**CE Pharm 2017 Troubleshooting Report: CZE and CE-SDS the Discussion Continues**

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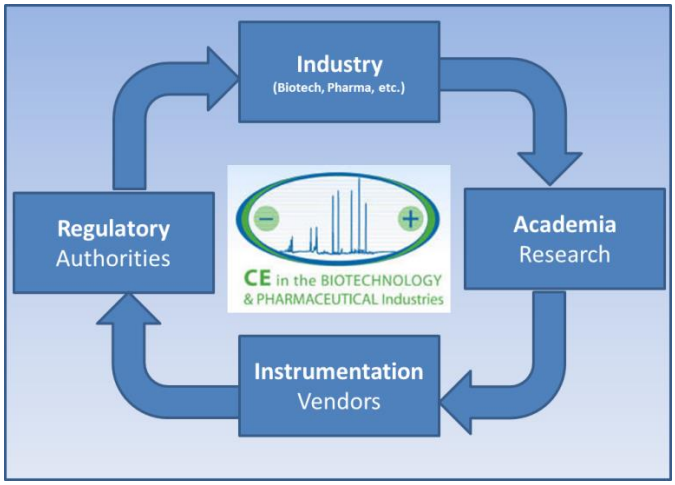
## Introduction

Capillary electrophoresis (CE) methods have been essential to the analytical control strategy of monoclonal antibodies and most biopharmaceuticals, in general. However, considering that the first commercial capillary electrophoresis instruments became available circa 1990, the routine use of CE methods as an essential part of the analytical control strategies was slow to come to fruition[1]. Two points are important to note about the gap in time between when CE instruments became commercially available and when CE methods became an essential part of analytical control strategies. The first is that a community of separation scientist dedicated to CE toiled for years to refine instruments, methods, practices, and reagents to bring them to their current state allowing for routine use. The second point is that with the more recent implementation of CE methods there is a whole new generation of separation scientists that need to become expert in troubleshooting these methods. And it is those two points that fuel the CE Pharm committee's goal of connecting experienced CE professionals who hold much of the CE 'tribal knowledge' together with the next generation of CE scientists. The goal of uniting these two groups is to enable the effective implementation and use of CE methods.

At CE Pharm we strive to bring together a community of academic CE experts, instrument vendors and regulatory authorities with CE users from industry, both novice and advanced, to talk through evolving CE issues in real time. This is accomplished through podium presentations, poster presentations, vendor sessions, and round table discussions. While these forums are effective and are used to share valuable experiences and the *bigger picture* stories, it is the troubleshooting workshop that the committee receives the most feedback to continue and expand. Perhaps the one factor that leads to the success of this session is the engagement of attendees who submit troubleshooting examples prior to the conference that can be discussed in the workshop.

[1] This is not unique to CE. It took for instance about 25 years for HPLC to establish itself properly in the pharmaceutical industry.




Attendees anonymously submitted troubleshooting issues prior to the conference. The CE Pharm committee sorted these submissions and guided the one-hour workshop.

**Topic I: CZE**

The two most common modes that CE Pharm attendees inquire about are size-based capillary gel electrophoresis (or CE-SDS) and charge-based isoelectric focusing (cIEF or icIEF) CE methods. These methods are largely 'kit-based' platform applications supported by various CE vendors. These two applications have been largely covered on previous workshops[2] though each year the discussion continues. Capillary zone electrophoresis (CZE) is the most fundamental mode, and perhaps the most underrated mode of CE, used in various applications throughout the industry. Since past years were focused on CE-SDS and cIEF applications, we intended to promote CZE the front and center of the 2017 workshop. In CZE, the capillary is filled with a continuous background electrolyte (usually a buffer) and separation of analytes is based on electrophoretic mobility differences under an applied electric field. The mobility for a given analyte is directly proportional to the charge/size ratio; thus separation in CZE is driven by the difference in mobility or speed between two analytes. But for most proteins, their high molecular weights and small changes on size effectively enables separation based on charge-differences. This mode has proven to be a rapid method for the resolution of charge heterogeneity of proteins and has gained in popularity[3] One topic presented at the 2017 CE Pharm troubleshooting workshop was in regard to the frequently used CZE background electrolyte:

<u>400 mM eACA, adjusted to pH 5.7 with acetic acid, 2 mM TETA and 0.05% HPMC</u>

(eACA: ε-amino caproic acid; TETA: triethylenetetramine; HPMC: hydroxypropylmethylcellulose)

*HPMC and HPMC quality*

[2] See http://www.casss.org/page/CEPharmTroubleshoot

[3] Chromatographia 53 Supplement (2001) and the chapters and references there in; Yan He et al., J Sep Sci 34 (2011) 548, also called the "Pfizer method"; J Chromatogr. B 983-984 (2015) 101, inter-company study.

Attendees reported that a supplier change of HPMC (sold by Sigma) resulted in poor method performance and loss of mAb peak resolution. As the CZE method is used for identity testing, the HPMC issues have a major impact to the release of drug product. The HPMC material from alternate vendor (Alpha Aeser) has addressed that issue and provided expected CZE profile. The question was raised how critical HPMC is for the method and how to distinguish between different batches and suppliers. The example of the problems with the change of supplier of HPMC sold by Sigma illustrates that the HPMC quality is critical. In general, it is good practice not to use the last bit of a batch of a chemical until comparison with the new lot has been made and approved. Can we request for the vendor to test? Certainly always ask for the vendor's Certificate of Analysis for the new batch, but do realize that the vendor's analytical procedure might not test for an attribute that is critical for your CE method. Retain a sufficient amount of a good batch, so that when problems arise, you can distinguish between instrumental problems and reagent issues. Quality control of ingredients does not seem to be current practice within biopharma, although in small-molecule pharma this certainly is. Is this issue unique for certain mAbs or is it a general problem? This issue might be unique for the use of HPMC for the CZE of mAbs, but change in reagents and batch-to-batch variability is a very common problem and needs to be addressed during method development and robustness testing. Then still one needs to do acceptance testing for critical reagents. Generally speaking, polymers always vary from batch to batch, as the polymerization process is not easy to control during manufacturing, and purification procedures are not very specific or in scope for the type of product and its general use. CE application is very minor market for these types of products.

Additional remarks on HPMC: Yan He reduced the HMPC from 0.1% (Stacey Ma's method[3]) to 0.05% to reduce the sieving effect that is seen when too much HPMC is used. The appropriate amount of HPMC might be dependent on the hydrophobicity and adsorption to the capillary wall of a certain mAb. In literature, there is a report that HPMC can be replaced with HPC (hydroxypropyl cellulose) for more basic mAbs[4]

*Quality of eACA*

In addition attendees have observed issues with the eACA quality. Baseline stability issues (drift) and loss of peak resolution were observed with bad lot of eACA compared to an established good lot of eACA used in the CZE assay. It was suggested that the issue might be connected to the purity of eACA. Ultra-pure eACA should be better. Some of the attendees offered general suggestions to resolve this issue including thorough capillary conditioning with BSA and storing the capillary in acidic conditions (with HCl or phosphoric acid) between runs while avoiding strong bases such as NaOH for conditioning. Although any strong acid rinse might serve to remove adsorbed material, HCl leaches out metal ions from silica[5], so a rinse with HCl instead of $H_3PO_4$ might have different effects on the wall and hence mobilities. Adjusting the molarity (due to high concentration of eACA used in the running buffer) and/or pH of BGE may also be helpful as this issue could be mAb molecule specific. Other factors that could help with baseline drift are the use of single wavelength UV detector instead of PDA due

[4] Moritz et al., Electrophoresis 38 (2017) 3136
[5] Gomez et al., Electrophoresis 29 (2008) 381

to its higher emission energy and/or decreasing capillary column temperature to minimize the effect of Joule heating. A company who uses only the single-wavelength detectors for their method observed no baseline issues. One attendee commented that they took out the TETA and the baseline was then normal.

**Topic II: CE-SDS**

Since CE-SDS is so commonly used, we of course had issues to discuss.

Attendees reported that:

Increasing migration time for the main IgG peak accompanied with an approximately 2 µA lower current was observed intermittently in NR CE-SDS runs. In addition, a post- Main peak shoulder was observed. In response to this question, it was pointed out that is not atypical to see delay in migration time for long sequence runs (over 24 hours period) and the presence of shoulder could be due to inadequate denaturation step.

Some other potential solutions to this issue were discussed as well. They included:

1. Replacement of the electrodes could help with improving consistency of migration time.
2. Use of LC grade water instead of MS spec grade water helped to minimize presence of the shoulder.
3. Verification of stability of power supply, either internal for instrument or external for the lab could help with migration time control as well.
4. Evaporation of water from gel solutions (i.e. gel becoming more concentrated as the sequence progresses).

A brief discussion about the purpose of 10 kDa Internal Standard (IS) used in the CE-SDS assay ensued. The utility of the IS marker to calculate relative migration times was contrasted with the potential that it could obscure small product fragment peaks. Several attendees mentioned a bad lot of material resulted splitting of IS peak. Companies should establish the intended purpose of their CE-SDS method and determine if such IS marker is needed. For example, does a purity method require calibration of size variants or the use of relative migration times for system suitability requirements if the method is designed to compare to test samples to a well-characterized reference standard?

**Concluding Remarks**

The long-term use among a large community has built a knowledge base for troubleshooting, and the CE Pharm workshop is committed to ensuring this knowledge is shared with all users. It is the hope of all participants in the workshop that these discussions lead to improvements in user performance, analytical equipment, and application kits, with the ultimate goal being improved analytical sophistication in the quest to develop and produce safe and efficacious drugs.

**Acknowledgments**

This article is based on workshop discussions at the 19th CE in the Biotechnology & Pharmaceutical Industries Symposium, held 18th September 2017 in Boston, MA. Over 170 representatives from across industry, academia and government attended this conference. We would like to thank all attendees for their participation in the troubleshooting workshop, in particular those who submitted real-life examples from their own labs. We would also like to thank the members of the conference organizing committee for helpful suggestions and critical review during the preparation of this summary.

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# "The rules"

- This is a workshop and not an entertainment programme! Active contribution is required!
- An opportunity to share the expertise among the industry
  - "Tribal Knowledge"
- Lively and open discussions with all of us
  - Keep it plenary
- Freely ask questions, think out loud
- There can be different solutions to the same observation – Provide ways to go to the core
  - One symptom can have many causes

- We might not be able to answer all, but we expect valuable input for important issues
  - We will continue a successful series of workshops
- You can contribute also next year by sending suggestions in the survey and mail your questions to the CASSS office (for anonymity)

# Topics

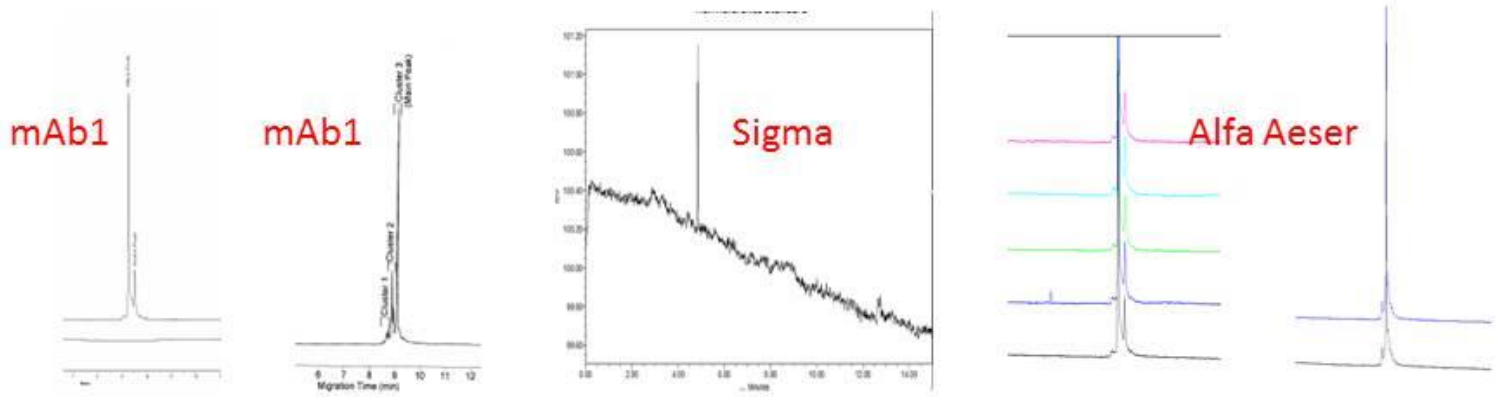
- CZE
  - Quality of chemicals
    - HPMC
    - εACA
  - Baseline issues
- CE-SDS
  - Shifting migration time in one or two runs
- cIEF
  - Missing pI 4.1 marker
  - Coagulation factor with online desalting method
- Troubleshooting Rules of Thumb

# CZE – HPMC quality

**Contribution from last year**

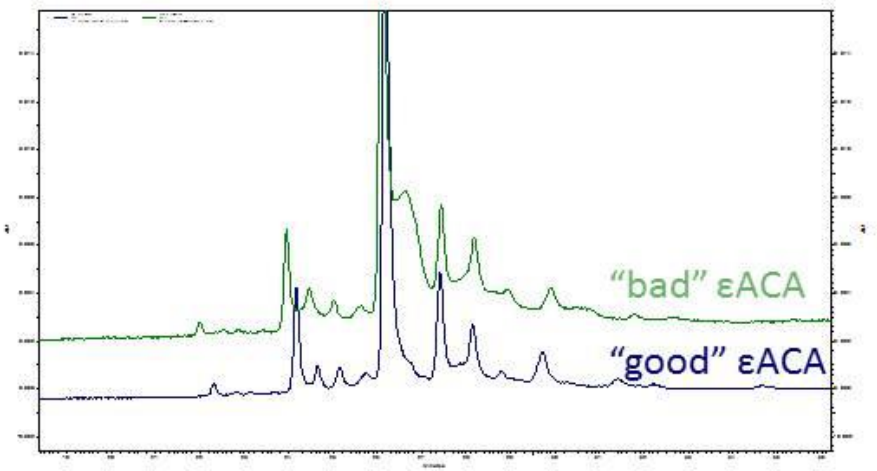
Observations:

- HPMC from Sigma (primary vendor) is a component of the CZE run buffer
- No protein signal obtained in recent CZE runs in multiple QC and development labs when using new HPMC lots
  - Sigma recently changed their vendor
  - Change could be the cause of the different performance
- Alternate vendor (Alfa Aesar) used and expected CZE profiles obtained



# CZE εACA method

- New batch of εACA
  - UV detection at 214 nm, fused silica capillary, BGE: 400 mM εACA, 2 mM TETA, 0.05% HPMC
  - Deterioration of resolution
    - Investigation: differences dependent on EACA lots, but also on some additional effect, that is not identified yet.
      - The effect is seen for some antibodies at a very significant extent, for others hardly any effect is detected.



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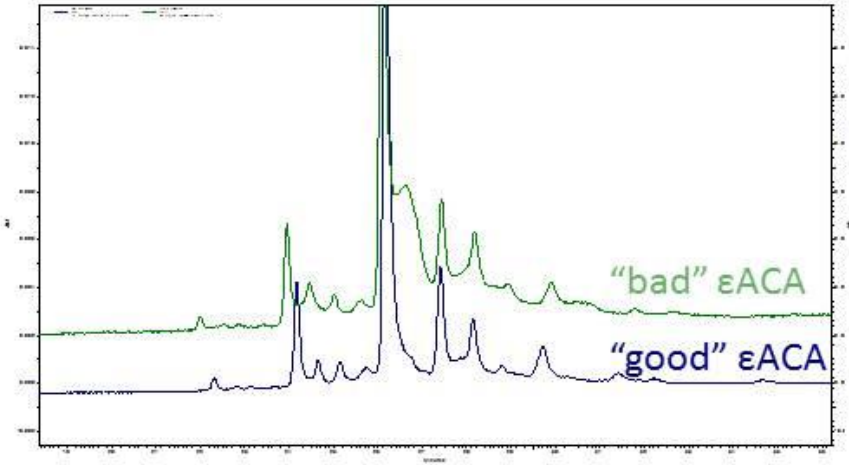
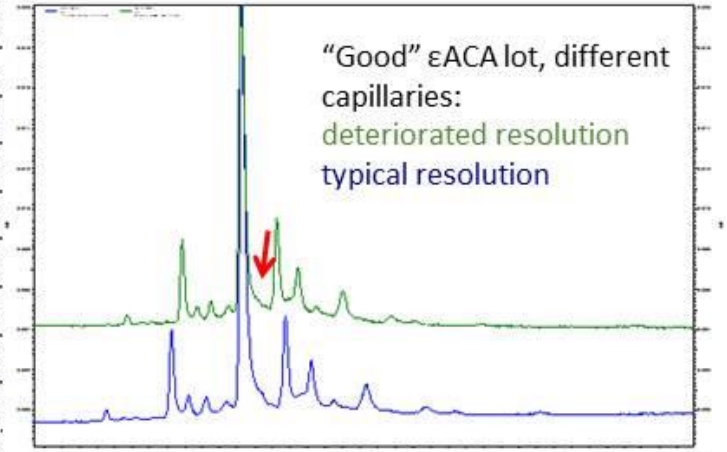
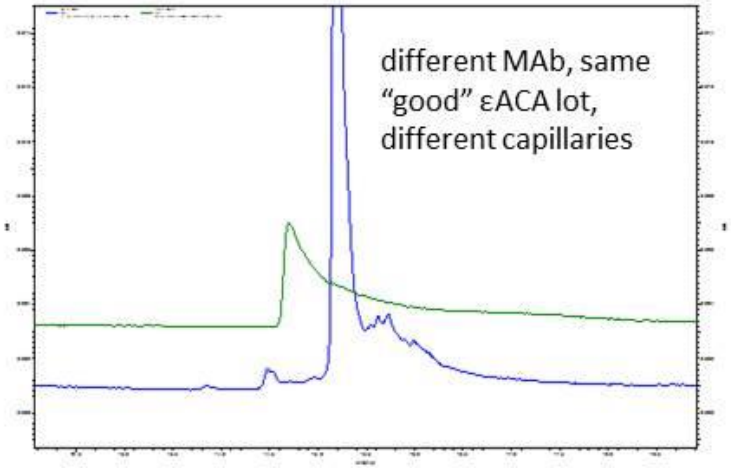
# CZE εACA method




- Could this be a problem in handling?
- Could a "bad" lot of εACA influence the following results by changing the capillary surface?
- Could pH of water have an effect?
- Any other suggestions or experiences with εACA lot variability?

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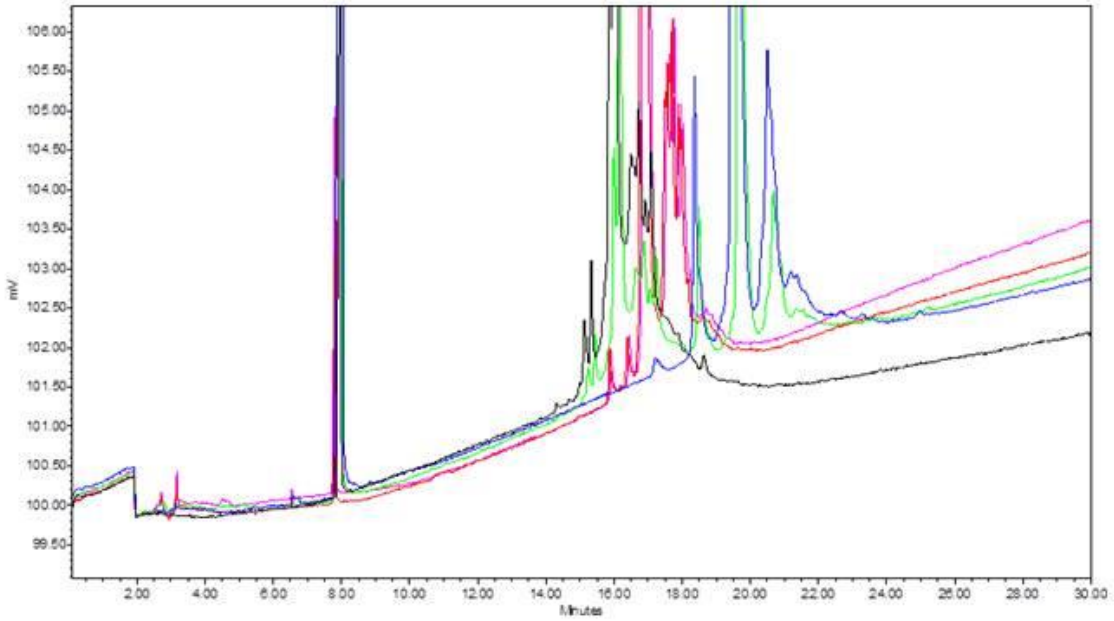
# CZE εACA method

- Baseline issues:




- PA800+, PDA, 50 µm x 40 cm capillary, following intercompany study protocol

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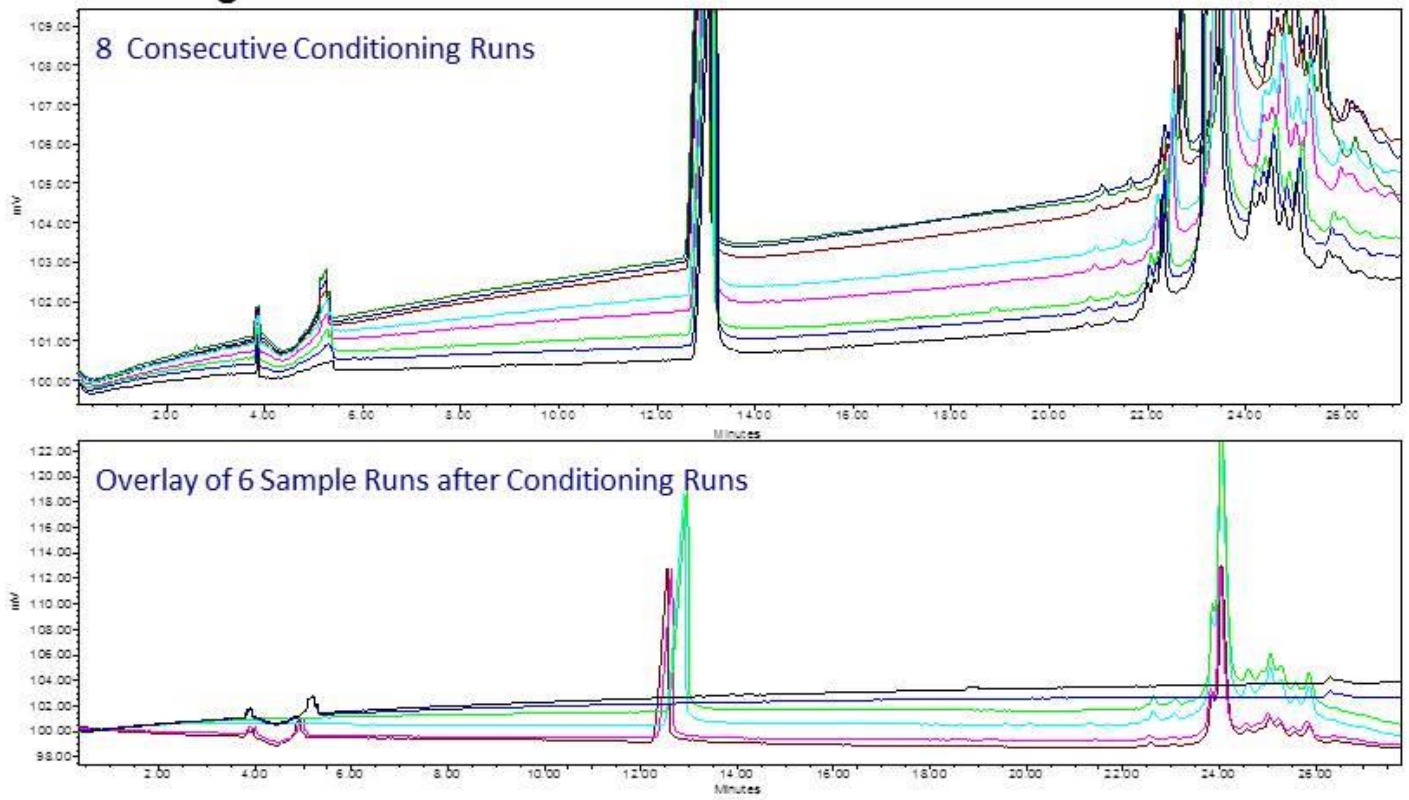
# CZE εACA method

- After investigating voltage, molarity and pH of the buffer
  - Change to 20 kV



# CE-SDS

- Sometimes, without a clear reason:
  - Peaks migrate later (by up to 1-2 min)
  - The product peak can in addition show an artefact shoulder
  - In this case, current is somewhat less negative (app. -24 µA instead of -26 µA)
- No further deviations or reasons for this problem could be detected. What could be the reasons and possible solutions?

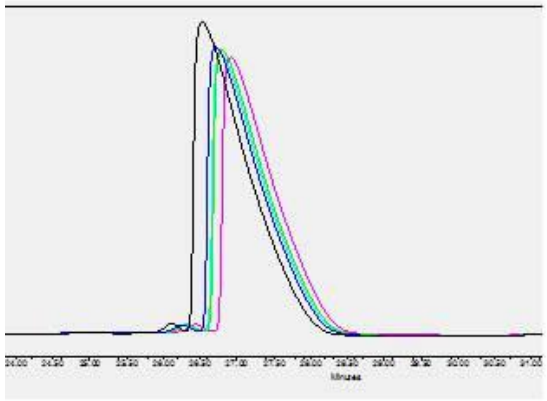
# CE-SDS

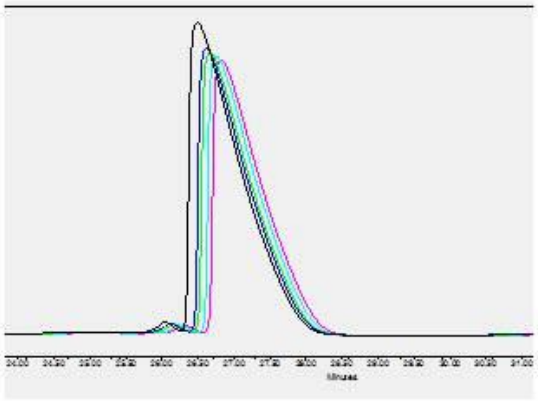
1. We use only 1 internal 10kDa marker in our CE-SDS. Do we have a MW ladder like we do in SDS-PAGE to make a calibration curve and identify the mass of the peaks? Or have two internal markers bracketing the profile like we do for cIEF?

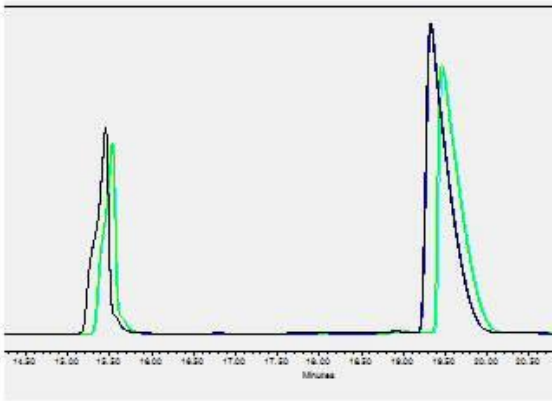
2. How we define the good resolution of the profile? We have a fusion protein whose peak width is three times wider than the regular mAb. Is the profile well resolved?

# CE-SDS

3. Five preparations of mAb sample from the same vial were prepared as individuals. They were pooled into one vial after heat incubation, vortexed and loaded into 5 vials individually for injection. The results obtained are not consistent in total correct area.
   - The %RSD > 7.3%
   - In Run2 The %RSD of system suit for the assay was >8%.
   - The Reduced Assay % RSD=9.75%.
   - The samples were injected in two different equipment. One of the equipment shows high %RSD for the 5 injection of system suit frequently.







# cIEF

- With some samples of the same product the pI marker 4.1 is missing (not focused??).
- Apart from the missing pI marker 4.1 and a somewhat broader product peak.
- All samples are desalted in the same way before the Test solution preparation and a single mix, inc. all components premixed, is used for all Test solutions ran in the same sequence.
- Samples come from the same process level, so no major differences in their composition are expected.
- No further deviations or reasons for this problem could be detected. What could be the reasons and possible solutions?

# cIEF Recombinant coagulation factor

- Sample solution diluted to 7 mg/ml with mQ water, no buffer exchange
- Master Mix 1: without pI markers, Master Mix 2 with pI markers
- Master Mix 2 used for sample
- Sequence:

| Repeats | Sample Inject Inlet | Sample Inject Outlet | Sample Inject Duration | Method |
|---|---|---|---|---|
| 2 | - | - | - | Conditioning |
| 1 | SI:A1 | BO:B1 | 99.0 | MM1 |
| 1 | SI:A2 | BO:B1 | 99.0 | MM2 |
| 1 | - | - | - | Conditioning |
| 2 | SI:B1 | BO:B1 | 99.0 | MM2 plus Sample |
| 1 | - | - | - | Conditioning |

# cIEF Recombinant coagulation factor

- Sample solution diluted to 7 mg/ml with mQ water, no buffer exchange
- Master Mix 1: without pI markers, Master Mix 2 with pI markers
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- Sequence:

| Repeats | Sample Inject Inlet | Sample Inject Outlet | Sample Inject Duration | Method |
|---|---|---|---|---|
| 2 | - | - | - | Conditioning |
| 1 | SI:A1 | BO:B1 | 99.0 | MM1 |
| 1 | SI:A2 | BO:B1 | 99.0 | MM2 |
| 1 | - | - | - | Conditioning |
| 2 | SI:B1 | BO:B1 | 99.0 | MM2 plus Sample |
| 1 | - | - | - | Conditioning |

# cIEF Recombinant coagulation factor

- Instrument set-up: online desalting

Instrument Setup

Initial Conditions | UV Detector Initial Conditions | Time Program

| | Time (min) | Event | Value | Duration | Inlet vial | Outlet vial | Summary | |
|---|---|---|---|---|---|---|---|---|
| 1 | | Rinse - Pressure | 50.0 psi | 3.00 min | BI:D1 | BO:E1 | forward, In / Out vial inc 8 | Capillary Cleaning 6M Urea Solution I |
| 2 | | Rinse - Pressure | 50.0 psi | 2.00 min | BI:B1 | BO:B1 | forward, In / Out vial inc 8 | Water Rinse |
| 3 | | Inject - Pressure | 25.0 psi | 99.0 sec | SI:A1 | BO:B1 | Override, forward | Sample injection |
| 4 | | Wait | | 0.17 min | BI:A1 | BO:A1 | In / Out vial inc 8 | Water Dip |
| 5 | 0.00 | Separate - Voltage | 10.0 KV | 5.00 min | BI:C1 | BO:C1 | 5.00 Min ramp, normal polarity, In / Out vial inc 8 | Online desalting - Phosphoric to NaO |
| 6 | 5.00 | Separate - Voltage | 25.0 KV | 17.00 min | BI:C1 | BO:C1 | 0.17 Min ramp, normal polarity, In / Out vial inc 8 | Focusing Step |
| 7 | 22.00 | Separate - Voltage | 30.0 KV | 30.00 min | BI:C1 | BO:D1 | 0.17 Min ramp, normal polarity, In / Out vial inc 8 | Chemical Mobilization Step - Phospho |
| 8 | 52.00 | Stop data | | | | | | Stop cIEF Separation |
| 9 | 52.10 | Rinse - Pressure | 50.0 psi | 2.00 min | BI:B1 | BO:B1 | forward, In / Out vial inc 8 | Water rinse |
| 10 | 54.20 | Wait | | 0.17 min | BI:A1 | BO:A1 | In / Out vial inc 8 | Water Dip |
| 11 | 54.40 | End | | | | | | Method End |
| 12 | | | | | | | | |

- (Normal method:)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1 | | Rinse - Pressure | 50.0 psi | 3.00 min | BI:D1 | BO:B1 | forward, In / Out vial inc 12 | Urea Solution rinse |
| 2 | | Rinse - Pressure | 50.0 psi | 2.00 min | BI:B1 | BO:B1 | forward, In / Out vial inc 12 | Water rinse |
| 3 | | Inject - Pressure | 25.0 psi | 99.9 sec | SI:A1 | BO:B1 | Override, forward | Sample Injection |
| 4 | | Wait | | 0.00 min | BI:A1 | BO:A1 | In / Out vial inc 12 | Water Dip. |
| 5 | 0.00 | Separate - Voltage | 25.0 KV | 15.00 min | BI:C1 | BO:C1 | 0.17 Min ramp, normal polarity, In / Out vial inc 12 | Focusing Step |
| 6 | 15.00 | Separate - Voltage | 30.0 KV | 30.00 min | BI:C1 | BO:D1 | 0.17 Min ramp, normal polarity, In / Out vial inc 12 | Chemical Mobilization Step |
| 7 | 45.00 | Rinse - Pressure | 50.0 psi | 2.00 min | BI:B1 | BO:B1 | forward, In / Out vial inc 12 | Water rinse |
| 8 | 45.00 | Stop data | | | | | | Stop cIEF separation |
| 9 | 47.00 | Wait | | 0.00 min | BI:A1 | BO:A1 | In / Out vial inc 12 | Water Dip |
| 10 | 47.10 | End | | | | | | Method End |

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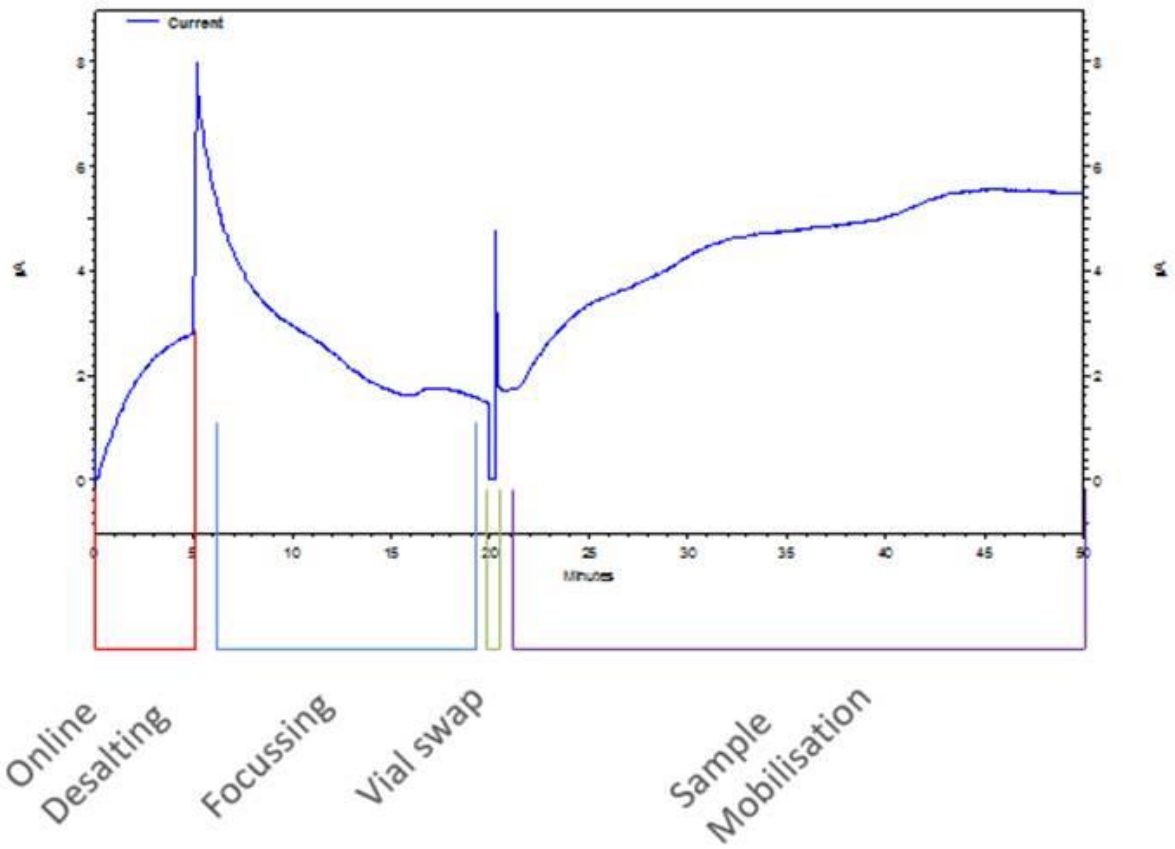
# cIEF Recombinant coagulation factor

- Current trace:



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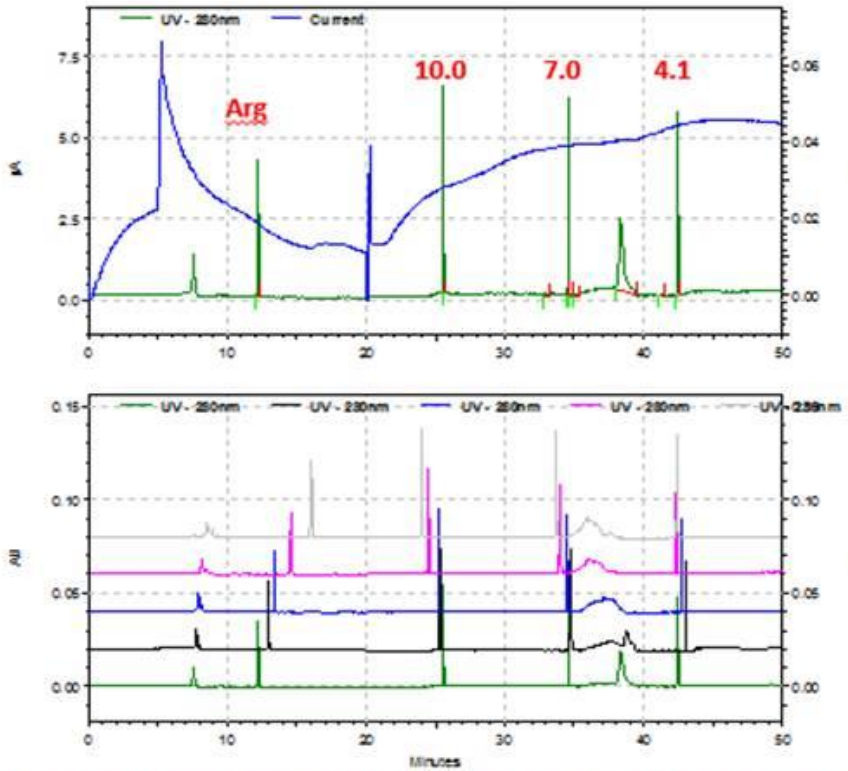
# cIEF Recombinant coagulation factor




3 M Urea / MM2 with pI Markers and Sample

Grey – 5.0 M Urea
Pink – 4.5 M Urea
Blue – 4.0 M Urea
Black – 3.5 M Urea
Green – 3.0 M Urea

Focussing seems completed, no pI marker/sample overlay and increasing sample separation with increasing Urea concentration

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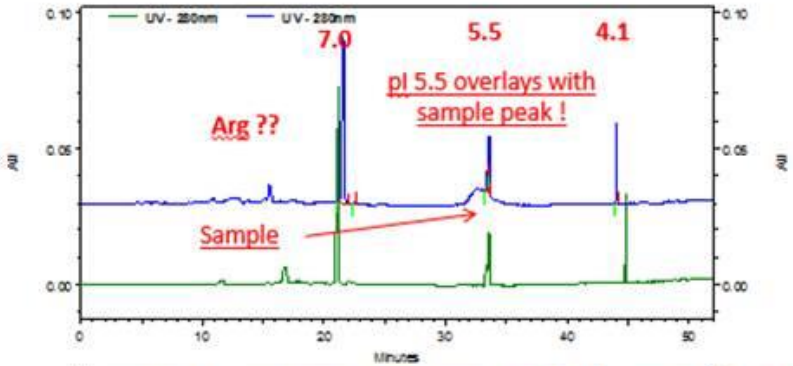
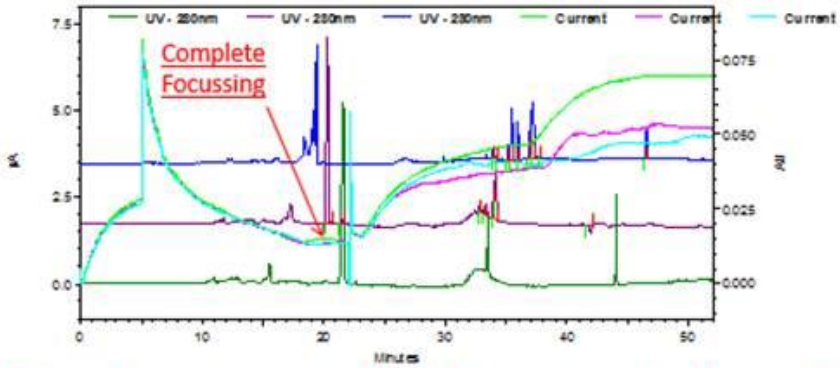
# cIEF Recombinant coagulation factor

- Pharmalyte pH 3 – 6.5




Blue – 5.0 M Urea / MM2 with pI Markers and Sample

Green - 5.0 M Urea / MM2 with pI Markers




Blue – 5.9 M Urea
Purple – 5.5 M Urea
Green – 5.0 M Urea

Focussing seems completed, however cathodic peaks "missing" (Arg solution stored at 4°C crystallised), pI 5.5 marker/sample overlay, no trend to sample separation

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# cIEF Recombinant coagulation factor

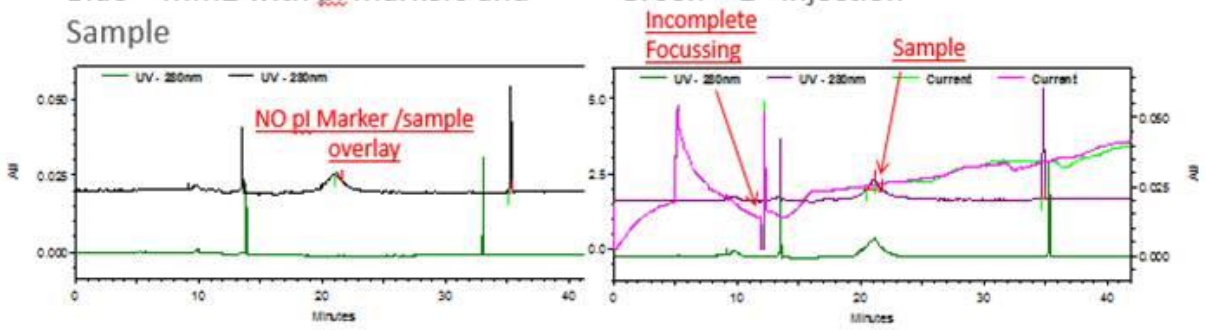
- Modified cIEF Test Conditions pH of 3 – 6.5

| MM1 Preparation | Reagent | Volume in µl |
|---|---|---|
| Reduced viscosity to facilitate mobilisation | 5.5 M Urea/cIEF Gel | 200.0 |
| | 500 mM Arg | **9.0** (20.0) |
| | 200 mM Iminodiacetic Acid | **5.0** (2.0) |
| | Pharmalytes | **6.0** (12.0) |
| MM2 = MM1 + pI | per each pI Marker | 2.0 |

- Focussing Time: 7 min

Green - MM2 with pI Markers
Blue – MM2 with pI Markers and Sample

Purple – 2nd Injection
Green – 1st Injection



# cIEF Recombinant coagulation factor

- Questions:
  - Is there any sample solubility issue?
  - What are suggestions on how to approach the sample separation?
  - What suggestion on how to approach the sample run conditions?

# Troubleshooting: 6 rules of thumb (Dolan)

1. Rule of one
   - Change one thing at a time
2. Rule of two
   - Can you repeat the problem, is it reproducible?
   - Is there a pattern?
   - Reinject
3. Divide and Conquer rule
   - Make an observation or experiment that allows to discard a large number of possible root causes
4. Model substitution role
   - Isolate the source of the problem:
     Replace a suspect part
   - Any level: BGE, capillary, instrument etc.
5. Put-it-Back rule
   - If the new part doesn't fix the problem, reinstall the old part

# Troubleshooting: 6 rules of thumb (Dolan)

6. Documentation rule
   - If it isn't documented, it didn't happen
   - Support for an audit
   - Avoid future problems
     - Establish failure pattern
     - Information for life time expectations
       - Replace parts proactively
     - Share the knowledge and experience!
   - Create a procedure that is easy simple to use
     - Compliance
     - Who, what, when, where, why, how?
     - Record part/serial/batch/lot numbers