DIONEX

Application Note 163

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Determination of Protein Concentrations Using *AAA-Direct*™

INTRODUCTION

Dye-binding assays such as bicinchoninic acid (BCA), Lowry, and Bradford (Coomassie) are the most common methods to determine total protein. The BCA assay depends on Cu (II) reduction to Cu (I) by cysteine, cystine, tyrosine, and tryptophan in alkaline conditions and the subsequent formation of bicinchoninic acid-copper (I) complexes that enable visual absorbance at 562 nm.[1,2] The Lowry assay depends on the reaction of copper sulfate and tartrate under alkaline conditions with the polypeptide backbone amino acids to form tetradentate copper-protein complexes, that then reduce Folin-Ciocalteu Reagent and produce a visible absorbance at 750 nm.[3,4] Amino acids with oxidizable side chains (tyrosine, tryptophan, cysteine) increase color yield, whereas amino acids that destabilize complex formation (i.e., proline and hydroxyproline) decrease color. The Bradford assay involves binding of the Coomassie dye under acidic conditions to protein amino acids having basic side chains (i.e., lysine, arginine, ornithine, and histidine) and the NH_2^- terminal amino group, producing a shift in visible absorbance from 465 to 595 nm.[5–7] The hydrophobicity of the amino acids adjoining the positively charged amino acids, and the length of the polypeptide, affect dye binding.

These assays require simple and inexpensive instrumentation, and can be performed in less than 3 h. For each of these assays, the response is dependent on the protein being measured. Protein measure in each assay is dependent on (1) accessibility of dye reagents to specific reactive sites on the protein, (2) similarity in amino acid composition to reference proteins (e.g., bovine serum albumin) used for calibration, and (3) lack of interferences in the sample matrix. For example, highly glycosylated, lipidated, or specifically engineered proteins with conjugated tethers (e.g., polyethylene glycol) may hinder access of dyes through steric hindrance. Proteins with an exceptionally low composition of dye-binding amino acids (e.g., collagen) produce lower color development and thus underestimate the protein content when calibrated against a dissimilar protein (e.g., BSA).

Under conditions where the accuracy of protein determination is adversely affected, or where additional information concerning the identity or quality of a protein is needed, amino acid analysis (AAA) can be used.[8] AAA measures the concentration of each free amino acid after the protein is hydrolyzed in acid, base, or other reagents. The hydrolysis procedure is important and must be properly executed to protect sensitive amino acids that can be destroyed during hydrolysis. After the hydrolysis, the hydrolysis reagents are removed, typically by evaporation, and then the hydrolysate is reconstituted in water (or another LC-compatible solvent) and analyzed by chromatography.

Because most free amino acids are poor chromophores, they cannot be directly detected at low concentrations using ultraviolet (UV), visible (Vis), or fluorescence detection, and therefore the hydrolysate must first be derivatized with a chromophoric or fluorometric reagent before detection. This derivatization can be performed either before chromatography (precolumn derivatization) or after chromatography (postcolumn derivatization). Both pre- and postcolumn derivatization methods are costly for both reagents and labor, add risk of toxic chemical exposure to personnel, and add concerns for hazardous waste removal.

AAA-Direct is a highly sensitive and direct detection type of AAA that does not require sample derivatization. *AAA-Direct* uses integrated pulsed amperometry (IPAD), a type of electrochemical detection, to directly detect amino acids. *AAA-Direct* also provides complete separation of all common amino acids using anion-exchange chromatography (AminoPac® PA10). Because *AAA-Direct* is a direct detection method, the hydrolyzed protein sample can simply be reconstituted in water and directly injected for analysis.

AAA-Direct has many advantages over derivatization methods for AAA. The derivatized amino acid products may not be stable, depending on the derivatization method, and the reagents can cause interferences during chromatography. Lengthy sample preparation and derivatization steps are often needed. Derivatization is not always suitable for methylated, halogenated, phosphorylated, or sulfonated amino acids. Di-substitutions can occur for tyrosine and histidine (using Fmoc chloride). Some derivatization methods (e.g., OPA) cannot directly analyze for secondary amino acids. For some derivatization methods, all hydrolysis reagents must be evaporated completely prior to addition of derivatization reagents. Many derivatization reagents are not stable, decompose, and have short shelf lives, adding uncertainty to the derivatization efficiency. Changes in response for the amino acids may occur after derivatization; amino acid response can change, increase, or decrease with sample storage. For *AAA-Direct*, the absence of toxic derivatization reagents eliminates safety hazards associated with exposure to toxic chemicals. The *AAA-Direct* eluents, sodium hydoxide and sodium acetate, only require a simple neutralization of the waste stream with hydrochloric acid (HCl) to produce harmless and ecologically safe waste products (sodium chloride and sodium acetate), reducing disposal costs.

In this application note, we describe a method to determine total protein using *AAA-Direct*. We used this method to participate in an Association of Biomolecular Resource Facilities (ABRF) Amino Acid Analysis Research Group collaborative study that compared total protein determinations using AAA to dye-binding assays.[9] We found that concentrations of all five protein solutions determined by *AAA-Direct* were equivalent to the determinations made by participants that used either pre- or postcolumn derivatization methods.

EXPERIMENTAL

Equipment

Dionex BioLC® Chromatography System configured for *AAA-Direct*, consisting of:

- GP50 Gradient Pump, microbore, PEEK, with degas option
- ED50 Electrochemical Detector with *AAA-Certified*™ disposable gold working electrode and combination pH/Ag/AgCl reference electrode in the pH Mode.
- AS50 Autosampler and Thermal Compartment, with 25-µL injection loop (0.010-in. i.d.).
- EO1 Eluent Organizer, including three 2-L plastic bottles and pressure regulator.

Chromeleon® Chromatography Workstation

Reacti-Therm™ III Heating Module with Reacti-Block™ H (Pierce Chemical Co., P/N 18940ZZ)

Vacuum Hydrolysis Tubes (8 × 60 mm, 1 mL, Pierce Chemical Co, P/N 29550ZZ; or equivalent)

Microcentrifuge tubes with detachable caps (plastic, 1.5 mL, Sarstedt, P/N 72.692.005; or equivalent)

SpeedVac® Evaporator system (Thermo Electron) consisting of:

SpeedVac model SVC100

- Refrigerator Vapor Trap model RVT400
- Vacuum Gauge model VG-5
- Welch Duo-Seal Vacuum Pump model 1402 capable of pulling 0.2 Torr (200 µm Hg) vacuum

Pasteur Pipettes, borosilicate glass (VWR Scientific, P/N 14673-043; or equivalent).

Nitrogen (4.8 Grade, 99.998%, <0.5 ppm oxygen)

Three-Way Stopcock Valve (VWR Scientific, P/N 59097-058)

Vacuum Tubing, 1/4 × 5/8 in. (VWR Scientific, P/N 63012-140)

Vacuum Pump (Gast Manufacturing Corp., P/N DOA-P104-AA; or equivalent)

Helium (4.5 Grade, high purity 99.5%)

Filter Unit, 0.2 µm, nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 164-0020; or equivalent)

Vial, 0.3 mL, polypropylene, microinjection, 12-32 mm screw thread with preslit Teflon®/silicone septum and polypropylene screw thread cap (Dionex P/N 055428)

REAGENTS AND STANDARDS

Reagents

Deionized water, 18 MΩ-cm resistance or higher

Sodium acetate, anhydrous *(AAA-Direct* Certified, Dionex Corp., P/N 059326)

Sodium hydroxide, 50%, low carbonate grade (w/w; Fisher Scientific, P/N SS254-500; or equivalent)

Hydrochloric Acid, Concentrated (12.23 M; Class 10, General Chemical Co., P/N 108-9651 or equivalent)

RBS-35 Detergent (Pierce Chemical Co., P/N 27950)

Standards

Amino Acids in 0.1 M Hydrochloric Acid; Standard Reference Material 2389, (National Institute of Standards & Technology)

Hydroxylysine (δ-; hydrochloride, mixed DL and DL-allo; Sigma-Aldrich Chemical Co., P/N H-0377)

Hydroxy-L-proline (hydroxyproline; Grade A, Calbiochem, P/N 3980)

Tryptophan (Trp; Sigma-Aldrich Chemical Co., P/N T-1029)

SAMPLES

Five ~2.5 mg/mL protein solutions in water were prepared by Alterman, M., et al., and provided to ABRF 2003 collaborators as unknown samples #1–5.[9] After the study was completed, their identities were revealed:

- Aprotinin (from bovine lung; Sigma-Aldrich Chemical Co.);
- Lactoglobulin (β-; from bovine lung; Sigma-Aldrich Chemical Co.)
- Fetuin (from fetal calf serum; Sigma-Aldrich Chemical Co.)
- Hemoglobin (Sigma-Aldrich Chemical Co.)
- Ubiquitin (from bovine red blood cells; Sigma-Aldrich Chemical Co.)

Bovine serum albumin (BSA) standard was also provided for calibration. BSA was in 14 mM NaCl, 71.57 ± 0.74 mg/mL; Standard Reference Material 927c (National Institute of Standards & Technology).

CONDITIONS

Columns:	AminoPac PA10 Analytical (2 × 250 mm, P/N 55406) with AminoPac PA10 Guard (2 × 50 mm, P/N 55407).
Flow Rate:	0.25 mL/min
Injection Volume:	10 µL
Temperature:	30 °C
Eluents:	A: Water B: 250 mM sodium hydroxide C: 1.0 M sodium acetate

Programmed Method:

Time (min)	*%A*	*%B*	*%C*	*Curve*	*Comments*
Init.	76	24	0	-	Autosampler fills the sample loop
0.0	76	24	0	-	Valve from load to inject
2.0	76	24	0	1	Begin hydroxide gradient
8.0	64	36	0	8	
11.0	64	36	0	8	Begin acetate gradient
18.0	40	20	40	8	
21.0	44	16	40	5	
23.0	14	16	70	8	
42.0	14	16	70	8	
42.1	20	80	0	5	Column wash with hydroxide
44.1	20	80	0	5	
44.2	76	24	0	5	Equilibrate to starting condition
75.0	76	24	0	5	End of run

Typical System Operating Backpressure:	2300–3000 psi
On-Line Degas:	30 s every 4 min

Waveform for the ED50:

Time (s)	*Potential (volts vs pH) (begin/end)*	*Integration*
0.00	+0.13	
0.04	+0.13	
0.05	+0.28	
0.11	+0.28	Begin
0.12	+0.55	
0.41	+0.55	
0.42	+0.28	
0.56	+0.28	End
0.57	–1.67	
0.58	–1.67	
0.59	+0.93	
0.60	+0.13	

* For the most current recommended waveform and gradient program, please consult the *Installation Instructions and Troubleshooting Guide for the* AAA-Direct *Amino Acid Analysis System*.[10]

PREPARATION OF SOLUTIONS AND REAGENTS

Water

The qualification of water for eluent use is less rigorous than for sample dilution. The presence of trace protein impurities becomes significant after hydrolysis because the free amino acids that are released appear as background peaks that compromise trace-level analysis. Without hydrolysis, the amino acids are not apparent. To qualify water for use in sample dilutions, hydrolyze an aliquot as you would a protein, and measure peaks having retention times of amino acids.

Filters are often manufactured using glycerol or other electrochemically active surfactants that appear as either high background levels when used as eluent or large interfering peaks when used as sample diluent. Any water in which filtration is used as part of its purification should be qualified. Water used for this application note was 18 MΩ-cm resistance or higher, and was purified by filters manufactured without electrochemically active surfactants. Water used for eluents was filter degassed (0.2 µm, nylon) under vacuum. Only nylon filters should be used.

250 mM Sodium Hydroxide

To prepare 2 L of eluent, combine 26 mL of 50% (w/w) low carbonate sodium hydroxide with 1974 mL purified filter-degassed water. Place this solution immediately under helium at 4–5 psi to reduce an accumulation of carbonate that can reduce retention times.[10]

1.0 M Sodium Acetate

To prepare 1 L of eluent, dissolve 82 g of *AAA-Direct* Certified anhydrous sodium acetate in ~800 mL purified water. Adjust the total volume to 1000 mL with additional water. Filter the solution through a 0.2-µm nylon filter unit to remove particulates and microbial contaminants, and to partially degas. Then place it under 4–5 psi helium or nitrogen to reduce microbial growth. The use of sodium acetate not certified for *AAA-Direct* use can result in contamination of the gold working electrode, resulting in loss of detector response.[10]

PROCEDURE

The procedure used for determination of total protein concentration consists of seven steps: (1) qualification of labware and reagents, (2) preparation of amino acid standards, (3) estimation of protein amount needed for hydrolysis, (4) preparation of protein solutions for hydrolysis, (5) hydrolysis of proteins, (6) quantification of amino acids in the hydrolysates, and (7) calculation of protein concentration. These steps are detailed below. Steps 1 and 2 may not be required for routine studies where reagents and labware have already been qualified and amino acid standards available. With experience testing similar protein solutions, the estimation of protein concentration needed for hydrolysis (Step 3) is not required.

(1) Qualification of Labware and Reagents Used for *AAA-Direct* Analysis

Labware and reagents used in AAA may have contaminating proteins, amino acids, or other compounds that, after hydrolysis, produce peaks that coelute with amino acids. The presence of these peaks in large amounts affects the accuracy of quantification, and should be reduced to the lowest practical levels.

Note: Technical Note 50 provides complementary information concerning factors important for optimizing AAA-Direct.[11]

Labware

To reduce carryover from previous samples and obtain low limits of detection for the method, clean the vacuum hydrolysis tubes with RBS-35 (Pierce Chemical Co.) according to the manufacturer's directions. Tubes should be rinsed at least three times with water before final use. Pipe cleaners (obtained from art supply stores) are useful for cleaning the bottom of hydrolysis tubes using the RBS-35. Problematic glassware may also be decontaminated with high temperature in a furnace heated to 450 °C for 2–3 h (without plastic caps and seals) to combust all organic compounds. Hydrolysis tubes are qualified by adding 150 µL of qualified 6 N HCl (see below) to the clean tube and performing the hydrolysis procedure followed by *AAA-Direct* analysis. Interfering peaks (coeluting with amino acid standards) are quantified based on calibration curves, and their concentrations should be less than about 1 µM in the reconstituted blank hydrolysate. Note that higher concentrations in the blanks dictate the need of more protein for hydrolysis. Pasteur pipettes used to transfer samples to and from the vacuum hydrolysis tubes, and microcentrifuge tubes used for evaporation, should be prerinsed with three volumes of purified water before use.

Reagents

Any new reagents, or reagents with suspected contamination, are qualified for AAA by using them in the hydrolysis procedure without protein, and measuring the background peaks obtained from the treatment-blank hydrolysate. Hydrolysis tubes previously unused for protein analysis should always be used for reagent screening. Varying levels of interferences are common for different manufacturers and lots of HCl. The hydrolysis of 150 µL 6N HCl in a clean hydrolysis tube followed by *AAA-Direct* analysis qualifies this reagent. To qualify water, evaporate 300 µL in a clean hydrolysis tube and hydrolyze with HCl as described above. Qualified reagents should be dedicated to AAA use, and qualified water may be stored frozen for future use. The potential for interference by sample excipients (e.g., Tris buffer), or excessive salt (>50 mM), should also be considered. Technical Note 55 provides extensive guidance on methods capable of screening sample ingredients for *AAA-Direct* suitability.[12]

(2) Preparation of Amino Acid Standards

Amino acid standard solutions are analyzed without hydrolysis by direct injection (10 µL), and their peak areas used for calibration and the quantification of amino acids in hydrolysates. The amino acid standard mix, obtained from the National Institute of Standards and Technology (NIST), consists of 17 amino acids (but not tryptophan (Trp), hydroxylysine, or hydroxyproline) at concentrations ranging from 1.2 to 2.9 mM (assume 2.5 mM for discussions below). Each amino acid concentration is defined on the Certificate of Analysis for this Standard Reference Material (see Table 1, line 1). We diluted this NIST amino acid standard mix with water to concentrations of 2.5, 10, and 25 µM. Trp, hydroxyproline, and hydroxylysine were weighed and reconstituted in purified water to a concentration of 5.11 mg/mL (25 mM), 1.31 mg/mL (10 mM), 19.9 mg/mL (100 mM), respectively. Trp, hydroxyproline, and hydroxylysine stock solutions were then diluted in water and combined at 2.5, 10, and 25 µM concentrations. It is unnecessary to use Trp, hydroxyproline, hydroxylysine, or any other uncommon amino acid standard when experience shows they are not expected to be present in the protein hydrolysate. All amino acid solutions were stored frozen until needed for use as calibration standards.

(3) Estimation of Protein Amount Needed for Hydrolysis

The estimated protein concentration for each hydrolysis condition is based on (1) a rough estimate of the protein concentration (e.g., dye-binding assay results or mass of solid dissolved), (2) the estimated molecular weight of the protein, and (3) the *AAA-Direct* results for prequalified reagents, labware, and other sample/assay components (see "Qualification of Labware and Reagents for Use in *AAA-Direct* Analysis"). Ideally, the protein concentration used for hydrolysis and subsequent protein determinations should be minimally based on the highest concentration (µM) of amino acid measured in the water hydrolysis blanks, times 10. For example, if the most abundant background peak coeluting with any amino acid was calculated to be 1 µM glycine, then the target minimum concentration of any single residue of amino acid in the protein hydrolysate should be 10 times greater, or 10 µM. This increase enables a better than 90% measurement accuracy for amino acids in the protein hydrolysate without subtracting background concentrations of amino acids. With background subtraction, accuracy is improved.

After the 10× blank concentration has been determined, then the concentration of protein solution required for hydrolysis is calculated. The equation below enables the analyst to vary the volume of protein solution to hydrolyze and the volume of hydrolysate reconstituted. The volume of protein solution to hydrolyze must be consistent with the volume of water (or other diluent) used to calculate the highest concentration (μM) of amino acid measured in the water hydrolysis blanks (see "Qualification of Labware and Reagents for Use in *AAA-Direct* Analysis"). The volume of hydrolysate reconstituted defines the number of injection replicates and injection volumes possible. The AS50 Autosampler may be used in three different modes of injection: full-loop, partial-loop, and partial-loop limited sample. The injection mode affects the volume of sample used, and the user's manual for the autosampler should be consulted to determine the number of injections possible for a given volume of reconstituted hydrolysate. The following equation can be used to calculate the concentration of protein solution needed for hydrolysis:

Protein Concentration (in μg/mL) = [(A × B × C)/1000]/V

A = target minimum concentration (in μM)

B = volume used to reconstitute evaporated hydrolysate (in mL)

C = molecular weight of the protein (daltons)

V = volume of protein solution evaporated (in mL)

For example, when a solution of a 50,000 dalton protein is used for protein determinations, a minimum target concentration of 10 μM is calculated from the blanks, and you wish to hydrolyze in 0.300 mL of that solution and reconstitute the dried hydrolysate in 0.300 mL of water, then 0.5 mg/mL protein would need to be hydrolyzed to ensure that a single amino acid residue present in the protein can be measured at 10 times more than the worse noise peak found in the water blank. Because proteins usually contain multiple residues of each amino acid, the measured concentration of these amino acids will be proportionally higher. In certain cases, some amino acids may be present at concentrations too high to quantify, and the sample will require dilution. When protein hydrolysates need to be diluted, the water blank hydrolysates used for subtraction require equivalent dilutions. The volume of protein solution (V) is also used in the equation for the option to directly add the protein solution to the HCl for hydrolysis without prior evaporation. The volume of the tube used for evaporation and the operational range of the pipettes used for transfer determine the practical limits for the volumes used in the equation above.

In this application note, the protein solutions were estimated to be ~2.5 mg/mL, (based on information provided by the supplier of the solutions who weighed out the mass of protein and dissolved in water) and the MW was assumed to be about 50,000 daltons. The estimated concentrations needed for all known and unknown protein samples were ~0.5 mg/mL, and the 300-μL aliquot of each undiluted hydrolysate allowed approximately 26 injections (10 μL, *partial limited-sample mode*).

(4) Preparation of Protein Solutions for Hydrolysis

The liquid protein samples were gravimetrically diluted to ~0.5 mg/mL with water (300 μL contains 150 μg protein), and the exact dilution was recorded for each sample.

Note: The use of accurate dilution factors helps improve the accuracy of the calculated protein content.

(5) Protein Hydrolysis Using 6 N HCl

In brief, HCl hydrolysis of protein solutions starts with the evaporation of the protein solution to dryness, followed by reconstitution in 6 N HCl and heat treatment (110–115 °C) for a period of time (16–17 h), evaporation to dryness, and reconstitution in water. In this application note, a 300-μL aliquot of each ~0.5 mg/mL protein solution was transferred to a clean 1.5-mL microcentrifuge tube. In addition, a 300-μL aliquot of purified water was also transferred to a clean microcentrifuge tube as a blank for subtracting background peaks. All tubes were dried using the SpeedVac Evaporator for 1–2 h. Samples (including blanks) were then reconstituted in 150 μL of 6 N HCl and the contents transferred to clean hydrolysis tubes using prewashed glass Pasteur pipettes. Hydrolysis tubes were evacuated using a vacuum (~25 in. Hg), and the headspace replaced with argon (low oxygen grade) by means of a three-way stopcock valve. The vacuum/argon cycle was repeated three times, and the

tubes were sealed with an argon headspace. Samples and blanks were hydrolyzed for 16 h in a 115 °C heating block, cooled to ambient temperature, and transferred to clean 1.5-mL microcentrifuge tubes. The HCl was evaporated to dryness using the SpeedVac Evaporator for approximately 2 h. Samples and blanks were reconstituted in 300 µL water. An aliquot of each sample and blank was also diluted 10-fold, accurately recording the dilution factor. Diluted and undiluted samples and blanks were either directly analyzed by *AAA-Direct* (10-µL injection), or stored frozen for later analysis. Amino acid standards used for calibration are not hydrolyzed.

Although not performed in this application note, the initial evaporation step of the protein may be eliminated by directly adding the protein solution to a slightly more concentrated HCl, so that after addition, the concentration is 6 N. Any variation in sample preparation requires equivalent treatment of the hydrolysis blanks. The use of an inert gas (e.g., argon or nitrogen) during hydrolysis helps to preserve sensitive amino acids. Hydrolysis time and temperature may be optimized to reflect the balance between underhydrolyzing, where incomplete cleavage of all peptide bonds leads to peptide fragments, and overhydrolyzing, which leads to chemical degradation of sensitive amino acids and lower amino acid recovery.

(6) Quantification of Amino Acids

For each nonhydrolyzed amino acid standard analyzed, the peak area of each amino acid minus the mean corresponding peak area of any peak eluting at the same retention time of the nonhydrolyzed water blanks is calculated. Linear regression is applied to this adjusted peak area (minus blank peak area), correlated with the corresponding molar concentration of the three amino acid standard concentrations injected. Slopes were based on three-point calibration, and the calibration was not forced through zero. A separate slope was determined for each amino acid, in units of peak area per µM. These slopes were used to calculate the concentrations of each amino acid in the hydrolyzed samples and hydrolyzed blanks. The peak area of each identified amino acid in the hydrolyzed protein samples and hydrolyzed water blanks, divided by the respective slope, equals the µM concentration of that amino acid in the solution injected. The concentration of each amino acid in the protein hydrolysate, minus the corresponding concentration in the hydrolyzed water blank, equals the final measured amino acid concentration for the diluted protein solution. This measured concentration of each amino acid in the protein hydrolysate solution injected for analysis is corrected for all dilution factors to calculate their starting concentrations. Each amino acid molar concentration is then converted to its mass concentration (mg/mL) by multiplying the µM (nmole/mL) concentration by the MW of its respective amino acid times 10^{-6}.

> Example: If the concentration of glycine (MW = 75.07) in the original 70 mg/mL BSA solution was determined to be 16537 µM (nmole/mL), then the mass concentration of this amino acid is $75.07 \times 16537 \times 10^{-6} = 1.24$ mg/mL.

(7) Determination of Protein Concentration.

The sum of all mg/mL concentrations for all amino acids is equal to the total mg/mL protein concentration. The ratio of this measured total protein concentration to the expected amount based on the known protein concentration is the percent recovery. A custom report template can be set up in the Chromeleon Workstation to automate all calculations and create a final summary report.

RESULTS AND DISCUSSION

This section provides a step-by-step example of the quantification of amino acids and determination of protein concentration.

Separations

Figure 1 presents two typical chromatograms of amino acid standards at the upper-range concentration (25 µM) used for peak identification and calibration. Panel A shows the 17 common amino acids (NIST SRM 2389). Panel B shows three uncommon amino acids, hydroxylysine (peak 2), hydroxyproline (peak 8), and Trp (peak 22). The uncommon amino acids selected for additional study in this note may also be resolved from the common amino acids, but were analyzed separately for convenience of handling the standard solutions.

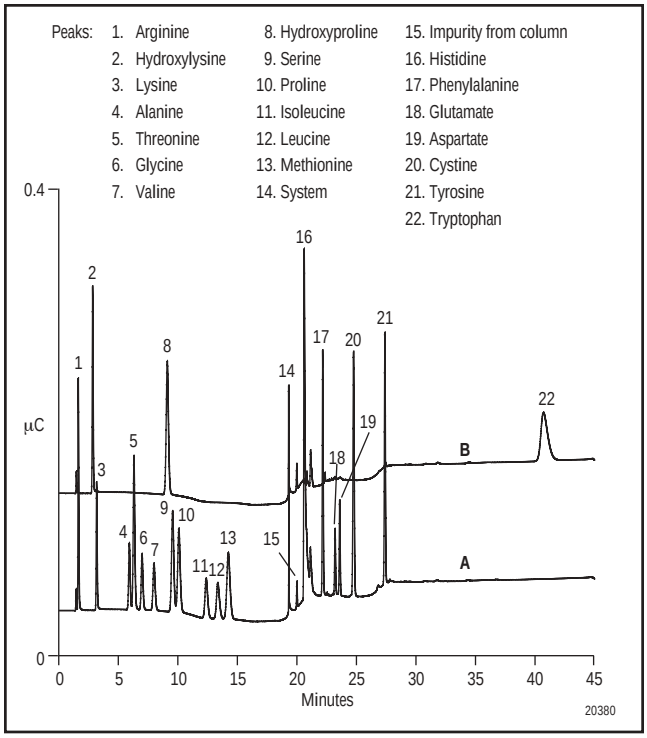

***Figure 1**. Separation of amino acid standards (25 μM, 10 μL injection) using* AAA-Direct. *(A) 17 common amino acids (NIST SRM 2389), and (B) hydrolysine, hydroxyproline, and tryptophan.*

Figure 2 shows the chromatogram of a typical water blank hydrolysate. The presence of significant peaks that coelute with amino acids is typical for reagents and labware contaminated with proteins, amino acids, or other electrochemically active compounds. A major objective for any laboratory engaged in AAA is to reduce these peaks to manageable concentrations, whatever the AAA technique. The larger the coeluting peaks in the blanks, the higher the protein concentration needed for hydrolysis to obtain an accurate measurement. In this study, the hydrolysates from the 300 μL of evaporated water contained additional peaks not found in either the HCl reagent blank hydrolysate (without water) or blank injection of water (without hydrolysis). This finding indicated that the water contained a trace amount of some impurities that were released upon acid hydrolysis. Furthermore, peaks found in the HCl reagent blank hydrolysate indicate either some impurities exist in the HCl reagent used for hydrolyzing samples, the hydrolysis tube was inadequately clean, or both. This undiluted water blank hydrolysate in Figure 2 contained minor peaks coeluting with all amino acids, except methionine and Trp.

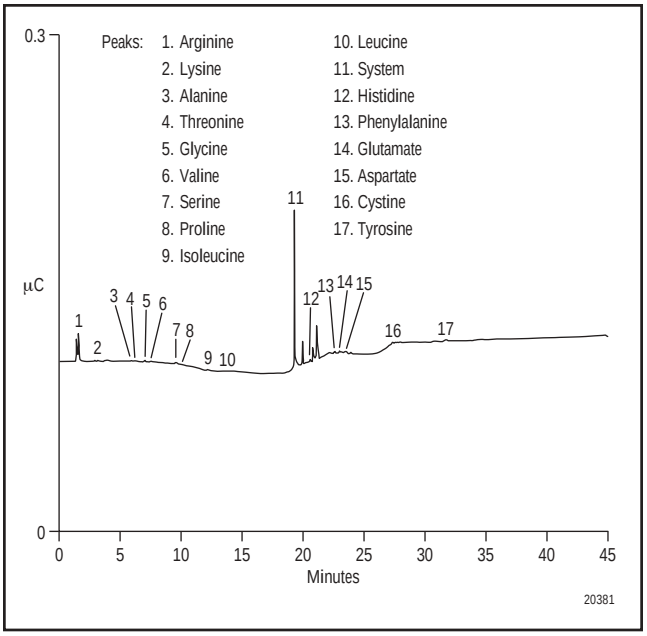

***Figure 2**. Typical water hydrolysis blank (undiluted) using* AAA-Direct *for which background peak area is subtracted from protein hydrolysate peak area for the accurate quantification of amino acid concentrations.*

Figure 3 presents the separation of amino acids released from the hydrolysis of BSA, aprotinin, β-lactoglobulin, fetuin, hemoglobin, and ubiquitin. All amino acid peaks were well resolved. Trp was poorly recovered using these hydrolysis conditions and therefore was not detected in these samples.

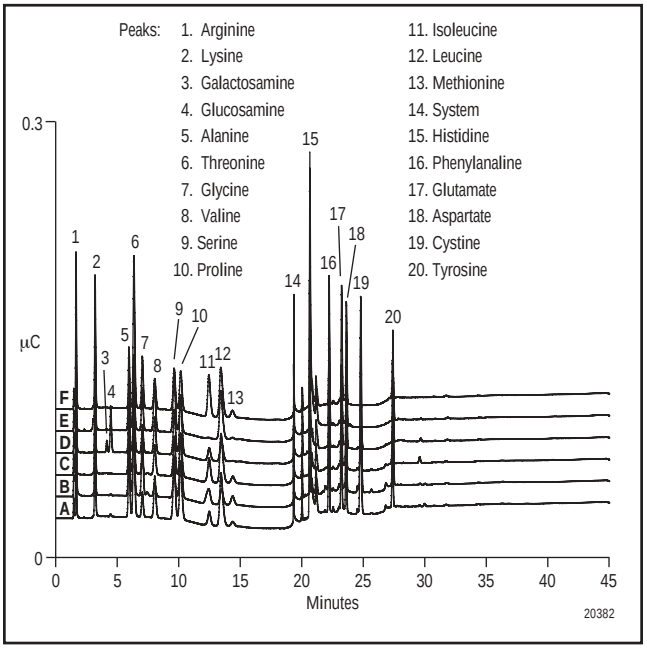

***Figure 3**. Separation of amino acids in undiluted HCl hydrolysates of 10-fold diluted protein solutions using* AAA-Direct *(10 μL injections). (A) BSA, (B) aprotinin, (C) β-lactoglobulin,(D) fetuin, (E) hemoglobin, (F) ubiquitin.*

Determination of Amino Acid and Total Protein Concentration of BSA

Table 1 describes the procedure for calculating total protein concentration. For simplification, the table was reduced to a single representative amino acid, lysine in BSA. However, the calculation procedure for all remaining amino acids (not presented) is identical. In this study, using only amino acid concentrations greater than 1 µM, but less than 25 µM, were used for reporting concentration from the undiluted BSA hydrolysate data. If the peak area for the undiluted BSA hydrolysate exceeded 25 µM (250 pmol per injection) level, then the measured amino acid concentrations from the 10-fold diluted sample was used. In this example, lysine peak area for the undiluted hydrolysate exceeded the level observed for the standard at the 25 µM level, and was therefore ignored (specified in Table 1 as "Out of Calibration"). Only the lysine concentration for the 10-fold dilution was used. Proper selection of data ensures that the best hydrolysate data is used between the two dilutions. Most useful data for the other amino acids were obtained from the 10-fold dilution data set for BSA. The best data between the undiluted and 10-fold diluted was selected (Table 1, line 29). The µM concentration of each amino acid was then converted to their mg/mL concentration (Table 1, line 31) using each respective amino acid molecular weight (Table 1, line 30).

Calculation of the Total Protein Concentration

Table 1 presents the calculations needed to determine all the amino acid concentrations of BSA (with only lysine presented). Slope is calculated from the corrected peak area of three concentrations of amino acid standards (e.g., lysine). Using the slope, the concentration of each amino acid is determined from the peak area of the measured BSA hydrolysate and the peak area of the water blank hydrolysate. The summation of all mg/mL amino acid concentrations is the total protein concentration of the starting undiluted protein solutions (see Table 2). Trp, cysteine, and cystine are normally recovered in low nonquantifiable yields using 6 N HCl (110 °C), and are omitted from the summation. The measured protein concentration for the BSA solution was 66.8 mg/mL.

Table 1. Calculation of Total Protein Concentration

Line No.	Sample/Condition	Lysine
1	Original NIST conc. (mM)	2.47
µM CONCENTRATIONS OF THE CALIBRATION STANDARDS AFTER DILUTION		
2	Water blank	0.00
3	Low level	2.47
4	Medium level	10.25
5	High level	24.70
AVERAGE PEAK AREA OF CALIBRATION STANDARDS AND WATER BLANK (nC*min)		
6	Water blank	0.01
7	Low level	1.50
8	Medium level	5.66
9	High level	11.58
AVERAGE PEAK AREA OF CALIBRATION STANDARDS AFTER WATER BLANK SUBTRACTION (nC*min)		
10	Low level	1.49
11	Medium level	5.65
12	High level	11.57
13	Slope (Area/µM)	0.448
14	R^2	0.9948
PEAK AREA OF HYDROLYSATES (nC*min):		
15	Water blank; undiluted hydrolysate	0.17
16	BSA; undiluted hydrolysate	65.00
17	Water blank; 10-fold diluted hydrolysate	0.02
18	BSA; 10-fold diluted hydrolysate	16.42
µM MEASURED CONCENTRATION IN HYDROLYSATES		
19	Water blank; undiluted hydrolysate	0.37
20	BSA; undiluted hydrolysate	Out of Calibration
21	Water blank; 10-fold diluted hydrolysate	0.05
22	BSA; 10-fold diluted hydrolysate	36.7
µM MEASURED CONCENTRATION IN HYDROLYSATES AFTER SUBTRACTION OF WATER BLANK		
23	BSA; undiluted hydrolysate	Out of Calibration
24	BSA; 10-fold diluted hydrolysate	36.6
DILUTION FACTORS FOR PROTEIN SAMPLES		
25	BSA; undiluted hydrolysate	145.7-fold
26	BSA; 10-fold diluted hydrolysate	1501-fold
µM CONCENTRATIONS IN ORIGINAL PROTEIN SAMPLE (Corrected for all dilutions)		
27	BSA; undiluted hydrolysate	Out of Calibration
28	BSA; 10-fold diluted hydrolysate	54927
SELECTING BEST SAMPLE DILUTION DATA (or averaging data)		
29	BSA; 10-fold diluted hydrolysate	54927
CONVERTING TO mg/mL CONCENTRATIONS		
30	Amino acid molecular weight	146.19
31	BSA	8.03

Determination of Amino Acid and Total Protein Concentration of the Test Proteins

The concentrations of five test proteins were determined in exactly the same manner as BSA. Table 2 summarizes these results. Both the undiluted and 10-fold diluted data set were useful for these proteins. BSA is provided by NIST as a 7% (w/w; 71.57 ± 0.74 mg/mL) solution, and the calculated concentration of this solution by *AAA-Direct* using HCl hydrolysis was 66.8 mg/mL, an accuracy of 93.3%. Because Trp is largely destroyed during the acid hydrolysis, its measure is not accurate. Trp concentration was also determined in BSA using an alkaline hydrolysis method described in Application Note 142.[13] The total protein concentration was adjusted to 67.2 mg/mL after the addition of the 0.42 mg/mL measured Trp, an accuracy of 93.9%. The addition of an accurate Trp measure increases the accuracy of this BSA measurement by 0.6%. Although cysteine and cystine are expected to be degraded during HCl hydrolysis,[14] an appreciable concentration (3.26 mg/mL) of cystine was measured. When both cystine and Trp are included, 70.5 mg/mL total protein was measured, or 98.5% of expected. These results suggest that better accuracy in total protein determinations is possible when Trp and cystine concentrations are included-even when cystine recovery may be lower than expected. For this note, Trp and cystine were not included in the concentrations tabulated. When the protein standard (e.g., BSA) is not 100% of the theoretical value, one may choose to adjust the other protein concentrations in proportion to the measured percent recovery for the protein standard (not done here).

Aprotinin solution concentration was 1.70 mg/mL, β-lactoglobulin was 1.63 mg/mL, fetuin was 1.22 mg/mL, hemoglobin was 1.55 mg/mL, and ubiquitin was 1.84 mg/mL. No significant amounts of hydroxylysine, hydroxyproline, or Trp were detected in any of the proteins tested. Cystine was measured in aprotinin (0.141 mg/mL), β-lactoglobulin (0.014 mg/mL), and fetuin (0.014 mg/mL), but neither hemoglobin nor ubiquitin.

Table 2. Amino Acid and Total Protein Concentrations

mg/mL Undiluted Samples						
AMINO ACID	**BSA**	**Aprotinin**	**β-Lactoglobulin**	**Fetuin**	**Hemoglobin**	**Ubiquitin**
Arginine	4.662	0.281	0.060	0.104	0.080	0.177
Lysine	8.030	0.169	0.200	0.081	0.188	0.208
Alanine	4.058	0.138	0.114	0.093	0.151	0.040
Threonine	3.319	0.080	0.068	0.058	0.066	0.139
Glycine	1.241	0.125	0.029	0.050	0.073	0.089
Valine	4.041	0.043	0.102	0.123	0.150	0.095
Serine	2.489	0.038	0.055	0.062	0.083	0.053
Proline	3.199	0.124	0.083	0.116	0.058	0.070
Isoleucine	0.676	0.055	0.073	0.018	0.002	0.133
Leucine	7.999	0.078	0.264	0.110	0.237	0.242
Methionine	0.374	0.027	0.021	0.002	0.004	0.018
Histidine	5.226	0.012	0.051	0.064	0.091	0.043
Phenylalanine	3.015	0.107	0.041	0.039	0.090	0.046
Glutamate	9.535	0.104	0.272	0.138	0.090	0.289
Aspartate	5.911	0.165	0.170	0.120	0.147	0.164
Tyrosine	3.032	0.151	0.025	0.037	0.036	0.030
TOTAL PROTEIN (mg/mL): (Excluding Trp and Cystine)	**66.81**	**1.70**	**1.63**	**1.22**	**1.55**	**1.84**
KNOWN CONC. (mg/mL):	**71.57**					
CALCULATED % RECOVERY:	**93.3%**					

The Chromeleon Chromatography Workstation enables report formats that may be easily customized to directly calculate these concentrations and generate reports that may be printed as permanent records.

Intermediate Precision of Protein Determinations Using *AAA-Direct*

To test the day-to-day variability of *AAA-Direct* for protein determinations, the same protein solutions were hydrolyzed and analyzed on three separate days (trials) over a period of 2 weeks. Amino acid concentrations were calculated for Trials 2 and 3 in the manner described for the data of Trial 1 (presented in Tables 1 and 2). Table 3 compares the protein determinations of each protein solution for each trial, and presents the between-day statistics for these data. The RSD for each protein solution ranged from 1.3 to 18% over the three trials. Excluding hemoglobin, RSDs ranged from 1.3 to 6.4%. Hemoglobin showed higher variability than the other five proteins tested, and upon closer examination appeared to be the result of an overall lower recovery for all hemoglobin amino acids after hydrolysis in trial 3, resulting in a 28% lower total protein measure in this trial compared to trials 1 and 2. The percent measured concentration relative to the expected concentration for the BSA protein standard ranged from 89% to 93% over the three trials.

Comparison of *AAA-Direct* with Other AAA Techniques

The report of the ABRF collaborative study[9] in 2003 presents the measured protein concentrations for the same five protein solutions examined in this note as determined by our lab and the 26 other collaborators using a variety of AAA techniques. In this study, our site 17, and another site 16, used *AAA-Direct* for protein determinations. The other 25 sites used non-*AAA-Direct* AAA techniques. Results for sites #16 and 17 were close to the study means. Table 4 presents the complete results for all 27 sites in the collaborative study, and the results compare favorably with the protein determina-

Table 3. Between-Day Variability in Measured Total Protein Concentration (mg/mL)

TRIAL	BSA	Aprotinin	β-Lactoglobulin	Fetuin	Hemoglobin	Ubiquitin
1	66.81	1.70	1.63	1.22	1.55	1.84
2	63.37	1.74	1.64	1.33	1.62	1.76
3	64.28	1.72	1.46	1.29	1.14	1.66
Mean:	**64.82**	**1.72**	**1.58**	**1.28**	**1.44**	**1.75**
Std Dev:	**1.78**	**0.02**	**0.10**	**0.06**	**0.26**	**0.09**
RSD:	**2.7%**	**1.3%**	**6.4%**	**4.5%**	**18.0%**	**5.0%**

Note: Results exclude cyctine and Trp measures from acid hydrolysis.

Table 4. Total Protein Measure for Five Test Proteins Compared to the Result of the ABRF 2003 Collaborative Study

	AAA-Direct (Site 17)*			ABRF 2003 Study[9]		
Protein	**Mean**	**Std Dev**	**RSD**	**Mean**	**Std Dev**	**RSD**
Aprotinin	1.72	0.02	1.3%	1.74	0.28	16.1%
β-Lactoglobulin	1.58	0.10	6.4%	1.67	0.23	13.8%
Fetuin	1.28	0.06	4.5%	1.18	0.17	14.4%
Hemoglobin	1.44	0.26	18.0%	1.64	0.23	14.0%
Ubiquitin	1.75	0.09	5.0%	1.72	0.20	11.6%

* Results from three trials performed on separate days.
All results exclude Trp and cystine.

tions using *AAA-Direct.* In this ABRF collaborative study, the mean ± standard deviation protein concentration for aprotinin was 1.74 ± 0.28 mg/mL, whereas *AAA-Direct* (Site 17) produced 1.72 ± 0.02 mg/mL for the same protein solution. Table 4 shows similar results for the other four proteins. Although we cannot perform a statistical comparison of *AAA-Direct* with the other AAA techniques because the data presented in the ABRF study include *AAA-Direct* data, the results for both *AAA-Direct* sites (16 and 17) clearly show that *AAA-Direct* produces results close to the mean of this study and within the variance seen for the other methods. Therefore, *AAA-Direct* can be considered equivalent to other AAA techniques for determining total protein concentrations.

Evaluation for Glycoprotein Monosaccharide Interference

AAA-Direct can simultaneously detect both amino acids and carbohydrates.[15,16] Because the acid hydrolysis conditions used for AAA allow the survival of some percentage of the original amino sugars, AAA by *AAA-Direct* can also identify glycosylated proteins. Fetuin is known to be highly glycosylated, and galactosamine and glucosamine are observed in its chromatogram (Figure 3, peaks 3 and 4).

Factors Affecting the Accuracy of Total Protein Determinations By AAA

(1) Chemical Degradation of Specific Amino Acids During Hydrolysis

Trp, cysteine, and cystine are sensitive amino acids often completely degraded during acid hydrolysis. Although normally present in small amounts in most proteins, their absence from the summation of amino acid concentrations contributes slightly to the inaccuracy of the total protein measure. Trp can be rapidly and accurately measured using *AAA-Direct* with alkaline hydrolysis, and this procedure is described in Application Note 142[13] and other publications.[17,18] Cysteine, cystine, and other sulfur-containing amino acids can be hydrolyzed and accurately measured using *AAA-Direct* with performic acid under oxidizing conditions.[19] The concentrations of Trp measured by this alternative method can be simply added to the list of amino acid concentrations to be summed. Other amino acids are also sensitive to HCl hydrolysis (methionine, serine, valine, and glutamate), and their recovery is sometimes less than 80%. If amino acid composition and concentration are accurately known for the protein control (e.g., BSA), it is possible to calculate the individual percent recovery of each amino acid, and correct for their respective concentrations in the test samples by this amount. However, caution is needed in applying these corrective steps because the recovery of amino acids from one sample may not be the same for another sample (e.g., higher protein concentration during hydrolysis improves amino acid recovery).

Glutamine (Gln) and asparagine (Asn) are converted to glutamate (Glu) and aspartate (Asp) during hydrolysis, respectively, and their concentrations are determined in proteins as Asp and Glu equivalents. The difference in mass between Asn and Asp, or Gln and Glu is 0.7%. Similarly, the pyro-glutamate present at the terminal end of some proteins is converted to Glu during hydrolysis, and is measured as Glu equivalents (12.3% mass error). Although the sum of the error for each residue of Asn, Gln, pyro-Glu, and Trp contributes to the total error in protein measure, this error contribution relative to the total amino acid composition is normally minor, and therefore the impact on protein concentration determination is usually insignificant. These effects may be significant for proteins that have unusually high concentrations of these amino acids.

It is common practice using AAA for protein determinations to exclude Trp (and Cys) from calculations due to their destruction during HCl hydrolysis. Also, Asn/Asp and Gln/Glu are each combined as Asx and Glx, respectively. The ABRF 2003 study[9] followed these practices.

(2) Omission of Uncommon Amino Acids in Protein Composition—Not Included in Analysis

Collagen (type 1) contains abundant amounts of the uncommon amino acids hydroxyproline (~9–10%) and hydroxylysine (~0.5–0.8%).[20] Elastin also contains hydroxyproline (~0.4–1.4%), in addition to isodesmosine, desmosine, and lysinonorleucine.[21] Prothrombin contains γ-carboxyglutamate, and some phosphorylated proteins and peptides contain phosphoserine, phosphotyrosine, and phosphothreonine.[22] Other connective tissue proteins or other proteins subject to posttranslational modifications can also contain these or other uncommon amino acids. When these amino acids are not tested for during AAA, their concentrations are not included in the summation, and

therefore the results underestimate protein concentration. *AAA-Direct* measures most uncommon amino acids, but standards must be included in the study to quantify these. Phosphoamino acids are sensitive to acid hydrolysis, and their recovery from proteins may require shorter hydrolysis times.[22]

(3) Conversion of Amino Acids During Chromatography

Cysteine dimerizes oxidatively during anion-exchange chromatography using alkaline eluent conditions and is converted to cystine. Cysteine is measured as cystine equivalents. The difference in mass between 2 moles cysteine and 1 mole cystine is 0.8%, and this minor difference for only the few residues typical for most proteins relative to the total amino acid composition-is normally insignificant for total protein determinations.

(4) Contamination of Samples, Reagents, or Labware

The quantification of amino acids and the accuracy of the total protein measure are affected by contamination from either amino acids, proteins, or other substances in either the blanks or samples. When the blanks are contaminated, and not representative of the background contribution to the protein hydrolysates, then their subtraction causes the total protein content to appear lower than actual. When the protein hydrolysate sample is contaminated, then the measured amino acid concentrations appear higher than actual. Replicate hydrolysates of blanks and samples can help detect sporadic contamination (e.g., dust particles, inadequately cleaned tubes, etc.). Qualification of labware, reagents, and careful laboratory practices helps to ensure low levels of contamination.

(5) Interferences

The presence of coeluting substances from the sample matrix may interfere with the accurate quantification of one or more amino acid peaks during chromatography. For example, Tris buffers will elute in the void as a peak interfering with arginine. Tris is also an interference for the Lowry protein assay. Glucose in large excess of 0.1–1 mM can interfere with alanine and threonine.[15] Using *AAA-Direct*, interferences are normally restricted to only one or two amino acids, and their omission from the total protein calculations will have only a minor effect on the accuracy of the assay compared with dye-binding assays, where the interfering substance cannot be resolved from the true substance to be measured. For most interfering carbohydrates, their selectivity on the AminoPac PA10 column can be altered to better resolve from coeluting amino acids, as described in Application Note 150.[15] Technical Note 55 provides detailed procedures for screening the suitability of substances for *AAA-Direct.*[12]

(6) Protein Ligands

Glycosylated or glycated proteins contain a fraction of their mass as a carbohydrate component. Dye-binding protein assays and AAA methods do not include this component in the mass concentration calculation, and therefore produce lower total protein concentrations than actual. *AAA-Direct* enables the detection of glycated proteins or glycoproteins by the presence of galactosamine and glucosamine peaks eluting between lysine and alanine. The accuracy of protein determinations for glycosylated proteins may be improved through inclusion of the carbohydrate component using HPAE-PAD.[11,23] Other attached ligands alter protein mass concentrations (e.g., lipids and polyethylene glycol conjugates). Accurate measures of these protein complexes depend on effective strategies for determination of the mass of these attached components and how you combine them with the total amino acid determinations.

(7) Dilution Errors

The greatest errors in protein determination come from poor pipetting techniques and inaccurate dilutions. Gravimetrically based dilutions help assure dilutions are true and their recordings help document any calculation error.

Generally, most of these factors have very minor effects on the accuracy of the total protein measure by AAA or are ignored, and steps to correct for these effects are normally unnecessary. An understanding of these variables is important for handling those special cases where AAA is needed. This application note presents procedures for using HCl for hydrolysis. Technical Note 50 should be reviewed when considering alternative hydrolyzing reagents.[24]

CONCLUSION

The use of AAA for total protein determinations provides advantages over colorimetric methods. Complete degradation of proteins to amino acids enables a complete accounting of the composition without bias from the specificity of dye-binding typical for colorimetric assays (BCA, Bradford, Lowry, etc). The compositional analysis also provides information about the identity and purity of the protein not achieved using dye-binding assays. The direct detection of amino acids using *AAA-Direct* results in lower cost in materials and labor, eliminates the risk of exposure to the hazardous chemicals used for pre- or postcolumn derivatization, and eliminates the hazardous waste stream created by pre- or postcolumn derivatization techniques. Accurate total protein measurements are achieved using *AAA-Direct,* with results equivalent to other AAA techniques.

REFERENCES

1. Smith, P. K.; et al. Measurement of Protein Using Bicinchoninic Acid. *Anal. Biochem.* **1985,** *150,* 76–85.
2. Wiechelman, K; Braun, R.; Fitzpatrick, J. Investigation of the Bicinchoninic Acid Protein Assay: Identification of the Groups Responsible for Color Development. *Anal. Biochem.* **1988,** *175,* 231–237.
3. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem.* **1951,** *193,* 265–275.
4. Legler, G.; Muller-Platz, C. M.; Mentges-Hettkamp, M.; Pflieger, G.; Julich, E. On the Chemical Basis of the Lowry Protein Determination. *Anal. Biochem.* **1985,** *150,* 278–287.
5. Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye-Binding. *Anal. Biochem.* **1976,** *72,* 248–254.
6. Tal, M.; Silberstein, A.; Nusser, E. Why Does Coomassie Brilliant Blue R Interact Differently with Different Proteins? *J. Biol Chem.* **1980,** *260,* 9976–9980.
7. Compton, S. J.; Jones, C. J. Mechanism of Dye Response and Interference in the Bradford Protein Assay. *Anal. Biochem.* **1985,** *151,* 369–374.
8. Schegg, K. M.; Denslow, N. D.; Andersen, T. T.; Bao, Y. A.; Cohen, S. A.; Mahrenholz, A. M.; Mann, K. Quantitation and Identification of Proteins by Amino Acid Analysis. In *Techniques in Protein Chemistry;* Marshak, D. R., ed.; Academic Press: San Diego, 1997; Volume VIII; pp 207–216.
9. Alterman, M.; Chin, D.T.; Harris, R.; Hunziker, P.; Le, A.; Linskens, S.; Packman, L.; Schaller, J. AAARG2003 Study: Quantitation of Proteins by Amino Acid Analysis and Colorimetric Assays. Oral and poster presentation at the ABRF 2003 in Translating Biology Using Proteomics and Functional Genomics, February 10–13, 2003, Denver, Colorado. http://www.abrf.org/ResearchGroups/AminoAcidAnalysis/EPosters/abrf2003_poster.pdf
10. Dionex Corporation. *Product Manual*: AAA-Direct *Amino Acid Analysis System.* Document 31481; Sunnyvale, CA.
11. Dionex Corporation. *Determination of Glycoprotein Monosaccharide Composition by HPAE-PAD Using On-Line Electrolytically Generated Eluents.* Technical Note 53; Sunnyvale, CA.
12. Dionex Corporation. *Screening of Sample Matrices and Individual Matrix Ingredients for Suitability in* AAA-Direct. Technical Note 55; Sunnyvale, CA.
13. Dionex Corporation. *Determination of Tryptophan Using* AAA-Direct. Application Note 142; Sunnyvale, CA.
14. Davidson, I. Hydrolysis of Samples for Amino Acid Analysis. In *Methods in Molecular Biology: Protein Sequencing Protocols,* Second Edition; B. J. Smith, ed.; Humana Press, Inc.: Totowa, NJ, 2003; p 120.
15. Dionex Corporation. *Determination of Amino Acids in Cell Cultures and Fermentation Broths.* Application Note 150; Sunnyvale, CA.
16. Hanko, V. P.; Rohrer, J. S. Determination of Amino Acids in Cell Culture and Fermentation Broth Media Using Anion-Exchange Chromatography with Integrated Pulsed Amperometric Detection. *Anal. Biochem.* **2003,** *324,* 29–38.
17. Hanko, V. P.; Rohrer, J. S. Direct Determination of Tryptophan Using High-Performance Anion-Exchange Chromatography with Integrated Pulsed Amperometric Detection. *Anal. Biochem.* **2002,** *308,* 204–209.

18. Hanko, V. P.; Rohrer, J. S. Determination of Tryptophan Content. *Gen. Eng. News* **2003,** *23 (3),* 38.
19. Jandik, P.; Pohl, C.; Barreto V.; Avdalovic, N. Anion-Exchange Chromatography and Integrated Amperometric Detection of Amino Acids. In *Methods in Molecular Biology;* Cooper, C., Packer, N., Williams, K., eds.; Humana Press, Inc.: Totowa, NJ, 2000; Vol. 159: Amino Acid Analysis Analysis Protocols.
20. Piez, K. A. Primary Structure. In *Biochemistry of Collagen;* Ramachandran, G. N., Reddi, A. H., eds.; Plenum Press: NY, 1976; pp 1–44.
21. Hay, E. D., ed. *Cell Biology of Extracellular Matrix.* Plenum Press: NY, 1981.
22. Hardie, D. G., ed. *Protein Phosphorylation: A Practical Approach.* Second edition. Oxford University Press: New York, 1999.
23. Dionex Corporation. G*lycoprotein Monosaccharide Analysis Using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD).* Technical Note 40; Sunnyvale, CA.
24. Dionex Corporation. *Determination of Amino Acid Content of Peptides by* AAA-Direct. Technical Note 50; Sunnyvale, CA.

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