INTRODUCTION

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyze a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatized for analysis.

APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have postcolumn derivatization capability, unless the sample is analyzed using precolumn derivatization. The detector is usually a UV-visible or fluorescence detector depending on the derivatization method used. A recording device (e.g., integrator) is used for transforming the analog signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High-purity reagents are necessary (e.g., low-purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

REFERENCE STANDARD MATERIAL

Acceptable amino acid standards are commercially available[*] for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analyzed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

CALIBRATION OF INSTRUMENTATION

Calibration of amino acid analysis instrumentation typically involves analyzing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analyzed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

Four to six amino acid standard levels are analyzed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nmol of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and is used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the nmol of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

REPEATABILITY

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that corresponds to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analyzing many replicates (i.e., six analyses or more) of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays conducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. Often, the amino acid composition of a standard protein (e.g., bovine serum albumin) is analyzed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results. Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to the quality of reagents and/or to laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits. All parameters involved are fully investigated in the scope of the validation work.

SAMPLE PREPARATION

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Methods that utilize postcolumn derivatization of the amino acids are generally not affected by buffer components to the extent seen with precolumn derivatization methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labor. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reverse-phase HPLC system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge; (2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); and (5) gel filtration.

INTERNAL STANDARDS

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids from the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis because their rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this particular point into consideration when interpreting the results. Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and α-aminobutyric acid.

PROTEIN HYDROLYSIS

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results. Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 hour in 1 N hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of concentrated hydrochloric acid and concentrated nitric acid (1:1). Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC grade methanol, dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500° for 4 hours may be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolyzing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids. Tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine, the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (i.e., *Methods 4–11*) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24, 48, and 72 hours) is often employed to analyze the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (i.e., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but it requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of 1 minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins. [NOTE—During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.]

Method 1

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis Solution: 6 N hydrochloric acid containing 0.1% to 1.0% of phenol.

Procedure—

Liquid Phase Hydrolysis—Place the protein or peptide sample in a hydrolysis tube, and dry. [NOTE—The sample is dried so that water in the sample will not dilute the acid used for the hydrolysis.] Add 200 µL of *Hydrolysis Solution* per 500 µg of lyophilized protein. Freeze the sample tube in a dry ice–acetone bath, and flame seal in vacuum. Samples are typically hydrolyzed at 110° for 24 hours in vacuum or inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 and 72 hours) are investigated if there is a concern that the protein is not completely hydrolyzed.

Vapor Phase Hydrolysis—This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimized by using vapor phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of *Hydrolysis Solution.* The *Hydrolysis Solution* does not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200 µm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110° for a 24-hour hydrolysis time. Acid vapor hydrolyzes the dried sample. Any condensation of the acid in the sample vials is minimized. After hydrolysis, dry the test sample in vacuum to remove any residual acid.

Method 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid (MESA) as the reducing acid.

Hydrolysis Solution: 2.5 M MESA solution.

Vapor Phase Hydrolysis—About 1 to 100 µg of the protein/peptide under test is dried in a hydrolysis tube. The hydrolysis tube is placed in a larger tube with about 200 µL of the *Hydrolysis Solution.* The larger tube is sealed in vacuum (about 50 µm of mercury or 6.7 Pa) to vaporize the *Hydrolysis Solution*. The hydrolysis tube is heated to between 170° to 185° for about 12.5 minutes. After hydrolysis, the hydrolysis tube is dried in vacuum for 15 minutes to remove the residual acid.

Method 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

Hydrolysis Solution**:** a solution containing 7 M hydrochloric acid, 10% of trifluoroacetic acid, 20% of thioglycolic acid, and 1% of phenol.

Vapor Phase Hydrolysis—About 10 to 50 µg of the protein/peptide under test is dried in a sample tube. The sample tube is placed in a larger tube with about 200 µL of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 µm of mercury or 6.7 Pa) to vaporize the TGA. The sample tube is heated to 166° for about 15 to 30 minutes. After hydrolysis, the sample tube is dried in vacuum for 5 minutes to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

Method 4

Cysteine-cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

Oxidation Solution—The performic acid is prepared fresh by mixing formic acid and 30 percent hydrogen peroxide (9:1), and incubating at room temperature for 1 hour.

Procedure—The protein/peptide sample is dissolved in 20 µL of formic acid, and heated at 50° for 5 minutes; then 100 µL of the *Oxidation Solution* is added. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine sulfone. The oxidation is allowed to proceed for 10 to 30 minutes. The excess reagent is removed from the sample in a vacuum centrifuge. This technique may cause modifications to tyrosine residues in the presence of halides. The oxidized protein can then be acid hydrolyzed using *Method 1* or *Method 2*.

Method 5

Cysteine-cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis Solution: 6 N hydrochloric acid containing 0.2% of phenol, to which sodium azide is added to obtain a final concentration of 0.2% (w/v). The added phenol prevents halogenation of tyrosine.

Liquid Phase Hydrolysis—The protein/peptide hydrolysis is conducted at about 110° for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the sodium azide present in the *Hydrolysis Solution*. This technique allows better tyrosine recovery than *Method 4,* but it is not quantitative for methionine. Methionine is converted to a mixture of the parent methionine and its two oxidative products, methionine sulfoxide and methionine sulfone.

Method 6

Cysteine-cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

Hydrolysis Solution: 6 N hydrochloric acid containing 0.1% to 1.0% of phenol, to which DMSO is added to obtain a final concentration of 2% (v/v).

Vapor Phase Hydrolysis—The protein/peptide hydrolysis is conducted at about 110° for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the DMSO present in the *Hydrolysis Solution*. As an approach to limit variability and to compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolyses of standard proteins containing 1 to 8 mol of cysteine. The response factors from protein/peptide hydrolysates are typically about 30% lower than those for nonhydrolyzed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

Method 7

Cysteine-cystine reduction and alkylation is accomplished by a vapor phase pyridylethylation reaction.

Reducing Solution—Transfer 83.3 µL of pyridine, 16.7 µL of 4-vinylpyridine, 16.7 µL of tributylphosphine, and 83.3 µL of water to a suitable container, and mix.

Procedure—Add the protein/peptide (between 1 and 100 µg) to a hydrolysis tube, and place in a larger tube. Transfer the *Reducing Solution* to the large tube, seal in vacuum (about 50 µm of mercury or 6.7 Pa), and incubate at about 100° for 5 minutes. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 minutes to remove residual reagents. The pyridylethylated protein/peptide can then be acid hydrolyzed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1 to 8 mol of cysteine to improve accuracy in the pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α-amino terminal group and the ε-amino group of lysine in the protein.

Method 8

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock Solutions—Prepare and filter three solutions: 1 M Tris hydrochloride (pH 8.5) containing 4 mM edetate disodium (*Stock Solution 1*), 8 M guanidine hydrochloride (*Stock Solution 2*), and 10% of 2-mercaptoethanol in water (*Stock Solution 3*).

Reducing Solution—Prepare a mixture of *Stock Solution 2* and *Stock Solution 1* (3:1) to obtain a buffered solution of 6 M guanidine hydrochloride in 0.25 M Tris hydrochloride.

Procedure—Dissolve about 10 µg of the test sample in 50 µL of the *Reducing Solution*, and add about 2.5 µL of *Stock Solution 3*. Store under nitrogen or argon for 2 hours at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 µL of 4-vinylpyridine to the protein solution, and incubate for an additional 2 hours at room temperature in the dark. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

Method 9

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

Stock Solutions—Prepare as directed for *Method 8*.

Carboxymethylation Solution—Prepare a solution containing 100 mg of iodoacetamide per mL of alcohol.

Buffer Solution—Use the *Reducing Solution,* prepared as directed for *Method 8*.

Procedure—Dissolve the test sample in 50 µL of the *Buffer Solution*, and add about 2.5 µL of *Stock Solution 3*. Store under nitrogen or argon for 2 hours at room temperature in the dark. Add the *Carboxymethylation Solution* in a 1.5 fold ratio per total theoretical content of thiols, and incubate for an additional 30 minutes at room temperature in the dark. [NOTE—If the thiol content of the protein is unknown, then add 5 µL of 100 mM iodoacetamide for every 20 nmol of protein present.] The reaction is stopped by adding excess of 2-mercaptoethanol. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The *S*-carboxyamidomethylcysteine formed will be converted to *S*-carboxymethyl-cysteine during acid hydrolysis.

Method 10

Cysteine-cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. [NOTE—The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.]

Reducing Solution: a solution containing 10 mg of dithiodiglycolic acid (or dithiodipropionic acid) per mL of 0.2 M sodium hydroxide.

Procedure—Transfer about 20 µg of the test sample to a hydrolysis tube, and add 5 µL of the *Reducing Solution*. Add 10 µL of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolyzed using *Method 1*. This method has the advantage that other amino acid residues are not derivatized by side reactions, and the sample does not need to be desalted prior to hydrolysis.

Method 11

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by *Asx,* while glutamine and glutamic acid residues are added and represented by *Glx.* Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing Solutions—Prepare and filter three solutions: a solution of 10 mM trifluoroacetic acid (*Solution 1*), a solution of 5 M guanidine hydrochloride and 10 mM trifluoroacetic acid (*Solution 2*), and a freshly prepared solution of dimethylformamide containing 36 mg of BTI per mL (*Solution 3*).

Procedure—In a clean hydrolysis tube, transfer about 200 µg of the test sample, and add 2 mL of *Solution 1* or *Solution 2* and 2 mL of *Solution 3.* Seal the hydrolysis tube in vacuum. Heat the sample at 60° for 4 hours in the dark. The sample is then dialyzed with water to remove the excess reagents. Extract the dialyzed sample three times with equal volumes of *n*-butyl acetate, and then lyophilize. The protein can then be acid hydrolyzed using previously described procedures. The α-, β-diaminopropionic and α-, γ-diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid assayed contents with un-derivatized and BTI-derivatized acid hydrolysis. [NOTE—The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatization; a hydrolysis without BTI will have to be performed if the analyst is interested in the protein/peptide content of these other amino acid residues.]

METHODOLOGIES OF AMINO ACID ANALYSIS

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by postcolumn derivatization (e.g., with ninhydrin or *o*-phthalaldehyde). Postcolumn detection techniques can be used with samples that contain small amounts of buffer components, such as salts and urea, and generally require between 5 and 10 µg of protein sample per analysis. The remaining amino acid techniques typically involve precolumn derivatization of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbonate; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenyl-methylchloroformate; and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reverse-phase HPLC. Precolumn derivatization techniques are very sensitive and usually require between 0.5 and 1.0 µg of protein sample per analysis but may be influenced by buffer salts in the samples. Precolumn derivatization techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Postcolumn derivatization techniques are generally influenced less by performance variation of the assay than precolumn derivatization techniques.

The following *Methods* may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, chromatographic systems, etc. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will utilize more than one amino acid analysis technique to exploit the advantages offered by each. In each of these *Methods*, the analog signal is visualized by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

Method 1—Postcolumn Ninhydrin Detection

Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis. As a rule, a Li-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster Na-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has characteristic purple or yellow color. Amino acids, except imino acids, give a purple color, and show maximum absorption at 570 nm. The imino acids, such as proline, give a yellow color, and show maximum absorption at 440 nm. The postcolumn reaction between ninhydrin and amino acid eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

The detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for proline. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

One method for postcolumn ninhydrin detection is shown below. Many other methods are also available, with instruments and reagents available commercially.

Mobile Phase Preparation—

Solution A—Transfer about 1.7 g of anhydrous sodium citrate and 1.5 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 3.0.

Solution B—Transfer about 1.7 g of anhydrous sodium citrate and 0.7 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 4.3.

Solution C—Prepare a solution containing 5% of sodium chloride, 1.9% of anhydrous sodium citrate, and 0.1% of phenol in water, and adjust to a pH of 6.

Column Regeneration Solution—Prepare a solution containing 0.8% of sodium hydroxide in water, and adjust to a pH of 13.

Mobile Phase—Use variable mixtures of *Solution A, Solution B,* and *Solution C* as directed for *Chromatographic system*.

Postcolumn Reagent—Transfer about 18 g of ninhydrin and 0.7 g of hydrindantin to 900 mL of a solution containing 76.7% of dimethyl sulfoxide, 0.7% of dihydrate lithium acetate, and 0.1% of acetic acid, and mix for at least 3 hours under inert gas, such as nitrogen. [NOTE—This reagent is stable for 30 days if kept between 2° and 8° under inert gas.]

Buffer Solution—Prepare a solution containing 2% of anhydrous sodium citrate, 1% of hydrochloric acid, 0.5% of thiodiglycol, and 0.1% of benzoic acid in water, and adjust to a pH of 2.

Chromatographic System—The liquid chromatograph is equipped with a detector with appropriate interference filters at 440, 570, or 690 nm and a 4.0-mm × 120-mm column that contains 7.5-µm sulfonated styrene-divinylbenzene copolymer packing. The flow rate is about 14 mL per hour. The system is programmed as follows. Initially equilibrate the column with *Solution A;* at 25 minutes, the composition of the *Mobile Phase* is changed to 100% *Solution B;* and at 37 minutes, the composition is changed to 100% *Solution C.* At 75 minutes into the run, the last amino acid has been eluted from the column, and the column is regenerated with *Column Regeneration Solution* for 1 minute. The column is then equilibrated with *Solution A* for 11 minutes before the next injection. The column temperature is programmed as follows. The initial temperature is 48°; after 11.5 minutes, the temperature is increased to 65° at a rate of 3° per minute; at about 35 minutes, the temperature is increased to 77° at a rate of 3° per minute; and finally at about 52 minutes, the temperature is decreased to 48° at a rate of 3° per minute.

Procedure and Postcolumn Reaction—Reconstitute the lyophilized protein/peptide hydrolysate in the *Buffer Solution,* inject an appropriate amount into the chromatograph, and proceed as directed for *Chromatographic System.* As the amino acids are eluted from the column, they are mixed with the *Postcolumn Reagent*, which is delivered at a flow rate of 7 mL per hour, through a tee. After mixing, the column effluent and the *Postcolumn Reagent* pass through a tubular reactor at a temperature of 135°, where a characteristic purple or yellow color is developed. From the reactor, the liquid passes through a colorimeter with a 12-mm flow-through cuvette. The light emerging from the cuvette is split into three beams for analysis by the detector with interference filters at 440, 570, or 690 nm. The 690-nm signal may be electronically subtracted from the other signals for improved signal-to-noise ratios. The 440-nm (imino acids) and the 570-nm (amino acids) signals may be added in order to simplify data handling.

Method 2—Postcolumn OPA Fluorometric Derivatization

Ion-exchange chromatography with postcolumn *o*-phthalaldehyde (OPA) fluorometric detection is used. The procedure employs an ion-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and derivatization using OPA and *N*-acetyl-L-cysteine. The sodium hypochlorite oxidation step allows secondary amines, such as proline, to react with the OPA reagent.

OPA reacts with primary amines in the presence of thiol compound to form highly fluorescent isoindole products. This reaction is utilized for the postcolumn derivatization in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as *Method I.* Instruments and reagents for this form of amino acid analysis are available commercially. Many modifications of this method exist.

Although OPA does not react with secondary amines (imino acids, such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and postcolumn derivatization using OPA and thiol compound, such as *N*-acetyl-L-cysteine and 2-mercaptoethanol. The derivatization of primary amino acids are not noticeably affected by the continuous supply of sodium hypochlorite.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes of pH and cation strength. After postcolumn derivatization of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limit is considered to be a few tens of pmol level for most of the amino acid derivatives. Response linearity is obtained in the range of a few pmol level to a few tens of nmol level. To obtain good compositional data, a sample greater than 500 ng before hydrolysis is best suited for the amino acid analysis of protein/peptide.

One method of postcolumn OPA fluorometric detection is shown below.

Mobile Phase Preparation—

Solution A—Prepare a solution of sodium hydroxide, citric acid, and alcohol in HPLC grade water having a 0.2 N sodium concentration and containing 7% of alcohol (w/v), adjusted to a pH of 3.2.

Solution B—Prepare a solution of sodium hydroxide and citric acid in HPLC grade water having a 0.6 N sodium concentration, adjusted to a pH of 10.0.

Solution C: 0.2 N sodium hydroxide.

Mobile Phase—Use variable mixtures of *Solution A,* Solution B, and *Solution C* as directed for *Chromatographic System*.

Postcolumn Reagent Preparation—

Alkaline Buffer—Prepare a solution containing 384 mM sodium carbonate, 216 mM boric acid, and 108 mM potassium sulfate, and adjust to a pH of 10.0.

Hypochlorite Reagent—To 1 L of *Alkaline Buffer,* add 0.4 mL of sodium hypochlorite solution (10% chlorine concentration). [NOTE—The hypochlorite solution is stable for 2 weeks.]

OPA Reagent—Transfer 2 g of *N*-acetyl-L-cysteine and 1.6 g of OPA to a 15-mL volumetric flask, dissolve in and dilute with alcohol to volume, and mix. Transfer this solution and 4 mL of 10% aqueous polyethylene (23) lauryl ether to a 1-L volumetric flask, dilute with 980 mL of *Alkaline Buffer*, and mix.

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set to an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.0-mm × 150-mm column that contains 7.5-µm packing L17. The flow rate is about 0.3 mL per minute, and the column temperature is set at 50°. The system is programmed as follows. The column is equilibrated with *Solution A;* over the next 20 minutes, the composition of the *Mobile Phase* is changed linearly to 85% *Solution A* and 15% *Solution B;* then there is a step change to 40% *Solution A* and 60% *Solution B;* over the next 18 minutes, the composition is changed linearly to 100% *Solution B* and held for 7 minutes; then there is a step change to 100% *Solution C,* and this is held for 6 minutes; then there is a step change to *Solution A,* and this composition is maintained for the next 8 minutes.

Procedure and Postcolumn Reaction—Inject about 1.0 nmol of each amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*. As the effluent leaves the column, it is mixed with the *Hypochlorite Reagent*. The mixture passes through the first postcolumn reactor which consists of stainless steel 0.5-mm × 2-m tubing. A second postcolumn reactor of similar design is placed immediately downstream from the first postcolumn reactor and is used for the OPA postcolumn reaction. The flow rates for both the *Hypochlorite Reagent* and the *OPA Reagent* are 0.2 mL per minute, resulting in a total flow rate (i.e., *Hypochlorite Reagent*, *OPA Reagent,* and column effluent) of 0.7 mL per minute exiting from the postcolumn reactors. Postcolumn reactions are conducted at 55°. This results in a residence time of about 33 seconds in the OPA postcolumn reactor. After postcolumn derivatization, the column effluent passes through the fluorometric detector.

Method 3—Precolumn Determination

Precolumn derivatization of amino acids with phenylisothiocyanate (PITC) followed by reverse-phase HPLC separation with UV detection is used.

PITC reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives, which can be detected with high sensitivity at 254 nm. Therefore, precolumn derivatization of amino acids with PITC followed by a reverse-phase HPLC separation with UV detection is used to analyze the amino acid composition.

After the reagent is removed under vacuum, the derivatized amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after three days.

Separation of the PTC-amino acids on a reverse-phase HPLC with ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

The detection limit is considered to be 1 pmol for most of the amino acid derivatives. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, a sample larger than 500 ng of protein/peptide before hydrolysis is best suited for this amino analysis of proteins/peptides.

One method of precolumn PITC derivatization is described below.

Mobile Phase Preparation—

Solution A: 0.05 M ammonium acetate, adjusted with phosphoric acid to a pH of 6.8.

Solution B—Prepare 0.1 M ammonium acetate, adjust with phosphoric acid to a pH of 6.8, and then prepare a mixture of this solution and acetonitrile (1:1).

Solution C: a mixture of acetonitrile and water (70:30).

Mobile Phase—Use variable mixtures of *Solution A, Solution B,* and *Solution C* as directed for *Chromatographic System*.

Derivatization Reagent Preparation—

Coupling Buffer: a mixture of acetonitrile, pyridine, triethylamine, and water (10:5:2:3).

Sample Solvent: a mixture of water and acetonitrile (7:2).

Sample Derivatization Procedure—Dissolve the lyophilized test sample in 100 µL of the *Coupling Buffer,* and then dry in a vacuum centrifuge to remove any hydrochloride if a protein hydrolysis step was used. Dissolve the test sample in 100 µL of *Coupling Buffer,* add 5 µL of PITC, and incubate at room temperature for 5 minutes. The test sample is again dried in a vacuum centrifuge, and is dissolved in 250 µL of *Sample Solvent*.

Chromatographic System—The liquid chromatograph is equipped with a 254-nm detector and a 4.6-mm × 250-mm column that contains 5-µm packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 52°. The system is programmed as follows. The column is equilibrated with *Solution A;* over the next 15 minutes, the composition of the *Mobile Phase* is changed linearly to 85% *Solution A* and 15% *Solution B;* over the next 15 minutes, the composition is changed linearly to 50% *Solution A* and 50% *Solution B;* then there is a step change to 100% *Solution C,* and this is held for 10 minutes; then there is a step change to 100% *Solution A,* and the column is allowed to equilibrate before the next injection.

Procedure—Inject about 1.0 nmol of each PITC-amino acid under test (10-µL sample in *Sample Solvent*) into the chromatograph, and proceed as directed for *Chromatographic System*.

Method 4—Precolumn AQC Derivatization

Precolumn derivatization of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reverse-phase HPLC separation with fluorometric detection is used.

AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reverse-phase HPLC. Therefore, precolumn derivatization of amino acids with AQC followed by reverse-phase HPLC separation is used to analyze the amino acid composition.

Separation of the AQC-amino acids on an ODS column is accomplished through a combination of changes in the concentrations of acetonitrile and salt. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent byproduct, 6-aminoquinoline. Excess reagent is rapidly hydrolyzed ($t_{1/2}$< 15 seconds) to yield 6-aminoquinoline-*N*-hydroxysuccinimide and carbon dioxide, and after 1 minute no further derivatization can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature, and the derivatives have more than sufficient stability to allow for overnight automated chromatographic analysis.

The detection limit is considered to be ranging from about 40 fmol to 320 fmol for each amino acid, except for Cys. The detection limit for Cys is approximately 800 fmol. Response linearity is obtained in the range of 2.5 µM to 200 µM with correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatized protein hydrolysates containing as little as 30 ng of protein/peptide.

One method of precolumn AQC derivatization is shown below.

Mobile Phase Preparation—

Solution A—Prepare a solution having a composition of 140 mM sodium acetate and 17 mM triethylamine, and adjust with phosphoric acid to a pH of 5.02.

Solution B: a mixture of acetonitrile and water (60:40).

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Sample Derivatization Procedure—Dissolve about 2 µg of the test sample in 20 µL of 15 mM hydrochloric acid, and dilute with 0.2 M borate buffer (pH 8.8) to 80 µL. The derivatization is initiated by the addition of 20 µL of 10 mM AQC in acetonitrile, and allowed to proceed for 10 minutes at room temperature.

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 250 nm and an emission wavelength of 395 nm and a 3.9-mm × 150-mm column that contains 4-µm packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with *Solution A;* over the next 0.5 minute, the composition of the *Mobile Phase* is changed linearly to 98% *Solution A* and 2% *Solution B;* then over the next 14.5 minutes to 93% *Solution A* and 7% *Solution B;* then over the next 4 minutes to 87% *Solution A* and 13% *Solution B;* over the next 14 minutes to 68% *Solution A* and 32% *Solution B;* then there is a step change to 100% *Solution B* for a 5-minute wash; over the next 10 minutes, there is a step change to 100% *Solution A;* and the column is allowed to equilibrate before the next injection.

Procedure—Inject about 0.05 nmol of each AQC-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System.*

Method 5—Precolumn OPA Derivatization

Precolumn derivatization of amino acids with OPA followed by reverse-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol and 3-mercaptopropionic acid can be used as thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reactions, make it amenable to automated derivatization and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in *Method 7* or *Method 8.*

Precolumn derivatization of amino acids with OPA is followed by reverse-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatized amino acids. Fluorescence intensity of the OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol. One method of precolumn OPA derivatization is shown below.

Mobile Phase Preparation—

Solution A: a mixture of 100 mM sodium acetate (pH 7.2), methanol, and tetrahydrofuran (900:95:5).

Solution B: methanol.

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Derivatization Reagent—Dissolve 50 mg of OPA in 1.25 mL of methanol (protein sequencing grade). Add 50 µL of 2-mercaptoethanol and 11.2 mL of 0.4 M sodium borate (pH 9.5), and mix. [NOTE—This reagent is stable for 1 week.]

Sample Derivatization Procedure—Transfer about 5 µL of the test sample to an appropriate container, add 5 µL of the *Derivatization Reagent,* and mix. After 1 minute, add not less than 20 µL of 0.1 M sodium acetate (pH 7.0). Use 20 µL of this solution for analysis. [NOTE—Use of an internal standard (e.g., norleucine) is recommended for quantitative analysis because of potential reagent volume variations in the sample derivatization. The sample derivatization is performed in an automated on-line fashion. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization.]

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.6-mm × 75-mm column that contains 3-µm packing L3. The flow rate is about 1.7 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with 92% *Solution A* and 8% *Solution B;* over the next 2 minutes, the composition of the *Mobile Phase* is changed to 83% *Solution A* and 17% *Solution B,* and held for an additional 3 minutes; then changed to 54% *Solution A* and 46% *Solution B* over the next 5 minutes, and held for an additional 2 minutes; then changed to 34% *Solution A* and 66% *Solution B* over the next 2 minutes, and held for 1 minute; then over the next 0.3 minute changed to 20% *Solution A* and 80% *Solution B,* and held for an additional 2.6 minutes; and then finally over 0.6 minute changed to 92% *Solution A* and 8% *Solution B,* and held for an additional 0.6 minute.

Procedure—Inject about 0.02 nmol of each OPA-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System.*

Method 6—Postcolumn DABS-Cl Derivatization

Precolumn derivatization of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reverse-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labeling of amino acids. Amino acids labeled with DABS-Cl (DABS-amino acids) are highly stable and show the maximum absorption at 436 nm.

DABS-amino acids, all 19 naturally occurring amino acids derivatives, can be separated on an ODS column of a reverse-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This method can analyze the imino acids, such as proline, together with the amino acids, at the same degree of sensitivity. DABS-Cl derivatization method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulfonic acids, such as mercaptoethanesulfonic acid, *p*-toluenesulfonic acid, or methanesulfonic acid, described for *Method 2* in *Protein Hydrolysis* under *Amino Acid Analysis.* The other acid-labile residues, asparagine and glutamine, can also be analyzed by previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI, described for *Method 11* in *Protein Hydrolysis* under *Amino Acid Analysis.*

The nonproteinogenic amino acid, norleucine, cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol. As little as 2 to 5 pmol of an individual DABS-amino acid can be quantitatively analyzed with reliability, and only 10 ng to 30 ng of the dabsylated protein hydrolysate is required for each analysis.

One method for precolumn DABS-Cl derivatization is shown below.

Mobile Phase Preparation—

Solution A: 25 mM sodium acetate (pH 6.5) containing 4% of dimethylformamide.

Solution B: acetonitrile.

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Derivatization Reagent Preparation—

Sample Buffer: 50 mM sodium bicarbonate, adjusted to a pH of 8.1.

Derivatization Reagent—Dissolve 1.3 mg of DABS-Cl in 1 mL of acetonitrile. [NOTE—This reagent is prepared fresh shortly before the derivatization step.]

Sample Dilution Buffer—Prepare a mixture of 50 mM sodium phosphate (pH 7.0) and alcohol (1:1).

Sample Derivatization Procedure—Dissolve the test sample in 20 µL of *Sample Buffer*, add 40 µL of *Derivatization Reagent*, and mix. The sample container is sealed with a silicon-rubber stopper, and heated to 70° for 10 minutes. During the sample heating, the mixture will become completely soluble. After the derivatization, dilute the test sample with an appropriate quantity of the *Sample Dilution Buffer.*

Chromatographic System—The liquid chromatograph is equipped with a 436-nm detector and a 4.6-mm × 250-mm column that contains packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 85% *Solution A* and 15% *Solution B;* over the next 20 minutes, the composition of the *Mobile Phase* is changed to 60% *Solution A* and 40% *Solution B;* over the next 12 minutes, the composition is changed to 30% *Solution A* and 70% *Solution B*, and held for an additional 2 minutes.

Procedure—Inject about 0.05 nmol of the DABS-amino acids into the chromatograph, and proceed as directed for *Chromatographic System.*

Method 7—Precolumn FMOC-Cl Derivatization

Precolumn derivatization of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reverse-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction of FMOC-Cl with amino acid proceeds under mild conditions, in aqueous solution, and is completed in 30 seconds. The derivatives are stable, with only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by reverse-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of acetic acid buffer, methanol, and acetonitrile (50:40:10) to a mixture of acetonitrile and acetic acid buffer (50:50), and 20 amino acid derivatives that are separated in 20 minutes. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low fmol range. A linearity range of 0.1 µM to 50 µM is obtained for most amino acids.

One method for precolumn FMOC-Cl derivatization is shown below.

Mobile Phase Preparation—

Acetic Acid Buffer—Transfer 3 mL of glacial acetic acid and 1 mL of triethylamine to a 1-L volumetric flask, and dilute with HPLC grade water to volume. Adjust with sodium hydroxide to a pH of 4.20.

Solution A: a mixture of *Acetic Acid Buffer,* methanol, and acetonitrile (50:40:10).

Solution B: a mixture of acetonitrile and *Acetic Acid Buffer* (50:50).

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Derivatization Reagent Preparation—

Borate Buffer—Prepare a 1 M boric acid solution, and adjust with sodium hydroxide to a pH of 6.2.

FMOC-Cl Reagent—Dissolve 155 mg of 9-fluorenylmethyl chloroformate in 40 mL of acetone, and mix.

Sample Derivatization Procedure—To 0.4 mL of the test sample add 0.1 mL of *Borate Buffer* and 0.5 mL of *FMOC-Cl Reagent*. After about 40 seconds, extract the mixture with 2 mL of pentane, and then extract again with fresh pentane. The aqueous solution with amino acid derivatives is then ready for injection.

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm and a 4.6-mm × 125-mm column that contains 3-µm packing L1. The flow rate is about 1.3 mL per minute. The system is programmed as follows. The column is equilibrated with *Solution A,* and this composition is maintained for 3 minutes; over the next 9 minutes, it is changed to 100% *Solution B;* then over the next 0.5 minute, the flow rate is increased to 2 mL per minute, and held until the final FMOC-amino acid is eluted from the column. The total run time is about 20 minutes.

Procedure—Inject not less than 0.01 nmol of each FMOC-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*. The FMOC-histidine derivative will generally give a lower response than the other derivatives.

Method 8—Precolumn NBD-F Derivatization

Precolumn derivatization of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) followed by reverse-phase HPLC separation with fluorometric detection is used.

7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatized with NBD-F by heating to 60° for 5 minutes.

NBD-amino acid derivatives are separated on an ODS column of reverse-phase HPLC by employing a gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives that are separated in 35 minutes. *E*-aminocaproic acid can be used as an internal standard because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as that for the precolumn OPA derivatization method (*Method 5*), excluding proline to which OPA is not reactive and might be advantageous for NBD-F against OPA.

The detection limit for each amino acid is about 10 fmol. Profile analysis was achieved for about 1.5 mg of protein hydrolysates in the final precolumn labeling reaction mixture for HPLC.

One method for precolumn NBD-F derivatization is shown below.

Mobile Phase Preparation—

Solution A: a solution of 10mM sodium citrate containing 75 mM sodium perchlorate, adjusted with hydrochloric acid to a pH of 6.2.

Solution B: a mixture of acetonitrile and water (50:50).

Derivatization Reagent Preparation—

Sample Buffer: a 0.1 M boric acid solution, adjusted with sodium hydroxide to a pH of 9.2.

Derivatization Reagent—Dissolve 5 mg of NBD-F in 1.0 mL of alcohol, and mix.

Sample Derivatization Procedure—Dissolve the test sample in 20 µL of *Sample buffer,* add 10 µL of *Derivatization Reagent,* and mix. The sample container is heated at 60° for 5 minutes. After the derivatization, dilute the test sample with 300 µL of *Solution A.*

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm and a 4.6-mm × 150-mm column that contains 5-µm particle size ODS silica packing. The flow rate is about 1.0 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 94% *Solution A* and 6% *Solution B;* over the next 16 minutes, the composition is changed linearly to 63% *Solution A* and 37% *Solution B;* over the next 5 minutes, the composition is changed linearly to 62% *Solution A* and 38% *Solution B;* over the next 9 minutes, the composition is changed linearly to 100% *Solution B*, and held for an additional 5 minutes; then finally over 2 minutes, the composition is changed linearly to 94% *Solution A* and 6% *Solution B;* and then the column is allowed to equilibrate before the next injection.

Procedure—Inject about 15 pmol of each NBD-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System.*

DATA CALCULATION AND ANALYSIS

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (less than 200 um µm of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapor phase hydrolysis can reduce the level of oxidative destruction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

Calculations

Amino Acid Mole Percent—This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular weight of the protein/peptide under investigation is unknown. This information can be used to corroborate the identity of a protein and has other applications. Carefully identify and integrate the peaks obtained as directed for each *Procedure*. Calculate the mole percent for each amino acid present in the test sample by the formula:

$$100r_U / r$$

in which r_U is the peak response, in nmol, of the amino acid under test; and r is the sum of peak responses, in nmol, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under test to data from known proteins can help establish or corroborate the identity of the sample protein.

Unknown Protein Samples—This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in µg, of each recovered amino acid by the formula:

$$mM_W/1000$$

in which m is the recovered quantity, in nmol, of the amino acid under test; and M_W is the average molecular weight, in mg, for that amino acid, corrected for the weight of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analyzed after appropriate correction for partially and completely destroyed amino acids. If the molecular weight of the unknown protein is available (i.e., by SDS-PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid by the formula:

$$m/(1000M/M_{WT})$$

in which m is the recovered quantity, in nmol, of the amino acid under test; M is the total mass, in µg, of the protein; and M_{WT} is the molecular weight, in mg, of the unknown protein.

Known Protein Samples—This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular weight and amino acid composition using the amino acid analysis data. When the composition of the protein being analyzed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine), and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nmol, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically, a greater than 5% variation from the mean is considered unacceptable, but this is arbitrary. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, by the formula:

$$100m/m_S$$

in which m is the experimentally determined quantity, in nmol per amino acid residue, of the amino acid under test; and m_S is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.■1S (*USP30*)