DIONEX

Technical Note 50

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Determination of the Amino Acid Content of Peptides by *AAA-Direct*

INTRODUCTION

The *AAA-Direct* ™ system separates amino acids on a high performance anion-exchange column and directly detects them by integrated amperometry.[1, 2] *AAA-Direct* has advantages over pre- and postcolumn derivatization methods beyond the reductions in sample preparation time and instrumentation complexity. Precolumn derivatization is highly susceptible to interference from the sample matrix and complex sample matrices can reduce derivatization efficiency, causing high variability in amino acid recovery.[3, 4] Furthermore, some amino acid derivatives are unstable.[5] Postcolumn derivatization using ninhydrin cannot be performed in samples containing high levels of ammonia because they form insoluble complexes that can plug the instrument's flow paths.[3] Urea, polyacrylamide, and ammonium ions form ammonia upon acid hydrolysis, which as described above, can complicate analysis using ninhydrin. The sample preparation considerations with *AAA-Direct* are different from those of cation-exchange/chemical derivatization methods. For example, certain surfactants present on lab filter devices that would not produce artifacts by ninhydrin techniques may produce artifacts (e.g., spurious peaks) when used with amperometric detection. This Technical Note describes the peptide hydrolysis procedures that lead to successful determinations of amino acid composition using *AAA-Direct*. Results for the amino analysis of two peptides, Melanocyte Stimulating Hormone (MSH) and Luteinizing Hormone-Releasing Hormone (LH-RH), using both hydrochloric acid and methanesulfonic acid (MSA) as hydrolyzing reagents are presented to demonstrate the performance of this method.

EQUIPMENT

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

- GP50 Gradient Pump, microbore, PEEK, with degas option
- ED40 Electrochemical Detector with AAA-Certified Gold Cell
- AS50 Autosampler and Thermal Compartment with 25-µL injection loop (0.010 in. i.d.)
- EO1 Eluent Organizer, including three 2-L plastic bottles and pressure regulator

PeakNet® Chromatography Workstation

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

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

Microcentrifuge tubes with detachable caps (plastic, 1.5 mL; Sarstedt, P/N 163/204 or equivalent)

SpeedVac Evaporator System (ThermoQuest Savant E/C Division) consisting of:

- SpeedVac, Model SVC100
- Refrigerator Vapor Trap, model RVT400
- Vacuum Gauge, Model VG-5
- Welch Duo-Seal Vacuum Pump, model 1402 capable of pulling 0.2 Torr (200 µm Hg) vacuum

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

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

Three-way stopcock valve (VWR Scientific, P/N 59097-058)

Vacuum tubing, $^1/_4$ in. x $^5/_8$ in. (VWR Scientific, P/N 63012-140)

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

Helium (4.5 grade, high purity 99.5%)

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

Vial, 0.3 mL, polypropylene, microinjection, 12-32 mm screw thread (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)

Amino Acid Abbreviations

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

REAGENTS AND STANDARDS

Reagents

Deionized water, 18 MΩ-cm resistance or higher

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

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

Hydrochloric acid, 6 M constant boiling sequenal (Pierce Chemical, P/N 24309)

Methanesulfonic acid, 4 M (Pierce Chemical, P/N 25600)

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

Standards

Amino acids in 0.1 M/L hydrochloric acid; Standard Reference Material 2389 (National Institute of Standards & Technology)

Norleucine, DL (Sigma, P/N N-6752)

Tryptophan (Sigma, P/N T-1029)

Threonine (Sigma, P/N T-8375)

Samples

α-Melanocyte Stimulating Hormone (Sigma, P/N M-4135); FW 1664.9

N-acetyl Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

Dry weight contained 78% peptide; peptide was 98% pure

Luteinizing Hormone-Releasing Hormone (Sigma, P/N L-7134); FW 1182.3

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

Dry weight contained 90% peptide; peptide was 98% pure

CONDITIONS

Columns:	AminoPac® PA10 Analytical (2 x 250 mm, P/N 055406) with AminoPac PA10 Guard (2 x 50 mm, P/N 055407)
Flow Rate:	0.25 mL/min
Injection Vol:	10 µL (Full or Partial mode)
Temperature:	30 °C
Eluents:	A: Water B: 250 mM Sodium hydroxide C: 1.0 M Sodium acetate
In-line Eluent Traps:	Anion Trap Column (IonPac® ATC-1, 9 x 24 mm, P/N 037151) installed between eluent bottles and degas module[6]
Typical System Operating Backpressure:	< 3000 psi

Programmed Method

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

On-line Degas: 30 sec every 4 min

Detection: Integrated pulsed amperometry AAA-Certified Gold Cell (P/N 055832); Ag/AgCl reference electrode, pH reference mode

Waveform for the ED40*

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, please consult the Installation Instructions and Troubleshooting Guide for the *AAA-Direct* Amino Acid Analysis System.

PREPARATION OF SOLUTIONS AND REAGENTS

Water

Water is used in eluent preparation and sample dilution. The qualification of water for eluent use is less rigorous than for sample use. The presence of trace protein impurities becomes significant after hydrolysis because the free amino acids that are released appear as background peaks that compromise trace level analysis. Without hydrolysis, the amino acids are not apparent.

To qualify water for use in sample dilutions, hydrolyze an aliquot using the chosen procedure and measure the background peaks. Filters are often manufactured using glycerol or other electrochemically active surfactants that appear either as high background levels when used as eluent or as large interfering peaks when used as sample diluent. Any water that is filtered during its purification should be qualified. Water used for this Technical Note was 18 MΩ-cm resistance or higher and was purified by filters manufactured without electrochemically active surfactants. Water used as eluent is filtered through a 0.2-µm nylon filter unit and then placed under helium at 4–5 psi to 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 at 4–5 psi to reduce an accumulation of carbonate that can result in shifting retention times.

1.0 M Sodium Acetate

To prepare 1 L of eluent, dissolve 82 g anhydrous *AAA-Direct* Certified sodium acetate in ~450 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 to reduce microbial contamination. The use of cellulose polysulfonic filters can introduce electrochemically active impurities into the eluent and should be avoided. Other filter brands and types should be qualified prior to use. The use of noncertified acetate can result in suddent or gradual loss in detector response.

SAMPLE PREPARATION

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

Clean the 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, vacuum hydrolysis tubes were cleaned with RBS-35 (Pierce Chemical) according to the manufacturer's directions. Pasteur pipettes used to transfer samples to and from the vacuum hydrolysis tubes were prewashed with three volumes of purified water.

Preparation of Amino Acid Standards

The amino acid standard mix, obtained from the National Institute of Standards & Technology (NIST), consists of 17 amino acids (but not Trp or NorLeu) at concentrations ranging from 1.2 to 2.9 mM. Each amino acid concentration is defined on the Certificate of Analysis. We diluted this NIST amino acid standard mix with water to concentrations ranging from 120–290 µM. Trp was weighed and reconstituted in purified water to a concentration of 5.11 mg/mL (25 mM), then diluted to 250 µM in water. NorLeu (internal standard) was reconstituted to a concentration of 1.0 mg/mL (7.62 mM), then diluted to 250 µM in water. These solutions were stored frozen until needed. The 120–290 µM NIST amino acid standard mix was combined with the 250 µM Trp and NorLeu standard solutions to make 12–29 µM, 4.8–11.6 µM, and 1.2–2.9 µM concentrations of the amino acid

standard mix for use as calibration standards. The 250 µM NorLeu solution was also added to blanks and peptide solutions at 10 or 25 µM concentrations as an internal standard. Standard solutions were analyzed without hydrolysis by direct injection (10 µL), and their peak areas used for calibration and the quantification of amino acids in hydrolysates.

A Thr solution (25 µM) for autosampler qualification was prepared by weighing 11.9 mg of solid and dissolving it in 10 mL of purified water to make a 1.19-mg/mL solution (10 mM). This was diluted to 1.0 mM by combining 1.0 mL with 9.0 mL water, and further diluted to 25 µM by combining 0.250 mL of 1.0 mM with 9.75 mL water.

Preparation of Peptide Solutions for Hydrolysis

Based on the percent mass of the peptide described on the label of each commercially prepared peptide, prequalified purified water was added to prepare a 1.0-mg/mL peptide solution. MSH and LH-RH solutions were prepared at concentrations of 605 µM and 846 µM, respectively. The peptide concentration selected for each hydrolysis condition was based on the results of prequalified reagents, labware, and other factors as discussed in the "Results and Discussion" section of this Note. For HCl hydrolysis, the peptides were diluted to 10 µM with water that contained 10 µM NorLeu (internal standard). For MSA hydrolysis, peptides were diluted to 100 µM and contained 100 µM NorLeu. Residual salt and buffer concentrations were assumed to be negligible (< 50 mM) at these peptide concentrations.

Hydrolysis of Peptides Using 6 M HCl

For each peptide, a 300-µL aliquot of the 10 µM solution was transferred, in triplicate, to 1.5-mL microcentrifuge tubes. Triplicate 300-µL aliquots of purified water, containing 10 µM NorLeu, were also transferred to microcentrifuge tubes as blanks for subtracting background peaks. All tubes were dried using a SpeedVac Evaporator for 1–2 h. Samples (including blanks) were then reconstituted in 100 µL of 6 M constant boiling HCl and the contents transferred to hydrolysis tubes using prewashed glass Pasteur pipettes. Hydrolysis tubes were evacuated using a vacuum (~25 in. Hg), and the headspace was replaced with nitrogen (low oxygen grade) using a three-way stopcock valve. The vacuum/nitrogen cycle was repeated three times and the tubes were sealed with a nitrogen headspace. Samples were hydrolyzed for 17 h in a 110 °C heating block. Samples and blanks were cooled to ambient temperature and transferred to 1.5-mL microcentrifuge tubes and the HCl was evaporated to dryness using the SpeedVac Evaporator for approximately 2 h. Samples and blanks were reconstituted in 300 µL purified water and stored frozen until analyzed by *AAA-Direct*. All samples and standards were directly analyzed (10-µL injection) by *AAA-Direct*. Each sample was injected four times.

Hydrolysis of Peptides Using 4 M MSA

MSA is used as a hydrolyzing reagent for the determination of acid-labile amino acids such as Trp, Ser, and Thr. Use of MSA is slightly more complicated than use of HCl in that it requires neutralization and at least a 10-fold dilution of the reconstituted hydrolysate. For each peptide, a 300-µL aliquot of the 100-µM peptide and NorLeu solution was transferred in triplicate to 1.5-mL microcentrifuge tubes. Triplicate 300-µL aliquots of purified water containing 10 µM NorLeu were also transferred to microcentrifuge tubes as blanks for subtraction of background peaks. The 10 times higher concentration of peptides and NorLeu used in MSA hydrolysis compared to the HCl hydrolysis accounted for the 10-fold dilution needed for MSA hydrolysates. All tubes were dried using the SpeedVac Evaporator for about 2 h. Samples (including blanks) were then reconstituted in 30 µL of 4 M MSA and then transferred to hydrolysis tubes using prewashed Pasteur pipettes. Hydrolysis tubes were evacuated by vacuum and the headspace was filled with nitrogen as described for the HCl hydrolysates. Hydrolysis was performed for 1 h at 165 °C. Samples were cooled to ambient temperature and transferred to 1.5-mL microcentrifuge tubes. Additional nonhydrolyzed blanks for use in preparing amino acid standards with equivalent residual salt (without hydrolysis) are also prepared by pipetting 30 µL of 4 M MSA into the 1.5-mL microcentrifuge tubes. Samples and blanks were neutralized with 30 µL of 4 N sodium hydroxide and evaporated to dryness using the SpeedVac Evaporator (approximately 2 h). Hydrolyzed samples and water blanks were reconstituted in 300 µL purified water and then diluted 10-fold by combining 50 µL with 450 µL of water. Amino acid standards were prepared by combining 300 µL of 120–290, 48–116, and 12–29 µM standard solutions

containing, respectively, 250, 100, and 25 µM Trp and NorLeu to the nonhydrolyzed MSA/NaOH blanks, and then diluting 10-fold by combining 50 µL with 450 µL of water. All samples, blanks, and standards were stored frozen until analyzed by *AAA-Direct*. All samples and standards were directly analyzed (10-µL injection) by *AAA-Direct*. Each sample was injected four times.

Quantification of Amino Acids

For each nonhydrolyzed amino acid standard analyzed, the peak area of each amino acid minus the mean corresponding peak area of any peak eluting at the same retention time of the nonhydrolyzed water blanks is divided by the corresponding molar concentration of the amino acid standard injected to calculate its respective response factor (RF). RF is presented in terms of area units per µM. The RF is calculated for each of the three concentrations of standards that are within their linear range, and the average RF is used to calculate the concentrations in hydrolyzed samples and blanks. The peak area of each identified amino acid in the hydrolyzed peptide samples and hydrolyzed water blanks divided by the respective RF equals the concentration of that amino acid in the solution injected. The concentration of each amino acid in the peptide hydrolysate minus the concentration in the hydrolyzed water blank equals the final measured amino acid concentration for the peptide. When samples and blanks are diluted to concentrations different from the standards, then the measured concentrations are corrected for the dilution factor to calculate the final concentration. The ratio of this measured concentration to the theoretical amount that would be expected based on known amino acid sequence data is the percent recovery.

When an internal standard (IS) correction is included in the calculations, the peak area for the IS measured in each hydrolyzed sample or blank is divided by the mean peak area of that IS in the nonhydrolyzed blank containing an equivalent concentration of the IS. This ratio of sample IS peak area to blank IS peak area is used as a multiplier for all other peaks in that sample chromatogram, and the derived area unit for each peak is then divided by the RF to determine "IS-corrected concentrations" of hydrolysates. The subtraction of IS-corrected concentrations in hydrolyzed water blanks from the IS-corrected concentrations of peptide hydrolysates yields the final concentrations. The use of IS should automatically correct for any dilutions.

To estimate the minimum concentration of amino acids that can be analyzed in hydrolysates using the adopted procedure (and reagents), water blank hydrolysates are analyzed and the concentration of each amino acid is determined. The highest of the amino acids concentrations measured in the blanks should set the background level for the other amino acids. In this Technical Note, approximately 10 times the highest background concentration is the minimum target concentration for hydrolysate samples analyzed by *AAA-Direct*.

RESULTS AND DISCUSSION

Analysis of Standards and System Prequalification

Figure 1A (page 6) shows a typical separation of the 12–29 µM amino acid standard mix, including 25 µM Trp and NorLeu, using a gradient method designed for the analysis of hydrolysates. Figure 1B shows a 1.2–2.9 µM amino acid standard mix using the same gradient method. When the acetate gradient begins at 11 min, the baseline rises about 30 nC and a system peak elutes. If the rise exceeds 50 nC, wash the column with 250 mM sodium hydroxide (100% B) for 30 min. The presence of extra peaks during a blank injection could indicate that the sample water is contaminated, the ATC-1 traps were not installed[6] or have exhausted their capacity, or the eluents contain contaminants. Steps should be taken to minimize these problems before analyzing samples. When baseline rise and extraneous peaks are reduced to an acceptable level, the injection of a standard mix should resemble the chromatogram in Figure 1A or 1B. The *Installation Instructions and Troubleshooting Guide for the* AAA-Direct *Amino Acid Analysis System* offers detailed troubleshooting guidelines.[6]

Autosampler performance is the single most important factor for assuring high precision during analysis of replicate injections. The performance of an autosampler can be evaluated easily with the following test: Equilibrate the AminoPac PA10 column with 90 mM sodium hydroxide for about 1 h at 0.25 mL/min. Inject a 25 µM solution of Thr and discard the first injection. Then inject this solution four times using injection volumes of 2, 5, 10, 15, and 20 µL. Injections are made with the Partial injection mode from a 25-µL loop. Run time is 10 min. The offset function of the

Column:	AminoPac™ PA10 Analytical and Guard
Eluent:	60 mM NaOH for 2 min, convex gradient to 90 mM NaOH over 6 min, and then 90 mM for 3 min. Convex sodium acetate gradient from 0 to 400 mM with concomitant NaOH gradient of 90 to 50 mM over 7 min, then a linear NaOH gradient from 50 to 40 mM in 400 mM sodium acetate over 3 min, followed by a linear sodium acetate gradient from 400 to 700 mM in 50 mM NaOH over 2 min, and then held for 19 min. The column is washed with 200 mM NaOH for 2 min, and then equilibrated to the starting eluent conditions of 60 mM NaOH for 31 min.
Temperature:	30 °C
Flow Rate:	0.25 mL/min
Inj. Volume:	10 µL A: Partial mode injection B: Partial LS mode injection
Detection:	Electrochemical (IPAD), *AAA-Direct* gold electrode
Sample:	A: 12-29 µM NIST Amino Acid Standards with 25 µM Trp and NorLeu B: 1.2-2.9 µM NIST Amino Acid Standards with 2.5 µM Trp and NorLeu

Peaks:			
	1. Arg	8. Pro	15. Phe
	2. Lys	9. Ile	16. Glu
	3. Ala	10. Leu	17. Asp
	4.Thr	11. Met	18. Cys
	5. Gly	12. NorLeu	19. Tyr
	6. Val	13. System	20. Air
	7. Ser	14. His	21. Trp

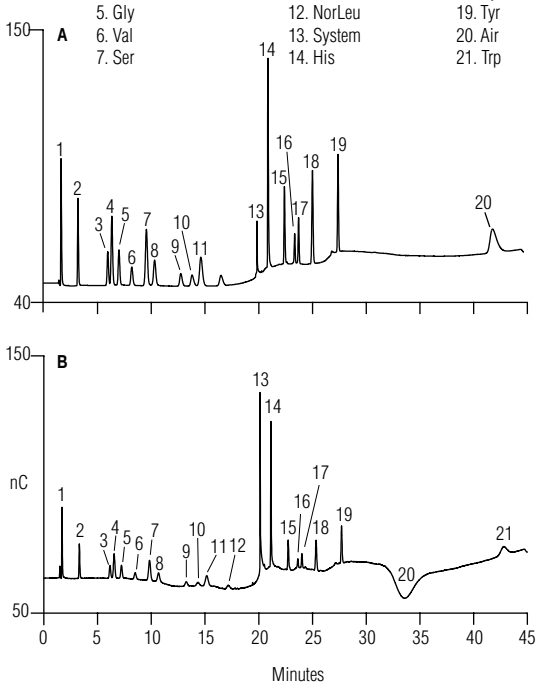

Figure 1. Chromatograms of non-hydrolyzed amino acid standards, A) 12–29 µM and B) 1.2–2.9 µM.

ED40 is turned off. The Thr peak area RSD should be less than 2% for 5- to 20-µL injections, and less than 4% for 2-µL injections. The r^2 for the linear regression line of peak area vs. injection volume using the complete data set should be greater than or equal to 0.998. High RSD values at any of the injection volumes or a low r^2 value for the line indicate that the autosampler should be repaired. Consult the autosampler operator's manual for troubleshooting guidelines. The Thr retention times for all injection volumes should be equivalent and have an RSD of < 1%. High retention time RSDs usually indicate pump performance problems.

Record the background level, measured in nC, at a time zero minutes for each injection (90 mM NaOH eluent). The average background should be less than 90 nC and the RSD should be ≤ 5%. Background levels exceeding this upper limit indicate probable contamination of the eluent (water or hydroxide). Peak-to-peak noise is measured in each chromatogram for the interval between 0–1 min using the Report function in PeakNet. Noise exceeding 800 pC for any of the chromatograms may indicate pump or detector malfunction. Consult the gradient pump and electrochemical detector operator's manuals for troubleshooting guidelines.

When a new column is installed, reproduce the test chromatogram to confirm expected performance. GP50 Pump and ED40 Electrochemical Detector performance can be evaluated using procedures described in their operating manuals. Full validation services are available from Dionex.

Preparation of Peptide Samples for Hydrolysis

Acid Hydrolysis Methods

Many amino acid hydrolysis procedures are described[7] that were designed primarily to improve recoveries of sensitive or hard-to-release amino acids. By far the most common method used is hydrochloric acid (6 N) hydrolysis.[7-9] However, it is limited by the poor recoveries expected for Trp, Cys, Ser, and Thr due to their partial decomposition; for Ile and Val due to slow release; for Met due to oxidation; and because Asn and Gln hydrolyze to Asp and Glu, respectively. For many applications, the poor recovery (or conversion to free acid form in the cases of Asn and Gln) of these amino acids is an acceptable compromise for the ease of use.

Alternatively, methanesulfonic acid (4 M MSA) hydrolysis[10,11] is used to measure Trp. However, when this method is used with *AAA-Direct* it requires a minimum 10-fold dilution because of the residual sodium hydroxide and the MSA remaining after hydrolysis and neutralization; consequently, larger amounts of sample are needed to perform an analysis. MSA hydrolysis can be performed in 1 h at 165 °C, making it faster than the typical 16–17 h HCl hydrolysis. Alkaline hydrolysis with 4.2 M NaOH for 18 h at 110 °C has also been used for Trp analysis.[8] Its application with *AAA-Direct* has also been demonstrated in the *Installation Instructions and Troubleshooting Guide* in

the "Analysis of Tryptophan" section.[6] For this Technical Note, HCl and MSA hydrolysis methods were compared for recoveries, but other hydrolyzing conditions using 50:50 (v/v) propionic acid hydrochloric acid[12-14] have been shown to be feasible using *AAA-Direct* (results not presented).

Note: Mercaptoethanesulfonic acid hydrolysis[14-16] must not be used because it irreparably damages the AminoPac PA10 column.

Estimating the Minimum Amount of Sample to Hydrolyze

The preparation of peptide samples for hydrolysis begins with a prediction of the quantity of sample needed for analysis. Generally, it is desirable to minimize the amount of peptide required. This minimum amount depends on the lower method limits of detection or background levels for each amino acid, number of each amino acid residue in the peptide, dilution required, number of injections desired, and type of injector used. The extent of release and degradation of amino acids during hydrolysis is also a consideration but should not be a criterion for predicting the amount needed for analysis. If poor recovery is observed, an alternative hydrolysis procedure should be selected rather than increasing the amount of sample hydrolyzed. In this Technical Note, 300 µL of a 10 µM peptide (3 nmol) solution was more than adequate for the two peptides (MW 1200 and 1700) studied. This quantity of sample can be reduced when the contributing factors for estimating starting amounts are found to be favorable. These contributing factors, discussed below, include:

- Background levels of amino acids
- Molar concentration of amino acids in peptides
- Dilution requirements
- Replicate analysis
- Injector requirements

Background Levels of Amino Acids—The sensitivity of the detector for each amino acid (baseline noise together with gradient-related artifacts for a given column) influences the instrumental detection limits. The presence of minor background peaks in the blanks from labware, reagents, and other sources after complete sample preparation represents the most important contribution to experimental error. The size of minor background peaks is the best guide to estimating the minimum levels of amino acids required for sample hydrolysis. The size of minor background peaks should always be documented by analyzing hydrolyzed water blanks using the chosen hydrolysis procedure and measuring the concentrations of each residual amino acid or peaks coeluting with amino acids in these blanks (Figure 2B).

Figures 2A and 2B (page 8) show the increase in background peaks before and after hydrolysis, respectively. The highest amino acid concentration measured in the hydrolyzed blanks serves as the worst-case estimate for the background concentration of all amino acids. Table 1 presents concentrations of amino acids found in 6 M HCl and 4 M MSA hydrolyzed water to exemplify the appearance of background peaks in blanks for a typical laboratory operation. Arg was the amino acid with the highest concentration (0.88 µM) in the HCl-hydrolyzed water blank, and Phe had the highest concentration (1.25 µM) in the MSA hydrolysate. These levels (and types of amino acids) will vary from lab to lab and over time. For this Technical Note, approximately 10 times the highest background concentration observed in the hydrolyzed blank (10 x 1 µM = 10 µM) served as the minimal target concentration for hydrolysate samples analyzed by *AAA-Direct.* Higher concentrations of peptide samples can further increase accuracy, but the derived amino acid concentrations should not be greater than their upper limit of linearity. Most are linear to 100 µM for a 10-µL injection. Arg, Lys, His, Gln, and Asn are linear to about 10–20 µM for a 10-µL injection.[1]

When exceedingly high background peaks are observed, it is necessary to investigate their source (e.g., water, hydrolyzing reagents, hydrolysis tubes, volumetric labware, etc.) and make necessary corrections. For example, the use of improperly selected filters, desalting columns, or other sample preparation devices used to pretreat samples can lead to high background concentrations (Figure 2C). Prerinsing the filters can reduce these interfering peaks, but selecting filters without electrochemically active surfactants used during their manufacture is the best choice. Other labware (such as tubes and pipettes), hydrolyzing reagents, and water can have protein or peptide contaminants that are observed only after hydrolysis. The direct detection capability of *AAA-Direct* makes investigations of background sources easy to perform.

Column: AminoPac™ PA10 Analytical and Guard

Eluent: 60 mM NaOH for 2 min, convex gradient to 90 mM NaOH over 6 min, and then 90 mM for 3 min. Convex sodium acetate gradient from 0 to 400 mM with concomitant NaOH gradient of 90 to 50 mM over 7 min, then a linear NaOH gradient from 50 to 40 mM in 400 mM sodium acetate over 3 min, followed by a linear sodium acetate gradient from 400 to 700 mM in 50 mM NaOH over 2 min, and then held for 19 min. The column is washed with 200 mM NaOH for 2 min and then equilibrated to the starting eluent conditions of 60 mM NaOH for 31 min.

Temperature: 30 °C

Flow Rate: 0.25 mL/min

Inj. Volume: 10 µL

Detection: Electrochemical (IPAD), *AAA-Direct* gold electrode

Sample: A: Nonhydrolyzed water blank
B: Hydrolyzed water blank with 25 µM NorLeu
C: Nonhydrolyzed water filtrate

Peaks:
1. Arg/surfactant
2. Lys
3. Unknown
4. Thr
5. Gly
6. Val
7. Ser
8. NorLeu
9. System
10. Unknown
11. His
12. Unknown
13. Phe
14. Unknown
15. Asp
16. Unknown
17. Unknown

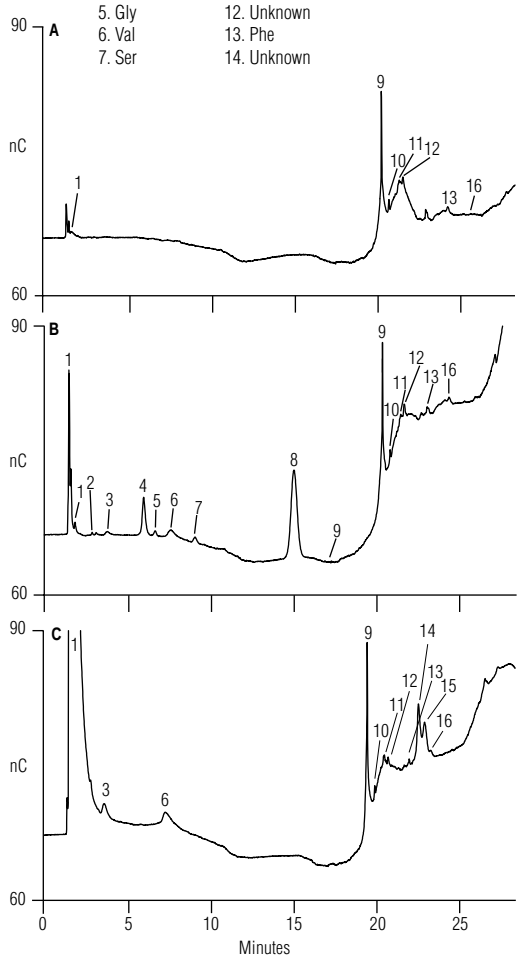

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Figure 2. Chromatogram of water A) without hydrolysis, B) with hydrolysis, and C) surfactant bleed-off from filters.

Table 1 Background Levels of Amino Acids in an HCl and MSA Hydrolyzed Water Blank

Amino Acid	HCl (6M)		MSA (6 M)	
	Average Conc.[a] (µM)	10X (µM)	Average Conc.[a] (µM)	10X (µM)
Arg	0.88	8.80	0.62	6.20
Lys	ND[b]	ND	ND	ND
Ala	ND	ND	0.54	5.40
Thr	ND	ND	ND	ND
Gly	ND	ND	ND	ND
Val	ND	ND	ND	ND
Ser	0.06	0.60	ND	ND
Pro	ND	ND	ND	ND
Ile	ND	ND	ND	ND
Leu	ND	ND	ND	ND
Met	ND	ND	ND	ND
His	ND	ND	0.10	1.00
Phe	0.03	0.30	1.25	12.50
Glu	0.02	0.20	ND	ND
Asp	0.22	2.20	ND	ND
Cys	ND	ND	0.03	0.30
Tyr	0.01	0.10	ND	ND
Trp	ND	ND	0.20	2.00

[a]Concentrations not corrected for internal standard
[b]ND = Not detected

Molar Concentration of Amino Acids in Peptides—Another relevant factor for predicting the amount of peptide sample to hydrolyze is the molar concentration of the amino acids in the peptide. Some peptides contain one or more moles of amino acids per mole of peptide. When these peptides are hydrolyzed, the molar concentration of these amino acids increases in proportion to the number of each residue present. The molar concentration of peptide used for hydrolysis can be lower when the molar concentrations of amino acids are high, but the level selected should be based on the molar concentration of the lowest amino acid concentration expected.

Dilution Requirements—The amount of sample dilution required will also affect the mass of peptide required to perform an analysis. The amount of dilution may depend on the concentration of salts and buffers present in the initial peptide sample, the type of hydrolysis reagent and additives selected, and the availability of an evaporation apparatus. For example, the retention times

decreased less than 5% when the total sample salt injected was less than 50 mM. The percentage of change increased in proportion to the concentration of salt. At 100 mM, the retention times decreased approximately 8–13%, and at 500 mM, 23–59%. Peaks eluting after the start of the acetate gradient, such as His, Phe, Glu, Asp, cystine, Tyr, and Trp, were unaffected by sample salt up to at least 500 mM. Ile, Leu, and Met appeared most affected by sample salt. In general, if desalting techniques cannot be used we recommend that the samples be diluted and/or the standards placed in the equivalent salt solutions.

Some buffers commonly used in biological samples may co-elute with some amino acids. For example, HEPES (10 mM) co-elutes with Ile, Leu, and Arg, and TRIS (10 mM) co-elutes with Arg, hydroxylysine, and Lys. When these buffers are diluted to 1 mM, their interference is reduced to a single amino acid (Leu and Arg, for HEPES and TRIS respectively). Some buffers do not cause any interference at 10 mM (e.g., MES and citrate) or at 1 mM (CHAPSO). When buffers produce interference, they must either be removed by desalting techniques or diluted to reduce the interference.

Another case when samples must be diluted is when the hydrolyzing reagent produces chromatographic interference or when salt is produced from the neutralization of the hydrolyzing reagent. For example, when MSA is used as a hydrolyzing reagent, a peak elutes just before the acetate gradient at 15–20 min (see Figure 3, Peak 12). MSA cannot be removed by evaporative centrifugation and, without neutralization, must be diluted 1000-fold prior to analysis. However, the MSA peak is reduced in size by preneutralizing the MSA by equimolar sodium hydroxide and evaporative centrifugation of the sample. Residual salt remains after evaporation, along with some of the MSA, but now only a 10-fold dilution is required. Even after 10-fold dilution, the amount of residual salt is still high enough to shorten retention times of early-eluting amino acids; consequently, amino acid standards must be placed in NaOH-neutralized MSA matrices to simulate the sample matrix if identification of early eluting amino acids is desired.

Column:	AminoPac™ PA10 Analytical and Guard
Eluent:	60 mM NaOH for 2 min, convex gradient to 90 mM NaOH over 6 min, and then 90 mM for 3 min. Convex sodium acetate gradient from 0 to 400 mM with concomitant NaOH gradient of 90 to 50 mM over 7 min, then a linear NaOH gradient from 50 to 40 mM in 400 mM sodium acetate over 3 min, followed by a linear sodium acetate gradient from 400 to 700 mM in 50 mM NaOH over 2 min, and then held for 19 min. The column is washed with 200 mM NaOH for 2 min and then equilibrated to the starting eluent conditions of 60 mM NaOH for 31 min.
Temperature:	30 °C
Flow Rate:	0.25 mL/min
Inj. Volume:	25 µL (Full Loop mode injection)
Detection:	Electrochemical (IPAD), *AAA-Direct* gold electrode
Sample:	A: 1.2–2.9 µM NIST amino acid standards in water B: 1.2–2.9 µM NIST amino acid standards in 1000-fold diluted MSA (4 mM) C: 1.2–2.9 µM NIST amino acid standards in 100-fold diluted MSA (40 mM)

Peaks:

1. Arg	8. Pro	15. Phe
2. Lys	9. Ile	16. Glu
3. Ala	10. Leu	17. Asp
4. Thr	11. Met	18. Cys
5. Gly	12. MSA	19. Tyr
6. Val	13. System	
7. Ser	14. His	

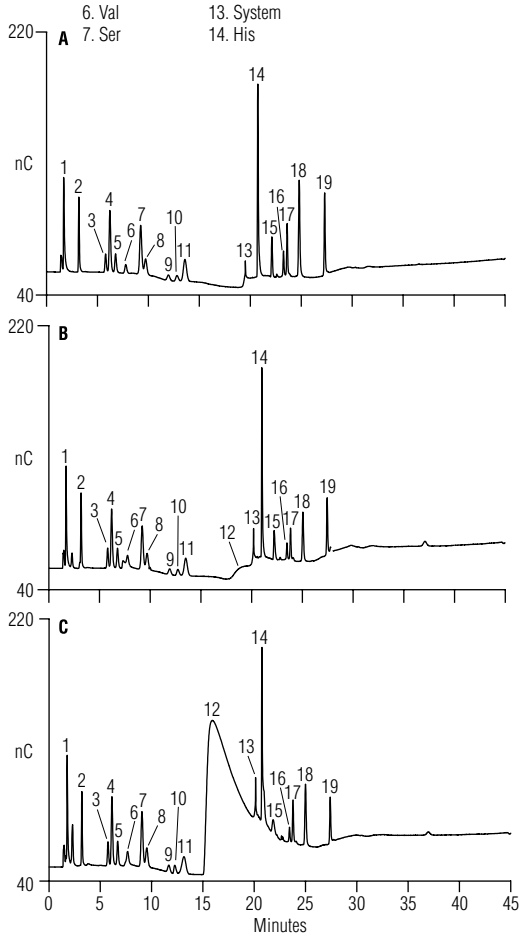

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Figure 3. Chromatograms of NIST AA standards in A) water, B) MSA diluted 1000X, and C) MSA diluted 100X.

Replicate Analysis—The amount of sample required also depends on the number of replicate samples hydrolyzed and the number of replicate injections desired for each sample. Replicate injections provide added confidence in the accuracy and precision of the measured levels for each sample but require a greater amount of each sample. The hydrolysis of replicate samples provides greater assurance that samples are accurately hydrolyzed. The number of replicates is arbitrary but a minimum of duplicate hydrolysates is highly recommended. In this Technical Note, triplicate hydrolysates were analyzed with four injections each. Table 2 shows the precision in amino acid concentration obtained for replicate injections and replicate HCl hydrolysates for two model peptides. For replicate injections of each MSH hydrolysate sample, peak area RSDs ranged from 0.6–5.5% for the amino acids recovered at high levels (excluding Met and Trp). For replicate injections of each LH-RH hydrolysate sample, the area RSDs ranged from 0.7–11% for the amino acids recovered at high levels (excluding Trp). The results showed that precision for replicate injections can be quite high when the system is performing normally. Table 2 also shows the precision for the measured peak area of replicate hydrolysate samples of each peptide. The RSDs for the mean amino acid peak area across replicate hydrolysate samples ranged from 0.9–12% for MSH and 2.5–6.1% for LH-RH. These results show that some variance exists between hydrolysate samples, and suggest that performing replicate hydrolysates is valuable for improving accuracy. Retention time RSDs ranged from 0.0–0.7% for either peptide sample hydrolysate (Table 3).

Table 2 Area RSDs for Replicate Injections of MSH and LH-RH HCl Hydrolysates

Amino Acid	% RSD for Replicate Injections[a]			% RSD of Replicate Hydrolysis[b]
	Replicate Hydrolysis Sample			
	1	2	3	
MSH				
Arg	4.9	0.9	2.3	3.3
Lys	3.3	4.8	3.6	2.7
Gly	1.8	1.5	1.3	11.8
Val	3.8	1.8	1.6	3.2
Ser	1.6	0.7	0.6	1.5
Pro	0.9	2.2	1.8	4.2
Met	LR[c]	LR	LR	LR
His	2.9	3.9	5.5	0.9
Phe	2.0	1.7	1.0	5.5
Glu	3.9	1.3	4.1	6.3
Tyr	1.9	1.8	1.2	1.9
Trp	ND[d]	ND	ND	ND
LH-RH				
Arg	2.8	7.3	4.9	5.0
Gly	2.3	7.0	9.1	3.8
Ser	0.7	7.1	7.2	6.1
Pro	1.3	4.5	6.4	2.7
Leu	3.8	10.7	7.8	3.7
His	1.4	6.7	6.1	3.8
Glu	3.2	5.1	7.6	2.5
Tyr	1.8	5.8	4.1	2.7
Trp	LR	LR	LR	LR

[a]Replicates of four injections of the same hydrolysis sample
[b]Averaged replicates of three different hydrolysis samples
[c]LR = Low recovery
[d]ND = Not detected

Table 3 Retention Time RSDs for Replicate Injections of MSH and LH-RH HCl Hydrolysates

Amino Acid	Retention Time RSD (%) for Replicate Injections[a]			% RSD of Replicate Hydrolysis[b]
	Replicate Hydrolysis Sample			
	1	2	3	
MSH				
Arg	0.7	0.0	0.6	0.3
Lys	0.3	0.3	0.0	0.2
Gly	0.1	0.2	0.1	0.2
Val	0.1	0.3	0.1	0.3
Ser	0.0	0.2	0.1	0.1
Pro	0.1	0.2	0.2	0.2
Met	0.2	0.3	0.5	0.3
His	0.1	0.0	0.0	0.1
Phe	0.0	0.1	0.0	0.1
Glu	0.0	0.0	0.0	0.1
Tyr	0.0	0.0	0.1	0.0
Trp	ND[c]	ND	ND	ND
LH-RH				
Arg	0.6	0.0	0.6	0.5
Gly	0.0	0.0	0.2	0.2
Ser	0.0	0.1	0.1	0.1
Pro	0.1	0.1	0.2	0.2
Leu	0.1	0.1	0.2	0.2
His	0.0	0.1	0.1	0.1
Glu	0.1	0.1	0.1	0.1
Tyr	0.0	0.0	0.0	0.1
Trp	0.1	0.2	0.1	0.2

[a]Replicates of four injections of the same hydrolysis sample
[b]Averaged replicates of three different hydrolysis samples
[c]ND = Not detected

Injector Requirements—The type of injector and the mode of injection also affect the amount of sample needed. The most accurate and reproducible injection mode is the Full Loop mode, but it requires the most sample. A 10-µL Full Loop mode injection requires 50 µL (2.5 x loop size + 25 µL) of sample using the AS50 Autosampler, and 83 µL (1.3 x loop size + 70 µL) using the AS3500 Autosampler (Thermo Separation Products). Partial injection modes can significantly reduce sample waste but may compromise precision and introduce a small amount of air that affects the baseline. The AS50 Autosampler in Partial LS (limited sample) mode has no sample waste; in Partial mode it uses the injection volume plus twice the cut volume. Partial mode injections performed in this Application Note used a 2-µL cut volume; however, 4 µL is recommended to improve reproducibility and further reduce the chance of injecting air. The chromatographic baselines for amino acid standards injected using both injection modes are presented in the *Installation Instructions and Troubleshooting Guide for* AAA-Direct *Amino Acid Analysis System*.[6] The baseline dip observed at 32–35 min results from the injection of a trace amount of air using the Partial LS mode (Figure 1B). This is eliminated when the injection mode is switched to Partial (Figure 1A) or Full Loop mode. The dip did not interfere with the elution of amino acid standards using the gradient conditions described in this Technical Note.

Calculating Final Concentration and Volume of Peptide Sample for Hydrolysis—In preparing for the hydrolysis of peptides, it is necessary to first determine background levels of amino acids and raise this concentration by at least 10-fold to estimate the minimum amino acid concentration needed for this method. The procedure used to determine the worst-case estimate of the background concentration of amino acids should include any dilution or concentration steps needed. For a peptide of unknown composition, this minimal amino acid concentration is normally equivalent to the targeted concentration of the final peptide hydrolysate solution used for analysis (i.e., assume one mole of amino acid per mole of peptide). However, if the amino acid composition of the peptide being hydrolyzed is known, then adjustments to this concentration are made according to the minimal mole ratio of the amino acids present in the peptide. Next, estimate the final volume needed for the peptide concentration. For autosamplers, the total volume is equal to the volume injected plus the amount wasted per injection, times the number of replicate injections. Add an extra volume to the microinjection vial beyond the volume being injected to submerge the injection needle in the vial (~40 µL for a 0.3-mL vial). This is the minimum final volume needed per hydrolysis sample. For example, a four-replicate 10-µL Partial mode injection requires 10 µL plus 8 µL extra (using a 4-µL cut volume) per injection, equivalent to 72 µL (18 µL x 4) for the replicates. The 40 µL of extra volume needed to accommodate the injection needle increases the total volume to 112 µL. This volume of 10 µM peptide hydrolysate equates to 1.12 nmol of peptide for hydrolysis when no dilutions are required. The 10-µM concentration was selected from the results of the worst-case estimate discussed previously in the "Background Levels of Amino Acids" section. This concentration may vary from lab to lab and over time. In this Note, 300 µL (3 nmol) of each peptide hydrolysate was prepared for injection. Replicate hydrolysates will increase this amount of peptide by the number of replicates (e.g., triplicate hydrolysates require 9 nmol). A single 10-µL injection in the Partial LS mode would require 0.5 nmol of peptide for hydrolysis without dilutions.

Analyses of amino acids above the recommended limit of linearity result in the broadening of peaks and deviation in linearity. Dilution of sample to levels that are within the linear range is recommended. Nonlinear curve fitting techniques should be used to improve quantification accuracy when dilution is not possible. Figure 4 (page 12) shows the effect of injecting 10 µL of 100 µM MSH hydrolysate (1 nmol); the inset shows the peak distortion of His when the system is overloaded. Other peak distortions can occur with Arg and Lys. The resolution of Ser/Pro and Glu/Asp can also be decreased when their mass injected exceeds about 100–200 pmol. Even before any peak distortion and/or excessive coelution are observed, the calculated results for some amino acids (Arg, Lys, His) may be too low because the range of linear calibration was already exceeded for those amino acids.

Column: AminoPac™ PA10 Analytical and Guard

Eluent: 60 mM NaOH for 2 min, convex gradient to 90 mM NaOH over 6 min, and then 90 mM for 3 min. Convex sodium acetate gradient from 0 to 400 mM with concomitant NaOH gradient of 90 to 50 mM over 7 min, then a linear NaOH gradient from 50 to 40 mM in 400 mM sodium acetate over 3 min, followed by a linear sodium acetate gradient from 400 to 700 mM in 50 mM NaOH over 2 min, and then held for 19 min. The column is washed with 200 mM NaOH for 2 min and then equilibrated to the starting eluent conditions of 60 mM NaOH for 31 min.

Temperature: 30 °C

Flow Rate: 0.25 mL/min

Inj. Volume: 10 µL

Detection: Electrochemical (IPAD), *AAA-Direct* gold electrode

Sample: 100 µM MSH Hydrolysate (HCl) with 25 µM NorLeu

Peaks:
1. Arg
2. Lys
3. Gly
4. Val
5. Ser
6. Pro
8. NorLeu
9. System
10. His
11. Phe
12. Glu
13. Tyr

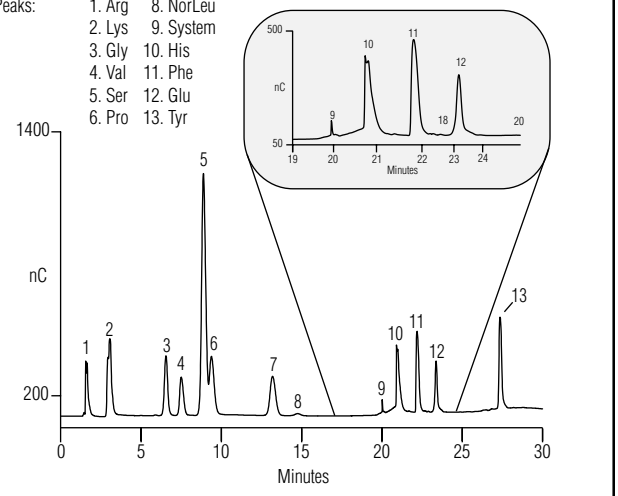

Figure 4. Chromatogram of 100 µM MSH HCl hydrolysate, an overloaded injection.

Table 4 Comparison of Good and Bad Injection Data with and without IS Correction

Amino Acid	Area RSD (%) for Replicate Injections[a]			
	Unadjusted Autosampler		Adjusted Autosampler	
	Without IS Correction	With IS Correction	Without IS Correction	With IS Correction
MSH				
Arg	10.5	6.5	0.9	3.9
Lys	12.4	4.6	4.8	3.6
Gly	11.8	5.6	1.5	4.5
Val	12.6	4.9	1.8	4.6
Ser	12.2	4.8	0.7	3.8
Pro	13.0	4.1	2.2	5.0
Met[b]	123.0	123.9	45.8	47.3
His	10.6	6.8	3.9	4.6
Phe	11.8	5.0	1.7	4.7
Glu	12.4	5.3	1.3	4.1
Tyr	11.2	6.0	1.8	4.9
Trp	ND[c]	ND	ND	ND
LH-RH				
Arg	11.0	8.5	2.8	3.9
Gly	3.8	6.5	2.3	2.8
Ser	7.7	5.3	0.7	3.3
Pro	9.8	6.4	1.3	4.5
Leu	7.2	6.1	3.8	3.3
His	12.6	7.9	1.4	3.9
Glu	10.0	5.4	3.2	2.7
Tyr	8.5	5.6	1.8	2.7
Trp	10.1	6.7	19.3	20.2

[a]Replicates of four injections of a single hydrolysis sample.
[b]Low recoveries (small peak area) of Met were observed and therefore the RSDs were large.
[c]ND = Not detected

Selecting the Appropriate Internal Standard

The use of internal standards is optional, but they provide added assurance if the autosampler or injector fails unexpectedly during an automated schedule or if significant sample losses occur during transfer. Table 4 shows the improvement in area precision obtained for replicate injections of amino acids when an internal standard correction is performed on data collected from an improperly operated (unadjusted) autosampler. If the data is already precise (< 3–4% RSD), the application of an IS will not improve the data and can sometimes make it less precise (see Table 4, adjusted autosampler). However, the use of internal standard correction provides a means to normalize data between different hydrolysate samples. Table 5 shows the mean concentrations for triplicate hydrolysate samples with and without correction for internal standards. Internal standard corrections are recommended for normalizing slight differences in replicate hydrolysate preparation, but to be valuable they must be added at the very beginning of the process when samples are prepared for evaporation before acid hydrolysis. The use of an internal standard is not useful for correcting amino acid degradation during hydrolysis because decomposition rates vary among the amino acids. The best internal standard is one with high stability during hydrolysis, with a retention time that is not the same as that of other peaks or baseline disturbances, and that is not present in the sample.

Table 5 Comparison of Replicate MSH-Hydrolysate Samples with and without IS Correction							
Amino Acid	**Theoretical Conc. (μM)**	**Measured AA Conc. (μM) Replicate Hydrolysates**			**Measured Mean Conc. (μM)**	**Mean Percent Recoverey**	**RSD (%)**
		1	**2**	**3**			
		Without Internal Standard Correction					
Arginine	10	9.0	9.5	9.7	9.4	94%	3.8%
Lysine	10	9.2	9.0	9.5	9.2	92%	2.7%
Glycine	10	8.7	10.8	10.8	10.1	101%	12.0%
Valine	10	8.6	8.8	9.1	8.8	88%	2.8%
Serine	20	16.3	16.3	16.7	16.4	82%	1.4%
Proline	10	8.8	8.9	9.5	9.1	91%	4.2%
Methionine	10	2.0	0.4	0.1	0.8	8%	122.6%
Histidine	10	8.9	8.8	8.9	8.9	89%	0.7%
Phenylalanine	10	8.2	8.6	9.2	8.7	87%	5.8%
Glutamate	10	10.2	11.4	11.3	11.0	110%	6.1%
Tyrosine	10	9.1	8.9	9.2	9.1	91%	1.7%
Tryptophan	10	0.0	0.0	0.0	0.0	0%	NA[a]
		With Internal Standard Correction					
Arginine	10	9.6	9.7	9.9	9.7	97%	1.6%
Lysine	10	9.7	9.2	9.7	9.5	95%	3.0%
Glycine	10	9.3	11.0	11.0	10.4	104%	9.4%
Valine	10	9.1	9.0	9.3	9.1	91%	1.7%
Serine	20	17.2	16.7	17.1	17.0	85%	1.6%
Proline	10	9.3	9.1	9.7	9.4	94%	3.3%
Methionine	10	2.1	4.0	0.1	2.1	21%	94.4%
Histidine	10	9.4	9.0	9.2	9.2	92%	2.2%
Phenylalanine	10	8.7	8.8	9.4	9.0	90%	4.2%
Glutamate	10	10.8	11.7	11.6	11.4	114%	4.3%
Tyrosine	10	9.7	9.1	9.4	9.4	94%	3.2%
Tryptophan	10	0.0	0.0	0.0	0.0	0%	NA

[a] NA = Not applicable

For *AAA-Direct* analysis of HCl hydrolysates, NorLeu is a good choice. However, for MSA hydrolysates, NorLeu may not be completely resolved from the MSA peak, thus citrulline is a better choice. Ornithine does not baseline resolve from lysine or hydroxylysine and norvaline is poorly resolved from serine and proline. For this Technical Note, NorLeu was used as the primary internal standard for both HCl and MSA hydrolysates. Peak 10 of Figure 5 shows the elution of NorLeu standard in a peptide HCl hydrolysate. Peak 11 in Figure 6 shows how NorLeu sometimes is poorly resolved from the MSA (Peak 12); consequently it is not recommended for this type of hydrolysate. Alternatively, citrulline has demonstrated satisfactory resolution from all amino acids, eluting between Lys and Ala, and is recommended for MSA hydrolysates. However, citrulline is acid labile and should be added to the sample after acid hydrolysis.

Drying Samples for Hydrolysis

In this Technical Note, all peptide samples were dried using a SpeedVac Evaporator. Alternatively a lyophilizer can be used, but much less conveniently. Concentrated peptide solutions can be added directly to the hydrolyzing reagent if the final concentration of hydrolyzing reagent is not diluted by more than about 5%. Greater dilutions may require different hydrolysis times or temperatures.

Hydrochloric Acid (6 M HCl) Hydrolysis

Figure 5A shows a separation of amino acids released from the HCl hydrolysis of MSH and Figure 5B presents the separation from the HCl hydrolysis of LH-RH. Figure 5C presents the corresponding water hydrolysis blank. The concentrations of amino acids measured after hydrolysis are presented in Tables 5 and 6 for these two peptides. These tables also present the percent recovery for these amino acids based on the concentrations expected for these hydrolysates. Stable amino acids were recovered at high rates, ranging from 82–110% for MSH and 60–99% for LH-RH. As expected, the percent recovery was much less for unstable amino acids, such as Trp and Met, using HCl.[7] Trp was recovered at only 5–14% for LH-RH and could not be detected in MSH. For MSH, the recovery of Met ranged from 1–20% for replicate hydrolysate samples. Although Ser and Val are regarded as problematic amino acids, their recoveries in this study (65–91%) were similar to the stable amino acids. The use of HCl can be optimized by varying hydrolysis time and temperature and comparing the amounts of amino acids recovered.[5,7] In some cases it may be necessary to perform hydrolysis using two or more incubation times. The use of HCl for hydrolysis can be an acceptable technique when sensitive amino acids such as Trp or Met are known to be absent from the peptide of interest. When Trp must be measured, other hydrolyzing conditions such as MSA are a better choice.

Table 6 LH-RH Amino Acid Concentrations and % Recoveries after HCl Hydrolysis

Amino Acid	Theoretical Conc. (μM)	Measured AA Conc. (μM)* Replicate Hydrolysates			Measured Mean Conc. (μM)	Mean Percent Recoverey	RSD (%)
		1	2	3			
Arg	10	8.0	7.2	7.7	7.6	76%	5.3%
Gly	20	15.6	14.5	15.4	15.2	76%	3.8%
Ser	10	6.9	6.5	7.4	6.9	69%	6.2%
Pro	10	7.8	7.4	7.6	7.6	76%	2.6%
Leu	10	8.4	7.8	8.1	8.1	81%	3.7%
His	10	7.8	7.3	7.4	7.5	75%	3.5%
Glu	10	9.9	9.5	9.8	9.7	97%	2.4%
Tyr	10	6.4	6.0	6.2	6.2	62%	3.2%
Trp	10	0.5	0.8	1.4	0.9	9%	53.1%

*Without internal standard corrections

Column:	AminoPac™ PA10 Analytical and Guard
Eluent:	60 mM NaOH for 2 min, convex gradient to 90 mM NaOH over 6 min, and then 90 mM for 3 min. Convex sodium acetate gradient from 0 to 400 mM with concomitant NaOH gradient of 90 to 50 mM over 7 min, then a linear NaOH gradient from 50 to 40 mM in 400 mM sodium acetate over 3 min, followed by a linear sodium acetate gradient from 400 to 700 mM in 50 mM NaOH over 2 min, and then held for 19 min. The column is washed with 200 mM NaOH for 2 min and then equilibrated to the starting eluent conditions of 60 mM NaOH for 31 min.
Temperature:	30 ºC
Flow Rate:	0.25 mL/min
Inj. Volume:	10 µL (Partial mode injection)
Detection:	Electrochemical (IPAD), *AAA-Direct* gold electrode
Sample:	A: 10 µM MSH Hydrolysate (HCl) with 10 µM NorLeu B: 10 µM LH-RH Hydrolysate (HCl) with 10 µM NorLeu C: Water hydrolysate (HCl) with 10 µM NorLeu

Peaks:

1. Arg	8. Leu	15. Tyr
2. Lys	9. Met (trace)	16. Trp (trace)
3. Ala	10. NorLeu	
4. Gly	11. System	
5. Val	12. His	
6. Ser	13. Phe	
7. Pro	14. Glu	

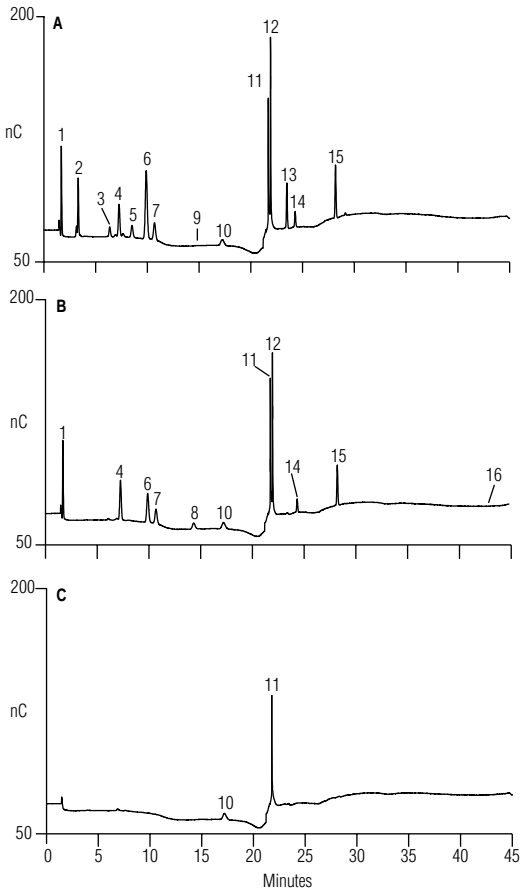

Figure 5. Chromatograms of A) MSH, B) LH-RH, C) water after HCl hydrolysis.

Column:	AminoPac™ PA10 Analytical and Guard
Eluent:	60 mM NaOH for 2 min, convex gradient to 90 mM NaOH over 6 min, and then 90 mM for 3 min. Convex sodium acetate gradient from 0 to 400 mM with concomitant NaOH gradient of 90 to 50 mM over 7 min, then a linear NaOH gradient from 50 to 40 mM in 400 mM sodium acetate over 3 min, followed by a linear sodium acetate gradient from 400 to 700 mM in 50 mM NaOH over 2 min, and then held for 19 min. The column is washed with 200 mM NaOH for 2 min and then equilibrated to the starting eluent conditions of 60 mM NaOH for 31 min.
Temperature:	30 ºC
Flow Rate:	0.25 mL/min
Inj. Volume:	10 µL (Partial mode injection)
Detection:	Electrochemical (IPAD), *AAA-Direct* gold electrode
Sample:	A: 10 µM MSH Hydrolysate (MSA) with 10 µM NorLeu B: 10 µM LH-RH Hydrolysate (MSA) with 10 µM NorLeu C: Water hydrolysate (MSA) with 10 µM NorLeu

Peaks:

1. Arg	8. Pro	15. Phe
2. Unknown	9. Leu	16. Glu
3. Lys	10. Met	17. Tyr
4. Unknown	11. NorLeu	18. Trp
5. Gly	12. MSA	
6. Val	13. System	
7. Ser	14. His	

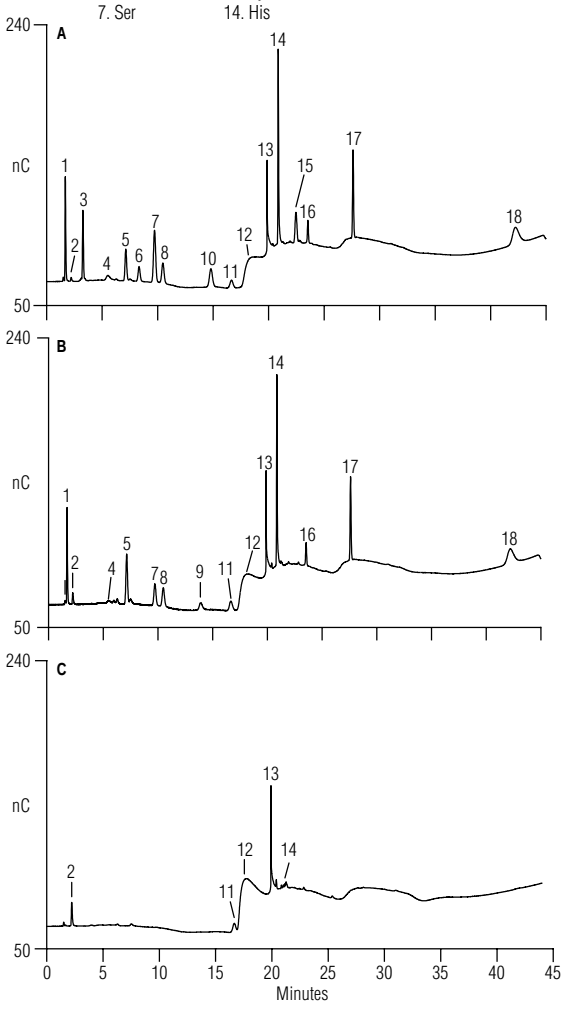

Figure 6. Chromatograms of A) MSH, B) LH-RH, C) water after MSA hydrolysis

Methanesulfonic Acid (4 M MSA) Hydrolysis

Figure 6A shows a separation of amino acids released from the MSA hydrolysis of MSH and Figure 6B presents the separation for LH-RH. Figure 6C shows the water hydrolysate blank. Table 7 presents the concentrations and recoveries of amino acids measured after hydrolysis of these two peptides. Stable amino acids were recovered at high rates, ranging from 95–139% for MSH and 88–101% for LH-RH. The normally unstable amino acids (including Trp, Met, and Glu along with Val, a normally difficult-to-release amino acid) were recovered at about the same levels as the stable and easily hydrolyzed amino acids. Trp was recovered from 85–114% for MSH and 78–86% for LH-RH. Met was recovered from 78–110% for MSH. Ser showed a lower recovery using MSA compared to the HCl hydrolysis method: 58–66% for MSH and 54–55% for LH-RH. It is unknown why Ser recovery was lower than Trp. Ser is normally regarded as more stable than Trp. Use of MSA can be optimized by varying hydrolysis time and temperature and comparing the amounts of amino acids recovered. In some cases it may be necessary to perform hydrolysis using two or more incubation times.

Data Reduction

The data presented in this Technical Note were designed to compare measured levels of amino acids in hydrolyzed model peptides with their theoretical composition. To do this, the expected concentration of each amino acid in the final hydrolysate was calculated from the molar concentration of the peptide used and with the mole percent of each amino acid. For a peptide of unknown composition, the calculation is similar except it is necessary to solve for the mole percentage by measuring the molar concentrations of amino acids and dividing each by the molar concentration of the peptide. Alternatively, for a peptide of unknown

Table 7 MSH and LH-RH Amino Acid Concentrations and % Recoveries after MSA Hydrolysis

Amino Acid	Theoretical Conc. (μM)	Measured AA Conc. (μM)* Replicate Hydrolysates			Measured Mean Conc. (μM)	Mean Percent Recoverey	RSD (%)
		1	2	3			
MSH							
Arg	10	10.6	11.9	13.9	12.1	121%	13.7%
Lys	10	10.2	11.3	12.5	11.3	113%	10.2%
Gly	10	11.1	11.6	13.0	11.9	119%	8.3%
Val	10	9.8	10.8	12.7	11.1	111%	13.3%
Ser	20	11.5	12.1	13.3	12.3	62%	7.5%
Pro	10	9.6	10.7	12.1	10.8	108%	11.6%
Met	10	7.8	9.5	11.1	9.5	95%	17.4%
His	10	10.9	11.6	13.7	12.1	121%	12.1%
Phe	10	9.8	11.7	13.7	11.7	117%	16.6%
Glu	10	10.2	11.7	13.8	11.9	119%	15.4%
Tyr	10	10.6	11.7	13.5	11.9	119%	12.3%
Trp	10	8.5	9.8	11.4	9.9	99%	14.7%
LH-RH							
Arg	10	10.1	10.1	9.7	10.0	100%	2.3%
Gly	20	18.4	18.1	17.8	18.1	91%	1.7%
Ser	10	5.4	5.5	5.4	5.4	54%	0.7%
Pro	10	9.1	8.9	8.8	8.9	89%	1.8%
Leu	10	9.8	9.8	9.3	9.6	96%	2.9%
His	10	10.0	10.0	10.0	10.0	100%	0.0%
Glu	10	10.1	10.6	10.4	10.4	104%	2.4%
Tyr	10	10.0	9.8	9.8	9.9	99%	1.2%
Trp	10	8.6	7.8	8.1	8.2	82%	4.8%

* Without internal standard corrections

composition and molar concentration, the mole percent can be calculated by measuring the molar concentration of one of the amino acids chosen as a reference. This reference amino acid ideally should exist as a single residue and should be well resolved and recovered after hydrolysis. The integer value obtained from this ratio describes the molar ratio of these amino acids. The interpretation of these data assumes that the measured values reflect complete recovery, which occasionally is not accurate, as presented in the percent recovery studies for the model peptides (Tables 6 and 7). These limitations are inherent in any amino acid analysis method where hydrolysis is used.

Other Considerations

A few other factors should be considered when performing amino acid analysis by *AAA-Direct*. When peptide samples require concentration to analyze for amino acids, it is important to consider the simultaneous concentration of any salts that may be present. Concentrations of salt exceeding 50 mM in samples analyzed by *AAA-Direct* cause shorter amino acid retention times.

Some peptides contain amino acid derivatives such as *N*-acetyl serine or pyroglutamate. When peptides contain any derivatives, it is necessary to confirm that the hydrolysis products can be measured by *AAA-Direct*. In the case of *N*-acetyl serine and pyroglutamate, Ser and Glu are the primary products following acid hydrolysis, respectively, as indicated by the high recoveries presented in Tables 6 and 7 for these amino acids. Other amino acid derivatives not studied in this Note should be investigated on a case-by-case basis.

AAA-Direct uses microbore tubing for eluent delivery. Sample loops larger than 25 µL result in appreciable loss of peak resolution and therefore should not be used.

Loss of some proteins and peptides to adsorption to glass surfaces (e.g., transfer pipettes, vials, etc.) can occur, resulting in a loss of apparent recovery. The use of plastic laboratory devices in place of glass or the addition of organic solvents sometimes reduces these adsorptive losses.

On rare occasions, Trp elutes later than normal and may interfere with the baseline distortion associated with the column wash. To correct for this, extend the programmed gradient method described at 42.0 min (14% A, 16% B, and 70% C) by 5 min, changing 42.0 min to 47.0 min. Adjust all subsequent steps in the program by adding 5.0 min to each timed event. The total run time therefore becomes 80 min. Making these changes will assure consistent Trp resolution.

Dionex Corporation is committed to continuous process improvements and therefore continues to make changes in *AAA-Direct* leading to better performance. Consult the most current version of the *Installation Instructions and Troubleshooting Guide for the* AAA-Direct *Amino Acid Analysis System*[6] for any updated operating conditions (e.g., waveform programs, gradient programs, troubleshooting instructions, etc.).

SUMMARY

In this Technical Note, two different hydrolysis methods that can be used with *AAA-Direct* were presented. The hydrolysis using 6 M HCl showed the advantage of easily hydrolyzing samples that have the ability to be completely evaporated without producing excessive residual salt. The disadvantage of this method was the poor recovery of some sensitive amino acids such as Trp and Met. Alternatively, 4 M MSA hydrolysis showed the advantages of the recovery of Trp and Met and also provided very rapid hydrolysis times. Recovery of Ser was low using MSA hydrolysis. The disadvantages of the MSA method were the production of residual salt from neutralization with NaOH and the appearance of the MSA peak, which required the sample to be diluted at least 10-fold.

These procedures demonstrate the capability of *AAA-Direct* to analyze these types of hydrolysates. This Note also presents guidelines for developing hydrolysis methods and offers an overview of important considerations for using *AAA-Direct* for amino acid analysis. The effect of sample salt on chromatography and the presence of electrochemically active substances from filtration devices are important factors that must be corrected for in developing *AAA-Direct* procedures.

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