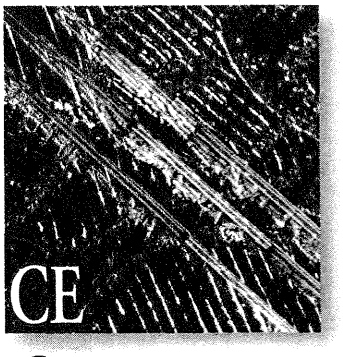

CE Currents

Guest Authors
Miroslav Macka, Cameron Johns, Philip Doble, and Paul R. Haddad

In the first part of this two-part series, the guest authors explained the principles and approaches to indirect detection and stressed the importance of buffering for method ruggedness. This second part outlines the different ways electrolytes can be buffered while maintaining compatibility with indirect detection and gives rules and practical guidelines for method development. The authors also discuss some instrumental aspects of indirect photometric detection that affect the method performance.

Kevin D. Altria
CE Currents Editor

Indirect Photometric Detection in CE Using Buffered Electrolytes — Part II, Practical Rules

The first part of this two-part series elucidated the basic theory of indirect photometric detection and outlined the need for buffering electrolytes to optimize method ruggedness and performance (1). This second part will deal with ways of making electrolyte buffering compatible with indirect detection and with the practicalities of its operation, including some instrumental aspects.

Making the Method Work

Four schemes for successful buffering of electrolytes for indirect detection: An electrolyte used in indirect photometric detection can be buffered in four ways to be compatible with indirect detection. These buffering systems are summarized in Table I, along with their comparative advantages and disadvantages.

The simplest method is to use a buffering co-ion, but the mobility of the buffer ion should be as different from that of the probe as possible. To separate high-mobility analytes such as inorganic anions, a probe of high mobility such as chromate is necessary to ensure good peak shape, and this requires the use of a low-mobility buffer anion such as borate. Analyte anions tend to displace chromate rather than borate because chromate and analytes have a closer match of mobilities. Whenever a borate ion is displaced by an analyte anion, no indirect detection signal will occur.

Another relatively simple buffering scheme is using the probe itself. As an example, the pK_{a2} of phthalate is 5.4, so using phthalate as the probe at pH 6.5 (Figure 2 in Part I [1]) provides some degree of buffering. Many probes such as nitrate and chromate do not have pK_a values that allow their buffering capacity to be accessed in the normal working range of capillary electrophoresis (CE). In these cases, buffering using counterions is a useful option. In the case of anion separations, counterion buffering can be achieved easily by titrating the acid form of the probe with a buffering base. For example, chromic acid titrated with Tris will yield an electrolyte that contains chromate anions and a mixture of Tris cations and neutral Tris, the latter being a good buffer. No interfering co-ions are present. Figures 1 and 2 illustrate the use of a chromate electrolyte buffered with a counterion. In comparison to the widely used unbuffered chromate electrolyte, the buffered chromate electrolyte provided a more stable baseline and significant improvements in method reproducibility (especially drift of migration times, Figure 1) and ruggedness (better tolerance to alkaline sample matrix, Figure 2) (2).

The last and most recently introduced buffering method (3–5) involves the use of an isoelectric buffer. These isoelectric buffers comprise ampholytic molecules, which have two pK_a values distributed relatively closely (within 1 pH unit) on either side of the pI of the molecule; therefore, they exhibit some buffering capacity at the pI, where the molecule has a zero net charge and does not interfere with indirect detection. The disadvantage of this scheme is that few of these isoelectric buffers are available.

Electrolyte formulation and optimization: Impurities in the electrolyte can introduce system peaks as well as co-ionic buffers. For example, carbon dioxide is absorbed by alkaline solutions and causes a system peak from the presence of carbonate, so alkaline electrolytes should be prepared freshly using bases free of carbonate. This problem does not occur with Tris, which does not absorb carbonate, but it can be a problem with other bases such as diethanolamine. Similarly, co-ionic impurities in the probe or other chemicals will cause system peaks, so only high-purity reagents should be used (5).

Electroosmotic flow modification: Anion analysis usually is performed in a fused-silica capillary in coelectroosmotic mode,

Table I: Buffering systems that are compatible with indirect detection*

Electrolyte Is Buffered By	Probe Character	Buffer Character	Advantages	Disadvantages
A co-ion of much lower mobility than the probe	High-mobility probe such as sodium chromate	Low-mobility co-ion such as borate or 2-(*N*-cyclohexylamino)-ethanesulfonic acid (CHES)	Flexibility in electrolyte composition	System peaks limit applicability for low- to medium-mobility analytes
The indirect detection probe ion itself	Buffering probe such as phthalate	The probe itself at pH ~ pK_a, for example phthalate	Simplicity	Low probe effective mobility giving poor peak shapes for high-mobility analytes; limited operating pH range
A counterion	Any probe	Counterion such as Tris or diethanolamine	Universality	Probe must be available as free acid or base
An isoelectric compound	Any probe	Isoelectric compound at pH ~ pI such as glutamic acid (pI 3.2), histidine (pI 7.7), or lysine (pI 9.7)	Simplicity; probe need not be available as free acid or base	Limited number of buffering ampholytes; limited operating range of pH

*In these examples, electrolytes are for the analysis of anions; that is, the co-ion is an anion and the counterion is a cation.

which means the electroosmotic flow has the same direction as the migration of the anions in the electric field; that is, to the anode at the detector side. The benefits of coelectroosmotic separations are short analysis time and overall high separation efficiencies. This modification requires a reversal of the electroosmotic flow using cationic additives to the electrolyte such as long-chain aliphatic quaternary ammonium surfactants or cationic polymers that adsorb onto the fused-silica capillary wall. Co-ions often are introduced to electrolytes as a component of such an additive, and these co-ions will cause system peaks. For example, bromide from cetyltrimethylammonium bromide causes a system peak, and it is preferable to use cetyltrimethylammonium hydroxide because excess OH^- is converted in the electrolyte to water and does not introduce a co-ion into the electrolyte. Unfortunately, stock solutions of cetyltrimethylammonium hydroxide tend to absorb carbon dioxide over time and introduce a system peak caused by carbonate.

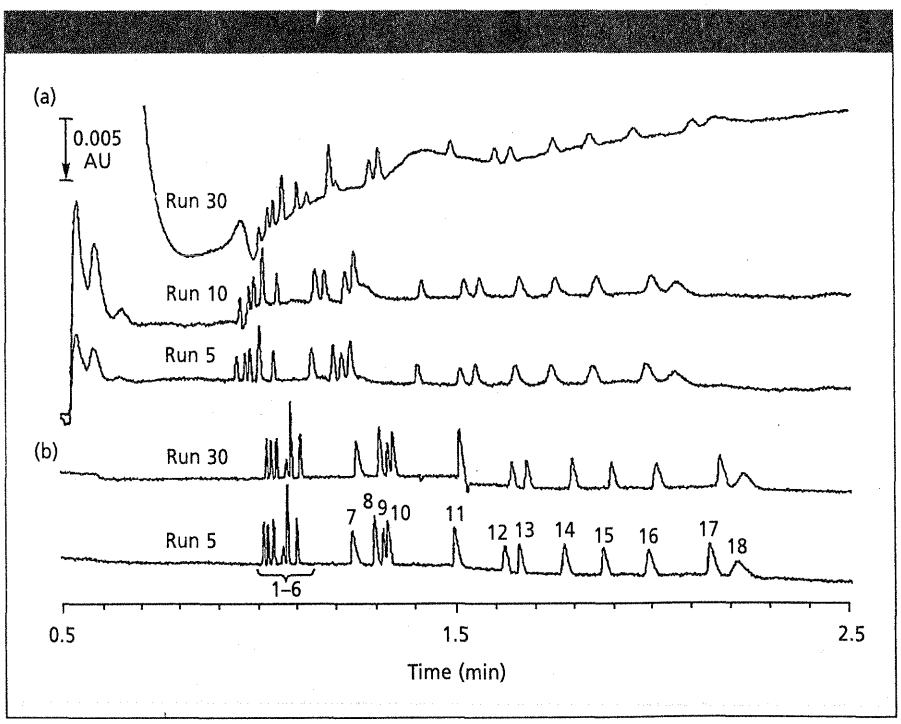

Figure 1: Electropherograms of a standard mixture of common anions separated in (a) unbuffered sodium chromate electrolyte and (b) buffered chromate electrolyte. Capillary: 0.485 m × 75 μm, 0.400 m to detector; electrolyte: (a) 5.0 mM sodium chromate, 0.5 mM tetradecyltrimethylammonium hydroxide (pH 8.5); (b) 5.0 mM chromic trioxide, 20 mM Tris, 0.5 mM tetradecyltrimethylammonium hydroxide (pH 8.5); voltage: −30 kV; injection: hydrodynamic at 20 mbar for 3 s, 0.1 mM of each anion; detection wavelength: 254 nm (8-nm bandwidth); temperature: 25 °C. Peaks: 1 = thiosulfate, 2 = bromide, 3 = chloride, 4 = iodide, 5 = sulfate, 6 = nitrate, 7 = citrate, 8 = tartrate, 9 = succinate, 10 = formate, 11 = methanesulfonate, 12 = iodate, 13 = ethanesulfonate, 14 = propanesulfonate, 15 = butanesulfonate, 16 = pentanesulfonate, 17 = gluconate, 18 = hexanesulfonate.

Probe solubility and adsorption on the capillary wall: A common problem regarding probe solubility occurs in chromate electrolytes that contain cetyltrimethylammonium bromide or similar cationic surfactants for electroosmotic-flow reversal, which causes precipitation of the water-insoluble ion pair (cetyltrimethylammonium–chromate) at pH levels lower than pH 8. If precipitation occurs, elevating the pH by as little as 0.1 pH unit usually helps. Preparing the electrolytes freshly each day from separate stock solutions also is advisable. The Tris-buffered chromate electrolyte used in our laboratory was free of the precipitation problem at pH 8.3. The diethanolamine-buffered chromate electrolyte has an approximate pH of 9.2, and precipitation does not occur at this pH.

Similar problems occur with some lipophilic organic analyte anions that form ion pairs with cetyltrimethylammonium. Under these circumstances, cetyltrimethylammonium and similar surfactants should be avoided. Using a less hydrophobic cationic polymer coating on the capillary wall can be an alternative, but it often leads to adsorption of the probe onto the wall. These problems sometimes result in an inability to reverse the electroosmotic flow,

and this result seems to be a limitation to the use of hydrophobic organic probes such as highly adsorbing dyes (5).

Separation of anions: Inorganic anions cover a wide range of electrophoretic mobilities, ranging from greater than -100×10^{-9} m^2/Vs to approximately -40×10^{-9} m^2/Vs, and organic ions have mobilities between -60×10^{-9} m^2/Vs and -20×10^{-9} m^2/Vs (6). Most of these ions have unique mobilities, and if comigration occurs, separation selectivity can be manipulated by additives such as organic solvents or by introducing ion-exchange-type interactions with cationic water-soluble polymers added to the electrolyte. For anions that exhibit protonation equilibria in CE's pH range, substantial selectivity changes can be achieved by pH changes of the electrolyte (see equation 1 in Part I [1]). However, the electrolyte must be well buffered to avoid pH inhomogeneity within the migrating sample zone for this approach to be successful.

The most popular method by far for anions is a coelectroosmotic separation performed in a fused-silica capillary with the electroosmotic flow reversed by a cationic surfactant such as cetyltrimethylammonium and with chromate as the probe (7). Although many other probes have been evaluated (most of which were listed in Figure 2 in Part I [1]), chromate is used most for inorganic analyses because of its high mobility. Its main disadvantage, however, is that it is classified as a hazardous substance. In that respect, molybdate, with a somewhat lower mobility than chromate, appears to be a good alternative for indirect detection at 230 nm (8). (Readers are referred to some of the detailed reviews in reference 9 for applications of indirect detection methods to anion analysis.)

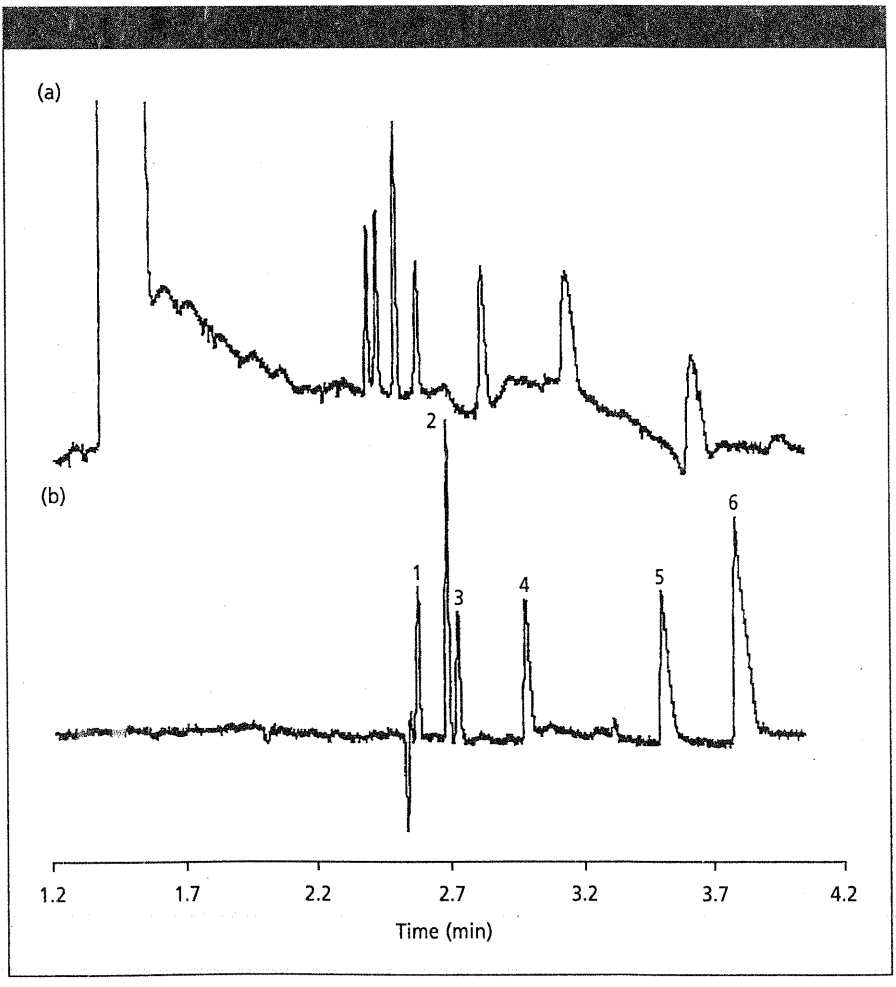

Figure 2: CE separation of analytes in an alkaline matrix using (a) unbuffered and (b) buffered chromate electrolytes. Capillary: 0.600 m × 75 µm, 0.500 m to detector; electrolyte: (a) 5.0 mM sodium chromate (pH 8.5), 0.5 mM tetradecyltrimethylammonium bromide; (b) 5.0 mM chromic acid, adjust pH with Tris to pH 8.5, 0.5 mM tetradecyltrimethylammonium bromide; separation voltage: −20 kV; injection: hydrostatic at 10 cm for 10 s, 0.1 mM of each anion in 50 mM sodium hydroxide; detection wavelength: 254 nm; temperature: 25 °C. Peaks: 1 = chloride, 2 = sulfate, 3 = nitrate, 4 = chlorate, 5 = phosphate, 6 = carbonate. (Reprinted with permission from reference 2.)

Separations of cations: The most powerful and straightforward tool for governing separation selectivity of metal cations is controlling the degree of complexation with auxiliary ligands such as α-hydroxycarboxylic acids (hydroxyisobutyric, lactic, and citric acids) or crown ethers, thereby controlling the effective mobility of the ion (3,6). Typically, the degree of complexation is kept low, so the effective charge on the metal ion averaged across the various species in equilibrium is still positive, and the analytes can be detected as cations using indirect detection with a cationic probe such as methylbenzylamine or imidazole. A different situation occurs when using auxiliary complexing ligands such as EDTA that form strong complexes with the metal ions and are mostly detected by direct detection. For cationic species exhibiting protolytic equilibria at the pH of the electrolyte (for example, weak organic bases), the same advice given for anionic analytes also applies, namely that a buffered electrolyte should be used. Overviews of CE applications for the determination of cations are available elsewhere (9,10).

Simultaneous determination of anions and cations: Anions and cations can be determined simultaneously by indirect detection if the electrolyte contains both positively and negatively charged probes. In the context of electrolyte buffering in a manner compatible with indirect detection, the probes should be mixed in their free acid and free base forms to avoid the presence of competing ions in the electrolyte. Buffering with a counterion (Table I) is impossible because both the electrolyte anion and the cation are probes, so buffering by one of the probes itself or by an isoelectric buffer are the only choices.

Despite the potential advantages of reduced analysis time and costs that come from analyzing both anions and cations in one run, a separate analysis of each group still is much easier and more robust. The simple fact that anions and cations migrate in opposite directions in an electric field means that a simultaneous analysis cannot be realized with one point each of sample introduction and detection. However, two approaches have proved successful. The first uses a sample injection simultaneously in two capillaries operated using the same electrolyte with both capillaries equipped with independent detectors located near their ends. A simpler approach developed by Kuban and Karlberg (11) uses one capillary, but the sample is injected at both ends and the detector is located approximately in the middle of the capillary. The indirect detection electrolyte was prepared from chromic acid and 4-aminopyridine, so no additional co-ions were introduced by mixing an anionic probe in free acid form and a cationic probe as a free base. The electrolyte (pH 8) was slightly buffered by the base itself (pK_a 9.1). This approach has been applied to the analysis of a range of real samples.

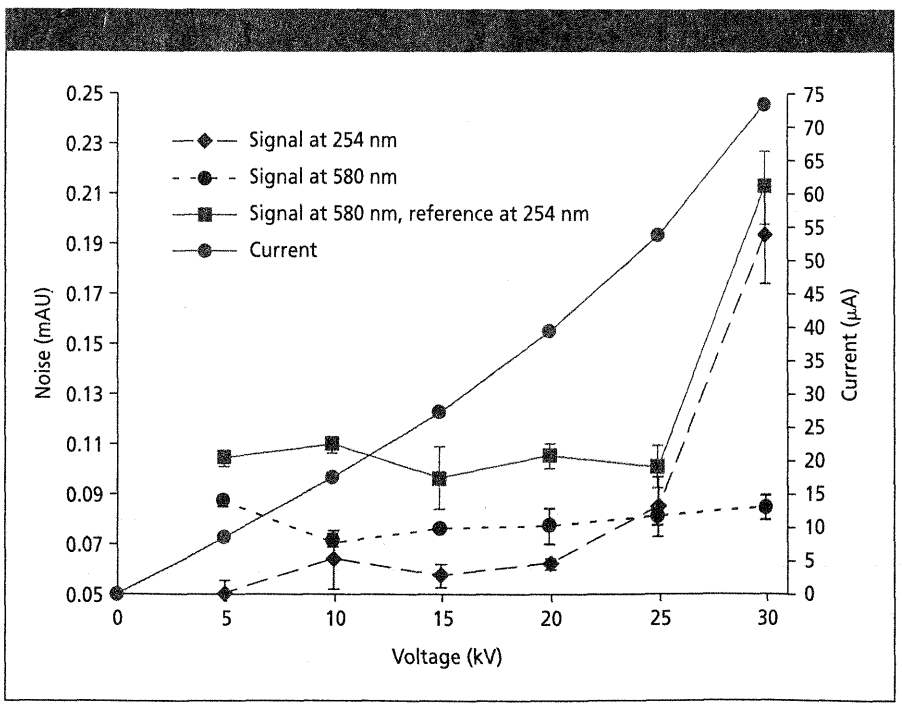

Figure 3: Plots of noise versus voltage for sodium chromate electrolyte at 254 and 580 nm. Capillary: 0.485 m × 75 μm, 0.400 m to detector; electrolyte: 5.0 mM sodium chromate (pH 8.0), 0.5 mM tetradecyltrimethylammonium bromide; detection wavelength: 254 nm; temperature: 25 °C.

Instrumental aspects — detection performance: *Two limitations of probe concentration — separation current and background absorbance:* In the "Mobility and concentration of the probe and the separation performance" section in Part I (1), we discussed that the maximum probe concentration should be used to minimize electromigrational peak broadening and sample zone broadening caused by the destacking of samples that have comparable conductivity to that of the electrolyte. The electrolyte concentration normally is limited primarily by the separation current, with too high a current causing decreased separation efficiency because of capillary overheating. However, the maximum probe concentration can be governed instead by the background absorbance, which may exceed the linear range of the detector, for some highly absorbing probes or on instruments with a limited-linear-range detector. Under these conditions, nonlinear calibration curves and poor detection sensitivity would be observed for the analytes. The limiting value of background absorbance can be determined by the method described below.

Checking baseline noise versus voltage: A standard test for determining the maximum current to be used for a particular electrolyte is to plot current versus voltage. A deviation from a linear relationship between these parameters indicates the maximum voltage that can be used without overheating the capillary. However, electrolytes to be used for indirect detection always have a substantial background absorbance, and we have found that an excessive current usually is indicated by an increase in baseline noise well before peak broadening is observable. Figure 3 illustrates this situation for a sodium chromate electrolyte. The increased noise may be a result of capillary overheating that causes the formation of microbubbles in the electrolyte — even though rigorous degassing of the electrolyte did not bring an improvement — or of refractive index changes. Increased noise occurs at separation currents of as little as approximately 50 μA (corresponding to 2.6 W/m), which is considerably less than the currents commonly used in CE with direct photometric detection. The Tris- or diethanolamine-buffered chromate electrolytes under the same conditions do not show this increase in baseline noise because the separation currents for the same chromate concentration are lower because of lower mobility of

signal and the reference wavelength. Although using a referencing wavelength often provides a less noisy and more stable baseline, Figure 5 illustrates the opposite case in which a reference wavelength did not improve baseline stability and actually caused considerably more noise (caused by the lower light level at 590 nm compared with 254 nm). In the end, the referenced signal showed a noise level approximately twice that of the unreferenced signal.

Conclusion

Buffering electrolytes for indirect detection should be realized in a way that does not add co-ionic impurities to the electrolyte. This buffering can be achieved in four ways: to a limited extent by using co-ionic buffers of substantially different mobility to that of the probe, by buffering with the probe itself if it has a suitable pK_a by using counterionic buffers, and by using isoelectric buffers. Analysts must consider additional, practical aspects of method development such as the formulation of a background electrolyte that provides probe and analyte solubility and reversal or suppression of the electroosmotic flow. Finally, workers must account for instrumental factors such as maintaining the background absorbance of the electrolyte within the linear range of the detector and keeping the separation current below the threshold at which baseline noise becomes problematic.

Acknowledgment

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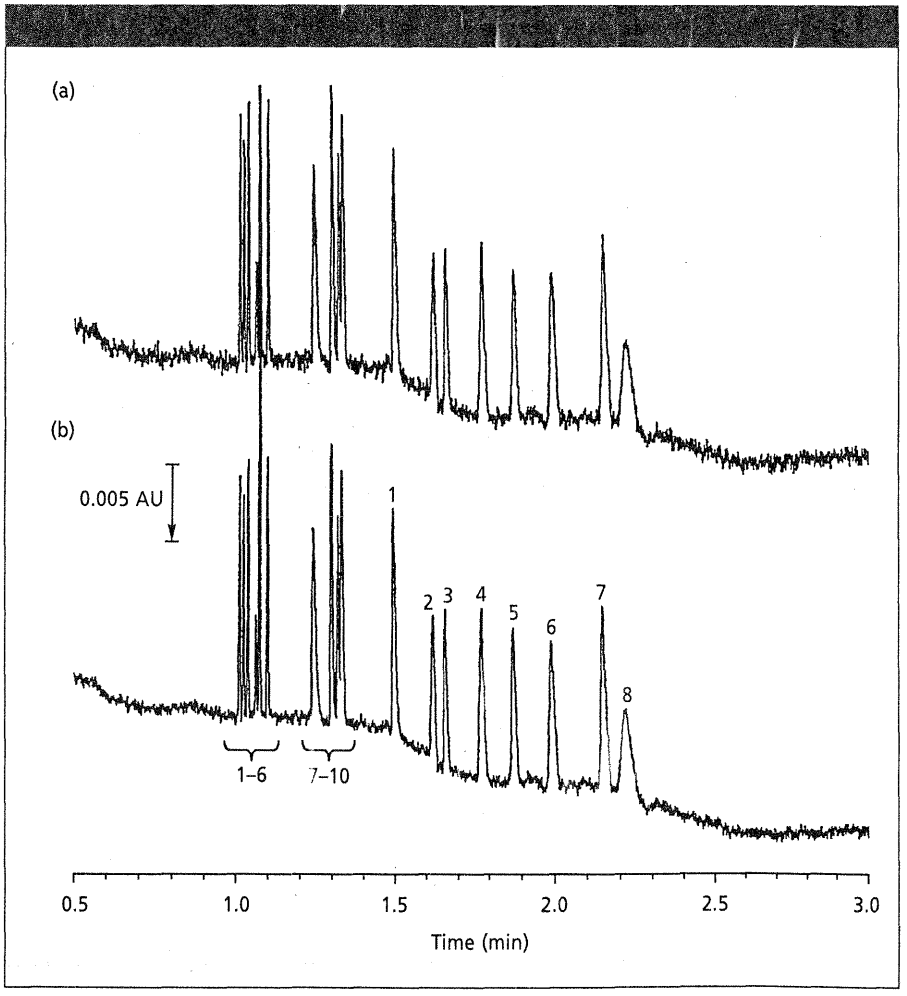

Figure 5: Electropherograms obtained using a 254-nm signal wavelength (a) with and (b) without a reference wavelength. Detection: (a) signal at 254 nm with an 8-nm bandwidth, reference at 590 nm with a 20-nm bandwidth; (b) signal at 254 nm with an 8-nm bandwidth, no reference. Other conditions were the same as in Figure 1a. Peaks: 1 = methanesulfonate, 2 = iodate, 3 = ethanesulfonate, 4 = propanesulfonate, 5 = butanesulfonate, 6 = pentanesulfonate, 7 = gluconate, 8 = hexanesulfonate.

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Erratum

The numbers on Figure 1's time axis in "Indirect Photometric Detection in CE Using Buffered Electrolytes — Part I, Principles" (*LCGC* **19**[1], 40 [2001]) were incorrect. The correct numbers are 2.2, 4.2, and 6.2.