**Roundtable Session - Table 7 - From Peaks to Product Knowledge: Characterizing CE Variants and Product-Related Impurities**

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**Abstract**

Capillary electrophoresis has largely replaced conventional techniques and has become a primary platform for high-resolution separation of protein size and charge variants over the past decade. However, characterization of CE-separated variants remains challenging, despite the development and implementation of various online and offline CE–MS approaches. This roundtable will discuss current techniques used to characterize CE variants, key challenges associated with these workflows, and potential solutions to address them.

**Discussion Questions:**

1. What are the primary techniques used in your lab to separate charge variants?
2. How do you characterize charge variants separated by CE?
3. What are the key challenges in characterizing charge variants using current techniques, and what potential solutions have you explored
4. What types of biotherapeutic modalities have you analyzed using CE-MS, including mAbs, ADCs, bispecifics, fusion proteins, AAVs, and vaccines
5. How do you characterize size variants separated by SDS-CGE?
6. At what stage do you perform heightened characterization of CE variants, and when do you include these data in regulatory filings?

**Notes:**

1. What are the primary techniques used in your lab to separate charge variants?
    - **Ion-exchange chromatography (IEX)** is used primarily when charge-variant fractions must be collected for subsequent characterization.
    - **Imaged capillary isoelectric focusing (icIEF)** is the most commonly used technique for routine analytical assessment of charge variants.
    - **Capillary zone electrophoresis (CZE)** is used less frequently, but it can support charge-variant characterization during early-phase development.

2. How do you characterize charge variants separated by CE?
    - **IEX fractionation** is a primary approach for isolating charge variants; however, an appropriate chromatographic gradient must first be developed to achieve sufficient separation.
    - **Peptide mapping** is typically performed on the isolated fractions to identify molecular attributes associated with each variant.
    - Peptide mapping alone may not fully explain a charge-variant peak, particularly when fragments or aggregates contribute to the observed profile; complementary characterization may therefore be needed.
    - The extent and depth of characterization should be **phase-appropriate**, with the analytical package expanding as the program advances.
    - Some participants use the **Intabio ZT** platform for early-stage characterization.
3. What are the key challenges in characterizing charge variants using current techniques, and what potential solutions have you explored?
    - **MauriceFlex:** The platform is commonly used and considered user-friendly, but loss of resolution during mobilization can be a limitation. Refocusing was suggested as a possible way to improve resolution.
    - **CEInfinite:** Participants noted improved reproducibility, but urea may remain in collected fractions and the user interface can be difficult to navigate.
    - **Arginine-containing conditions:** Avoiding arginine can be important for ADCs because high-pI conditions may promote payload degradation. Using a different ampholyte formulation was suggested as a potential alternative.
    - **CZE–MS:** Approaches such as ZipChip can support direct characterization, but execution may be challenging because numerous MS adducts can be observed and applicability may be limited by the molecule's pI.
    - **CZE as a complementary method:** CZE can provide information about charge gradients, but participants questioned whether it can substitute for characterization of the cIEF profile; direct characterization of the cIEF profile may still be required.

4. What types of biotherapeutic modalities have you analyzed using CE-MS, including mAbs, ADCs, bispecifics, fusion proteins, AAVs, and vaccines?
    - No modality-specific CE–MS examples were captured in the session notes for this question.
5. How do you characterize size variants separated by SDS-CGE?

- **CE-SDS as a foundational technique:** The discussion considered whether CE-SDS should be treated as an important skill for students to learn. Participants noted its practical importance as a replacement for traditional SDS-PAGE in biotherapeutic size-variant analysis.

6. At what stage do you perform heightened characterization of CE variants, and when do you include these data in regulatory filings?
    - **Characterizing CE-SDS size variants:** Teams should consider whether individual size variants require structural characterization. Intact-mass analysis may provide useful identification data, although it is typically less sensitive than CE-SDS for detecting low-level species.
    - **Enrichment of low-molecular-weight (LMW) species:** A preparative size-exclusion chromatography (SEC) column can be used to enrich low-abundance LMW species, enabling more reliable quantitation and subsequent characterization.
    - **Development stage and filing strategy:** The timing and extent of heightened characterization depend on the molecule's known or suspected degradation pathways and on whether the CE method is shown to be stability-indicating.
    - **Process relevance:** Characterization results should be considered in the context of whether identified variants or degradation pathways influence process-development decisions.

**Additional discussion topics**

- **Use of collected fractions in potency testing:** Participants raised the possibility of using isolated fractions in later potency studies. This would require fractionation method controls or handling strategies that demonstrate the fractions remain stable and retain functional activity.
- **Enzymatic treatment:** Enzyme treatment may help evaluate variants associated with glycosylated molecules.
- **Orthogonal characterization:** Native analysis paired with multi-attribute method (MAM) testing was identified as another potential strategy.
- **Native cIEF fractionation:** The group asked whether native cIEF fractionation is being used. High protein loads can create solubility challenges, and urea is often required to keep the protein soluble during fractionation. No examples of native cIEF fractionation.