Application Note

Biopharmaceuticals

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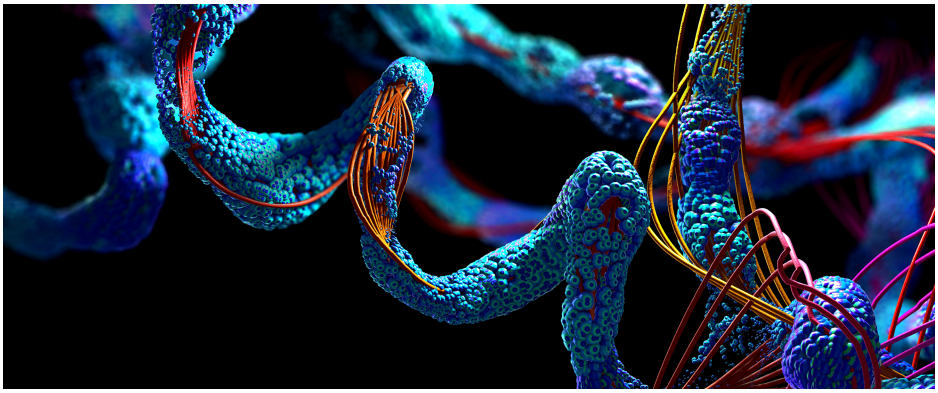
Determination of Protein Extinction Coefficients and Concentration by UV-Vis

Enhancing lab efficiency and productivity with the Agilent Cary 3500 Multicell UV-Vis Spectrophotometer



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Abstract

This study highlights the advanced capabilities of the Agilent Cary 3500 Multicell UV-Vis spectrophotometer and Agilent Cary Workstation software for determining protein extinction coefficients. These measurements enable accurate calculation of protein concentrations. Automated software features are used to streamline the measurement of protein concentration, enhancing the efficiency and accuracy of the application. These measurements are critical at every stage of drug development, from discovery to manufacturing and quality control (QC) of monoclonal antibodies (mAbs) or vaccines. The study also compares extinction coefficients of two biosimilars (Reditux and Truxima) with an innovator (Ristova), enabling similarities in amino acid compositions to be assessed.

Introduction

Measurement of protein concentration is crucial when studying protein–protein and protein–ligand interactions, as well as for evaluating enzyme activity. It is also vital in biopharmaceutical development and the manufacturing of protein-based therapeutics, such as monoclonal antibodies (mAbs) and vaccines. The extinction coefficient of a protein enables the determination of its concentration through absorbance measurements at 280 nm using UV spectroscopy. Aromatic amino acids, such as tryptophan, tyrosine, and cysteine, strongly absorb UV light at 280 nm, making it a reliable indicator of protein concentration in solution. The calculation of protein extinction coefficients can be automated using software, enabling research scientists in protein characterization labs to enhance lab efficiency and productivity. In this note, we demonstrate the effectiveness of the automated custom equation calculation feature of Agilent Cary UV Workstation software for determining protein extinction coefficients.

Biosimilars are biopharmaceutical drugs designed to be similar in quality, safety, and efficacy to an already licensed innovator product.[1] Establishing analytical similarities to the innovator is essential in the development and regulatory approval of biosimilar products.[2] This study also outlines the analysis of the extinction coefficients of innovator and biosimilar products using the Agilent Cary 3500 Multicell UV-Vis.

Experimental

Instrumentation

Data acquisition was performed by the Agilent Cary 3500 Multicell UV-Vis using the parameters shown in Table 1. Ultra-microvolume rectangular cells with a path length of 10 mm and 70 µL fill volume (Agilent part number 5062-2496) were used. A sample volume of 50 µL was used for each cell. The highly collimated and permanently focused beam of the Cary 3500 UV-Vis easily passes through small apertures, ensuring analytical accuracy for small-volume measurements. The stationary multicell holder requires no alignment, allowing repeatable measurements of up to eight microcuvettes in a single experiment without operator adjustment.

Table 1. Agilent Cary 3500 Multicell UV-Vis spectrophotometer parameters.

Parameter	Value
X Mode	nm
Y Mode	Absorbance
Collect Mode	Scan
Scan Range Start	325 nm
Scan Range Stop	240 nm
Averaging Time	0.020 s
Data Interval	1.00 nm
Scan Rate	3,000 nm/min
Spectral Bandwidth	2.00 nm
Detector Module	Multicell Peltier UV-Vis

Reagents and materials

Lysozyme (chicken egg white, L-6876), β-casein (C6905), ovalbumin (A5503), guanidine hydrochloride (G4505) were bought from Sigma-Aldrich (St. Louis, MO, USA). The innovator (Ristova) and two rituximab biosimilars (Reditux and Truxima) were bought from a local distributor in Singapore.

Fresh ultrapure water was obtained from a Milli-Q Integral system (Millipak, Merck-Millipore, Billerica, MA, USA) equipped with a 0.22 µm membrane point-of-use cartridge.

Experiments

Determination of protein extinction coefficients using Beer–Lambert law: The Cary UV Workstation software features a built-in equation function that automatically calculates equations from UV-Vis scans, making it easier to determine protein extinction coefficients using the Beer–Lambert law. Lysozyme, β-casein, ovalbumin, and Ristova were prepared at a concentration of 1 mg/mL in 6 M guanidine hydrochloride. The protein extinction coefficients were determined using the Beer–Lambert law.

The Equation feature of the Cary Workstation software automatically calculated the protein extinction coefficient as shown in Equation 1.

Equation 1.

$$A = \varepsilon cL$$

$$\varepsilon = A/cL$$

where A is absorbance at 280 nm, c is molar concentration, L is pathlength, and ε is the molar extinction coefficient, $M^{-1}cm^{-1}$.

It is recommended to correct absorbance at 320 nm to account for potential scattering caused by precipitates, if present.

Absorbance measurements: The absorbance of the various protein samples was measured at 280 nm, with baseline correction implemented using 6 M guanidine hydrochloride as the blank. All measurements were conducted by the Cary 3500 UV-Vis in a single zone comprising seven sample channels and one reference channel (Figure 1). Given the eight cell positions of the Cary 3500 Multicell, lysozyme, β-casein, ovalbumin were measured in duplicate, while single positions were used for Ristova and the blank.

The built-in equation function of the Cary UV Workstation software allows for the customization of equations (Figure 2). For this study, parameters such as concentration (mg/mL), molecular weight ($g.mol^{-1}$), and path length (cm) were defined in the software. The extinction coefficient equation was then entered using the equation editor as follows: ValueAt(280)/(Pathlength × Concentration/MolecularWeight). The equation enables the automatic calculation and reporting of the protein extinction coefficient of a sample.

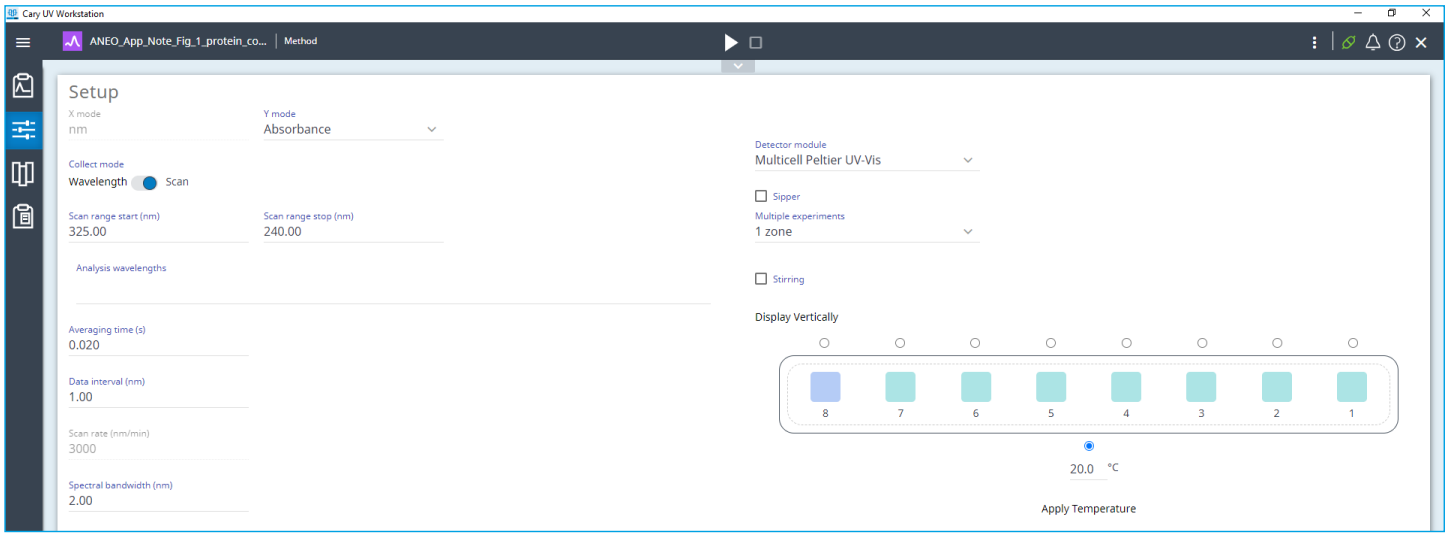

Figure 1. Screenshot of single zone multiple experiment feature of the Agilent Cary 3500 Multicell UV-Vis. The green and purple positions represent the samples and reference positions within the single zone, respectively.

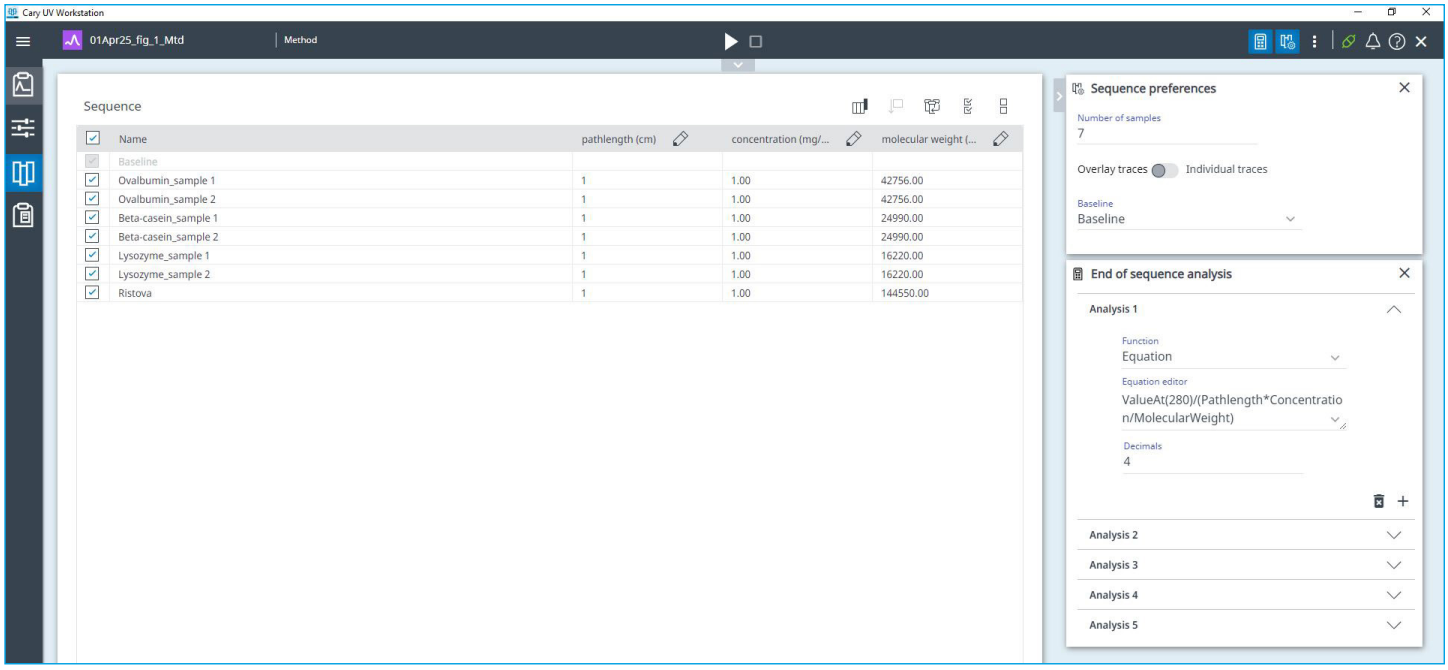

Figure 2. The built-in equation function within the Agilent Cary UV Workstation software allows the calculation of protein extinction coefficient to be performed automatically.

Determination of protein extinction coefficients using sequence: The extinction coefficient of a protein can be estimated using known amino acid composition or protein sequence data. For example, the molar extinction coefficients of a denatured protein in 6 M guanidine hydrochloride can be calculated using Equation 2.

Equation 2.

$$\varepsilon \text{ guanidine hydrochloride} = a\varepsilon \text{ Tyr} + b\varepsilon \text{ Trp} + c\varepsilon \text{ Cys}$$

where:

- ε Tyr, ε Trp, and ε Cys are the molar extinction coefficients of tyrosine, tryptophan, and cysteine residues at the wavelength used. The molar extinction coefficient values are shown in Table 2.[3]
- a, b, and c are the number of each type of residue per molecule of protein.[3]

Table 2. Extinction coefficients of tryptophan, tyrosine, and cysteine in 6.0 M guanidine hydrochloride and 0.02 M phosphate buffer, pH 6.5, determined using sequence.[3]

Amino Acid Residue	Extinction Coefficient at Various Wavelengths				
	276 nm	278 nm	279 nm	280 nm	282 nm
ε Trp	5,400	5,600	5,660	5,690	5,600
ε Tyr	1,450	1,400	1,345	1,280	1,200
ε Cys	145	127	120	120	100

Comparing innovators and biosimilars using extinction coefficients: In this study, the extinction coefficients of the biosimilars and the innovator were assessed for similarity. Ristova (innovator) and Reditux and Truxima (biosimilars) were analyzed at concentrations of 0.5 and 1 mg/mL in 6 M guanidine hydrochloride. Absorbance at 280 nm was measured at 20 °C, and the extinction coefficient was calculated using the Cary UV Workstation software.

Protein concentration estimation: The built-in calibration curve software function of the Cary Workstation facilitates the estimation of mAb concentration using UV absorbance at 280 nm, making it useful during the bioprocessing steps of mAb production. A calibration curve was created using Ristova with 6 M guanidine hydrochloride as the diluent, at concentrations of 0.1, 0.25, 0.5, 0.75, 1, 1.5, and 2 mg/mL.

Results and discussion

Easy measurement of protein extinction coefficients

The Cary Workstation software automatically calculated the protein extinction coefficients for ovalbumin, β-casein, lysozyme, and Ristova simultaneously. As shown in Figure 3, the results include an absorbance-versus-wavelength graph and the calculated extinction coefficient values, which are presented in the "Results" column for each sample.

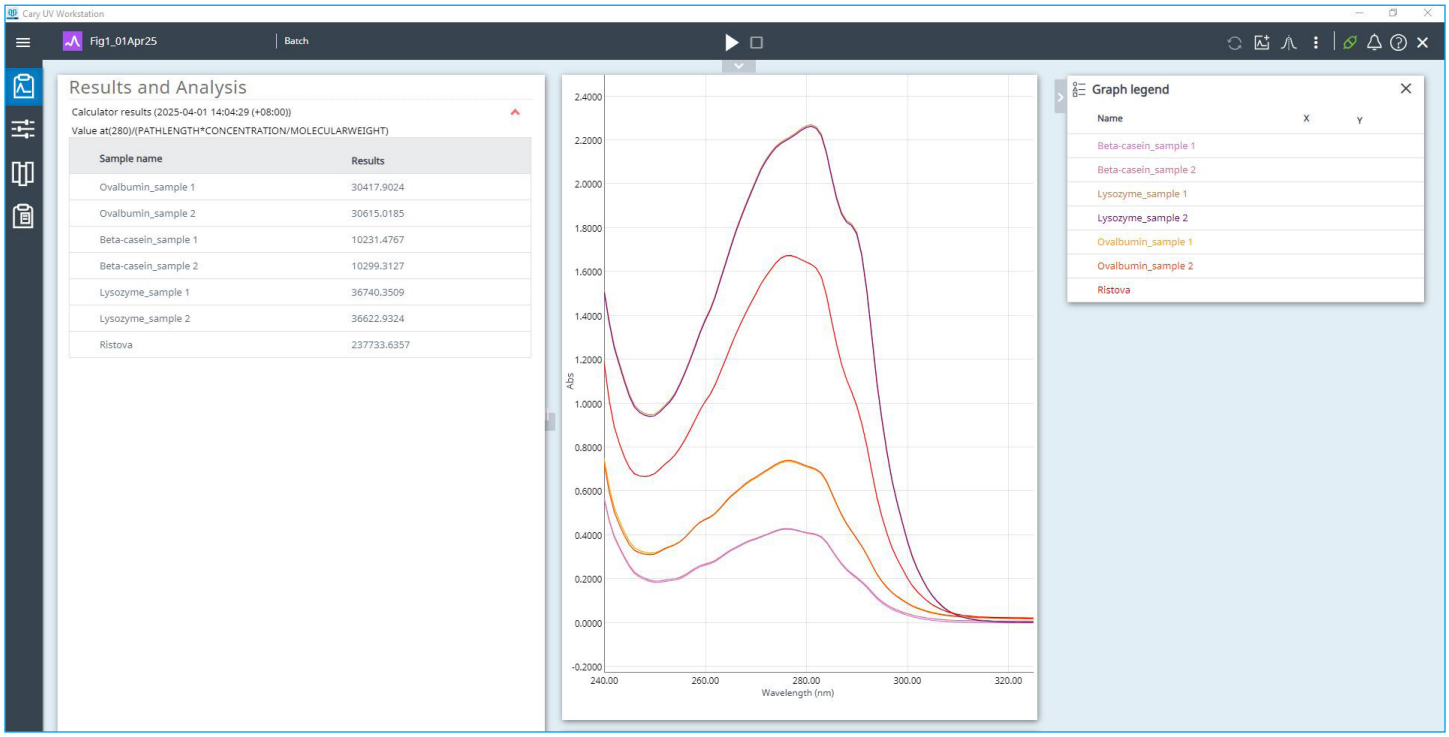

Figure 3. The absorbance scans and protein extinction coefficient results for different proteins. The protein extinction coefficients were automatically calculated by the Agilent Cary UV Workstation software.

Known protein sequences and a freely available online tool[4] were used to calculate the molar protein extinction coefficients of the seven samples. The experimentally determined extinction coefficients from this study were consistent with the molar extinction coefficients calculated using the protein sequence and the values reported in the literature (Table 3). The close agreement of the results demonstrate the suitability of the Cary 3500 UV-Vis method for the accurate determination of molar extinction coefficients.

Table 3. Comparison of calculated molar extinction coefficients using Agilent Cary 3500 UV-Vis measurement of A_{280}, protein sequence, and literature values.

Proteins	Molecular Weight ($g.mol^{-1}$)	Molar Extinction Coefficient ($M^{-1}cm^{-1}$)		
		Calculated Using A_{280}	Calculated Using Protein Sequence and Online	Literature Reported
Ovalbumin Sample 1	42,756	30,418	30,590	29,972[3]
Ovalbumin Sample 2	42,756	30,615	30,590	29,972
B-Casein Sample 1	24,990	10,231	11,460	12,142–14,191[5]
B-Casein Sample 2	24,990	10,299	11,460	12,142–14,191
Lysozyme Sample 1	16,220	36,740	37,470	37,932[3]
Lysozyme Sample 2	16,220	36,623	37,470	37,932
Ristova	144,550	237,734	235,380	196,166–224,104[6]

Matching innovator and biosimilars by comparing their extinction coefficients: The extinction coefficients of the biosimilars (Reditux and Truxima) matched those of the innovator (Ristova) at both tested concentrations of 1 and 0.5 mg/L, as shown in Table 4. The measured values were consistent with the extinction coefficients calculated from the protein sequence, demonstrating the robustness of this analysis at different concentrations. These results indicate that the biosimilars and the innovator proteins are likely to have similar aromatic amino acid compositions.

Table 4. Comparison of the absorbance and molar extinction coefficients of innovator (Ristova) and biosimilars (Reditux and Truxima). The measured extinction coefficients were comparable with values calculated from protein sequencing.

Proteins	Concentration (mg/mL)	Absorbance at 280 nm	Molar Extinction Coefficient ($M^{-1}cm^{-1}$)	
			Calculated Using A_{280}	Calculated Using Protein Sequence
Ristova	1	1.6042	231,887	235,380
Truxima	1	1.5794	228,302	235,380
Reditux	1	1.5843	229,011	235,380
Ristova	0.5	0.8447	244,203	235,380
Truxima	0.5	0.7975	230,557	235,380
Reditux	0.5	0.8054	232,841	235,380

Calibration curve function for estimation of unknown protein concentration: A concentration calibration curve was generated for the innovator (Ristova) using the "Concentration" function in the Cary UV Workstation software. Ristova shows good linearity from 0.1 to 2 mg/mL, with an R^2 value of 0.9918 (Figure 4). This application is valuable in bioprocessing, where it is necessary to measure the concentration of mAbs produced at various stages of the process.

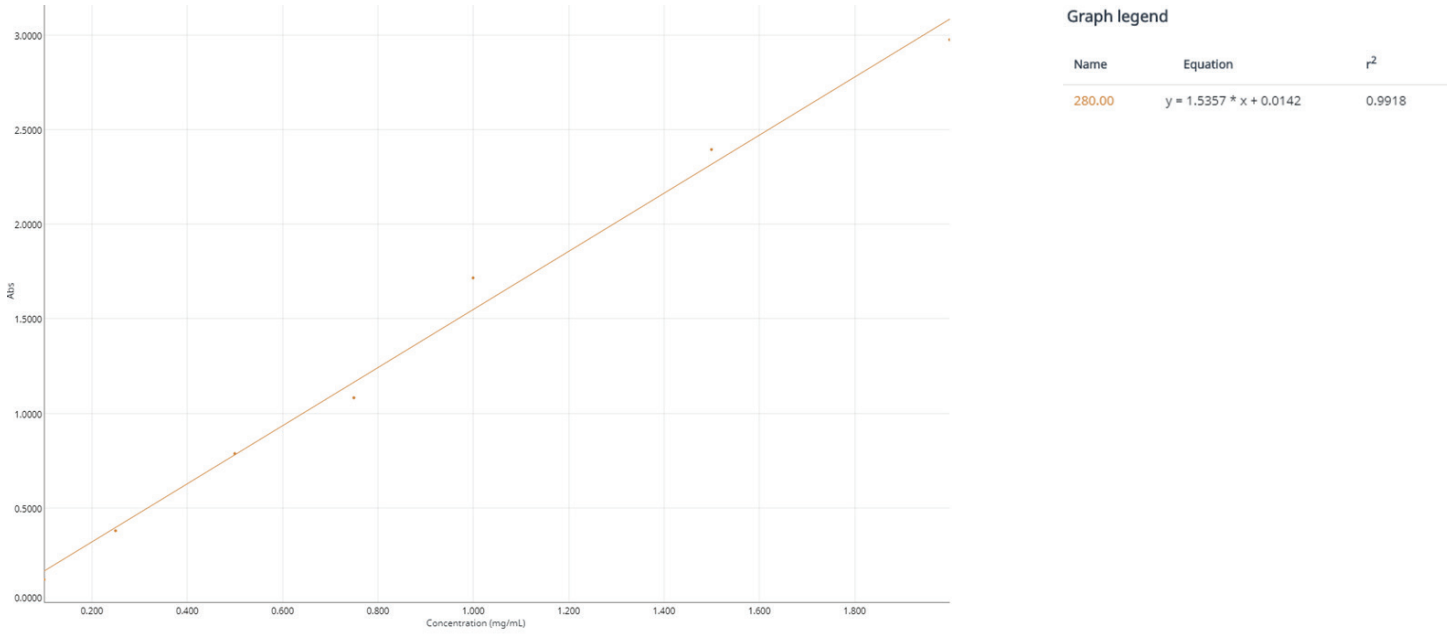

Figure 4. The calibration curve of Ristova from 0.1 to 2 mg/mL with R^2 = 0.9918.

Conclusion

The Agilent Cary 3500 Multicell UV-Vis spectrophotometer is a highly effective tool for biopharmaceutical applications, as demonstrated by its ability to determine protein extinction coefficients and concentrations. With its user-friendly design and advanced functionality, the Cary 3500 UV-Vis enabled the simultaneous measurement of seven samples and a reference. The equation function of the Agilent Cary UV Workstation software further enhanced the efficiency of the measurements, delivering immediate results for each sample.

To assess the similarity of amino acid profiles between an innovator (Ristova) and two biosimilar proteins (Reditux and Truxima), the Cary 3500 Multicell UV-Vis method swiftly calculated extinction coefficients at two concentrations.

The results confirmed the similarity of the proteins while also aligning closely with extinction coefficients derived from protein sequencing.

The Cary UV Workstation software's concentration and calibration curve function can estimate antibody concentrations at various times during the manufacturing of monoclonal antibodies, helping to ensure the quality of the final product.

With its advanced features and innovative software, the Cary 3500 Multicell UV-Vis offers significant value to the biopharmaceutical industry by streamlining workflows and ensuring accurate, reliable measurements of proteins.

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Further information

- Cary 3500 Multicell UV-Vis Spectrophotometer
- Cary UV Workstation Software
- UV-Vis Spectroscopy and Spectrophotometry Overview

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