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Journal of Chromatography A, 907 (2001) 21–37

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

How to increase precision in capillary electrophoresis

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Received 25 July 2000; received in revised form 9 October 2000; accepted 17 October 2000

Abstract

This review surveys approaches on how to improve precision in capillary zone electrophoresis and micellar electrokinetic chromatography. Many different techniques have been employed successfully to improve instrument precision and to facilitate method transfer between instruments and laboratories. Operational parameters as well as theories will be discussed in detail.

Keywords: Reviews; Precision; Reproducibility; Migration time; Peak area

Contents

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0021-9673/01/$ – see front matter
PII: S0021-9673(00)01057-8

1. Introduction

As commercial capillary electrophoresis (CE) instruments have been available more than 10 years, CE has become a mature and well-established analytical tool. CE is extensively used for routine analysis as an alternative and complementary technique to high-performance liquid chromatography (HPLC). CE offers a broad range of selectivity in combination with high separation efficiency, working with minute sample volume.

One of the major weak points of CE is poor precision. Increased precision was the most frequently mentioned need for improvement of CE systems in a survey [1]. In CE, injection repeatability is generally not as good as that of HPLC [2–5]. This is a major obstacle for the widespread use of CE in routine analysis [6].

The performance of HPLC and CE was compared in some quantitative studies. In a bioassay, a higher precision for HPLC (relative standard deviation, RSD, 2.7%) than for CE (RSD 6.0%) was reported [7]. When insulin was determined in dosage forms, both HPLC and CE provided reasonable accuracy. However, an RSD value as high as 13% was found for the CE method. Consequently, both methods were able to achieve reasonable accuracy, although HPLC exhibited a clear advantage in terms of accuracy as well as precision [8].

Low intermediate precision for migration time was found for four different CE systems of one manufacturer (Beckman Coulter, Fullerton, CA, USA). High differences in instrument precision were reported for a capillary zone electrophoresis (CZE) method as well as for a micellar electrokinetic chromatography (MEKC) method, with RSD values ranging from 0.6 to 17.7% and 2.0 to 14.6%, respectively [9].

Some inter-laboratory studies using CE have been published with highly inconsistent findings concerning accuracy and precision [10–13]. Three different inter-company cross-validation exercises between six or seven pharmaceutical companies were conducted by Altria and co-workers. Successful method transfer and good performance in terms of precision and accuracy were obtained for a chiral separation [10], for an MEKC method [11], and for determination of drug stoichiometry [12]. For all three assays, the RSD values for migration time were under 1% and the peak area precision about 1–2.6%.

In contrast to these reports, an inter-laboratory study conducted by the Laboratory of the US Food and Drug Administration showed an unacceptable level of variability. Eleven collaborators obtained highly variable results in terms of accuracy and precision. In a system suitability test, the migration times were rather precise (RSDs between 0.1 and 2.0%), but the peak area precision was low (RSD 1.8–7.1%). Moreover, accuracy for recovery data ranged from 37 to 281%. The authors complained about many instrumental limits, such as unstable

temperature, overloading, analyte–wall interactions, current instability and buffer depletion [13].

Just because of low precision, many original publications focussed on improvement of it. A lot of different experimental parameters affect the precision of both migration time and peak area. The total variance of migration time is the sum of variance of electroosmotic flow (EOF), analyte mobility, wall interaction and temperature. The total variance of peak area is the variance of injection, diffusion, temperature, wall interaction and peak area integration [3].

The method validation criteria for CE methods are similar to those used in HPLC [1,14]. The International Conference on Harmonization has defined parameters for the validation of chromatographic methods [15,16]. Additionally to these parameters, some CE-specific requirements are needed [17].

2. Injection

In CE, the injection process has a very important impact on precision. Imprecision is largely attributable to the difficulties involved in precise injecting nanoliter sample volumes into the capillary [2]. Several unwanted effects like siphoning, ubiquitous injection, sample carryover, and sample loss may affect quantitative results.

2.1. Injection mode

In early CE systems the sample was manually injected, causing high imprecision. Injection repeatability was greatly increased with the introduction of automated injection systems [18,19].

2.1.1. Hydrostatic injection

Very reproducible results were obtained with the hydrostatic injection mode. RSD values for peak area between 1.3 and 0.55% were reported [20]. A disadvantage of this injection method is that a positive deviation of sample amount introduced is observed, therefore, a correction factor for the injection time is necessary [19,20]. In an early comparison between hydrostatic and electrokinetic injection, hydrostatic injection showed better results (RSD 2.9%) than automated electrokinetic injection (RSD 4.1%) [19].

2.1.2. Electrokinetic injection

Electrokinetic injection was popular among CZE users largely because it requires little or no instrumentation other than that required for the separation itself. Therefore, electrokinetic injection equipment should be simpler and more reliable than hydrodynamic and hydrostatic injection equipment [21]. Nevertheless, only low reproducibility for peak areas (RSDs between 4.1 and 10%) were found [19,22]. Acceptable analytical accuracy was obtained for quantitation of insulin in commercial dosage forms (with a recovery of 85.8–108.1%), but high imprecision was reported (RSD 13%) [8].

The amount of sample introduced by electrokinetic injection depends on the EOF and the electrophoretic mobility of the analyte. Therefore, precision is influenced by many parameters.

(i) Factors that alter the EOF will affect the amount of analyte injected.

(ii) Discrimination occurs for ionic species since the more mobile ions are loaded to a greater extent than those that are less mobile. This leads to an uncertainty in quantitative accuracy even if an internal standard is used.

(iii) Changes of the sample solution in ionic strength and matrix composition will affect conductivity. Thus, different quantities of the analyte are loaded due to variations in sample concentration and ionic strength [23]. Biological matrices such as urine or plasma can have very variable composition and conductivity [24,25].

(iv) As current flows through the sample solution during injection, electrochemical reaction products may be produced. This can damage the sample or contaminate the sample solution [19].

Despite these limitations, electrokinetic injection is useful for some applications or even unavoidable, e.g., in capillary electrochromatography or when gel-filled capillaries are used.

2.1.3. Hydrodynamic injection

The hydrodynamic injection mode is more precise and robust than electrokinetic injection. The injection conditions are generally only affected if the viscosity of the buffer is drastically changed by temperature.

In a recent comparison between hydrodynamic and electrokinetic injection, the injection repeatability gave RSD values of 1.59% and 5.13%, respectively [25]. In general, analysts should prefer hydrodynamic to electrokinetic injection, especially when analyzing biological matrices like plasma or urine with varying composition and conductivity.

2.2. Injection plug length

Because of the small sample size the proper choice of injection time and, in turn, injection plug length and injection volume is important for precise quantitation. The effect of injection time on precision of migration time as well as peak height is depicted in Fig. 1.

(i) Too small injection plug length: several investigators reported large errors when small sample volumes were introduced [20,21,26–28]. Different explanations were given: first, the precision of the injection mechanism of the CE instrument itself can limit the precision of the analysis [3]. Precise control of applied pressure or vacuum is difficult, especially at short injection times [29]. At larger injection times the differences in vacuum or pressure level are averaged out [28]. Second, the contribution of spontaneous injection can be significant [29], see Section 2.5. Third, diffusion of the analyte into or out of the capillary is responsible for the reduction of accuracy [30,31]. Diffusion processes are more important at shorter injection times and at small molecules with a higher diffusion coefficient than larger molecules [32].

(ii) Too large injection plug length: if the injection plug length is too large, separation is destroyed [33]. Sample overloading leads to poor peak shape, low separation efficiency and poor precision [3]. For a 50-μm capillary, the injection plug length should be smaller than 0.79 mm (=1.55 nl) [34]. The volume introduced into the capillary increases with capillary diameter. Therefore the injection volume can be maintained with a shorter injection length using a wider bore capillary [33]. In contrary, recent investigations showed that short time injections at high pressure are better than long time injections at low pressure. The injection repeatability of short time injections at high pressure (3 s, 15 mbar) was found to be better than long time injections at a lower pressure (15 s, 3 mbar), with RSDs of 1.6% and 2.4%, respectively [25].

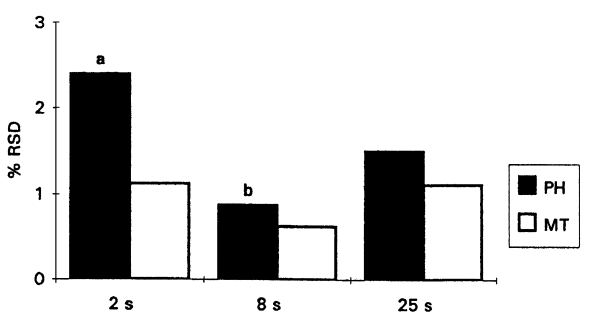

Fig. 1. Effect of sample injection time on RSD for theophylline. MT=Migration time; PH=peak height. (Reproduced with permission from Ref. [3]).

For successful method transfer it is necessary to determine the injection volume. Each instrument supplier has its own specific design with different options and with unique settings, e.g., variable or fixed injection pressure or other injection modes (pressure or vacuum). When transferring methods between instrument types, the analyst should determine the injection volume [35]. Some very useful theoretical and experimental approaches to determine the injection volume were published [7,23,36].

(iii) Sample volume: the volume of sample from where injection is performed has also some impact on precision. Rose and Jorgenson investigated the sample amount introduced by hydrodynamic flow from very small volumes, ranging from 0.25 to 10 μl. As the sample volume was reduced, the amount of sample introduced decreased [19].

2.3. Siphoning

The cause for siphoning is a pressure difference across the capillary arising due to (i) different heights of liquid levels in the two reservoir vials and (ii) formation of droplets at the capillary ends during exposure of the capillary end to air [14]. Siphoning results in low accuracy due to variable injection volumes and in band broadening during separation [33,37]. Siphoning can be suppressed observing some simple operational parameters:

(i) Several investigators stressed the importance of leveling the electrolyte solutions to reduce siphoning effects [14,32,38–40]. The use of different elec-

trolyte vials for conditioning and separation is recommended to prevent unleveled liquid heights after the preconditioning steps [29].

(ii) Altria and Fabre investigated the situation where the capillary outlet immersed in an empty vial or in a vial containing running buffer. Although no variations in injection volumes were observed, it was recommended to immerse the capillary outlet into a vial containing running buffer [29].

(iii) Theoretical considerations led to the idea that siphoning can be suppressed by using long, narrow or gel-filled capillaries and a viscous background electrolyte [32]. Another approach to reduce siphoning effects is the use of restrictors at the ends of wide-bore capillaries [33].

2.4. Sample carryover

In CE, the capillary is routinely rinsed with washing and re-equilibration solutions between each injection [41]. These steps remove adsorbed analyte and buffer components from the inside surface of the capillary. Nevertheless, the outside surface of the capillary and the electrode may cause sample carryover and contamination of sample and buffer solutions.

Several simple suggestions were made to avoid sample carryover:

(i) The removal of the polyimide coating at the tip of the capillary reduces the amount of adherent sample and buffer liquid at the outside of the capillary ends [40].

(ii) The capillary inlet end should be washed with water prior to injection. After the sample solution plug, a short plug of water can be introduced [12].

(iii) The outside surface of the capillary inlet and the electrode should be washed after each injection process. Residual sample can be removed by dipping the capillary inlet end and electrode into a vial containing fresh buffer [42]. This procedure prevents contamination of the inlet electrolyte solution.

(iv) Frequent renewal of electrolyte, washing and re-equilibration solutions eliminates all sources of sample carryover.

2.5. Sample loss and ubiquitous injection

Numerous unwanted events occur during sample introduction and electrophoresis. As the capillary ends enter and leave different solutions, interfaces between different phases are generated.

(i) The capillary end is exposed to air during the changes of the vials at the injection process. Several events occur at the interface between air and liquid such as droplet formation, siphoning, movement of the sample on the outside, and evaporation [14].

(ii) When the capillary enters or leaves a solution, several phenomena occur due to the entrance shock leading to siphoning or to diffusion into or out of the capillary.

One phenomenon, which affects precision seriously at short injection times, is called ubiquitous or spontaneous injection. Rose and Jorgenson observed a positive intercept for the plot peak area versus injection volume, because the peak area is greater than zero at zero injection time. This positive intercept was explained as diffusion of the analyte into the capillary inlet and as siphoning during injection [19]. Grushka and McCormick showed that a dye solution penetrate into the capillary with a length of about 700 μm. This ubiquitous injection occurs over and above the conventional injection [43]. Dose and Guiochon presented computer simulations, which illustrate the nature of quantitative errors caused by spontaneous injection [32,44]. Net diffusion at the zone boundary between solutions in the capillary and in the vials may be the explanation for ubiquitous injection. Whenever the analyte concentration in the injection zone differs from that in the electrolyte solution with which it is in contact, diffusion of analyte into or out of the capillary occurs. Diffusion differs among sample species, leading to an uncertainty in quantitative accuracy even if internal standards are used.

Diffusion processes can be suppressed by using long, decreased I.D. or gel-filled capillaries and background electrolyte with increased viscosity [32]. Furthermore, the duration of the capillary end exposed to air (=delay time) should be short and reproducible [45]. Therefore, the reservoir vials should be changed very rapidly and potential should be applied as fast as possible [32]. A trick, which minimizes the delay time is to place the electrolyte vials used as inlet reservoirs in the autosampler in the positions directly adjacent to sample vials [14]. However, this parameter seems not to be very

important, as other researchers did not find a decrease in precision even if a delay time of 6 s was inserted [29].

Fishman and co-workers explained the ubiquitous injection as spontaneous fluid displacement. When the capillary leaves the sample solution, the solution forms a droplet that adheres to the end of the capillary. Spontaneous, the entire droplet enters into the capillary due to interfacial pressure difference [45,46]. Fishman et al. suggested several approaches to reduce spontaneous injection:

(i) Modifying the surface roughness can control the shape and amount of droplet at the capillary inlet.

(ii) A straight-edged capillary end assures a reproducible morphology of the capillary wall at the inlet.

(iii) The speed at which the capillary leaves the sample solution may influence the droplet.

(iv) The amount of sample adheres at the capillary end can be reduced by removing the protective polyimide coating and by reducing the outer diameter of the capillary. They suggested to use capillary etched with hydrofluoric acid or thin-walled capillaries.

(v) The noise from pump sources and other vibrations influence the form of the injection droplet. Therefore, the instrument should be isolated from external vibrations [46].

Another mechanism responsible for sample loss is thermal expansion of the buffer and expulsion of sample from the capillary. Instantaneous application of the running voltage at the beginning of an analysis may lead to rapid heating, thermal expansion of buffer and expulsion of the sample from the capillary [47]. Therefore, injection of the sample is normally made at low or no applied voltage [48]. Mathematical analysis indicates a maximum permitted ramp-up rate of 900 V/s for a typical CZE experiment [47]. It is a common practice to ramp the voltage at the beginning of the separation within the first 30 s from no voltage to the desired level of 20 or 30 kV. Co-injection of running buffer also prevents sample loss due to expansion after applying voltage [4,29].

A further approach to minimize sample loss is electrostacking in air described by Sjögren and Dasgupta. In this technique, the electric field is turned on while the capillary is transported from one vial to another. Therefore, problems connected with diffusion processes during injection are eliminated, resulting in improved precision and band asymmetry [49].

2.6. Sample stacking

Several investigators showed that stacking techniques are beneficial for quantitative results [3–6,50,51]. The Shihabi and co-workers observed that acetonitrile and other stacking techniques indirectly improve the precision of peak area and height [3,6,51]. As peak areas are increased, the integration related errors are reduced. The proper choice of the dissolving solvent can have a significant impact on precision. A 10-fold dilution of the running buffer as the sample diluent was often found to be optimal [4,5]. The injection solvent is not only of importance to obtain stacking effects but also to obtain repeatable migration times as well as quantitative results by stabilizing the electrophoretic current [4].

In contrast to these observations, Thomas et al. observed the opposite effect of stacking on precision. They reported that stacking techniques which used differences in ionic strength between sample and buffers provided increased sensitivity but decreased peak area precision [14].

3. Separation

Some CE instruments offer the option to perform the separation in three different modes: constant voltage, constant current or constant power. When operating at constant current, a self-compensating effect can be observed. As the temperature of the capillary increases during electrophoresis, conductivity and, in turn, applied voltage decreases. Therefore, temperature changes are smaller, and more constant migration times are obtained [52]. Wynia et al. observed that the migration time of a component is highly correlated to the current going through the capillary [4]. Therefore, the use of constant current improves migration time precision and gives better repeatability within a single sequence. Nevertheless, the constant voltage mode is preferred in most cases due to a better inter-day repeatability [29]. A stable electrical current profile at constant voltage operation is desirable and can be obtained, e.g., by the correct

use of running buffer, sample diluent or capillary thermostating [4].

The expected operating current should be stated in every method. Furthermore, the level of the electrophoretic current should be monitored and stored for every run. Irregular current profiles can help to discover the reasons for air bubbles in the sample solution, capillary blockage or capillary breakage [53].

4. Capillary

4.1. Capillary diameter

One manufacturer specifies deviations from 4 to 6% for fused-silica capillary tubes with 50 to 250 μm I.D. [54]. Deviations around 4% are specified for capillaries with 150 to 360 μm external diameter. These specifications were experimentally confirmed by measuring the actual I.D. of the capillary ends under a microscope [55]. Although temperature has an impact on a lot of different parameters in CE, the external or internal diameter of the fused-silica tube is not affected due to the very small coefficient of expansion of silica [47,50].

4.2. Capillary ends

As many disturbing events take place at the capillary end, several investigators stressed the importance of a well trimmed capillary tip. A number of problems arises due to a poorly cut capillary end. The capillary edge affects the size of the injected sample plug and may greatly distort the EOF profile resulting in excessive zone broadening [57]. Different cuts or annuli at the capillary end lead to different sizes of emersion peaks, baseline shift and spontaneous injection [38,39]. Therefore, a straight-cut capillary tip results in a smooth baseline, increased peak symmetry, and increased separation efficiency. Several techniques are described to make a straight-edged cut and to remove the polyimide coating at the capillary tip [58].

4.3. Heat generation

During electrophoresis, electrical energy is partially converted into Joule heat. As the temperature increases, the conductivity will increase and the current will rise, leading to a further increase of the temperature [52,55,59]. Depending on the application and the electrophoretic conditions, temperature changes may range from a few degrees above the ambient temperature to boiling or outgassing of the solution [30,55,60,61]. While the absolute rise in temperature is big, the temperature difference within the tube itself will be only a matter of few degrees [62–64].

An increased column temperature can have an impact upon analyte (net charge, stability, protein denaturation, configuration changes), buffer (viscosity, conductivity, pH, air and vapor bubble formation) and chemical equilibria (ionization of the capillary surface, EOF, micelle partitioning) [52,60]. Conductivity and, in turn, mobility vary by about 2%/K [52]. Therefore, the fluctuating temperature affects the precision of the migration time as well as peak area [64,65]. In addition, quantitative errors may occur due to variable injection volumes, as the viscosity of the buffer is changed [50,66].

Heating effects can be compensated for by lowering the electrophoretic current and enhancing heat dissipation. Modified experimental parameters are: decreased ionic strength of buffer; low conductivity buffer; smaller I.D. of the capillary, as a higher surface-to-volume ratio improves heat dissipation [22,34,62,63]; larger external diameter of capillary [52]; shorter capillary; lower separation voltage [3,48,59]. Unfortunately, these options can be detrimental to separation and have only limited capabilities to reduce temperature changes [52].

4.3.1. Thermostating of the capillary

A problem, which had caused irreproducible results during the development of CE, was inefficient thermostating of the capillary in early CE instruments. Today, all commercial available CE systems offer the possibility to thermostat the capillary. Different cooling systems are used to realize capillary thermostating, e.g., liquid cooling or forced air convection:

(i) Forced air convection at a velocity of 10 m/s reduces the temperature excess about fivefold compared to a non-cooled system [62].

(ii) Solid state thermostating is reported to be

superior to forced air convection. Under the same buffer conditions, the capillary temperature increased by 1 K for a solid-state cooling system, whereas the temperature increased by about 10 and 34 K for forced air convection with a velocity of 1.5 m/s and for a capillary without cooling, respectively [52].

(iii) Beckman Coulter uses a liquid-cooled system for capillary thermostating. As the thermal contact between a liquid and the capillary tube is better than between air and the capillary tube, more precise migration times as well as peak areas are obtained compared to forced air circulation [59,67]. In another experimental setting, only little differences were found between liquid-cooled and forced air convection thermostated systems [68]. However, liquid thermostating seems to be slightly superior to forced air convection and may be necessary for some applications.

4.3.2. Capillary ends

The design of a cooling system should minimize the length of uncooled capillary external to the temperature control system [52]. One fact that will gain in significance in future is that the capillary ends are not subjected to temperature control in all existing instruments [42,69]. Temperature changes occur in the short sections of the inlet and outlet end of the capillary. The electrolyte vials act as a heat sink for the respective ends of the capillary and serve as a source of cooled buffer for the pumping action of EOF [59]. The uncooled capillary ends may be less important when using on-column detection. In fraction collection techniques or hyphenated detection methods such as mass spectrometers and laser induced fluorescence detectors, the capillary must be lead out of the CE instrument. The tiny capillary with low thermal capacity is prone to rapid thermal fluctuations. Therefore, room air conditioning is recommended to avoid temperature differences between day and night or between seasons [50,52].

5. Chemistry of the capillary surface

An unstable surface of the capillary wall causes fluctuations of the EOF and migration times [70–72]. The EOF depends on the pretreatment and the previous history of the capillary. In addition, the EOF may change during analysis, as the wall surface is altered by adsorption of analyte and buffer components as well as electrophoresis. Observed run-to-run variations in migration times are due to fluctuations of the EOF rather than differences in the electrophoretic mobility of the analytes [73]. In CZE, migration times of anionic compounds are affected to a greater degree than these of cationic species, because anionic compounds are longer exposed to the variable EOF. Further, where a significant EOF is needed, e.g., in sodium dodecyl sulfate (SDS) MEKC, its reproducibility is of great importance to the precision of the assay [74].

Adsorption of material onto the inner capillary wall causes several unwanted effects, such as a non-homogeneous EOF, peak tailing, and loss of analyte which contribute to imprecise migration times and quantitation [56,75,76]. Towns and Regnier described the characteristics of protein adsorption in more detail. Positively charged proteins are quantitatively adsorbed onto the negative charged fused-silica surface. Anionic proteins also adsorb in some cases. Any protein having a region of net positive charge or an external hydrophobic domain can interact with the surface of a fused-silica capillary [77]. Four major strategies are adopted to circumvent problems caused by adsorption and fluctuations of the EOF [56].

5.1. Separation at pH extremes

Several investigators tried to overcome the problem of adsorption of proteins on the capillary wall simply by working at extremes of pH [48,77–79]. At pH values below 3, ionization of surface silanols is suppressed, and there is little driving force for the adsorption of cationic proteins. At very high pH (above 11.5), all proteins are anionic and repelled from the capillary wall. However, while these approaches are useful in some cases, there are several limitations and disadvantages, especially as a wide range of run conditions at varying pH values needs to be available [77,80].

5.2. Preconditioning

A number of investigators recognized the importance of preconditioning for high separation ef-

ficiency as well as high run-to-run repeatability. They stressed that rinsing should be performed before each run [28,41,56,65,73,81]. Rinsing returns the system to ''first run'' conditions leading to less migration time error, as the influence of the EOF variation is reduced [73].

Today, it is common practice to employ two different preconditioning steps before each run. First, the capillary is rinsed with different solutions to remove adsorbed material from the capillary wall. In many bioassays, sodium hydroxide and/or strong acids in the range of 0.1 to 1 *M* are used as a between-run wash step [28,81]. For example, a 1 *M* solution of sodium hydroxide, maybe in combination with an acid washing step, is likely to provide adequate washing with directly injected plasma. SDS dissolved in run buffer can also provide excellent removal of adsorbed proteins [82]. If clean samples are injected, this washing step can be omitted. In the second step, the capillary surface is re-equilibrated by rinsing with electrolyte buffer. Sometimes a rinsing step with water is performed between the washing and re-equilibration step.

Some facts must be considered, when a preconditioning method is developed:

(i) The selection of the flush solvents affects migration time. This is more important in the case of systems with reversed EOF [83].

(ii) Different combinations of water, buffer, hydrochloric acid, and sodium hydroxide should be experimentally tested to find optimum conditions for rinsing [83].

(iii) The re-equilibration time needed can be reduced to a minimum, if great pH differences between washing and re-equilibration solutions are avoided.

(iv) Individual vials with buffer solutions for rinsing and separation should be used [84].

(v) Some cases were reported, where preconditioning was detrimental to precision. For example, a combination of sodium hydroxide and an organic rinsing solution such as methanol appears to alter migration time and should be avoided [73]. No sodium hydroxide washing step seems to be required when plasma samples are deproteinized by acetonitrile [3].

Rigorous washing conditions extend the total run time. Long re-equilibration times between each run under strong conditions (e.g., 1 *M* sodium hydroxide) are sometimes necessary to reduce EOF variations [79,85–87]. This can lead to the unfortunate situation that the washing and re-equilibration steps may take as long as the separation itself [81].

The capillary surface undergoes modification until a steady-state condition is established. It may require several runs to reach this condition [53,73,83]. Therefore, it was proposed to discard the data from the first runs to allow the system to come to equilibrium [11,29,68].

Despite long preconditioning, the EOF may not be stable. Even with excessive rinsing no reproducible EOF was realized until the capillary underwent one month of regular use, another capillary required four months of use [38]. Another problem complicating method transfer is that the EOF decreases as the capillary ages [71].

Rinsing before the first use of a capillary: batch-to-batch reproducibility of the capillaries is highly dependent on the nature of the fused silica itself. Surface charge and EOF can vary by 5% RSD or more between capillary batches [24]. A rinse step prior to the first use of a capillary is highly recommended to reduce variations between fused-silica capillaries from different batches or manufacturers. In common, rinsing with 1 *M* sodium hydroxide is performed, sometimes at elevated temperatures.

Another approach to stabilize the capillary wall is called ''voltage conditioning''. A short duration voltage (e.g., 20 kV for 2 min) is applied to equilibrate the column. Highly reproducible migration times (RSD$<$0.25%) were observed when the capillary was preconditioned by voltage condition combined with rinsing with fresh running buffer, see Fig. 2 [74]. A similar approach is an electro-conditioning step, where the capillary is conditioned for 1 h at 30 kV before analysis [83].

5.3. Buffer additives

The use of buffer additives is an approach to modify the capillary wall. Buffer additives can reduce or even reverse the charge of the wall from negative to positive. This method offers two advantages on precision: (i) buffer additives reduce the adsorption of dirt and proteins onto the capillary surface and diminish analyte–wall interactions. (ii)

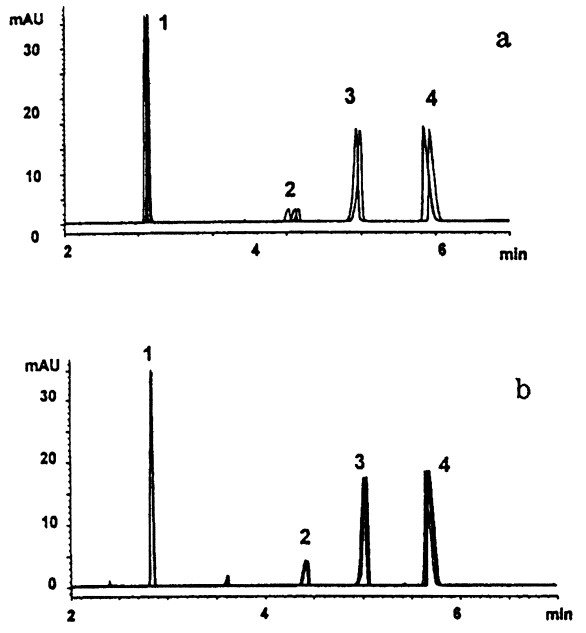

Fig. 2. Overlay of separations of four analytes. Pre-treatment: (a) capillary washed 1 min with buffer; (b) inlet and outlet vials replenished, capillary washed 1 min with fresh buffer, and application of 30 kV for 120 s. (Reproduced with permission from Ref. [74]).

Buffer additives stabilize the EOF and lead to more repeatable migration times.

One approach to mask adsorption sites of the capillary surface is the use of high-salt or high-ionic strength running buffers [50]. High injection repeatability in terms of migration time (RSD 0.4–1%) and peak area (RSD 0.7–2.3%) was obtained for a protein mixture separated with a concentrated running buffer (0.5 *M* phosphate buffer) [87]. Additives such as Tween20, Brij35, urea and ethylene glycol are useful for protein separation to suppress adsorption [79,80]. The addition of EDTA helps to remove metal ions in some buffer solutions, as even pure buffer salts are contaminated [74].

5.4. Coated capillaries

In particular applications, the three above described strategies may not be successful enough to prevent adsorption or to stabilize the surface of the capillary. Especially for protein separation capillaries with a permanent coated surface are advisable, as quantitative results are improved by reducing analyte–wall interaction [88]. Although coatings dramatically reduce adsorption, sometimes rather strong conditions are required to clean the surface between runs. The main drawback of wall coatings is its long-term stability. Laboratory-made capillaries are not stable in the long run and do not yield stable migration times, whereas commercially available coated capillaries yield excellent stability [80].

6. Evaporation effects

In CE, the sample and electrolyte vials cannot be tightly closed because the caps must allow the entrance of the capillary and electrode into the vials. This requirement means, that evaporation of sample and solvent occurs and is aggravated during long-term measurements. Consequences of evaporation of analyte and solvents are quantitative errors and changes of separation performance.

The most effective precaution to minimize evaporation is to close the vials while the capillary is still able to enter the vials. Instrument manufacturers have developed various approaches to this problem. The system from Beckman Coulter uses caps with cross-slotted openings, which allow the capillary and electrode to enter the vial. These caps reduce evaporation losses to 5 nl/h compared to 50 nl/h of an uncapped vial [89]. Plastic caps offered by Agilent Technologies tightly close the sample vials; therefore, evaporation from the sample vials is prevented. During injection, a sharp metal cutter makes a hole in the plastic cap, through which the electrode and capillary enter the vial. Only after the hole is punched, evaporation of the sample can occur [90]. The evaporation rate ranges between 10 and 60 nl/h at 8°C in a vial closed with a cap perforated one time or many times [53].

Several additional efforts were made to reduce evaporation of solvent and sample:

(i) The temperature of the autosampler tray should be controlled [50,90]. Thermostating is required both to reduce evaporation effects and to improve thermal stability of the analyte. Today, the autosampler tray thermostating is incorporated in some instruments, but alternatively temperature control can be achieved by using an external water bath. Temperature fluc-

tuations of the samples or electrolyte buffer vials may occur due to temperature fluctuations of the room or due to heat generation of the instrument, e.g., from capillary or detector lamp [53].

(ii) The air of the sample compartment can be saturated with water vapor from several small water-filled beakers to prevent significant evaporation from the running electrolyte reservoirs [39]. Analogously, it was suggested to saturate the atmosphere of sample micro-vials with solvent mixture [50,91].

(iii) In an open CE vial, an evaporation rate of 180 nl/h was found [89]. This means, that 4 μl of a 5-μl sample volume will evaporate within 24 h. As larger sample volumes exhibit a relatively lower but absolutely higher evaporation rate, larger sample and buffer volumes are recommended [50]. The use of less volatile solvents also reduces evaporation effects [90].

(iv) In some systems, sampling takes place from a microtiter plate, where evaporation effects can be significant. Evaporation can be minimized by covering the microtiter plate with a household food wrap, which is punctured by the capillary and the electrode during sample introduction [28]. Another approach is to cover the solutions in the wells with a film of light mineral oil [82].

(v) The use of an internal standard is a widespread technique to compensate solvent evaporation from sample solutions.

7. Buffer handling

7.1. Buffer preparation

CE users must be very careful when preparing electrolyte solutions, as CE is not a robust method with regard to buffer composition. Small changes of buffer pH or composition may result in large changes of migration time [92]. Analysts can purchase accurately produced buffers and reagents from several CE suppliers. As an alternative, buffer solutions are prepared within the laboratory. This is a much cheaper, but implies a number of sources of variety and bias. It is most important for repeatable analyses to prepare accurate buffers from day-to-day or from laboratory-to-laboratory. Variations happen due to varying buffer preparation techniques.

(i) Buffer reagents: buffer selection depends greatly on desired selectivity. Nevertheless, some tricks should be observed to improve precision. The use of high-purity water and HPLC-grade reagents and solvents is highly recommended. Zwitterionic buffers are recommended, e.g., biological buffers, as these buffers generate less electrophoretic currents. The counter-ion of the buffer should also be cited [93].

(ii) pH adjustment: the method for pH adjustment can be a source of variability and should be described in detail. The pH of a buffered system should be determined only after all components are added and the temperature is allowed to stabilize [93].

(iii) Filtration: many different techniques are used for filtration leading to variable buffer solutions. For example, variable SDS concentrations were reported, as SDS was adsorbed onto filter units [94].

(iv) Degassing: several different methods are used to degas electrolyte solutions, e.g., sonication, vacuum filtration or bubbling helium through the buffer. Formation of air bubbles in electrolyte solutions must be avoided, as they change short-term EOF and current leading to imprecise results of peak areas [25].

Detailed procedures for accurate and reproducible buffer preparation and approaches to improve their performance were described very usefully in literature [93,94]. In every method, a detailed description of the buffer preparation procedure should be given.

7.2. Changes of buffer solutions during analysis

Unfortunately, buffer solutions do not remain unaffected during CE analysis. Several effects like buffer depletion [95,96], contamination of the inlet electrolyte vial or volume reduction change both pH and composition [84]. The extent of buffer electrolysis is dependent on the buffering capacity, buffer concentration, ionic strength, pH, volume of the buffer vials, temperature, electrophoretic current and total run time. Changes of the electrolyte affect separation efficiency and migration times. Even peak splitting was observed [97]. Different efforts are made to minimize buffer depletion:

(i) The use of larger electrolyte reservoirs is recommended. The system of Beckman Coulter is equipped with 4-ml vials or eight 30-ml reservoirs.

(ii) The correct choice of buffer prolongs the time

to use the same pair of run buffer vials. An electrolyte with sufficient buffering capacity can provide consistent migration times and selectivities. It is important to choose a strong buffering system at the relevant pH. It is recommended to determine the maximum number of injections that can be performed using a single pair of run buffer vials without significant depletion effects. Eighteen injections using the same run buffer vials gave migration times with an RSD of less than 0.6% [97]. With higher concentrated buffers over 70 injections can be possible with the same pair of buffer vials. However, when the choice of buffer is incorrect, only five injections are possible [37].

(iii) Buffer renewal: the complete renewal of buffer solutions eliminates all problems concerning buffer depletion, contamination and evaporation. Therefore, high precision of migration time and peak area can be obtained [24,28]. Fig. 3 shows migration time fluctuations for a series of nine replicate separations. Migration times are affected by buffer renewal and different preconditioning steps. Best precision is obtained when both inlet and outlet buffer solutions are refreshed prior to each separation [96].

A disadvantage of frequent buffer renewal is that the additional electrolyte vials need space in the autosampler tray. This reduces the number of samples, which can be analyzed unattended. A compromise proposal is to refresh only the inlet vial, because the renewal of the inlet vial is more important than of the outlet vial. Frequent changes of inlet buffer vials yield significant improvement of RSD, see Fig. 3 [14,96]. However, both inlet and outlet buffer solutions should be replaced before each run, if analytes have pK_a values close to pH of the electrolyte solution [71].

Certain instruments offer the possibility to empty and fill electrolyte vials automatically (Agilent Technologies). This replenishment system facilitates buffer renewal before each run and improves precision of migration time and peak area [33].

Beside the renewal of electrolyte solutions, the renewal of preconditioning solutions is also unavoidable in long-term measurements. Renewal of these solutions is necessary during a long sequence. This leads to higher precision in migration time, but to a further loss of space in the autosampler tray [53].

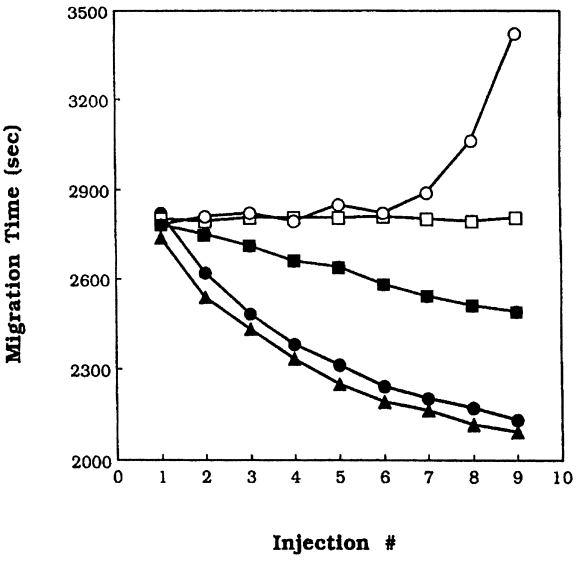

Fig. 3. Migration time of trypsinogen plotted versus separation replicate for a series of nine replicate separations. Separation was performed under 25 kV constant voltage, if not stated otherwise, employing the following preconditioning steps prior to each separation: (■) two column volume inlet rinse; (○) two column volume rinse from a fresh inlet buffer, followed by separation using the fresh inlet buffer, outlet buffer not changed; (●) two column volume inlet rinse, separations performed using 12.5 μA constant current; (▲) two column volume inlet rinse, separations performed using 0.338 W constant power; (□) complete buffer replenishment, i.e., fresh inlet and outlet buffers and two column volume inlet buffer rinse. (Reproduced with permission from Ref. [96]).

8. Quantitation

8.1. Data-sampling rate

A proper data-sampling rate of the detector must be set in the software to assure accurate quantitation by collecting enough data points. As a rule of thumb, 10 data points per peak should be acquired [98]. This means, that the sampling rate must be increased for narrow peaks. For example, a sampling rate of 100 Hz is recommended for peaks with a peak width of 100–200 ms. On the other side, the data-sampling rate should not be higher than 30 points per peak to prevent large data files and a high background noise [99].

8.2. Software

Faller and Engelhardt compared different commercially available softwares for CE [83]. The first generation of software used in CE was originally designed for control of HPLC systems. The new generation of software is designed exclusively for the use with CE systems. In general, higher values of the RSD for peak areas are obtained with older softwares than with newer ones. Especially for strongly tailing or leading peaks, the imprecision is higher than for symmetrical peaks. No significant differences could be observed when recent CE softwares were compared.

8.3. Normalized peak area

In contrast to chromatography, different samples do not pass the detector cell with the same velocity. Thus slower migrating compounds will remain longer in the detector cell and give a longer response, resulting in larger peak areas and quantitative bias. However, this effect can be compensated for by the use of normalized (also called corrected) peak areas. The normalized peak area is the peak area divided by the corresponding migration time. This quantity is proportional to the sample concentration [3,30,50,84,88,100,101].

Normalized peak areas are unavoidable in certain cases:

(i) Migration time shift: a drift in migration time increases quantitative errors if the peak areas are used without normalization for quantitation [101].

(ii) Analysis of drug-related impurities: without normalization, peaks migrating before the main peak will be underestimated whilst later migrating peaks will be overestimated [84].

(iii) Chiral separations: the later migrating enantiomer will be overestimated [2,10,101,102].

(iv) Comparison of peak areas within a single separation [103].

However, normalized peak areas are not necessary, if the variation in migration time is very small [4,14,50]. In this case, the compensation makes hardly any difference. Similar values were reported for corrected peak areas and for peak areas without normalization [29]. Therefore, many analysts dispense with corrected peak areas, since it is easier to obtain precise migration times than peak heights or peak areas [68].

8.4. Peak area versus peak height

The area or height of a peak can be used for quantitation. Analogous to HPLC and gas chromatography (GC), peak area shall be preferred in most cases in CE, as peak areas show less variation than peak height and have a wider range of linearity [3,6,22,82].

The calibration function, that is, sample amount versus peak area or peak height, exhibits a typical behavior in CE. The calibration function is a straight line, if normalized peak areas are used, see Fig. 4. On the other hand, the calibration function is curved when peak heights are used, obviously due to peak broadening caused by overloading [20,104]. Therefore, the sensitivity of peak height calibrations is very low at high sample concentrations. In addition, peak heights give lower precision because the peak height is more affected by stacking conditions or

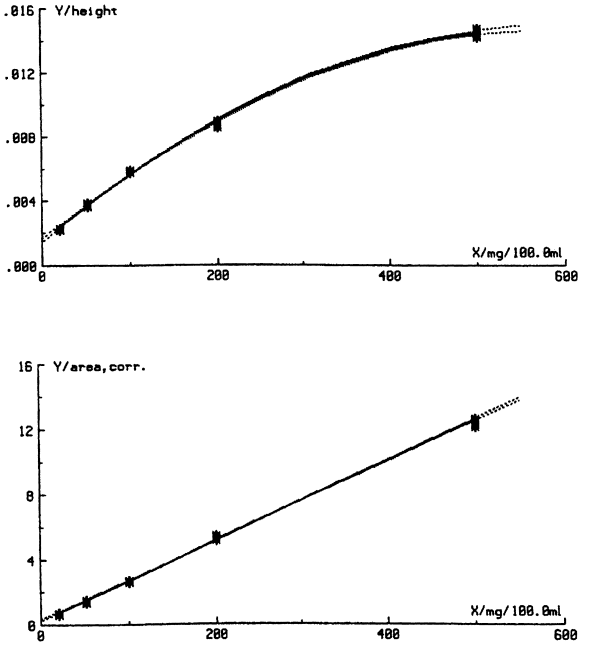

Fig. 4. Calibration functions of *N*-acetylcysteine; (a) peak height and (b) normalized peak area versus sample concentration. (Reproduced with permission from Ref. [104]).

peak distortion [29]. Thus the RSD for the analytical results is much better, if peak area calibrations are used. Honda et al. reported RSD values for peak height between 2.4 and 0.84%, whereas the RSD values for the peak area were much smaller, i.e., between 1.3 and 0.55% [20].

There are exceptions, where peak height shall be preferred to peak area. The peak height gives better precision than the peak area for sharp peaks at low concentration [3,29], because the determination of peak heights is less influenced by integration errors than the determination of peak areas [82]. Because of numerous variables it was recommended to access experimentally accuracy and precision for peak height and peak area for each new analysis [3].

8.5. Increased peak area

As in HPLC, the precision of the peak area is highly related to the sample concentration [84]. Wätzig and Dette [50] reported that the precision for peak data depends on the sample concentration to a large extent. A significant increase of the standard deviation with concentration is observed. As the standard deviation increases a bit slower than the signal, the RSD decreases with higher concentration [50,82,105]. The improvement of precision is attributed to the reduction of integration errors [41]. Precision about 5% RSD was reported for low-concentration test mixtures [10,65]. In contrast to that, typical peak area precision in the order of 1–2% RSD can be obtained when employing high sample concentrations [10]. Therefore, the injection of higher sample concentrations or larger sample volumes improves precision [3,41,51,82]. This approach is limited by column overloading which leads to overlapping peaks and a decrease in separation efficiency [50]. Another possibility to increase the peak area is field programming. In this technique, the voltage level is reduced immediately before the analyte zone reaches the detector. Consequently, the analyte migrates with a slower rate through the detector cell and gives rise to an increased signal [101,106].

8.6. Regression algorithm

The signal standard deviation increases a bit slower than the signal, therefore, the RSD decreases with higher concentrations. As a consequence a systematic error occurs, if ordinary least-squares regressions are used. If one wants to calibrate over a concentration range of one order of magnitude or more, the systematic error made can be significant. For CE data, weighted least-squares regressions are recommended. However, this does not cause problems, if the concentration range of the calibration curve is small [105].

9. Marker techniques

9.1. Internal standard

In CE, internal standards are used in the same way as in chromatography. Several analysts reported the benefit of using internal standards by correcting errors, which are introduced by variable injection volume, voltage, or EOF [11,12,27,29]. In addition, evaporation losses of solvents are compensated for [90]. For example, the use of an internal standard improved the CE and MEKC precision from 7 to 10% RSD to 1% [5,107]. In a more recent study an internal standard improved the injection repeatability to 2.38–1.88% RSD [25].

For the hydrodynamic injection mode, a single internal standard offers a precision advantage, whereas only poor precision can be obtained with a single internal standard at electrokinetic injection. Therefore, two internal standards are required to improve the precision for electrokinetic injection mode. RSD values for peak area of 1.4–3.6% and even below 1% were reported with this technique [21,65].

Nevertheless, care must be taken when choosing an internal standard. In certain cases it was reported, that the peak areas of the internal standards were less precise than the peak areas of the analyte itself [8,14,92]. No precision advantage was noticed as the sample and internal standard concentrations were both low doubling the integration errors. Therefore, internal standards should be used at high concentrations.

9.2. Mobility marker

Many investigators complained that the re-

peatability of analyte migration times is poor in CZE. Fluctuations in the migration times are not due to any characteristics of the test compounds but are mainly related to the unstable EOF. Several different mathematical and experimental efforts were made to eliminate the influence of a variable EOF.

(i) Mobility: electroosmotic mobility shows greater stability than migration times by removing the influence of the EOF variation. Therefore, mobility is a much more rugged migration parameter than migration time [73].

(ii) Mobility scale transformation enables a better comparison between electropherograms than with the primary time-scale. Besides possible qualitative peak tracking, a quantitative improvement can be achieved [108].

(iii) Migration index: CE is highly repeatable if markers with known electrophoretic mobilities are used for identification of the analytes. Good values for the mobility can be obtained (RSD 0.01–0.07%) even if the absolute migration times are highly irreproducible [69,70]. Extremely high repeatability for the electrophoretic mobility (RSD ranging from 0.01 to 0.03%) was obtained when employing two to four different markers [71]. The application of a migration index shows superior performance compared to migration time in terms of precision and reproducibility, as the migration index is independent of a number of experimental conditions. Therefore, data and method transfer between laboratories is facilitated [69].

(iv) Mobility ratio and migration time ratio: these parameters are independent of such items as operating voltage, electric field, length, diameter, and positioning of the capillary, temperature, solvent viscosity, and buffer additives. Therefore, good within-day precision can be obtained [69]. Fig. 5 compares migration time and migration time ratios of several injections for an amino acids test mixture. In this example, the RSD for the migration time ratio ranged from 0.6 to 1.1%, while the RSD for the migration time ranged from 2 to 5% [72].

(v) Effective mobility and corrected effective mobility: again, these parameters are more precise than migration time. For both a CZE and MEKC method the RSD values of 6.5% and 8.6% for migration time were reduced to 1.1% and 0.6% for effective mobility, respectively [9].

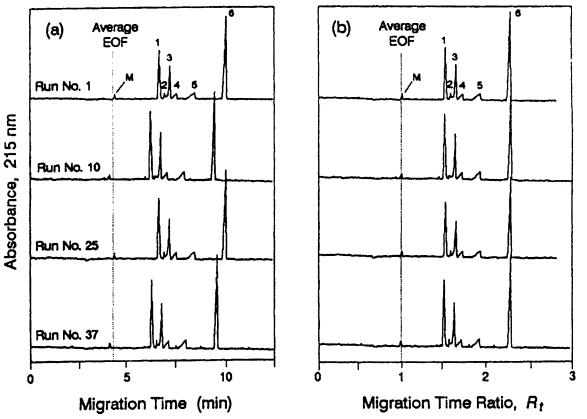

Fig. 5. Variation in the (a) migration times and the (b) migration time ratios for several injections of an amino acid mixture (=1–6) onto a CE system. Mesityl oxide (=M) was added to all samples as a marker for EOF and for use in the calculation of migration time ratios. The dashed vertical line indicates the average position of the mesityl oxide peak. (Reproduced with permission of Ref. [72]).

10. Conclusions

CE users must pay attention to a vast number of experimental parameters and technical details to obtain high precision. The method validation criteria for CE methods are similar to those used in HPLC. Specific requirements for the validation of a CE method are capillary variation (lot-to-lot, suppliers), electrolyte stability, instrument transfer, or capillary rinsing. Method transfer is complicated because CE instruments are not standardized. Each instrument supplier has its own specific designed capillary lengths and its own unique settings and procedures for both injection and rinsing [35]. Therefore, every method should be described in detail to facilitate method transfer from laboratory to laboratory and to improve reproducibility.

Today, personal computer-controlled and highly automated CE systems are commercially available. Many instrumental improvements have been made during the last several years to assure sufficiently precise quantitative results. Better instrumental equipment, more experienced users, and better-described methods should yield more precise quantitative results.

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