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Review

Sample stacking of cationic and anionic analytes in capillary electrophoresis

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Abstract

The behavior of charged species along concentration boundaries in capillary zone electrophoresis (CZE) that was first described in detail by Everaerts et al. in 1979 assured the possibility of concentrating charged solutes inside the capillary. The concentration effect is based on the sudden change in analyte electrophoretic velocity brought about by the difference in the magnitude of the electric field. Furthermore, this on-line method could be the needed solution to the problem of low concentration sensitivity in CZE. Sample stacking, which is now its well known name, has then found valuable use in applying CZE in many fields, especially after the in-depth studies performed in the early 90s by Chien and Burgi. This article reviews the theory and methodological developments of sample stacking developed for charged analytes in CZE and also in electrokinetic chromatography. A table conveying the reported applications especially in the biomedical and environmental fields is given. On top of this, other on-line concentration methods for charged species, namely, sample self-stacking, acetonitrile stacking, sweeping, cation selective exhaustive injection–sweeping, and use of a pH junction, are briefly discussed.

Keywords: Reviews; Sample stacking; Cationic analysis; Anionic analysis

Contents

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1. Introduction and basic theory of sample stacking in capillary electrophoresis

During the latter part of the 20th century, scientists in distinct fields have shown that their individual analytical problems can be solved by capillary electrophoresis (CE) [1–6]. Here, the CE mode capillary zone electrophoresis (CZE), which is primarily used for the separation of charged analytes is mainly considered [1,2]. Separation of charged analytes in CZE is achieved primarily by virtue of the difference in analyte electrophoretic mobility. Improvement of separation is easily obtained by adjusting the buffer pH, alteration of the buffer ionic strength, addition of special substances into the buffer used, among other things [3–6].

A major area of interest in CE is to improve the low concentration sensitivity. The concentration sensitivity problem comes from two sources, namely the low sample injection volume and the short optical pathlength for on-capillary detection. Ingenious manipulation of the on-line capillary detection window afforded up to 10-fold response improvement with the most common UV detector [7–9]. Powerful laser induced fluorescence, electrochemical, and amperometric detectors can improve detection sensitivity several orders, however are not widely applicable and are very expensive for a common laboratory [10–13].

A more practical and moderate way to concentrate samples is the on-line (or on-capillary) chemical approach [14–112]. This is done by manipulating the composition of the sample and background solutions together with simple injection procedures without alteration of present commercial instrumentation. An on-line chemical approach example is sample stacking. Sample stacking phenomenon in electrophoresis was probably first suggested by Tiselius and coworkers (in polyacrylamide gel electrophoresis) [16]. Several workers have already shown that sample stacking can provide from 10 to >1000-fold improvement in detector response, thus expanding the applicability of CZE to many fields involving trace analysis [17–91].

Briefly, sample stacking results from the movement of sample ions across a boundary that separates the region containing the sample ions from the rest of the capillary containing the background buffer solution [18]. The basic principle of sample stacking of anions is summarized in Fig. 1. The region containing the sample ions is a low conductivity solution while the background region is a high conductivity solution (Fig. 1A). If a low and a high conductivity solution are present inside a capillary

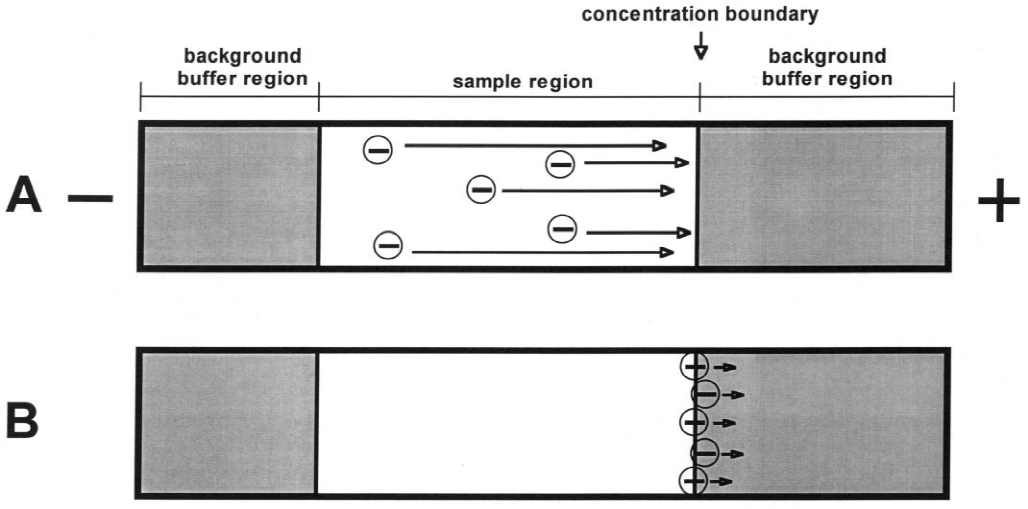

Fig. 1. General sample stacking model for anions. Discussion is found in the text.

upon application of voltage, the low conductivity region will experience a higher electric field compared to the background region. Sample ions will then move faster in the low conductivity region than in the high conductivity region. The abrupt or sudden change in sample ion movement across the concentration boundary then results in the reduction of sample zone length thus providing zones with concentrations higher than the original (Fig. 1B). The electroosmotic flow (EOF) here is assumed zero.

This article attempts to review the latest developments in the field of sample stacking. Considerable work has been published regarding the theory of sample stacking including the consequences of the low conductivity zones on EOF and broadening, and the stacking boundary [17–30]. Several sample stacking variations have been developed for the analysis of anions or cations alone or in mixtures [31–37,64,71–72,82]. Use of sample stacking in electrokinetic chromatography, non-aqueous CE, and electrophoresis on chip were also reported [67–70,86–88]. Many applications of sample stacking in the biomedical and environmental science fields have been reported showing the usefulness in real world analysis. Sample stacking has also been reviewed several times and has appeared in portions of reviews dealing with pharmacokinetics, trace analysis, etc. [18,38–39,89–94]. Additionally, other chemical approach methods including sample self-stacking, stacking with acetonitrile, sweeping, cation selective exhaustive injection–sweeping, and use of a pH junction will be discussed.

Sample stacking can be performed in both the hydrodynamic (e.g., gravity or pressure) and electrokinetic (e.g., voltage) injection modes. Unless stated, fused-silica capillaries are referred to throughout the text. In the simplest case with hydrodynamic injection, the samples are dissolved in a low conductivity matrix (e.g., water) and the resulting solution introduced using pressure into the capillary. Here, the mechanism works in a straightforward manner [31–70]. With electrokinetic injection, however, sample stacking works whether the matrix is or is not a low conductivity solution [70–87]. For example, in dealing with a moderate or high conductivity matrix, a water plug introduced before electrokinetic injection induces sample stacking. Note that the sudden change in electrophoretic velocity across the water plug and the background solution is responsible for the focusing effect. Here, hydrodynamic injection modes will be dealt with more since a separate review regarding sample stacking with electrokinetic injection will appear in this issue.

2. Hydrodynamic injection

The initial length of injected zone (l_{inj}) in CZE sample stacking with hydrodynamic injection is narrowed by a value roughly equal to the reciprocal of γ (Eq. (1)):

$$l_{stack} = l_{inj}\frac{1}{\gamma} \quad (1)$$

where l_{stack} is the length of the analyte zone after sample stacking and γ is equal to the quotient of the field strength in the sample solution (S) and background solution (BGS) zones ($\gamma = E_S/E_{BGS}$) [18,105]. The resulting concentration after sample stacking of the injected sample can also predicted by Eq. (2) [18]:

$$C_{stack} = C_{inj}\gamma \quad (2)$$

where C_{inj} and C_{stack} are the concentrations of an analyte in the injected S zone and the resulting stacked zone, respectively. If the conductivity or resistivity of the S and BGS zones are preserved during application of voltage, γ is equal to the conductivity of the BGS divided by the conductivity of the S. The greater the γ, the higher the sensitivity enhancement that can be expected.

Since sample stacking is defined as the movement of sample ions across the stacking boundary, the narrowing factor due to sample stacking in CZE [NF_{stack} (CZE)] is correctly defined as equal to the quotient of the electrophoretic velocities of an analyte in the BGS and S zones. This is at the time when focusing occurs. The stacking boundary, where focusing occurs, originates from the initial concentration boundary between the S and BGS zones before application of voltage. The stacking boundary

composition changes with time and has a velocity equal to the EOF [29,30].

Assuming again that the conductivity of the S and BGS zones are preserved during application of voltage, the value of NF_{stack}(CZE) is equal to $1/\gamma$. In the real case, the value of γ changes right after application of voltage due to mixing of the S and BGS zones. Mixing leads to changes in conductivity values and lower γ values. Mixing may be caused by the difference in local electroosmotic velocities between the S and BGS zones. The decrease in γ can be implied in Fig. 2, where separation of stacked

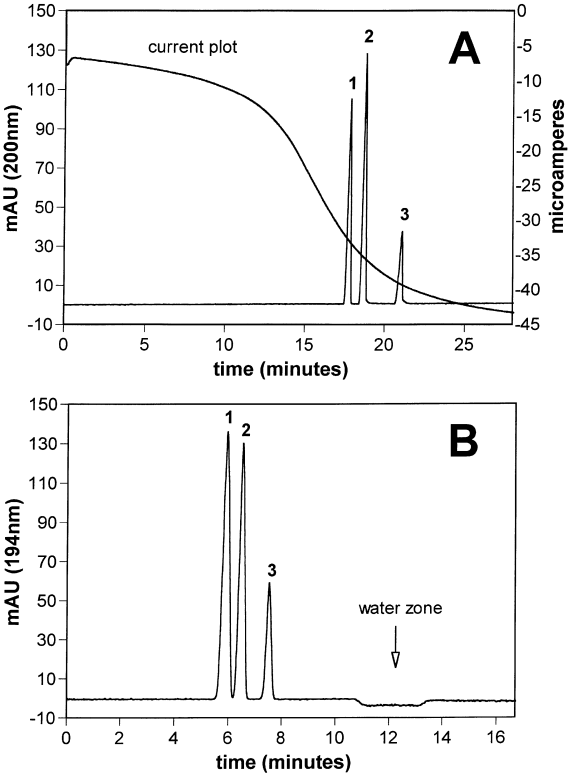

Fig. 2. Evidences of the separation of anions prior to the total removal of sample matrix. Conditions: BGS, 100 m*M* phosphate buffer at pH 2.5; peaks, 1 (bromide), 2 (nitrate), 3 (bromate); S, samples in water; injection, 200 s (50 mbar); stacking and separation regimen, −16 kV throughout the run (A), −16 kV for 10 min followed by pressure at 50 mbar until all the peaks are detected (B); capillary (untreated fused-silica), 50 μm (I.D.), 64.5 cm (total), 56 cm (effective); data collection, from the application of voltage (A), from the application of pressure (B). Reprinted with permission from Ref. [34].

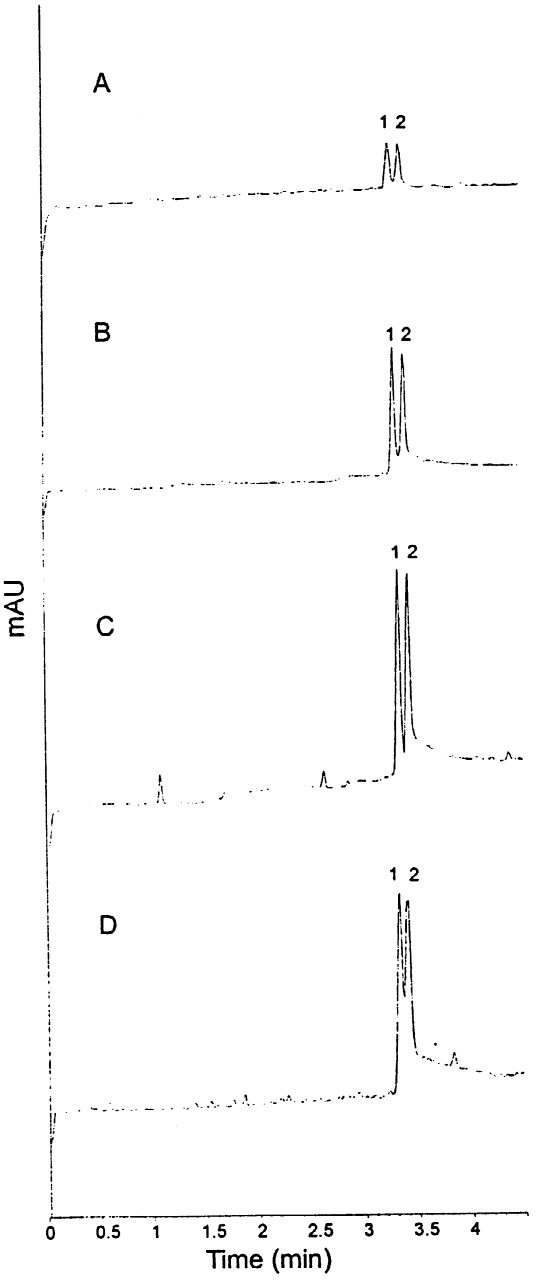

Fig. 3. Effect of injection time on NSM. Conditions: BGS, 20 m*M* phosphate at pH 5.2 with organic modifier (40% v/v acetonitrile); S, samples in water (0.01 m*M* each); identification of peaks, benzyldimethyldodecyl ammonium ion (1), benzyldimethyltetradecyl ammonium ion (2); injection (50 mbar), 5 s (A), 10 s (B), 20 s (C), 30 s (D); capillary, 50 μm (I.D.), 48.5 cm (total), 40 cm (effective); applied voltage, 25 kV. Reprinted with permission from Ref. [66].

zones occurs even before the total removal of the low conductivity sample matrix. Note that the EOF is directed toward the injection end. A substantial electric field in the BGS zones leads to the separation of stacked zones. If the γ was constant from the time voltage was applied to the time the sample matrix was removed from the capillary, the electric field in the BGS zone should be very low not to allow separation of stacked zones. Furthermore, focusing of all sample ions does not occur simultaneously since they are contained at different distances from the stacking boundary. These are the reasons why enrichment or sensitivity improvement factors found experimentally were usually smaller than that expected from Eq. (2).

As a note, most CE researchers use the word 'stacking' to define any on-capillary mode of concentrating or focusing analytes. It is suggested here that sample stacking should be limited to methods that concern the use of electrophoretic velocity change due to the electric field across concentration boundaries.

2.1. Normal stacking mode

Normal stacking mode (NSM) is the simplest among sample stacking modes. It is done by dissolving the sample in a low conductivity matrix and by injecting the resulting sample solution hydrodynamically. As stated earlier, focusing happens at the interface between the low conductivity matrix and the BGS due to the abrupt change in electrophoretic velocity. A limitation in NSM is the short optimum sample plug length that can be injected into the capillary without loss of separation efficiency or resolution. This is due to the broadening of stacked zones that result from the mixing of low and high conductivity zones in the concentration boundary and the laminar flow generated inside the capillary as a

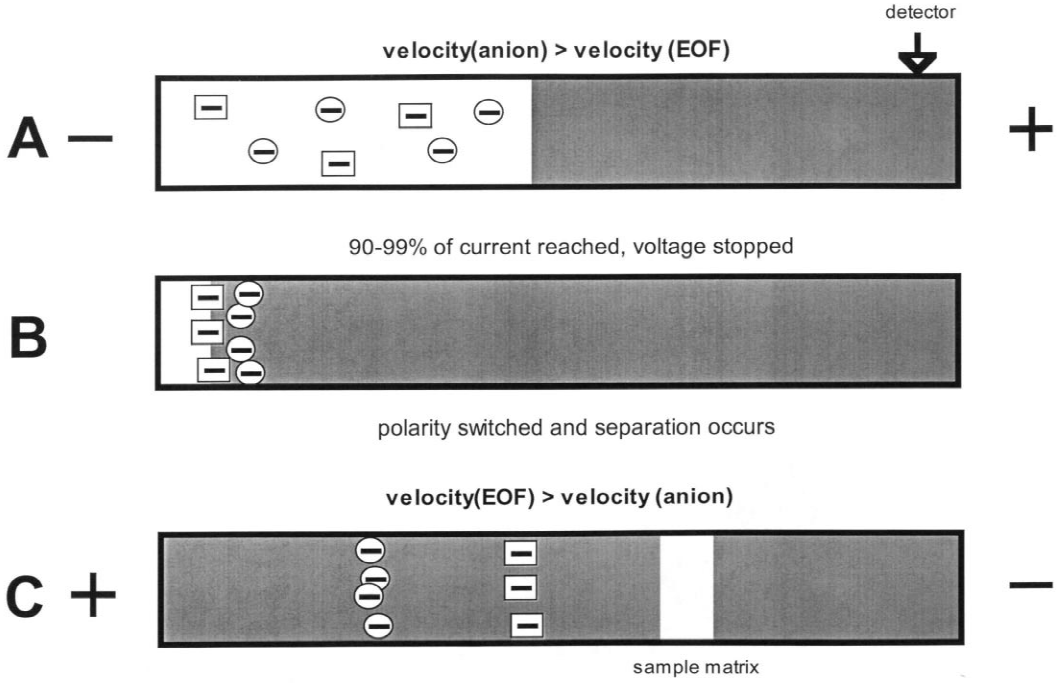

Fig. 4. LVSS with polarity switching of anions model. (A) starting situation, injection of sample prepared in a low conductivity matrix and application of voltage at negative polarity for focusing of zones and removal of sample matrix; (B) anions are completely focused and most of the sample matrix are removed, voltage is stopped and polarity is reversed; (C) application of voltage at positive polarity for separation and detection of focused zones.

result from the mismatch of local and bulk EOF velocities [31,32,27]. Similar to stacking effects, mixing and laminar flow effects are higher with higher values of γ. The electropherograms in Fig. 3 show the effect of injection time on peak shapes. Significant decrease in resolution and widening of peak widths occurred with the 30 s injection of sample solution. Concentration factors of around 10 are usually obtained with NSM, improving concentration limit of detection (LOD) a whole order of magnitude. Concentration factors are ordinarily obtained by dividing the peak height obtained with sample stacking by the peak height obtained with usual injection (e.g., 1 mm or 2 s pressure injection of sample solution).

2.2. Large volume sample stacking

When the volume of sample introduced is greater than that found optimum in NSM, the sample matrix must be pumped out from the capillary in order to preserve separation efficiency. This mode is termed as large volume sample stacking (LVSS). Pumping may be performed with external pressure or with EOF. The direction of pumping is always opposite that of the electrophoretic movement of charged solutes. The velocity of pumping should also be lower than the electrophoretic velocity of the charged solutes. A limitation of LVSS is that only positive or negative solutes can be effectively concentrated at one time. Concentration factors of more than 100 are reported for LVSS, improving LOD from two orders of magnitude.

Pumping with EOF is considered here. With EOF pumping, the maximum fraction of the capillary filled with sample solution (χ_{max}) is given by Eq. (3):

$$\chi_{max} = |\mu_{ep}/\mu_{eof}| \quad (3)$$

where μ_{ep} and μ_{eof} is the electrophoretic mobility of the charged solutes and the coefficient of EOF, respectively. If the fraction of the capillary filled with sample solution exceeds the value of χ_{max}, loss of sample ions into the inlet vial will occur [18].

2.2.1. LVSS with polarity switching

LVSS with polarity switching, like NSM, is done by dissolving the sample in a low conductivity matrix and by injecting the resulting sample solution hydrodynamically but for a longer period of time. A diagram showing the steps done in LVSS with polarity switching of anions is found in Fig. 4. Polarity switching as a means of controlling the EOF is performed in CZE systems involving high EOF conditions to carry the separated analytes to the detector. The polarity of voltage during sample stacking is chosen based on the charge of the sample ion, which is the reverse polarity used for separation and detection. For example, a positive electrode at the outlet end is used for anionic samples. A buffer

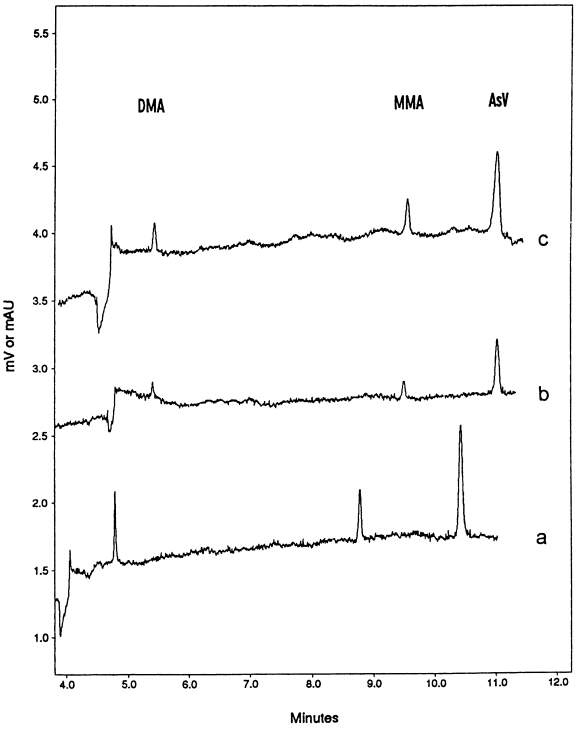

Fig. 5. LVSS with polarity switching of three arsenious compounds. Conditions: BGS, 20 m*M* phosphate buffer at pH 6; 5, samples in 0.2 m*M* phosphate buffer (pH 6); identification of peaks, dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), arsenic acid (AsV); capillary, 75 μm (I.D.), 78 cm (total), 70 cm (effective); applied voltage during stacking and matrix removal, −25 kV; applied voltage, 25 kV; (A) classical hydrodynamic injection of 10 ppm samples (1% of the capillary volume); (B) stacking of an injected volume representing 50% of the capillary volume of 250 ppb samples; (C) same as in (B) but with 500 ppb samples. Reprinted with permission from Ref. [43].

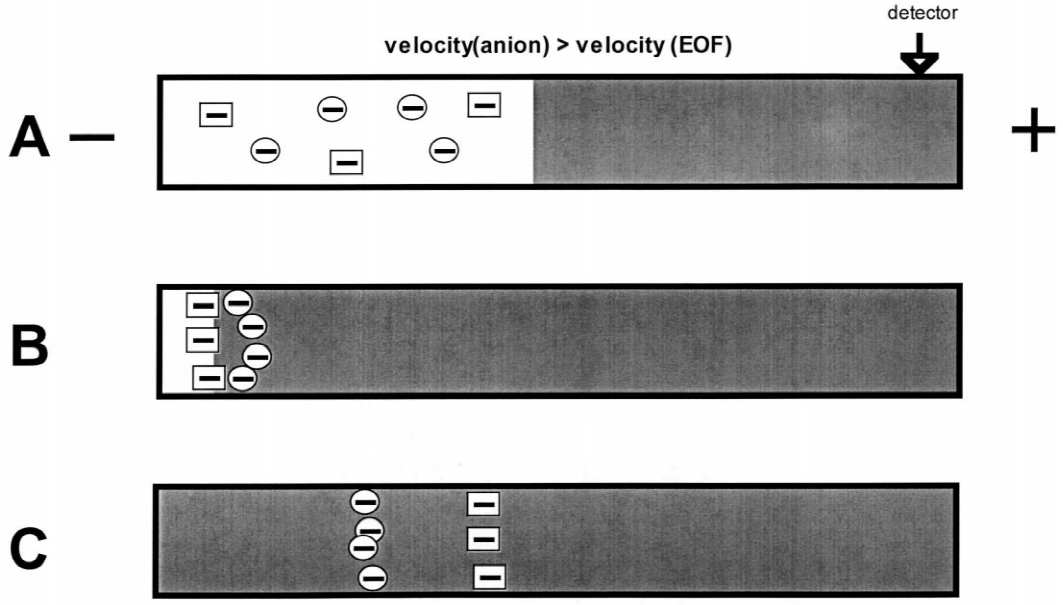

Fig. 6. LVSS without polarity switching of anions model. (A) starting situation, injection of sample prepared in a low conductivity matrix and application of voltage at negative polarity for focusing of zones and removal of sample matrix; (B) anions are completely focused and most of the sample matrix are removed, voltage is retained since the EOF velocity is always slower than the electrophoretic velocity of the anions; (C) separation and consequent detection of focused zones.

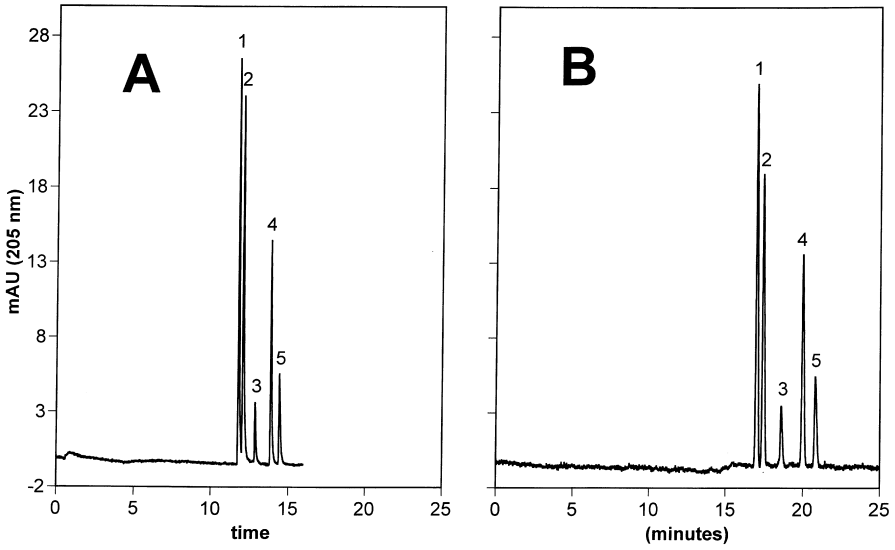

Fig. 7. LVSS without polarity switching of some beta-blocker drugs. Conditions: BGS, 2 m*M* CTAB in 100 m*M* phosphoric acid/20% acetonitrile; S, samples in 20% acetonitrile; samples (concentration in A and peak identification), bunitrolol (190 ppm, 1), propranolol (270 ppm, 2), metoprolol (210 ppm, 3), procaterol (190 ppm, 4), acebutolol (290 ppm, 5); concentration in B, 1/120 dilution of A in 20% acetonitrile; injection, 0.64 mm (A), 8.0 cm (B); capillary, 50 μm (I.D.), 64.5 cm (total), 56 cm (effective); applied voltage, 25 kV. Reprinted with permission from Ref. [37]. © 2000 Wiley-VCH Verlag GmbH.

Table 1
Applications of sample stacking in capillary electrophoresis

	Compound	Sample matrix[a]	LOD[b]	CF[c]	Ref.
A. Hydrodynamic injection					
1. Anionic					
1.	Phenol derivatives	40% MeOH (pH 11)	low ppb	>100	[41]
2.	Phenoxy acid herbicides (2,4-D, 2,4,5-T)	Water	ppb	200	[69,89]
3.	Sodium dodecyl sulfate	Simulated stream water	low ppm		[54]
4.	Aldehydes as hydrazone derivatives	Rain samples	n*M* w/z-cell	10	[45]
5.	Arsenious acid (dimethylarsinic acid. monomethylarsonic acid, arsenic acid. monophenylarsonic acid)	0.2 m*M* disodium hydrogen Phosphate (pH 6)	ppb	40	[43,44]
6.	Bromobenzoic acid. benzoic acid. benzene sulfonic acid		ppt	2000	[52]
7.	Anionic dyes with SO_3 (cibacron, pemazol dyes)	Standards	10^{-7} *M*	20	[63]
8.	Boron chelate with Azomethine H	Artificial river water	10^{-8} *M*		[63]
9.	*Serratia marcescens* nuclease	25 m*M* phosphate buffer			[40]
10.	DNA adducts (PAH-DNA adducts)	In-vitro DNA reaction (calf thymus)	10^{-8} *M*[d]	1000	[42]
11.	PTH-aspartic acid. PTH-glutamic acid			>100	[32]
12.	Underivatized protein and nonprotein amino acids (carnosine. aminobenzoic acid. aminonicotinic acid, mimosine, aminosalicylic acid, tryptophan, hydroxytryptophan, homophenylalanine, phenylalanine, dithydroxphenylalanine tyrosine).		n*M*	500	[36]
13.	Urinary porphyrins (uroporphyrin)	Urine	pmol/ml	4	[67]
14.	Styrene oxide adducts in DNA and DNA components[e]				[47]
15.	DNA oligonucleotides	Calf thymus DNA, DNA reaction mixtures	10^{9} *M*	200	[48–51]
16.	Inorganic and organic anions (chloride, nitrate, sulfate, oxalate, maleate, fumarate, bromide, bromate)	Analytical grade KBr and boric acid	10 ppb	300	[33–35]
17.	Iso-α-acids	Beer	low ppm	10	[68]
2. Cationic					
1.	Cobalt (5-Br-PADAP chelate)		10^{-8} *M*	10	[62,63]
2.	Cobalt, iron, copper, zinc derivatized with 4-(2-pyridylazo)resorcinol	Pond water vitamin supplement	10^{-8} *M*	100	[61]
3.	Platinum ions [Pt(IV) and Pt(II) chloride complexes]	Samples of catalytic converters	ppb	13	[60]
4.	Chromium (VI)				[59]
5.	Methylmercury	Methylmercury reference materials, human hair sample	12 ng/g (after clean up)		[57]
6.	Aromatic organic amines (aniline, benzylamine, dimethylaniline. phenylethylamine, naphthylamine)	1 m*M* phosphoric acid/ 30% ACN		100	[37]
7.	Selected drugs (quinine, chlorpheniramine. trimipramine, tipepidine, salbutamol, laudanosine, noscapine)	1 m*M* phosphoric acid/ 30% ACN		>100	[37]
8.	Beta-blocker drugs (propranolol, procaterol. acebutolol, bunitrolol, metoprolol)	20% ACN	10^{-8} *M*	l00	[37]
9.	Terbutaline, bambuterol, physostigmine	Human plasma	n*M* (With extraction)	400	[64,65]
10.	PTH-Arginine, PTH-Histidine		10^{-8} *M*	>100	[31,32]
11.	Peptides (leu-enkephalin, lutenizing hormone releasing hormone, somatotatin, angiotensin II, bradykinin II)	Water	n*M*[f]	50	[87]
12.	Tryptic peptides from digestion of membrane protein extract from *H. influenzae* Rd^- strain	2-D gel electrophoresis spot	n*M*		[87]
13.	Alkylbenzyl quaternary ammonium compounds (*N*-benzyl-*N*-alkyl-*N*,*N*-dimethylammonium chloride compounds)	Medicinal alcohol, cleaning lotion	10^{-7} *M*[f]	10	[66]

Table 1. Continued

	Compound	Sample matrix[a]	LOD[b]	CF[c]	Ref.
B Electrokinetic injection					
1. Anionic					
1.	PTH-aspartic acid, PTH-glutamic acid			>100	[71,72]
2.	Inorganic and organic anions (bromide, nitrate, bromate. fluoride, nitrite, chloride, phosphate. carbonate, formate, acetate)	Moderate ionic strength matrix, soil extract, bore water, estuarine water	ng/ml	1000	[34,73,74]
2. Cationic					
1.	Metal ions (thallium, nickel, lead, cadmium, zinc, silver, cobalt, mercury copper)	Spiked and real snow samples	10^{-7} *M*[g]	10	[76]
2.	Arsenic compounds (arsenocholine and arsenobetaine)		μg/1	10	[85]
3.	Herbicides (paraquat, diquat, difenzoquat)		μg/1	35	[75]
4.	Antiarrhythmic drugs (amiodarone. desethylamiodarone)	Serum	n*M*	1000	[77,78]
5.	Antimalarial drugs				[79,80]
6.	Opiates (pholcodine, 6-monoacetylmorphine, morphine, heroin, codeine. dihydrocodine)	Human plasma, serum, urine	ng/ml	1000	[81,82,84]

[a] If blank, samples were only standards in water.
[b] Concentration Limit of Detection (LOD) using UV detection.
[c] Concentration factor (CF).
[d] Detection using continuous fast flow atom bombardment MS.
[e] Detection using ESI-MS.
[f] Chip with MS detection.
[g] Detection using anodic and cathodic pulse amperometry.

additive (e.g., cationic surfactant) is used to reverse the EOF and a negative electrode at the outlet end is used for cationic samples. When the observed current reached 90–99% of the actual current (current obtained when the capillary is filled with BGS only), the polarity is switched such that the direction of the EOF is toward the detector. An electropherogram showing LVSS with polarity switching of arsenious compounds is shown in Fig. 5.

2.2.2. LVSS without polarity switching

Polarity switching is not possible in some commercial instrumentation. It is troublesome, a possible source of irreproducibility, and a hindrance to automation since the observed current should be manually monitored for polarity switching unless a reproducible EOF is observed. To eliminate the above concerns, the magnitude and/or direction of EOF can be manipulated properly in order to perform LVSS without polarity switching. For anions, the EOF should be reduced or should always be lower than the electrophoretic velocity of the sample. For example, a buffer additive (e.g., cationic surfactant) may be added to a neutral buffer or low pH buffer may be used [33–35]. A diagram showing the steps done in LVSS without polarity switching of anions using a low pH buffer is found in Fig. 6. For cations, aside from reducing the EOF the direction of the EOF should also be reversed. A reduced and reversed EOF can be achieved by using a low pH buffer containing a low concentration of cationic surfactant or using specially coated capillaries [36–37]. An electropherogram showing LVSS without polarity switching of several beta-blocker drugs is shown in Fig. 7.

3. Electrokinetic injection

Sample stacking with electrokinetic injection is first described by Chien and Burgi [71]. A useful theoretical treatment is given by Chien [20]. The sample is prepared in a low conductivity matrix and the resulting sample solution is injected using volt-

age. Usually positive or negative ions can be concentrated effectively using a single electrokinetic injection. This is a similar problem to that of LVSS. For example, only negative ions can be injected and concentrated when the positive electrode is at the outlet end during electrokinetic injection. If the direction of the EOF is opposite that of the sample ions, the velocity of the EOF should be lower than that of the sample ions. This assures the entry of sample ions into the capillary. Furthermore, there will be a bias in favour of high mobility analytes [71,113]. A larger amount of high mobility ions will be introduced and focused compared to lower mobility ions.

On one hand, sample stacking with electrokinetic injection provided larger sensitivity enhancements compared with hydrodynamic injection [77]. This is explained by the fact that in hydrodynamic injection, the volume of the sample solution that can be injected into the capillary limits the injected amount of sample. This is not a problem in electrokinetic injection since analyte molecules are introduced electrophoretically. Concentration factors of more than 1000 are reported for LVSS, improving LOD up to three orders of magnitude.

3.1. Role of a water plug and other special solution plug

A short plug of water before electrokinetic in-

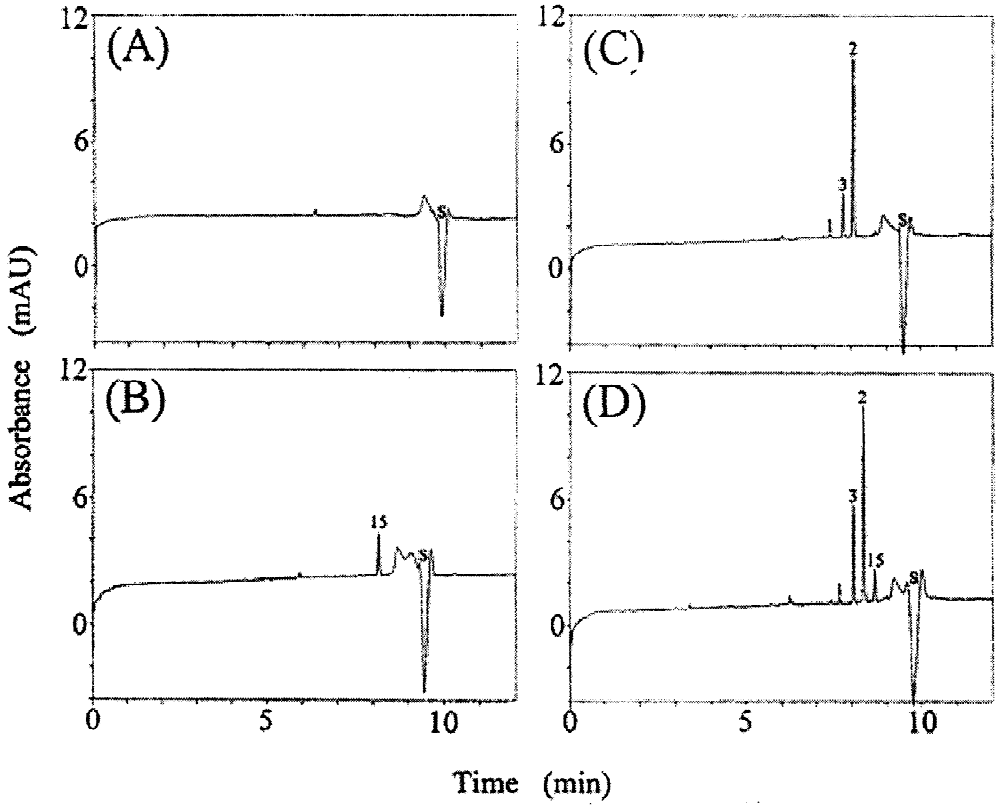

Fig. 8. Sample stacking after sample treatment. Typical electropherograms obtained from a total (effective) plasma volume of 20 μl (8 μl) having (A,B) blank plasma of (A) a subject who did not receive any drugs and (B) a subject who had been pretreated with quinidine, and (C,D) plasma taken 3.25 h after administration of 60 mg of dihydrocodeine from (C) a subject who had no premedication and (D) a subject who had been pretreated with quinidine. The extraction procedure, identification of peaks, and other conditions can be found in Ref. [82]. Reprinted with permission from Ref. [82]. © 1998 American Chemical Society.

jection of the sample provides proper electric field enhancement at the injection point and renders an empty region to concentrate ions deeper into the column and away from the inlet end [20,71]. The sample ions will focus at the injection point without the water plug and cause degradation of the field enhancement. We conducted experiments where the direction of the EOF and electrophoretic mobility of the sample ion are the same and found that the presence of a water plug did not improve the peak shape or the corrected peak areas. The sample is dissolved in water. Note that corrected peak areas are directly related to the number of ions injected. The use of the water plug may be more useful when samples are contained in high conductivity matrices, and the need to study this is evident. Carnielo et al. also has pointed out that sample conductivity affects accuracy and precision and sample volume during injection affects the signal [75]. Moreover, Wey and Thormann noted that a water plug is necessary for reproducibility [83]. Literature on systems where the direction of the EOF is opposite that of the electrophoretic mobility of the analytes is also needed to fully address the use of the water plug in sample stacking with electrokinetic injection.

Zhang and Thormann have shown that introduction of a short plug of high conductivity, high pH, and high viscosity solution before the water plug can further increase about 2-fold sample focusing [82]. The special plug serves as a temporary trap for the solutes. The increase in sensitivity was caused by the further decrease in electrophoretic velocity due to the increase in viscosity and pH and not on the lower electric field in the special plug. In a separate study, it was observed that the amount of sample injected into the capillary, as seen from the increase in corrected peak areas, is increased when a special solution having a conductivity greater than the BGS is injected before the water plug. It was also found that due to destacking, the peak heights were not significantly changed whether the special solution was injected or not [108].

3.2. Polarity switching

In the presence of a water plug or diluted BGS, both cations and anions can be injected and concentrated in the capillary column in a single run by selecting the proper polarity of the electrodes during injection [72]. For example, in a system where all

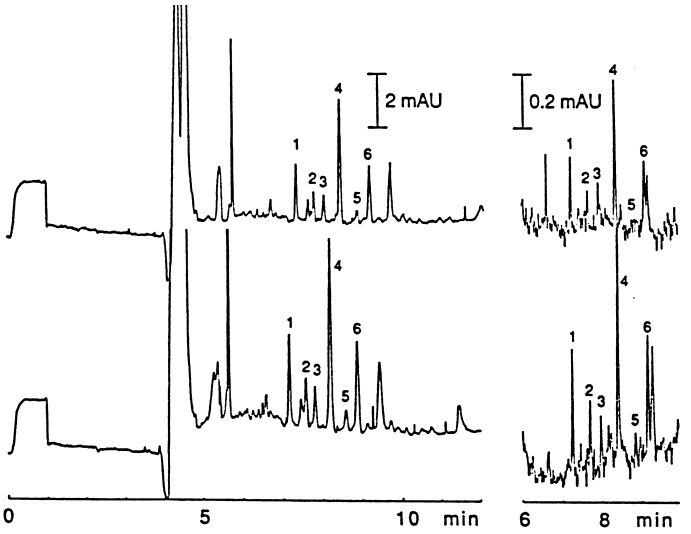

Fig. 9. Sample stacking with direct injection. Conditions: BGS, 65 m*M* phosphate buffer at pH 7.6, 40 m*M* SDS; injection time (hydrodynamic), 25 s (top left), 2 s (top right), 45 s (bottom left), 4 s (bottom right); peak identification, 1=*cis*-isocohumulone, 2=*trans*-isocohumulone, 3=*cis*-isoadhumulone, 4=*cis*-isohumulone, 5=*trans*-isoadhumulone, 6=trans-isohumulone; capillary, 50 μm (I.D.), 57 cm (total), 50 cm (effective); applied voltage, 23 kV. Reprinted with permission from Ref. [68].

sample ions are brought to the detector by the EOF, cations are introduced first with the negative electrode at the outlet end. Anions are then introduced with the positive electrode at the outlet end, followed by application of voltage with the negative electrode at the outlet end. Concentration factors obtained here are significantly higher for either the cations or the anions. This is caused by the loss of anions or cations to the sample vial during an electrokinetic injection. Unlike the other forms of sample stacking, application of this technique by others was not found.

4. Sample stacking of charged solutes in electrokinetic chromatography

Although most charged analytes are easily separated by CZE on the basis of differences in electrophoretic mobility or velocity, electrokinetic chromatography (EKC) provides added separation selectivity and sometimes the necessary separation power for charged analytes that cannot be separated by CZE. Charged analytes in EKC are separated on the basis of differences in analyte affinities between the pseudostationary phase [often a micelle, termed as micellar EKC (MEKC)] and aqueous phase and electrophoretic migration of the analyte. Principle of sample stacking for charged analytes in EKC is similar to that in CZE. Several groups give interesting reports on the sample stacking of charged analytes in MEKC [67–70,82].

5. Applications of sample stacking

Instead of describing specific publications in the text, a table listing the compounds analyzed, matrix used, limit of detection (LOD), concentration factor, and references is given in Table 1. This would hopefully help the reader in finding literature that is suited for their specific needs. Compounds were grouped to separate sample stacking methods performed by hydrodynamic or electrokinetic injection. Under each injection mode, compounds were separated based on their charge, cations or anions.

Although many of the published works dealt with standard samples, an ample number has been performed on real world samples. Many of the real world problems are related to environmental (e.g., water analysis) and biomedical analysis (e.g., human fluids, DNA). In dealing with real samples, authors often perform a sample preparation step (e.g., solid-phase extraction or other forms of extraction) before injection. This is inevitable since real sample matrices often contain substances that could interfere with sample stacking, especially substances that increase the conductivity of the matrix. An example of an analysis requiring a sample preparation scheme (i.e., liquid–liquid extraction) is given in Fig. 8. The matrix is human plasma that contains proteins and ions that could interfere with the sample stacking procedure. Nice electropherograms (Fig. 8) can be

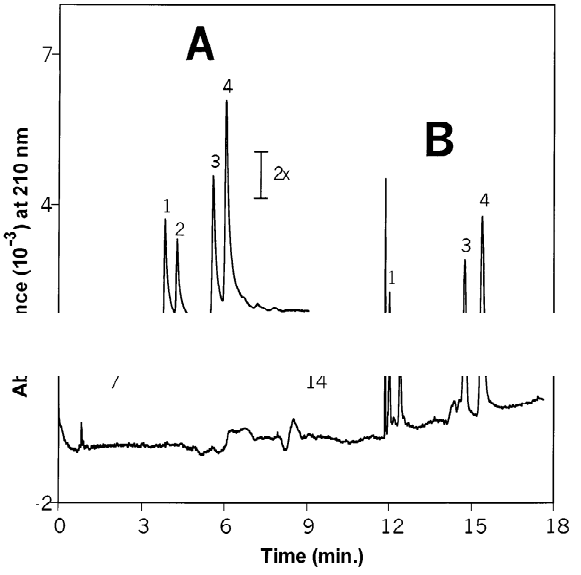

Fig. 10. Optimized electropherogram of selected biologically active compounds with sweeping and comparison with usual injection. Conditions: BGS, 100 m*M* SDS in 100 m*M* phosphoric acid/20% acetonitrile/2% methanol; S, trimipramine (1), nicardipine (2), noscapine (3), laudanosine (4) in phosphoric acid having a conductivity similar to the BGS; concentration of analytes, 190–265 ppm (A), 19–26.5 ppb (B); length of injected S, 0.064 cm (A), 42 cm (B); capillary, 50 μm (I.D.), 64.5 cm (total), 56 cm (effective); applied voltage, −23 kV. Reprinted with permission from Ref. [103]. © 1998 American Association for the Advancement of Science.

obtained if a sample preparation scheme is performed. On one hand, some matrices are compatible with sample stacking allowing direct injection. An example of an analysis that did not require a sample preparation scheme is given in Fig. 9. In this example, the sample matrix (i.e., beer) has some conductivity; however, the γ value can be effectively increased by increasing the conductivity of the BGS to a reasonable level that will not incur high currents. The compounds of interest were also nicely separated allowing sample stacking with direct injection.

6. Other chemical approaches for on-line concentration of charged species

The other chemical approaches for on-line concentration in CE that will be discussed here are sample self stacking, acetonitrile stacking, sweeping, cation selective exhaustive injection–sweeping, use of pH junction. Sample self stacking effect in CZE applies to minor ions in samples that contain a high concentration of an ionic component of like charge and high mobility. The sample components are stacked at the rear of the zone of the major component by an isotachophoretic mechanism [95–97]. Concentration factors obtained with sample self stacking are comparable to that with NSM.

Acetonitrile stacking is a form of focusing which occurs in mixtures of acetonitrile and high concentrations of inorganic salts in the sample. In contrast with sample stacking described above, focusing is improved by the presence of a high concentration of ions [98–102]. The exact focusing mechanism is unknown and could probably be due to the change in electrophoretic velocity caused by the change in viscosity as the sample ions move from the acetonitrile zone to the BGS zone [114]. Concentration factors obtained with acetonitrile stacking are comparable to that with NSM. Acetonitrile stacking

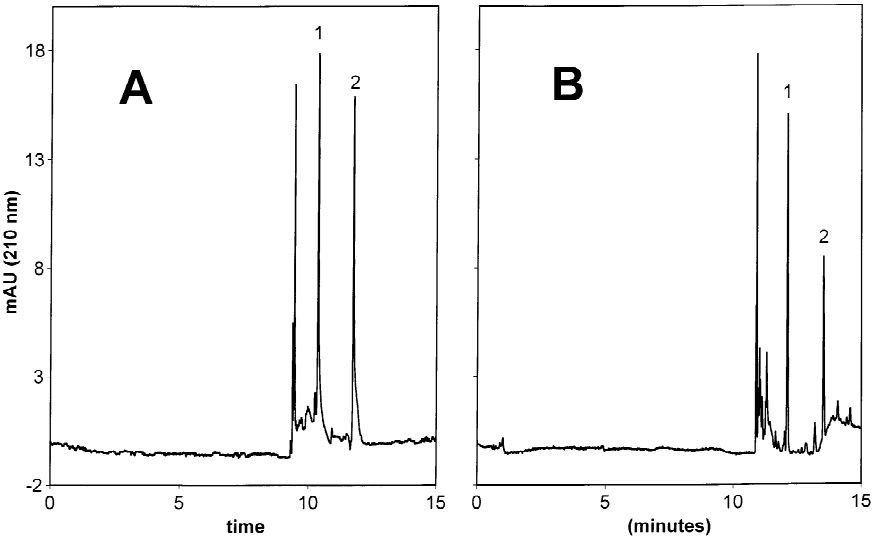

Fig. 11. CSEI–sweep and MEKC analysis of two cations. Conditions: nonmicellar BGS, 1 m*M* triethanolamine/15% acetonitrile/100 m*M* phosphoric acid; micellar BGS, 100 m*M* SDS/1 m*M* triethanolamine/15% acetonitrile/50 m*M* phosphoric acid; high conductivity buffer devoid of organic solvent (HCB), 100 m*M* phosphoric acid; sample solution, laudanosine (1) and 1-naphthylamine (2) in water; sample concentration, ~240 ppm (A), ~240 ppt (B); injection scheme, 0.6 mm of sample solution (A), 30 cm of HCB then 3 mm of water followed by 23 kV electrokinetic injection of sample solution for 1000 s (B); sweeping and MEKC voltage, −23 kV with the micellar BGS at both ends of the capillary; capillary, 50 pm (I.D.), 64.5 cm (total), 56 cm (effective). Reprinted with permission from Ref. [108]. © American Chemical Society.

is useful for biological samples containing unwanted proteins and ions since acetonitrile removes proteins and counterbalances the harmful effects of high concentrations of inorganic ions present in the sample [95].

Sweeping in EKC is the picking and accumulating of sample molecules by the pseudostationary phase that enters and fills the sample zone upon application of voltage [103]. It obviously occurs whenever the sample matrix does not contain the pseudostationary phase used. The matrix could be a low conductivity, similar conductivity, or high conductivity solution [103–107]. It was initially described primarily for neutral species and then further expanded to charged species [105]. Concentration factors obtained for some charged solutes are more than 1000. Fig. 10 shows the more than 1000-fold increase in peak height for some biologically important cations by sweeping.

Recently, we reported the combination of sample stacking with electrokinetic injection and sweeping for the on-line concentration of cations (cation selective exhaustive injection–sweeping, CSEI–sweeping) [108]. Many molecules of cations from a very dilute sample solution are electrokinetically injected and focused by sample stacking for a long period of time. The resulting stacked zones are then focused for the second time by sweeping using micelles. Focused zones are separated by MEKC. Concentration factors obtained for some cations are more than 100 000. Fig. 11 shows the more than 100 000-fold increase in peak height for naphthylamine and laudanosine by CSEI–sweeping and MEKC analysis.

Using a dynamic pH junction between S and BGS zones, focusing occurs due to the change in the electrophoretic velocity as the charged solutes encounter the pH junction upon application of voltage.

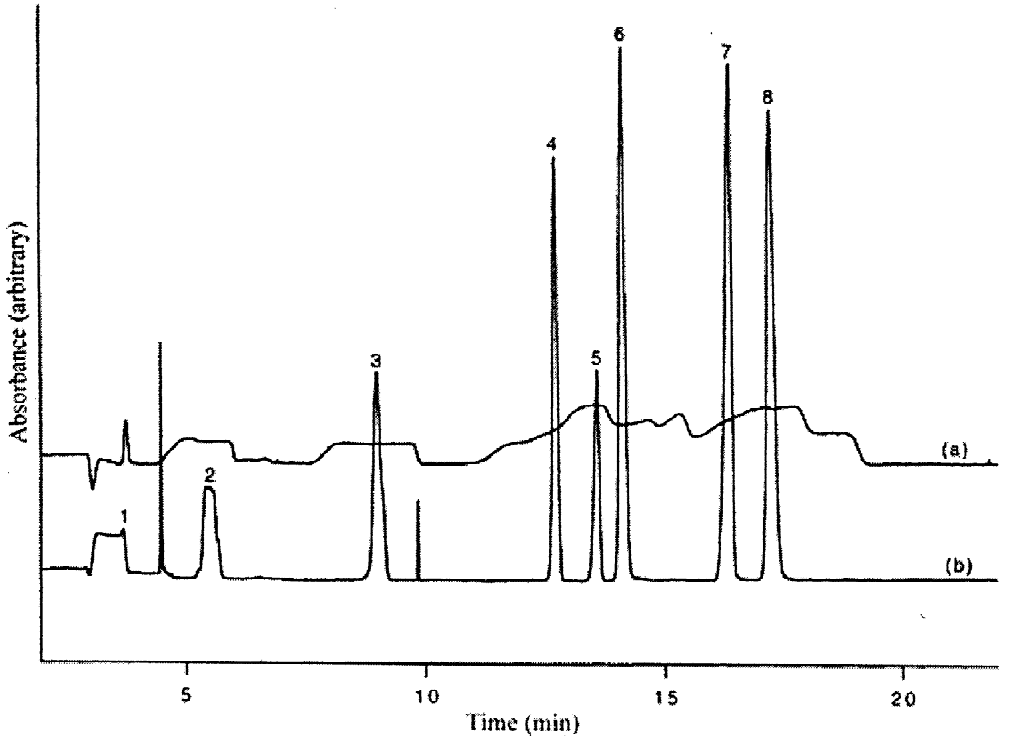

Fig. 12. Electropherograms showing the effect of using (a) continuous electrolyte (b) pH junction system. Sample solutions contained 160 m*M* borate, 150 m*M* NaCl, (a) pH 9.7 or (b) pH 7.0. Conditions: aqueous 160 m*M* borate BGS, pH 9.7; voltage, 12 kV; capillary, 50 pm (I.D.), 37 cm (total); injection, 30 s. Analyte peak numbering: 1 = dA; 2 = A; 3 = dAMP; 4 = dADP; 5 = dATP; 6 = AMP; 7 = ADP; 8 = ATP. Reprinted with permission from Ref. [112]. © 2000 American Chemical Society.

Differences in pH and concentration of complexing agent in the S and BGS zones affect the focusing of the zones [110–113]. Concentration factors obtained for some zwitterionic catecholamines, weakly acidic compounds, and nucleotides are more than 50-fold. Fig. 12 shows the significant improvement in peak height of several nucleotides by using a pH junction.

7. Conclusion

Over the past few years, we have seen some enthusiasm in the CE community in pushing the limits of detection in CE for trace analysis. This is seen in the many publications dealing with on-line concentration in CE especially on sample stacking, sometimes more than 10 publications in a year. Whether hydrodynamic or electrokinetic injection is used, at least an order of magnitude increase in concentration detection sensitivity is achieved that is sometimes sufficient for some analysis. New methods and mechanisms have also emerged, which are very promising and hopefully will be used and further studied. Translating all these methods to the chip format where detection vessels are even smaller is surely expected more in the future. A major hurdle in chips will be the introduction of sample solutions into the channels, since EOF is used for sample injection. But of course, we expect new technologies to emerge in this area. Applications to real samples and reproducibility or validation studies are still areas of importance. Hopefully more CE methods will eventually complement or be an alternative to existing separation methods like high-performance liquid chromatography or gas chromatography.

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