

Determining Protein Concentration

Part 1: Methodology

Determining protein concentration is one of the most important analytical methods used in the discovery, development, and manufacturing of protein products. Part 1 discusses a number of methods for analyzing samples ranging from complex mixtures to highly purified proteins.

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Whether a product consists of crude tissue homogenates, semipurified biological fluids, or highly purified recombinant proteins, the amount of protein in a given amount of sample is valuable information.

Protein concentration is analyzed to monitor the amount of material recovered at each step in a purification process and to determine the appropriate quantity of material to use in subsequent process steps. In biopharmaceutical production, determining protein concentration is one way to measure the manufacturing consistency of each batch or the stability of a protein solution over time. Protein concentration values are also needed when preparing test samples for analytical methods to assess product purity, identity, and potency. Because of its wide-ranging use in operations from discovery and development through commercial manufacturing, the measurement of protein concentration is one of the most useful and significant test methods performed on biotechnology products. In Part 1 of this discussion, we look at a number of methods for determining protein concentration.

The nature of the protein preparation to be tested is a critical factor in the selection of an appropriate concentration determination method. Some methods, such as colorimetric assays, can be used with both crude and purified protein solutions to determine a total protein concentration. Others, such as UV absorbance, require solutions of relatively pure protein for accurate results.

Unless methods are chosen that can isolate the signal specific for a single protein, heterogeneous protein mixtures will generate a combined response from the proteins present. Solutions containing host cell elements such as lipids or

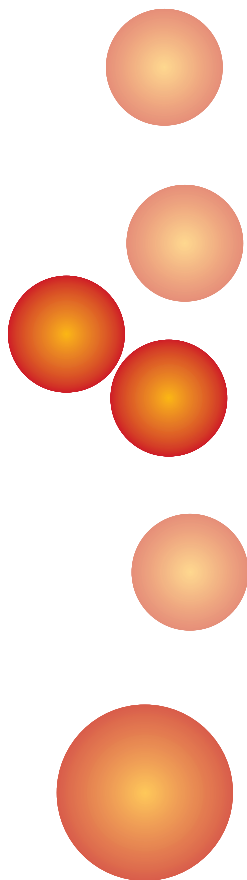
nucleic acids, cell culture media containing dyes or surfactants, and formulations with high concentrations of reducing agents, detergents, or salts may each require different types of protein concentration analysis to obtain accurate results in the presence of interfering matrix components.

The amount of sample available for testing (both volume and concentration) is also a driving factor in selecting the appropriate method. Protein assays can vary significantly in their minimum required volume, their linear working range, and their ability to measure highly diluted samples accurately. There is often a trade-off between sensitivity and accuracy, although vendors of commercial protein assay kits are working to improve both.

Matrix components can significantly affect protein concentration determination. Therefore, with most protein concentration assays you should analyze the sample buffer alone to evaluate and correct for background signals. If the interference is too great, then either remove or dilute the matrix or select another assay. Even when no individual components interfere, the synergistic effect of some substance combinations can create assay artifacts. When in doubt, it is always wise to confirm the effects of the specific buffer component or components at the concentrations expected for the product with the protein concentration assay chosen.

COLORIMETRIC METHODS

Colorimetric assays are commonly used in biomolecular laboratories for determining protein concentration because the procedures and their instrumentation requirements are simple. In such assays, the protein is reacted with one or more chromogenic substances to yield a detectable signal.



Two basic forms of colorimetric assays are used. The first form involves reactions between the protein and chemicals in solution that yield colored, fluorescent, or chemiluminescent products. In the second form, a colored dye is bound to the protein and an absorbance shift is generated. Within the linear working range of both types of assays, the intensity of the color quantitatively correlates to the amount of protein present.

These methods are well established, proven with many different protein preparations, and relatively easy to use. When optimized for specific applications, most are precise and reproducible. However, their disadvantages include limited sensitivity at very low ranges (for example, below 1 µg/mL), interferences from buffer components, unstable chromogenic reaction products, and potential inaccuracies if a protein standard does not react similarly to the test protein.

Standards. The quantitation of these assays is based on the preparation of a standard curve with known concentrations of standards. Standards can be nonproduct surrogate proteins (such as bovine serum albumin, BSA) or a well-characterized reference standard of the product itself. A set of *x,y* data points is generated from the known standard concentrations and their corresponding absorbance readings, and a best-fit regression line is drawn through them. The test protein absorbance readings are plotted against this best-fit line, and the concentration results are calculated by linear regression analysis.

Because protein standards drive the quantitative results, the accuracy of colorimetric assays depends on the validity of the standard curve. Key elements in preparing accurate and precise standard curves include the protein chosen as the known standard (either a surrogate or a reference protein), the preparation of the standard curve dilutions, establishment of the assay's linear working range, and the stability of the stock standard solution.

Not all proteins react similarly with the various chromogenic reagents used in colorimetric assays. For the most accurate results, use a protein standard that will react as closely as possible to the protein or proteins being tested. Ideally, a highly purified solution of the protein being tested will provide the most accurate standard curve values because it will react in the assay exactly as will the test article. When a well-characterized product reference standard is unavailable, a representative protein such as BSA can be used as a surrogate standard.

In either case, you must know the exact concentration of the standard to have confidence

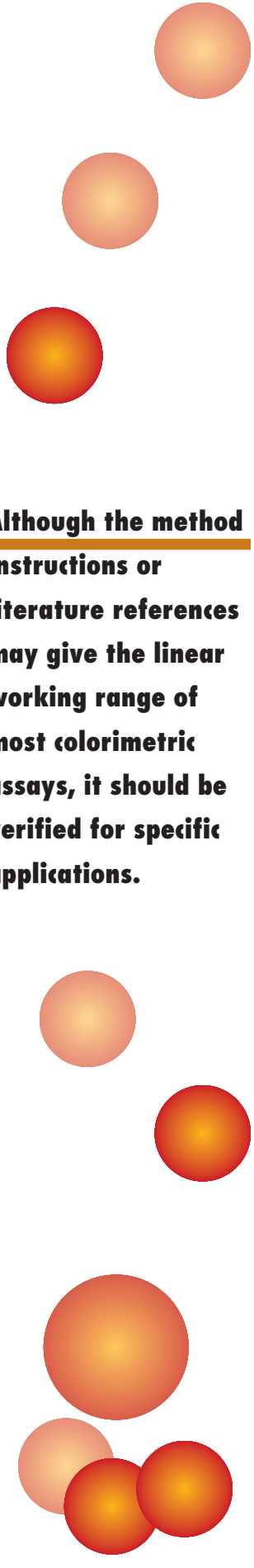
in data from a standard curve. Generally, a stock solution of the standard protein is prepared and its concentration accurately determined (by amino acid analysis or microanalytical total nitrogen, for example).

For long-term use (and when validating a colorimetric assay), it is important to consider the stability of the standards under their intended storage conditions. If a stock solution of the standard shows precipitation or microbial growth upon storage, its protein concentration will no longer be as expected. Assays performed with the solution will not be accurate. To increase confidence in the stock standard solution concentration accuracy over time, perform stability tests and establish appropriate storage conditions.

Buffers and reagents. In addition to stable standard solutions, accurate assay results use the same type of buffer in preparing the standard curve dilutions as in the test sample. If the concentration of buffer components affects a colorimetric assay, adjust the standards to contain the same concentration of buffer constituents across the dilution range of the standard curve. For some colorimetric assays (such as the Lowry method), chromogenic reactions are a function of time. Therefore, the timing of the reaction incubations for individual assay tubes can be critical to obtaining accurate and precise protein concentration results. You may have to coordinate the addition of reagents to ensure that each vial is incubated for the same length of time at the same temperature before measuring its absorbance reading.

The effect of reagents on spectrophotometric cuvettes used for holding assay solutions can affect precision. Reagents such as colloidal gold (Aurodye) or Coomassie blue dye can bind to some cuvettes, causing a drift in absorbance readings from the first to the last sample. Also, the use of glass rather than plastic cuvettes can affect assay results. Procedures for cleaning quartz or glass cuvettes between samples must be adequate to prevent sample carry over. When using disposable plastic cuvettes, manufacturing quality and consistency from lot to lot are critical for obtaining reliable data.

Procedures. Although the method instructions or literature references may give the linear working range of most colorimetric assays, it should



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We describe here six colorimetric protein concentration assays that may be used with biopharmaceutical products. For additional colorimetric assays, refer to Thorne (1). Many of these assays are available as commercial kits containing all assay reagents and, in some cases, surrogate standard proteins. In each section, we compare their general operational ranges (combining their original and microscaled versions).

Check current vendor literature for the latest kits available because improvements are continuously being made to enhance the limit of detection, limit of quantitation, and assay robustness to matrix components. Not all proteins react the same in every assay, so it may be useful to evaluate several methods to determine the appropriate one to use. Regardless of which method is chosen, confirm the actual working ranges when first testing an assay.

Biuret assay. The biuret assay is among the oldest of the colorimetric assays, with elements originating in the early 1900s (2–4). Protein reacts with copper (II) sulfate that, when reduced by the nitrogen atoms in the peptide bonds to Cu^{1+} , undergoes a color shift to 540 nm. The assay is performed at room temperature with 30 minutes of incubation. Its working range of protein detection is 0.5–10 mg/mL. Hence it is unsuitable for highly dilute protein preparations. Buffer components such as Tris (5) and glycerol (6) can interfere with accurate concentration determination in the assay.

Lowry assay. The Lowry assay and its modifications are enhancements of the biuret reaction (7–9). Intensified color results from the reaction of Folin–Ciocalteu reagent: Alkaline copper reacts with a protein (as in the biuret test), then the phosphomolybdate-phosphotungstate salts in the reagent are reduced to produce a maximum absorbance at 750 nm. This

assay's working range is 0.01–1 mg/mL. Color reaction development is time-dependent and light-sensitive and can vary significantly from protein to protein. In addition, numerous buffer components can interfere with the Lowry assay, including salts (10,11), Tris (11), detergents (8,12,13), sucrose (8,13), and EDTA (13).

Bicinchoninic acid assay. The bicinchoninic acid assay (BCA) is also a modification of the biuret reaction (13). This assay uses the reduction of Cu (I) by protein to Cu (II) in an alkaline solution, then bicinchoninic acid detects the cuprous ion to form a colored product absorbing at 562 nm. The working range is 0.001–1 mg/mL. The BCA assay has many advantages over the biuret and Lowry assays: The color reaction is stable for hours, the assay is simple (one step), and it is rugged to many common buffer components (13). However, like the Lowry assay, it exhibits some protein-to-protein variation in reactivity (13). Use an orthogonal concentration method to evaluate the selected standard against the test protein for reactivity.

Bradford dye-binding assay. The Bradford assay (also known as the dye binding or Coomassie blue assay) uses Coomassie brilliant blue G-250 dye, which undergoes a color shift from 465 nm to 595 nm when bound to protein (14–16). The binding reaction is rapid and is stable for up to an hour. This assay's working range is 0.001–0.100 mg/mL, so it can be used with dilute protein solutions. Like the BCA assay, it is a simple, one-step protocol, rugged to most common buffer components (14,15). But as with the Lowry and BCA, protein to protein differences in reactivity can occur (15,16).

Colloidal gold assay. Colloidal gold has a maximum absorbance at 530 nm (17). When mixed with protein, the absorption maximum shifts and can be optimally detected at 595 nm. The reaction involves

chloroauric acid (Aurodye), which is highly sensitive to protein. The working range of this assay is 20–200 ng/mL. The reaction occurs in 30 minutes, and the color is stable for up to 24 hours. This assay is rugged to a wide range of buffer components but is slightly sensitive to pH, performing optimally at pH 3 (17). As with the colorimetric assays above, reactivity differs among proteins, so it is best to analyze standards by a noncolorimetric method to verify their suitability for use with the test protein(s).

O-phthalaldehyde (OPA) assay. When *o*-phthalaldehyde reacts with amines, it generates an adduct that fluoresces on excitation at 340 nm and can be detected at 450 nm (18). The OPA assay procedure is significantly more involved than the other colorimetric assays. It requires extensive sample preparation and a fluorometer rather than a spectrophotometer. However, in some applications where you cannot avoid interfering matrix elements, this may be the most suitable assay.

You may get more accurate results with some protein samples if you first hydrolyze them with NaOH at high temperature. The working range using unhydrolyzed samples is 1–25 $\mu\text{g/mL}$. Hydrolysis can increase sensitivity 10-fold. However, you may introduce significant assay imprecision during hydrolysis and subsequent neutralization and dilution. Because the fluorescent adducts are stable only for up to 15 minutes, this assay requires timed coordination of active reagent additions. It is less affected than the Lowry or Bradford methods by many common buffer components such as detergents, EDTA, thimerosal, cesium chloride, and guanidine hydrochloride. But Tris, sucrose, thiol reagents, and urea do affect OPA assay results, and as with other colorimetric assays, reactivity differences among proteins have been seen (18).

be verified for specific applications — especially if the buffer contains components not shown in a published method. Common practice is to prepare a minimum of five standard points in duplicate or triplicate across a five- to tenfold range of concentration. Using linear regression, determine the best-fit line through the data points. An acceptable linear fit would yield a coefficient of determination (r^2) of 0.98 or greater. The precision among replicates should be within $\pm 5\%$.

If the precision among replicates is acceptable, but the coefficient of determination is less than 0.98, the standard curve points might not reflect the linear range of the assay. Repeat the standard

curve within a narrower concentration range, or shift the range higher or lower. Once you have established the linear working range of an assay, the absorbance readings of test samples should fall within the range of those generated by the standard curve. If they are too high, it may be possible to dilute and re-assay the protein solution. If they are too low, the protein solutions should be quantitatively concentrated and if possible, re-assayed.

UV ABSORBANCE MEASUREMENTS

Determining protein concentration by measuring a solution's absorbance at ultraviolet (UV) wave-

lengths can be rapid and precise. Apart from a blank or placebo solution, no additional assay reagents are needed. Protein concentration is calculated using the extinction coefficient of the protein. Again, concentration accuracy depends on linear regression from a standard curve. Measuring concentration by UV absorbance is hypothetically nondestructive to the sample. Once measurement is completed, the sample can be recovered and reused (although this practice is not recommended in typical biopharmaceutical applications).

UV absorbance methods have certain limitations. They can detect only those proteins that contain aromatic residues (predominantly tyrosine and tryptophan). Because calculation of concentration uses the extinction coefficient of a specific protein, this method is more accurate with solutions of purified proteins than with complex solutions. It is critical that a buffer blank be analyzed in parallel without protein because certain solvents and buffer components exhibit UV absorption. Other biomolecules present in the sample, such as nucleic acids that also absorb in the UV region, can confound the results.

Calculating the results. The following equation can be used to calculate protein concentration from UV absorbance values: concentration (mg/mL) = absorbance/molar extinction coefficient (19). The extinction coefficients of numerous proteins have been published; however, those of most new biopharmaceutical products have not. Therefore it may be necessary to determine it empirically for a given protein if it is highly purified and quantities are sufficient to analyze accurately (20). Highly purified preparations can be estimated by gravimetric (dry weight) analysis if the protein preparation is free of exogenous substances such as salts or detergents (21).

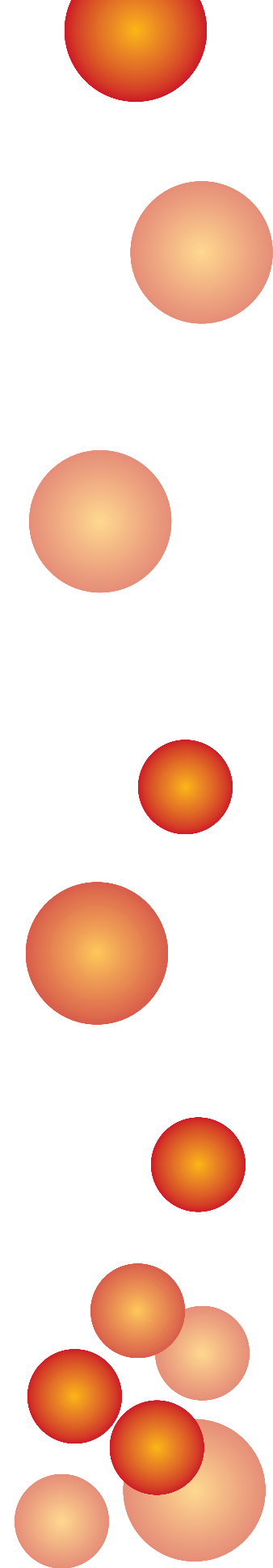
For the greatest accuracy, amino acid analysis can determine a protein's composition and weight (see below). If there is a significant discrepancy between the dry weight and the weight derived from amino acid analysis, your protein preparation is likely to contain additional, nonproteinaceous material. If the amino acid composition of your protein is known, you can estimate the theoretical extinction coefficient based on the number of tyrosine and tryptophan residues present (20) and using a simple formula: extinction coefficient = (number of tyrosines x 1,280) + (number of tryptophans x 5,690)/protein molecular weight. Although it may be useful to estimate this value early in product development, the actual value should be determined experimentally for use in product characterization.

A protein solution's concentration can be calculated from a standard curve measured directly by UV absorbance, similar to the colorimetric methods. Using a reference solution with a known concentration of the same protein to be analyzed, you can generate a standard curve. Measure the absorbances at 280 nm of each standard point and plot those against its known concentration, then draw a best-fit line. Measure the test sample at A_{280} , then evaluate against the standard curve using linear regression. The accuracy of this technique depends on both the reference protein solution and the test sample protein solution having the same absorbance maxima and containing the same quality and quantity of impurities.

When first using the UV absorbance method on any test sample, you should perform spectral scans be performed from 200 to 600 nm on a buffer blank or placebo, and then scan the test solution. If the test solution is too concentrated, it can be diluted in buffer or placebo to read in the linear range of the spectrophotometer (typically 0–2 AU). By comparing the absorbance profiles among blanks and samples, you should discover any potential interfering signals present in the matrix. If you find overlapping absorbance peaks, you may be able to adjust the protein's absorbance values by subtracting the interference values (if they are at low levels). This is particularly important in solutions containing nucleic acids, where the A_{260} value is subtracted from the A_{280} value to correct for the presence of DNA or RNA (22). Use the corrected absorbance value to calculate protein concentration.

AMINO ACID ANALYSIS

Amino acid analysis is one of the most accurate ways to determine protein concentration and a protein's extinction coefficient (23). This technique also yields the amino acid composition of purified protein samples. When analyzing biopharmaceutical products, amino acid composition can be used in conjunction with other techniques (such as protein sequencing) to establish protein identity (24). Determining concentration by amino acid analysis is an important product characterization method and provides an orthogonal technology to colorimetric assays or UV absorbance. Although it requires hydrolysis and HPLC instrumentation and an experienced analyst, it is sensitive enough to be used with very low-level samples (200–500 pmol) including eluates from electrophoretic gels or PVDF blots (25,26,27). We give a brief description of the



method and some of its limitations here. A comprehensive review of amino acid analysis technology by Anders begins on page 32 of this issue. Other detailed reviews of amino acid analysis derivatization chemistries can be found elsewhere (20,28).

Hydrolysis. In amino acid analysis, you hydrolytically destroy the peptide bonds between the individual amino acids in the protein, and then measure the quantity of recovered amino acid residues. Hydrolysis is typically performed with strong acid and high temperature. This procedure adequately liberates most of a protein's amino acids, with certain limitations. Aspartamine and glutamine are converted to aspartic and glutamic acids, respectively; tryptophan, cysteine, serine, and threonine are partially or completely destroyed; peptide bonds between some residues (isoleucine, leucine, and valine) are more resistant to hydrolysis than others; many modified amino acids are not reliably recovered.

By modifying hydrolysis conditions or derivatization chemistry, one or the other of those issues can be addressed, but (to date) no single hydrolysis procedure can quantitatively yield every amino acid simultaneously. In fact, hydrolysis is the greatest source of error in amino acid analysis (29). Compositional accuracy averages 15% error overall; individual error associated with difficult residues can approach 30% with some derivatization chemistries (25). For maximum accuracy, perform multiple hydrolysis time points (24, 48, and 72 hours, for example), and extrapolate the data back to estimate time-zero yields of each amino acid (30). To improve assay accuracy and precision, compounds such as α -amino-*N*-butyric acid, norvaline, or norleucine spiked into samples can be used as quantitative internal standards to correct for potential sample losses during hydrolysis.

Quantitation. After sample hydrolysis, the released amino acid residues are resolved by HPLC and quantitated. To be detected by UV or fluorescence, the residues must be derivatized with a chromagenic agent. That can be done before (precolumn) or after (postcolumn) HPLC. In precolumn analysis, the residues are derivatized, chromatographically separated, then detected; in postcolumn analysis the residues are separated, derivatized, then detected. Currently, the four predominant derivatization chemistries are phenylisothiocyanate (PITC) (31), 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (32) (both precolumn), OPA (33), and ninhydrin (34) (both postcolumn). In general,

precolumn methods offer greater assay sensitivity, but postcolumn methods are more robust (25). The benefits and limitations of different hydrolysis procedures, derivatization reagents, and chromatographic resolution systems are detailed elsewhere (20,28).

Certain buffer components can interfere with the accuracy of amino acid analysis. Samples containing amines (such as Tris or glycine) may yield peaks that coelute with amino acid residues during HPLC, making accurate quantitation difficult. Detergents or high amounts of salts can interfere with the solubilization and recovery of hydrolyzed residues and may negatively affect chromatography. Protein properties can also interfere with analysis. Glycoproteins and lipoproteins may be incompletely hydrolyzed or incompletely solubilized after hydrolysis. Environmental conditions also contribute to contaminating substances: Residue on glassware, impure reagents, even powder from an analyst's gloves can contribute to artifacts during amino acid analysis.

Calculating the results. Protein concentration can be calculated from amino acid results in different ways (35). In each case, determine or estimate the amount of protein present in the tested aliquot. Calculate concentration using the amount of protein per volume of sample, adjusting for all dilution steps used for sample preparation in the method. Most analysts omit cysteine and tryptophan from consideration because of the imprecision associated with those residues (36). The simplest method is single residue normalization, in which the data from one stable amino acid (reliably recovered by hydrolysis) is used to represent all amino acids in the protein (37). Phenylalanine, alanine, and leucine are typically stable residues. This method requires knowledge of the theoretical amino acid composition of the protein. The percentage of a chosen residue contained in the protein is calculated. The actual amount obtained of the chosen residue is multiplied by the known amount of that residue in the protein. The data are then normalized to represent 100% of the protein's mass, yielding an estimate of recovery based on weight percent.

Best-fit analysis is a more accurate estimation method. It requires data from all the amino acids present (except cysteine and tryptophan) (36). Like normalization, this method requires theoretical compositional values for the protein. Calculate an initial average yield by dividing the amount obtained by the known (theoretical) residue amount, then calculate the mean of those data. Compare the amount obtained of each residue to

Nitrogen Assays

Kjeldahl assay. The Kjeldahl assay involves digestion of the protein sample by a combination of acid, catalysts, and high temperature, which converts the nitrogen to ammonium sulfate (39). Then, steam-distill the ammonium sulfate to liberate ammonia gas. Quantitate the ammonia by indirect titration against an acid or direct titration against a base. Use the amount of ammonia to calculate the nitrogen in the sample, and then mathematically convert that to protein mass. To account for potential environmental contaminants, measure the reagent blanks (without protein), and subtract that from the test sample data. Test an ammonium sulfate reference preparation of known concentration as a check solution to ensure accuracy of the assay.

Nessler assay. The Nessler assay is similar to the Kjeldahl assay. Protein samples are digested with acid, but hydrogen peroxide is used to accelerate the oxidation of nitrogen to ammonium sulfate (40). After digestion, add Nessler reagent (mercury and potassium iodide in sodium hydroxide) to the reaction to produce a colored complex with ammonia. Use an ammonium sulfate standard reference solution to quantitate the yield of ammonia, then evaluate the test sample data against that. Calculate total nitrogen from the ammonia recovered, which indicates the mass of protein present in the samples. As above, use a reagent blank as an environmental control for ammonia.

the initial average yield value and discard any residue deviating more than 15%. Use the remaining residues to recalculate a mean value, which is considered the best-fit (most accurate) data indicating the protein amount recovered.

If the theoretical composition for a protein is unknown, you can use relative weight percent to calculate concentration (20). First, multiply the obtained amount of each residue by the molecular weight of that residue. Then add individual residue weights to yield total residue weight, which indicates the mass of protein recovered. You can also use this method to estimate amino acid composition if the molecular weight of the protein is known. Divide the mass of protein recovered by the molecular weight to yield the moles of protein obtained. Then divide the amount of each amino acid residue by the moles of protein for the number of amino acids in the protein.

NITROGEN DETERMINATION

Total nitrogen determination is another protein concentration measurement technique. Two similar methods are used: the Kjeldahl and the Nessler assays (see box above). Both exploit the observation that nearly all proteins contain approximately 16% nitrogen (38). Although these methods can be quite accurate, they are not as sensitive as the techniques described earlier, and relatively large (mg) amounts of sample are required for analysis.

Both assays are particularly useful with crude mixtures of proteins. Because these analyses are based on total nitrogen content, they do not demonstrate the protein-to-protein variations of

colorimetric methods. However, you will get inaccurate results using samples that contain non-proteinaceous sources of nitrogen or ammonia unless you can remove the exogenous material before analysis. Incomplete digestions can result in imprecision, so you may need to optimize digestion conditions for individual applications. Also, precision can be affected by the loss of material to the sides of tubes and flasks. Take care to completely recover liquid droplets adhering to glass vessels, especially after digestion. The Kjeldahl method requires specific apparatus for the quantitative distillation of ammonium sulfate. The Nessler reactions do not require a distillation step.

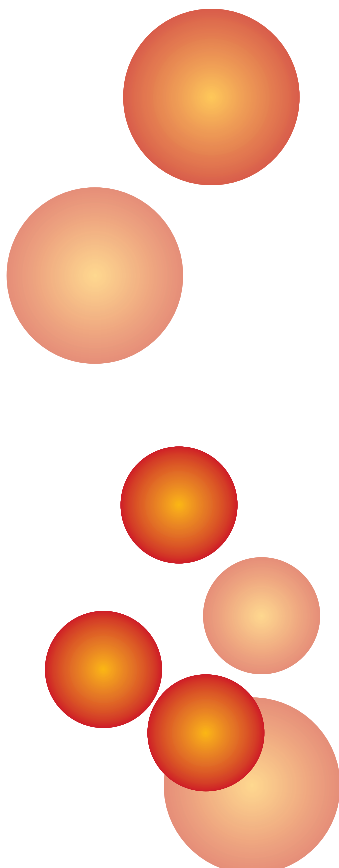
APPLICATIONS AND VALIDATION

Early in the product development cycle, simple and rapid methods for estimating protein concentration can be used to establish purification process parameters. As product development progresses, more sophisticated concentration methods may be employed for protein characterization. When used in release and stability programs, protein concentration methods should be selected that are as accurate and precise, simple and robust as possible. These features will assure the method can be validated for its intended use, and can be conducted successfully by trained QC analysts. Part 2 of this article will examine strategies for performing validation of the methods discussed here.

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