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High-salt stacking principles and sweeping: comments and contrasts on mechanisms for high-sensitivity analysis in capillary electrophoresis

James F. Palmer*

Department of Physiology and Biophysics, University of California, Medical Sciences I, C-337, Irvine, CA 92697, USA

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Abstract

High-salt stacking in electrokinetic chromatography (EKC) is defined and contrasted to the sweeping method. A recent paper argued the two methods are identical, where high concentrations of micelle in the sample were intended to mimic the effect of high-salt stacking. However, high micelle concentration in the sample matrix in EKC is analogous to using a high-conductivity sample instead of a low-conductivity sample in field amplified stacking. High-salt stacking does not require a sample free of pseuostationary phase, only a sample with a high-mobility co-ion compared to the separation buffer electrokinetic vector. High-salt stacking uses a discontinuous buffer system and should not be confused with continuous buffer stacking systems such as sweeping.

Keywords: High-salt stacking; Sweeping; On-line sample concentration; Electrokinetic chromatography

1. Introduction

Capillary electrophoresis is a high-resolution analytical separation method [1]. Small sample injection volume has been addressed with stacking techniques for charged analytes [2,3]. Neutral analytes can be separated by a method known as electrokinetic chromatography (EKC) [4]. High-salt stacking in EKC was originally developed as an on-line concentration method for neutral corticosteroids [5]. The conditions for this electrophoresis technique include a separation buffer consisting of an anionic micelle (sodium cholate) and an electrolyte (disodium phosphate) at pH ∼ 9 that provide a consistent electroosmotic flow with bare fused silica capillaries. It was found that a sufficient concentration of either sodium chloride or sulfated β-cyclodextrin dissolved in the sample matrix provided unprecedented stacking of neutral molecules in EKC. Concentrations of sulfated β-cyclodextrin from 0.25 to 5% in the sample matrix were examined. When compared to sample matrix sodium chloride concentrations of 0.0–180 mM, it was found the stacking effect was similar with stacking evident only when the sample matrix conductivity was higher than the separation buffer conductivity. It was postulated [5] and later affirmed [6] the effect of the high-salt sample matrix was to cause a stacking of micelles at the sample/separation buffer interface. Some conclusions of the original study [5] follow.

"It is postulated the critical factors for sample matrix concentration are (1) the sample anion has a larger charge-to-mass ratio than the run buffer micelle; and (2) the ionic strength of the sample matrix is higher than the run buffer. When the sample matrix has these characteristics, the run buffer micelles are initially concentrated at the detector end of the sample plug. This occurs through isotachophoresis of the run buffer micelles in the lower-ionic strength run buffer." Two studies followed the initial findings of the original study [5]. The first examined the effect of adding sodium chloride to the sample matrix [6]. The second examined the effect of different high concentration, high-mobility cyclodextrins in the sample matrix to initiate stacking [7].

The purpose of high-salt stacking is to provide sample matrix conditions that influence the separation buffer micelle concentration to induce a stacking effect. In each of the

* Tel.: +1-949-824-7784; fax: +1-949-824-8540.
E-mail address: jfpalmer@uci.edu (J.F. Palmer).

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previous high-salt stacking studies [5–7], it was shown that manipulation of the sample matrix constituents was critical to implement stacking. In a further study [8], the parameters required for high-salt stacking were represented by the equation:

$$\mu_{\text{sample}} E_{\text{sample}} < \mu_{\text{ev}} E_{\text{ev}} \quad (1)$$

where μ_{sample} and μ_{ev} are the electrophoretic mobilities of the sample matrix co-ion (ion with the same charge as the electrokinetic vector) and the electrokinetic vector in the separation buffer (commonly a micelle), and E_{sample} and E_{ev} are the electric field strengths in the sample matrix and separation buffer. In addition, the mobility of the sample matrix co-ion must be higher than that of the electrokinetic vector ($\mu_{\text{sample}} > \mu_{\text{ev}}$) [8]. For high-salt stacking, a common situation would be to use sodium chloride in the sample matrix, and a micelle such as sodium cholate in the separation buffer ($\mu_{\text{chloride}} > \mu_{\text{cholate}}$). After the high-salt sample matrix is injected and electrophoresis begins, the micelles stack at the interface with the sample matrix. When the sample matrix has a higher conductivity than the separation buffer, $E_{\text{sample}} < E_{\text{ev}}$. The micelle velocity will be greater in the separation buffer ($\mu_{\text{ev}} E_{\text{ev}}$), and less at the sample matrix interface ($\mu_{\text{ev}} E_{\text{sample}}$), causing micelles to stack. At the chloride/micelle interface, cholate micelles will accumulate, but are excluded from the chloride zone due to their lower intrinsic anodic mobility [6]. The micelle approaching the high-conductivity zone will never pass the sample co-ion in the high-conductivity zone because $\mu_{\text{sample}} E_{\text{sample}}$ is greater than $\mu_{\text{ev}} E_{\text{sample}}$. Stacking of the analytes is secondary to the stacking of the micelles: analytes experience a reduction in velocity upon encountering the stacked micelle/sample zone interface. This is due to the different velocity of analytes in the sample zone (EOF velocity) versus the velocity of analytes in the stacked micelle zone (enhanced counter-EOF mobility and reduced velocity due to a high local micelle concentration) [6]. In sweeping, there is no micellar stacking observed, and the velocity of analytes in the sample zone in the presence of micelles is the same as the velocity of the analytes in the separation buffer.

In contrast to the high-salt stacking method described above, the sweeping method ignores the importance of the sample matrix composition. In sweeping, the sample matrix was designed to be continuous with the separation buffer and equivalent in conductivity [9]. This is implemented by adjusting the sample matrix to an equal conductivity with the separation buffer using the same electrolyte as found in the separation buffer (e.g., phosphate). When the sample matrix ions have the same mobility as the separation buffer ions, the system is termed “continuous”. When the sample matrix ions have a different mobility than the separation buffer, as in high-salt stacking, the system is termed “discontinuous”. For simplicity, cations are generally continuous in either system, with hydrogen or sodium cations in both the sample matrix and the separation buffer. In EKC, the electrokinetic vector in the separation buffer (e.g. SDS, cholate) is usually anionic. The anion in the sample matrix is then termed as the co-ion.

In a recent publication [10], the theory of high-salt stacking was questioned. It was suggested that while stacking of the micelles occurs with high-salt sample matrixes, there is also an equal and opposite destacking process. This was represented in two equations, the first of which describes the increase in concentration of a micelle solution when it enters a high-conductivity sample zone [2]:

$$C_{\text{PS}}(\text{S}) = C_{\text{PS}}(\text{BGS})\gamma' \quad (2)$$

where $C_{\text{PS}}(\text{S})$ is the concentration of the pseudostationary phase entering the sample zone, $C_{\text{PS}}(\text{BGS})$ is the concentration of the pseudostationary phase in the separation buffer, and γ' is the enhancement factor which is equal to the ratio of the conductivity of the sample matrix to the separation buffer. The next equation suggests the stacked pseudostationary phase undergoes a destacking process [10] described by:

$$C_{\text{PS}}(\text{destacked}) = \frac{C_{\text{PS}}(\text{S})}{\gamma'} \quad (3)$$

It was further stated that the final swept zones in a homogeneous (the sweeping condition) and reduced electric field (high-salt stacking) system should give similar lengths. According to [10], the resulting analyte zone length in a reduced electric field system can be approximated by:

$$l_{\text{sweep}} = l_{\text{inj}} \left(\frac{1}{1+k} \right) \quad (4)$$

where l_{inj} is the length of the injected sample zone, l_{sweep} is the resulting length of the swept zone, and k is the retention factor [9]. To explain the sweeping effect in a sample matrix with a low conductivity [10], Eq. (5) predicts an initial destacking:

$$C_{\text{PS}}(S) = \frac{C_{\text{PS}}(\text{BGS})}{\gamma} \quad (5)$$

and Eq. (6) suggests and equal and opposite restacking:

$$C_{\text{PS}}(\text{stacked}) = C_{\text{PS}}(\text{S})\gamma \quad (6)$$

These equations indicate the sample matrix conductivity, whether lower, equal to or higher than the separation buffer, has no effect on the subsequent stacking or focusing of analytes in EKC. According to sweeping theory, the concentration of the sample matrix co-ion does not affect the outcome of stacking, and the resulting stacking effect is described by Eq. (4) regardless of the salt content of the sample matrix. Stacking efficiency according to Eq. (4) is dictated by the k-value of a given analyte in a given separation buffer.

Based on Eqs. (2)–(6), Quirino et al. [10] used several experiments to argue a similarity between high-salt stacking and sweeping. Their first experiment used a high concentration of micelle in the sample matrix to emulate high-salt stacking and a postulated destacking of analytes. A second experiment examined stacking in low, equal, and

high-conductivity sample matrixes with a high-mobility co-ion in the sample. The *k*-value of an analyte in mixtures of cholate and sodium chloride was used to explain that high-salt stacking is caused by an increase in *k*-value due to the presence of chloride, not by micelle stacking. These experiments are examined below in greater detail, and several distinctions between high-salt stacking and sweeping are demonstrated.

2. Results and discussion

2.1. Destacking

Eqs. (2) and (3) suggest futility in optimizing stacking in EKC by increasing sample matrix conductivity. Eq. (2), adapted from [2], was used to describe field-amplified stacking of charged analytes. It is important to note [2] deals specifically and solely with stacking under continuous conditions where analytes are dissolved in a sample matrix consisting of separation buffer and water. As defined in Eq. (1), high-salt stacking relies on a discontinuous buffer system, and is not described by Eqs. (2)–(6). The validity of the experiments based on continuous buffer systems to emulate high-salt stacking [10] is examined below.

Quirino et al. [10], injected neutral analytes in a sample matrix consisting of separation buffer with low and high concentrations of SDS relative to the separation buffer and observed the stacking effect after electrophoresis. The separation buffer was 40 mM phosphate at pH 2.5 with 80 mM SDS, and the sample matrix was 40 mM phosphate with 80 or 240 mM SDS. It was found the analyte peak width at half height nearly doubled when using the 240 mM SDS sample matrix. This was given as evidence of analyte destacking with high-salt sample matrixes. However, stacking in a continuous buffer system is afforded by dissolving samples in a dilute separation buffer [2], not a concentrated one. For an example of this type of on-column stacking in EKC, Liu et al. [11] dissolved neutral analytes in a low-concentration micellar solution to achieve field-amplified stacking of neutral analytes. Adding a high concentration of micelles to the sample matrix in a continuous buffer system in EKC is not analogous to high-salt stacking. It is analogous to using a high-conductivity sample instead of a low-conductivity sample in field amplified stacking [2].

In the Quirino et al. experiment described above, the micelle in the sample matrix was the same as the micelle in the separation buffer. Therefore, the discontinuous conditions required to cause the high-salt stacking did not exist. For high-salt stacking, a higher mobility pseudostationary phase or co-ion would be used in the sample matrix. In the original manifestation of high-salt stacking, highly-sulfated β-cyclodextrin was used as the sample matrix co-ion, and sodium cholate was used as the separation buffer micelle [5]. It was determined that a sulfated β-cyclodextrin concentration of approximately 3% provided an optimum stacking of the neutral corticosteroids with a separation buffer of 80 mM cholate with 10 mM phosphate. The separation of the analytes using only 3% sulfated β-cyclodextrin in the separation buffer was also observed, validating the sulfated β-cyclodextrin as a pseudostationary phase.

Stacking with sulfated β-cyclodextrin in the sample was further studied using three estrogens as neutral analytes [7]. Using a separation buffer of 80 mM sodium cholate, 5 mM borate/phosphate and 20% acetonitrile, the effect of sulfated β-cyclodextrin concentration in the sample matrix can be

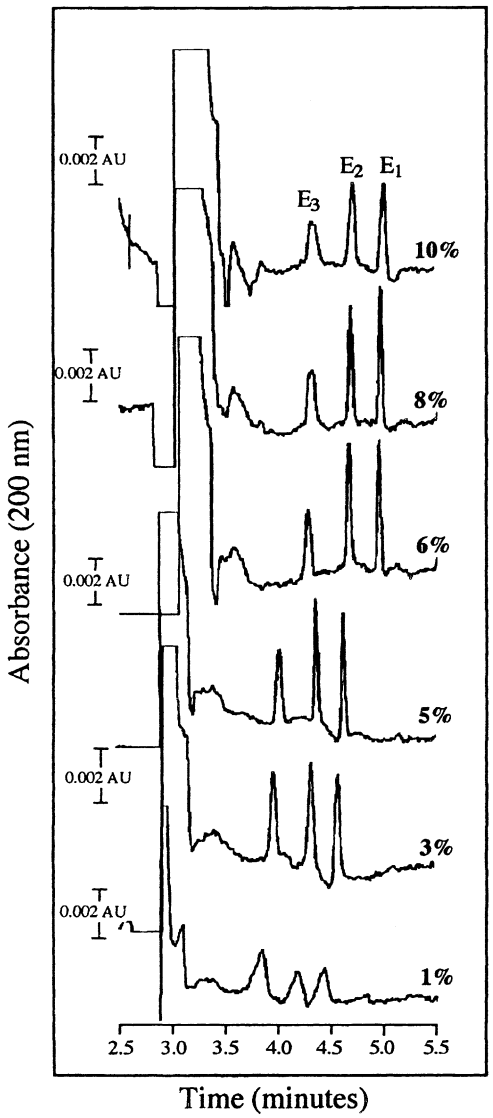

Fig. 1. Optimal sβ-CD concentration for stacking. Standard injection of estrogens in 1, 3, 5, 6, 8, and 10% sβ-CD. 64-s Hydrodynamic injection. Separation buffer: 5 mM borate–5 mM phosphate–80 mM sodium cholate–20% acetonitrile, pH 8.9 (reprinted from Fig. 2, p. 373 [7], with permission from Elsevier). Other conditions [7]: polyimide-coated, bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) used in these experiments were 47 cm (40 cm to the detector) × 50 μm i.d. × 375 μm o.d. Polarity was normal (the inlet was the anode), the capillary temperature was maintained at 20 °C and detection was by UV absorbance at 200 nm.

seen in Fig. 1. Each sulfated β-cyclodextrin sample matrix concentration that has higher conductivity than the separation buffer (above the 1% concentration) stacks the three analytes. This phenomenon can be explained by Eq. (1): With sulfated β-cyclodextrin as the sample co-ion, and the mobility of the sulfated β-cyclodextrin greater than that of cholate, the stricture $\mu_{sample} > \mu_{ev}$ is fulfilled. For Eq. (1) to be true, the electric field in the sample must be diminished, because the mobility of the sample matrix co-ion is greater than the electrokinetic vector mobility. The electric field in the sample zone is diminished by increasing the concentration of the sample matrix co-ion, and stacking is observed above the 1% sulfated β-cyclodextrin concentration as seen in Fig. 1. With sulfated β-cyclodextrin as the sample co-ion, one would not expect any stacking according to Eq. (4), where the critical condition for sweeping is "a sample matrix free of the additive (*pseudostationary phase*)" [10]. This critical condition is not a prerequisite for high-salt stacking.

The destacking concept described by Eqs. (2)–(6) has not been shown to be applicable to high-salt stacking with a discontinuous buffer system. If destacking caused a decrease in analyte velocity as stated in [10], it would actually cause a secondary stacking effect. This would have to occur under ITP conditions or the destacking micelles would have an offsetting increase in velocity. If the micelles could be made to destack to lower than the critical micelle concentration, infinite stacking would be possible for analytes with any *k*-value (although a secondary separation mode would have to be found). If analytes migrated into a trailing solution of lower-concentration, higher velocity micelles, a secondary sharpening effect would likewise occur. According to Eqs. (2)–(6), high-salt stacking under continuous conditions could be accomplished by injecting the high-conductivity solution in the detector-end of the capillary so that it formed a zone that encompassed the detector window.

2.2. Stacking

Quirino et al. [10] implemented the high-salt stacking conditions prescribed in [6] with a final experiment. With 80 mM cholate separation buffer, sample matrixes containing 25, 50, and 150 mM sodium chloride at injected lengths of 14, 21, and 35 mm were used. The effect on stacking three neutral analytes can be seen in Fig. 2. The examples of 25 and 50 mM sodium chloride sample matrixes (panels (A) and (B)) exhibit similar analyte peak height. It has been noted previously that the 50 mM sodium chloride sample matrix is similar in conductivity to the 80 mM cholate separation buffer [6], making the only example of high-salt stacking that fulfills Eq. (1) the injection with 150 mM sodium chloride shown in panel (C). There is a sharp increase in peak height for the progesterone analyte (#3) in panel (C).

The sharp increase in peak height for progesterone with the 150 mM high-salt sample matrix was attributed by Quirino et al. [10] to the fact the retention factors are significantly increased in high-salt concentration matrixes. This suggests the analyte, cholate, and chloride are in the same zone as they pass the detector. They examined the retention factor for progesterone in separation buffer containing 25 mM sodium chloride and 150 mM sodium chloride.

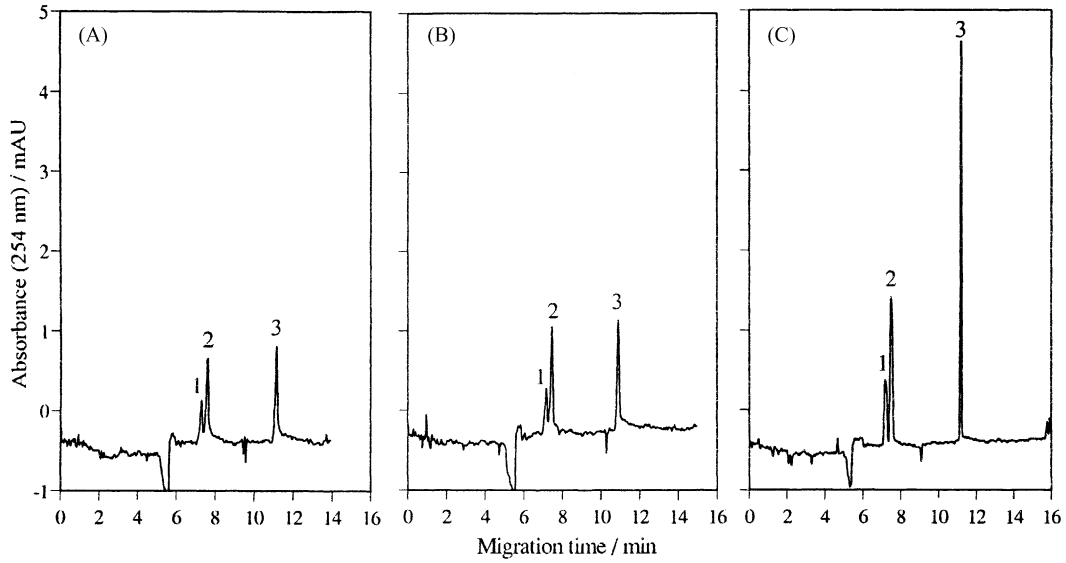

Fig. 2. Effects of salt concentration in sample matrices on maximum sample injection lengths to obtain highest concentration efficiency in sweeping. Peak identification: (1) cortisone (3.3 ppm); (2) hydrocortisone (4.0 ppm); (3) progesterone (5.0 ppm). BGS or separation solution, 80 mM sodium cholate in 10 mM sodium tetraborate containing 10% ethanol; sample matrix, 25 mM NaCl (A), 50 mM NaCl (B), 150 mM sodium chloride (C); injected sample plug length, 14 mm (pressure injection for 20 s at 50 mbar) (A), 21 mm (30 s) (B), 35 mm (50 s) (C); applied voltage 30 kV; detection, UV absorbance at 254 nm (reprinted from [10] with permission from Elsevier). Other conditions [10]: capillary 50 μm i.d. × 60 cm, temperature 25 °C, CE instrument, Hewlett-Packard 3D-CE.

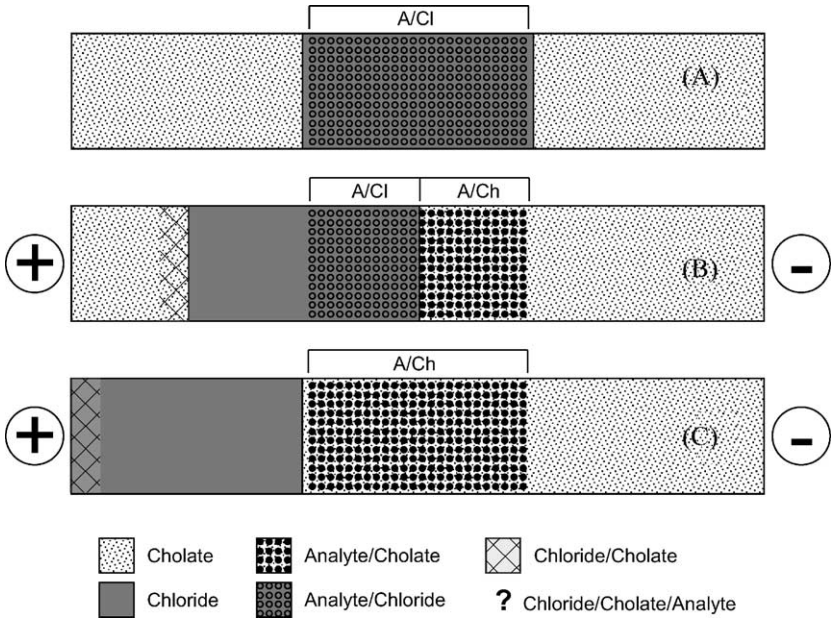

Fig. 3. Movement of anionic species with high-salt sample stacking conditions. In panel (A), a high-salt sample matrix is depicted in dark shade in the middle of a capillary. The stippled regions to either side correspond to micellar separation buffer. Neutral analytes are depicted as open circles. In panel (B), electrophoresis is initiated, with the anode to the left. The movement of the anodic components is shown. Chloride moves to the left, and diffuses into the cholate on the left side. On the right side of the chloride region, the lower-concentration cholate will stack, but not overtake the higher mobility chloride region. In panel (C), neutral analytes complex with micelles (dark circles) upon emerging from the chloride region into the cholate region.

They reported a retention factor of ∼24 for 80 mM sodium cholate with 25 mM sodium chloride, and a retention factor of ∼40 for 80 mM cholate with 150 mM sodium chloride [10]. This reasoning implies that high-salt stacking is from a separation buffer effect, not a sample matrix effect, thus identical to sweeping.

However, there is no time during high-salt stacking or subsequent separation that the analytes, micelles, and chloride are in the same region at the same time. This is illustrated in Fig. 3. In the first panel (A), a sodium chloride sample with neutral analytes is depicted in the dark region in the center. The stippled regions on either side correspond to micelles. The movement of chloride and cholate are toward the anode (to the left), and the neutral analytes (depicted by circles) do not move. Stacking is not shown in this diagram to make interpretation simpler. In panel (B), electrophoresis has begun and the chloride and cholate move to the left. The chloride has a higher mobility but also a higher concentration than the cholate. The boundary between the chloride and cholate at the cathode side of the sample region should be sharp, non-diffusive, and pseudosteady-state [12]. Cholate does not penetrate this boundary, but stacks up against it [6]. In order for cholate to penetrate the chloride region, the mobility of the cholate would have to be greater than chloride, but this is not the case ($\mu_{ev}E_{sample} < \mu_{sample}E_{sample}$). The analytes pass from the left of the boundary to the right. Analytes are never in a mixture of cholate and chloride at the same time. As stated previously [6], there may be mixture of the cholate and chloride at the anode-side of the high-salt sample matrix due to diffusion of the higher mobility chloride into the micellar buffer, however, there are no analytes in that region. Analyte/chloride and analyte/cholate zones are indicated by A/Cl and A/Ch in the figure. The high-salt stacking effect clearly seen in Fig. 2 is certainly a sample matrix effect, not a separation buffer effect. It is clear that Eqs. (2)–(6) do not explain this high-salt stacking effect. A more complete examination of the effect of salt concentration in the sample matrix on stacking can be found in [6].

3. Conclusions

The original conclusions for high-salt stacking are valid to afford stacking in EKC [5]. The use of a high-mobility sample co-ion at a higher concentration than the separation buffer micelle affords high-salt stacking. Emulating high-salt stacking with high concentrations of separation buffer micelles [10] is actually analogous to loading charged analytes in a high-salt sample in field-amplified stacking [2]. It is not valid to use continuous buffer conditions to emulate high-salt stacking. It is shown when a high-mobility co-ion that is also a pseudostationary phase (sulfated β-cyclodextrin) is added to the sample matrix in accordance with high-salt stacking procedures, stacking is achieved. This is not predicted by sweeping theory, where the critical condition is a sample matrix free of the additive (pseudostationary phase) [10]. The stacking effect observed with high-salt sample matrixes is also not due to a higher retention value in the presence of chloride. It is shown that chloride, micelles, and the analyte are never in the same zone at the same time.

The original conditions suggested for high-salt stacking [5] have not changed [6–8] and are not dependent on sweeping theory developed since [9,10,13]. High-salt stacking was the first stacking method in EKC to use a sample matrix devoid of any pseudostationary phase [5]. It was the first stacking method in EKC to be translated to the microchip format, and the first electrophoretic injection of any type to successfully exceed the effective length of the capillary [8]. Future references concerning high-salt stacking should not incorrectly be referred to as sweeping. For independent corroboration of the high-salt stacking effect, see [14,15].

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