Roundtable Session 1 – Table 4 – Best Practices of Extended Characterization Mass Spec Methods

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Abstract:

Mass spectrometry (MS) is a powerful technique widely used for characterizing biopharmaceuticals, and the combination of high sensitivity, selectivity, and specificity make it indispensable. Often paired with liquid chromatography (LC), MS simplifies complex mixtures and accurately identifies proteoforms, playing a key role in understanding many aspects of the drug development process.

Extended and in-depth characterization is crucial for monitoring features that ensure drug safety and efficacy, such as post-translational modifications (PTMs), glycosylation and the determination of unknown features. Supporting a Quality by Design approach, by enhancing upstream characterization, adjustments can be made promptly, resulting in better yields and overall product quality in downstream operations.

Additionally, novel modalities like multi-specific monoclonal antibodies, vaccines, gene therapies, and RNA-based therapeutics pose challenges for existing mass spectrometry approaches. As these modalities gain market share, continued development of in-depth characterization techniques becomes increasingly important.

Discussion Questions:

- How does MS characterization differ for new modalities compared to established modalities?
- What is the current limiting factor for MS-based characterization? Expertise? Hardware? Software?
- What advantages might AI offer in the area of MS-based characterization? Would this be an appropriate tool for ‘up-skilling’ lower experience analysts to perform more in-depth analyses?
- What level of automation is most beneficial in characterization?
- How do you judge the level of acceptable risk when performing characterization? Does this change when considering a new modality versus a traditional antibody?

Notes:

Participants at the table worked across development, from early to late stage. The discussion centered around in-depth characterization, which is non-routine and oftentimes requires expert knowledge acquired over many years, rather than faster, higher throughput more routine analysis. The need for orthogonal assays, flexibility in chromatographic LC and MS dimensions as well as robust sample preparation was also explored, particularly for tricky applications, such as the analysis of acidic variants.

The Need for Orthogonal Assays

One challenge discussed was how well orthogonal assays agree with each other – this is relevant even for common PTMs, like deamidations.

Examples were given to illustrate example use cases. For example, an observed percentage increase in acidic species suggests a deamidation, but the mass vs percent deamidation may not correlate. Then, a scientist needs to figure out what is contributing to that underlying acidic species and determine its relevance. This was discussed further later in our session (see notes below).

Participants shared that one way to address this challenge is through comparison with the bioassay result and observing the impact on the potency. This opened up another discussion topic. If one assay says 20% and another says 30%, the potency measurements may well cover the range and not offer significant insights.

Therefore, understanding the biological impact of measurements will help further guide relevancy in terms of comparability between orthogonal methods.

Alternatively, different LC separation modes and column chemistries can be used to enhance separation for different attributes e.g. for charge variance separation. In addition, from a mass spectrometric perspective different fragmentation techniques should also be considered e.g. ETD, HCD.

From a practical and time perspective, we also need to consider where the boundaries should be in terms of how deep we should go in characterization and how much time and effort should be spent? At what point are we in the scope of diminishing returns? To help assess this several questions and perspectives are often posed and considered by experienced analysts, including:

- When these experiments are done and they don't agree, could you try a different mass spec technique to investigate further e.g. intact, pep map, charge variant, subunit
- Have different stress conditions been considered? What variations have been observed?
- What about sample preparation and fractionation considerations.

The consensus of the table participants was that typically we get to about 90% of the answer with one approach, which is usually enough. However, we may employ other approaches to go further. Therefore, orthogonal techniques are needed for extended characterization as

sometimes there are worries about how trace variants could affect product efficacy/stability. The hope is that orthogonal approaches can provide these greater insights.

Use Cases: Analysis of Acidic Variants

The opening discussion above led directly into a discussion focused specifically on the analysis of acidic variants.

The analysis of acidic variants is complex, primarily because the acidic variants themselves are complex. When they are analyzed by intact MS very “grassy” deconvoluted spectra are observed. For this reason, it’s important to utilize multi-dimensional LC-MS.

One challenging case is when non-specific modifications are observed. For example, sometimes modifications appear primarily on lysine and may also be observed on some arginine residues and perhaps on some other non-specific residues. In this case, quantification is difficult due to the lack of site specificity, which is compounded when you look to quantify at a site level, because then it could be below the detection point. In this case, analysts must look at the modification at a global level, often utilizing different techniques, which are not MS based. This is a limitation.

Use Cases: Glycation

Glycation is another example of a challenging use case. Often different flavors of MS are needed to ensure the totality of evidence required to get the overall picture when it comes to quantitative analysis. Sometimes a mixture of approaches is needed including peptide mapping and subunit-based approaches. Particularly, as it can be common that to observe glycation near a CDR region but not in it. For this reason, different enzymatic digestion approaches are often used e.g. trypsin, chymotrypsin, etc.

Additional complexity stems from the fact that there are different ways to quantitate the percentage of glycation. With peptide mapping, site specific quantitation is sometimes employed to identify and quantify one potential glycation site. However, there are 2 parent ions per peptide, and you will get different values based on what you are using. How can you tell what is correct? A different protease is necessary, as single measurements are hard to trust. This led into a discussion about monitoring glycation in the context of MAM, which the table believed is hard to achieve. In the NIST mAb study it was mentioned that quantification of the percentage glycation should be done using peptides of different length. Someone who was part of the EFPIA MAM consortium for QC tools mentioned that this consortium had stated the caveats for the quantitation of glycation in their paper, but that it needs to be spelled out more clearly. In particular, when quantitation of the parent peptide must be specified, otherwise the data is meaningless.

Throughput vs Depth: The Challenge

Moving the discussion forward the facilitator summarized the discussion: “What I’m hearing is that extended characterization can be long and involved. Different combinations of separation approaches, enzymes and sample preparation, as well as the MS fragmentation. All come together to build the overall picture. So, the question then becomes how much time do we put into doing this? When is enough of an answer enough?

The participants commented: “For drug discovery you are only focusing on the product, so it isn’t too bad.” “Maybe you don’t have to go that deep?”.

The consensus was that for discovery the focus is on micro variants, which are prone to degradation and hotspots. The same goes for clone selection. In these cases, higher throughput doesn’t hurt. But for extended characterization, you want to go really deep to have a more detailed understanding of the molecule. In this way, the risk of problems downstream is reduced. It is important to balance the risk later down the line. Patient safety is paramount.

Sample Preparation Challenges

The discussion moved into discussing additional challenges. It was apparent that there are different challenges at different companies including updating sample preparation protocols. One roundtable participant noted that their current company doesn’t do alkylation as standard. At first, they didn’t like this protocol but now think it is easier to implement.

Software Challenges

Software for MS remains an integral aspect in extended characterization studies. Comparatively data collection takes a small amount of time compared to data processing and data analysis.

One person mentioned that they used vendor software for data processing. For routine analyses, not a lot of time is spent, but for SVA, HCP, etc, it’s necessary to go into the weeds. Looking at 1% of max intensity and below takes time, and they choose to manually verify results because they don’t always trust the software.

Every software weighs false positives versus false negatives differently. It’s better to have false positives scientifically, but it requires more manual review. The consensus was that manual verification is best practice, whilst so called waterfall approaches can help by narrowing the search space, but expert knowledge is still needed for confirmation. Others thought it would be great if software could predict and validate trypsin using orthogonal approaches. Some thought it would be great if software could have logic and expert knowledge built-in via artificial intelligence. Others believed the task of manual review can’t be solved with AI as “it needs to be right,” and the healthy skepticism of an expert human is necessary for this.

The Role of MS Experts and Transferring Institutional Knowledge in Extended Characterization

The need and role of MS experts in extended characterization was undisputed and extended characterization is typically only performed by experts. The expert needs to be able to

understand the limits of methods and have total visibility. They also need to know which MS method to use, as going down the wrong route could waste a lot of time.

The consumers of the data resulting from extended characterization studies are often the MS experts themselves. Some workflows may be more accessible to non-experts. However, others found it necessary to consolidate the results of the data analysis so that a non-expert can digest them.

The question was then posed around how to capture institutional knowledge and ensure this knowledge is accessible either for training or for future studies. This is particularly important in these times of high turnover, as you will lose expertise and there is no substitute for expertise. Scientific knowledge is very important and if people don't have the right background, it is much harder to train them.

Several suggestions to mitigate this were made including:

- Written guidelines and appendices.
    - Even if this results in a burden to keep these updated.
- Training the team is another way to capture this knowledge, but it's hard.
    - For experts much of the data interpretation is done instinctively or intuitively and sometimes it can be time-consuming to break the steps down so someone else can follow.
    - People do need to learn on their own in some ways.
    - It's even difficult when folks just have small molecule experience and then go to peptide mapping, as there is a lot more to be aware of.
- Open Communication
    - Group meetings where scientists who do analysis show others what they do, and others can learn by example.
    - Open office hours where more junior analysts can ask expert scientists questions are also helpful.

<u>Summary</u>

Extended characterization analytics is the domain of the MS expert. Flexibility in the types of approach taken as well as time to review the data is key. The ability to transfer knowledge or build in knowledge or logic into the software analysis platforms could be beneficial, but at the end of the day, manual verification will still be needed.