

Long-Term Analyses with Capillary Electrophoresis

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This month's instalment of "CE Currents" deals with problems that may arise when using capillary electrophoresis for unattended, long-term operation. The guest authors look at issues such as sample carryover, evaporation effects, capillary conditioning, capillary surface changes, buffer handling, capillary breakage and detector-lamp deterioration. They provide several technical recommendations for stabilizing the electrophoretic system and increasing reproducibility.

Because commercial capillary electrophoresis (CE) instruments have been available for a decade, CE has become a mature and well-established analytical tool. CE is extensively used for routine analysis as an alternative or complementary technique to high performance liquid chromatography (HPLC). Many of the technical problems have been solved, and handling the instruments has become effortless. Nevertheless, several problems may arise during long-term sequences, but they are unnoticeable when only a few runs are performed.

One major disadvantage of CE in comparison to HPLC is its poor precision. The experimental conditions must be controlled accurately during an extended period; for example, when performing stability tests, observing the course of a reaction or analysing a large number of samples. CE users can improve reproducibility by paying attention to several, sometimes simple, technical details.

Sample Introduction

During long-term measurements, the caps of frequently used sample vials may become worn and need replacement. Precision may decrease because of two effects: leakage during preconditioning and hydrodynamic injection that results in varying flush and injection volumes and, therefore, irreproducible migration times and peak areas; and analyte and solvent evaporation from sample and buffer vials that cause concentration changes and unequal liquid heights. The caps of frequently used vials containing

conditioning buffers and inlet and outlet buffer solutions should be replaced during long-term analyses. Cleanliness of the vial caps is also important. The voltage can skim across dirty vial caps and go to ground rather than pass through the capillary. This skimming would result in very late or no peaks. This problem was more common with the original CE instruments, but it can still cause problems, so users should take care.

Thermostating

Capillary: Today, temperature control of capillaries is a feature of most CE instruments. Compared with forced air convection, liquid thermostating seems to be slightly superior and may even be considered necessary for some separations (1). Nevertheless, capillary ends cannot be thermostated because they protrude from the cartridges (2).

Autosampler tray: The temperature of the autosampler tray may vary, because the instrument generates Joule heating during electrophoresis and from the detector lamp. For example, the power consumption of a deuterium lamp (Agilent Technologies GmbH, Waldbronn, Germany) is 20 W. The autosampler tray thermostating is incorporated in some instruments, but alternatively temperature control can be achieved by using an external water bath. Although certain instruments display the temperature of the autosampler tray, users must be careful. The actual temperature of the sample vials should be checked with an external thermometer, because the displayed

temperature may deviate from the real temperature.

Cooling provides several advantages: Because the viscosity of buffer and sample solutions depends on temperature, the injection volumes are more reproducible. Air bubble formation in buffer, preconditioning and sample solutions is suppressed. Degradation of thermally labile analytes is reduced. Evaporation effects of solvent and sample solutions are minimized.

If cooling is unavailable consider placing the samples in the autosampler 30 min before analysis to equilibrate.

Room: Typical analytical sequences are started during the day and run unattended overnight. Temperature-constant rooms can be helpful to avoid temperature differences between day and night or the seasons (3).

Evaporation of Sample and Solvent

The evaporation of sample and solvent can become a serious problem during long-term measurement. The vials cannot be tightly closed because the caps should allow the entrance of the capillary and electrode. The evaporation rate of water has been found to be 50–180 nL/h in an open vial at ambient temperature (3, 4). The system manufactured by Beckman Coulter Inc. (Fullerton, California, USA) uses caps with cross-slotted openings, which reduce evaporation losses to 5 nL/h (4). Plastic caps from Agilent Technologies tightly close the sample vials until the cap is perforated during injection. The evaporation rate was 1060 nL/h at 8 °C in

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vials closed with a cap and perforated one or more times (5). For example, when the cap was perforated only once by the prepuncher, the evaporation rate was 10 nL/h. When the cap was perforated repeatedly by the prepuncher, the evaporation rate was 60 nL/h.

Several additional strategies can reduce the evaporation of solvent and sample:

- The air of the sample compartment can be saturated with water vapour from several small water-filled beakers to prevent significant evaporation from the running electrolyte reservoirs (6). Similarly, others have suggested saturating the atmosphere of sample microvials with solvent mixture (3, 7).
- An evaporation rate of 180 nL/h means that 4 μ L of a 5 μ L sample volume will evaporate within 24 h. Because larger sample volumes exhibit a relatively lower but absolutely higher evaporation rate, Wätzig and Dette (3) recommend larger sample and buffer volumes. Using less volatile solvents also reduces evaporation effects (8).
- In some systems, sampling can take place from a microtitre plate. Because microtitre plates have small volumes and cannot be closed tightly, evaporation can occur. Evaporation has been minimized by covering the microtitre plate with a household plastic wrap, which was punctured by the capillary and the electrode during sample introduction (9). Another approach is to cover the solutions in the wells with a film of light mineral oil (10).
- The use of an internal standard is a common technique for analysis, and it is helpful to compensate for solvent evaporation from sample solutions.

Capillary

A straight-edge capillary is helpful to increase peak symmetry and to reduce spontaneous-marker peaks (6, 11, 12). Removing the outer polyimide coating at the capillary tips can reduce sample carryover (13). After the injection process residual sample from the outside surface of the capillary can be removed by dipping the injection end into a vial containing fresh

buffer (2). Many other useful hints concerning the care and maintenance of capillaries have been described by Altria (14).

Regenerating the capillary: Washing and reequilibration of the capillary is commonly used after each run to remove adsorbed material and to reequilibrate the column (9). Furthermore, the process avoids sample carryover (15).

Washing is necessary to remove material, such as precipitated proteins, adsorbed on the capillary wall from the last injection. One to three rinse steps may be necessary before each separation. When analysing samples in a biological matrix, the washing steps must be performed under strong conditions; for example, using 0.1–1 M sodium hydroxide, 0.1–1 M hydrochloric acid, phosphoric acid, buffer with surfactants, water or organic solvents such as methanol (16). Rinsing under these strong conditions — 0.1 M hydrochloric acid for a neutral coating or 1 M hydrochloric acid and 1 M sodium hydroxide for a polyamine-coated capillary — may become necessary even with coated capillaries (17).

Reequilibration can be performed to obtain a buffer-ion double layer at the capillary surface. A common practice is to rinse the capillary with running buffer. If alkaline and acid wash steps are used, considerable reequilibration time is necessary between runs to increase reproducibility of the electroosmotic flow, migration times and peak areas (16). This reequilibration can lead to the unfortunate situation in which capillary washing and reequilibration steps may take longer than

the separation itself (16, 18).

In addition to the loss of time, regenerating steps have a second disadvantage. Several positions of the autosampler tray are occupied by vials that contain solutions for washing and reequilibration. These vials must be replaced from time to time, which results in a greater loss of space in the autosampler tray. This replacement is necessary, especially for long-term measurements, because the preconditioning solutions are consumed, liquid levels decrease, preconditioning solutions may become contaminated and solvents may evaporate.

The electroosmotic flow and migration times are very sensitive to conditions of the capillary surface. Figure 1 shows how changes during preconditioning influence the migration times of an analyte. For the analysis shown in this figure, we rinsed a fused-silica capillary before each run with 0.1 M sodium hydroxide for 7 min and running buffer (20 mM citrate buffer that contained 50 mM sodium dodecyl sulfate [pH 2.8]) for 10 min. We separated an analyte standard solution of 20 μ g/mL cefpirome in Ringer's solution in the reversed mode by applying a 20 kV voltage. The inlet and outlet vials containing running buffer and conditioning buffer were automatically replenished before each run to prevent buffer depletion and sample carryover (19). During the sequence, the liquid level of the sodium hydroxide vial decreased, whereas the liquid level of the waste vial was kept constant by refilling the vial with running

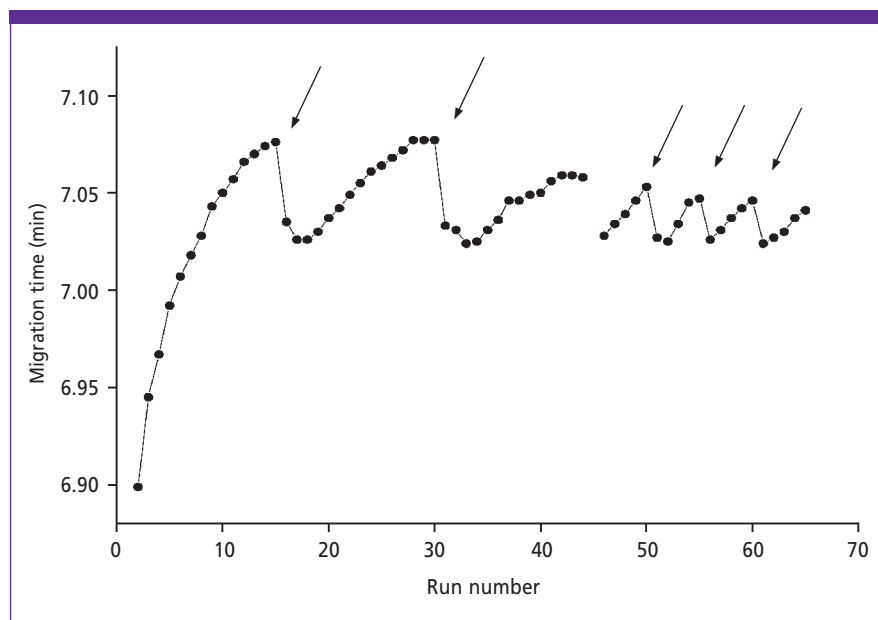


Figure 1: Plot of migration time versus run number for an analyte separated by MEKC. Arrows mark renewals of the washing solutions.

buffer after each run. The vial containing sodium hydroxide was replaced after every 15th run (marked with arrows in Figure 1) during run numbers 1–45. The relative standard deviation (RSD) of analyte migration time was 0.24% for run numbers 7–45; that is, without the deviations of the first few runs. The vial containing sodium hydroxide was replaced after every fifth measurement during run numbers 46–65. As the washing solution was renewed more frequently, we obtained a decrease of RSD to 0.12%. We observed the same phenomenon when analysing approximately 2000 samples in a bioassay; however, a complete explanation is missing.

Changes of capillary surface: If you need more than one capillary for the same application, we recommend purchasing capillaries from the same brand and batch. Differences of capillary surface can be eliminated by rinsing the capillary with a 1 M sodium hydroxide solution at elevated temperatures before its first use.

Even if the capillary is preconditioned in the same way before each run, the capillary surface undergoes modification until a steady-state condition is established. It may require several runs to reach this condition (20). Figure 1 gives an example in which the migration times of the first few runs are lower than at steady state. A similar phenomenon is well known in HPLC.

When a capillary is used for an extended period of time, the surface of the capillary wall may become altered. Colyer and

co-workers (12) reported that a reproducible electroosmotic flow is not realized until the capillary has undergone approximately one to four months of regular use, even if excessive rinsing has been performed. In general, the electroosmotic flow decreases gradually as the capillary ages (21).

Breakage: Usually, a capillary breaks during manipulation; that is, during installation of the capillary into the cartridge or the CE instrument. To reduce mechanical stress, we recommend storing every capillary in its own cartridge. This practice allows a faster and safer exchange of capillaries and methods.

Although rather unlikely, a capillary can break or crack without manipulation. Figure 2 shows an example of spontaneous breakage during unattended, overnight operation. Without manipulation, the capillary broke near the detection window, a site that is prone to breakage because of the lack of the outer polyimide coating. The figure shows the electrophoretic currents of three consecutive measurements (run numbers 15–17). Run number 15 shows a typical electrophoretic current level. Because of the highly variable electrophoretic current, we assumed that the breakage occurred during run number 16 at approximately 2 min. The voltage was ramped from 0 to 30 kV within the first 0.5 min, so heat generation probably was the cause of breakage. Because the current of run number 16 was very variable, it was surprising that run number 17 displayed a current level that was

similar to the currents observed in normal runs. After automated flushing and conditioning steps, the electrophoretic current seemed to find its way through the leakage with the help of buffer or flushing solvent ions. Although the current level was hardly changed in this run, the corresponding electropherogram was not useful for interpretation. To discover, and attempt to explain, the reason for these anomalies, it is good practice to record the expected operating current in a method and compare it with that achieved in routine analysis.

Buffer Solution Handling

The correct and reproducible preparation of buffer solutions has been described in detail in earlier instalments of "CE Currents" (22, 23). Unfortunately, buffer solutions are altered during electrophoresis:

- The composition and pH of the running buffer can be altered during electrolysis, a phenomenon called buffer depletion (24).
- The electroosmotic flow pumping action causes unequal liquid heights, and siphoning effects can occur during injection (13, 25).
- Sample carryover during injection and electrophoresis contaminates the electrolyte vials with the analyte.

As a result, separation efficiency can vary, and the reproducibility of migration times and peak areas may decrease. The extent of alterations depends upon electrolyte composition, pH and volume, electrophoretic current, temperature and total run time. Therefore, some applications do not require buffer renewal after each run. Renewing the inlet buffer is more important than renewing the outlet buffer (26). However, fresh buffer solutions are recommended after each run if analytes have pK_a values close to the pH of the electrolyte solution (21). A frequent change of the buffer vials requires space in the autosampler tray and reduces the number of samples that can be analysed unattended.

One approach to reduce buffer depletion is using large-volume buffer reservoirs (27). A system from Beckman Coulter has eight 30 mL buffer reservoirs. Another approach is using an automated replenishment system, which is available on some CE instruments. Renewing the buffer solutions does not waste time if an automated replenishment system can provide capillary conditioning and replenishment in a parallel mode. Nevertheless, users must take care to avoid some situations:

- Crystal formation in buffer solutions can block the tubes of the replenishment

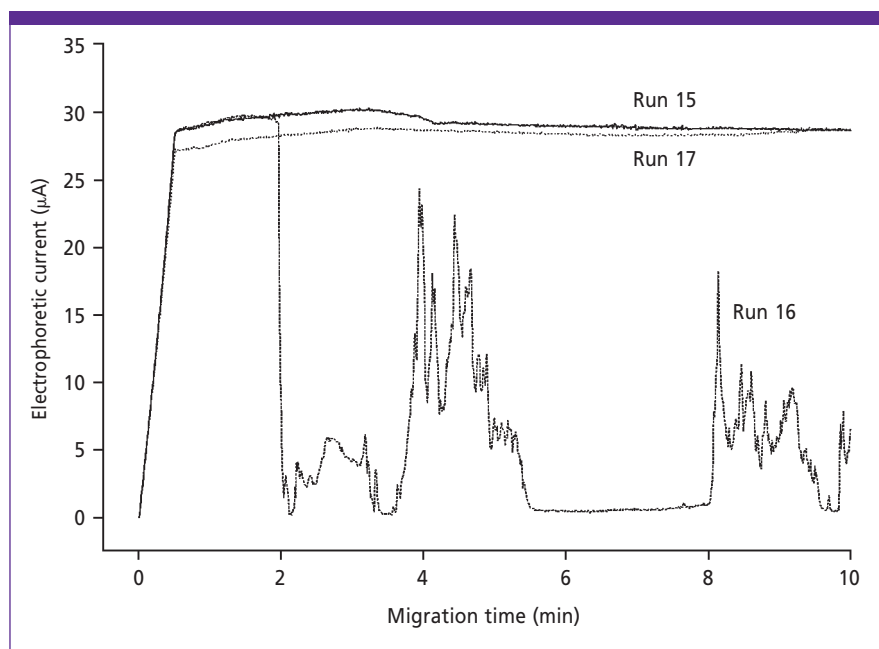


Figure 2: Changes of the electrophoretic current of three consecutive runs caused by spontaneous capillary breakage. The breakage occurred at the detector window during run 16 of an unattended overnight operation.

system. Therefore, when the system is idle, we recommend storing the replenishment needle in a vial that contains water. The level-sensor needle of the replenishment system should be cleaned with water or an appropriate solvent after each sequence.

- Some instruments operate with vacuum systems. If problems with vacuum systems arise, the caps of the electrolyte and waste bottles should be checked for leaks. Clogged inlet filters in the electrolyte bottle or used air inlet filters can cause failure of a vacuum system.
- The buffer solution reservoir of the replenishment system is stored under slight pressure in the electrolyte bottle at room temperature. Under those conditions, the buffer solutions can be used during continuous operation throughout a week. If no problems — air bubbles or salt crystal formation — arise, the system is well suited for long-term operation.
- In micellar electrokinetic capillary chromatography (MEKC) mode, buffer solutions can contain surfactants, such as sodium dodecyl sulfate, which tend to foam excessively. The waste bottle may be filled with bubbles within a few runs, and the replenishment system can malfunction. A powerful precaution to prevent bubble formation of the waste solution is to coat the inner wall of the waste bottle manually with silicone grease (19).

Detection

Lamp deterioration: One major problem of long-term measurements with ultraviolet (UV) and fluorescence detectors is the short lifetime of the detector lamp — approximately 1000–2000 h. The deterioration of the lamp performance occurs slowly, so it is not easy to distinguish between lamp deterioration and other problems. Figure 3 shows three electropherograms that display typical changes of the baseline caused by lamp deterioration: an oscillating baseline at approximately 3 min and a spike at 8 min (Figure 3(a)); a baseline shift at 3 min and a noisy baseline (Figure 3(b)); and recurring negative spikes in the first few minutes and large negative peaks at approximately 4 and 10 min (Figure 3(c)). These three electropherograms were recorded on the same day using a UV lamp with approximately 1000 h of operation in an Agilent Technologies CE system. Between these runs, we performed numerous measurements with low baseline noise, apparently showing an acceptably operating lamp.

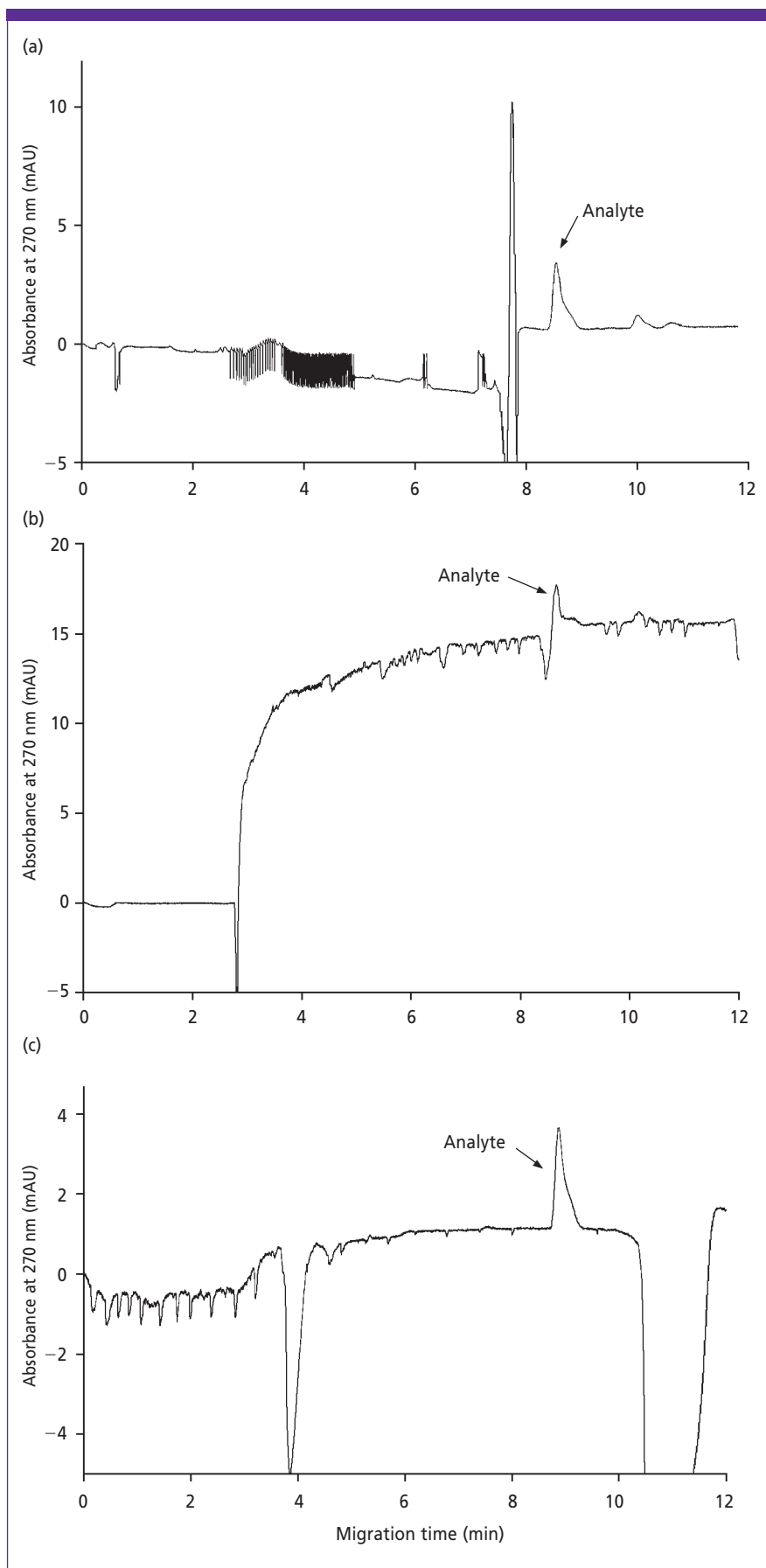


Figure 3: Electropherograms displaying (a) baseline oscillations, (b) a baseline shift and (c) negative spikes and peaks caused by UV lamp deterioration after approximately 1000 h of operation.

Table 1: Checklist of parameters for long-term measurements.

	Action	Effect
Hydrodynamic injection	Dip capillary into vial containing fresh buffer	Prevents sample carryover
Buffer solution handling	Replenish buffer vials used for conditioning and electrophoresis Replenish buffer in parallel mode Renew flushing and conditioning solutions Coat waste bottle with silicone grease in MEKC	Prevents buffer depletion, siphoning effects and sample carryover Saves time Keeps migration times constant Suppresses bubble and foam formation of waste solution
Thermostating	Thermostat capillary, sample tray and room	Minimizes viscosity changes, suppresses air bubble formation and reduces evaporation
Capillary	Remove polyimide coating from the tips and straight edges Flush and condition capillary after each run Keep every capillary in its own cartridge	Increases injection precision and avoids sample carryover Removes absorbed material and equilibrates capillary wall Avoids mechanical stress to capillaries
Detector	Monitor lamp durability, replace if necessary Avoid hits, pressure or vibrations to CE instrument	Decreases baseline noise Prevents position changes of the capillary in detector cell
Vial caps	Replace caps of frequently used vials	Avoids leakage during injection

If you observe any of these baseline changes and can exclude other reasons — air bubbles, a broken capillary, or capillary blockage — you should consider replacing the lamp. To distinguish between lamp deterioration and other phenomena, flush the capillary with fresh buffer and monitor the baseline with no applied voltage. If the baseline is still noisy, the lamp should be replaced.

Signal changes: CE detectors must be very sensitive because they are required to analyse minute amounts of samples. Therefore, the proper position of the capillary cartridge, which houses the fused-

silica capillary in the detector, is important. We found that the position of the cartridge device and capillary could be manipulated mechanically on certain CE instruments. Gentle pressure on the top cover of the instrument may cause a spike or a complete baseline change (see Figure 4). You should avoid other disturbances such as vibrations from an external water bath or a knock against a laboratory workbench, to prevent displacement of the cartridge and the capillary within the detector.

Corrected peak areas: In CE, unlike chromatography, different samples pass

the detector window with different velocities. Analytes that migrate slowly exhibit a larger peak area. If it is impossible to keep the migration time constant, accuracy can be improved by using corrected peak area; that is, the division of the peak area by its corresponding migration time (3).

Maintenance

Although CE instruments operate well for a long time, maintenance is necessary between long-term sequences. We recommend cleaning prepunchers, electrodes, and replenishment system tubes (if available) with water and isopropanol to remove buffer solution crystals and other deposits. Air inlet filters in a dusty atmosphere must be changed frequently and capillary detection windows and interfaces must be cleaned very carefully to remove dust and debris. For more instructions and details, refer to the instrument manuals.

Economy

Economic considerations may be a reason for using CE during long-term operation, because CE offers several advantages. Homemade buffer solutions are very cheap, whereas commercially available buffer solutions are more expensive. Nevertheless, additives such as detergents or chiral selectors can be very expensive. The costs of the filter devices for buffer preparation can be significant.

Low solvent consumption and reduced organic waste are other advantages of CE. For example, an MEKC determination of a

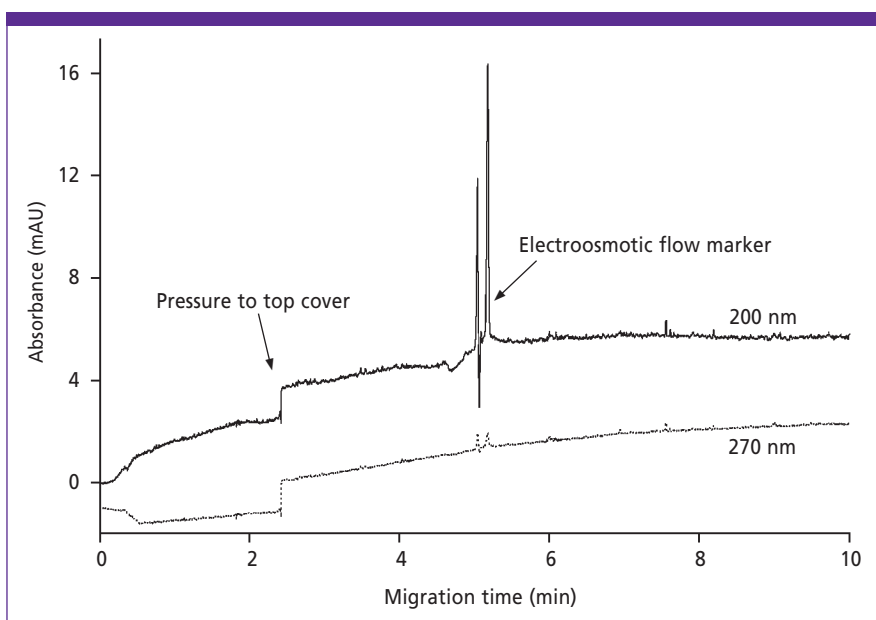


Figure 4: Detector signal changes caused by a slight pressure to the top cover of the CE instrument that displaced the capillary within the detector.

cephalosporin required 1000 mL of running buffer and 20 mL of preconditioning solutions for one week of continuous operation with a 26 min run time, 50 runs per day, 6 overnight measurements and 300 samples. In comparison, an HPLC determination with a 1 mL/min flow would require 8500 mL of eluent per week, which is an eightfold increase of solvent consumption compared with that needed for CE.

The costs of the capillaries are another economic aspect. Uncoated fused-silica capillaries are inexpensive compared with HPLC columns, especially when the longer durability of CE columns is considered. In addition, CE users may consider purchasing fused-silica tubing as a stock item. In contrast to uncoated capillaries, coated capillaries are more expensive and less durable.

Conclusion

CE instruments are well suited for unattended long-term measurements in routine analysis. Nevertheless, knowledge of analytical and instrumental pitfalls is important. Table 1 provides a short checklist that summarizes several experimental parameters. Proper methods, care and maintenance are necessary to maintain high performance and high reproducibility.

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