

Using Amino Acid Analysis to Determine Absorptivity Constants

A Validation Case Study Using Bovine Serum Albumin

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Amino acid analysis of well-recovered residues offers an easy way to calculate the absorptivity constant for a known protein. The method provides an absolute measure of protein concentration, free from interference from water, excipients, and bound salts. This article demonstrates a qualified method for determining protein content by AAA.

With protein biologics, it is critical to accurately determine an absorptivity constant. The constant is then used when routinely measuring protein concentration by ultraviolet (UV) spectrophotometry. The yield of product during manufacture and the product content and potency on the label are dependent on this constant. There have been several approaches in the past to determining the constant, but all have drawbacks — primarily poor accuracy, or interference from excipient salts, or overly tedious methods.

Our study sought to demonstrate a method for determining specific absorptivity constants (a_s) by amino acid analysis (AAA) that provides accurate and precise quantitation of protein

concentrations. (The full name of amino acids abbreviated in this article can be found in the “Amino Acid Code” sidebar.)

BASICS AND HISTORY

Although AAA techniques used today have been around since the late 1950s, remarkable progress has made those methods more sensitive, automated, and available for use in any biochemical laboratory. In AAA, protein and peptide bonds are hydrolyzed in hot hydrochloric acid leaving free amino acid residues. Amino acids are then analyzed by quantitative ion-exchange chromatography (1).

Determination of a_s for a small organic molecule is often a minor undertaking. The purified and dried molecule is quantitated by gravimetric means, followed by dissolution in an appropriate buffer or solvent and simple UV-vis spectrophotometric analysis at its λ maximum. However, for protein-based biopharmaceuticals this is not as easily accomplished. The limiting step is quantifying the protein, which may contain contaminating proteins, bound salts, metals, or excessive amounts of bound water. Quantification of purified proteins to determine the a_s in a defined solution has been accomplished in the past by gravimetric, Kjeldahl, and colorimetric techniques (2,3) and by amino acid analysis (4). As an alternative,



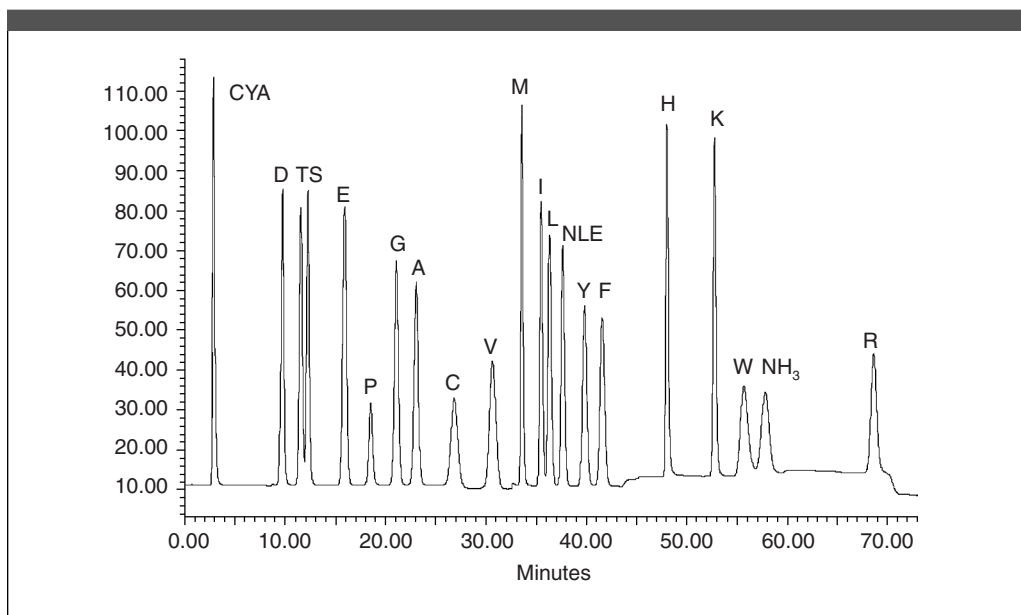


Figure 1. A representative chromatogram of 18 amino acids and norleucine (NLE); internal standard resolved by cation-exchange chromatography with postcolumn ninhydrin derivatization

statistically derived approaches for predicting a_s of proteins are also widely used. These predictive models are based on the UV absorptivities of tryptophan, tyrosine, and cystine residues at 280 nm. With the exception of the predictive spectrophotometric methods and AAA, the above methods are not commonly used in biopharmaceutical applications today because of the large quantities of protein needed or the large degrees of error associated with their use.

History of a_s for proteins and peptides. Determining a_s for synthetic or recombinant proteins or peptides is a necessary component of biopharmaceutical production and formulation. Once an a_s value is determined for a particular protein in a well-defined formulation matrix, the concentration of that protein in solution can be rapidly determined from its measured absorbance and the specific absorptivity constant using a derivative of Beer's law (see Equation 1).

The a_s is expressed in $\text{mL mg}^{-1} \text{cm}^{-1}$ units and can be converted to the molar absorptivity (a_m , formerly known as ϵ) using the relationship $a_m = a_s \times MW$, where MW is the molecular weight of the protein. Commonly used estimates of a_s are based on predictive ultraviolet spec-

trophotometric models. Gill and von Hippel developed such a predictive model for determining the a_m (or ϵ) of denatured proteins in 6.0 M guanidine hydrochloride (Gdn) (5), based on the earlier work of Edelhoch (6) (see Equation 2).

Using the model in Equation 2, Gill and von Hippel calculated the a_m of 18 globular proteins and compared the results to measured values found in the published literature (5). Reasonably good agreement was found between predicted a_m values for proteins in Gdn and those values listed for Gdn in its native aqueous solutions. The average difference between predicted and actual values was 4.9%, with a maximum difference of -14.9%.

Pace et al. further refined the Gill and von Hippel equation by testing the predictive capability of a modified equation on 80 different proteins in aqueous solutions (7). From that body of data, they refined the average a_m values for Trp, Tyr, and Cys by statistical means (Equation 3). Examination of their data revealed that for 47 out of 80 proteins, the predicted a_m agreed with the literature values within 5%. The 33 other proteins were found to agree between 5% and 17%.

The Amino Acid Code

Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic Acid
Asx		Asp + Asn ^a
Cys	C	Cysteine ^b
Gln	Q	Glutamine
Glu	E	Glutamic Acid
Glx		Glu + Gln ^c
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Nle		Norleucine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

^aResults from deamidated Asn to Asp during acid hydrolysis

^bCystine is degraded to cysteine and several unidentified, mixed, disulfide compounds during acid hydrolysis; also cystine and cysteine are converted to cysteic acid (cya) during oxidative hydrolysis with dimethylsulfoxide.

^cResults from deamidated Gln to Glu during acid hydrolysis

$$\text{protein concentration (mg/mL)} = \frac{\text{absorbance of protein or peptide solution (1-cm path length)}}{a_s}$$

Equation 1. A derivative of Beer's law for determining protein concentration in a solution when the absorptivity constant has been determined for a particular protein in a well-defined formulation matrix, where $a_s = \text{mL mg}^{-1} \text{cm}^{-1}$

Table 1. BSA concentration in each serial dilution was estimated using biuret reaction. Each dilution was then spectrophotometrically scanned and absorbance at 280 nm recorded. (No correction for wavelength scatter at longer wavelengths was necessary.)

Sample and Treatment	Putative Protein Concentration in BSA Solutions			
	Dilution 1 ~1.0 mg/mL	Dilution 2 ~0.8 mg/mL	Dilution 3 ~0.6 mg/mL	Dilution 4 ~0.4 mg/mL
Hydrolysis volume (μL)	66.4	83	110.7	166
Hydrolysis putative amount (μg)	~66.4	~66.4	~66.4	~66.4
Hydrolysis putative amount (nmol)	~1.0	~1.0	~1.0	~1.0
Day 1: absorbance ^a 280 nm AU ^b and %RSD ^c	0.6466 0.26	0.5207 0.08	0.3856 0.03	0.2549 0.15
Day 2: absorbance ^a 280 nm AU ^b and %RSD ^c	0.6432 0.16	0.5078 0.06	0.3750 3.59	0.2540 0.12
Day 3: absorbance ^a 280 nm AU ^b and %RSD ^c	0.6433 0.19	0.5040 0.16	0.3841 0.13	0.2495 0.21

^aEach absorbance value is the mean of three determinations per day.
^bAU = absorbance units
^c%RSD = percent relative standard deviation

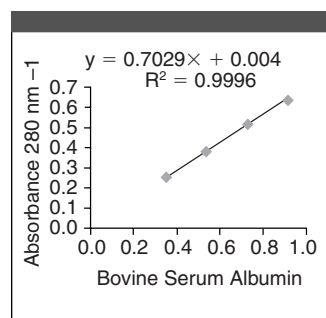


Figure 2. Absorptivity by well-recovered amino acid residues for day 1

The predicted protein concentrations, estimated by these statistical models, were based on the solution's content of Trp, Tyr, Cys. Therefore, using the method in Equations 2 and 3 requires that Trp, Tyr, and Cys residues be present in sufficient quantities to ensure adequate absorption at 280 nm. Using such methods also requires that the number of residues be known. Such predictive spectrophotometric methods served biochemists for many years because of their ease of use. However, the underlying assumption in that method is that the absorbance characteristics of Trp, Tyr, and Cys residues as determined in the model are equivalent to those observed in the test solvent. If no such residues exist, Scopes (8) described an alternate spectrophotometric method using absorption of the peptide bond at 205 nm.

A less commonly used method for protein quantitation to determine a_m is by dry weight, as described by Kupke and Dorrier (9) and later by micro-dry weight determination (10). As the name suggests, the mass of the protein or peptide is determined gravimetrically after exhaustive drying. The method is reliable but requires a large amount of purified protein and great care in sample handling. Additionally, the presence of nonvolatile salts or excipients in the protein formulation can require dialysis to remove them, thereby reducing the usefulness of the dry weight

approach for most biopharmaceutical formulations. Additionally, the drying process can lead to oxidation of labile residues, such as Trp and Met, altering the absorptivity of the protein (11).

QUANTIFYING PROTEINS BY AAA

Proteins and peptides quantified by AAA to determine the a_s are free of common assumptions (such as equivalent absorbance characteristics between models and test solvents) and interferences (such as those from excipients, salts, and oxidized residues) — the drawbacks of earlier methods. This work supports the quantitative approach described by Sittampalam et al. using AAA as a reference for quantifying highly purified proteins (4).

Materials. Our study was undertaken to offer a qualified method for accurate and precise protein quantitation for determining the absorptivity coefficient using AAA. We used bovine serum albumin (BSA) as the model protein solution for our quantitative approach. In our chromatographic quantitation, we compared peak responses in the samples to hydrolysate standard preparations containing known amounts of all amino acids and internal standards. We chose a traceable reference source of BSA from the National Institute of Standards and Technology (NIST, www.nist.gov) to minimize concerns about the quality and purity of the test article. The BSA in the NIST standard was previously quantified using the biuret reaction (12,13) by the sponsor.

Methods. Most biopharmaceuticals are relatively well characterized, and the primary sequence, amino acid composition, and MW of the proteins are known. For such protein products, determining the a_s by quantitation of well-recovered amino acids is sufficient for determining the concentration of protein in a solution. However, for protein products that are less well characterized, in which the amino acid composition and MW are not accurately known (such as immunoglobulins), AAA quantitation of total amino acid residues may be required. The same is true of products composed of an ill-defined mixture of proteins or peptides.

Therefore, we quantified the amount of BSA by total compositional AAA and by analysis of well-recovered amino acid — Ala, Asx, Gly, Glx,

$$\epsilon(6\text{ M Gdn}, 280\text{ nm}) = (\# \text{ of Trp residues}) \times 5,690 + (\# \text{ of Tyr residues}) \times 1,280 + (\# \text{ of Cys residues}) \times 120$$

Equation 2. The predictive model developed by Gill and von Hippel for determining the molar absorptivity constant of denatured proteins (2)

Table 2. A comparison of the concentration of bovine serum albumin protein results using well-recovered amino acid residues and total amino acid residues (mg/mL)^a

Dilution	Day 1		Day 2		Day 3	
	Well-Recovered Residues	Total Residues	Well-Recovered Residues	Total Residues	Well-Recovered Residues	Total Residues
Dilution 1	0.920	0.919	0.946	0.930	0.928	0.904
Dilution 2	0.729	0.728	0.751	0.739	0.743	0.725
Dilution 3	0.537	0.537	0.562	0.554	0.546	0.535
Dilution 4	0.363	0.362	0.369	0.364	0.364	0.356

^aAll data are the mean of three determinations.

Table 3. Absorptivity constants for BSA using amino acid analysis and either well-recovered residues or total residues

Dilution	Day 1		Day 2		Day 3	
	Well-Recovered Residues	Total Residues	Well-Recovered Residues	Total Residues	Well-Recovered Residues	Total Residues
Slope ^a	0.7029	0.7033	0.6773	0.6906	0.6885	0.7092
Y-intercept	0.00405	0.00430	0.00002	-0.00162	0.00097	-0.00158
Correlation Coefficient	0.9996	0.9996	0.9997	0.9996	0.9992	0.9992
Mean slope	0.6896	0.7010				
%RSD ^b	1.86	1.36				

^aIn mL mg⁻¹ cm⁻¹

^b%RSD = percent relative standard deviation

Leu, and Lys. The concentration data on BSA (found through either total composition AAA or well-recovered AAA) were then correlated with measured absorbance data to obtain a_s values.

We expected the two AAA methods to yield an equivalent a_s for BSA. This would serve to qualify AAA quantitation by either approach for purposes of obtaining an accurate a_s value.

Dilutions and absorbance. For this study, we diluted a 7% reference solution (72.17 g/L) of NIST BSA to a putative 1.0 mg/mL concentration in 150 mM NaCl. The BSA was serially diluted further to approximately 0.8, 0.6, and 0.4 mg/mL in 150mM NaCl (see Table 1 for UV_{280 nm} data for all BSA dilutions). In our qualification study, the protein concentration of BSA was previously estimated by a biuret reaction. However, determining protein concentration at this early stage in the process requires only a crude estimate of concentration, so a variety of means could have been used: Colorimetric techniques

such as those described by Lowry et al. (2) or Bradford (3) would be sufficient for this purpose. The important factor is that the dilutions result in UV absorbance readings at the desired λ of the sample and within the linear range of the spectrophotometer used (generally 0.1 to 1.0 absorbance units, AU).

We spectrophotometrically scanned all BSA dilutions from 200 to 400 nm using 150 mM NaCl to establish a baseline in a 1-cm quartz cell. Absorbance at 280 nm was recorded, and correction for light scatter at longer wavelengths was found to be unnecessary.

Protein hydrolysis. We removed triplicate aliquots corresponding to 1 nmol of BSA protein based on putative concentration from each of the serial dilutions following UV_{280 nm} spectrophotometric analysis. To each aliquot, we added a known amount of internal standard and prepared it for acid hydrolysis and subsequent AAA. The internal standard used for analysis of all residues, except Trp, was norleucine. For Trp analysis, we used L- α -amino- β -guanidinopropionic acid as the internal standard. To calculate BSA concentrations, we used a MW of 66,430 Da (14).

AAA of well-recovered residues. To quantitate BSA by analyzing well-recovered amino acid residues, we used a single method of hydrolysis. Approximately 1 nmol of BSA was hydrolyzed in 6 N HCl with 1% phenol at 150°C for four hours under vacuum. All residues except Cys and Trp were detected with this method. The focus of our analysis was the quantitation of the well-recovered residues: Ala, Asx, Gly, Glx, Leu, and Lys.

AAA of total amino acids. Total compositional AAA required three additional analyses for the labile residues — Ser, Thr, Trp, Pro, and Cys (primarily in the form of cystine) — in conjunction with liquid-phase hydrolysis for all other residues. Accurate quantitation of Ser and Thr residues required a time-course hydrolysis study in which the BSA samples were hydrolyzed at 150°C in 6 N HCl with 1% phenol for 4, 8, and 12 hours. Yields for Ser and Thr were plotted versus time: Total yield for Ser and Thr were then determined by extrapolation to time zero. That provided the best mass estimate of these residues because of their rapid degradation during

$$\mathcal{E}(280 \text{ nm}) = (\# \text{ of Trp residues}) \times 5,500 + (\# \text{ of Tyr residues}) \times 1,490 + (\# \text{ of Cys residues}) \times 125$$

Equation 3. The predictive model refined by Pace et al., which refines the molar absorptivity constant average values by statistical means (4)

acid hydrolysis. For AAA of Trp in BSA, we used liquid-phase hydrolysis in 3 N mercaptoethanesulfonic acid (MESA) as described by Penke et al. (15).

We analyzed Cys and Pro residues by a third method using oxidative hydrolyses of BSA in 6 N HCl with 2% (v/v) dimethyl sulfoxide (DMSO) as described by Spencer and Wold (16). Analysis of Pro by oxidative hydrolysis prevents the formation of Cys degradants, which partially coelute with Pro, falsely elevating yields of that residue by more than 25%. Use of this battery of specialized methods offers the best available recovery of labile residues Trp, Cys, Ser, Thr, and Pro.

Chromatographic method. We separated the free amino acids contained in these hydrolysates by cation-exchange chromatography on a sodium column using a Beckman 6300 amino acid analyzer. The mobile phase was composed of 1.7% sodium citrate with an increasing pH step gradient from 3.3 to 4.3 and then to 6.3 and a temperature gradient from 48°C to 64°C and then to 77°C.

Chromatographic conditions were identical for all hydrolysis methods employed in this study with the exception of the Trp analysis. Chromatography for Trp was extended from 82 to 92 minutes to obtain better resolution of basic residues. Amino acids eluting from the column were derivatized by mixing them in a stream of ninhydrin and heating them to 135°C. All residues were detected by a summation of signal at 440 and 570 nm. Figure 1 shows a typical AAA cation-exchange chromatogram.

Calculation of the a_s . The a_s is derived from the slope of the line formed by plotting UV absorbance at 280 nm versus the concentration of BSA quantitated by AAA. In this study, six such plots were constructed, half for quantitation of BSA using well-recovered amino acids and the other three using total amino acids (see Figures 2 and 3 for representative plots). The reported a_s is the mean of the three determinations for each analytical approach (Table 3).

Experimental results using well-recovered residues. On three different days, the BSA was serially diluted from a putative concentration of 1.0 to 0.4 mg/mL and analyzed spectrophotometrically at 280 nm followed by AAA for well-recovered residues. All samples were prepared and analyzed in triplicate. The calculated concentrations of BSA (mg/mL) based on the yield of Ala, Asx, Glx, Gly, Leu, and Lys are shown in Table 2, and Figure 2 gives a representative plot

Table 4. A comparison of absorptivity values from literature sources (using observed values except where noted as predicted) versus quantitation of well-recovered residues using amino acid analysis of bovine serum albumin at 280 nm

	Molar Absorptivity (a_m) $M^{-1} \text{ cm}^{-1}$	Absorptivity Constant ^a (a_s) $\text{mL mg}^{-1} \text{ cm}^{-1}$	Percent Difference Between Literature ^b and AAA
Predicted value Pace et al. (1995)	42925	0.6462	-6.3
Wetlaufer (1962)	43890	0.6607	-4.2
Gill and von Hippel (1989)	43291	0.6517	-5.5
Nozaki (1986) ^c	41568	0.6257	-9.3
Pace et al. (1995) ^d	42961	0.6467	-6.2
Amino acid analysis of well-recovered residues ^e	45810	0.6896	0.0
Amino acid analysis of total residues ^e	46567	0.7010	1.7

^a Absorptivity constant (a_s) = molar absorptivity (a_m) ÷ molecular weight (MW), where the MW of BSA = 66,430 Da.
^b Complete reference citations can be found in the reference section of the text: Pace et al. (7), Wetlaufer (19), Gill and von Hippel (5), Nozaki (10).
^c Determined by dry weight
^d Average of 10 a_s determinations
^e Work performed at aaiPharma (www.aaiintl.com)

of absorbance at 280 nm versus concentration of BSA as determined by AAA using only well-recovered residues.

Experimental results using total residues. Serial dilutions of BSA were quantitated by the yield of total amino acids. This required the compilation of data from analyses of the 6 N HCl hydrolysis at 150°C for four hours for most residues, the Trp analyses in 3 N MESA, the Cys and Pro analyses in 6 N HCl with 2% DMSO, and the time course data for Ser and Thr (Table 2). A representative plot of absorbance at 280 nm versus concentration of BSA as determined by AAA using total amino acid residues for the first day of analysis is depicted in Figure 3.

Experimental calculation of a_s . A specific a_s was calculated each day for both AAA quantitative approaches (Table 3). Quantitation of BSA at 280 nm yielded an a_s of 0.6896 mL mg⁻¹ cm⁻¹ using well-recovered residues and of 0.7010 mL mg⁻¹ cm⁻¹ by AAA of total residues. The a_s for each of the three days of analysis revealed that the results were highly reproducible for both AAA approaches (Table 3). The absorptivity coefficients determined by the two methods were in close agreement with each other and agreed reasonably well with previously published results for BSA (Table 4).

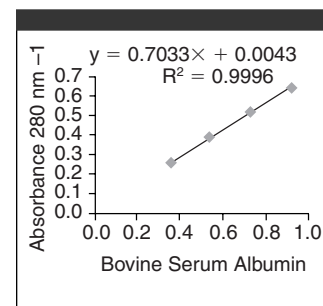


Figure 3. Absorptivity by total amino acid residues for day 1

ACCURATE AND RAPID RESULTS

Predictive spectrophotometric methods are easy to use in biopharmaceutical applications for proteins of known composition. Such models generally work well for most proteins; however, as described, many factors can lead to inaccurate quantitation when such predictive models are used. Regulatory testing of bulk and finished protein-based biopharmaceuticals have often disclosed significant discrepancies in protein concentration determined by UV methods that are based on predictive spectrophotometry rather than on methods using quantitative AAA. AAA provides an absolute measure of amino acids free of interferences.

When quantitating BSA, a protein of known composition and *MW*, well-recovered amino acids are an accurate measure of protein concentration. Analysis by total amino acids is nearly equivalent to the well-recovered method (Tables 3 and 4) but more labor intensive. In our laboratory, quantitation by total amino acids is rarely done if a sufficiently accurate *MW* and a theoretical amino acid composition are known for the protein of interest. In biopharmaceutical applications, tests to determine a valid a_s using either AAA approach should be repeated on three different days to provide data with sufficient accuracy and precision.

Study results. Sittampalam et al. (4) concluded that AAA of well-recovered residues provides an absolute measure of protein concentration when care is taken to optimize the method. In our study, we optimized the quantitation of all amino acids with care. Recovery of the well-recovered residues in our study was found to be 99.9% with a relative standard deviation (RSD) of 1.4%. Recovery of all amino acids was only slightly lower at 97.6% but less precise with a resulting RSD of 16.5%. The lowered precision was because recovery of Met (43.3%), His (116%), and Trp (117%) was poor (data not shown). Met residues are heavily degraded during acid hydrolysis, and Cys degradation often interferes with recovery of His and Trp. Recovery of Pro by oxidative hydrolysis was found to be satisfactory at 104%.

Data presented in our work indicate that quantitation of BSA by AAA is accurate. For well-recovered residues, recovery was complete, resulting in an accurate and precise determination of BSA concentration and a_s . AAA of total amino acids resulted in a slightly lower BSA concentration and therefore a slightly larger a_s (Table 4).

COMPARING THE RESULTS

Pace et al. (7) reported a predicted a_s of 0.6462 mL mg⁻¹ cm⁻¹ for BSA, approximately 6.3% lower than the AAA result we obtained using well-recovered amino acids. Determining the a_s using concentrations calculated from dry weight analysis yielded the greatest difference to our well-recovered data (-9.3%) (10). An average of 10 absorptivity values compiled from the literature for BSA agreed within -6.2% of our data (Table 4). In all cases, a_s cited in the literature for BSA were lower than those found by AAA.

Our data suggest that predictive spectrophotometric and other measurements of BSA concentration described in the literature may be overestimating BSA concentration, leading to a lower a_s value (Table 4). The presence of non-volatile salts or bound water may be the factors contributing most greatly to the undetected protein dilutions. The predictive models described by Gill and von Hippel (5) and Pace et al. (7) use experimentally derived absorptivities of many proteins that could also be contaminated with bound salts or water resulting in undetected protein dilution.

Sittampalam et al. (4) cited instances of quantitation by total nitrogen using the Kjeldahl method (17), which was likely compromised by the presence of ammonium ions in the lyophilization buffer causing an overestimated protein concentration. Quantitation by dry weight can easily overestimate protein concentration by not accounting for presence of non-volatile salts. Even protein-bound water can be a significant source of error. Perkins demonstrated that bound water was such a significant factor that he suggested that predicted absorption coefficients should be increased on average 3% for the 23 proteins and glycoproteins he studied — which included BSA (18).

Absorptivity measurements can be significantly influenced by the composition, temperature, and pH of the aqueous buffer solution used to solubilize proteins. Wetlaufer presented considerable UV spectral data on Trp, Tyr, and Cys that suggested that the composition, temperature, and pH of the solubilization buffer must be well defined to calculate absorptivities (19). **BPI**

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