Introduction

HPLC is used to separate components of a mixture, allowing for the purification, identification and quantification of macromolecules. Coupling this with light scattering (MALS-QELS) and refractive index (RI) detectors determines the MW and size of sample components.

Features

Bio-Inert Agilent 1260 Infinity II HPLC System

Components include:

- Quaternary pump (60 MPa, 0.001—10 mL/min)
- High capacity multi-sampler (vials and plates)
- Peltier-controlled column compartment
- UV-Vis diode array detector (DAD)
- Fluorescence spectra and phosphorescence detectors
- Versatile fraction collector
- Optional coupling to MALS-QELS-RI detectors

Wyatt miniDAWN TREOS MALS/QELS Detector

- Uses light scattering detectors at 3 angles (49˚, 90˚, 131˚) combined with an integrated Quasi-Elastic Light Scattering (QELS) module to calculate the absolute molecular mass, RMS radius and hydrodynamic radius (R_h).

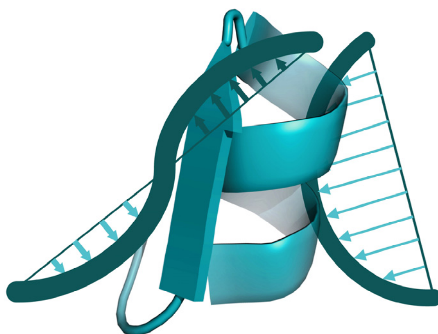
Wyatt Optilab T-rEX RI Detector

- Measures differential RI and dn/dc (refractive index increment) of the solvent at the same wavelength as the miniDAWN TREOS.
  RI detection is often used for samples with weak or no UV absorption.

- Temperature range from 4°C - 65°C with ±0.005°C regulation.

Applications

- A wide array of columns available for Bio-HPLC allow for versatility in sample analysis.
- The in-line T-rex RI detector can be applied to analyze polymers, saccharides, fatty acids and samples with low/no UV absorption, to accurately quantify the relative amounts and masses of species present in a polydisperse sample.



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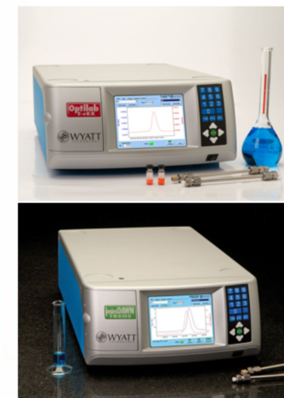


Bio-HPLC-MALS-QELS-RI

Agilent 1260 Infinity II Bio-Inert High Performance Liquid Chromatography with Wyatt Multi-Angle & Dynamic Light Scattering and Refractive Index Detection

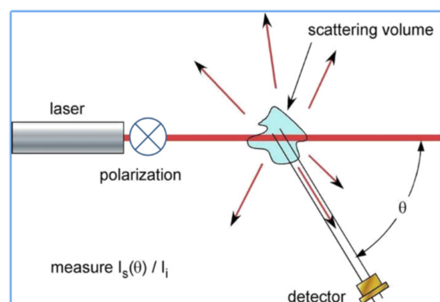
- Molecular Mass and Radius Determination of Polydisperse Samples including Proteins, Protein Conjugates (Glycosylation) and Polymers

- Protein Purification, Purity Verification and Quantification using Size Exclusion Chromatography (SEC)

MALS-QELS-RI Analysis of BSA for MW and Size Distribution Determination

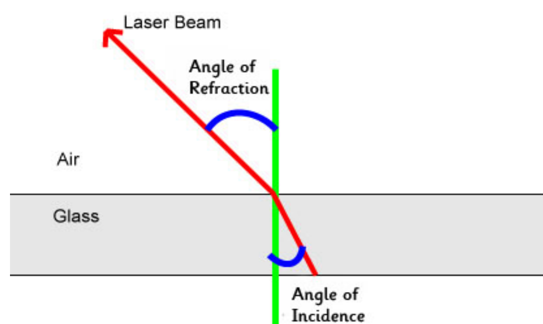
Light Scattering (miniDAWN TREOS)

When oscillating polarized light hits a macromolecule, the resulting intensity of light scattered is proportional to concentration and the molar mass which can be determined. Random Brownian motion is corrected by averaging intensity over time while the root mean square (RMS) radius (mass-weighted mean distance from the core to each mass element) can be calculated.



The hydrodynamic radius (R_h) can be calculated using QELS.

Refractive Index (Optilab T-rEX)



Differential RI can be reduced to a measure of how much light bends when it enters a medium of a different density. Absolute RI refers to when the difference is between a medium and a vacuum.

As the sample enters the flow cell, the change of the refraction angle is measured against that of the reference cell, containing the desired solvent. Using Snell's Law, this appears as a peak or a trough in the resulting chromatogram.

BSA Quantification

HPLC-MALS-RI Specifications

Protein: 2mg/mL BSA

Column: Advancebio SEC 300A 2.7µm 4.6x300mm (PL1580-5301)

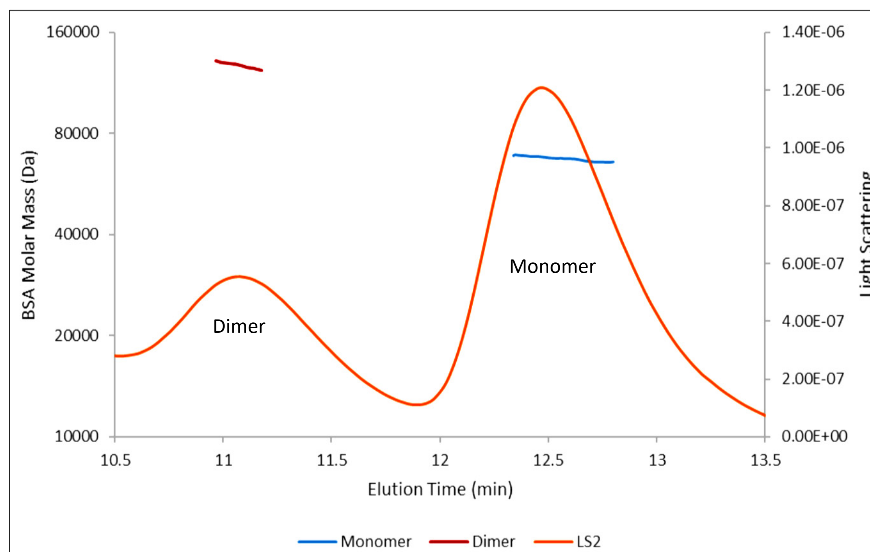
Buffers: Standard PBS Buffer

Flow rate: 0.2 mL/min for 30 minutes

Temperature: 4°C

Injection volume: 10 µL

Detection signals: Ab_{280}, Light Scattering (MALS/QELS), RI at 658nm



A 2mg/mL BSA solution was injected into the Bio-Inert HPLC system, its constituent conformations separated in the SEC column before entering the detectors. The MW values calculated from both RI and UV detector source data are expected to be roughly equal.

	Data Source: UV-Vis DAD		Data Source: T-rEX RI Detector	
Peak Number	Mass Fraction (%)	Calculated MW (g/mol)	Mass Fraction (%)	Calculated MW (g/mol)
Peak 1 (Monomer)	90.5	67.9 KDa ±2.5%	90.7	66.9 kDa ±2.5%
Peak 2 (Dimer)	8.5	128.3 KDa ±4.5%	8.4	127.8 kDa ±4.5%

The BSA sample elutes as multiple peaks from the SEC column, under the given conditions, demonstrating that a standard BSA sample is polydisperse with the major species (>90%) being the monomer and ~8% being the dimer while the remaining proportion is a tetramer (data not shown).

Software Analysis Specifications

- The Zimm Model was used to analyze the light scattering data.
- The dn/dc value represents the rate of change of the refractive index at a given temperature, wavelength and solvent. The accepted dn/dc value for an unmodified protein, including BSA, is 0.185 mL/g.
- The Fit Degree used during the fitting protocol was 1, representing a linear function.

The calculated molecular mass of the major elution peak is ~66-68 kDa, which corresponds closely with the known MW of the BSA monomer (66.5 kDa). For peak 2, the calculated molecular mass is ~128 kDa, approximately double that of the known weight of BSA monomer, suggesting that the peak corresponds to a dimer.

The Agilent 1260 Infinity II Bio-HPLC is a flexible and reliable instrument for purification and analysis. This instrument, coupled with the MALS-QELS-RI detection system, allows for an even more advanced analysis including molecular weight and size distribution determination.