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An Absolute Method for Protein Determination Based on Difference in Absorbance at 235 and 280 nm

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Because of the importance of quantitative determination of protein in the research laboratory as well as in the food and feed industries (1), search for the ideal method continues unabated after many years. Methods available include nitrogen determination (Kjeldahl (2) and Dumas (3)), hydrolysis of the protein, derivatization of the amino acids with phthalaldehyde and fluorescence determination (4), determination of bound or free lysine (5) or glutamate (4), and the Lowry (6), biuret (7) dye-binding (8–11) turbidity (12) and spectral methods (13). With the exception of the spectral methods, the methods involve destruction of the sample.

In this paper we report the use of difference in absorbance between 235 and 280 nm for determination of protein concentration.

MATERIALS AND METHODS

Bovine trypsin (twice crystallized, Lot 104c-0302), bovine chymotrypsinogen A (six times crystallized, Lot 124c-8200), bovine globulin (Cohn Fraction II, >99% γ-globulin, Lot 70c-2700), *Bacillus subtilis* α-amylase (four times crystallized, Lot 64c-0262), bovine pancreatic ribonuclease A (five times crystallized, Lot 47c-0422), and bovine serum albumin (crystallized and lyophilized, Lot 98c-8050, 15.2% N) were obtained from Sigma Chemical Company, St. Louis, Missouri. Chicken ovalbumin was obtained from Pharmacia Fine Chemicals Inc., Piscataway, New Jersey (Lot OCA) as part of a kit for molecular weight determination. *Clostridium perfringens* enterotoxin was purified by the method of Granum and Skjelkvåle (14) to electrophoretic homogeneity. All other reagents were of analytical grade.

The protein samples were weighed on a Cahn electrobalance Model 4100 and dissolved in 20 mM sodium phosphate buffer, pH 6.8. The organic solids concentration was determined by the chromic acid oxidation method (15) using analytical grade, moisture-free sucrose as the standard. The protein concentrations used permitted determination of the spectra from 340 to 230 nm without dilution at the lower wavelengths.

Spectra were determined on a Beckman 25 spectrophotometer with recorder. The spectrophotometer had been recently serviced with check of wavelength and linearity. These were verified in addition with several concentrations of bovine serum albumin and tryptophan.

RESULTS AND DISCUSSION

The measured extinction coefficients of eight proteins at 235 and 280 nm are given in Table 1. The average measured extinction coefficient at 235 nm is 2.82 times that at 280 nm while the average calculated extinction coefficient based on amino acid composition gave an average ratio of 0.849 at these two wavelengths. There is a much larger variation of the extinction coefficients of the proteins at 280 than at 235 nm.

The measured extinction coefficients

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differ from the calculated values primarily at 235 nm. This difference is due to absorbance of the peptide bond, possibly with a small contribution from secondary and tertiary structure. Therefore, the difference in absorbance between 235 and 280 nm, due primarily to the peptide bond, can be used to calculate protein concentration by the equation

$$\text{Protein concentration (mg/ml)} = (A_{235} - A_{280})/2.51,$$

where the factor 2.51 is the difference between the average measured extinction coefficient ($E^{0.1\%}$) at 235 and 280 nm (Table 1). The last column of Table 1 gives the cal-

TABLE 1

MEASURED AND CALCULATED EXTINCTION COEFFICIENTS ($E^{0.1\%}$) FOR SEVERAL PROTEINS AT 235 AND 280 nm

	$E^{0.1\%}$				
	Measured		Calculated[b]		
Protein[a]	235 nm	280 nm	235 nm	280 nm	$(E_{235}^{0.1\%} - E_{280}^{0.1\%})$/2.51[c] (mg/ml)
Trypsin	3.64	1.60 (1.54[d]; 1.56[e])	1.30	1.56	0.810
Chymotrypsinogen	3.92	2.02 (2.00[f]; 2.06[g])	1.12	1.98	0.757
γ-Globulin	4.36	1.38 (1.35[h]; 1.39[i])	1.14	1.39	1.19
α-Amylase	4.83	2.42 (2.53[j])	1.52	2.05	0.960
Ovalbumin	3.28	0.789 (0.735[k])	0.606	0.642	0.992
Ribonuclease A	3.69	0.769 (0.722[l]; 0.695[m])	0.927	0.597	1.16
Bovine serum albumin	3.07	0.701 (0.820[n]; 0.661[l])	0.744	0.590	0.944
Enterotoxin	4.34	1.33	1.22	1.24	1.20
Average ± SD[o]	3.89 ± 0.59	1.38 ± 0.62	1.07 ± 0.30	1.26 ± 0.60	1.00 ± 0.17
Range/average	0.452	1.25	0.854	1.16	0.434
Relative to BSA					
Average ± SD[o]	1.27 ± 0.19	1.96 ± 0.89	1.50 ± 0.39	2.29 ± 0.98	1.06 ± 0.18
Range	1.00–1.57	1.00–3.45	0.815–2.04	1.00–3.48	0.804–1.27

[a] The amino acid compositions used were from: trypsin, Ref. (16); chymotrypsinogen, Ref. (17); γ-globulin, Ref. (18); α-amylase, Ref. (19); ovalbumin, Ref. (20); ribonuclease, Ref. (21); bovine serum albumin, Ref. (22); enterotoxin, Ref. (14).

[b] Calculated contributions of tryptophan, tyrosine, phenylalanine, histidine, methionine, cystine, and cysteine to absorbance. $E_{280}^{0.1\%}$ values used: tryptophan, tyrosine and half-cystine were 30.6, 7.84, and 0.582 mg^{-1} cm^2, respectively (Ref. (23)); histidine, methionine, and cysteine were 0.036, 0.034, and 0.19 mg^{-1} cm^2, respectively (Ref. (24)). $E_{235}^{0.1\%}$ values used: tryptophan, tyrosine, and phenylalanine were 13.4, 11.1, and 0.27 mg^{-1} cm^2, respectively, determined in this study; half-cystine, 1.25 mg^{-1} cm^2 (Ref. (25)); and histidine, methionine, and cysteine were 0.80, 0.63, and 1.85 mg^{-1} cm^2, respectively (Ref. (24) as determined at 233.3 nm).

[c] The factor 2.51 is the difference between the average measured values of the proteins at 235 and 280 nm.

[d] Ref. (16).

[e] Ref. (26); $E_{280}^{0.1\%}$ values for trypsin in the literature range from 1.29 (Ref. (27)) to 1.72 mg^{-1} cm^2 (Ref. (28)).

[f] Ref. (29), at 282 nm.

[g] Ref. (30).

[h] Ref. (18), for rabbit γ-globulin.

[i] Ref. (31), for human γ-globulin.

[j] Ref. (32).

[k] Ref. (33).

[l] Ref. (25).

[m] Ref. (34).

[n] Ref. (26).

[o] Calculated as $\pm(\sum d^2/(n-1))^{1/2}$.

culated concentrations of a 1 mg/ml protein solution of each of the eight proteins by this equation. The standard deviation of the calculated values is 17% as compared with 45 and 15%, respectively, for measurements made at 280 and 235 nm alone.

Advantages of the method include: (a) determination of protein concentration without use of a reference protein and standard curve; (b) lack of interference by nucleic acids (see below); (c) independent of specific amino acid composition of a protein (see below); (d) measurements at wavelengths readily accessible to all uv spectrophotometers where there is little interference by the usual buffers and where both absorption determinations can be made on the same sample preparation without dilution; and (e) bovine serum albumin, often used as a reference protein in other methods, gives a typical value by this method while it does not at 280 and 235 nm alone (Table 1).

Nucleic acids have essentially the same absorbance at 280 and 235 nm (24,35) and therefore do not affect calculations of protein concentration by the equation above. Of equal importance, the combined absorbance values of tryptophan, tyrosine, phenylalanine, histidine, methionine, cysteine, and cystine are nearly identical at 235 and 280 nm, based on the amino acid composition of 208 proteins (36) (A_{235}/A_{280} = 0.853; for the eight proteins used here the calculated ratio is 0.849). Based on the 208 proteins, the combined absorbance values of the above amino acids in an "average" protein would be identical at 233.6 and 280 nm.

Previously proposed methods for calculation of protein concentration based on the difference in absorbance at two wavelengths were designed to correct for interference by nucleic acids (13,24,35,37) or for specific amino acid composition (38), but not for both. Some methods (39–41) do not correct for contributions by either nucleic acids or specific amino acids.

The average calculated protein concentration (based on absorbance of a 1 mg/ml solution) for the eight proteins in Table 1 is 1.00 ± 0.17 mg/ml by the proposed equation, 1.45 ± 0.67 mg/ml by the Kalckar and Shafran equation (42), and 1.57 ± 0.73 mg/ml by the Layne equation (13). Relative to bovine serum albumin, the values (mg/ml) are 1.06 ± 0.18, 2.03 ± 0.93, and 2.02 ± 0.93 for the proposed equation, the Kalckar and Shafran equation, and the Layne equation, respectively.

The difference of 2.51 between $E_{235}^{0.1\%}$ and $E_{280}^{0.1\%}$ is 11% lower than the expected value of 2.82 based on the difference of $E_{235}^{0.1\%}$ (measured) and $E_{235}^{0.1\%}$ (calculated) (Table 1). This difference is probably due to effect of secondary and tertiary structure on the absorbance of tryptophan, tyrosine, phenylalanine, histidine, methionine, cystine, and cysteine as indicated by the difference of 9.5% between the measured and calculated extinction coefficients at 280 nm (Table 1).

Protein concentration can be determined from absorbance at 235 nm alone with a 2.82-fold increase in sensitivity over that at 280 nm alone (Table 1). The variation among proteins is also smaller at 235 than at 280 nm (15 vs 45% SD for the eight proteins used). However, a standard curve would be needed and corrections for specific amino acid composition and for nucleic acids would not be made.

The proposed method has a sensitivity of 45% of the usual Lowry method.

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