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APPLICATION NOTE

AN1622: SEC-MALS Analysis of Proteins with Empower™ Chromatography Data System

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Summary

Empower™ Software is globally adopted as a compliant-ready Chromatography Data System (CDS) used to submit novel drug data to regulatory agencies. It now integrates data acquisition and processing for certain applications using size exclusion chromatography with multi-angle light scattering (SEC-MALS). This integration bridges the reliability and traceability of Empower Software with the versatility and precision of SEC-MALS. This new capability can streamline the transfer of SEC-MALS methods from development, where the full suite of analyses available in ASTRA™ software is required, to a simplified workflow for quality control (QC). In this application note, we used Empower 3.10.0 software to characterize the NIST monoclonal antibody (NISTmAb) standard reference material (SRM 8671)[1] and the BEH™ 200 SEC Protein Standard Mix by SEC-MALS.

Introduction

Waters Empower CDS software streamlines select workflows of SEC-MALS data acquisition analysis related to proteins, peptides, and similar biomolecules. With this functionality, the molar mass and total eluted mass of biotherapeutics, like monoclonal antibodies (mAbs) and GLP-1 analogs, can be monitored in a regulatory-friendly environment enhancing quality, long-term data integrity, and productivity.

SEC-MALS can be used to determine the molar mass of therapeutic proteins, independent of elution time. The molar mass is measured directly at each point across the chromatogram, enabling direct quantification of heterogeneity across the peak, which may be caused by coeluting aggregates or fragments or on-column dissociation of reversible oligomers. Analysis with SEC-MALS can be particularly advantageous compared to mass spectrometry as it enables analysis in a variety of mobile phases, including formulation buffers or physiological conditions. The incorporation of SEC-MALS data into the Empower Software platform can mediate the transfer of methods from development into QC workflows to simplify the qualification process and meet regulatory needs.

In this study, two protein standards were analyzed by SEC-MALS using Empower 3.10.0 software:

1. NISTmAb, which is used as a benchmark molecule in method qualification for characterization of mAbs
2. BEH200 SEC Protein Standard Mix, which is a mixture of protein standards used to assess column and system performance.

We utilize Empower Software to determine the identity and purity of these samples as a demonstration of the software's capabilities and underscore its suitability in QC.

Materials and Methods

Materials and reagents

BEH200 SEC Protein Standard Mix (Waters part number 186006518) was reconstituted in 1 mL of mobile phase and filtered to 0.2 µm. NISTmAb at 10 mg/mL was obtained from Millipore Sigma and was used as-is.

SEC-MALS Analysis

SEC-MALS was performed using a Waters Arc™ Premier HPLC system, which includes a quaternary solvent manager and a sample manager. The mobile phase consisted of 50 mM sodium phosphate pH 6.7 with 50 mM sodium chloride and 250 ppm sodium azide. Samples were separated using a Waters XBridge™ Premier Protein SEC column (250 Å, 2.5 µm, 7.8 x 300 mm) at a flow rate of 0.5 mL/min and a column temperature of 25 °C. Filtered BEH200 SEC Protein Standard Mix was injected at 12 µL or 25 µL. NISTmAb was injected neat at 10 µL. For

detection, a Waters Arc Premier 2489 UV/vis detector, a DAWN™ MALS photometer, and an Optilab™ differential refractive index (dRI) detector were employed. Data were collected and analyzed in Empower 3.10.0 software.

Empower Data Processing

The Empower Software method set consists of the instrument method, processing method, reporting method, along with additional system constants and channel sets needed to analyze SEC-MALS data. The key features of the processing method are the peak "Integration" table and the "MALS Components" table.

System Constants

System constants including band broadening parameters and normalization coefficients were first determined using a monodisperse and isotropic standard, such as BSA (data not shown). These system constants, along with the MALS calibration constant, concentration source, and laser wavelength were all saved to the method set and used for all samples within this dataset. For more information about establishing system constants, please visit the Wyatt Support Center.

SEC-MALS Processing Method – Integration

The Integration table specifies the baseline region and region of interest where peaks should be identified. The same integration table can be used for all injections in a sample set or sequence, regardless of the number of peaks expected to be found. These parameters must be defined for both the concentration source ("Concentration" radio button, Figure 1 left) and the MALS detectors ("SEC-MALS" radio button).

SEC-MALS peak integration requires a minimum of three inputs for both the concentration and MALS channels (Figure 1 left): "Inhibit Integration" before (Line 1) and after (Line 3) the relevant elution volume and "Force Baseline by Time" to enable *absolute* quantitation of both concentration and light scattering intensity (Line 2). It is important to note the "Force Baseline by Time" function, which is required for accurate SEC-MALS analysis, is only available within the "Traditional" Integration Algorithm and is not available in ApexTrack. The "Inhibit Integration" time points must match for the concentration and MALS detector. The "Force Baseline by Time" endpoints may differ between the concentration and MALS detector channels to accommodate differences in baseline noise and drift. Additional lines may be added, such as "Force Dropline" to fix peak endpoints, as needed. The peak width, threshold, minimum area, and minimum height may also be adjusted as needed to optimize peak finding.

SEC-MALS Processing Method – MALS Components

The "MALS Components" tab specifies the peak name, retention time, refractive index increment (dn/dc), and applicable extinction coefficient which assigned to the appropriately found peaks (Figure 1, right). These component parameters are unique to each type of sample in a sample set or project. Thus, individual processing methods may be required for different injections in a particular Sample Set, although some information can be carried over from one processing method to another, including the peak integration table. In this study, the MALS Components table for the BEH200 Protein Standard Mix was defined as shown in Figure 1 (right). For NISTmAb, a single monomer component was specified with dn/dc of 0.185 mL/g and UV extinction coefficient at 280 nm of 1.42 mL mg^{-1} cm^{-1}.

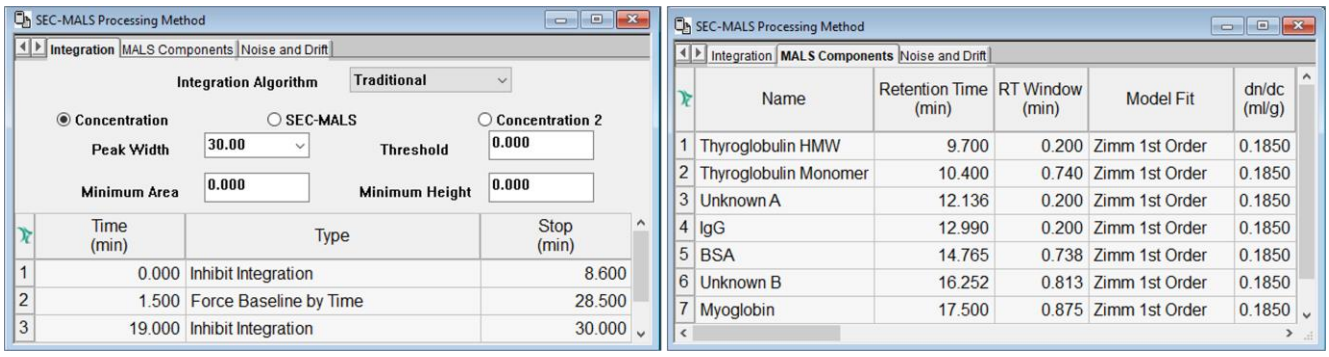

Figure 1: Example SEC-MALS processing method for the BEH200 SEC Protein Standard Mix. Left: Integration tab and inputs required to find peaks in the elution region between 8.6 and 19 minutes using the Traditional Integration Algorithm. Right: MALS components tab with relevant information for identified peaks in the BEH200 Protein Standard Mix

Results and Discussion

BEH200 SEC Protein Standard Mix

To evaluate column performance and assess Empower peak detection capabilities, we first quantified the molar mass each peak in the Waters BEH200 SEC Protein Standard Mix. To characterize peaks with unspecified extinction coefficients and resolve their identities, data were obtained using a dRI detector in combination with MALS, which provided insights that could not be achieved from elution time alone.

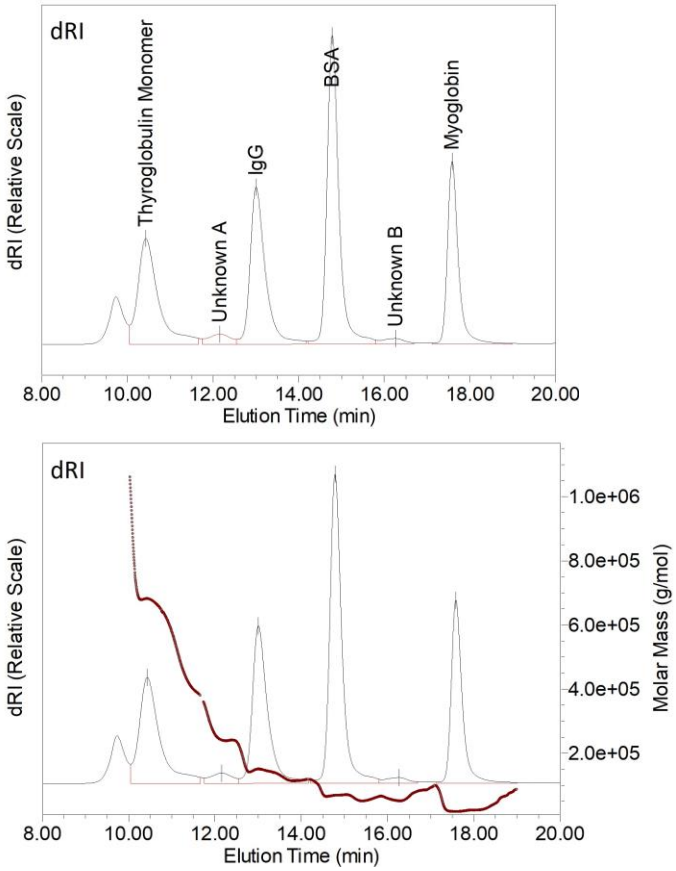

Figure 2: SEC-MALS analysis of the BEH200 SEC Protein Standard Mix. Top: Differential refractive index (dRI) chromatogram depicting the full elution region with each component labeled. Bottom: Measured molar mass overlaid on dRI chromatogram.

This mixture includes thyroglobulin, IgG, BSA, and myoglobin. The XBridge Premier Protein SEC column provided excellent separation and resolution in agreement with the certificates of analysis for the Protein Standard Mix and the column (Figure 2). A total of seven peaks were identified and processed—the four protein peaks specified in the certificate of analysis, a high-molecular weight (HMW) thyroglobulin peak, and two unknowns.

The measured molar mass and eluting concentration of every major peak was in good agreement with the certificate value (Table 1). The first two peaks consist of thyroglobulin monomer and a HMW species. MALS confirmed that the solution weight-average molar mass (M_w) of the thyroglobulin monomer to be 652 kDa, which agrees with the expected certificate and sequence value of 660 kDa. The M_w of the HMW species was 1895 kDa which is much higher than the expected molar mass of the dimer (1320 kDa) (Table 1). In addition, the total concentration of thyroglobulin (1.41 mg/mL for combined monomer and HMW) is in good agreement with the specified concentration of thyroglobulin on the certificate of analysis (1.5 mg/mL), providing additional insights into column health and system suitability. Inspection of the molar mass distribution of the HMW species instantly reveals heterogeneity in the measured molar mass (Figure 3) unlike the thyroglobulin monomer and other species which exhibit a constant molar mass across the peak. These results suggest coelution of dimer, trimer, and/or higher order oligomers within this single HMW peak. Relying only on peak shape and retention time, as in traditional analytical SEC-UV, would lead a user to wrongly assign the thyroglobulin dimer as the identity of the peak.

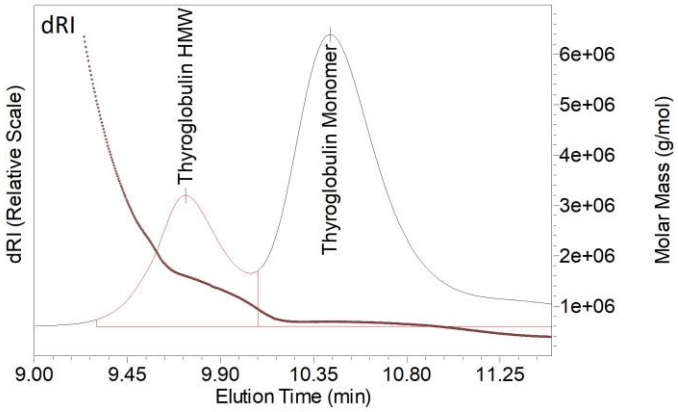

Figure 3: Measured molar mass overlaid on dRI chromatogram for the BEH200 SEC Protein Standard Mix. Only the first two peaks (eluting between 9 min and 11.5 min) are shown to highlight the thyroglobulin monomer and HMW species.

In addition to the four proteins specified on the certificate of analysis, two additional unknowns were identified and quantified. Unknown A, eluting at 12.2

minutes, accounts for <2% of the total eluted mass with M_w of 252 kDa (Table 1). The molar mass suggests that this could be co-elution of the IgG dimer and the BSA dimer. Unknown B, eluting at 16.3 minutes, comprises ~1% of the total eluted mass with M_w of 64 kDa. This peak may contain an oligomer of myoglobin, a BSA fragment, a low molecular weight (LMW) IgG species, or co-elution of any of the aforementioned species (Figure 2). The molar mass distribution of unknown B exhibits heterogeneity throughout the peak making coelution likely. With just SEC alone, the identity of these unknown peaks would be limited. However, the addition of MALS gives deeper insights into the peak identity and heterogeneity.

Table 1: Retention time, weight-average molar mass (M_w), calculated eluting concentration (Conc.), and mass fraction measured by SEC-MALS for proteins in the BEH200 SEC Protein Standard Mix.

Species	Exp. MW (kDa)[1]	Exp. Conc. (mg/mL)[1]	Retention Time (min)	Meas. M_w (kDa)[2]	Meas. Conc. (mg/mL)[2]	Mass Fraction (%)[2]
Thyroglobulin HMW	NS	NS	9.7	1895 ± 19	0.35 ± 0.01	6.3 ± 0.2
Thyroglobulin Monomer	660	1.5	10.4	652 ± 5	1.06 ± 0.01	19.3 ± 0.2
Unknown A	NS	NS	12.1	252 ± 9	0.10 ± 0.00	1.9 ± 0.1
IgG	150	1.0	13.0	146 ± 2	1.16 ± 0.01	21.1 ± 0.2
BSA	66.4	2.5	14.8	67 ± 1	1.78 ± 0.01	32.4 ± 0.4
Unknown B	NS	NS	16.3	64 ± 2	0.07 ± 0.02	1.3 ± 0.0
Myoglobin	16.7	1.0	17.5	18 ± 0.1	0.97 ± 0.02	17.7 ± 0.3

NS: Not specified
1. Expected values, as specified on Certificate of Analysis, calculated using UV detector
2. All values are the average and standard deviation of triplicate measurements, calculated using dRI detector

NISTmAb

As expected, NISTmAb eluted as a single, well-defined main peak along with a small fraction (<5% by mass) of HMW species (Figure 4). The XBridge Premier SEC column provided excellent mass recovery of the NISTmAb sample (95.5%). The measured M_w of the main monomeric peak is 153± 0.2 kDa (Figure 5) , in excellent agreement with the expected molar mass and previously confirmed intact mass spectrometry results[2]. The NISTmAb monomer was the predominant species with a mass fraction of 97.6%, in agreement with the certificate mass fraction value.

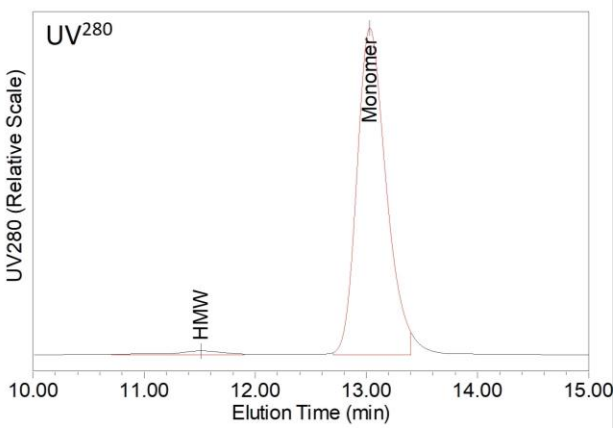

Figure 4: UV Chromatogram of NISTmAb depicting the monomer and HMW species

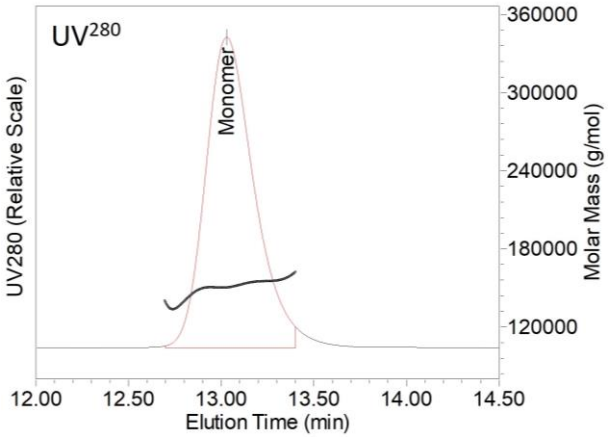

Figure 5: UV Chromatogram of NISTmAb depicting a zoomed-in view of the monomer peak. The molar mass distribution is overlaid.

Conclusions

In this study, we identified the solution molar mass of multiple protein standards, quantified their abundance, and confirmed the identity of oligomers with excellent reproducibility and accuracy. Empower Software peak detection capabilities enabled streamlined processing and analysis. MALS enabled direct quantification of the molar mass across the chromatogram, revealing heterogeneity across some peaks and giving deeper insight into sample identity. These results highlight how SEC-MALS with

Empower Software can be adopted in QC for reliable characterization and data integrity, accelerating the production of safe and efficacious biotherapeutics.

The integration of molar mass analysis by MALS into Empower CDS software enables the implementation of SEC-MALS workflows into QC in regulated environments. With Empower 3.10.0, acquisition and processing methods developed using the full suite of analyses available in ASTRA software can be transferred into Empower Software for enterprise-wide integration into QC.

References

(1) NISTmAb, Humanized IgG1κ Monoclonal Antibody., National Institute of Standards and Technology. https://tsapps.nist.gov/srmext/certificates/8671.pdf (accessed 2025-06-27).

(2) Kashi, L.; Yandrofski, K.; Preston, R. J.; Arbogast, L. W.; Giddens, J. P.; Marino, J. P.; Schiel, J. E.; Kelman, Z. Heterologous Recombinant Expression of Non-Originator NISTmAb. *mAbs* **2018**, *10* (6), 922–933. https://doi.org/10.1080/19420862.2018.1486355.

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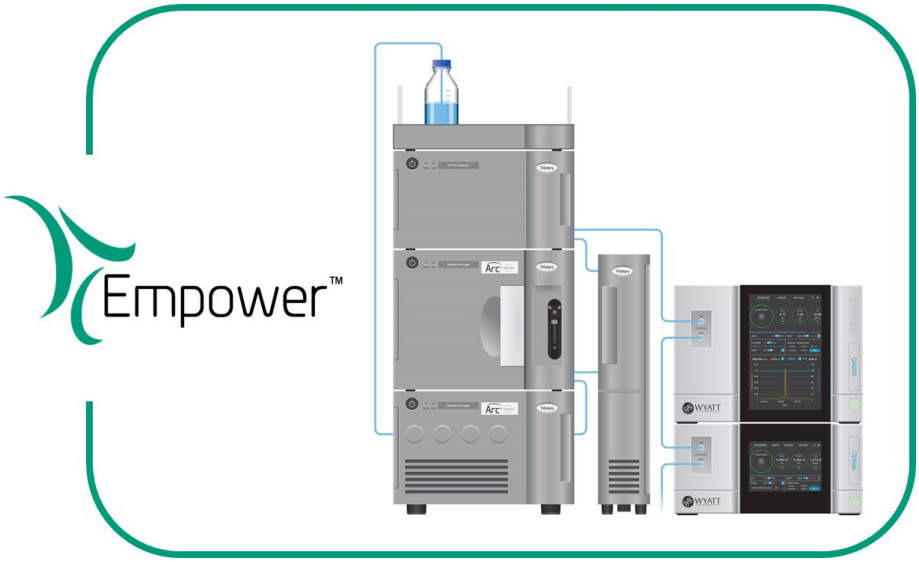

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