DIONEX

Application Note 130

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Identification of a Hydroxylysine-Containing Peptide Using *AAA-Direct*™

INTRODUCTION

One type of protein microheterogeneity is a result of the posttranslational modification of lysine (Lys) to hydroxylysine (Hyl). This modification normally occurs in collagen and a few other proteins with domains similar to collagen.[1] Recently, the hydroxylation of lysine has been discovered in recombinant proteins that do not contain collagen-like domains.[2–3] Posttranslational modifications may impact the safety or efficacy of a biotherapeutic product. Therefore, process development and quality control scientists need techniques to measure the type and extent of posttranslational modifications.

This application note shows that *AAA-Direct*™ can be used to determine peptides with lysine hydroxylation. A contract laboratory attempted synthesis of two identical peptides, differing only in the presence of Lys or Hyl. These peptides were intended to have an amino acid sequence identical to a CD4 fragment known to contain either Hyl or Lys. In this application note, the two synthetic peptide samples were hydrolyzed with 6N hydrochloric acid and the amino acid composition determined using *AAA-Direct*. Because the amino acid compositional analysis indicated possible peptide impurities, the peptides were also analyzed using reversed-phase high-performance liquid chromatography (RP-HPLC) and cation-exchange chromatography (ProPac® WCX-10 column). The cation-exchange separation provided greater separation than RP-HPLC, and therefore the major peaks were collected from the cation-exchange column. The high salt content in these samples shifted amino acid retention times when the acid-hydrolyzed samples were analyzed by *AAA-Direct*. Therefore, sample salt was removed prior to hydrolysis. The collected fractions were desalted by microdialysis, and the extent of salt removal was measured using an anion-exchange separation with suppressed conductivity detection of chloride and phosphate in the dialysate. The desalted samples were acid-hydrolyzed and analyzed for amino acids using *AAA-Direct*. All common amino acids, including Hyl, were separated by *AAA-Direct*, and the presence or absence of Hyl in the fractions confirmed peak identities. The techniques described in this application note show several ways that peptides can be evaluated for identity and purity, how peptides can be desalted, and how much desalting is needed for successful *AAA-Direct* amino acid analysis.

EQUIPMENT

Dionex BioLC® Chromatograph System consisting of:
- GS50 or GP50 Gradient Pump, standard bore and microbore, PEEK, with degas option
- AD25 variable wavelength absorbance detector
- ED50 Electrochemical Detector with
  - *AAA-Certified*™ gold cell and standard combination pH/Ag/AgCl reference electrode
  - DS3 conductivity cell
  - ASRS® ULTRA Suppressor
- AS50 Autosampler
- AS50 Thermal Compartment or LC30 Chromatography Oven
- EO1 Eluent Organizer, including three 2-L plastic bottles and pressure regulator

Dionex Summit™ HPLC system consisting of:
- P580 Gradient Pump
- UVD 340S Absorbance Detector
- GINA 50 Autosampler
- STH585 Column Oven

Chromeleon® Chromatography Workstation

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

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

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)

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

Nitrogen; 4.8 Zero-Grade, 99.998%, <5 ppm oxygen (Praxair)

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

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

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

Nylon filter unit, 0.2 µm (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 liner and polypropylene screw thread cap (for the microinjection vial; Sun International, P/N 500-061)

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

Biodialyser™ microdialysis system with 100- and 500-dalton molecular weight cutoff (MWCO) cellulose acetate membranes (AmiKa, Inc., The Nest Group, P/N SSM0500)

REAGENTS AND SAMPLES

Reagents

Deionized water, 18 MΩ-cm resistance or higher

Monobasic sodium phosphate, monohydrate, crystal (J. T. Baker)

Dibasic sodium phosphate, anhydrous, powder (J. T. Baker)

Sodium chloride, ACS grade (VWR)

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

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

AS14A Eluent Concentrate (100X, 800 mM sodium carbonate/100 mM sodium bicarbonate; Dionex Corp., P/N 56937)

Acetonitrile (HPLC Grade; Burdick and Jackson, P/N 015-4)

Trifluoroacetic Acid (TFA; Pierce Chemical, P/N 28901)

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

Standards

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

Hydroxylysine (Hyl; Sigma, P/N H-0377)

Tryptophan (Trp; Sigma, P/N T-1029)

Seven Anion Standard (Dionex, P/N 56933)

Samples

CD4 Peptide Fragment Containing Lysine (Lys-peptide)
Gly-Ser-Phe-Leu-Thr-**Lys**-Gly-Phe-Ser-Lys-NH_2

CD4 Peptide Fragment Containing Hydroxylysine (Hyl-peptide) Gly-Ser-Phe-Leu-Thr-**D/L Hyl**-Gly-Phe-Ser-Lys-NH_2

CONDITIONS

AAA-Direct System for Amino Acid Analysis

Column:	AminoPac® PA-10 (2 × 250 mm, P/N 055406) with AminoPac PA10 Guard (2 × 50 mm, P/N 055407)
Temperature:	30 °C
Flow Rate:	0.25 mL/min
Inj. Volume:	25 µL
Detection:	Integrated pulsed amperometry (reference electrode in pH mode)
Eluents:	A: Water B: 250 mM NaOH C: 1.0 M sodium acetate

Programmed Method for *AAA-Direct**

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, Valve from inject to load

Waveform for the 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 method and waveform, see the *Installation Instructions and Troubleshooting Guide for the* AAA-Direct *Amino Acid Analysis System*.[4]

Reversed-Phase HPLC System for Peptide Separations

Column:	Acclaim® C18, 5 µm, 120 Å, 4.6 × 250 mm (P/N 059149)
Temperature:	25 °C
Flow Rate:	1.0 mL/min
Inj. Volume:	20 µL
Detection:	220 nm
Eluents:	A: 0.1% TFA in water B: 0.1% TFA in acetonitrile

Programmed Method for Acclaim C18 Separation of Peptides

Time (min)	*%A*	*%B*	*Comments*
0.0	100	0	Inject Sample, Begin Gradient
20.0	0	100	End Gradient, Hold
40.0	0	100	
40.1	100	0	Equilibrate to Starting Conditions
55.0	100	0	End Run

Cation-Exchange System for Peptide Separations

Column:	ProPac WCX-10, 4 × 250 mm (P/N 54993)
Temperature:	30 °C
Flow Rate:	1 mL/min
Inj. Volume:	10 µL
Detection:	UV, 210 nm
Eluents:	A: 10 mM sodium phosphate, pH 6.0 B: 10 mM sodium phosphate with 500 mM sodium chloride (pH 6.0)

Programmed Method for ProPac® WCX-10 Separation of Peptides

Time (min)	*%A*	*%B*
0.0	96	4
30.0	30	70
30.1	96	4
45.0	96	4

Anion-Exchange System for Inorganic Anion Separations

Column:	IonPac® AS14A (3 × 150 mm, P/N 056901) with AG14A Guard (3 × 30 mm, P/N 056899)
Temperature:	30 °C
Flow Rate:	0.8 mL/min
Inj. Volume:	5 µL
Detection:	Anion Self-Regenerating Suppressor ULTRA (P/N 053947), 2 mm; 100 mA, AutoSuppression® Recycle Mode using a DS3 Conductivity Cell
Eluent:	8 mM sodium carbonate/1.0 mM sodium bicarbonate
Run Time:	6 min

PREPARATION OF SOLUTIONS AND REAGENTS

AAA-Direct Separation of Hydrolyzed Peptide Amino Acids

Water

Water used in eluent preparation must be qualified for *AAA-Direct* use. Qualification of water is described in Technical Note 50.[5] Water is filtered through a 0.2-µm nylon filter apparatus manufactured without electrochemically active surfactants or leachable residue. Use of cellulose or polysulfone-based filters for any of the eluents used in *AAA-Direct* may introduce electrochemically active impurities into the eluent and should be avoided. Other filter brands and types should be qualified prior to use. Water is placed under nitrogen at 4–5 psi to reduce adsorption of carbon dioxide and microbial contamination.

250 mM Sodium Hydroxide

To prepare 2 L of eluent, combine 26 mL of 50% (w/w) low-carbonate NaOH with 1974 mL purified and prefiltered water (0.2-µm nylon filter, see comments above) as per the instructions described in the *Installation Instructions and Troubleshooting Guide for the* AAA-Direct *Amino Acid Analysis System*.[4] Place this solution immediately under helium or nitrogen at 4–5 psi to limit 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-Direct* Certified anhydrous sodium acetate in ~800 mL purified water, as per the instructions described in the *Installation Instructions and Troubleshooting Guide for the* AAA-Direct *Amino Acid Analysis System*.[4] Adjust the total volume to 1000 mL with additional water. Filter the solution through a 0.2-µm nylon filter unit (see comments above) and place it under 4–5 psi helium or nitrogen.

Reversed-Phase HPLC Separation of Peptides

0.1% TFA in Water or Acetonitrile

Combine 1.0 mL of TFA with 999 mL degassed water or 999 mL degassed acetonitrile. Solutions are placed under helium or nitrogen at 4–5 psi.

Eluents made with TFA may degrade during long-term storage, increasing UV absorbance, and therefore should not be used for longer than two weeks.[6]

Cation-Exchange Separation of Peptides

Two eluents are prepared for the cation-exchange chromatography of peptides: 10 mM sodium phosphate (pH 6.0) and 10 mM sodium phosphate (pH 6.0) with 1.0 M sodium chloride (NaCl). The sodium phosphate buffer system is prepared by diluting appropriate quantities of monobasic and dibasic sodium phosphate concentrate solutions with water to obtain the desired pH 6.0. The relative amount of monobasic and dibasic sodium phosphate solution added differs between an eluent with sodium chloride compared to that without because NaCl

is usually not pH neutral. The following procedure is a recommended starting point for obtaining the desired eluents, but some deviation from this formula may be necessary when using a different source (or lot) of sodium chloride with differing pH. If the pH of the sodium phosphate buffer is not 6.0, then adjust the proportions of monobasic and dibasic solutions added. The combined total amount should remain 100 mL to produce 2 L of 10 mM sodium phosphate.

2M Sodium Chloride

Dissolve 116.90 g of sodium chloride in water, and fill to a final volume of 1.0 L. Filter through a 0.45-µm filter.

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate in 1.0 L of water. Filter through a 0.45-µm filter. Store frozen until needed.

200 mM Sodium Phosphate, Monobasic

Dissolve 27.60 g monohydrate monobasic sodium phosphate in 1.0 L of water. Filter through a 0.45-µm filter. Store frozen until needed.

10 mM Sodium Phosphate, pH 6.0

Combine 14 mL 200 mM dibasic sodium phosphate, 86 mL 200 mM monobasic sodium phosphate, and 1900 mL water.

10 mM Sodium Phosphate with 1 M Sodium Chloride, pH 6.0

Combine 35 mL 200 mM dibasic sodium phosphate, 65 mL 200 mM monobasic sodium phosphate, 1000 mL 2 M sodium chloride, and 900 mL water.

Anion-Exchange Separation of Inorganic Anions

8.0 mM Sodium Carbonate/1.0 mM Sodium Bicarbonate

Combine 20.0 mL of AS14A Eluent Concentrate (100×) with 1980 mL degassed water.

SAMPLE PREPARATION

Preparation of Labware for *AAA-Direct*

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

Preparation of Standards

The amino acid standard mix, obtained from the National Institute of Standards & Technology (NIST), consisted of 17 amino acids (but not Hyl and Trp) at concentrations ranging from 2.4 to 2.9 mM (except 1.2 mM cystine). Each amino acid concentration was reported on the Certificate of Analysis. We diluted this NIST standard with water to concentrations ranging 240–290 µM (except cystine, 120 µM). Hyl and Trp were weighed and dissolved in water to 25 mM concentrations, then diluted to 250 µM in water. These solutions were stored frozen until needed. The 240–290 µM NIST standard was combined with the 250 µM Hyl and Trp standard solutions to make a 9.6–11.6 µM concentration (4.8 µM cystine) of the amino acid standard mix for use as a calibration 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 acid-hydrolysates. To determine the effect of sample salt on amino acid retention times, amino acid standards were diluted with salt solutions to final concentrations of 0, 10, 50, 100, 250, and 500 mM sodium phosphate/NaCl mix, and final amino acid concentrations of 2.4–2.9 µM (except 1.2 µM cystine).

The seven anion standard was diluted 3-, 4-, and 10-fold in water and these standards were used for the experiments that evaluated the effectiveness of desalting.

Preparation of Peptide Solutions

The synthesized peptides were received from the manufacturer in solid form. Based on the Certificate of Analysis, each peptide was dissolved in water to 5.0 mg/mL. The molarity of the solution was calculated from the targeted molecular weight of each peptide. The Lys-peptide and Hyl-peptide solutions had concentrations of 3.5 and 3.4 mM, respectively. Each peptide solution was diluted to its required concentration with water.

Collecting Peptide Fractions

Each peptide was analyzed by cation-exchange chromatography, and the major peaks were manually collected, as they eluted from the UV detector cell, into 1.5-mL microcentrifuge tubes. A single peak was collected from the separation of the Lys-peptide preparation (Figure 4), and four peaks were collected from the separation of the Hyl-peptide preparation (Figure 5).

Desalting Peptide Fractions

The salt concentration of each peak collected from the cation-exchange separation was calculated from the salt gradient applied to the column. Evaporation to increase peptide concentration also increases the salt concentration. To reduce the salt concentration, evaporated samples were reconstituted in 500 µL water and placed into 500-µL-capacity Biodialyzer microdialysis cells (The Nest Group) sealed with 100- and 500-dalton MWCO membranes. These cells were placed into 4 L of water and dialyzed overnight at room temperature. An aliquot of the dialysate (5 µL) was analyzed by anion-exchange chromatography for chloride and phosphate content. The extent of desalting was evaluated against the expected levels of chloride and phosphate present in the dialysate. Desalting devices are qualified for *AAA-Direct* use by analyzing sample blanks after acid hydrolysis by *AAA-Direct*. The absense of interfering peaks qualify the device for *AAA-Direct* use.[5]

Acid Hydrolysis of Peptide Samples and Collected Column Fractions

For each synthesized peptide, a 300-µL aliquot of a 10 µM solution was transferred to a 1.5-mL microcentrifuge tube, evaporated to dryness in the SpeedVac Evaporator, and reconstituted in 100 µL 6 M constant boiling HCl. Each dialyzed column fraction was dried and acid-hydrolyzed with the same protocol. A hydrolysis blank was prepared using 100 µL of 6 M HCl. All samples and blanks were transferred into glass hydrolysis tubes using Pasteur pipettes, evacuated using a vacuum (~25 in. Hg), and the headspace filled with nitrogen (low oxygen grade) using a three-way stopcock valve. The vacuum/nitrogen cycle was repeated ten times and tubes were sealed with nitrogen in the headspace. Samples and blanks were hydrolyzed for 16 h in a 110 ˚C heating block, cooled to ambient temperature, and transferred to 1.5-mL microcentrifuge tubes. The HCl was evaporated to dryness using the SpeedVac Evaporator. Samples and blanks were reconstituted in 300-µL water and either diluted or directly injected (10 µL) for *AAA-Direct* analysis.

Peaks observed in the *AAA-Direct* chromatograms of sample and blank hydrolysates were identified by retention times determined from 10-µL injections of the 9.6–11.6 µM amino acid standard mix (except 4.8 µM cystine). Concentrations of amino acids in samples and blanks were determined from peak area response factors calculated from the standards.[5] Sample concentrations were corrected for any amino acid contribution found in the blank hydrolysate. For desalted samples, the reported amino acid concentrations were corrected by subtraction of amino acids measured in the hydrolyzed dialyzed-water blank. The theoretical mole ratio was the expected number of amino acid residues based on the amino acid composition of each peptide synthesized. The measured mole ratio is the integer number calculated from the concentration ratio of each amino acid determined in the hydrolysate, relative to the concentration measured for glycine.

RESULTS AND DISCUSSION

The acid hydrolysis and *AAA-Direct* amino acid compositional analysis of the two peptide samples showed that the AminoPac PA10 column completely resolved Hyl and all other amino acids (Figure 1). The Lys-peptide composition agreed with the target composition (Table 1), as only the expected amino acids were observed at high concentrations, and the theoretical mole ratio was the same as the measured mole ratio.

The amino acid compositional analysis did not show the same agreement for the Hyl-peptide. Although the expected amino acids were observed at high levels, the theoretical mole ratio was not the same as the measured mole ratio. One mole of Lys was expected per mole of peptide, yet 2 moles were measured. Two moles of Ser were expected, but 3 were measured. This discrepancy suggested the Hyl-peptide was either incorrectly synthesized or impure.

Although the chromatograms provided by the contract lab (unspecified C18 column, RP-HPLC) revealed no impurities, we analyzed the peptide samples by RP-HPLC (Acclaim C18 column). The Lys-peptide sample appeared pure as a single major peak (Figure 2). The Hyl-peptide sample resolved into 9 peaks (Figure 3), confirming that the preparation was impure.

The RP-HPLC analysis did not show baseline resolution of these peptide impurities. For this reason, cation-exchange separation of the peptide samples using the ProPac WCX-10 column was attempted. The Lys-peptide remained as a single major peak at 27.7 min and 2 minor peaks (Figure 4). This major peak was collected as a fraction (27.4 to 28.4 min elution time, see Figure 4) for further characterization by amino acid compositional analysis using *AAA-Direct*. The Hyl-peptide was also analyzed on the WCX-10 column, yielding 12 peaks (Figure 5). Four fractions (F1, F2, F3, F4) were collected, as shown in Figure 5, for amino acid analysis.

Table 1. Measured Concentrations of Amino Acids in the Lys- and Hyl-Peptides						
	Lys-Peptide			Hyl-Peptide		
Amino Acid	Amino Acid Conc. (μM)	Theoretical Mole Ratio	Measured Mole Ratio	Amino Acid Conc. (μM)	Theoretical Mole Ratio	Measured Mole Ratio
Arginine	0.03	0	0	ND	0	0
Hydroxylysine	ND*	0	0	2.63	1	1
Lysine	8.58	2	2	6.70	1	2
Alanine	ND	0	0	0.47	0	0
Threonine	4.32	1	1	2.30	1	1
Glycine	9.54	2	2	6.40	2	2
Valine	ND	0	0	0.03	0	0
Serine	7.89	2	2	8.32	2	3
Proline	ND	0	0	0.05	0	0
Isoleucine	0.06	0	0	ND	0	0
Leucine	4.93	1	1	3.07	1	1
Methionine	ND	0	0	ND	0	0
Histidine	0.33	0	0	ND	0	0
Phenylalanine	8.31	2	2	7.89	2	2
Glutamate	ND	0	0	ND	0	0
Aspartate	ND	0	0	ND	0	0
Cystine	ND	0	0	ND	0	0
Tyrosine	ND	0	0	ND	0	0

** ND = Not Detected.*

Because this cation-exchange chromatography method used sodium phosphate and sodium chloride to elute the peptide impurities, fractions collected from this separation also contained relatively high levels of salt. The salt concentration was further increased during the evaporative steps prior to acid hydrolysis. The presence of high salt in any peptide hydrolysate analyzed by *AAA-Direct* caused most amino acids to elute earlier (Table 2). This produced uncertainty about amino acid peak identity and affected peak resolution. Furthermore, a baseline dip occurred between 20–24 min, and increased with salt concentration. Glu peak height and peak area were affected by this baseline dip. Figure 6 shows chromatograms of amino acids in water (Panel A), 50 mM salt (Panel B), and 100 mM salt (Panel C). We recommend a maximum of 50 mM total salt concentration in samples (10-μL injection) to ensure retention time shifts for the early eluting amino acids are <5%. Because Glu peak area is affected even at lower salt concentrations, accurate quantification requires that this amino acid standard be tested in the same salt concentration as the sample.

A variety of techniques were investigated to remove salt from peptides prior to acid hydrolysis. Microdialysis delivered the best performance of the techniques evaluated. MWCO as low as 100 and 500 daltons are commercially available. Using the 100 dalton MWCO membrane, 94% of the sodium chloride, and 27% of the sodium phosphate were removed. Using the 500 dalton MWCO membrane, 87% of the NaCl and 47% of the sodium phosphate were removed. Increasing the porosity of the membrane to 500 daltons increased phosphate removal and resulted in better desalting. No interfering *AAA-Direct* chromatographic peaks were observed using this technique. Although not investigated in this application note, fractions from ion-exchange separations can also be desalted by RP-HPLC.

The desalted peptide fractions were acid-hydrolyzed and analyzed by *AAA-Direct* for amino acid composition. The major peak collected from the cation-exchange separation of the Lys-peptide analysis showed amino acid composition (Table 3) identical to that observed for the starting sample and theoretical composition (Table 1).

Fractions 1 and 2 (F1 and F2) from the Hyl-peptide separation had amino acid compositions that were inconsistent with the expected composition, including the absence of Hyl in both. Both the F3 and F4 composition agreed perfectly with the expected composition of the synthesized Hyl-peptide. Both peaks (fractions) contained one mole each of Lys and Hyl, where only one mole was expected in each. It is unclear why peptides containing identical amino acid composition were separated on the cation-exchange column. This peptide was synthesized using a mixture of D- and L-isomers of Hyl, and the preparation should contain both D- and L-Hyl-peptides. Therefore, it is possible the ProPac WCX-10 column resolves these two peptides. It is also possible that the two peptides (F3 and F4) have identical composition, but do not have identical sequences. No further characterization was performed to resolve this question.

Trace levels of many unexpected amino acids were also observed in these desalted acid-hydrolyzed samples, and may be the result of impurities in the peptide preparation, trace contamination of the dialysis system, or trace contamination of the hydrolysis glassware and reagents. A correction for most artifacts was possible by subtraction of the amino acid concentrations measured in appropriate blanks. The amino acid mole ratios presented in Table 3 were corrected by subtraction of amino acids measured in the acid-hydrolyzed dialyzed-water blank.

The concentrations of amino acids observed for the fractions collected from the Hyl-peptide separation, accounting for most of the peaks observed, were summed, and a mole ratio for this sum was calculated (Table 3). This mole ratio of the sum of the fractions is the same as the mole ratio observed for the Hyl-peptide sample before separation (Table 1). The close agreement between the sum of the collected fractions and the starting material helps support the conclusion that the Lys-peptide preparation is pure, whereas the Hyl-peptide preparation is not; the unusual amino acid composition observed in the Hyl-peptide preparation can be explained by the mixture of peptide impurities. These results also show that the Lys-peptide elutes on the ProPac WCX-10 column at 27.7 min, while the desired Hyl-peptide elutes at either 27.4 or 28.2 min using the conditions described in Figures 4 and 5.

Table 2. Effect of Sample Salt on *AAA-Direct* Retention Times (10-µL injections) of Amino Acids (~10 µM each)

Amino Acid	Salt Concentration in Sample Injected (mM)					
	0	10	50	100	250	500
	Retention Time (min)					
Arginine	1.7	1.7	1.7	1.8	1.9	2.2
Lysine	3.2	3.2	3.1	2.9	2.8	2.4
Alanine	5.8	5.8	5.7	5.3	5.1	4.4
Threonine	6.2	6.2	6.0	5.6	5.4	4.6
Glycine	6.8	6.8	6.6	6.1	5.9	5.0
Valine	7.8	7.7	7.6	6.9	6.6	5.4
Serine	9.2	9.2	9.0	8.1	7.8	6.2
Proline	9.7	9.6	9.4	8.5	8.1	6.4
Isoleucine	11.9	11.8	11.6	10.4	6.2	4.9
Leucine	12.8	12.6	12.2	10.6	9.8	7.1
Methionine	13.6	13.5	13.1	11.2	10.6	7.6
Histidine	21.1	21.0	21.0	20.7	20.5	19.3
Phenylalanine	22.4	22.3	22.2	21.9	21.5	23.2
Glutamate	23.5	23.3	23.2	24.4	24.8	24.7
Aspartate	23.8	23.6	23.5	24.0	23.9	23.6
Cystine	25.0	24.8	24.6	27.1	27.1	26.5
Tyrosine	27.5	27.3	27.4	27.3	27.4	27.4
Tryptophan	40.4	40.1	40.1	39.5	40.2	40.3

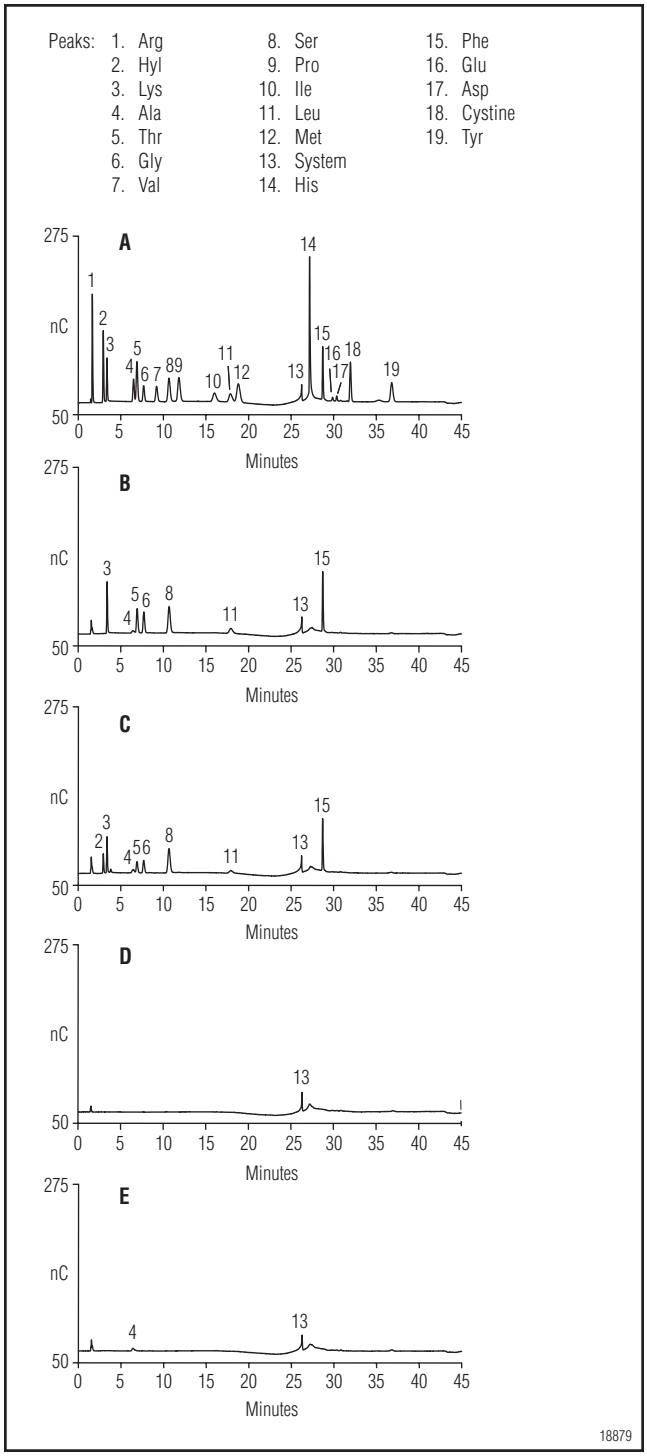

***Figure 1 (A–E)**. The separation of amino acids in amino acid standard mix (A), acid-hydrolyzed Lys-peptide (B), acid-hydrolyzed Hyl-peptide (C), nonhydrolyzed water blank (D), and an acid-hydrolyzed water blank (E) using AAA-Direct.*

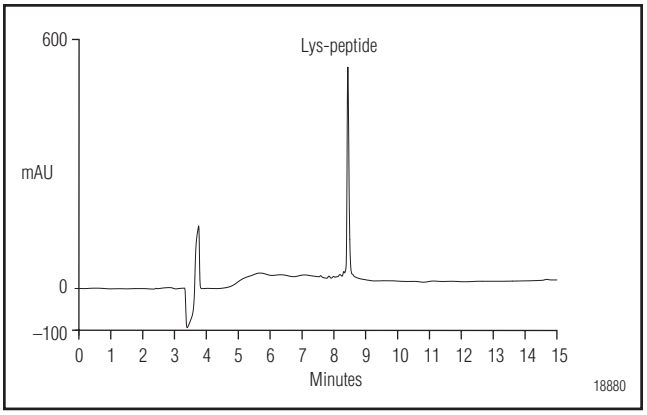

***Figure 2**. RP-HPLC of the Lys-peptide using the Acclaim C18, 5 µm, 120 Å 4.6 × 250 mm column.*

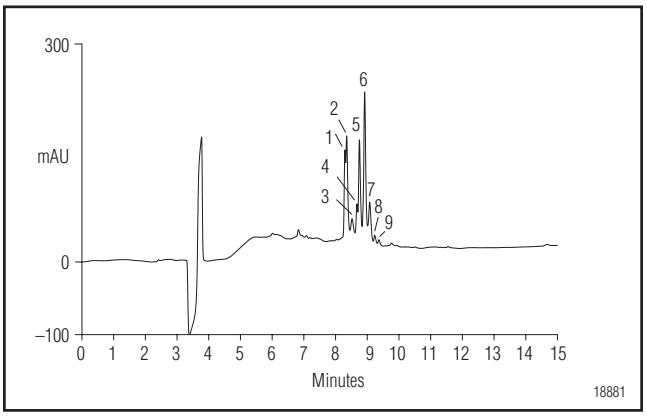

***Figure 3**. RP-HPLC of the Hyl-peptide using the Acclaim C18, 5 µm, 120 Å 4.6 × 250 mm column.*

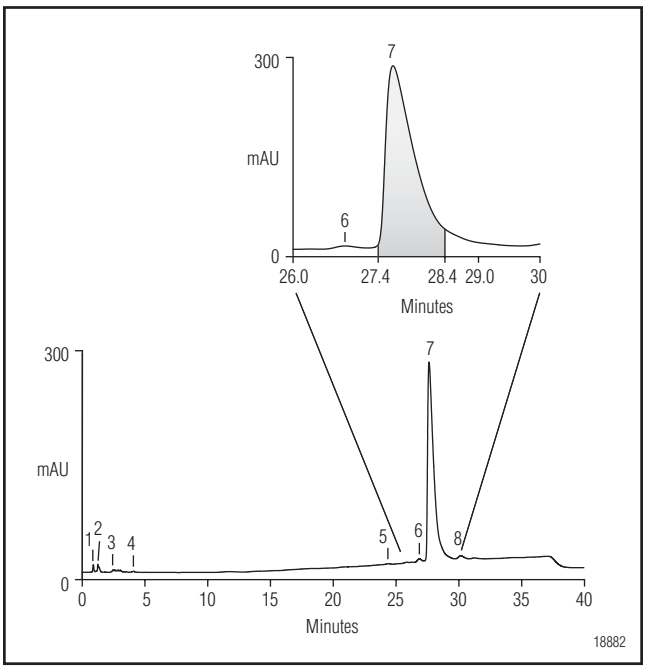

***Figure 4**. The ProPac WCX-10 cation-exchange separation of Lys-peptide with a fraction collected between 27.4 and 28.4 min.*

Table 3. Calculated Mole Ratios						
	Lys-Peptide	Hyl-Peptide				
Amino Acid	Fraction 1	Fractions 1	2	3	4	Sum of Fractions
Arginine	0	0	1	0	0	0
Hydroxylysine	0	**0**	**0**	**1**	**1**	**1**
Lysine	**2**	**5**	**2**	**1**	**1**	**2**
Alanine	0	0	0	0	0	0
Threonine	**1**	**0**	**0**	**1**	**1**	**1**
Glycine	**2**	**2**	**2**	**2**	**2**	**2**
Valine	0	0	0	0	0	0
Serine	**2**	**6**	**3**	**2**	**2**	**3**
Proline	0	0	0	0	0	0
Isoleucine	0	0	0	0	0	0
Leucine	**1**	**0**	**0**	**1**	**1**	**1**
Methionine	0	0	0	0	0	0
Histidine	0	0	0	0	0	0
Phenylalanine	**2**	**5**	**1**	**2**	**2**	**2**
Glutamate	0	0	0	0	0	0
Aspartate	0	0	1	1	0	0
Cystine	0	0	0	0	0	0
Tyrosine	0	0	0	0	0	0

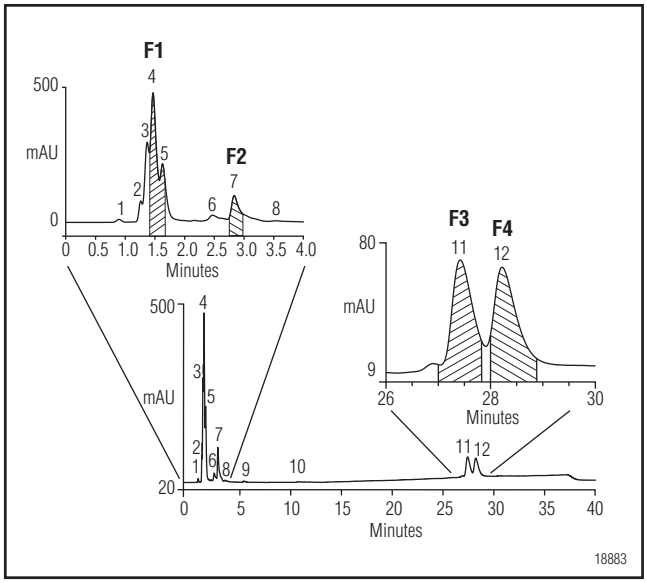

Figure 5. *Propac WCX-10 cation-exchange separation of Hyl-peptide with fractions collected between 1.4 and 1.7 min (F1), 2.7 and 3.1 min (F2), 27.0 and 27.8 min (F3), 28.0 and 28.8 min (F4).*

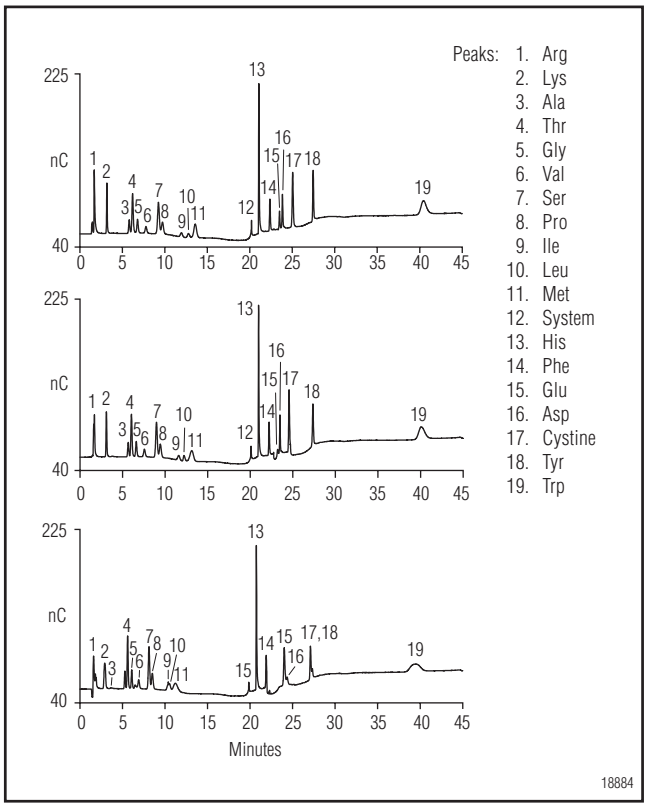

Figure 6. *Separation of 2.4–2.9 μM amino acids (10 μL injections) using AAA-Direct with samples absent of salt (A), with 50 mM salt (B), and with 100 mM salt (C).*

CONCLUSION

AAA-Direct can be used to evaluate peptide samples for identity and purity, either as starting material or as fractions collected from a chromatographic separation. The AminoPac PA10 separates Hyl from all other common amino acids, and IPAD directly detects the amino acids. This technique allows the identification of protein and peptide microheterogeneity due to posttranslational modification of Lys to Hyl. The ProPac WCX-10 and the Acclaim C18 columns are shown to separate peptides differing by the hydroxylation of a single lysine residue. This application note shows that microdialysis can be used to desalt peptides prior to *AAA-Direct* amino acid compositional analysis. Desalting efficiency was evaluated by ion chromatography.

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