BECKMAN COULTER
Life Sciences

Analytical Ultracentrifugation: Analyzing Sedimentation and Diffusion for Enhanced Molecular Characterization

Introduction

Analytical ultracentrifugation (AUC) is an analytical method that can be used to perform quantitative analysis of macromolecules in solution, by observing how the sample sediments. It enables the determination of the sedimentation and diffusion coefficients of a molecule, from which various parameters – such as molecular weight, size, shape, fullness, aggregation, heterogeneity, stoichiometry and binding constants – can be obtained. This makes AUC perfect for studying proteins and nucleic acids, drug delivery vehicles, antibodies and other biologics in pharmaceutical applications. Experiments are conducted under biologically relevant conditions, without the use of any supporting matrices or resins. This ensures that therapeutics can be characterized in the lab in the same state in which they will ultimately be administered to the patient. However, extracting valuable insights from AUC data requires an understanding of the analytical process, and the two main phenomena occurring during the centrifugation process, which are sedimentation and diffusion.

Sedimentation

Sedimentation happens naturally as gravity pulls particles downward. However, this takes an extremely long time, so centrifuges are used to speed up the sedimentation process. Centrifugation involves loading particles suspended in a liquid medium into a centrifuge tube, which is placed in a rotor and spun at a user-specified speed. Several forces are acting on the particles during the sedimentation process, including frictional, buoyant and centrifugal forces (Figure 1). The centrifugal force pulls a particle outward from the axis of rotation, while the buoyant and the frictional forces are acting against this particle motion.

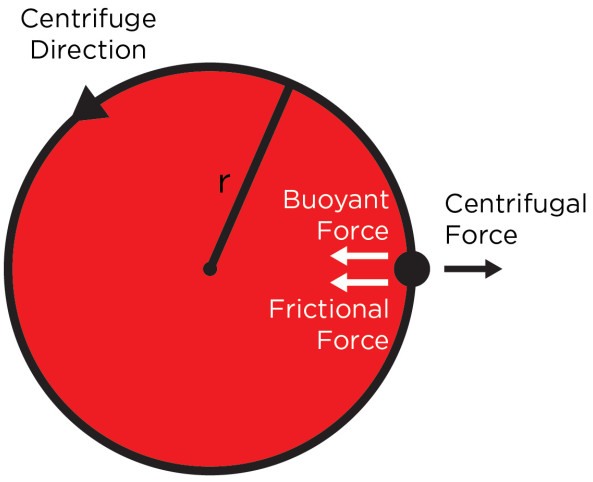

Figure 1: Forces acting on a particle during sedimentation.

Centrifugal force

Centrifugal force appears when an object is travelling on a circular path, and is proportional to the mass of the particle (*m*), the radius of the path (*r*) and the angular frequency of the motion (*w*):

$$F_C = \omega^2 r m$$

This means that heavier particles will experience a greater force than lighter particles, and that the centrifugal force becomes stronger when the radius and angular velocity are increased.

Buoyant force

Buoyant force is the upward force exerted by a fluid on an object placed in it, opposing the gravitational force pulling the object down. Its strength is dictated by the mass of the fluid displaced by the particle (m_w), and the more fluid an object can displace, the easier it floats:

$$F_b = -\omega^2 r m_w$$

Frictional force

Frictional force is the resistance experienced by an object moving on a surface or through a medium, acting opposite to the direction of motion. The frictional force depends on the shape of the particle and its velocity (*v*) as it travels through a viscous fluid:

$$F_f = -fv = 6\pi\eta R_0 \frac{f}{f_0}$$

η is the solvent viscosity, R_0 is the radius of a perfect sphere, *f* is the frictional coefficient and f/f_0 is a shape factor indicating how close the particle's shape is to a sphere, with 1 corresponding to a perfect sphere. Lower *f* values indicate less opposition to a particle's motion through a fluid, allowing it to move more freely.

Sedimentation coefficient

Assuming instantaneous equilibrium where the sum of F_c, F_b and F_f is equal to zero, it is possible to derive the Svedberg equation:

$$S = \frac{m(1-v\rho)}{f} = \frac{M(1-v\rho)}{6\pi\eta R_0 \frac{f}{f_0}} = \frac{v}{\omega^2 r}$$

S is the sedimentation coefficient, ρ is the buffer density and M is the molar mass of the solute. The sedimentation coefficient is a measure of how fast a particle sediments under the influence of centrifugal force. The rate of sedimentation can be analyzed, and the sedimentation coefficient provides information about the particle's mass, shape, and hydrodynamic properties. Figure 2 demonstrates the impact that molar mass and shape factor have on the sedimentation coefficient.

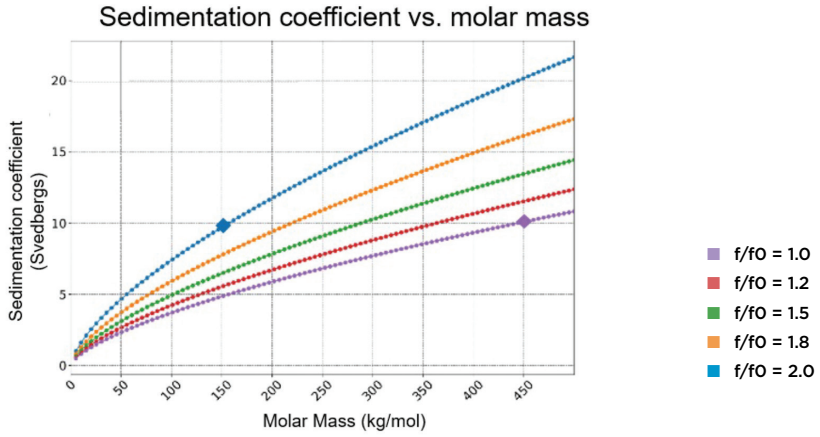

Figure 2: Sedimentation coefficient shown as a function of molar mass for different values of the particle radius and shape factor.

Diffusion

Diffusion gives information about the size and shape of solute particles relative to the medium they are dispersed in, and can be modelled using the Stokes-Einstein relation:

$$D = \frac{k_B T}{6\pi\eta R_0 \frac{f}{f_0}}$$

The diffusion coefficient is inversely proportional to the effective radius (r) the particle; a smaller and more spherical particle experiences less resistance, allowing it to move farther in the fluid, resulting in a higher diffusion coefficient.

Experimental set-up

There are two main types of AUC experiments; sedimentation velocity AUC (SV-AUC) and sedimentation equilibrium AUC (SE-AUC).

EXPERIMENTAL SET-UP		
	SV-AUC	**SE-AUC**
Experimental time	Hours	Days
Rotor speed	High (>10,000 rpm)	Low (<10,000 rpm)
Process	A sedimentation boundary is established, moving radially outward in the direction of the centrifugal force.	Low rotor speed allows an equilibrium between sedimentation and diffusion to be established, generating a radial concentration gradient of the analyte.
Analysis	Analysis of the shape and velocity of the boundary enables the determination of the sedimentation and diffusion coefficients, from which it is possible to obtain molecular mass, shape and size distributions/ heterogeneity.	Analysis of the equilibrium concentration gradient allows precise quantification of molecular weight and binding constants.

Table 1. A comparison of the two main AUC experimental set-ups..

Crucial parameters

It is crucial for manufacturers of compounds for pharmaceutical applications to know the molecular weight, size, shape, fullness, aggregation, heterogeneity, stoichiometry and binding constants of their particles. This information is vital for quality control, ensuring reliable and reproducible manufacture of consistent particle products. These parameters also directly influence the biological activity of pharmaceuticals. Factors such as binding affinity and stoichiometry in drug-receptor interactions play a crucial role in determining the efficacy and safety of a pharmaceutical product. Moreover, the size and shape of particles significantly impact their behavior within the human body, affecting absorption, distribution, metabolism and excretion. This, in turn, influences the bioavailability and therapeutic efficacy of the drug.

Sedimentation velocity AUC

Theory

During SV-AUC, the rate at which boundaries of molecules move during sedimentation is tracked. Analyzing the velocity and the shape of the boundary allows determination of both the sedimentation and diffusion coefficients using the Svedberg equation:

$$S = \frac{v_{bnd}}{\omega^2 r_{bnd}} = \frac{1}{\omega^2 r_{bnd}} \frac{dr_{bnd}}{dt}$$

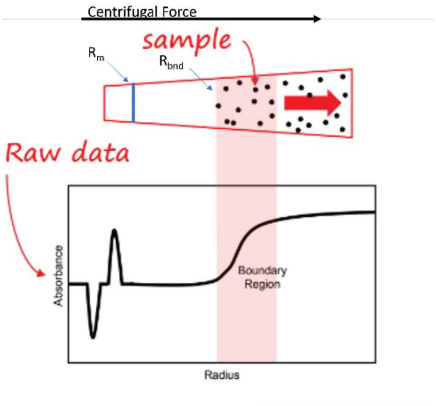

Figure 3: A schematic representation of the sedimentation boundary created during centrifugation, with r_m being the position of the meniscus and r_{bnd} the position of the boundary. The lower part of the figure illustrates the resulting absorbance data.

The velocity (v) is set to the velocity of the sedimentation boundary (v_{bnd}), which is equal to dr_{bnd}/dt with r_{bnd} being the position of the boundary. This equation can be integrated and, by plotting the logarithm of r_{bnd}/rm against time, makes it possible to obtain *S* from the slope of the resulting line:

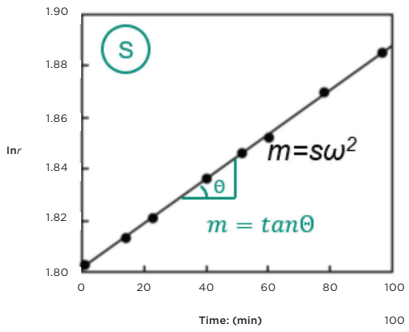

Figure 4: The sedimentation coefficient (*S*) can be obtained by plotting the natural logarithm of the sedimentation boundary velocity against time.

Practical considerations

Sedimentation coefficients depend on the mass of the solute and the density and viscosity of the solvent, so each solute in a solution will give rise to a separate sedimentation boundary. The shape of the boundary is affected by several factors, including heterogeneity, diffusion, and self-sharpening due to concentration dependence. However, if two species have similar sedimentation coefficients, it might be difficult to resolve the two corresponding boundaries.

Band sedimentation AUC

Band sedimentation AUC (BS-AUC) is a type of SV-AUC that allows the formation of a sedimentation band inside the centrifuge. The set-up involves a small reservoir connected near the top of the sample sector via thin capillaries. Samples are loaded in the reservoir, and a buffer is loaded into the sample sector. The sample is then pushed through the capillary channel by centrifugal force when the rotor begins to spin. The sample is then overlayed over the buffer solution, forming a thin layer. Upon overlay, species in the sample layer start to diffuse and sediment in bands at their corresponding sedimentation velocities. In this manner, BS-AUC allows the formation of a physical separation of macromolecular mixtures when there are sufficient differences in sedimentation velocities. One of the benefits of this approach is that it requires smaller sample volumes than typical SV-AUC experiments.

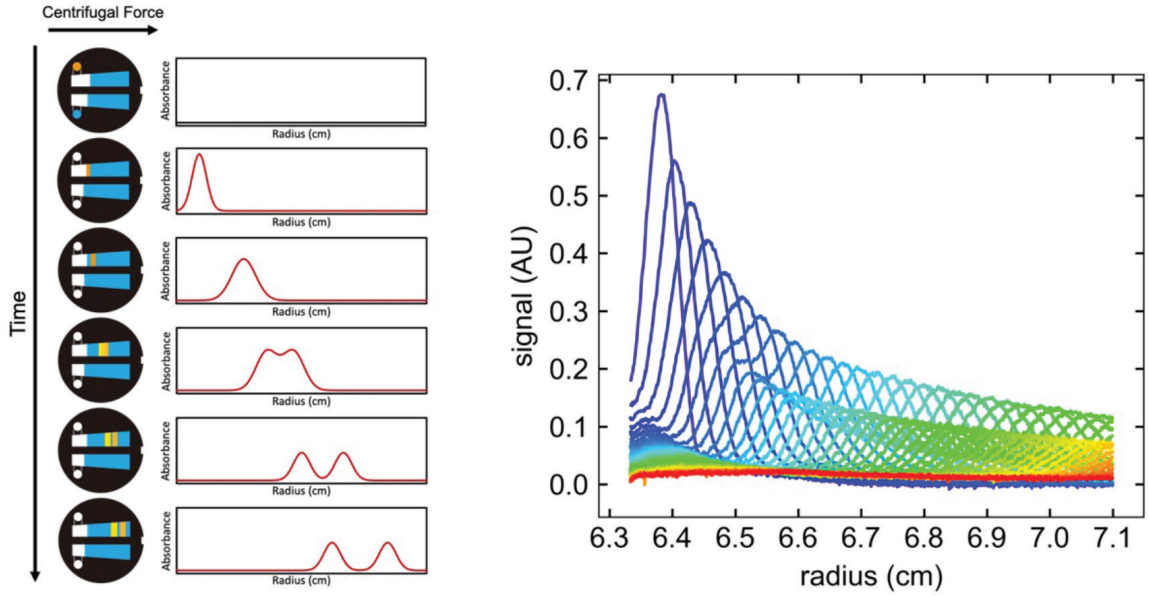

Figure 5: Formation of sedimentation bands during BS-AUC.[1]

Sedimentation equilibrium AUC

Theory

Standard SE-AUC has a much lower rotor speed and, therefore, significantly smaller centrifugal forces than SV-AUC, which enables the formation of an equilibrium concentration distribution where sedimentation (j_{sed}) and diffusion (j_{diff}) fluxes are equal. Figure 6 shows an equilibrium profile with concentration versus radius, as well as the directions of the two fluxes.

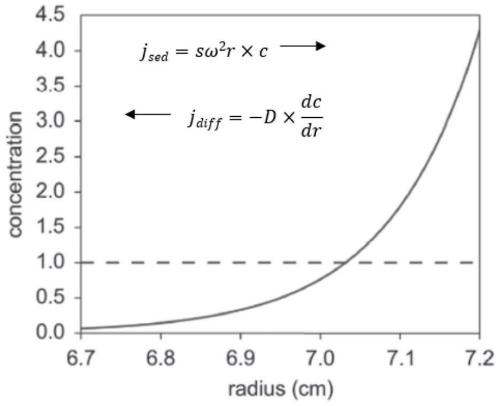

Figure 6: Equilibrium profile with concentration plotted versus radius.

For an ideal, single, non-interacting species, the equilibrium radial concentration gradient is:

$$c(r) = c_0 exp\left(\frac{M_b \omega^2}{RT}\frac{(r^2 - r_0^2)}{2}\right)$$

c_0 is the reference concentration at r_0, M_b is the buoyant mass, and R is the gas constant.

Practical considerations

A rotor speed is usually chosen so that the concentration at the base of the solution is four to 10 times greater than at the meniscus after equilibrium has been reached. Small volumes and shorter pathlengths are commonly used to reduce the time it takes for the process to reach equilibrium, which can be approximated by:

$$t_{eq} = \frac{0.7 l^2_{sector}}{D}$$

l_{sector} is the sector length, and D can be roughly estimated as $3 \times 10^{-5}/M^{1/3}$. If several molecular species are present, each individual species will form an equilibrium distribution of its own, based on its molecular weight.

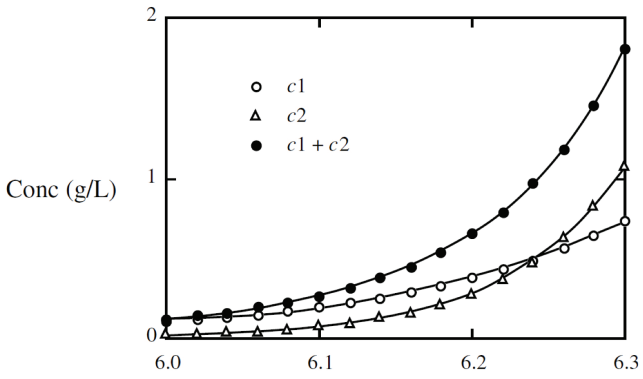

Figure 7: Different species form separate equilibrium distributions.

Density gradient equilibrium AUC

Density gradient equilibrium AUC (DGE-AUC) is a type of SE-AUC, where the sample is mixed with a density gradient medium (often a cesium chloride (CsCl) solution) in the sample cell. The sample cell is then spun at high speeds in the AUC and, over time, the particles in the sample will migrate to their position of equilibrium within the density gradient. The resulting concentration profile is measured by monitoring the absorption of light across the tube. The positions of the banded particles directly correlate to the buoyant density of each species.

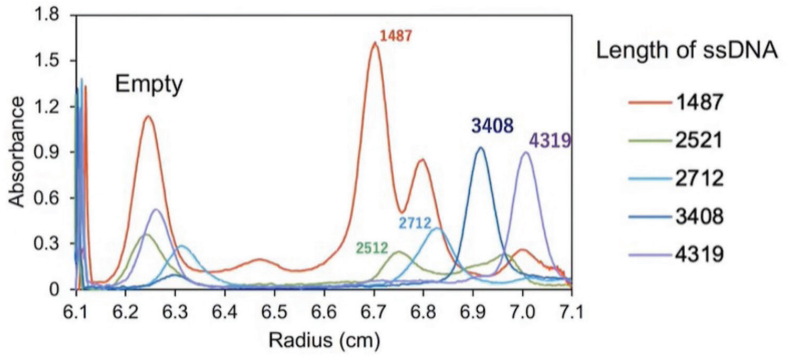

Figure 8: Data obtained using DGE-AUC for different viral vectors.

Optics

The samples are measured in a specialized tube called an AUC sample cell. The sample cell is equipped with windows above and below the sample chamber, allowing light to pass through the sample during centrifugation. This makes it possible to observe the sedimentation and diffusion patterns created, which can be achieved with either absorbance or interference optics. Absorbance optics relies on the detection of changes in the optical absorbance of a solution as particles sediment under the influence of a centrifugal field. In contrast, interference optics allow the analysis of interference patterns generated by the interaction of monochromatic light with the sample boundary. Both approaches are summarized in Table 2.

OPTICS		
	ABSORBANCE	**INTERFERENCE**
Light source:	Xenon flash lamp wavelength from 190 to 800 nm, dynamic range 0-3 AU	120 mW diode laser, 660 nm wavelength
Data collection:	Photomultiplier tube, slit scanning.	Low (<10,000 rpm)
1,500 scan steps, linear resolution of 10 microns.	Camera with 2048 x 1088 pixels, FFT to generate scan data	Low rotor speed allows an equilibrium between sedimentation and diffusion to be established, generating a radial concentration gradient of the analyte.
Wavelength:	Wavelength increment: 1 nm	Fixed wavelength
Speed:	1 scan point every 10 ms (depends on rotor speed), 1 sector every 20 seconds	1 image every 5 seconds
Applications	When the analyte has one or more well defined absorption peaks (e.g. proteins, nucleic acids, AAV)	When the analyte has a refractive index distinct from its buffer (e.g. nanoparticles, vesicles)
Strengths:	Only measures analyte, eliminates signal from ligands, multi-wavelength measurements allows stoichiometric analysis	Faster than absorbance, large baseline is acceptable, measurement has large dynamic range
Weakness:	Large baselines can complicate measurement of small molecules, slower than interference	Everything is measured

Table 2. A comparison of the absorbance and interference methods in AUC.

Absorbance

Absorbance (A) can be visualized using Lamber-Beer's law, which states that the signal is directly proportional to solute concentration (c):

$$A=\varepsilon cl=log\left(\frac{I_0}{I}\right)$$

ε is the absorptivity of the sample, and l is the optical path length, which in this case is the width of the sample tube. I_0 is the intensity of the incoming light, and I is the intensity of the light after it has passed through the sample. Measuring the differences in these intensities allows changes in concentration as the sample sediments to be monitored.

Interference

Interference optics approaches involve the use of monochromatic light that passes through two fine parallel slits; one centered on a cell containing the sample solution, and the other one on a cell that holds just the solvent.

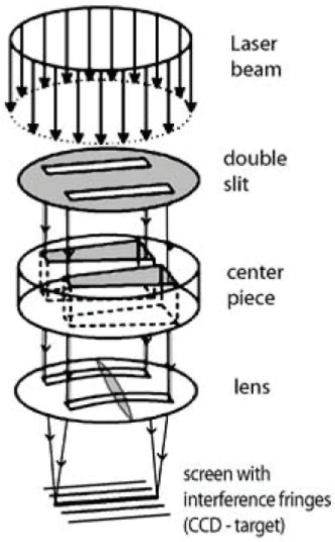

Figure 9: A schematic representation of interference optics.

Light passing through the slits is scattered, and the resulting light waves interfere with each other, creating interference patterns known as 'fringes'. As particles move through the detection area, the change in refractive index shifts the position of the fringes in the vertical direction, and the resulting pattern can be used to determine concentration distribution and sedimentation properties.

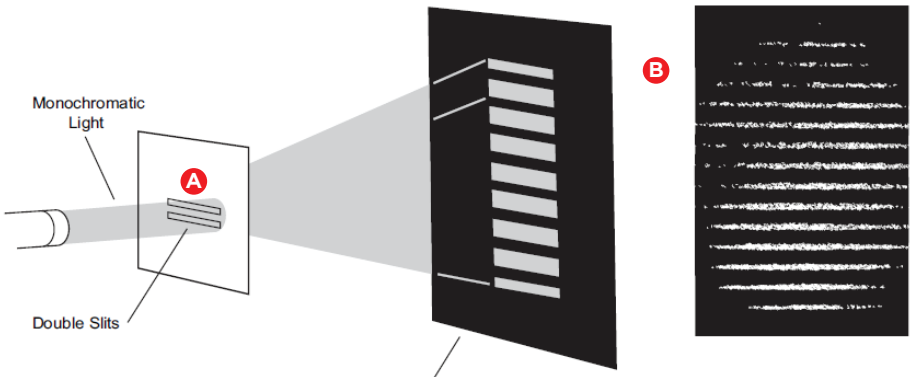

Figure 10: A Light passing through two parallel slits interferes, resulting in fringes. B The difference in refractive index shifts the position of the fringes vertically as particles in the sample sediment.

Application example: viral vector characterization

Adeno-associated virus vectors are commonly used for gene therapy and vaccines, but the efficacy and safety of these therapeutics requires stringent purification during the manufacturing process to eliminate empty particles (vectors that lack a nucleic acid payload). SV-AUC has been successfully used to analyze adeno-associated virus vectors, determining their full/empty ratio[1]. Normalized concentration can be plotted against S, resulting in peaks at 90-100 S and 60-70 S that correspond to full and empty particles, respectively.

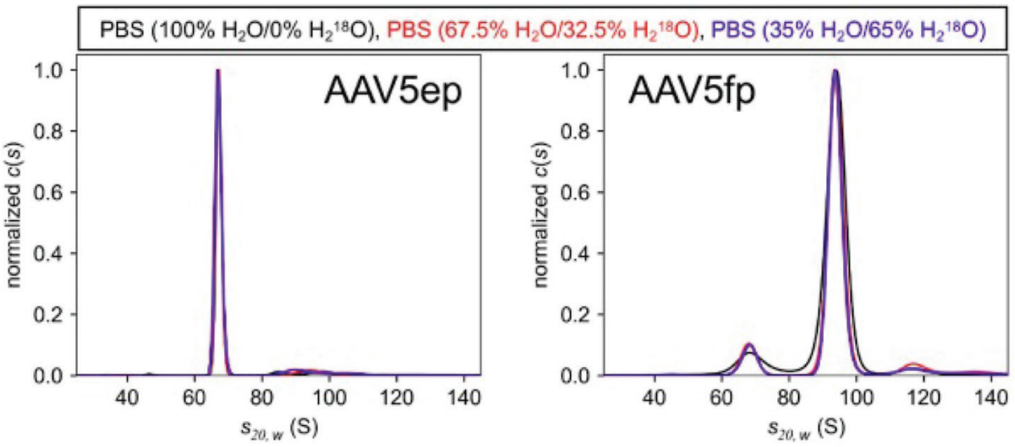

Figure 11: Normalized concentration as function of S produces peaks at 67 S and 94 S corresponding to empty (AAV5ep) and full particles (AAV5fp), respectively.[2]

However, this type of analysis has an intrinsic uncertainty, due to partial-specific volume and frictional ratio (f/f_0). Moreover, the smaller peaks visible in the two figures cannot be characterized. Fortunately, multiwavelength SV-AUC allowed the determination of the unknown components through their UV absorption profiles.

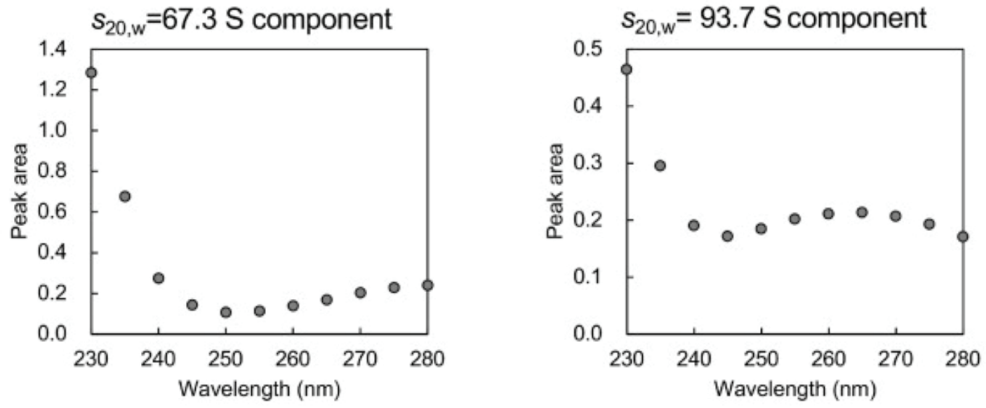

Figure 12: Peak area at each wavelength obtained through MW-SV-AUC. [2]

Optima AUC

Optima AUC from Beckman Coulter Life Sciences can perform all types of AUC experiments, allowing the analysis of a diverse array of particles in native, matrix-free conditions with minimal buffer constraints. Offering a maximum rotational speed of 60,000 RPM, it supports both absorbance and interference optics to provide versatile, robust and accurate characterization of proteins, nanoparticles, peptides, polymers, micelles, liposomes, extracellular vesicles, drug conjugates, and viral payloads. The resulting data can provide critical information about shape, mass, diameter, stoichiometry, heterogeneity, association, aggregation, purity, and formulation of the sample.

The system is ideally suited to academic and biopharma research, as it enables the analysis of macromolecules across a wide concentration range in low volumes (down to 0.1 mL), allowing processing of up to seven samples in a single run. Crucially, this analysis does not require any standards, as it relies on the first principles of thermodynamics. It is also non-destructive, offering the potential for sample recovery.

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