Differences in Absorbance Levels as Found in Capillary Zone Electrophoresis under Stacking Conditions

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Summary
During some capillary zone electrophoresis (CZE) experiments, the baseline UV absorbance signal at 200 nm “jumped” from one stable level prior to the water plug (marking the flow of neutrals) to another stable level after the water plug. The phenomenon was further examined with distilled water as the sample and with different buffers, applied potentials, and salt concentrations in the buffer. It seems that there is an “isotachophoretic effect” on top of the CZE separations when running under stacking conditions. The effect results in a higher pH value of the buffer after the water plug compared to the pH prior to the plug. The nature of the buffer, the salt concentration in the buffer, and the applied potential all affect this phenomenon.

1 Introduction
Stacking of the sample zone has been used to increase detectability in capillary zone electrophoresis [1, 2]. Sample stacking is the process occurring when applying a voltage along a capillary tube containing a sample zone with a lower conductivity than the surrounding running buffer. As the electric field is inversely proportional to the specific conductivity of the liquid, the field strength is higher along the sample zone compared to the running buffer. A “reversed dispersion” occurs, which results in a narrowed sample zone.

During some of our CZE experiments, where stacking conditions were used, a peculiar effect was observed: The baseline UV absorbance signal at 200 nm “jumped” from one stable level before the water plug (marking the electro-osmotic flow = flow of neutrals) to another stable level after the water plug.

In order to further examine this phenomenon, we performed a series of experiments, where the sample plug was distilled water and the running buffer was either tricine or tris containing 0, 12.5, 25, or 50 mM NaCl.

This report summarizes these experiments and the results we obtained.

2 Materials and Methods

2.1 Materials and Equipment
A fused silica capillary with an inner diameter of 50 μm, 100 cm total length and 75 cm to the detector was obtained from Polymicro Technologies (Phoenix, AZ, USA). Tris (Tris(hydroxy-
methyl)aminomethane), tricine (N-[Tris(hydroxymethyl)meth-
yl]glycine) and NaCl were from Fluka (Buchs, Switzerland), while HCl and NaOH were from Merck (Darmstadt, FRG). All the experiments were carried out on an Applied Biosystems Model 270A Capillary Electrophoresis apparatus (Foster City, CA, USA), and recorded on a BBC Goerz Metrawatt SE120 chart recorder (Vienna, Austria).

2.2 Methods
All experiments were performed at 27 °C, the absorbance signal was measured at 200 nm, and the sample was distilled water. Two sets of experiments were performed.

a) Absorbance jump as a function of NaCl concentration in the buffer
The capillary was washed for 2 min with a 0.1 M NaOH solution, and then for 5 min with the running buffer. Sample was introduced for 1 s by applying a vacuum (ca. 16.8 kPa). The sample consisted of distilled water. The increased baseline absorbance signal from the leading to the terminating edge of the water plug was measured.

The experiments were carried out with a 10 mM tricine buffer adjusted to pH 8.0 with 1 M NaOH as well as a 10 mM tris buffer adjusted to pH 8.0 with 1 M HCl. Tricine and tris buffers were made containing 0, 12.5, 25, or 50 mM NaCl, respectively. The experiments were run with an applied potential of 30 as well as 15 kV.
b) Absorbance of tricine, tris and NaOH at various pH values

10 mmol tricine ($M_w = 179.18$, $pK_a = 8.15$) was dissolved in 1 liter distilled water. pH was adjusted with $1 \times$ NaOH in the pH range 5.55–11.60 and the absorbance of the buffer measured at intermediate pH values by introducing it into the capillary by means of vacuum.

10 mmole tris ($M_w = 121.14$, $pK_a = 8.3$) was dissolved in 1 liter distilled water. pH was adjusted with $1 \times$ HCl in the range 9.89–3.16 and the absorbance of the buffer was measured at intermediate pH values as described above.

A NaOH solution was prepared by dissolving a NaOH pellet in distilled water and diluting the solution to a pH value of approximately 12. $1 \times$ HCl was added, thus adjusting the pH of the solution in the range 11.99–3.57. Absorbance was measured at intermediate pH values as described above.

3 Results and Discussion

Figures 1 and 2 show examples of the electropherograms which were obtained during the experiments with the tricine and tris buffers, respectively. The "jump" in baseline signal from the leading edge of the water plug (the negative peak) to the terminating edge of the plug is observed in all eight electropherograms. The jump is highest when the tricine buffer is employed as the running buffer. Besides, increasing the applied voltage or the concentration of NaCl seems to result in increased absorbance jumps.

Figure 1
Examples of the absorbance jumps observed when a pH 8.0 10 mM tricine was the running buffer. The absorbance baseline signal increases from one stable level prior to the water plug (the negative peak, marking the neutrals) to another stable level after the plug. Distilled water was the sample zone. For further details, see the text. a) 30 kV applied, 25 mM NaCl in the buffer, b) 15 kV applied, 25 mM NaCl in the buffer, c) 30 kV applied, no NaCl in the buffer, d) 15 kV applied, no NaCl in the buffer.

Figure 2
Examples of the absorbance jumps observed when a pH 8.0 10 mM tris was the running buffer. The absorbance baseline signal increases from one stable level prior to the water plug (the negative peak, marking the neutrals) to another stable level after the plug. Distilled water was the sample zone. For further details, see the text. a) 30 kV applied, 25 mM NaCl in the buffer, b) 15 kV applied, 25 mM NaCl in the buffer, c) 30 kV applied, no NaCl in the buffer, d) 15 kV applied, no NaCl in the buffer.
Absorbance jump of the tricine and tris buffers at 15 and 30 kV as a function of the NaCl concentration in the buffer. See the text for further details.

Figure 3 depicts the absorbance jumps as a function of the applied potential and the concentration of NaCl. With the tricine buffer it is observed that the increasing absorbance jump levels off as the NaCl concentration increases, thus indicating some kind of saturation mechanism.

The jump in absorbance baseline signal means that the composition of the running buffer prior to the neutral zone is not identical to the composition of the running buffer after the zone. But what are the differences between the two "plugs"? The absorbance of the 10 mM tricine and 10 mM tris buffers as well as a NaOH solution (see the experimental section for details) was measured as a function of pH. The results are shown in Figure 4 as the "uncorrected curves".

The absorbance increases dramatically for both the tricine and the tris buffer in the proximity of the pKₐ value (tricine: pKₐ = 8.15, tris: pKₐ = 8.3). Absorbance of the NaOH solution was only measurable for pH values exceeding 10. From Figure 4 it is concluded that the tricine and tris buffers absorb more light at 200 nm as bases compared to the corresponding acids. The reason for the exponentially increasing absorbance of the tricine buffer with increasing pH at high pH values lies in the combined absorbance of the tricine base as well as OH⁻. Hence, if the absorbance of OH⁻ is subtracted from the absorbance of the tricine and tris buffers at each measured pH value, the "corrected" curves in Figure 4 result.

The base concentration is calculated at each measured pH value by the use of the acid-base equation

$$[B] = \frac{C₀}{10^{pH - pKₐ} + 1} \tag{1}$$

while the OH⁻ concentration is calculated by

$$[OH⁻] = 10^{pH - 14} \tag{2}$$

[B] is the base concentration, C₀ is the total buffer concentration, and Kₐ is the acid constant.

If the absorbance of the three solutions are depicted vs. the base concentration, the curves reveal a linear relationship as shown in Figure 5. Linear regression of the values in Figure 5 yields Figures 6a and 6b, where the absorbance of the tricine and tris buffer are depicted vs. the base concentration and the pH, respectively.

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Figures 4–6 indicate that the jump in absorbance as observed in Figures 1 and 2 could be due to a pH effect resulting in a higher pH value after the water plug than before the plug. The absorbance jump is higher for the tricine buffer runs compared to the tris buffer runs, among other things due to the fact that the tricine
As an example, an absorbance jump equal to 2 mAU would then give a pH jump equal to 0.05 if the pH value prior to the water plug is 8.0. If the peculiar effect is due to a pH jump, it seems as though the jump is lower for the tris buffer compared to the tricine buffer.

The results as discussed above reveal that during CZE with stacking conditions, the buffer zone prior to the neutral plug is not identical with the buffer zone after the plug. Hence, zone electrophoresis of the analytes is not the only process taking place in the capillary. To us it seems most likely that isotachophoresis occurs on top of the CZE separations, thus resulting in a pH shift. Both low concentration levels of a sample plug [3, 4] and high concentration(s) of mobile compounds in the sample plug [5] lead to a "sharpening up" effect, due to "Köhrausch regulation functions" as he called it "die beharrliche Funktionen" [6]. In isotachophoresis it is well known that concentrations of the sample zones and the terminator zone are all adjusted to the concentration of the leading zone [7]:

$$\frac{C_1}{C_0} = \frac{m_1(m_2 + m_0)}{m_2(m_0 + m_1)}$$  \hspace{1cm} (4)

In this equation $C_1$ is the concentration of the leading ion, $C_0$ the concentration of the sample ion (or terminating ion), following the leading ion, $m_1$ is the mobility of the leading ion, $m_0$ is the mobility of the sample ion (terminating ion), and $m_2$ is the mobility of the (buffering) counter ion. All mobilities are given as absolute mobilities (not vectorial quantities) at infinite dilution.

In isotachophoresis such a situation leads, by proper sampling, to a so-called steady-state; in CZE it leads to a stationary state, prior to the "elution phenomenon".

Immediately after the introduction of sample, the sample plug only contains $H^+$ and $OH^-$, while the surrounding buffer contains $H^+$ and $OH^-$ as well as $Na^+$, $Cl^-$ and the tricine or tris buffer in both the acid and base form. It is believed that the $H^+$ and $OH^-$ in the sample plug accounts for the "isotachophoretic effect", as the $H^+$ zone pulls the cations towards the cathode, while the $OH^-$ zone pulls the anions towards the anode. Prior to the sample plug, the acid/base ratio of the buffer increases due to the electroneutrality principle, while the ratio decreases after the sample plug. Hence, compared to the pH value of the buffer as measured outside the capillary, the pH of the surrounding buffer is lower prior to the sample plug and higher after the plug.

**References**


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