DIONEX

Application Note 142

Determination of Tryptophan Using *AAA-Direct*™

INTRODUCTION

Tryptophan (Trp) is a difficult amino acid to determine in proteins and peptides because it chemically decomposes during acid hydrolysis. Consequently, many procedures have been developed for the independent preservation and determination of this amino acid. Mercaptan antioxidants such as thioglycolic acid, thiodiglycolic acid, or dodecanethiol[1,2] have been added to 6 N hydrochloric acid (HCl) to preserve Trp. The addition of phenol has also been reported.[3-5] Alternatively, *p*-toluenesulfonic acid[6], methanesulfonic acid[7] containing 3-(2-aminoethyl) indole, and mercaptomethanesulfonic acid[8,9] have been used in place of HCl for hydrolysis, leading to some improvements in the recovery of Trp.

Alkaline hydrolysis has also been studied and was shown to produce higher Trp recovery than acid hydrolysis.[10] The high recoveries found with alkaline hydrolysis can be improved by the addition of reducing agents such as stannite[11], antioxidants such as starch[12], and other protectants such as a lead acetate/histidine mixture.[13] The use of polypropylene tubes instead of borosilicate tubes also improved recoveries.[14] Alkaline hydrolysis was improved by using sodium hydroxide (NaOH) instead of barium hydroxide to prevent problems with both precipitation and adsorption of Trp.[15] Performing the hydrolysis under vacuum or inert gas also improved Trp recovery.

Early in the development of hydrolysis techniques that led to improved recoveries of sensitive amino acids, chromatographic separations were recognized as a means of increasing specificity and eliminating interferences. The pioneering work of Spackman, Moore, and Stein[16] established cation-exchange chromatography as the most widely used method for amino acid analysis.[12,15-22] This postcolumn derivatization method used a dimethylsulfoxide-ninhydrin reagent developed by Moore[23] to enhance detection. Later separations were made using starch columns[14], gel filtration[24], and gas chromatography.[25] Developments in reversed-phase high-performance liquid chromatography (RP-HPLC) in the 1970s led the way to precolumn derivatization methods and rapid separations of amino acids.[26-28]

One of the current procedures for determination of Trp is published in the AOAC Official Methods of Analysis.[29] The AOAC method uses NaOH hydrolysis with pH adjustment and centrifugation followed by analysis by an amino acid analyzer. The amino acid analyzer is not defined in this procedure, but generally involves either cation-exchange chromatography and postcolumn derivatization with ninhydrin or reversed-phase liquid chromatography with UV or fluorescence detection. Cation-exchange separation typically takes 1 h of run time, while reversed-phase methods take 45 min.[30] Both of these techniques require either pre- or postcolumn derivatization of Trp to achieve high- to mid-level picomole sensitivity. Derivatization adds complexity to the assay, requires additional labor, and increases uncertainty in the results. In this Application Note, the use of *AAA-Direct* for Trp determinations is investigated in proteins, peptides, and in cell cultures or fermentation broths. *AAA-Direct* technology provides both complete separations of all common amino acids using anion exchange chromatography (AminoPac™

PA10) and the direct detection of amino acids by integrated pulsed amperometric detection (IPAD).[31, 32] We present a new isocratic method designed to rapidly elute Trp while separating it from free amino acids, carbohydrates, and peptide fragments. Trp is then directly detected with good sensitivity (low picomole level quantities injected). This method reduces the run time to as short as 12 min, significantly increasing throughput compared to conventional techniques.

EQUIPMENT

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

- GP50 or GS50 Gradient Pump, microbore, PEEK, with degas option
- ED50 Electrochemical Detector with AAA-Certified Gold Cell
- AS50 Autosampler and Thermal Compartment with 25-µL injection loop (0.0100-in. i.d.)
- EO1 Eluent Organizer, including three 2-L plastic bottles and pressure regulator

PeakNet® Chromatography Workstation

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

Vacuum hydrolysis tubes (8 x 60 mm, 1 mL; Pierce, P/N 29550ZZ or equivalent)

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

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

Nitrogen; 4.8 Zero grade, 99.998%, < 5 ppm oxygen (Praxair Specialty Gases); optional

Argon; 5.0 Ultra High Purity grade, 99.999%, < 1 ppm oxygen (Praxair)

Helium; 4.5 grade, 99.995%, < 5 ppm oxygen (Praxair)

Three-way stopcock valve (VWR, P/N 59097-58)

Vacuum tubing, 1/4 in. x 5/8 in. (VWR, P/N 63012-140)

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

Filter unit, 0.2 µm Nylon (Nalgene 90-mm Media-Plus, Nalge Nunc International, P/N 164-0020 or equivalent nylon filter apparatus)

Vial, 0.3 mL, polypropylene, microinjection, 12-32 mm screw thread (Sun International, P/N 500-118)

Septum, pre-slit Teflon®/silicone and polypropylene screw thread cap (for the microinjection vial; Sun International, P/N 500-061

REAGENTS AND STANDARDS

Reagents

Deionized water, 18 MΩ-cm resistance or higher

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

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

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

Dextrose, monohydrate powder (glucose; J.T. Baker, P/N D08717)

Standards

Tryptophan (Sigma, P/N T-1029 or equivalent), FW 204.2

Samples

Luteinizing Hormone-Releasing Hormone (LH-RH; Sigma, P/N L-7134); FW 1182.3 *p*Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. Dry weight contained 90% peptide and peptide was 98% pure; 1 Trp residue per molecule.

Bovine Serum Albumin (BSA), 7%; National Institute of Standards and Technology (NIST), Standard Reference Material 927c; MW 66430, 71.57 g/L; 2 Trp residues per molecule.

Escherichia coli (E. coli) cell culture (Strain A19, met(+), [delta]tonA; courtesy of Stanford University, Palo Alto, CA). The culture was grown in a defined glucose minimal media supplemented with glucose, inorganic salts, amino acids (including Trp), vitamins, metal ions, and buffer (MOPS, pH 7.2).

Bacto YPD Broth, dehydrated (BD Biosciences, P/N 242820YPDBROTH), consisting of Bacto Yeast Extract, Bacto Peptone, and Bacto Dextrose.

CONDITIONS

Columns:	AminoPac PA10 Analytical (2 x 250 mm, P/N 055406) with AminoPac PA10 Guard (2 x 50 mm, P/N 055407)
Flow Rates:	0.25 mL/min (Method 1) or 0.35 mL/min (Method 2)
Injection Volume:	10 µL
Temperature:	30 °C (Method 1) or 40 °C (Method 2)
Eluents:	A) Water B) 250 mM Sodium hydroxide C) 1.0 M Sodium acetate D) 1.0 M Sodium acetate with 50 mM sodium hydroxide
On-line Degas:	30 s every 4 min
Detection:	Integrated pulsed amperometry, AAA-Certified Gold Cell (P/N 055832) Standard combination pH–Ag/AgCl reference electrode, pH reference mode (P/N 042899)
Background:	60–160 nC
Typical System Operating Backpressure:	2300–2540 psi (Method 1) 3220–3560 psi (Method 2)

Programmed Method 1, 0.25 mL/min, 30 °C:

Time (min)	%A	%B	%C	%D	Comments
Init.	0	20	80	0	Autosampler fills sample loop
0.0	0	20	80	0	Valve from load to inject
30.0	0	20	80	0	End of run

Programmed Method 2, 0.35 mL/min, 40 °C:

Time (min)	%A	%B	%C	%D	Comments
Init.	0	0	0	100	Autosampler fills sample loop
0.0	0	0	0	100	Valve from load to inject
15.0	0	0	0	100	End of run

Waveform for the ED40/ED50*:

Time (seconds)	Potential (volts) vs. pH	Integration (begin/end)
0.00	+0.13	
0.04	+0.13	
0.05	+0.33	
0.21	+0.33	Begin
0.22	+0.60	
0.46	+0.60	
0.47	+0.33	
0.56	+0.33	End
0.57	–1.67	
0.58	–1.67	
0.59	+0.93	
0.60	+0.13	

* For the most current waveform, see the *Installation Instructions and Troubleshooting Guide for the* AAA-Direct *Amino Acid Analysis System*.[33]

PREPARATION OF SOLUTIONS AND REAGENTS

Eluents

Water

Water is used in eluent preparation and sample dilution. The presence of trace protein impurities become significant after hydrolysis because the free Trp that is released appears as a background peak that compromises trace level analysis. Without hydrolysis, the Trp is not apparent.

To qualify water used for sample dilutions, hydrolyze an aliquot using the chosen hydrolysis procedure and measure the background peaks. Filters for sample preparation should also be qualified. Water used for this Application Note was 18 MΩ-cm resistance or higher and was filtered by Nylon filters manufactured without electrochemically active surfactants or leachable residue. Water used as eluent is placed under helium or nitrogen at 4–5 psi to reduce adsorption of carbon dioxide and reduce microbial contamination.

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 water. Place this solution immediately under helium or nitrogen at 4–5 psi to reduce an accumulation of carbonate that can reduce retention times.

1.0 M Sodium Acetate

To prepare 1 L of eluent, dissolve 82 g of AAA-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 and then place it under 4–5 psi helium or nitrogen to reduce microbial contamination. Use of cellulose or polysulfone-based filters may introduce electrochemically-active impurities into the eluent, and should be avoided. Other filter brands and types should be qualified prior to use.

1.0 M Sodium Acetate with 50 mM Sodium Hydroxide

To prepare 1 L of eluent, dissolve 82 g of AAA-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 and then add 2.6 mL of 50% (w/w) low-carbonate sodium hydroxide. Place this solution immediately under helium or nitrogen at 4–5 psi.

SAMPLE PREPARATION

Preparation of Labware for Use in *AAA-Direct* Analysis

Clean the borosilicate glass vacuum hydrolysis tubes with suitable detergents prior to use to reduce carryover from previous samples and to obtain low limits of detection for the method. In this Note, hydrolysis tubes were cleaned with RBS-35 (Pierce) according to the manufacturer's directions.

Stock Standard

Trp (51.1 mg) was dissolved in 10.0 mL of purified water to make a 5.11 mg/mL or 25 mM Trp stock solution.

Working Standard

The 25 mM Trp stock solution was diluted 250-fold to make 100 µM Trp solution. This solution was further diluted in water to make calibration standard solutions, or in 4 M NaOH, protein, peptide, dextrose, or broth solutions to evaluate spike recovery from these matrices.

Stock Protein and Peptide Solution

Commercial proteins and peptides normally are available as solid materials that are weighed and then dissolved as needed. The solid mass consists of the specific protein or peptide of desired sequence and composition, and protein/peptide impurities of non-desired sequence and composition, salt (as counter-ions), buffer residue, and adsorbed water. Other "impurities" may also be present. The accuracy of the peptide (or protein) concentration is dependent on accurate measures of these components and correction for the mass of these components during preparation of solutions. Accurate protein or peptide concentrations are important for accurate quantitation of Trp in protein and peptide hydrolysates.

In this Note, the solid peptide (LH-RH) mass provided by the manufacturer was weighed and dissolved in purified water to a concentration of 1.0 mg/mL. The percent peptide composition reported on the certificate of analysis (90%) was used to correct for the buffers, salts, and water present in the solid, resulting in an assumed concentration of 0.90 mg/mL of peptide. The percent purity of the peptide reported on the certificate of analysis (98%) was used to correct for the mass of peptide of specified sequence and composition, yielding a 0.88 mg/mL of pure peptide. BSA was obtained from NIST as a 7% solution (certificate of analysis: 71.57 mg/mL). The resulting molarity of the peptide (or protein) solution was calculated using the following equation:

$$\frac{\text{(mg/mL of pure peptide or protein)}}{\text{(molecular weight of protein or peptide)}} = \text{molarity}$$

The molecular weights used for LH-RH and BSA were 1182.3 and 66430, respectively[34]. Protein and peptide solutions were diluted in purified water to yield 100 µM concentrations of stock protein or peptide solutions. Stock solutions were stored frozen until needed.

Hydrolyzing Reagents

4.4 M Sodium Hydroxide (NaOH)

Combine 2.3 mL of 19 M (50% w/w) sodium hydroxide with 7.7 mL of purified water.

Hydrolysates

The 100 µM protein, peptide, and Trp standards were diluted 10-fold in 4.4 M NaOH to make 10 µM solutions in 4.0 M NaOH. These 10 µM solutions were transferred in 300-µL aliquots into glass hydrolysis tubes using Pasteur pipettes. A vacuum (20–25 in. Hg) was applied to the hydrolysis tube/cap assembly,

followed by an argon or nitrogen (10 psi) replacement to the headspace using a three-way stopcock valve. After 10 cycles of vacuum and inert gas replacement, the tubes were sealed and heated at 110 °C for designated times ranging from 10 min to 24 h. Solutions that were not heated served as a zero time-point hydrolysate. Solutions were transferred into microinjection vials after hydrolysis and directly injected (10 µL) for analysis by *AAA-Direct*.

E. Coli Cell Culture

E. coli cell culture was grown in culture flasks incubated at 37 ºC and agitated at 280 rpm. Time points of 0, 1, 2, 3, 4, 5, and 6 h were removed from the culture, and immediately centrifuged at 16,000 x g for 8 min to remove particulates such as cells and cell debris. The supernatant was diluted in purified water 65-fold and directly injected for *AAA-Direct* Trp analysis (10 µL). The measured concentrations of Trp were corrected by the dilution factor to reflect the actual culture concentrations.

Yeast Broth

Bacto YPD Broth was dissolved (5.0 g) in water (100 mL) to make a 50 mg/mL solution, the standard concentration used for yeast cultures. Aliquots of the 50 mg/mL solution were centrifuged at 16,000 x g for 10 min, and the supernatant was diluted 100-fold for *AAA-Direct* analysis. The 50 mg/mL supernatant was also diluted 100-fold and spiked with 10 µM Trp to evaluate recovery from the broth matrix.

Dextrose

Dextrose (glucose, 2.0 g) was dissolved in water (10 mL) to make a 1.0 M (200 mg/mL) solution. This was diluted 10-fold to make 100 mM dextrose. The 1.0 M dextrose solution was also diluted 10-fold and spiked with 10 µM Trp to evaluate recovery from 100 mM dextrose.

Quantitation

Calibration

Calibration curves were produced using Trp standards dissolved in water at concentrations ranging from 1 to 1000 µM. The peak area slopes of these curves were used to demonstrate linearity and quantify unknowns. Peak height slopes were used to estimate lower limits of detection and quantitation (see "Limits of Detection and Quantitation" section below). Routine quantification was performed using the response factor derived from a single standard concentration of 10 µM Trp.

Limits of Detection and Quantitation

The limit of detection (LOD) is defined in this Note as the concentration of Trp calculated from three times the peak-to-peak noise (a height value) measured at the 1-min interval encompassing the retention time of Trp, divided by the slope of the peak height calibration curve. The LOQ is the concentration derived from 10 times this noise.

Recovery

Percent recovery is the ratio of measured Trp concentration in the peptide and protein hydrolysates or other matrices relative to the expected concentrations. The expected concentrations in protein or peptide hydrolysates were equal to the molar concentration of the protein or peptide times the number of Trp residues expected per molecule of protein or peptide. One Trp residue was expected for each LH-RH molecule, and two Trp residues were expected for each BSA molecule.[34] Percent spike recovery is defined as the concentration of Trp measured in a matrix, minus the endogenous concentration, relative to the known amount added.

RESULTS AND DISCUSSION

Chromatography (Selectivity)

The elution of Trp using a complex gradient designed to separate all the common amino acids has previously been published.[31, 33] Trp was eluted with 700 mM sodium acetate and 40 mM NaOH using the AminoPac PA10 column. In this work, we present isocratic conditions using high concentrations of acetate and NaOH optimized for retention time, detector response, baseline noise, and convenience. As acetate and NaOH concentrations in the eluent are increased, retention times shorten. As NaOH concentration increases, detector response (peak area or height) also increases until an optimal concentration of 50 mM is reached, at which point the response begins to decrease. When column temperature and/or flow rate increases, retention times are shortened. Increases in flow rate,

Table 1. Rapid Trp Analysis Methods

	Method 1	Method 2
Acetate (mM)	800	1000
NaOH (mM)	50	50
Flow Rate (mL/min)	0.25	0.35
Column Temperature (°C)	30	40
Retention Time (min)	18.30	9.53
Baseline Noise (pC)	62	121
Peak Area (x10^6)*	11.18	8.64
Peak Height (pC, x10^3)*	28.6	41.1

*10-µL injection of 10 µM Trp in water

temperature, and the application of eluent proportioning cause more baseline noise. Based on these observations, we developed the two methods in this Application Note (Table 1). Method 1 uses the recommended flow rate, column temperature, and eluents already available in the eluent bottles used for complete amino acid analysis described in the *AAA-Direct* manual.[33] Method 2 requires the preparation of a fourth eluent that is either placed in the fourth unused eluent channel or used to replace one of the other eluents. Method 2 increases the flow rate to 0.35 mL/min and the column temperature to 40 °C. Although this method requires additional eluent preparation and maintenance, it reduces run times to 12 min compared to 18 min for Method 1. The previously published method[31-33] for complete amino acid analysis (including Trp) has a run time of 75 min. Because Method 2 operates at higher flow rates and temperature, the baseline noise is elevated. On the other hand, the use of an eluent that is not proportioned from several eluent channels helps to lower the baseline noise level slightly. Therefore, Method 2 is better suited for high-throughput Trp analysis, while Method 1 is better suited for occasional Trp analysis and for using the *AAA-Direct* system for complete amino acid analysis. Figure 1 shows chromatograms of a 10 µM Trp standard using Methods 1 and 2, respectively.

Retention of many commonly known ingredients of biological samples have been documented using the gradient method for complete amino acid separation.[33] With the exception of urocanic acid, Trp was the latest eluting compound. The phosphoamino acids eluted much earlier than Trp using either of the two methods and did not interfere with Trp analysis. None of the common amino acids or carbohydrates co-eluted with

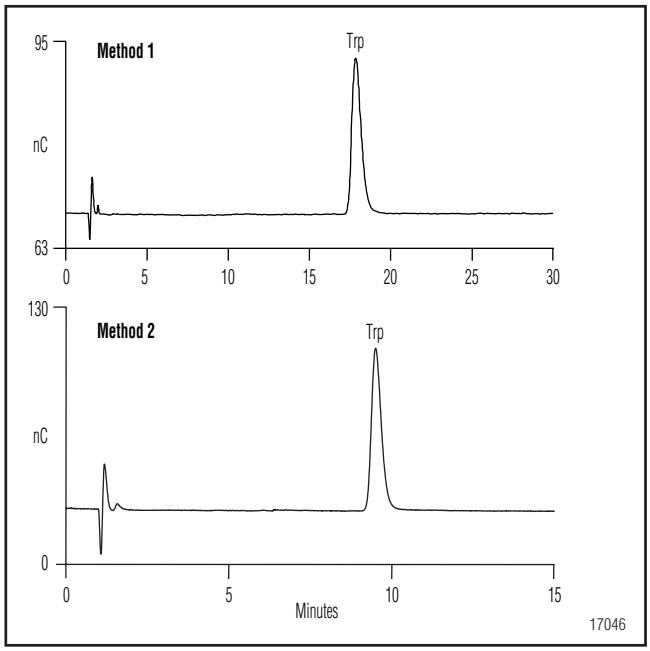

Figure 1. Fast separation of 10 µM Trp (10-µL) using the AminoPac PA-10 with high sodium acetate Eluents (Method 1 and Method 2, see Table 1).

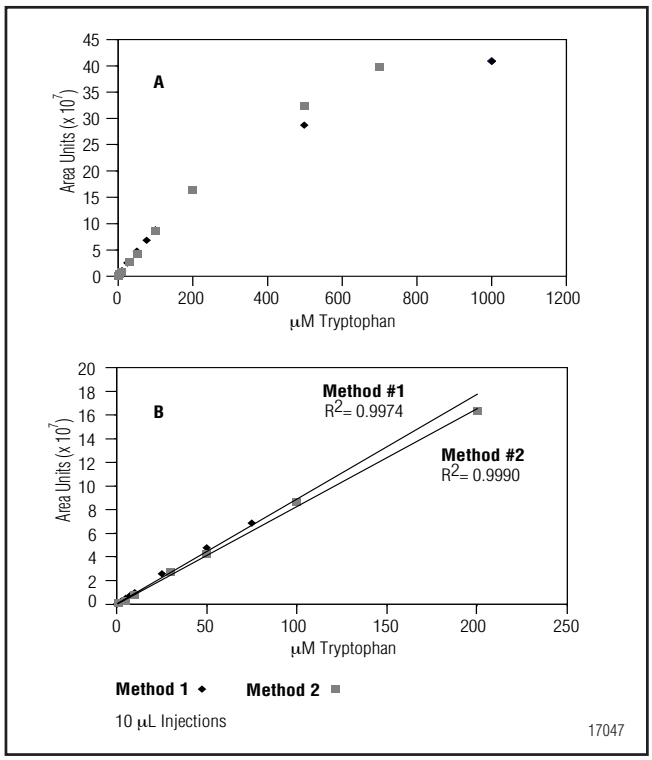

Figure 2. Trp calibration curves using AAA-Direct.

Trp. The retention times were the same for 10-µL injections of Trp dissolved in either 4 M NaOH or water. Retention time relative standard deviation (RSD) was 0.6% over 80 h, 144 injections.

Linearity

Figure 2A shows the peak area calibration curves for Trp analyzed by both Methods 1 and 2 between 1 and 1000 μM Trp. Between 1 and 200 μM, the r^2 values were 0.9974 and 0.9990 for Method 1 and 2, respectively. This indicates the possibility of linear calibration up to 100–200 μM (10-μL injections). The slopes for both methods are nearly identical. The Trp response factors for Method 1 decreased by more than 10% at concentrations between 75 and 100 μM Trp (750–1000 pmol per injection), indicating an upper limit of linearity for Trp. Method 2 has an upper limit between 200 and 500 μM Trp (2000–5000 pmol). Similar linearity results are observed for peak heights, although the actual height values (slopes) differed due to the shorter retention time of Method 2 compared to Method 1. Figure 3 shows a chromatogram of an overloaded Trp analysis (5000 pmol) and the resulting peak distortion that produces lower response. Non-linear curve-fitting techniques (e.g. second degree polynomials) can extend the accuracy above these levels for both peak area and height. Using sodium acetate not certified for *AAA-Direct* eluent can reduce the Trp response and may require a restoration of the working electrode[33].

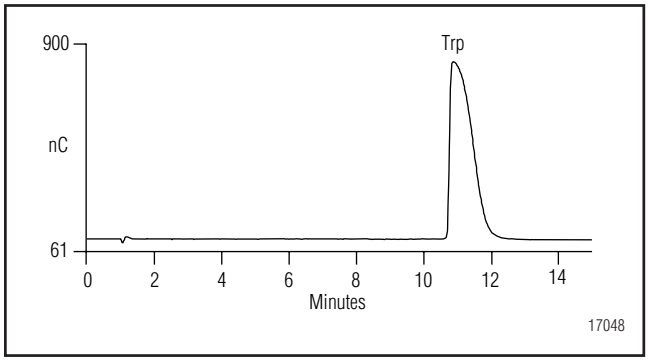

Figure 3. Separation of Trp (5000 pmol) at an overloaded condition.

Lower Limits of Detection and Quantitation

LOD in this Note is based on the measured baseline noise for a blank injection within the region of the chromatogram where Trp would elute. In this study, baseline noise for Method 1 ranged from 60 to 90 pC, and Method 2 ranged from 60 to 230 pC. Both methods show sensitivity down to the low pmol level for Trp. Table 2 shows the estimated LOD and LOQ. The LOD ranged from 0.12 to 0.14 μM (10-μL injection, 1.2 to 1.4 pmol per injection) for both methods. The LOQ ranged from 0.41 to 0.46 μM. Though the baseline noise is generally higher for Method 2, both methods yield similar detection limits because the greater peak height response in Method 2 compensates for the increase in noise.

Baseline noise can vary slightly for different reagents used to make eluents and for different states of system equilibration, but high deviations from the above LOD and LOQ values indicate that there are other problems (e.g. pump not primed, leaks, malfunctioning electrochemical cell, etc.). Consult the *AAA-Direct* manual for the recommended repair.[33]

Table 2. Lower Limits of Detection and Quantitation

	Mean ± SD (n = 3)			
	pmol		μM	
	LOD[a]	LOQ[b]	LOD[a]	LOQ[b]
Method 1	1.4 ± 0.5	4.6 ± 1.6	0.14 ± 0.05	0.46 ± 0.16
Method 2	1.2 ± 0.3	4.1 ± 0.9	0.12 ± 0.03	0.41 ± 0.09

[a]Based on three times the peak-to-peak noise from a 10-μL injection of 4 M NaOH blank
[b]Based on 10 times the noise

Reproducibility

The peak area RSD for 144 uninterrupted 25-μL injections at the 10 μM concentration extending over 80 h was 2.4 % using Method 1. The RSD for the first 12 injections was 1.7%. No upward or downward trending was observed (Figure 4), indicating the system response was stable over this period.

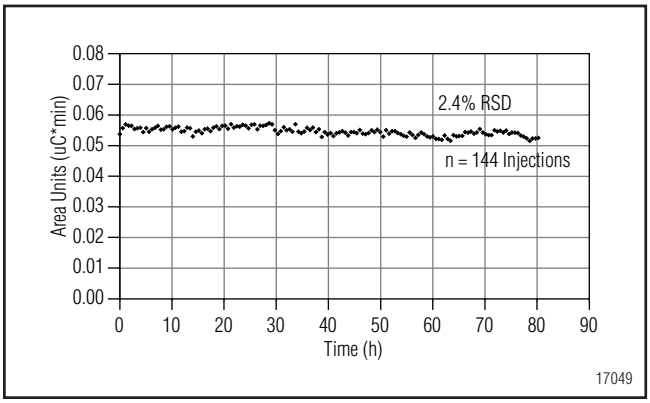

Figure 4. Trp peak area reproducibility over 80 h.

Percent Recovery from BSA Hydrolysates

Trp is completely recovered from a 4 M NaOH matrix (without heat treatment); however, Trp is destroyed in this matrix during hydrolysis at 110 ºC. Table 3 shows the loss of Trp over time in 4 M NaOH without the addition of an inert gas in the headspace.

Table 3. Recovery of Trp During Alkaline Hydrolysis of Bovine Serum Albumin (BSA) in the Absence of Inert Gas Headspace Protection

	% Tryptophan Recovery[a]	
Hydrolysis Time (h)	BSA Mean ± Standard Deviation[b]	Trp (Neat) Mean
0	0.5 ± 0.9	98.1
0.17 (10 min)	12.3 ± 0.9	95.3
0.5 (30 min)	38.2 ± 2.4	82.9
1	68.3 ± 1.5	68.7
2	75.3 ± 0.9	44.7
3	67.6 ± 0.7	38.1
4	56.2 ± 1.5	24.7
5	40.5 ± 0.7	17.3
6	37.5 ± 0.6	5.2
7	30.6 ± 1.0	5.2
8	24.4 ± 0.6	2.4
15	6.1 ± 0.4	2.6
24 (1 day)	4.0 ± 0.3	2.3
31	2.6 ± 0.2	NA
96 (4 days)	3.6 ± 1.0	NA

[a]Percent expected levels of Trp after hydrolysis in 4 M NaOH at 110 °C using plastic vials without inert gas headspace.

[b]Replicates of three injections of the same sample over 18 h with samples maintained at ambient temperature.

The Trp present in the protein BSA is released throughout the hydrolysis process, reaching a maximum recovery of 75% after 2 h of hydrolysis before it decreases. Free Trp shows a steady loss in concentration during hydrolysis, and only 45% remains after 2 h. The indole group side chain of free Trp is known to react with dissolved oxygen, producing a number of oxidation products such as oxindolylalanine.[35-37] These products are not detected by *AAA-Direct*. Attempts to correct for the losses in Trp by calculation are not possible because the rate of loss is very different between free (neat) Trp in solution and the Trp liberated from proteins (Table 3). For example, the Trp recovery calculated after 8 h hydrolysis of BSA when correcting for the observed losses of neat Trp at 8 h would predict an erroneous 1001% recovery. The presence of other amino acids, peptide fragments, and proteins appears to reduce the rate of decomposition and introduce an additional level of error in corrections across different matrices. The replacement of air (oxygen) with an inert gas in the headspace reduces the concentration of oxygen in solution and reduces the degradation of Trp during hydrolysis. Argon preserved Trp slightly better (69%) than nitrogen (62%) after 2 h of hydrolysis, likely the result of its higher density and slightly lower oxygen content (1 ppm) than nitrogen (5 ppm). Argon can thus be the gas of choice for routine hydrolysis because of the higher recovery obtained.

The use of 1% (v/v) phenol in the 4 M NaOH as an oxygen scavenger[3-5], or the use of 4 M methanesulfonic acid[7] instead of NaOH both produced large baseline disturbances, causing extreme Trp peak distortions. Mercaptomethanesulfonic acid was apparently destructive to the AminoPac PA10 stationary phase. Thioglycolic and thiodiglycolic acids or dodecanethiol[1, 2] were not investigated in this Note as preservatives because sulfur-containing substances are electrochemically active and produce background disturbances. Starch, another preservative commonly used in alkaline hydrolysis,[12, 22] also was not investigated because its hydrolysis would produce extremely high levels of carbohydrates that would likely overload the column and produce baseline disturbances. Exposure of borosilicate glass hydrolysis tubes to 4 M NaOH at 110 ºC for more than than 2–3 h dissolved the glass and the silicate apparently competed for adsorption and/or reaction sites on the gold electrode, thereby reducing Trp detector response. The use of plastic containers for hydrolysis[22] reduced electrode fouling but appeared to extract compounds from the plastic, resulting in extra peaks. In some cases, a substance was extracted from the polypropylene tube during alkaline hydrolysis that eluted at 29 min using Method 1, requiring extended run times. Different sources of plastic vials were found to produce different sizes and types of peaks. However, none of these peaks co-eluted with Trp. Later-eluting peaks may be allowed to elute on the next injection to maintain high throughput. We were unable to find suitable reaction tubes made of plastic designed to hold a vacuum or inert headspace, but speculate that this would make the ideal container

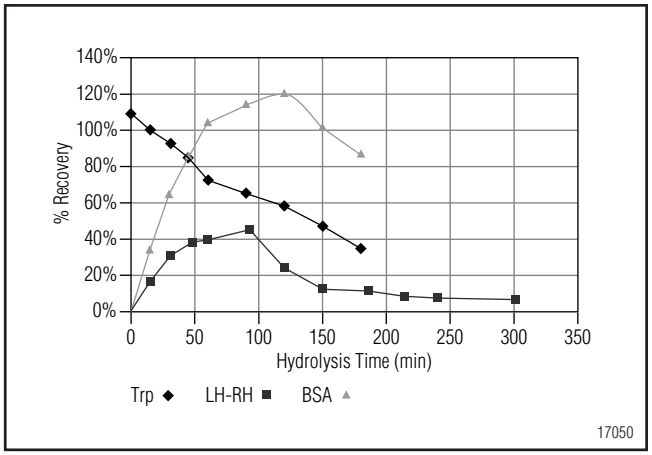

Figure 5. Recovery of tryptophan from the peptide LH-RH and the protein BSA during alkaline hydrolysis under argon.

for hydrolysis. We verified that for hydrolysis periods of less than 2–3 h, the dissolved silicate was not high enough to produce electrode fouling. Although not investigated in this Note, others have adapted glass hydrolysis tubes with plastic inserts. These tubes maintain the evacuation design of the glass tubes and provide a plastic surface to eliminate the dissolution of borosilicates by strong base. Tubes used for hydrolysis should be qualified prior to routine use.

Figure 5 shows the results for the hydrolysis of 10 μM concentrations of neat Trp and BSA in 4 M NaOH at 110 ºC sealed under argon. Figure 6 shows chromatograms of the protein hydrolysate after 0, 30, 60, 90, 120, 150, and 180 min. An initial increase in the concentration of Trp corresponds to its release from the protein's peptide bonds during hydrolysis. Chromatograms in Figure 6 reveal the presence of early eluting peaks that either disappear or are reduced in size during the course of the hydrolysis. These peaks are presumed to be peptide fragments that become digested into free amino acids during hydrolysis. Complete recovery (>100% of theoretical) of Trp was observed between 1 and 2.5 h of hydrolysis, with a maximum recovery of 120% at 2 h. After this time, the Trp is chemically destroyed and the recovery decreases, reaching 87% recovery at 3 h. The neat Trp control, hydrolyzed under identical conditions as the protein showed a steady loss in concentration over time. These percent recoveries are compared in Table 4. The presence of an argon headspace increased the $t_{1/2}$ (time for 50% recovery) from 107 min to 142 min.

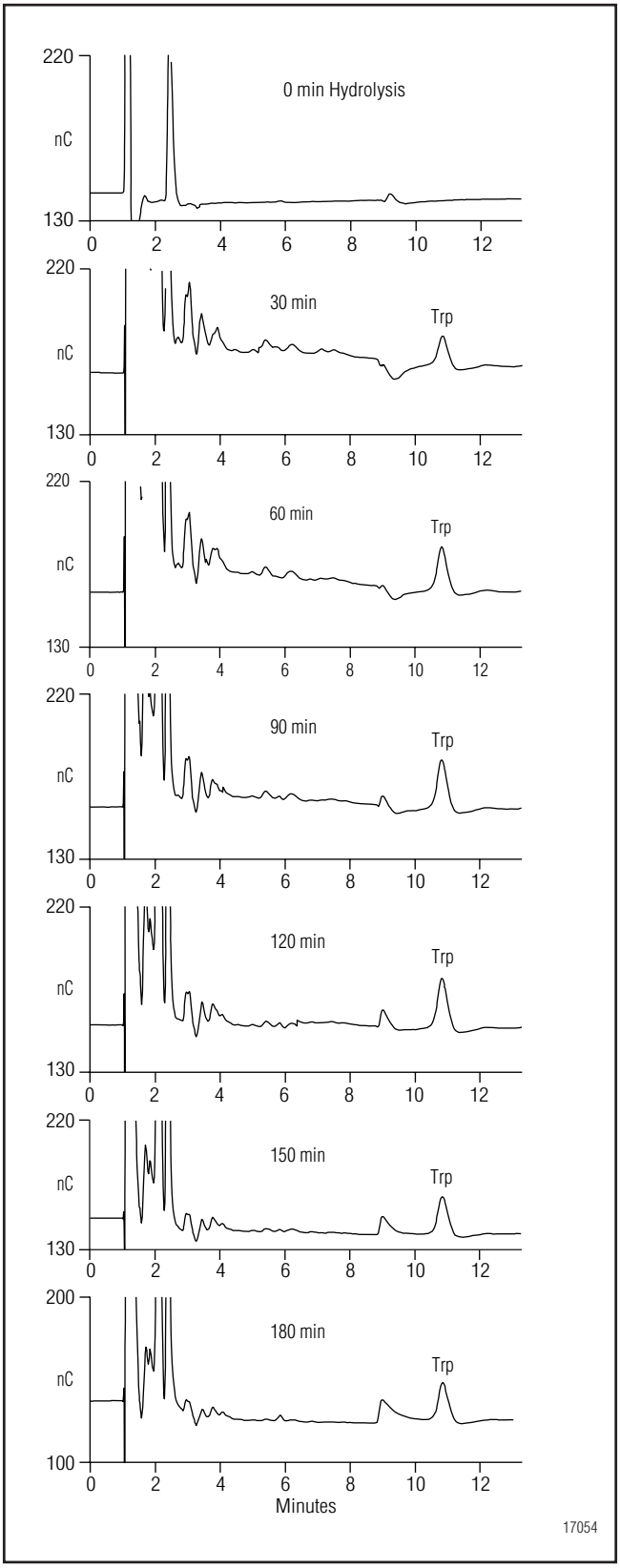

Figure 6. Separation of tryptophan from peptide fragments and other free amino acids during the alkaline hydrolysis of BSA protein under argon.

Table 4. Recovery of Trp During Alkaline Hydrolysis of Free (Neat) Trp, BSA, and LH-RH in the Presence of an Argon Headspace

Hydrolysis Time (min)	% Tryptophan Recovery*		
	Trp (Neat)	BSA	LH-RH
0	109	0	0
15	100	34	17
30	93	65	31
45	85	86	38
60	73	105	40
90	65	114	45
120	58	120	24
150	47	102	13
180	35	87	12

*Percent expected concentration of Trp after hydrolysis with 4 M NaOH at 110 °C using glass hydrolysis tubes with an argon headspace. Average of two replicate injections of the same sample.

Percent Recovery from LH-RH Peptide Hydrolysates

The peptide LH-RH was hydrolyzed under identical conditions as the protein (BSA). Figure 5 also plots the recovery of Trp during the alkaline hydrolysis of LH-RH. Figure 7 shows chromatograms for the separation of Trp from peptide fragments and free amino acids during the course of hydrolysis. At time 0, when no heat was applied to the peptide, a major unidentified peak (1) was observed in the void, and was also present in the 4 M NaOH blank. Minor unidentified peaks (2–5, 7) not observed in the blanks were also observed, indicating that some minor hydrolysis had occurred even without heat treatment. No Trp was detected. At 30 min, the major peak 1 and the minor peaks 2, 4, 5, and 7 decreased, and the minor peak 3 increased and Trp (peak 6) appeared. After 30 min, all unidentified peaks decreased. The recovery of Trp increased for the first 90 min, reaching a maximum recovery of 45%. The complete recovery of Trp from peptides could not be performed using these hydrolysis conditions. The concentration of amino acids in the peptide hydrolysate was probably insufficient to provide the same level of protection observed for the high concentration of amino acids (higher total mass) present in the protein hydrolysates. For the reasons above, the use of correction factors based on the recovery of neat Trp under identical hydrolysis conditions was not accurate.

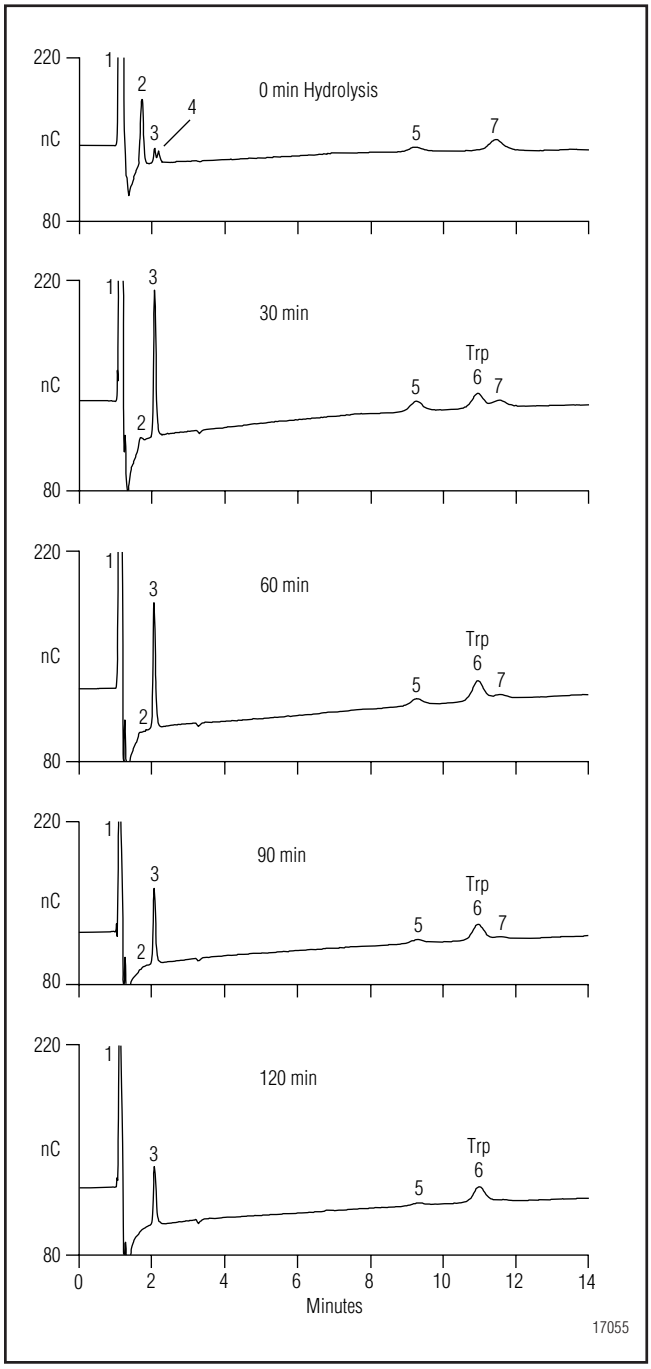

Figure 7. Separation of Trp from peptide fragments and other free amino acids (peaks 1-5, 7) during the alkaline hydrolysis of LH-RH peptide under argon.

Recovery of Trp in High Dextrose (Glucose)

Carbohydrates are also detected by *AAA-Direct*, so the possible interference of glucose was investigated. We selected glucose because it is the most common carbohydrate in food, beverages, and cell culture media and it is typically found at high levels. The measured recovery of 10 µM Trp from 100 mM glucose (2%; w/w) was 99%, and no chromatographic interferences or shifts in retention time were observed for the Trp peak.

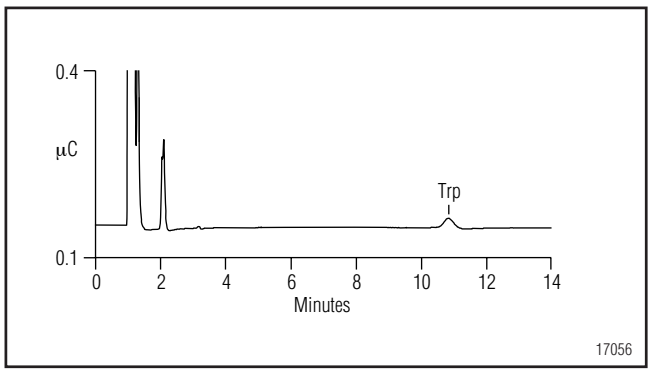

Figure 8. Separation of Trp in E. coli *cell culture using* AAA-Direct.

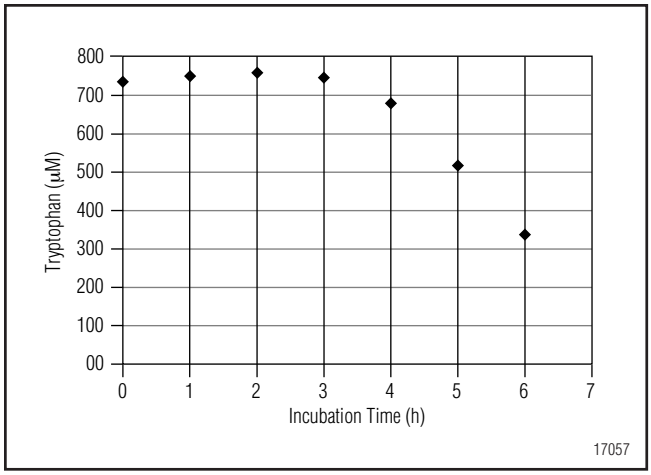

Figure 9. Determination of Trp in E. coli *fermentation broth (cell culture) using* AAA-Direct.

Determination of Free Trp in Cell Culture

The determination of free Trp in cell culture is easily performed using the procedures described in this Note. Unlike the measures of Trp for proteins and peptides that depend on both effective release from peptide chains and low losses from chemical decomposition during hydrolysis, the Trp in cell culture is free in solution and not subject to these constraints. Monitoring the levels of Trp available for use by microorganisms in culture simply involves centrifugation to remove particulates such as cells and cell debris, and dilution in water to adjust concentrations of free Trp to levels suitable for quantitation by *AAA-Direct*. The diluted supernatant is directly injected for *AAA-Direct* determination of Trp. A chromatogram of this separation is presented in Figure 8. Figure 9 plots the measured levels of Trp in an *E. coli* cell culture over its incubation time of 6 h. These results show the loss of Trp after 3 h of incubation resulting from its utilization in the synthesis of new proteins during cell growth. This strain of bacteria can synthesize Trp if necessary, and therefore the observed drop in concentration was apparently the result of this culture's reliance on extracellular resources of this amino acid provided by the culture media to synthesize new proteins before its synthetic pathways are activated. For organisms that cannot synthesize Trp, any limitation to its availability would limit cell growth and the production of any biological products requiring Trp. The limited sample preparation required for direct detection of Trp using *AAA-Direct*, and the short run times possible with the methods presented in this Note allow fermentation chemists to quickly monitor cell culture conditions in order to optimize their process by making timely media supplements.

Determination of Free Trp in Complex Cell Media

The measurement of free Trp in a complex undefined media was also investigated. Bacto YPD broth, containing yeast extract, peptone, and dextrose commonly used for yeast cultures, is a complex mixture of undefined biological substances. Figure 10 shows the chromatogram for a 25-µL injection of a 100-fold dilution of the broth supernatant, and demonstrates the complete separation of Trp from other components of this broth using Method 1. The spike recovery of 10 µM Trp from this 100-fold dilution was 110%. From this analysis, the concentration of free Trp in the undiluted broth was calculated to be 650 µM. The retention times for the Trp standards in water were nearly identical to those obtained in the broth matrix. The presence of high salt or greater sample load appear less critical using the methods presented in this Note than the gradient method. The high recovery of Trp, low chromatographic interferences from matrix components, and the minimal sample preparations possible through direct detection makes this method ideal for rapid quantification of Trp in cell media.

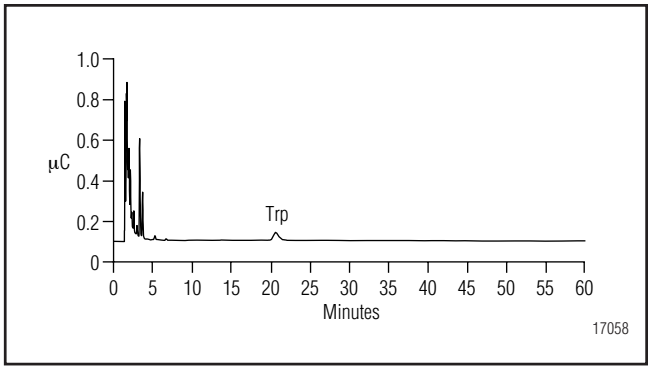

Figure 10. Determination of 6.5 μM Trp in YPD broth (100-fold dilution, 500 μg/mL, 25 μL injection) using Method 1.

CONCLUSION

The *AAA-Direct* system using the AminoPac PA10 column set under strong-eluting conditions provides a rapid method for direct determination of Trp. Two methods that can be used for rapid analysis of Trp in protein hydrolysates and in cell cultures are presented in this Note. The direct detection of *AAA-Direct* for amino acid analysis minimizes the sample preparation needed for the determination of Trp, making this technique less labor intensive than pre- or postcolumn derivatization methods. Additionally, rapid Trp determinations make the monitoring of cell cultures or fermentation processes possible, and provide the opportunity to optimize culture conditions for high productivity.

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