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Advancing native top-down MS analysis of non-covalent protein complexes: The Thermo Scientific Q Exactive UHMR mass spectrometer

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Introduction

Native mass spectrometry (MS) has emerged as a powerful technique for studying protein-ligand interactions and elucidating the structure of macromolecular assemblies, including both soluble and membrane protein complexes. Native MS relies on maintaining a protein's natural folded state and associated non-covalent interactions for analysis.

A few years ago, we introduced an Orbitrap™-based mass spectrometer called the Thermo Scientific™ Exactive™ Plus EMR mass spectrometer specifically designed for native MS. The instrument provided high mass resolution (>140,000), extended mass range up to 20,000, and the ability to perform all-ion fragmentation. The limited mass range up to 20,000 and low sensitivity at high *m/z* made it impossible to tackle large molecules such as whole viruses or protein-nucleic acid complexes. Also, this instrument was limited in terms of performing native top-down analysis due to the poor fragmentation of protein complexes into subunits in the front end of the mass spectrometer and due to the lack of a quadrupole to isolate specific subunit ions for further fragmentation in the HCD collision cell.

This technical note examines the technology innovations that enable the Thermo Scientific™ Q Exactive™ UHMR (Ultra High Mass Range) Hybrid Quadrupole-Orbitrap™ mass spectrometer to go beyond the capabilities of previous MS instruments in allowing interrogation of heteromeric protein assemblies using top-down pseudo-MS^3 approaches. Experiments were

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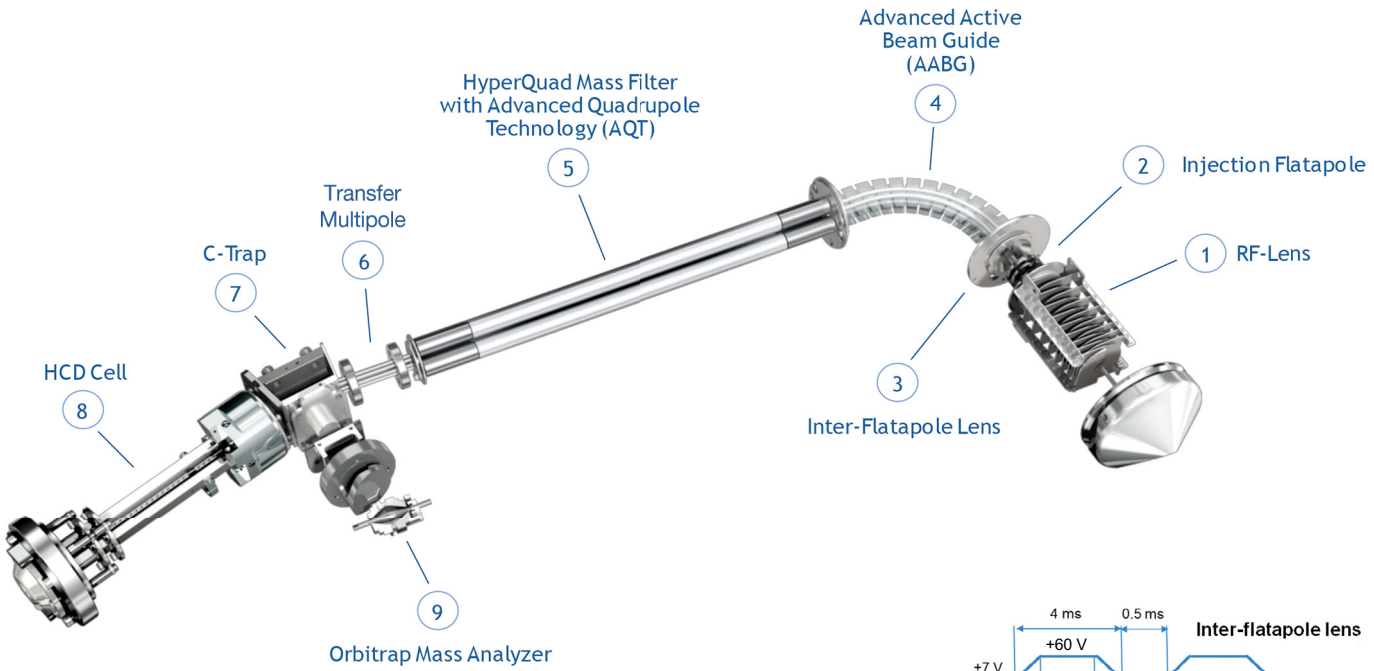
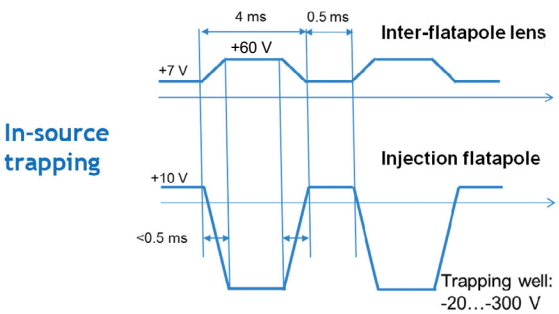
performed using *E. coli* GroEL, rabbit 20S proteasome, LmrP membrane protein, and *E. coli* AmtB membrane protein complex as model systems.

Optimized Q Exactive Hybrid Quadrupole-Orbitrap technology solves native MS challenges

As analytes have gotten larger and more complex, mass spectrometry capabilities have needed to advance rapidly to keep pace. A combination of enhancements to hybrid quadrupole-Orbitrap technology have come together in the Q Exactive UHMR mass spectrometer to allow direct detection of large, intact proteins and protein complexes with unprecedented resolution and sensitivity.

The Q Exactive UHMR mass spectrometer[3,4] provides the ability to perform pseudo-MS^3 for native top-down analysis and transmission of very high *m/z* ions through the implementation of both hardware and software enhancements (Figure 1). The most important of these enhancements were 1) pulsed trapping of ions in the injection flatapole region, a process called "in-source trapping" (Figure 1 inset) and 2) the reduction of the frequency of RF voltages applied to the injection and bent flatapoles, quadrupole, transfer multipole, C-trap, and HCD cell.





2, 3 Shown in the Figure 1 inset, the injection flatapole is pulsed down to a negative voltage to improve desolvation of large protein complexes, while the inter-flatapole lens is maintained at a high positive potential to prevent ions from eluting out. Trapping is followed by restoration of the voltage levels, allowing low-energy elution of ions into the bent flatapole (Advanced Active Beam Guide).

4 The bent flatapole guides and focuses ions using an axial DC field and a focusing RF field, enhancing sensitivity.

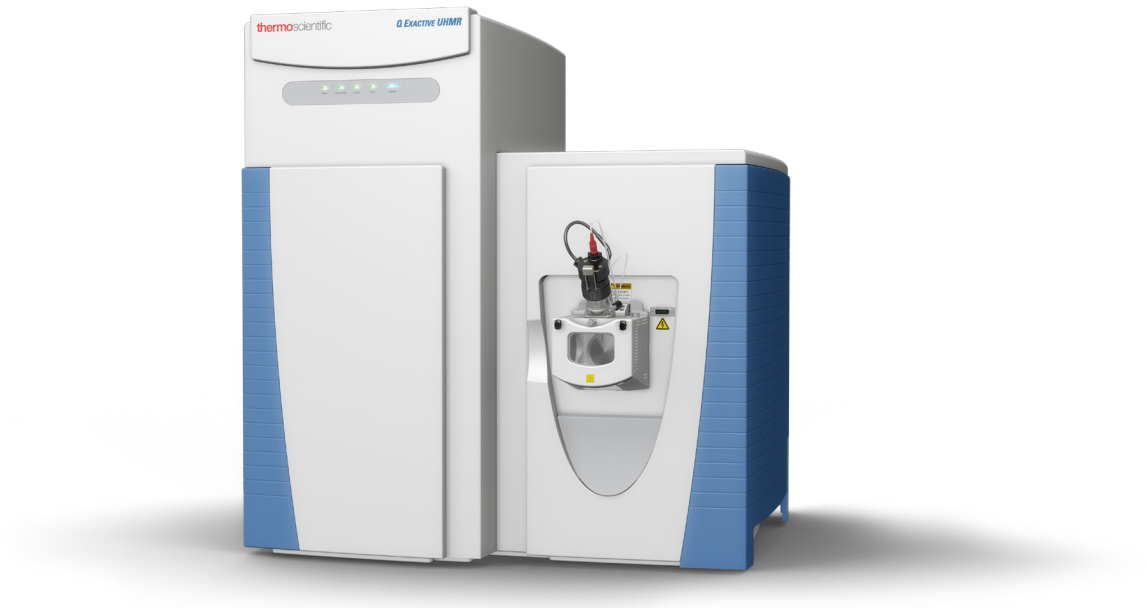
5 The high-mass quadrupole operates at a three-fold lower frequency than its predecessor and can be used to select ions with an *m/z* up to 25,000.

2, 4–8 The RF frequencies of all ion routing multipoles—the injection and bent flatapoles, quadrupole, transfer multipole, and HCD cell—are reduced to improve ion transmission.

9 High mass ions are efficiently injected into the Orbitrap mass analyzer by adjusting the slew rate of the high-voltage pulse that captures ions in the analyzer.

M. Belov, US2015340213 (2015), US9887074 (2018)

Figure 1. Schematic of the Q Exactive UHMR Hybrid Quadrupole-Orbitrap mass spectrometer with in-source trapping (inset)



Thermo Scientific Q Exactive UHMR system

In-source trapping allows controllable, highly efficient desolvation of large ions and the optional application of energy to dissociate these ions into subunits in the injection flatapole region prior to their release for subsequent mass analysis, including pseudo-MS^3 analysis.

The reduced RF voltages increase the efficiency of high *m/z* ion transfer and utilization, delivering orders of magnitude higher sensitivity, and thus much lower sample volume requirements. Multiply charged ions are detected with such efficiency that individual ions can be detected within milliseconds, providing the ultimate analytical sensitivity needed for minute sample volumes.

Other developments include gas pressure control, an increase in the maximum HCD energy from 200 to 300 V, and an adjustment of the voltage ramp rate of the Orbitrap mass analyzer, which facilitates transmission of very high *m/z* ions from the C-trap into the Orbitrap mass analyzer.

Pseudo-MS^3 analysis

The improvements in performance provided by the modifications described earlier can be demonstrated in pseudo-MS^3 experiments applied to non-covalent protein complexes. A pseudo-MS^3 analysis begins by using moderate desolvation energy and transferring the intact protein complex through the mass spectrometer without fragmentation to produce its MS^1 spectrum. Next, dissociation of the protein complex into its subunits in the injection flatapole region yields the MS^2 spectrum. Following that, quadrupole selection and subsequent fragmentation of individual subunits in the HCD cell provide the MS^3 spectrum that can be used for sequence analysis.

Native MS and native top-down analysis of the GroEL protein complex

The GroEL protein complex is a molecular chaperone required for the proper folding of many proteins in cells. Using in-source trapping, efficient desolvation and fragmentation of the GroEL 14-mer complex into its monomer and stripped complexes (13-mer and 12-mer) was achieved (Figure 2). When followed by quadrupole selection of the monomer and subsequent fragmentation in the HCD cell, a total of 112 b and y ions were identified, which represents 21% of the residue cleavages.

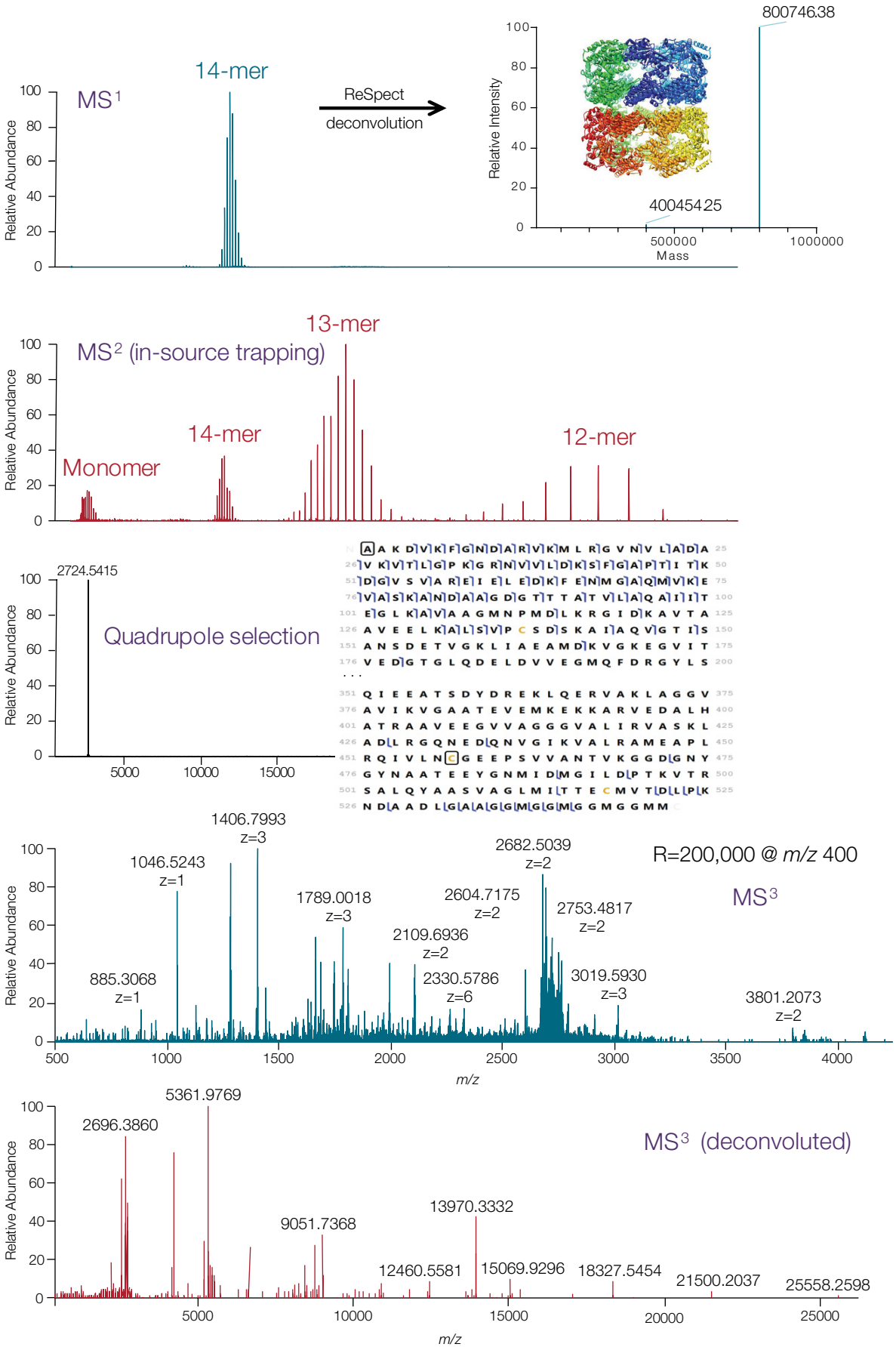


Figure 2. The native MS and native top-down analysis of the GroEL protein complex enabled identification of a total of 112 b and y ions, representing 21% of the residue cleavages.

Native MS and native top-down analysis of rabbit 20S proteasome

The rabbit 20S proteasome is a heterogeneous protein complex composed of 28 subunits arranged into four stacked heptameric rings. Its two outer rings contain seven non-identical alpha subunits and its two inner rings contain seven non-identical beta subunits.

The native MS analysis of the rabbit 20S proteasome produced a baseline resolved charge envelope for the intact, 28-subunit complex with a measured molecular mass of 717,023.187 Da after deconvolution using the ReSpect™ algorithm (Positive Probability Ltd.) (Figure 3 inset).[5] The deviation from expected mass was <0.082%. Native MS^2 analysis of the intact 20S proteasome complex using sequentially increased HCD energy revealed the dissociation of the alpha-6, alpha-2, and alpha-5 subunits (Figure 3).

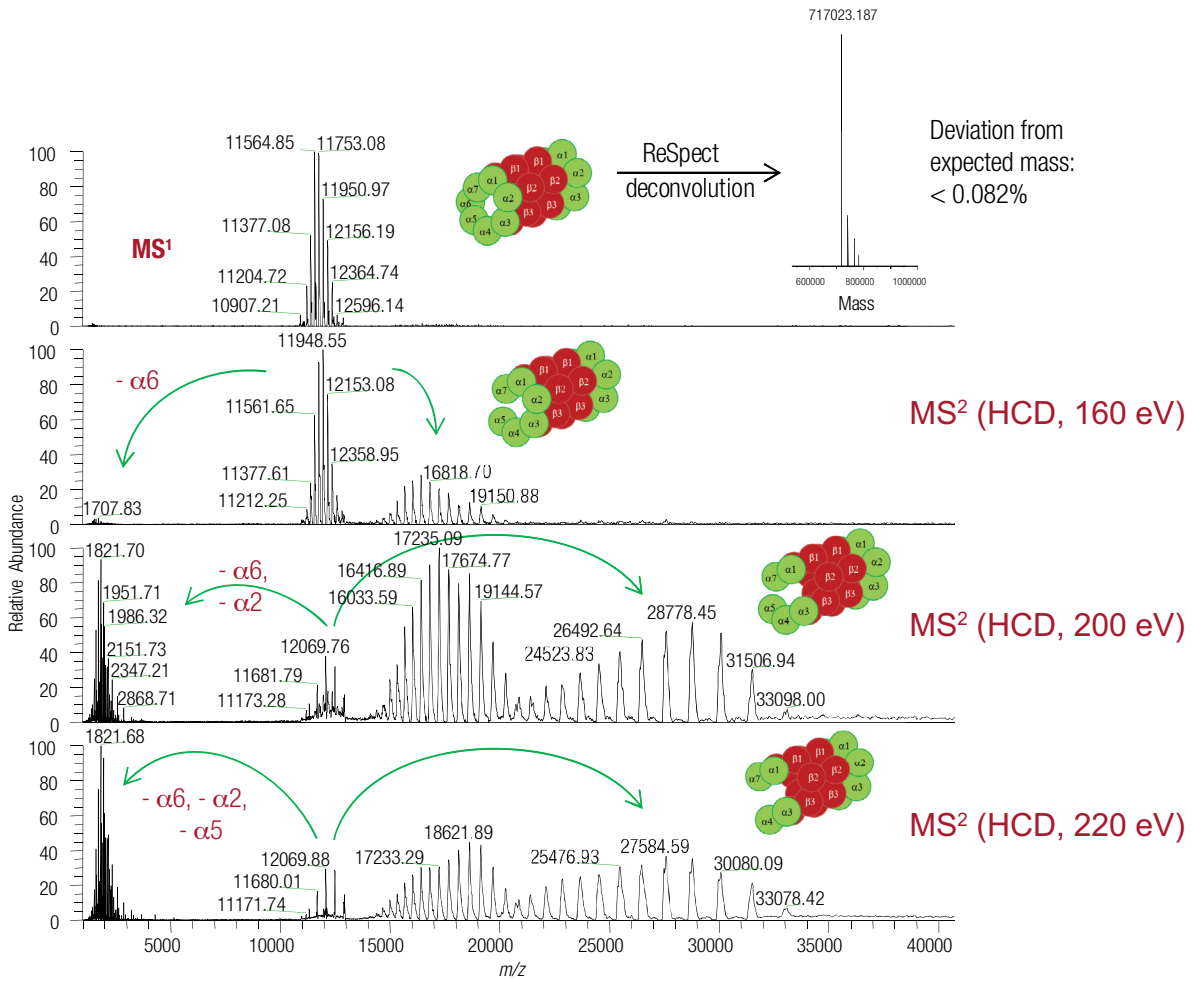


Figure 3. Native MS and MS^2 (HCD) analysis of the rabbit 20S proteasome complex with MS-level deconvolution results (top inset). Sequentially increasing the MS^2 HCD energy revealed the loss of the alpha-6, alpha-2, and alpha-5 subunits.

Native MS^2 analysis of the intact 20S proteasome complex using in-source trapping showed dissociation of six out of seven alpha subunits. The Q Exactive UHMR MS isotopically resolved all six dissociated subunits at a resolution setting of 200,000 and provided accurate monoisotopic masses (Figure 4).

Native top-down pseudo-MS^3 experiments allowed unambiguous identification of the alpha-6, alpha-2, and alpha-5 subunits. To obtain the data shown in Figure 5, the quadrupole was used to isolate the 16+ charged ion at *m/z* 1707, and then HCD fragmentation was performed in the HCD cell. The pseudo-MS^3 HCD spectrum acquired at a resolution setting of 200,000 at *m/z* 400 allowed unambiguous identification of the alpha-6 subunit.

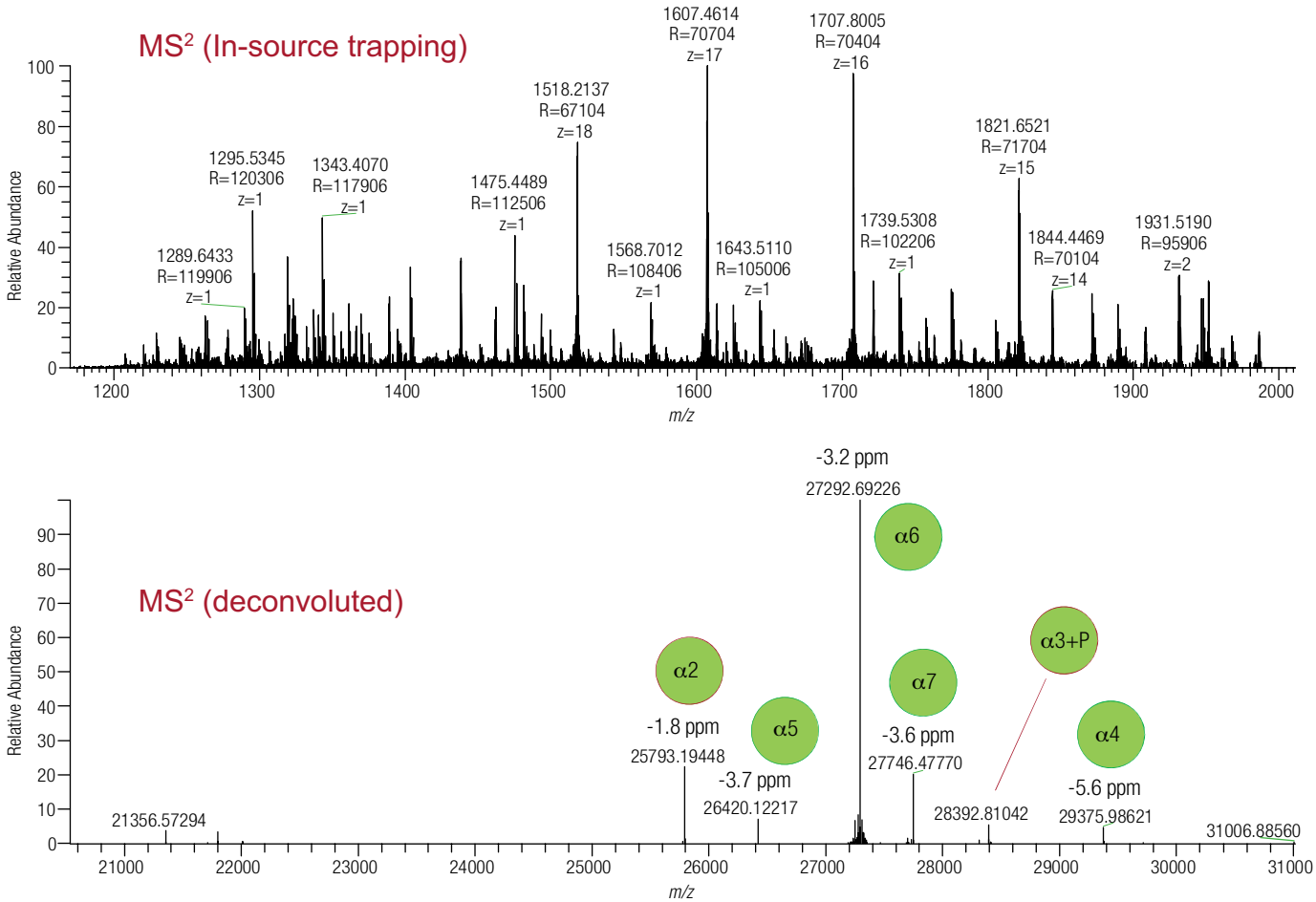


Figure 4. Native MS^2 (in-source-trapping) analysis of the rabbit 20S proteasome complex showing deconvoluted monoisotopic masses and mass accuracies. Six of the seven subunits were confidently identified (bottom).

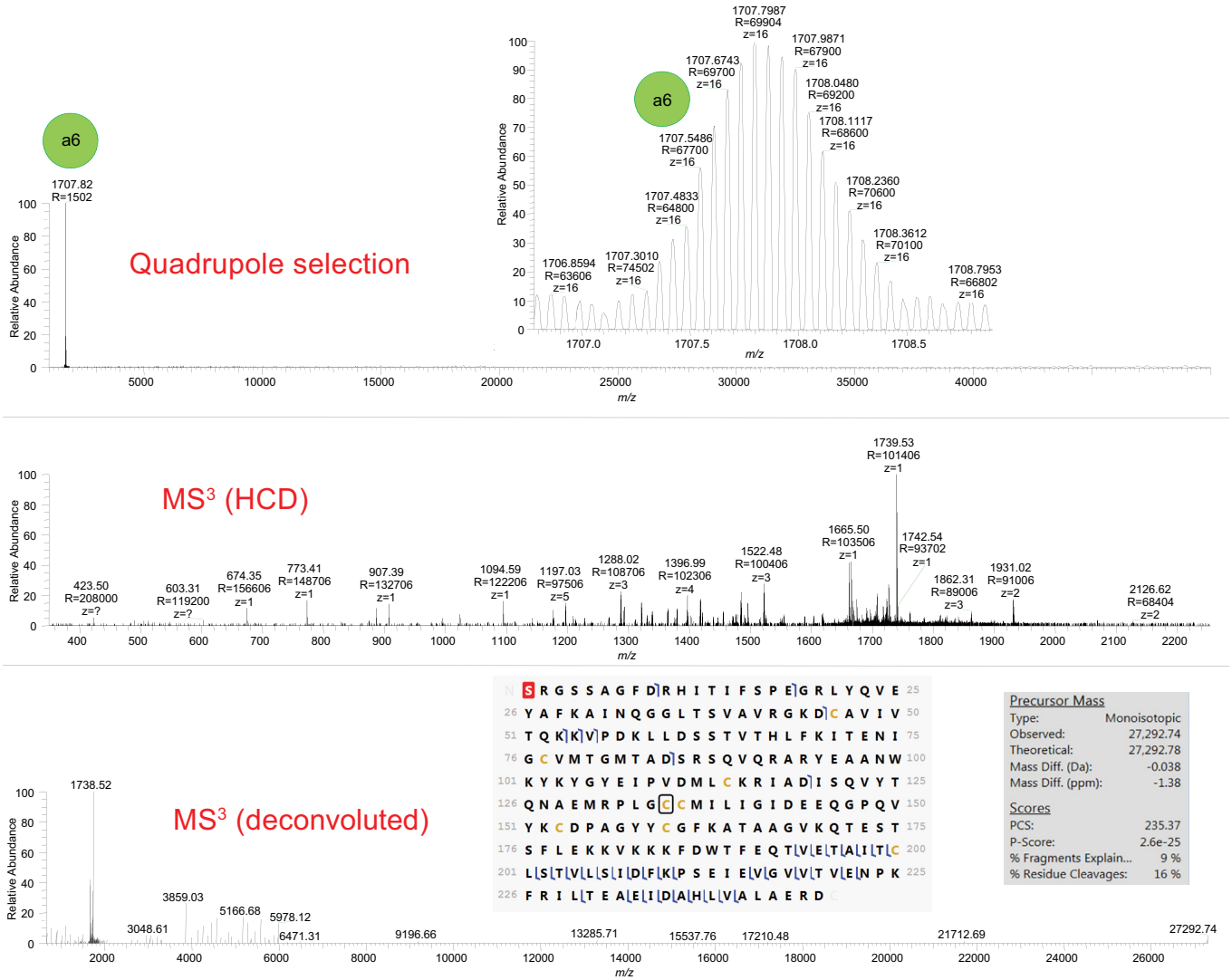
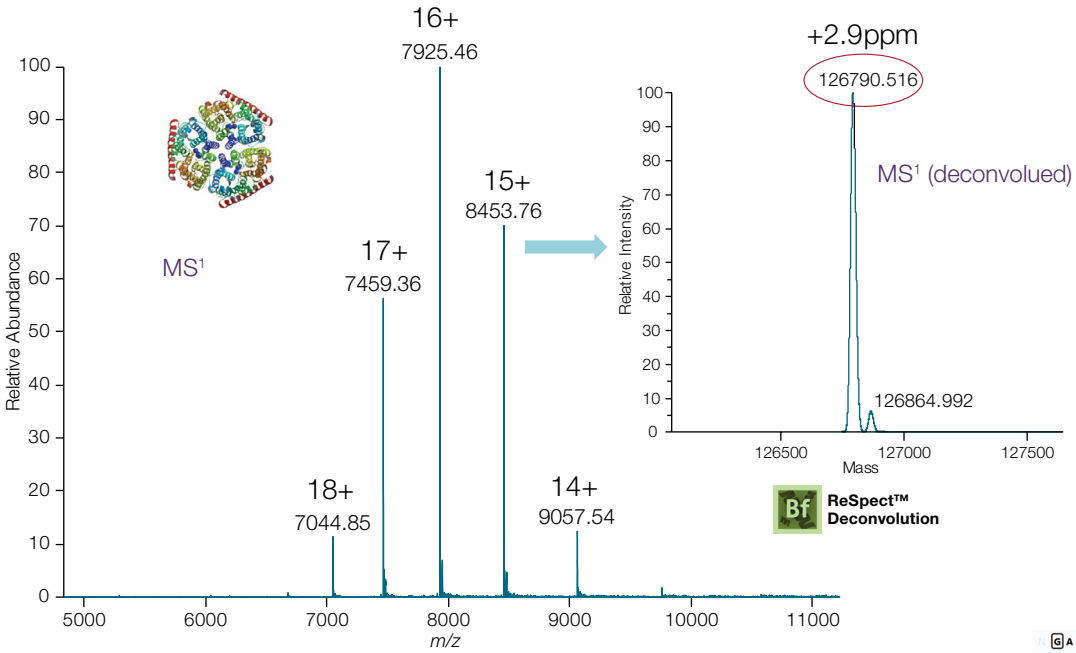


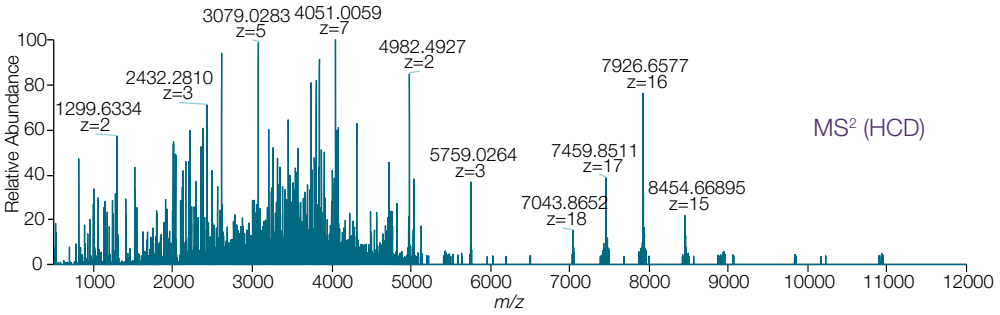
Figure 5. Native top-down pseudo-MS³ analysis of the rabbit 20S proteasome alpha-6 subunit

Accurate intact analysis of membrane proteins

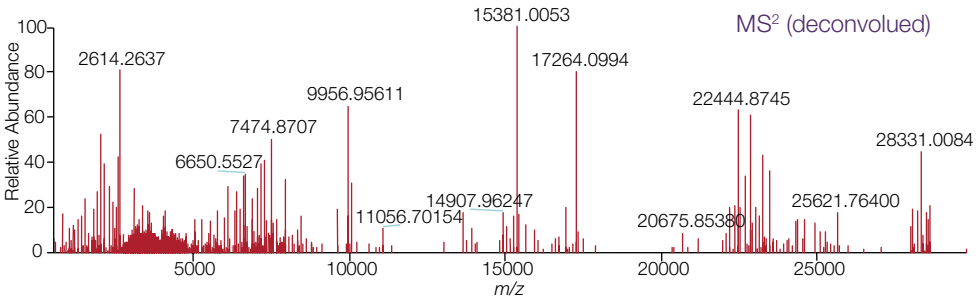
Membrane protein complexes are generally hydrophobic, requiring highly heterogeneous micelle assemblies for solubilization. This makes them extremely challenging for many biophysical analytical methods, including native MS. With in-source trapping, the Q Exactive UHMR mass spectrometer can release intact membrane-protein assemblies directly in the injection flatapole region from a variety of detergent micelles and membrane mimetics. By varying the desolvation energy, protein subunits can be released for top-down sequencing or, with very gentle activation, membrane proteins bound to multiple ligands can be retained for whole complex MS analysis.

Shown in Figure 6, in-source trapping allowed efficient removal of the detergent micelles for accurate intact mass determination of the AmtB trimer. Native top-down analysis of this membrane protein complex with low ppm mass accuracy resulted in high-confidence identification of AmtB protein, with 75 b and y matched ions representing 19% of the residue cleavages.





Scores	
PCS:	711.17
P-Score:	9.30E-63
% Fragments Explained:	24%
% Residue Cleavages:	19%



Matching Fragments (Count 77)

Name	Ion Type	Ion Number	Theoretical Mass	Observed Mass	Mass Difference (Da)	Mass Difference (ppm)
B230	B	230	24,349.81	24,349.80	-0.009	-0.38
B231	B	231	24,496.88	24,496.83	-0.043	-1.74
B239	B	239	25,252.29	25,252.36	0.062	2.45
B244	B	244	25,677.56	25,677.52	-0.040	-1.54
B270	B	270	28,330.98	28,331.01	0.031	1.10
B271	B	271	28,388.00	28,387.97	-0.026	-0.91

Figure 6. Native MS and native top-down analysis of the AmtB membrane protein complex. Low-ppm mass accuracy allowed confident identification of AmtB protein. Seventy-five b and y ions were identified, representing 19% residue cleavages.

Another example of efficient removal of detergent micelles and subsequent top-down analysis of a membrane protein is presented in Figure 7. Native MS and native top-down analysis of the multidrug transporter membrane protein LmrP from *L. lactis* using the Q Exactive UHMR mass spectrometer allowed identification of a total of 104 b and y ions, representing 33% residue cleavages.

Conclusion

The results of the native MS and native top-down experiments presented here demonstrate the unprecedented performance of the Q Exactive UHMR mass spectrometer when applied to the structural characterization of homomeric and heteromeric protein assemblies, including membrane protein complexes. With a unique combination of high mass resolution and accuracy, high sensitivity, and MS^2 and pseudo-MS^3 capabilities, the Q Exactive UHMR mass spectrometer pushes beyond the limits of today's native MS experiments.

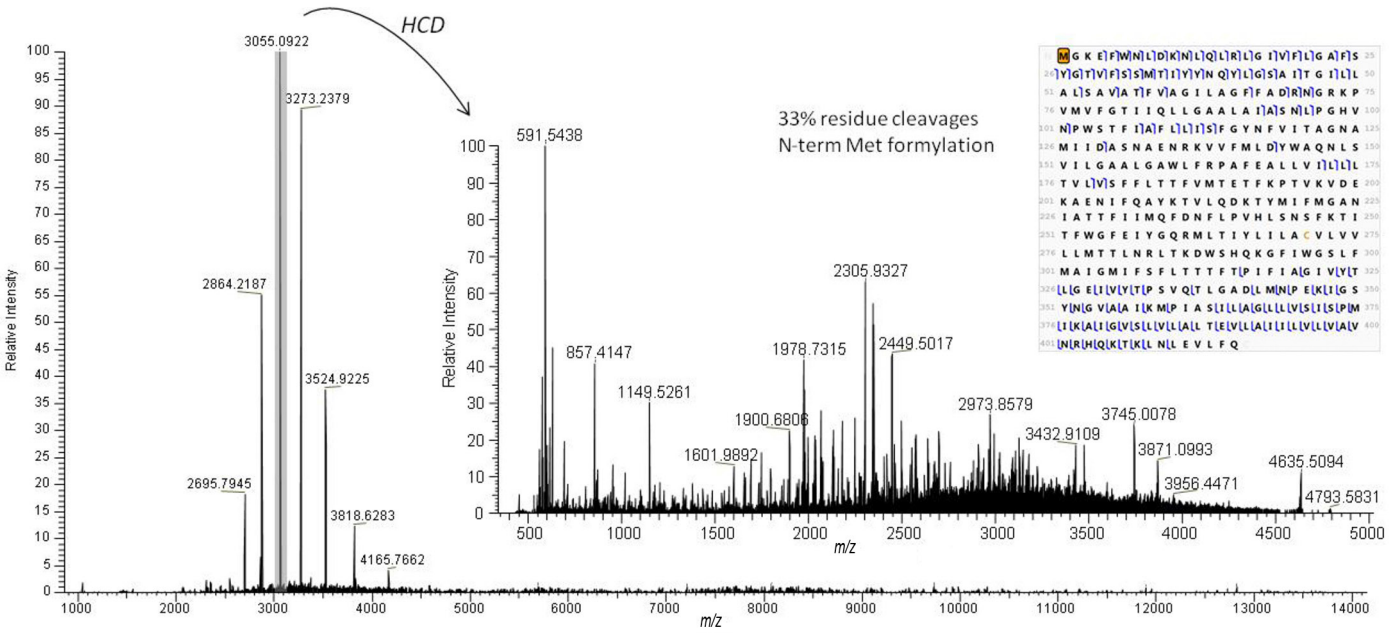


Figure 7. Native MS and native top-down analysis of the multidrug transporter membrane protein LmrP from *L. lactis* enabled identification of a total of 104 b and y ions, representing 33% residue cleavages

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