Enabling Mass Spectrometric Analysis of Intact Proteins in Native Conditions on A Hybrid Quadrupole-Orbitrap Mass Spectrometer

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INTRODUCTION

Analysis of proteins in native-like conditions free of organic solvents can allow proteins to preserve non-covalent interactions and retain high degrees of folding. This effect has analytical benefits: greater protein folding leads to reduced charge states leading to increased mass separation and increased signal at higher m/z. This strategy has been utilized for analysis of antibodies and antibody drug conjugates present in highly complex mixtures of different antibody/dosing combinations [1]. Requirements for performing native MS on antibody samples include scanning towards 8000 m/z and increased transmission optimization for large compounds. Such features are not compatible with current commercially available quadrupole-Orbitrap instruments. Here we show results obtained after successful implementation of modifications aimed at adding the capability to perform native MS analysis without compromising performance of normal operation modes.

The analysis of intact proteins under native conditions is more challenging than under denaturing conditions since the buffers used don’t contain any organic solvents. Performing electrospray from aqueous buffer solutions produces larger solvent droplets size and desolvation is less efficient. Moreover, for large proteins such as intact antibodies the required mass range for analysis under native conditions requires a mass range of more than 6000 m/z due to a smaller number of accepted charges. The increase of the upper mass range on the mass spectrometer was achieved by implementing instrument control software changes. The analysis of molecules across the full mass range including the detection of proteins under native conditions required the use of optimized parameter settings to ensure efficient desolvation in the front region of the instrument, the efficient transfer via multiplies, efficient trapping in the C-trap/HCD region and the sensitive injection and detection in the Orbitrap mass analyzer. Critical parameters are the optimization of in-source fragmentation that strongly influences the support of the desolvation process. Also, for the transmission efficiency specific voltages have been evaluated and optimized to ensure robust and sensitive performance in the higher mass range when performing analyses under native conditions, experiments that have not been possible so far on this type of mass spectrometer. Also, the standard calibration routine previously used was modified and adapted to ensure high mass accuracy across the full mass range.

With the data collected on different types of samples and presented in this study we demonstrate the successful analysis after implementation of the High Mass Range (HMR) mode, successful desolvation and optimized critical hardware operation settings. This makes the instrument an ideal platform to cover the three major workflows in BioPharma: intact mass analysis under denaturing and under native conditions in HMR mode, subunit analysis (reduced mAb and or Idel® digested mAb) in protein mode and peptide mapping in standard mode (see Figure 1).

Figure 1: Operating modes for the three major BioPharma workflows: Normal Mode, Protein Mode and HMR mode

MATERIALS AND METHODS

Samples:
Samples used in this study are ammonium heptanesulfonate (AHFS, Fisher Scientific, part number A0988370), and Triazolemethide (tradename Heropathin, Roche, UK).

Chromatography:
A Thermo Scientific™ Vanquish™ UHPLC system was used for all LC/MS experiments. For native analysis, 50 mM ammonium acetate buffer (90.95%, Sigma Aldrich) was used. Reversed phase chromatography was performed with water:0.1% formic acid and acetonitrile:1% formic acid on a Thermo Scientific™ MAXPeat™ RP 2.1x50 mm column.

Mass Spectrometry:
Mass spectrometers used in this study are the Thermo Scientific™ Q Exactive™ Plus and Q Exactive™ HF systems with BioPharma Option. The instruments were operated under Tune 2.8 instrument control software in HMR mode, in which the RF applied to the C-trap was increased from 2,400 V p-p to 2,900 Vp-p for better trapping of the high m/z ions. Also, to ensure better capture of high-m/z ions in the Orbitrap analyzer, the initial central electrode voltage was adjusted from ~3.7 kV to ~3.4 kV, while the setting during detection remained unchanged (~5 kV). The 5-lens RF level was allowed to be increased to a setting of 200 in HMR mode and set to that level for all experiments shown here.

Data Analysis:
Data analysis was performed with Thermo Scientific™ BioPharma Finder™ 1.0 SP1 software.

RESULTS

There are many factors that play a key role in the analysis of proteins, some of which relate to sample preparation (buffers, solvents, additives) while others relate to the mass spectrometer’s source conditions as well as the physical environment inside the instrument [2,3]. The Q Exactive Plus and Q Exactive HF mass spectrometers (Figure 2A) have previously been introduced with the Protein Mode option, which was one of many advancements for intact protein analysis on the Orbitrap platform. For these two instruments an automated HCD gas control was introduced by using an electronically controlled valve for nitrogen gas in the HCD cell for easier optimization of experimental conditions required for different types of analyses wished to run on a single platform.

In Normal Mode pressure settings are factory-optimized, suitable for most analyses and ions are cooled in the C-trap (Fig. 2B). The trapping gas pressure setting is 0.1 which corresponds to a high vacuum pressure delta (HVΔ) of ~3.1 e-5mbar. The HVΔ is defined as the difference between HV with HCD gas on minus HV with HCD gas off.

In Protein Mode the default trapping gas pressure setting is 0.2 and that corresponds to a HVΔ which is 5x lower than in Normal Mode. Additionally, ions are transferred and cooled in the HCD cell and thus have a longer flight path (Fig. 2C).

The combination of reduced C-trap and HCD cell gas pressures, and trapping ions in the HCD cell prior to mass analysis extends the life time of protein ions resulting in increased signal intensities of isotopically resolved species (Fig. 2E).
For higher gas pressures, high charge states of the same protein decay faster than lower charge states. This is because center-of-mass collision energy $K_e = E_m (M/2z)$

resulting in $K_e$ is proportional to the charge state $z$. This explains observations of charge envelope shifts on the m/z scale when comparing data acquired in different modes with different pressure regimes in the HCD cell and C-trap region, as shown in one example in Figure 6. Here we have investigated and implemented the new High Mass Range (HMR) Mode that is especially required for the analysis of proteins under native conditions when samples are kept in aqueous buffers with no organic solvents involved at near neutral pH.

For HMR mode the default trapping gas pressure setting is 1 but it can be slightly increased up to 1.5 for even improved trapping of certain species such as protein complexes and heterogeneous large proteins (e.g. antibody drug conjugates). The trapping path in HMR mode is the same as in Protein mode with ion cooling taking place in the HCD cell. And also, mass detection is enabled ranging up to m/z 8000 compared to m/z 6000 in the two other modes.

The trapping gas pressure in all modes is set and saved in the tune files and since a method allows for segmentation using different tune files different pressure settings can be used within one LC-MS run. In contrast the mass range setting is set in the method and the method editor allows for several modes with different experiment types using different mass ranges within one LC-MS run.

Table 1. Theoretical and measured masses (Figure 3) of ammonium hexafluorophosphate for calibration of HMR mode

<table>
<thead>
<tr>
<th>Mass (m/z)</th>
<th>Theoretical</th>
<th>Experimental</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>50.9786</td>
<td>50.9786</td>
<td>0.01 ppm</td>
</tr>
<tr>
<td>100</td>
<td>101.9572</td>
<td>101.9572</td>
<td>0.01 ppm</td>
</tr>
<tr>
<td>150</td>
<td>152.9359</td>
<td>152.9359</td>
<td>0.01 ppm</td>
</tr>
<tr>
<td>200</td>
<td>203.9146</td>
<td>203.9146</td>
<td>0.01 ppm</td>
</tr>
</tbody>
</table>

Further parameters that were found critical in optimizing source conditions for native analysis using size exclusion chromatography (SEC) were the capillary temperature (also referred to as transfer tube) and the probe heater temperature (also referred to as Aux heater temperature). Figure 8 shows one example of two different temperature settings resulting in differences in charge state distribution.
Figure 1: Operating modes for the three major BioPharma workflows: Normal Mode, Protein Mode, and HMR mode. Figure 2: Schematic of the Q Exactive Plus/HF mass spectrometers and differences for +17 charge state of a mAb light chain comparing Protein Mode and Normal Mode.

MATERIALS AND METHODS

Data analysis was performed with Thermo Scientific™ BioPharma Finder™ 1.0 SP1 software. A0368370), and Trastuzumab (tradename Herceptin, Roche, UK).

Moreover, for large proteins such as intact antibodies the required mass range for analysis under native conditions requires a mass range of more than 6000 m/z due to a smaller number of accepted signal at higher m/z. This strategy has been utilized for analysis of antibodies and antibody drug conjugates.

CONCLUSIONS

We have successfully implemented the High Mass Range Mode on the Q Exactive Plus and Q Exactive HF mass spectrometers allowing for mass detection up to m/z 8000.

This new operating mode extends the instrument’s capabilities to cover all three major workflows for BioPharma characterization.

Desolvation/declustering conditions, ions transfer and trapping have been optimized to allow for improved sensitivity in HMR mode for resolution settings as high as 70k.

Critical parameters for online LC-MS analyses under native conditions are capillary and probe heater temperatures as key factors in supporting desolvation/declustering.

REFERENCES