Application Note

Wyatt Technology Corporation

Absolute Molar Mass in UHPLC via the µDAWN and UT-rEX

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Size exclusion chromatography (SEC) is widely used for characterizing the size and composition of aggregates or fragments in a biomolecular sample. Conventional SEC relies on reference standards to calibrate column elution time as a function of molar mass. However, these standards often do not accurately represent the conformation of the sample, or the sample may exhibit column interactions that change its elution properties relative to the standard. Coupling a light scattering detector, such as the miniDAWN™ TREOS®, to SEC enables absolute quantification of a molecule's molar mass, irrespective of elution time.

Ultra-high performance liquid chromatography (UHPLC) enables fast, efficient separation of biomolecular samples. Compared to standard SEC, UHP-SEC provides improved resolution, higher throughput, less solvent and smaller sample consumption for analysis of precious biological samples. Detectors designed for standard HPLC, however, have proven incompatible with this technology. The Wyatt µDAWN™ multi-angle light scattering (MALS) detector and Optilab® UT-rEX™ refractive index (RI) detector, designed specifically for UHPLC applications, combine to measure the absolute molecular weight (or molar mass) and size of eluting species in UHPLC.

For a given protein, the entire UHPLC analysis can be completed in under 5 minutes (Figure 1, red) as compared to traditional HPLC (Figure 1, blue) which may require 20 minutes or more per sample. In both cases, the combination of light scattering intensity and concentration measured by RI are used to determine the molar mass for each peak. Figure 1 overlays the chromatograms with the monomer, dimer and aggregate molar masses as determined by the miniDAWN TREOS and Optilab T-rEX for standard SEC-MALS, and the µDAWN and Optilab UT-rEX for UHP-SEC-MALS. The chromatograms in Figure 1 (top) are rescaled as a function of column volume (bottom) for easier comparison. The perfect agreement in molar mass for each peak means that it is now even easier to migrate existing HPLC methods to UHPLC while maintaining the same accurate, high-quality molecular weight data.

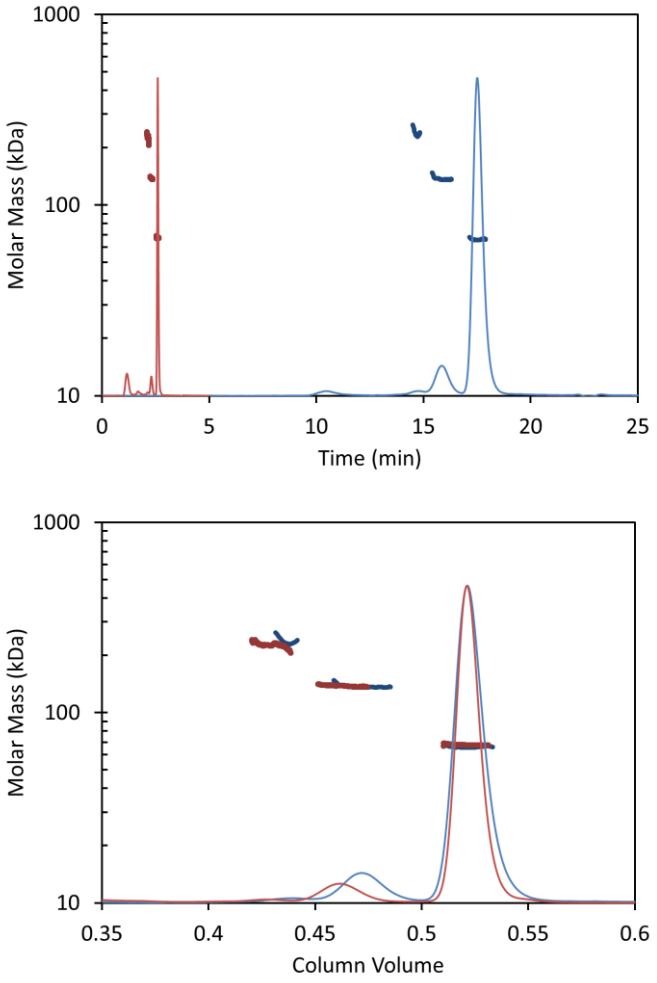

Figure 1: Light scattering data and measured molar mass for a protein separated by UHPLC and detected using the µDAWN and UT-rEX (red) overlaid with the separation by standard HPLC and detected with the miniDAWN TREOS and Optilab T-rEX (blue). The top plot shows the chromatograms as a function of elution time. The same data are rescaled in the bottom plot as a function of column volume.

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In addition to faster analysis time, the small particle size of the UHPLC stationary phase means enhanced resolution of aggregate and fragment peaks. Dispersion caused by the large internal volume of standard columns and detectors broadens out these small peaks, leading to the "vanishing" of certain poorly-resolved species, such as the fragment shown in Figure 2 (blue traces). The µDAWN is optimized for minimal internal volume in order to preserve the resolution in a UHPLC elution profile, enabling the detection and analysis of previously unknown species.

As shown in the bottom chromatogram of Figure 2 (red traces), the fragment peak is preserved as it travels from the UV detector to the µDAWN and UT-rEX in order to quantify the molar mass of the fragment. Since the refractive index increment (dn/dc) is nearly constant for all proteins, there is no need to know the extinction coefficient of the peak (and thus the identity of the fragment) in order to determine the eluting concentration and molar mass. In fact, the combination of UV and RI detection may be used to determine the extinction coefficient of each species and aid in their identification.

Not only does the µDAWN quantify absolute molecular weight, it also offers all the in-depth analyses available with the miniDAWN TREOS to UHPLC. As with standard HPLC-MALS, changes in molar mass across the UHPLC-MALS peak quantify polydispersity of a biopolymer or assess reversible protein oligomerization. Triple detection with UV, MALS, and RI enables the application of Protein Conjugate Analysis to identify the amount of post-translational modification, associated detergent, drug-conjugate, or other additions to a polypeptide background. Optional dynamic light scattering (DLS) detection with the WyattQELS™ module measures hydrodynamic radius in the same measurement volume as MALS detection, while the 10-50 nm range of rms radius available to the three-angle miniDAWN is replicated in the µDAWN. Finally, this complete set of detectors means enjoying the efficiency of UHPLC without sacrificing the data quality and the ability to measure absolute molar mass of polymers in solution via MALS.

Methods: UHPLC was performed using a Waters Acquity UPLC pump, autosampler, and UV detector coupled to the µDAWN and UT-rEX. The separation was achieved with a Waters BEH200 SEC column: 4.6 × 150 mm for the data in Figure 1 and 4.6 × 300 mm for the data in Figure 2. Bovine serum albumin was used for all samples.

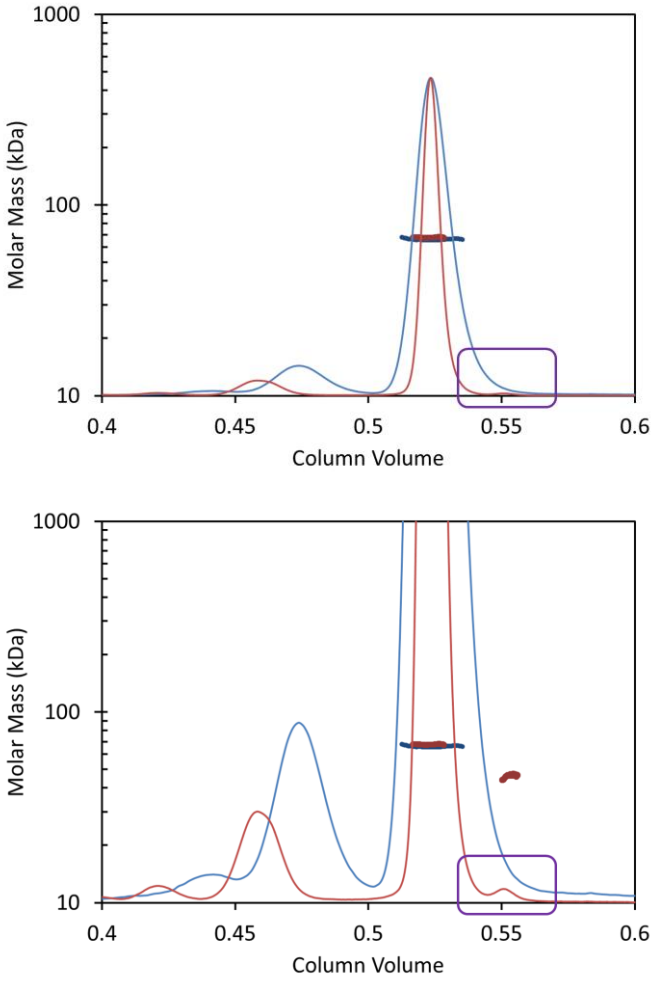

Figure 2: Light scattering data and measured molar mass for a protein separated by UHPLC (red) overlaid with the separation by standard HPLC (blue). The box indicates a fragment peak that was separable by UHPLC. The top figure shows the entire chromatogram. The bottom figure zooms in on the base of the peaks and shows the molar mass for the fragment peak, as measured by the µDAWN and UT-rEX.