Application Note

UV-0003

Quantitative Determination of Proteins

Generally, the concentration of proteins is measured using UV-Vis spectrophotometers. The JASCO V-630 Bio (Figure 1) is a UV/Vis spectrophotometer designed for biochemical analysis. The V-630 Bio is equipped with 6 quantitative calibration curves based on UV absorption spectrophotometry including the Lowry, Biuret, BCA, Bradford, and WST methods.

Figure 1 V-630BIO an iRM

Table 1 shows the features of the six different quantitation methods. As outlined, the preferred method can be selected by reviewing the sample and range of the quantitation and existence of any possible contaminants.
Five of the analysis methods are quantitative methods that utilize a chromogenic reaction. Reagent manufacturers produce chromogenic kits for BCA, Bradford or WST with an instruction manual explaining the measurement procedures*1). On the other hand, the chromogenic reagents for the Lowry and Biuret methods need to be prepared by the user. The measurement procedures for the Lowry and Biuret methods differ depending on each document. For that reason, this application data explains how to create the calibration curves for the Lowry, Biuret and UV absorption analysis methods. Fluctuations of the values depending on the type of proteins and the differences of the concentration ranges calculated as a result of the cell volume*2) were also investigated.

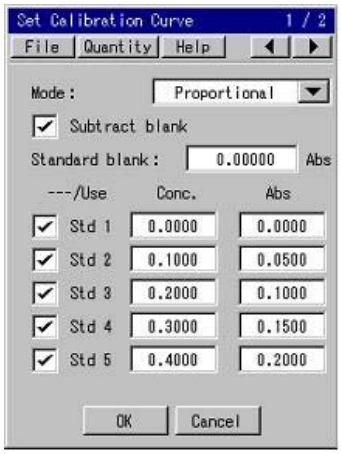

Figure 2 Quantitative Analysis program - Set Calibration Curve dialog

*1) *BCA method: BCA Protein Assy Kit by PIERCE*
Bradford method: Protein Quantification Kit - Rapid by DOJINDO
WST method: Protein Quantification Kit - Wide Range by DOJINDO

*2) *10 mm rectangular cells (quartz) and disposable cells (Figure 3) were used for the experiments. The optional cell holder accessory enables the measurement of a sample with a small volume (minium volume: 100µ)*

Figure 3 Disposable cuvette

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Table 1 Details for the protein quantitation methods

	Principle	Concentration range	Advantages	Disadvantages
UV Abs	The absorption maximum at 280 nm corresponds to the response of the tyrosine and tryptophan and is used for the analysis method.	50 to 2000 µg/mL (*BSA)	Simple method. Sample can be used after measurement.	The absorbance differs for each protein. Proteins such as collagen and gelatin that do not have absorption at 280 nm cannot be measured. Contamination by nucleic acids with absorption in the UV region obscures the measurement.
Biuret	Protein solutions turn purple after the polypeptide chain chelates with a copper ion. An alkaline solution of Biuret reagent including copper sulfate and Rochelle salt is added to a protein solution. Uses the absorption maximum at 540 nm to determine the quantity.	150 to 9000 µg/mL (BSA)	Simple procedure. The chromogenic rate is constant for most proteins.	Low sensitivity. Sample with a low protein concentration cannot be measured. The chromogenic reaction is influenced by high-concentrations of trisaminomethane, amino acids, and ammonium ion.
Lowry	Add an alkaline copper solution to a protein solution. Tyrosine, tryptophan, and cisteine of proteins reduce molybdenum acid and phosphotungstic acid of a phenol reagent, turning the solution blue. Uses the absorption maximum at 750 nm to determine the quantity.	5 to 200 µg/mL (BSA)	High sensitivity. Widely used.	Complicated procedure with a long preparation. Since the chromogenic reaction occurs by a reduction reaction, contamination of the reduction material interferes with the quantitative determination. The chromogenic rate differs for each protein.
BCA	The BCA method combines the Biuret method and Bicinchoninic Acid (BCA). BCA has high sensitivity and selectivity for copper ions. When a copper ion that is formed by the reduction action of protein reacts with 2 molecules of the BCA, the solution turns purple. Uses the absorption maximum at 560 nm to determine the quantity.	20 to 2000 µg/mL (BSA)	Simple procedure. High sensitivity with wide concentration range.	Thiol, phospholipid, and ammonium sulfate interferes with the measurement.
Bradford	The absorption maximum of a protein shifts from 465 nm to 600 nm when the protein binds to the Coomassie Brilliant Blue G250, one of triphenylmethanse blue pigment. Uses the absorption maximum at 600 nm to determine the quantity.	10 to 2000 µg/mL (BSA)	Very simple operation. Hardly influenced by the blocking materials.	The chromogenic rate differs for each protein. Contamination by a surfactant can interfere with the chromogenic reaction.
WST	Reduce WST-8 with proteins with a high pH, turning the sample blue. Uses the absorption maximum at 650 nm to determine the quantity.	50 to 5000 µg/mL (BSA)	Simple operation. Hardly influenced by a surfactant.	The chromogenic rate differs for each protein.

*BSA: Bovine Serum Albumin

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1. UV Absorption Method

Figure 4 illustrates the absorption spectrum of Human Serum Albumin (HSA). Simple UV Absorption spectrophotometry can determine the quantity of proteins in the sample by using the maximum absorption at 280 nm.

1.1 Samples

Bovine Serum Albumin (BSA): 0.02, 0.025, 0.05, 0.1, 0.2, 0.25, 0.4, 0.5, 1, (1.5, 2) mg/mL
Hen Egg Lysozyme: 0.02, 0.025, 0.05, 0.1, 0.2, 0.25, (0.4, 0.5) mg/mL
Chymotrypsin from bovine pancreas: 0, 0.1, 0.2, 0.25, 0.4, 0.5 mg/mL

Bracketed values are for concentrations used with the 10 mm rectangular cell. The other values are concentrations used with the 10 mm rectangular cell and micro cell.

1.2 Measurement Procedures

Measure the absorbance of the protein solution using the wavelength of 280 nm.

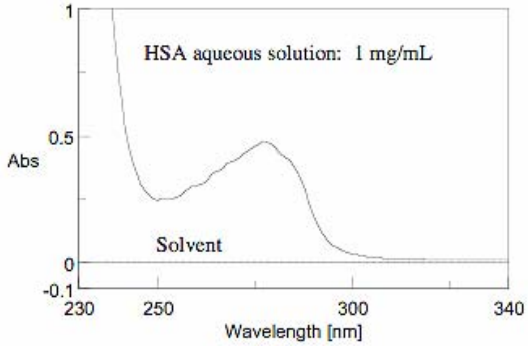

Figure 4 Absorption spectrum of HSA

Table 2 Results for the UV absorption method

	Cell	Concentration range	Calibration curve formula	Correlation function	Standard error	Detection limit	Determination limit
BSA	10 mm rectangular cell (quartz)	to 2 mg/mL	Y = AX + B A = 0.6652 ± 0.0079 B = -0.0130 ± 0.0064	0.9994	0.0219	0.0097 mg/mL	0.0470 mg/mL
	Micro cell	to 1 mg/mL	Y = AX + B A = 0.6713 ± 0.0043 B = -0.0016 ± 0.0008	0.9999	0.002	0.0012 mg/mL	0.0218 mg/mL
HEL	10 mm rectangular cell (quartz)	to 0.5 mg/mL	Y = AX + B A = 0.6474 ± 0.0459 B = -0.0150 ± 0.0109	0.9991	0.0076	0.0041 mg/mL	0.0680 mg/mL
	Micro cell	to 0.25 mg/mL	Y = AX + B A = 2.7499 ± 0.0429 B = -0.0060 ± 0.0055	0.9995	0.0031	0.0020 mg/mL	0.0096 mg/mL
α- Chymotrypsin	10 mm rectangular cell (quartz)	to 0.5 mg/mL	Y = AX + B A = 1.904 ± 0.0237 B = -0.0035 ± 0.0070	0.9997	0.0042	0.0037 mg/mL	0.0615 mg/mL
	Micro cell	to 0.5 mg/mL	Y = AX + B A = 2.1279 ± 0.0655 B = -0.0202 ± 0.0193	0.9983	0.0104	0.0091 mg/mL	0.1263 mg/mL

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2. Biuret method

Protein solutions turn purple with the absorption maximum of 540 nm when Biuret reagent is added (Figure 5).
The time course of the chromogenic reaction was measured at 540 nm absorption. The absorbance became stable approximately after 60 minutes (Figure 6). Figure 7 demonstrates the spectra of the HSA aqueous solutions that were measured after 60 minutes.
Biuret method determines the quantity by using the absorption maximum at 540 nm after reacting with the sample for 60 minutes.

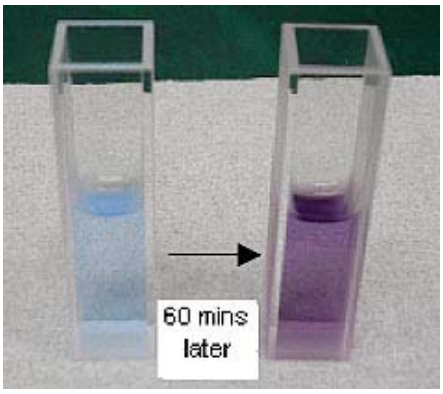

Figure 5 Color change after adding Biuret regent

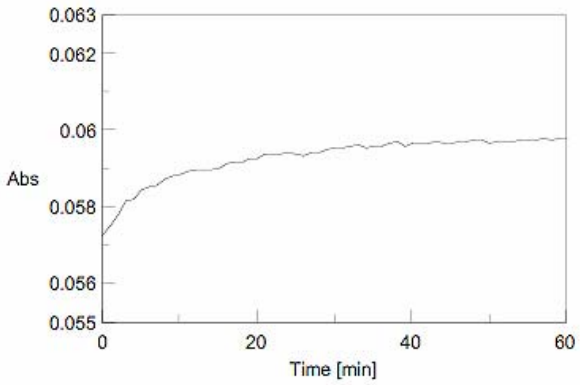

Figure 6 Time course trace of the chromogenic reaction

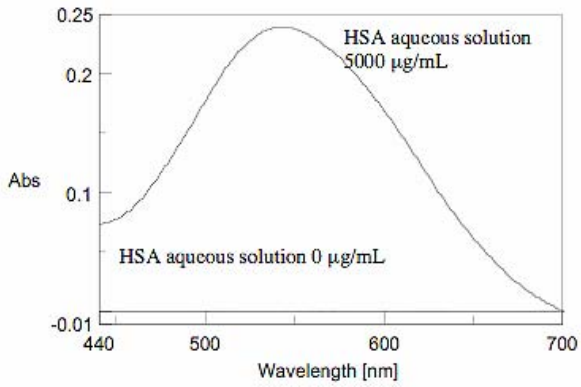

Figure 7 Spectra of the HSA solutions using the Biuret method

2.1 Chromogenic Reagent

Biuret reagent: Add 60 mL of 10% NaOH to 100 mL of aqueous solution to dissolve 0.3 g $CuSO_4$ and 1.2 g Rochelle salt, and then add water to 200 mL. (Standard reagent can be saved in a polyethylene bottle.)

2.2 Samples

Bovine Serum Albumin (BSA):	0, 0.25, 0.5, 1, 5, 9 mg/mL (10 mm rectangular cell (quartz))
	0, 1, 3, 5, 9 mg/mL (Micro cell)
Hen Egg Lysozyme:	0, 1, 3, 5, 9 mg/mL (10 mm rectangular cell (quartz), Micro cell)
Chymotrypsin from bovine pancreas:	0, 1, 3, 5, 9 mg/mL (10 mm rectangular cell (quartz), Micro cell)

2.3 Measurement Procedures

Add Biuret reagent (2.0 mg) to 500 µL of protein aqueous solution and mix them well. Then react the solution for 60 minutes. Measure the absorbance at the wavelength of 540 nm.

Table 3 Results for the Biuret method

	Cell	Concentration range	Calibration curve formula	Correlation function	Standard error	Detection limit	Determination limit
BSA	10 mm rectangular cell (quartz)	to 9 mg/mL	$Y = AX^2 + BX + C$ $A = -0.0005 \pm 4.3444 \times 10^{-5}$ $B = 0.0548 \pm 0.0004$ $C = 0.0505 \pm 0.0004$	1	0.0109	0.008 mg/mL	0.1483 mg/mL
	Micro cell	to 9 mg/mL	$Y = AX^2 + BX + C$ $A = -0.0016 \pm 0.0003$ $B = 0.0653 \pm 0.0027$ $C = 0.0450 \pm 0.0045$	0.9998	0.0775	0.0696 mg/mL	1.0127 mg/mL
Lysozume	10 mm rectangular cell (quartz)	to 0.9 mg/mL	$Y = AX^2 + BX + C$ $A = -0.0009 \pm 4.9343 \times 10^{-5}$ $B = 0.0591 \pm 0.0005$ $C = 0.0509 \pm 0.0008$	1	0.0144	0.0133 mg/mL	0.2420 mg/mL
	Micro cell	to 9 mg/mL	$Y = AX^2 + BX + C$ $A = -0.0053 \pm 0.0005$ $B = 0.0983 \pm 0.0048$ $C = 0.0476 \pm 0.0063$	0.9999	0.0786	0.0640 mg/mL	—
α-Chymotrypsin	10 mm rectangular cell (quartz)	to 9 mg/mL	$Y = AX^2 + BX + C$ $A = -0.0012 \pm 0.0004$ $B = 0.0064 \pm 0.0035$ $C = 0.0578 \pm 0.0060$	0.9996	0.0958	0.0934 mg/mL	1.2987 mg/mL
	Micro cell	to 9 mg/mL	$Y = AX^2 + BX + C$ $A = -0.0010 \pm 0.0003$ $B = 0.0614 \pm 0.0025$ $C = 0.0343 \pm 0.0043$	0.9998	0.0697	0.0695 mg/mL	0.9987 mg/mL

3. Lowry method

Protein solutions turn blue with an absorption maximum of 750 nm when Alkline copper solution and Phenol reagent are added to the protein solution (Figure 8).

The time course of the chromogenic reaction was measured at 750 nm absorption. The absorbance became stable approximately after 60 minutes (Figure 9). Figure 10 illustrates the spectra of the HSA aqueous solutions that were measured after 60 minutes.

Lowry method determines the quantity by using the absorption maximum of 750 nm after reaction of the sample for 60 minutes.

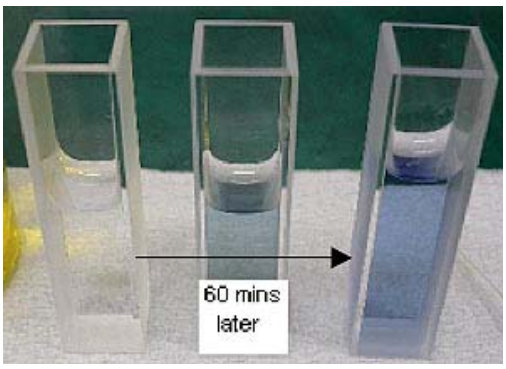

Figure 8 Color change after adding Phenol regent

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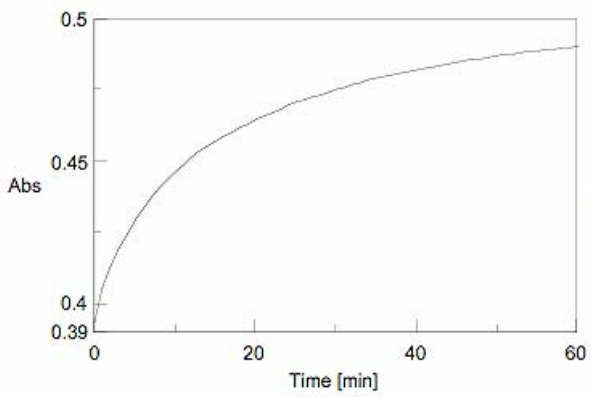

Figure 9 The time course trace of the chromogenic reaction

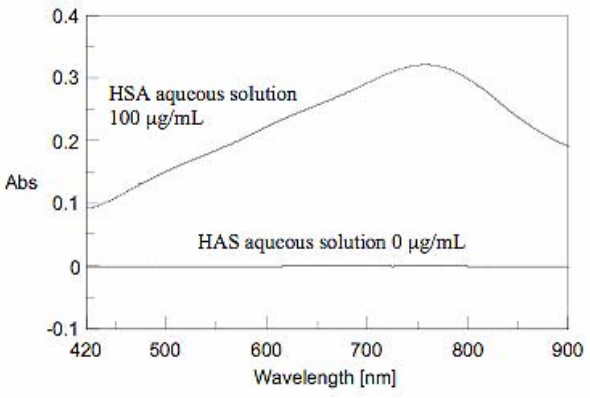

Figure 10 Spectra of HSA aqueous solution by Lowry method

3.1 Reagents

Alkaline copper solution: Mix 50 mL of 2% Na_2CO_3 solution*3) with 1 mL of 0.5% $CuSO_4$ solution*4) (can only be used for immediate analysis).

**3) 2% Na_2CO_3 solution: dissolve anhydrous sodium carbonate (2 g) and caustic soda (0.4 g) in 100 mL water.*

**4) 0.5% $CuSO_4$ solution: dissolve copper (II) sulfate pentahydrate (50 mg) and potassium sodium tartrate tetrahydrate (0.1 g) in 10mL of water.*

Phenol reagent: Dilute commercial solution (2N phenol chemical reagent of Kanto Chemical) to 1N.

3.2 Samples

Same concentrations are used both for the 10 mm rectangular cell and the micro cell of 10 mm

Bovine Serum Albumin (BSA) : 0, 2, 20, 50, 100, 200 µg/mL

Hen Egg Lysozyme : 0, 1, 5, 10, 20, 50, 100, 200 µg/mL

α-Chymotrypsin from bovine pancreas : 0, 2, 20, 50, 100, 200 µg/mL

3.3 Measurement Procedures

Add 2.5 mg of alkaline copper solution to 500 µL of protein solution. Allow to react for 10 minutes after mixing well. Then, add phenol reagent. Rapidly mix and allow the solution to react for 60 minutes. Measure the absorbance at 750 nm.

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Table 4 Results for the Lowry method

	Cell	Concentration range	Calibration curve formula	Correlation function	Standard error	Detection limit	Determination limit
BSA	10 mm rectangular cell (quartz)	to 200 µg/mL	$Y = AX^2 + BX + C$ $A = -4.4663 \times 10^{-6} \pm 5.5049 \times 10^{-7}$ $B = 0.0041 \pm 0.0001$ $C = 0.0250 \pm 0.0034$	0.9999	1.2336	0.8385 µg/mL	3.9441 µg/mL
	Micro cell	to 200 µg/mL	$Y = AX^2 + BX + C$ $A = -4.0578 \times 10^{-6} \pm 1.3689 \times 10^{-6}$ $B = 0.0041 \pm 0.0003$ $C = 0.0150 \pm 0.0097$	0.9994	2.5325	2.3903 µg/mL	10.1765 µg/mL
Lysozyme	10 mm rectangular cell (quartz)	to 200 µg/mL	$Y = AX^2 + BX + C$ $A = -5.6033 \times 10^{-6} \pm 7.2903 \times 10^{-7}$ $B = 0.0049 \pm 0.0001$ $C = 0.0293 \pm 0.0037$	0.9998	1.3861	0.7598 µg/mL	3.5722 µg/mL
	Micro cell	to 200 µg/mL	$Y = AX^2 + BX + C$ $A = -4.8873 \times 10^{-6} \pm 8.2675 \times 10^{-7}$ $B = 0.0047 \pm 0.0002$ $C = 0.00076 \pm 0.0042$	0.9997	1.6514	0.8911 µg/mL	4.1471 µg/mL
α-Chymotrypsin	10 mm rectangular cell (quartz)	to 200 µg/mL	$Y = AX^2 + BX + C$ $A = -1.0948 \times 10^{-5} \pm 4.3250 \times 10^{-7}$ $B = 0.0061 \pm 8.8164 \times 10^{-5}$ $C = 0.0112 \pm 0.0027$	1	0.685	0.4371 µg/mL	7.6764 µg/mL
	Micro cell	to 200 µg/mL	$Y = AX^2 + BX + C$ $A = -8.8298 \times 10^{-6} \pm 8.8527 \times 10^{-7}$ $B = 0.0054 \pm 0.0002$ $C = 0.0167 \pm 0.0055$	0.9999	1.3341	1.0214 µg/mL	16.3006 µg/mL

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