with other methods. The coupling with mass spectrometry is discussed in refs 124 and 125, and it is considered highly prospective for 2-D separations in, e.g., proteomics. A survey of the methods and applications of the FI−chip−CE (and also FI−classical CE) can be found in refs 126 and 127. An important tandem for speciation analysis and for the study of metal-biomolecule interactions is an online coupling of chip CE with atomic fluorescence spectrometry; for a survey of methods and applications, see ref 128.

Many reviews are devoted to applications of chip−CE. A survey of the methods for chiral and achiral separations and determinations of amino acids can be found in ref 129. Rapid and sufficiently reliable screening techniques are looked at for explosive materials and residues, in connection with terrorist activities. For the chip−CE possibilities in this area, see ref 130. Many applications of chip−CE are in analyses of foodstuffs[131] and environmental pollutants (phenols, aromatic nitrocompounds, hydrazines, etc., including small inorganic ions).[132] The chip−CE is most often used for determinations of organic analytes, but it can also be used to determine metal ions; a survey of the methods and their use for the speciation analysis can be found in ref 133.

5. Conclusions

It can be seen from this review that CE along short separation pathways permits rapid, effective, and reliable solutions of many analytical problems requiring separations

of analytes in both single- and multidimensional systems. The most important applications are in the field of rapid preliminary, orientative, and control analyses, especially in biomedicine and environmental studies; the possibility of monitoring the course of many complex reactions through fast separation and sensitive detection of selected reactants and products is highly significant for research in molecular biology, microbiology, and medicine. On the other hand, complete separations of complicated, multicomponent mixtures of substances still usually require longer CE capillaries.

The apparatus for short-pathway CE involves many possibilities of sample injection and a number of universal or specialized interfaces for coupling with a preceding analytical procedure, such as flow-injection, sequential-injection, dialysis, or liquid chromatography. In comparison with the closest relative, the CE microchip system, the instruments containing capillaries have certain advantages, namely, it is easier to optimize the internal diameter and the length of the separation channel, the capillary internal walls are modified more easily, and the connection of the separation capillary with external parts of the apparatus is usually simpler. The replacement of a damaged capillary is much simpler and cheaper than the replacement of a malfunctioning microchip.

On the other hand, these advantages must be paid for by certain drawbacks. Good separation efficiencies are attained in capillaries with very small internal diameters and with very small samples; therefore, the zones of the analytes separated are very narrow. This places high demands on the detection method sensitivity. This is the reason for frequent use of the LIF detection, which is sensitive and capable of detecting analytes within a very short capillary section. Furthermore, during separations along extremely short pathways, when the analyte migration times differ by fractions of a second, very fast techniques must be used to collect the detector data. The simple separation unit, a very short capillary, must thus be surrounded by rather complex instrumentation providing the sample introduction, hyphenation with other techniques in multidimensional systems, and the detection of the separated analytes; of course, this is a general problem, common for most microseparation methods.

6. List of Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CAPS	cyclohexyl-3-aminopropanesulfonic acid
CBI	capillary batch injection
CE	capillary electrophoresis
CTAB	cetyltrimethylammonium bromide
EMMA	electrophoretically mediated microanalysis
EOF	electroosmotic flow
FDI	falling-drop interface
FI	flow injection
FITC	fluorescein isothiocyanate
HDB	hexadimethrine bromide, polybren
HIBA	hydroxyisobutyric acid
HSCE	high-speed capillary electrophoresis
LC	liquid chromatography
LCW	light core waveguide
LED	light-emitting diode
LIF	laser-induced fluorescence
LOD	limit of detection
MC	methylcellulose
RSD	relative standard deviation
SDS-PAGE	sodium dodecyl sulfate−polyacrylamide gel electrophoresis
SEI	short-end injection
SI	sequential injection
TAPS	tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid
TEA	triethanolamine
TRIS	tris-(hydroxymethyl)aminomethane
WCID	whole-column imaging detection

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