Application Note

Proteomics

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Analysis of Native Intact Protein Complexes Using the Agilent 6545XT AdvanceBio LC/Q-TOF Mass Spectrometer

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Abstract

The analysis of intact, native protein complexes by mass spectrometry (MS) requires ionization and transmission of ions with low internal energies. Nano electrospray ionization (nESI) techniques such as static spray and direct infusion have been shown to promote the formation of low-energy ions over traditional high flow ESI. A static nESI tip holder amenable to the Agilent Nanospray source on a commercial unmodified Agilent 6545XT AdvanceBio LC/Q-TOF MS was developed to study several protein complexes. The results show that using only micrograms of protein, large biomolecules are well resolved on the commercial unmodified Agilent 6545XT AdvanceBio LC/Q-TOF MS. The large biomolecules analyzed include soluble proteins, membrane proteins, and chaperonin protein complexes with molecular weights above 800 kDa. This study demonstrated the excellent performance of the 6545XT AdvanceBio LC/Q-TOF MS for the analysis of a wide variety of large protein complexes.

Introduction

As proteomics and pharmaceutical research and development has shifted towards the analysis of larger biomolecules, native mass spectrometry, which preserves the noncovalent interactions of proteins in both solution and gas phases, has emerged to characterize the compositions, stoichiometry, and topology of proteins.[1] Because no organic solvents or acids are added to enhance protein desolvation and ionization, native protein MS generally produces lower charge states and lower MS signal compared to denatured conditions. This is especially true for large protein complexes where the mass-to-charge ratio is well over 5,000 *m/z*, which is beyond the mass range of standard mass spectrometers.

Commercial instrumentation has been developed to accommodate larger ions. The development of larger ion traps with lower pressures has allowed for acquisition up to 80,000 *m/z* with sampling mass resolutions of 200k; however, these instruments are not often used for native MS studies where quantitation is required. Conventional time-of-flight mass analyzers have been used to study intact protein complexes, but the resolving power is often limited by pulsing electronics and non-optimal flight tube vacuum. Recent commercial developments in Q-TOF design have circumvented these issues through enhanced vacuum capabilities (ca. 10^{-8} Torr) and increased flight tube length.

These physical improvements have been actualized on the Agilent 6545XT AdvanceBio LC/Q-TOF MS, which was used with a modified tip holder in the Agilent Nanospray source and static spray capillaries for nESI of protein complexes. These protein complexes included alcohol dehydrogenase (ADH) and pyruvate kinase (PK) as examples of soluble protein complexes, the bacterial ammonium transport channel (AmtB) as an example of a membrane protein complex, and the protein chaperonin GroEL from *E. coli* as an example of a very large protein complex. Characterization of each of these systems only required picomoles of material per spectrum demonstrating the utility of this platform for high-resolution MS of native intact protein complexes. The effects of collisional activation in both the source and collision cell regions of the instrument were also demonstrated for further characterization of complex stoichiometry and interrogation of low relative abundance contaminant species.

Experimental

Materials and methods

Ammonium acetate and lyophilized powders of yeast alcohol dehydrogenase (ADH1_YEAST; UniProt ID: P00330; MW 147 kDa as tetramer; pI 6.21; PDB 4W6Z) and pyruvate kinase from rabbit muscle (KPYM_RABIT; UniProt ID: P11974; MW 232 kDa as tetramer; pI 7.60; PDB 1F3W) were purchased from Sigma-Aldrich (St. Louis, MO). A double mutant (E87C, C312T) of the ammonium transport channel membrane protein complex (AmtB; AMTB_ECOLI; UniProt ID: P69681; MW 126.9 kDa as trimer; pI 6.25; PDB 1U7G) and GroEL chaperonin complex (GroEL; CH60_ECOLI; UniProt ID: P0A6F5; MW 800.7 kDa as tetramer; pI 4.85; PDB 1SS8) were expressed at Texas A&M University. Ultrapure water (18.2 MΩ·cm) water was from Thermo Barnstead (Waltham, MA).

Instrumentation

- Agilent Dual Nanospray Ion Source (G3253A)
- Agilent 6545XT AdvanceBio LC/Q-TOF

Sample preparation

All samples were buffer exchanged to 200 mM aqueous ammonium acetate using Micro Bio-Spin P-6 gel columns at working concentrations of 1 to 10 µM. AmtB samples were buffer exchanged into 200 mM ammonium acetate supplemented with 0.5% C_8E_4.

MS analysis

Borosilicate capillary tips were pulled in-house using a Flaming/Brown micropipette puller (P-1000, Sutter Instruments), loaded with aqueous protein solution, and placed in a custom-made capillary tip holder with a Pt wire inside to ground the solution and facilitate nESI (Figure 1). This tip holder was then placed in the Agilent capillary holder, loaded into the Agilent Nanospray source, properly grounded, and position adjusted to within ~5 to 10 mm of the spray shield. The Nanospray source is coupled to an Agilent 6545XT LC/Q-TOF MS. Capillary voltages were adjusted to maximize ionization efficiency for each protein complex. Fragmentor and skimmer potentials were screened to maximize ionization and ion transmission through the source region while simultaneously minimizing dissociation. Further collisional activation was performed in the collision cell using N_2 as a bath gas at the manufacturer recommended backing pressures (22 psi). Before analysis of protein samples, the instrument was tuned in high-mass mode (*m/z* 30,000) with appropriate mass ranges selected for different experiments. Sliding scales for different mass ranges are available for *m/z* 90 to 10,000 through *m/z* 6,830 to 30,000 and were adjusted and retuned appropriately for different experiments.

Data processing

All experimental MS spectra were viewed and extracted from Agilent MassHunter Qualitative Analysis and BioConfirm (version 10). Experimental molecular weights (MW) were determined from MS peak maxima with average MW and standard deviations calculated from identified charge states. Theoretical molecular weights were calculated based on the appropriate UniProt sequences using ChemCalc.[2]

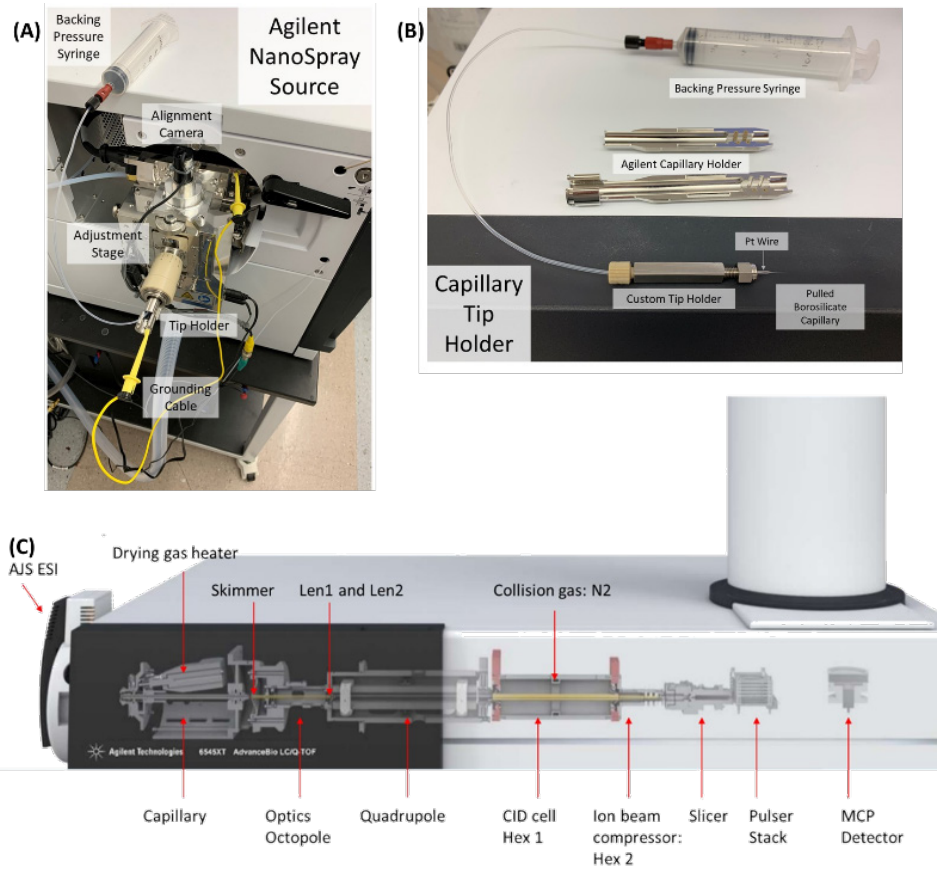


Figure 1. Instrument schematic for the Agilent 6545XT AdvanceBio LC/Q-TOF. (A) The Agilent Nanospray source includes a tip holder with an adjustment stage. (B) The tip holder was modified to incorporate a Pt wire in the borosilicate capillary with backing pressure manually applied by a syringe. (C) A schematic of the Agilent 6545XT with ion optics labeled.

Results and discussion

Soluble protein complexes

Optimization of source potentials was performed using the homotetrameric protein complexes alcohol dehydrogenase (ADH) and pyruvate kinase (PK) by varying the capillary exit and skimmer potentials (data not shown) and observing changes in relative abundances of ejected monomer ions and intact protein complex ions. At high source potentials, in-source dissociation leads to ejection of high charge monomer ions. At low source potentials, ion transmission decreases due to poor desolvation and ion transmission efficiencies. A compromise between maximizing ion transmission and minimizing dissociation was reached using similar source potentials for both protein complexes (capillary exit: 220 V; skimmer: 140 V).

The resulting native mass spectra of intact ADH and PK are shown in Figure 2. For ADH, the signals for the intact homotetrameric complex observed with a charge-state distribution (CSD) centered at 27^+. The measured zero-charge MW of 147.5 kDa is in good agreement with the theoretical MW (146.9 kDa). Mass spectra of native pyruvate kinase (PK) contain ions that are assigned to three different complexes. The intact homotetrameric complex is centered at the 36^+ charge state with a measured zero-charge mass of 232.0 kDa, which agrees well with the MW of PK following removal of the initiator methionine and acetylation of the N-terminal serine (231.8 kDa).[3] Low abundance signals, centered at 36^+, yield a measured MW of 229.8 kDa, and these ions are tentatively assigned to a truncation the first five residues (^{1}MSKSH^5) from each subunit corresponding to a molecular weight of 229.9 kDa for the resulting complex. A third CSD is observed that corresponds to a 153.9 kDa complex centered at 27^+ is also observed. The increased mass peak widths, significantly reduced zero-charge MW, and unidentified stoichiometry suggest that this complex is not a simple sequence modification of the native PK complex. While the specific sequences of this contaminant remain unknown, similar signals have been observed previously from samples prepared similarly to here.[4]

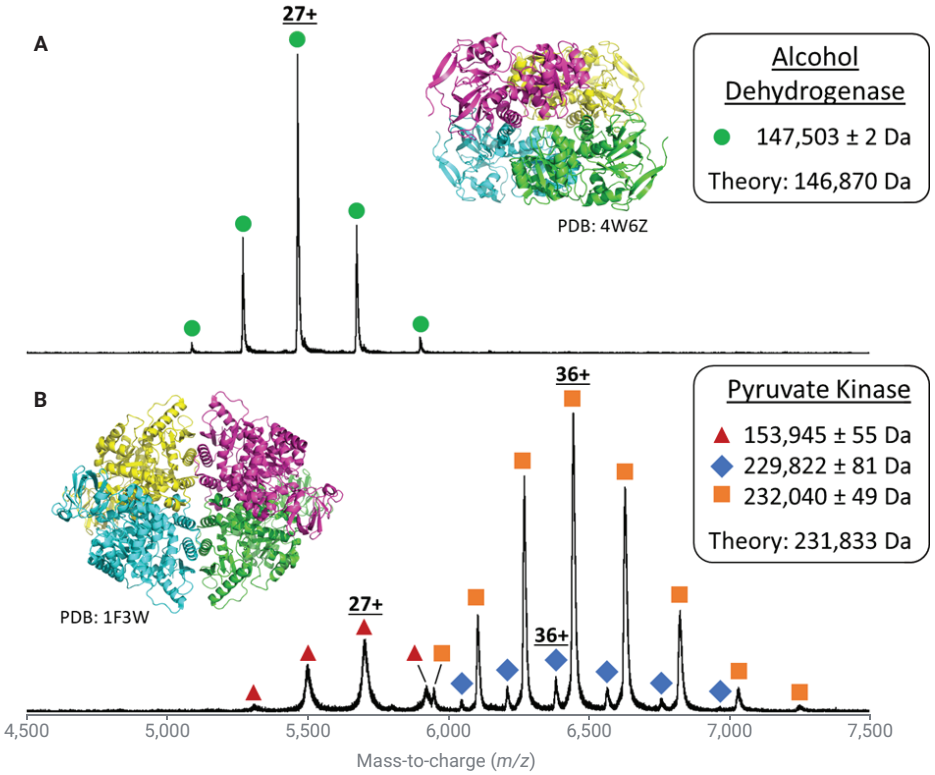


Figure 2. Native MS spectra of (A) alcohol dehydrogenase and (B) pyruvate kinase reveal the intact, native protein complexes. Zero-charge molecular weights for identified charge-state distributions are shown in the inset boxes. Differences between theoretical and zero-charge molecular weights are largely attributed to unresolved, noncovalent adducts and are a common feature of native mass spectra.

Membrane protein complexes

Native mass spectra of a double mutant (E87C, C312T) of the bacterial ammonium transport channel (AmtB) membrane protein complex revealed the intact homotrimeric complex centered at 16^{+}, as shown in Figure 3. At low collision energies, zero-charge mass spectra (Figure 3b) show a distribution of four species, viz. the apo-AmtB trimer complex and signals for to up three adducts that are shifted by 77 Da. These mass shifts are attributed to the covalent binding of β-mercaptoethanol (β-ME) molecules to the cysteine side chain of the E87C mutant. Increasing collision energy leads to monomer ejection, and the ejected monomer signals provide further evidence for the covalently bound β-ME molecules. The measured mass of the ejected apo-AmtB monomer ions (42,235 Da) is in good agreement with the theoretical MW of the mutant (42,273 Da).

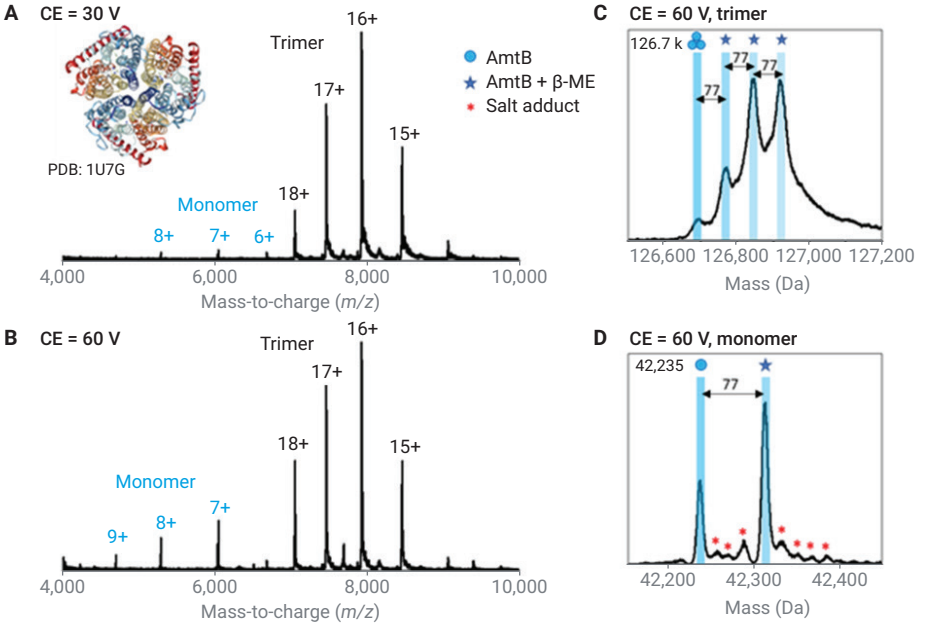
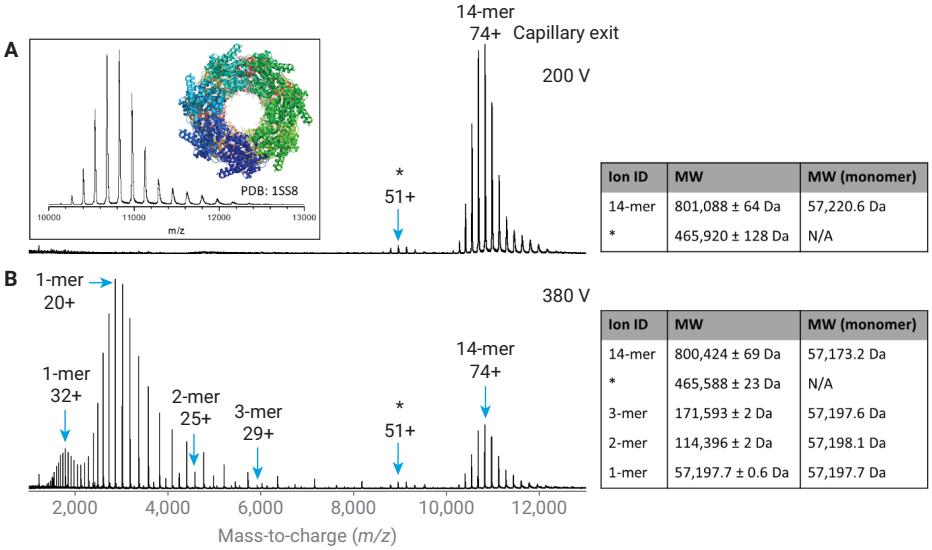


Figure 3. Native MS of a trimeric membrane protein complex. (A) MS spectrum of AmtB at low collision energy (30 V) reveals the native intact trimer complex with a mass of 126.7 kDa. (B) Increasing the collision energy (60 V) leads to increased monomer ejection. (C) The zero-charge mass spectrum shows up to three β-mercaptoethanol (β-ME) adducts (77 Da) on the intact trimer ions. (D) The zero-charge mass spectrum of the ejected monomer ions reveals low abundance salt adducts and one β-ME molecule per subunit and low abundance salt adducts.

Chaperonin complex (GroEL)

GroEL is a tetradecameric (14-mer) protein chaperone, composed of two stacked heptameric rings, which assists protein refolding. Native mass spectra for the GroEL 14-mer complex are observed in high relative abundance with well-resolved charge states when electrosprayed using mildly activating source potentials (fragmentor voltage 200 V, Figure 4A). The measured mass determined under these conditions (801,088 Da) agrees well with the theoretical MW of the chaperonin complex (800,760 Da). In-source dissociation (ISD) of GroEL was then performed by increasing the fragmentor voltage to 380 V (Figure 4B), resulting in the ejection of low-order oligomers and a shift in the measured mass of the intact GroEL by ~664 Da. Signals for ejected oligomers include monomer, dimer, and trimer ions with monomer ions observed with a bimodal CSD. The measured subunit masses of the ejected ions



Ion ID	MW	MW (monomer)
14-mer	801,088 ± 64 Da	57,220.6 Da
*	465,920 ± 128 Da	N/A

Ion ID	MW	MW (monomer)
14-mer	800,424 ± 69 Da	57,173.2 Da
*	465,588 ± 23 Da	N/A
3-mer	171,593 ± 2 Da	57,197.6 Da
2-mer	114,396 ± 2 Da	57,198.1 Da
1-mer	57,197.7 ± 0.6 Da	57,197.7 Da

Figure 4. Native mass spectra for GroEL. (A) Mass spectra obtained using low source potentials, nESI of GroEL contain abundant signals for the intact 14-mer complex centered at 74+. The inset in (A) contains a narrow *m/z* range that illustrates the high resolution obtained for the intact 14-mer complex shown and the crystal structure of GroEL (PDB 1SS8). (B) Contains the in-source dissociation (ISD) mass spectrum of GroEL. The mass spectrum contains strong signals for unfolded and native-like monomer ions (charge states 20+ and 32+, respectively) as well as di-, and trimer ions centered at 25+, and 29+, respectively. The measured MWs for each of the GroEL species are reported in the boxes. The measured mass for the CSD label with an *, assigned as 51+ ions with MW of 465.9 kDa) does not agree with any known ions derived from GroEL (see text). It is possible that this species could be an 8-mer of GroEL, as an intact 7-mer with a monomer trapped inside the cavity along with water and other small salt molecules. Further identification of the stoichiometry and topology of these ions is ongoing.

(57,198 Da) are in excellent agreement with the theoretical GroEL subunit mass (57,197 Da).

CID was performed on the 14-mer GroEL complex by increasing the collision cell voltage while sampling in a higher *m/z* range to observe signal for the charge-stripped 13-mer ions (Figure 5). Increasing the collision voltage from 20 to 60 V results in shifts in the measured mass of GroEL owing to the removal of adducted species before dissociation. At higher CID energies, charge stripping of the 14-mer ions also occurs as the CSD expands to lower charge with increased CE; alternatively, the relative abundance of lower-charge ions (higher *m/z*) increases at higher CE owing to depletion of higher charge state ions. At 80 V, the charge-stripped 13-mer ions appear centered at 45^{+}. When charge-balanced, the CSD of the native 14-mer (CE 0 V, centered at 75^{+}) and charge-stripped 13-mer ions (CE 100 V, centered at 43^{+}) suggests the ejected monomer ions would be centered at 32^{+} (*m/z* 1787.6), which agrees well with CSD of ejected monomer ions from ISD shown in Figure 4B. Also, signals for a possible octamer are observed at both high and low collision energies (labeled with * in Figures 4 and 5), which suggests that the ions are not a dissociation product of GroEL 14-mer ions. These ions are tentatively assigned as an unfolded monomer trapped in a single heptameric ring. The subunit mass differences between these ions (~58.2 kDa) and native GroEL and its ejected subunits (~57.2 kDa) is attributed to salt, water, and/or other small molecules binding to the interior and exterior of this complex. Further identification of the stoichiometry and topology of these ions is ongoing.

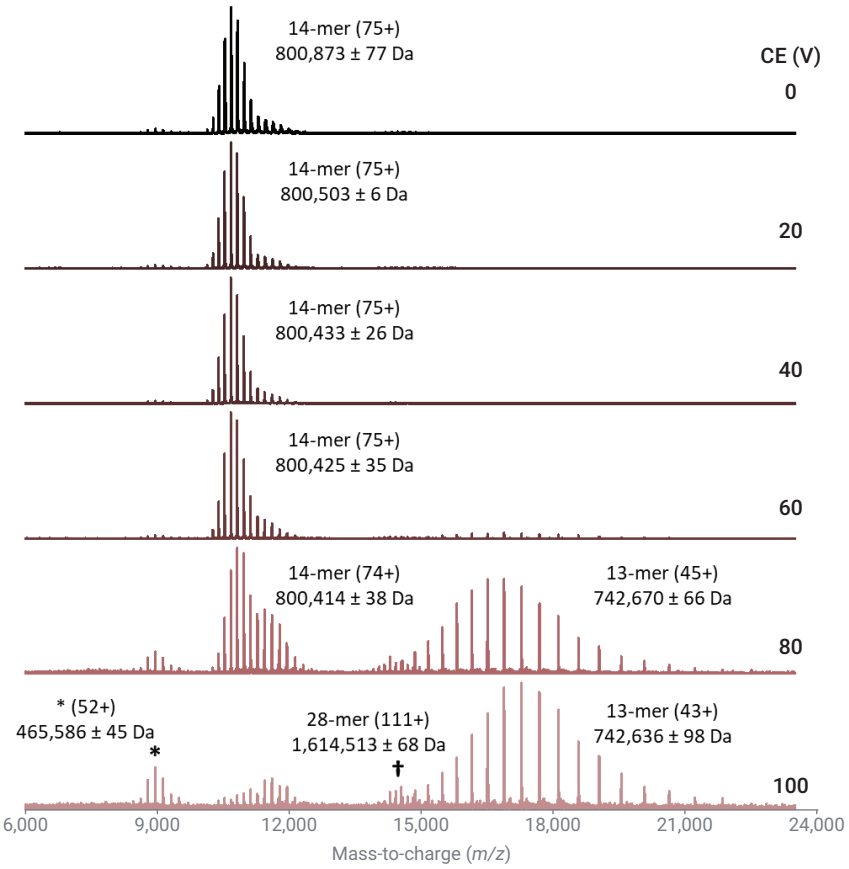


Figure 5. MS spectra of GroEL over a range of collision energy (CE) voltages. As the CE increases, both charge-stripping and CID are observed. Low abundance GroEL 28-mer ions appear at higher relative abundance at high collision voltage due to lower abundance of neighboring signals.

Conclusion

MS analysis of native, intact protein complexes requires the preservation of noncovalent interactions when transitioning from nonvolatile biological buffers to a volatile aqueous buffer solution and ionization methods, which retain those native contacts to minimize unfolding and/or dissociation before detection. The custom static nESI source allows the acquisition of well resolved mass spectra for large biomolecules on the commercial Agilent 6545XT AdvanceBio LC/Q-TOF MS, including soluble protein, membrane protein, and chaperonin protein complexes with molecular weights above 800 kDa using only micrograms of protein. Low-abundance contaminants were identified of native protein mass spectra, and native, intact chaperonin complexes and ejected low-order oligomeric products of in-source dissociation were observed, demonstrating the superior sensitivity and resolving power of the 6545XT AdvanceBio LC/Q-TOF for intact large molecules.

References

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