Ghostly Peaks and other Haunting Aberrations in CE

Troubleshooting workshop Tuesday 11 September at CEPharm 2018

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Capillary electrophoresis (CE) has become a well-established technique within the pharma and biotech industries. With its increased use, the knowledge base of rare phenomena and unexpected issues has increased as well. Therefore, it makes good sense to have a forum to share troubleshooting experiences and best working practices. Every year at the CEPharm conference, the committee organizes a troubleshooting workshop where some of industry's most experienced CE users share, discuss, and strive to solve the practical issues we encounter in applying CE technologies. This community shares acquired "tribal knowledge" and seeks to determine root causes of issues currently being encountered by participants. The hope is that this sharing will reduce time spent on analytical deviations and the invalid assay rate of CE Methods.

The troubleshooting examples discussed were submitted by workshop participants to the CEPharm Manager (CASSS.org) prior to the conference. Those issues discussed during the troubleshooting workshop are summarized here. No names or affiliations are mentioned so that everyone could feel free to add their thoughts, questions and experiences.

Below (Figure 1) is an example of a submission by a conference attendee. Note that it includes an electropherogram that allows for easy visualization of the issue. As well, there is a brief written description that provides the details necessary to understand the circumstance under which the issue was observed.

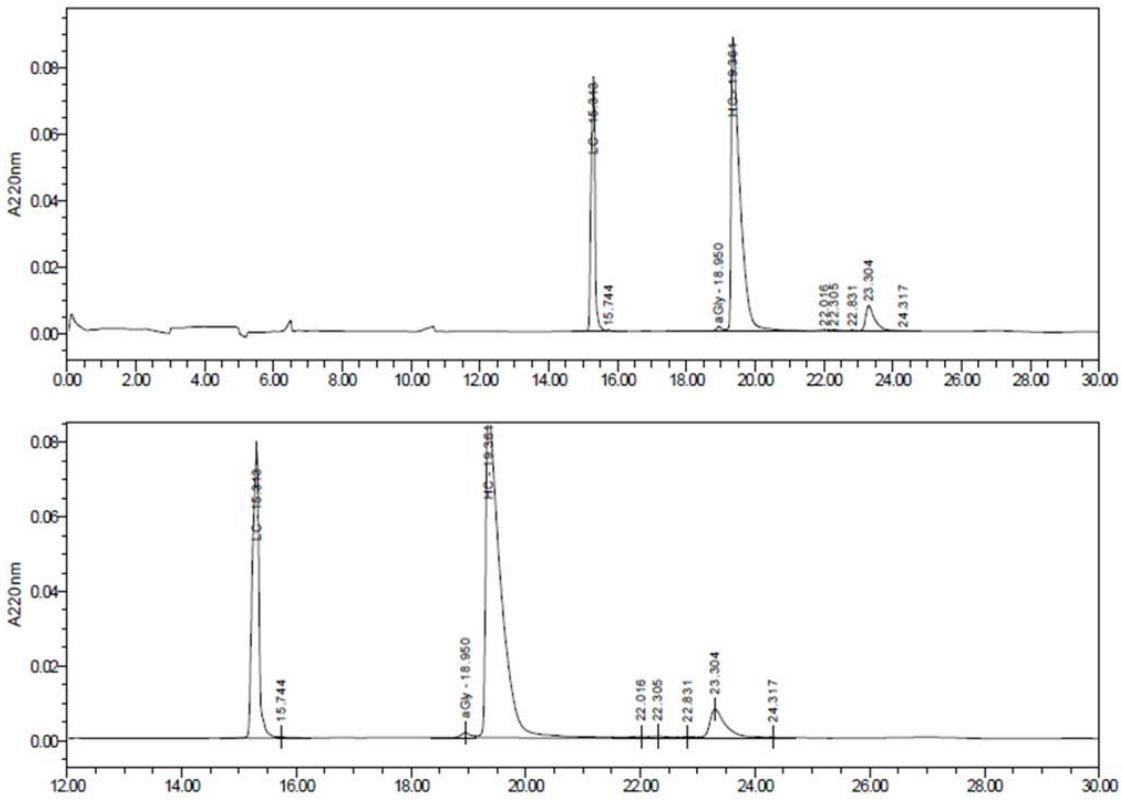

*Figure 1: Ghost peak in reduced CE-SDS. CE-SDS E-gram of a reduced mAb run on a PA800 Plus system (top: full scale; bottom: zoom-in view). Intitially the E-gram was as expected, typical for the mAb, on freshly prepared sample. However, a newpeak appeared at ~23.3 min after the prepared CE-SDS sample was stored for a few hours (> ~8hr). This peak was more obvious from sample reduced with **higher** b-ME concentration. Default sample preparation condition: 1 mg/mL of mAb, 100 uL Tris sample buffer (pH 9.0, 1% SDS), 10 uL of b-ME stock (14.3M), total volume: 200 uL, heated at 65 °C for 10 min. We screened different volumes of b-ME from 2 uL to 12 uL. The more b-ME, the more likely the additional peak would appear. We also tried higher heating temperature (70 °C) but it didn't solve this issue.*

Ghostpeaks in rCE-SDS – case 1

Ghost peaks are a recurring discussion topic for the troubleshooting workshop (Figure 1 is a common example of a ghost peak). A show of hands revealed that an overwhelming majority had encountered these aberrant peaks and that they continue to haunt labs throughout the Biopharma industry. Many Labs have tried to solve this issue independently and together the Troubleshooting Workshop has dedicated time to work through the issue the last several years [1].

A summary of the characteristics of ghost peaks is:

- Ghost peaks occur randomly, seemingly without correlation to experimental conditions.
- A sample that showed a ghost peak frequently does not show it on reinjection, it is hard to reproduce. This makes troubleshooting extremely difficult
- With multiple injections of the same sample, it is not always the first injection that shows the ghost peak. A range of frequencies have been reported from a round one in ten injection and even less frequent.
- At previous CEPharm meetings, there have been presentations that attributed the problem to certain types of vials, but these vials have been used by others without showing differences in occurrence [2–4]

- Ghost peaks have been observed in different types of detection and different formats (CE-SDS, MCE).
- Those that studied the issue in more detail could not attribute the occurrence to a specific surfactant, detector, vial, instrument, capillary or analyst.
- Examples of typically observed ghost peaks are given in Figure 1.

Random issues can occur no matter how well we understand and are in control of a method. If such random issues occur at a rate of 1 in a 1,000, for example, then it may be reasonable to conclude that they are truly random issues. However, if the frequency is closer to 1 in 100 or 1 in 10, then that is too frequent to assign randomness as a cause and calls for troubleshooting the method. At high frequencies (e.g. 1 in 100 or greater), serious consideration must be given that this atypical peak could be a sample real component (e.g. impurity), albeit it non-reproducible. There are some indications that the ghost peak is related to the heavy chain and could be due to incomplete denaturation during sample prep or possibly a re-oxidation linking two chains .

The following observations were mentioned by various participants:

- The participants were asked, by a show of hands, if that had encountered this ghost peak issue in Reduced CE-SDS of a mAb(s). The response was a sea of raised hands companied audibly with groans.
- Varying incubation conditions can increase or decrease the frequency that the ghost peaks are observed.
- A few participants noted that increased β-mercaptoethanol concentration increased the frequency that ghost peaks were observed.
- Increased SDS concentration in the sample buffer was mentioned to reduce the frequency.
- Chip-based platforms are more amenable to increased SDS in the sample because sample introduction into the capillary (i.e injection) is less effected than it is in the capillary format.
- Poorly cut capillaries can lead to random ghost peaks due to the trapping of minute amounts of protein at the capillary/polyimide coating juncture.
- There was some discussion and anectodes shared suggesting that the ghost peak could be occurring in the capillary (post sample introduction) and be a reaction product of protein (sample) and gel components (e.g. polymer).

Incomplete denaturation or reduction of the heavy chain should be studied during method development by trying to stress the sample prep to its extremes in an attempt to force the observation. A multifactorial design with high and low levels of temperature, SDS, β-mercaptoethanol etc. should be run with sufficient replicates to determine conditions that induce problems like the ghost peak.

In summary, the ghost peak is a persistent phenomenon that this community of CE Users has encountered extensively, made progress to control, but has yet to fully understand and eliminate. It was proposed that an inter-company collaboration be formed to once and for all determine the mechanism of this phenomenon and how to modify CE-SDS methodology to eliminate it.

Ghost peak (shoulder) in front of LC – case 2

A second example shows a randomly appearing ghost peak in front of the light chain (Figure 2). Multiple injections from the same vial shows this peak occurring in some injections while not in others. It occurs in standards as well as in samples, in normal and stressed samples, and without obvious trend or apparent correlation to experimental conditions.

The submitter noted that troubleshooting included:

- Confirmed that the capillary had good straight cut with the polyimide uniformly removed.
- Confirmed that the vials looked proper, the vial were correctly capped, the liquid levels were appropriate, and no air bubbles were observed.
- Confirmed that the instrument was clean and that routine maintenance procedure had been adhered to.
- Re-injection the old standard prep in a new sequence showed no issues (i.e. could not repeat the observed issue).

Some suggestions from the audience focused on the reduction of the LC. In reduced CE-SDS, both inter-chain and intra-chain disulfide bonds need to be reduced. Inter-chain disulfide bonds tend to reduce easier than intra-chain disulfide bonds and intra-chain disulfide bonds need stronger denaturing conditions for complete reduction [5]. The accessibility of the intra-chain bridges can be different depending on structural differences of the protein. This line of reasoning lead to the proposal that incomplete reduction of intra-chain could be the source of this shoulder. Varying the concentration and type of reducing agent was suggested to investigate further.

Another explanation could be the presence of hydrophobic patches on the protein that make denaturation with SDS insufficient. Increasing the SDS concentration might help, or as suggested in a talk the previous day [6, 7], the strong denaturant Sodium Hexadecyl Sulfate (SHS) reagent could be evaluated.

Another suggestion concerned the presence of solvents in the sample that might induce conformational changes in the protein.

All of suggested conditions underline the importance of thoroughly studying sample preparation during method development. We must always keep in mind that molecules are different, and invest time to investigate the particularities of each molecule to avoid needing to invest extensive time troubleshooting or re-development at an inconvenient time in the future. Finally, be vigilant to maintain a clean instrument, ensure capillaries have straight cut with the polyimide coating uniformly removed, be precise in pipetting and mixing (Figure 3).

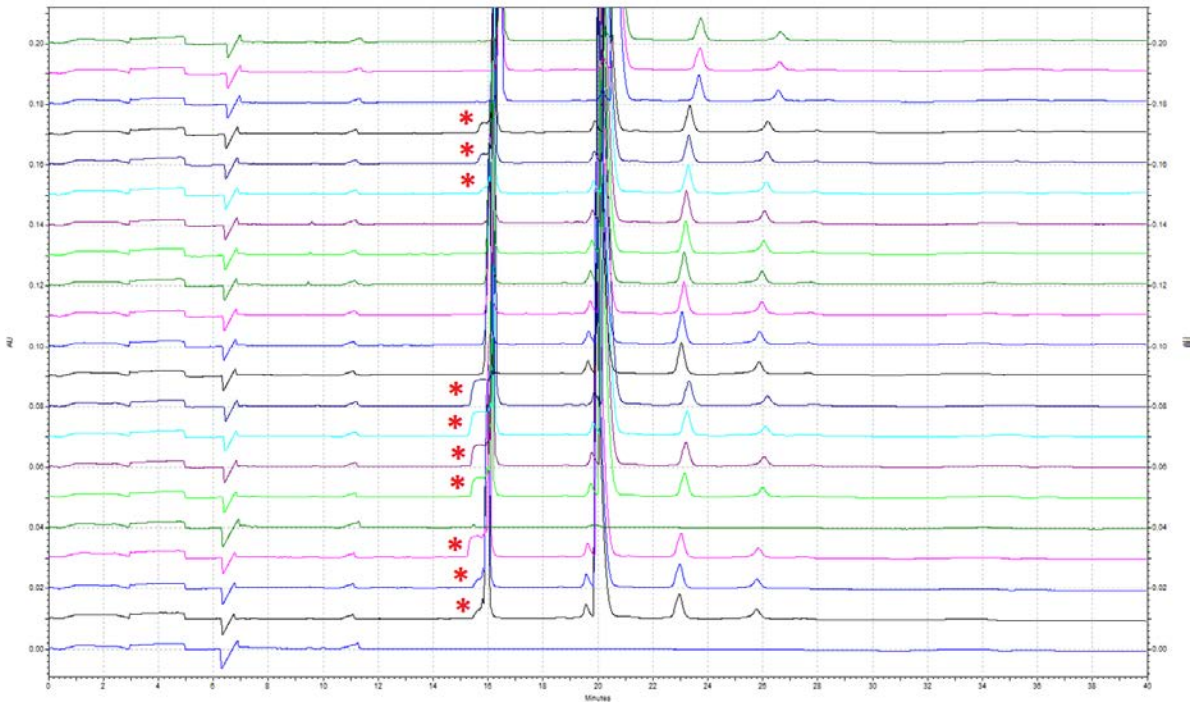

*Figure 2: ReducedCE-SDS of a mAb – A front side shoulder is observed on LC peak for some analyses (marked with *) but not others.*

Figure 3: The lucky four-leaved clover for successful CE-SDS analysis: Attention to a clean instrument, a capillary with straight cut ends and polyimide removed, precise pipetting and mixing (© Kantisto).

CZE - εACA quality

During the 2017 troubleshooting workshop [8] several participants reported that a change in the quality of ε-aminocaproic acid (eACA) had adversely impacted the performance of their CZE separations. They reported loss of resolution when using certain lots of εACA and indicated that they sought alternative suppliers. This year (2018) after investigating various vendors, several attendees communicated that alternative vendors also had "good" and "bad" batches of εACA. To make matters worse, one attendee reported that εACA buffers that had previously demonstrated stability for a year can now deteriorate within a week with current batches of εACA. This for a current batch that was initially screened and determined to be "good" batch.

Some participants shared that they had contacted εACA suppliers. Sigma confirmed that they had changed their raw material supplier for the US market, not for Europe. However, the suppliers are not highly motivated to thoroughly investigate CE community's issue apparently becasue their economic benefits would not outweigh the cost. Additionally, the Certificate of Analysis fails to provide any information useful in troubleshooting this issue. For the moment, the advice from last year remains: Test new lots before using them, and have a new approved lot ready before you completely consume the previous lot. Regardless, our community has a problem that we need to come together to solve.

It was debated if the root cause of this resolution issue could be due to insufficient capillary conditioning (as discussed in 2017) and whether the issue is consistent for coated as well as uncoated capillaries. Most companies indicate working with uncoated, bare fused silica capillaries for this method. Someone using the method with coated capillaries commented that the combination of HPMC and εACA and capillary conditioning together influence the resolution. HPMC has also been noted for having lot variability issues where viscosity differences are noticeable from lot to lot.

Starting a collaboration within the CE community to band together to resolve the εACA quality issues was suggested.

Migration time shift in CZE method

The next case submitted reported shifting migration times for a method with a 100 mM $(NH_4)_2HPO_4$ adjusted to pH 6.0 with phosphoric acid background electrolyte (BGE). Successive injections show a marked shifted to earlier migration times, suggesting an increased in EOF with each injection (Figure 4). An audience member spoke up and mention she would be presenting a poster [9] with a seemingly identical problem. She went on to describe that the NaOH storage container was crucial to preventing a trend in migration times with each subsequent injection. Experimentally, she found that temporarily storing the NaOH wash solution in borosilicate glass vials for at least 2 hours resulted in stable migration times. This period of time in glass vials may allow the NaOH wash solution to become saturated with silica and, in turn, facilitate more reproducible conditioning of the

capillary between runs.

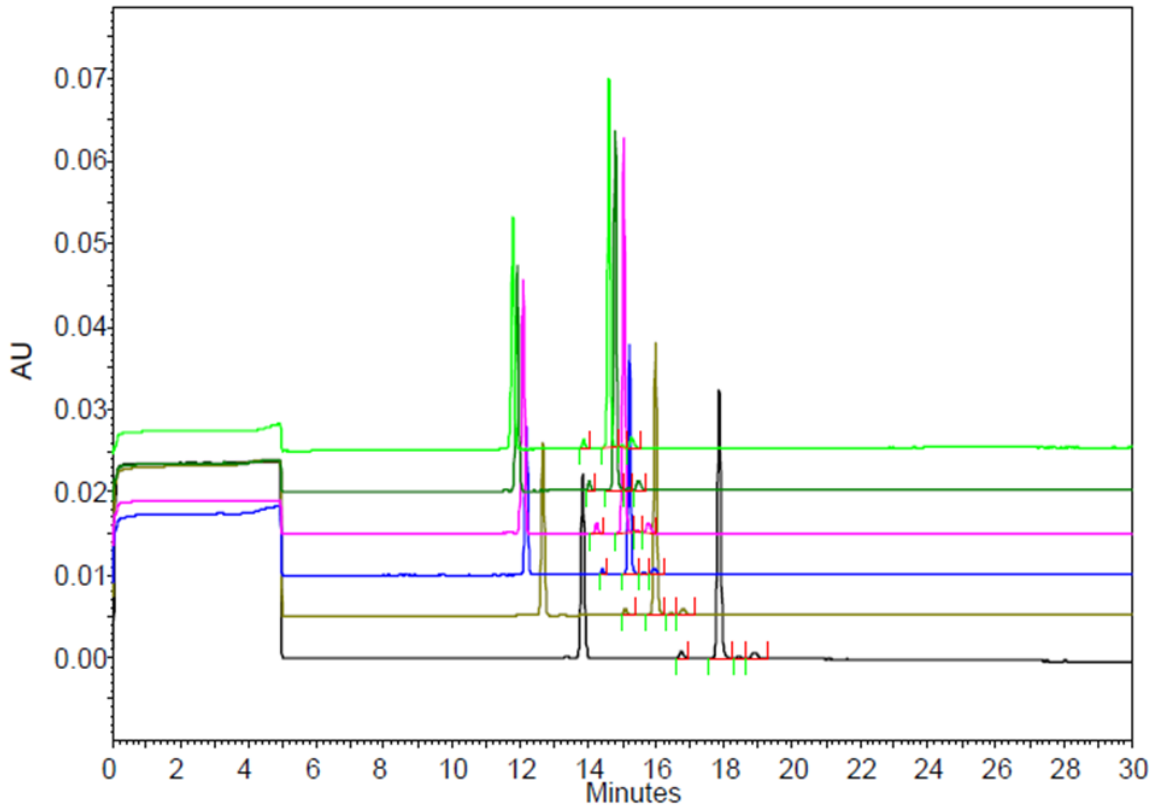

Figure 4: Migration time shifts in a CZE method. CZE buffer: 100 mM di-ammonium hydrogen phosphate adjusted to pH 6.0 with ortho-phosphoric acid (increment every 5 injections). Bare fused-silica capillaries with a total length of 60 cm / effective length of 50 cm and 50 µm inner diameter. Capillary Temperature: 30 °C. Voltage: 217 V/cm = 13 kV. The capillary is conditioned as follows: 1 M NaOH at 30 psi for 4.8 min, Pure water at 30 psi for 2.4 min, 0.1 M NaOH at 30 psi for 4.8 min, followed by an equilibration run: 0.1 M NaOH at 30 psi for 0.5 min, Electrolyte solution at 30 psi for 1.45 min. The equilibration run is performed before each sample injection. (anonymous submission)

While this method shows desirable peak shapes and resolution, there are some "good CE working practices" worth considering. This serves as an opportune time to review some:

- Phosphate is a buffer with pK_as at pH 2.16, 7.2 and 12.7. At pH 6, more than one pH unit away from the pK_a, the buffering capacity is low.
- The current traces are indicative of excessive Joule heating. The current at the beginning of the injection is 110 µA and gradually increases during the run. Both the high current and the gradual increase indicate excessive Joule heating. Excessive Joule heating results in temperature gradients across the capillary, which in turn result in convective liquid currents, band broadening and reproducibility issues. As a general rule of thumb, if the current is over 50 µA, there will be excessive Joule heating. An Ohm's Law plot, that is a plot of the current *vs.* the applied voltage, is no longer linear if there is excessive Joule heating (Figure 5) [10]. To generate a Ohm's Law Plot, fill the capillary with BGE and apply the voltage (manually) in increasing steps, measure the current and make a plot.
- Ammonium is volatile, and in combination with the high circulating air and open caps in the PA800plus instrument, there is a significant risk that not only ammonium but also water

evaporates during the sequence. Similar observations have been made for CE-MS. For that, a layer of mineral oil on top of the BGE reduced evaporation significantly [11].

- The pH of the BGE is very close to the "pK_a" of the silanol groups of the capillary wall and thus the EOF is not robust.
- Different conditioning approaches cause pH hysteresis [12] on silica and EOF reproducibility issues.

Current

Current is created by the movement of charges particles in an electric field. The current for a certain CE system depends on the capillary diameter, the field strength and the conductivity of the buffer. The latter is directly proportional to the ionic strength and mobility of the buffer components. The ionic strength not only depends on the buffer concentration, but also on the charge of the buffer ions. Mobility is size related. So multiple-charged, small buffer components give rise to relatively high currents in CE buffers. Zwitterionic buffers result in low currents. The best way to check whether a certain current is too high and results in excessive Joule heating in our system, is by making an Ohm's plot. An Ohm's plot is made by varying the applied voltage and measuring the resulting current. A plot of the results should be a straight line. If the current starts deviating from the straight line, more heat is generated than can be dissipated, which will result in band broadening. The combination of applied voltage, buffer and capillary diameter is unfavourable. To reduce the current and with that the excessive Joule heating for our system, we can either reduce the voltage, the capillary diameter or the buffer conductivity. The buffer conductivity is reduced by reducing the concentration or by choosing another type of buffer, like zwitterionic buffers.

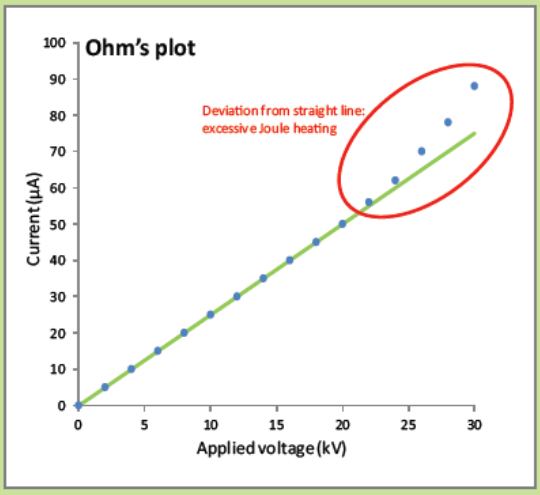

Figure 5: Current and excessive Joule heating, from [10].

A nice tool to study and design buffers is PeakMaster, freeware developed by the University of Prague [10]. The software simulates electropherograms rather accurately. The database contains a broad array of molecules that can be selected as electrolyte or analyte component. While proteins are not included in the database, even those doing primarily protein work will find the software useful as an aid to understanding characteristics of various buffer systems. With PeakMaster, the ammonium phosphate pH 6 buffer was calculated (Figure 6).

ituents

Name	c (mM)	u_eff	c(-3) (mM)	c(-2) (mM)	c(-1) (mM)	c(0) (mM)	c(+1) (mM)
AMMONIUM	200.000	61.632				8.61e-02	199.914
PHOSPHORIC ACID	178.000	-25.372	1.84e-05	21.932	156.051	1.71e-02	

System parameters

pH	6.003
Ionic strength (mM)	221.847
Conductivity (S/m)	1.695
Resistivity (Ohmm)	0.590
Buffer capacity (mM)	45.035
EOF marker time (min)	N/A
EOF mobility (1e-9 m2/V/s)	0.000
1. system eigenmobility	0.001
2. system eigenmobility	-30.723

Figure 6: Snap shots from PeakMaster for the calculation of 100 mM di-ammonium hydrogen phosphate adjusted to pH 6.0 with ortho-phosphoric acid.

At pH 6, the ionic strength, conductivity and mobility of ammonium are very high (hence the high current), while the buffering capacity is moderate to low. The low buffering capacity means that frequent BGE refreshments/increments are needed to reduce the effects of electrolysis.

The pH 6 is on the steep section of the EOF *vs.* pH curve for fused silica capillaries (Figure 7). This means that a tiny shift in pH results in a relatively large change in EOF. The effect is not mitigated by buffering of the selected BGE, as the buffering capacity is low. Generally, working in the pH 4 – 7 range requires caution and special capillary wall treatment in order to develop robust methods.

This combination of the low buffering capacity, excessive Joule heating, buffer volatility and working near the pK_a of the silanol groups amplifies variability and leads to poor robustness in the EOF.

Blindly conditioning a bare fused silica capillary with an acidic or basic wash can often result in different EOF due to the "pH hysteresis" effect. Hysteresis is the phenomenon in which the value of a physical property lags behind changes in the effect causing it. The equilibration of the capillary surface (i.e surface charge) is a slower process than one might think [13]. As a consequence, as simple acidic wash or a basic may not result in consistent EOF. There are many citations in the scientific literature that describe extensive capillary conditioning between injections. Unfortunately, many of these wash procedures have not been development with a deep understanding of the underlying principles. Generally, start with BGE rinses only. If this is not sufficient to obtain stable migration, experiment to improve reproducibility by modifying conditioning steps systematically. Do not overdo the conditioning and remember that what worked well of one method might not work as well for another.

If pH 6 phosphate buffer is required for the separation, both the capillary conditioning and the BGE could be optimized. One way of getting a more stable EOF is to work with SMIL-coatings (Successive Multiple Ionic-polymer Layers), where the capillary wall is coated with successive layers of charged polymers. The pK_as of these polymers differ from the pK_a of silica, and the pH 6 region is less sensitive to pH-dependent EOF drifts. Additionally, a less volatile (relative to ammonium phosphate in the example) and lower conducting BGE could be selected. For instance, selecting tris (base) as the buffer co-ion instead of ammonium would reduce the conductivity significantly (Figure 8). However, buffering capacity will still require some attention.

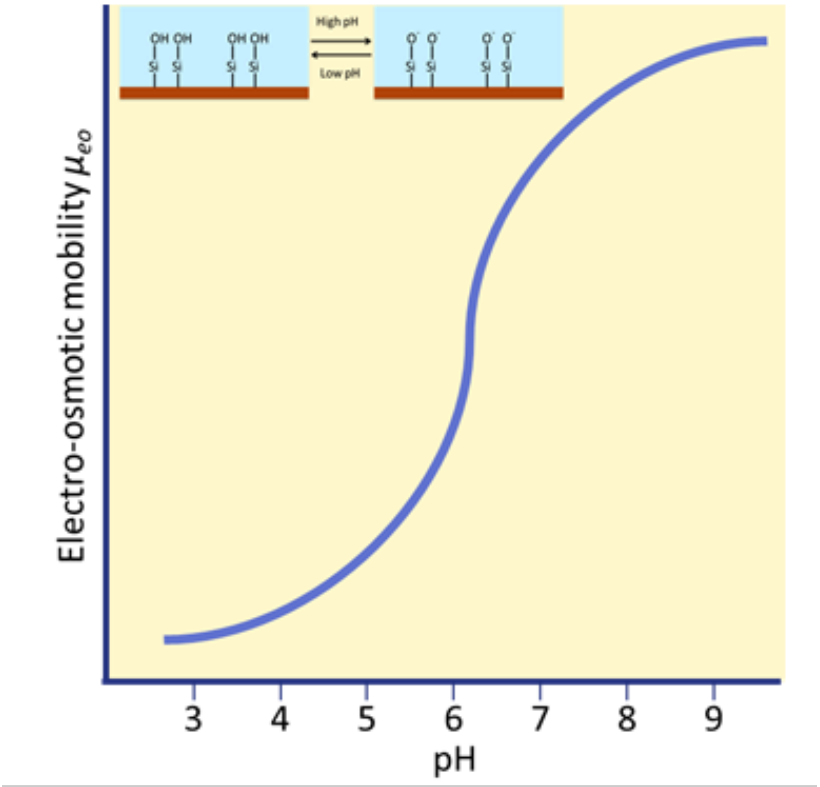

Figure 7: The electroosmotic flow in fused silica capillaries as a function of pH.

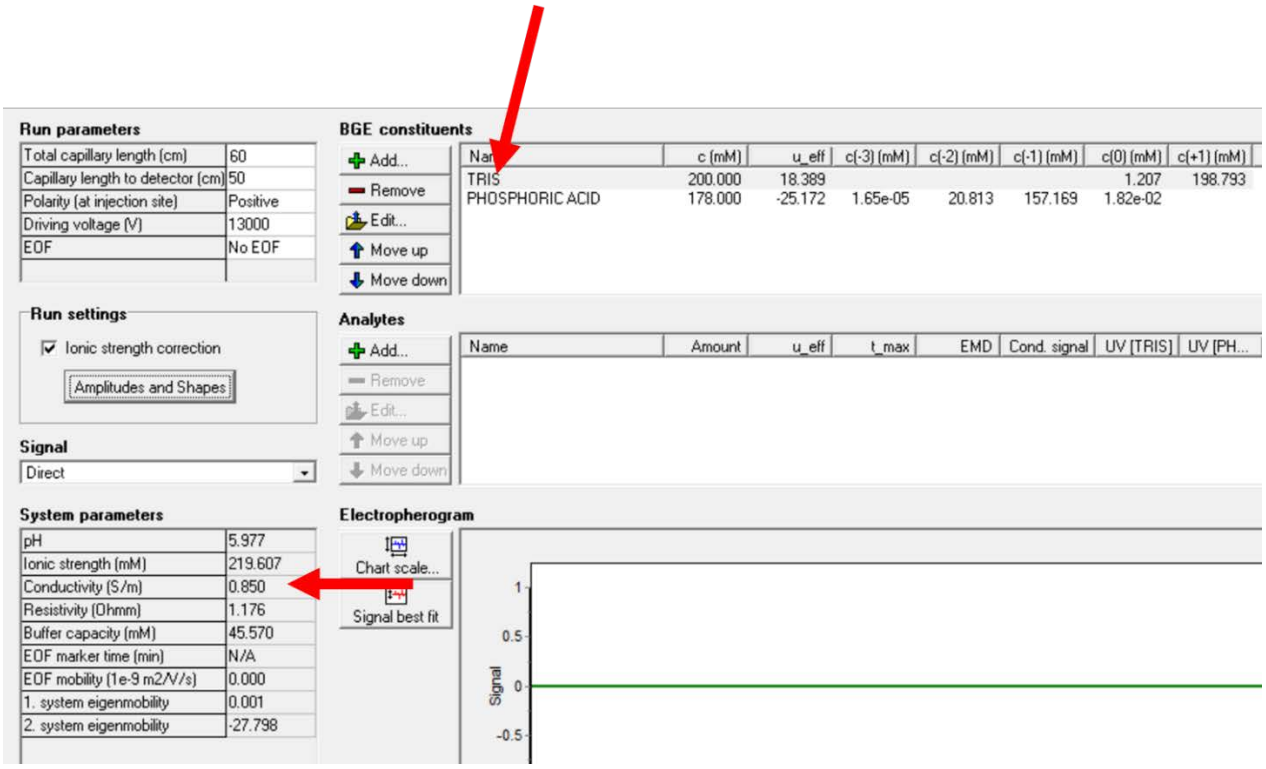

Figure 8: When selecting tris base as co-ion in the phosphate buffer instead of ammonium, the conductivity of the BGE reduces significantly and the current during the run will as well.

Occasional dips/patterns in the current trace

The last issue discussed during the 2018 workshop was a regular sawtooth pattern observed in the current trace that occurred in some injection but not others (Figure 9). Several workshop participants stated having seen this phenomenon. Cleaning the instrument was one of the first things that participants suggested, but in this case the instrument was well-kept and clean. Someone offered that they observed issues like this when there is hairline fracture in the capillary. The capillary was cracked rather than completely snapped in two and it was difficult to see with the naked eye because the capillary was still intact. Bending the capillary is an effective way to confirm the integrity of the capillary; the capillary will break if there is a hairline fracture. Such fractures can be caused by liquid in the holes of the interface block where the capillary is guided through. Liquid can gather where the capillary passes through the interface block if the capillary is not wiped off with a tissue before the capillary cassette is removed.

Someone else had observed similar patterns when the CE instrument was plugged in to the same electric circuit as a vortex. Every time someone used the vortex, the current trace in the CE was affected.

A participant questioned if the current or current trace should be a part of the system suitability criteria. Several participant stated using the current as a check whether the BGE is prepared properly. It is advised to prepare the BGE with fixed concentrations rather than titrating to a certain pH to have a more constant ionic strength and hence current, which improves reproducibility [10].

With that, the time for the troubleshooting session run out. We would like to thank all that contributed with cases, questions and tips. The slides used at the session are added at the end of the document.

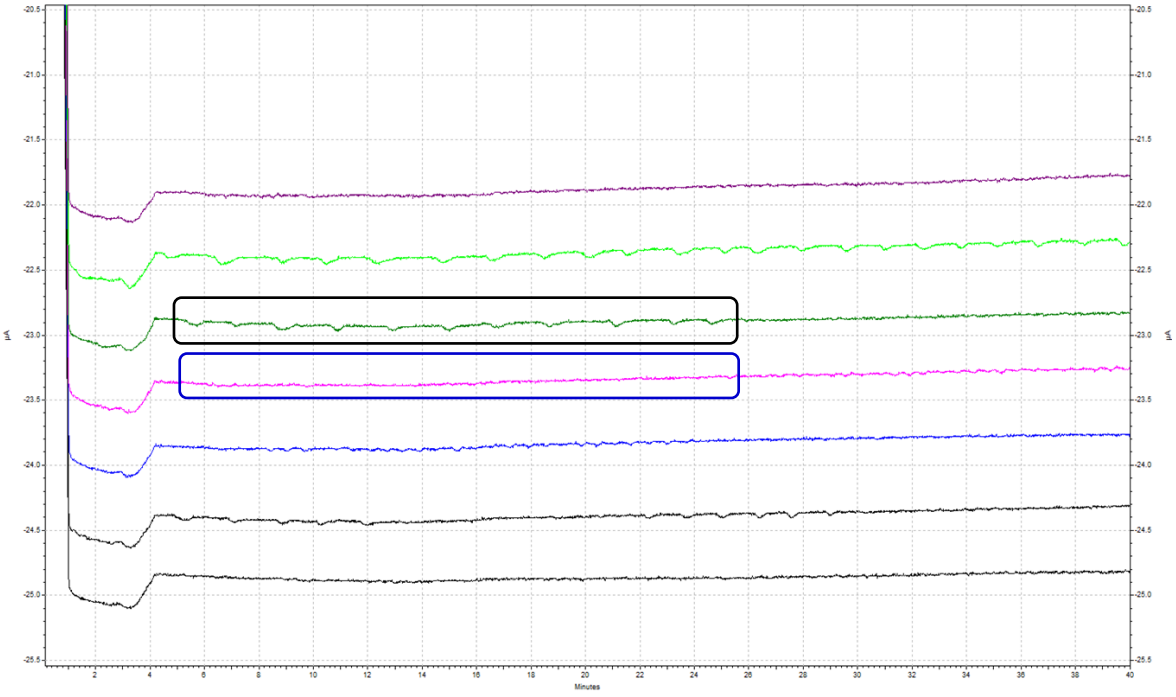

Figure 9: Sawtooth waves in some current traces. "Sometimes we see these wave patterns in the current trace. Although this sequence had some issues, there was no relation between wavy currents and our issues. Still, we'd like to understand where this comes from." Note- Blue box highlights normal current profile and black box highlights "Sawtooth" current profile.

References

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2. 2013 Oral Presentations. Nancy Bernier Amgen Inc West Greenwich, RI. Abstract Quote: "Some examples of troubleshooting that were performed to resolve the source of aberrant peaks identified will be shared along with general best practices for consistent, high quality Capillary Electrophoresis results" Discussion: Colored sample Prep tubes were implicated by the investigation and determine to cause or contribute to the "Aberrant" or "Ghost " peak.
3. David Fishers' 2012 presentation "*MythBusters: Could a fragment peak be identified in a reduced CE-SDS profile*"
4. 2012 Oral Presentation: Tami Wu Seattle Genetics. "*CE Applications in ADC Analysis*" found that the Aberrant Peak appeared in 5 of 8 injection when the sample was prepared in Tubes from brand "A", but 0 of 8 injections when prepared in tubes of Brand "B" (Eppendorf PCR Clean). It was questioned if SDS could leach something from the plastic that contributes to occurrence of the Ghost peak
5. Liu, H.; Chumsae, C.; Gaza-Bulseco, G.; Hurkmans, K.; Radziejewski, C. H., Ranking the Susceptibility of Disulfide Bonds in Human IgG1 Antibodies by Reduction, Differential Alkylation, and LC−MS Analysis. Analytical Chemistry 2010, 82 (12), 5219-5226.
6. Jeff Beckman, BMS, *Capillary Electrophoresis Sodium HexadecylSulfate (CE-SHS): A Novel Approach to Evaluate the Purity of Therapeutic Proteins*, CEPharm 2018, slides available at https://www.casss.org/page/CE1819

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11. Cari Sänger – van de Griend, *CE Solutions 2: Method development in CE: selecting your background electrolyte*, 2012, available at http://www.kantisto.nl/index.php/ce-solutions/12-ce-solutions
12. Angela ten Pierick, Cari Sänger – van de Griend, Govert Somsen and Rob Haselberg, *Towards Robust Analysis of Human Growth Hormone by Capillary Electrophoresis-Mass Spectrometry – Essential Considerations*, Poster P-149-W presented at ATEurope 2017. Quote from abstract: "Due to the volatile nature of the BGE, evaporation was a significant problem for long-term stability of the method, resulting in a pH drop of almost 1 unit, a considerable shift in migration times (MT) and loss of the analyte peak. A mineral oil overlay applied on top of the BGE prevented evaporation of acetonitrile and ammonium, leading to prolonged stable MTs and peak areas. Additionally, the nature and order of conditioning showed to have a big impact on MT stability. After optimization, MT RSDs remained constant and were below 0.5% for 48 consecutive injections."
13. William J Lambert and David M Middleton, *pH hysteresis effect with silica capillaries in capillary zone electrophoresis*, Anal. Chem. 62 (1990) 1585-1587