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Application Note 211

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Hydrophobic Interaction Chromatography for Separation of Tryptophan and Methionine Oxidized Peptides from Their Native Forms

INTRODUCTION

Hydrophobic interaction chromatography (HIC) is a technique used to separate peptides, proteins, and other biological molecules based on their degree of hydrophobicity. The HIC mobile phase comprises a high-concentration salting-out agent—typically ammonium sulfate—that increases the hydrophobic interaction between the solute and the stationary phase.[1]

HIC and reversed-phase chromatography are closely related techniques. Both are based upon interactions between solvent-accessible nonpolar groups (hydrophobic patches) on the surface of the solute and the hydrophobic ligands of the stationary phase. In practice, however, HIC and reversed-phase chromatography are different, as reversed-phase stationary phases are more highly substituted with hydrophobic ligands than HIC stationary phases. The techniques also use different mobile phases. Protein binding to reversed-phase stationary-phases is usually very strong, and requires polar solvents for elution, which can denature the proteins during the separation. Reversed-phase HPLC has found extensive application in analytical and preparative separations of peptides and low molecular weight proteins that have stable primary structure in aqueous-organic mobile phases. HIC is an alternative to exploiting the hydrophobic properties of proteins, working with nonpolar, non-denaturing mobile phases.[2]

Proteins and peptides are sensitive to oxidative damage. Oxidizing proteins and peptides can alter their biological activity, half-life, and immunogenicity. Natural biological and environmental oxidants have been suggested as causative or contributory factors in many diseases. Oxidation of proteins has also been reported as a natural posttranslational event mediated enzymatically by amines and oxidases.[3] Methionine, cysteine, histidine, tryptophan, and tyrosine residues are most susceptible to this oxidation.[4] Methionine is easily oxidized by atmospheric oxygen to form methionine sulfoxide, and tryptophan can be oxidized into four different oxidation products by peroxide. Peroxide is an impurity generated during protein storage, or from polysorbates commonly used for protein purification and solubilization. Other peroxide-contaminated materials include polyethylene glycol (PEG) or silicon rubber from vial stoppers. When proteins are used as pharmaceutical ingredients, methionine and tryptophan oxidation during processing or storage can affect protein activity.[5]

In Application Note 129, the authors used a weak cation-exchange chromatography method with the ProPac® WCX-10 to separate oxidized and native Luteinizing Hormone-Releasing Hormone (LH-RH)—a tryptophan-containing peptide, and oxidized and native α-Melanocyte Stimulating Hormone (MSA)—a methionine-containing peptide. Here, the authors demonstrate separation of these oxidized and non-oxidized variants on the ProPac HIC-10 (hydrophobic interaction) column and the ProPac WCX-10 (weak anion-exchange) column using ICS-3000 PEEK™ and UltiMate® 3000 Titanium systems. The ProPac HIC-10 column separates LH-RH isoforms not found using weak the cation-exchange column and chemistry, and the resolution of the oxidized variants is improved over the aforementioned method.

EQUIPMENT

ICS-3000 Liquid Chromatography System

- ICS-3000 SP (P/N 061707)
- ICS-3000 VWD (P/N 064653)
- ICS-3000 TC (P/N 064444)
- AS Autosampler (P/N 056859)
- ProPac HIC-10 column, 4.6 × 100 mm, (P/N 063655)
- ProPac WCX-10 column, 4.6 × 100 mm, (P/N 054993)

UltiMate 3000 Titanium Liquid Chromatography System

- SRD-3600 Solvent Rack with six degasser channels (P/N 5035-9230)
- LPG-3400AB Quaternary Analytical Pump (P/N 5037.0015), or
- DPG-3600AB Dual Ternary Analytical Pump (P/N 5037.0014)
- WPS-3000TBPL Biocompatible Analytical Autosampler (P/N 5823.0020)
- TCC-3200B Column Compartment with two PEEK 10-port 2-position valves (P/N 5723.0025), or
- TCC-3000 Column Compartment (P/N 5722.0000)
- Detector VWD-3400 Variable Wavelength (P/N 5074.0010), or
- PDA-3000 PhotoDiode Array Detector (P/N 5080.0020)
- Biocompatible Analytical Flow Cell (P/N 6074.0200), or
- Biocompatible Analytical Flow Cell for PDA (P/N 6080.0200)

Chromeleon® Data Management Software

MSQ Plus™ Mass Spectrometer with Data System (P/N 063116)

AXP-MS Auxiliary Pump Kit (P/N 060684)

Chromeleon MS Support (MSQ MS control software) (P/N 060726)

REAGENTS AND SAMPLES

Deionized water 18.2 (MΩ-cm)

Ammonium sulfate, Molecular biology grade (Sigma-Aldrich, A4418)

Sodium phosphate, dibasic, anhydrous, powder (J.T. Baker, 3826)

Sodium phosphate, monobasic, monohydrate (Sigma-Aldrich, S8282)

Ammonium bicarbonate (Sigma-Aldrich, S9638)

Ethylenediaminetetraacetic acid (EDTA), disodium dihydrate (Sigma-Aldrich, E1644)

Hydrogen peroxide, 30% (Sigma-Aldrich, H1009)

Dimethyl sulfoxide (DMSO), ACS reagent (Sigma-Aldrich, 472301)

Hydrochloric acid (HCl), 11–12 N, Ultrex® II Ultrapure Reagent (J.T. Baker, 6900-05)

Acetic acid, glacial, HPLC grade (J.T. Baker 9515-03)

Luteinizing hormone-releasing hormone (LH-RH) Human (Sigma-Aldrich, L7134), 97% of the peptide material contains the following sequence: *p*-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

α-Melanocyte Stimulating Hormone (α-MSH), (Sigma-Aldrich, M-4135), 78% of dry weight is peptide, 98% of the peptide material contains the following sequence

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

CONDITIONS

Conditions for Hydrophobic Interaction Chromatography

Column:	ProPac HIC–10 column, 4.6 × 100 mm (P/N 063655)
Flow Rate:	1.0 mL/min
Temperature:	30 °C
Inj. Volume:	10 µL

Detection: UV, 214, 254, and 280 nm

Eluents: A: 2 M Ammonium sulfate in 0.1 M sodium phosphate (pH 7.0)

B: 0.1 M Sodium phosphate, monobasic (pH 7.0)

Gradient Program:

Time (min)	A%	B%	Comments
–10.0	100.0	0.0	Precondition column before sample injection
0.0	100.0	0.0	Sample injection
2.0	100.0	0.0	Isocratic mobile phase during sample injection
17.0	0.0	100.0	Gradient
20.0	0.0	100.0	Wash
22.0	100.0	0.0	Re-equilibration

Prewash column with 100% B for 15 min prior to the first injection.

Conditions for Weak Cation-Exchange Chromatography

Column: ProPac WCX–10 column, 4 × 250 mm (P/N 054993)

Flow Rate: 1.0 mL/min

Temperature: 30 °C

Inj. Volume: 10 *μ*L

Detection: UV, 214, 254, and 280 nm

Eluents: A: 10 mM Sodium phosphate (pH 6.0)

B: 10 mM Sodium phosphate with 500 mM sodium chloride (pH 6.0)

Gradient Program:

Time (min)	A%	B%	Comments
0.0	98.0	2.0	Sample injection
30.0	84.0	16.0	Gradient
30.1	98.0	2.0	Wash
45.0	98.0	2.0	Re-equilibration

Mass Spectrometry Conditions

An AXP-MS auxiliary pump was used to manually infuse the oxidized and non-oxidized LH-RH samples at a flow rate of 0.2 mL/min for optimization in the MSQ Plus mass spectrometer.

During optimization, various cone voltages were examined, with 70V determined as optimal. As these were charged species, ESI was the preferred ionization mode, with positive ionization providing the best response.

Ion Source: Positive ESI

Needle voltage: 3000V

Cone voltage: 70V

Probe temp: 350 °C

Scan mode: Full scan at 1000 *m/z* per second

Scan range 15~2000 *m/z*

AXP-MS flow 0.2 mL/min CH_3CN/H_2O (50/50, v/v)

Infused Volume 10 µL

PREPARATION OF SOLUTIONS AND REAGENTS

2 M Ammonium Sulfate in 0.1 M Sodium Phosphate, Monobasic, pH 7.0

Dissolve 264.2 g ammonium sulfate and 12 g sodium phosphate monobasic in 650 mL DI water (18.2 MΩ cm) in a 1 L volumetric flask. Adjust the pH to 7.0 with approximately 2.5 mL 50% sodium hydroxide and bring to volume with DI water. Filter through a 0.22 µm filter.

0.1 M Sodium Phosphate, Monobasic, pH 7.0

Dissolve 12 g sodium phosphate monobasic in 900 mL DI water in a 1 L volumetric flask. Adjust the pH to 7.0 with approximately 1.5 mL of 50% sodium hydroxide and bring to volume with DI water. Filter through a 0.22 µm filter.

1.0 M Ammonium Bicarbonate, pH 8.8

Combine 7.91 g ammonium bicarbonate with 90 mL DI water in a 100 mL volumetric flask. Adjust the pH to 8.8 with 11-12 N HCl solution. Bring to volume with DI water. Filter through a 0.22 µm filter.

75 mM EDTA, pH 8.0

Combine 78.8 g EDTA disodium dihydrate with 900 mL DI water in a 1 L volumetric flask. Adjust the pH to 8.0 using a 0.05% NaOH (w/w) solution. Bring to volume with DI water. Filter through a 0.22 µm filter.

400 mM Hydrogen Peroxide

Combine 0.455 mL hydrogen peroxide (30%, 8.79 M) with 9.55 mL water in a 10 mL volumetric flask. Make this solution fresh daily.

1 M Sodium Chloride

Dissolve 58.45 g sodium chloride in deionized water in a 1 L volumetric flask and bring to volume. Filter through a 0.22 µm filter.

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate in 1 L DI water in a volumetric flask. Filter through a 0.22 µm filter.

200 mM Sodium Phosphate, Monobasic

Dissolve 27.60 g of monohydrate monobasic sodium phosphate in 1 L DI water in a volumetric flask. Filter through a 0.22 µm filter.

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 in a 2 L volumetric flask. Adjust the relative proportions of 200 mM dibasic and monobasic sodium phosphate used to achieve a pH of 6.0 while maintaining a total volume of 100 mL.

10 mM Sodium Phosphate with 500 mM Sodium Chloride, pH 6.0

Combine 35 mL 200 mM dibasic sodium phosphate, 65 ml 200 mM monobasic sodium phosphate, 1000 mL 1 M sodium chloride, and 900 mL DI water in a 2 L volumetric flask. Adjust the relative proportions of 200 mM dibasic and monobasic sodium phosphate to achieve a pH of 6.0 while maintaining a total volume of 100 mL.

SAMPLE PREPARATION

Oxidation of Tryptophan in LH-RH

A vial containing 1 mg LH-RH was reconstituted with a solution consisting of 48.6 µL glacial acetic acid, 6.5 µL 11–12 N HCl, and 1.3 µL DI H_2O, and labeled Non-Oxidized LH-RH. Another vial containing 1 mg LH-RH was reconstituted with a solution consisting of 48.6 µL glacial acetic acid, 6.5 µL 11–12 N HCl, and 1.3 µL DMSO and labeled Oxidized LH-RH. A non-oxidized buffer control was prepared by combining 41.7 µL glacial acetic acid, 5.6 µL 11-12 N HCl, and 1.1 µL water. An oxidized buffer control was prepared by combining 41.7 µL glacial acetic acid, 5.6 µL 11–12 N HCl, and 1.1 µL DMSO. All vials were incubated at room temperature for 15 min. After incubation, 94.7 µL DI H_2O was added to each vial. The samples were diluted 100-fold by adding 10 µL of sample to 495 µL of eluent B, then adding 495 µL of eluent A. The samples were maintained at room temperature and analyzed within 48 h. When stored at 4 ºC, the injection solution can be used for up to one week. Samples can be stored in a -40 °C freezer for up to one month, with one thaw cycle. Samples stored frozen should be stored in single-use size aliquots.

Oxidation of Methionine in α-MSH

A vial containing 1 mg MSH was reconstituted with 533 µL water to make a 1.5 mg/mL MSH solution. A 1 mg/mL non-oxidized MSH control was prepared by combining 105 µL 1.5 mg/mL MSH solution with 15 µL 1M ammonium bicarbonate, 10 µL 75 mM EDTA, and 20 µL water. A 1 mg/mL Met-Oxidized MSH sample was prepared by combining 105 µL 1.5 mg/mL MSH solution with 15 µL of 1M ammonium bicarbonate, 10 µL 75 mM EDTA, and 20 µL 400 mM hydrogen peroxide. A buffer control was prepared by combining 105 µL water with 15 µL 1M ammonium bicarbonate, 10 µL 75 mM EDTA, and 20 µL 400 mM hydrogen peroxide. All the oxidized samples had a final concentration of 0.1 M ammonium bicarbonate, 5 mM EDTA, and 53 mM hydrogen peroxide. All samples were incubated for 30 min in an ice water bath. After incubation, each sample was diluted 10-fold by adding 30 µL to 135 µL eluent B, then adding 135 µL eluent A. The samples are stable for 48 h at room temperature, one week at 4 °C, and one month at -40 °C (with one thaw cycle).

RESULTS AND DISCUSSION

Figure 1A shows the elution of two non-oxidized LH-RH peaks at 11 and 18 min (peaks 5 and 6) on the HIC-10 column at 214 nm. (Note, data was collected at 254 and 280 nm as well, but 214 nm provided optimum response for this analysis.) The peak eluting within the first 3 min was also seen in the buffer control (data not shown). Mass spectrometric analysis of the non-oxidized LH-RH (Figure 2B) revealed only a single major component with a mass-to-charge ratio (*m/z*) equal to that expected for LH-RH. Figure 2B shows the presence of two MS peaks. The first MS peak was identified as LH-RH $[M+2H]^{2+}$, and the second peak was identified as an acetonitrile adduct of LH-RH.

Fractions of peaks 5 and 6 in Figure 1A were collected, dialysed against water, vacuum dried to concentrate, and infused for MS analysis. The *m/z* of these

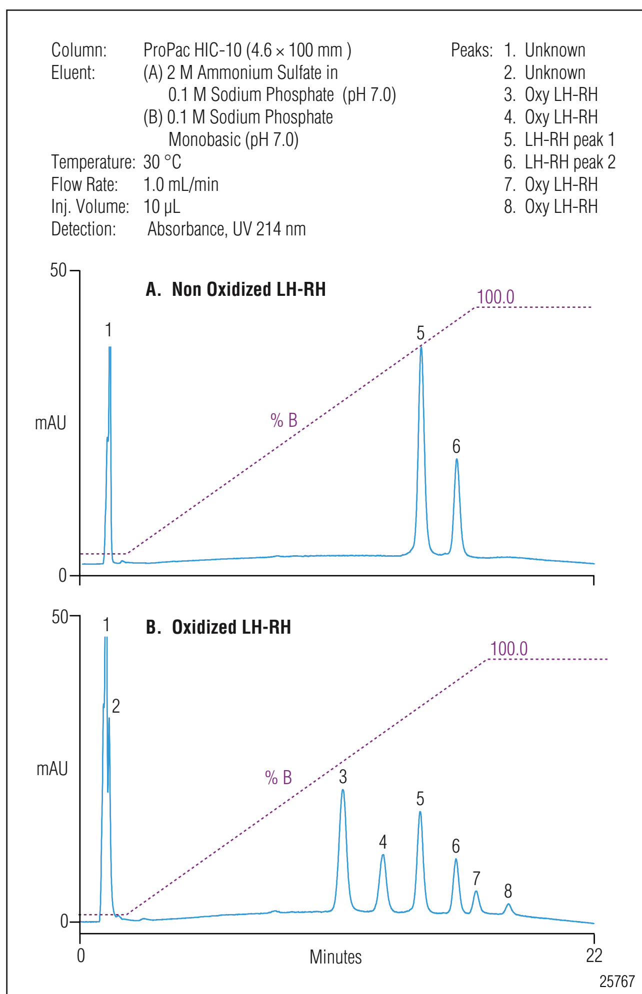

Figure 1: The separation of (A) non-oxidized LH-RH and (B) oxidized LH-RH using the ProPac HIC-10 column.

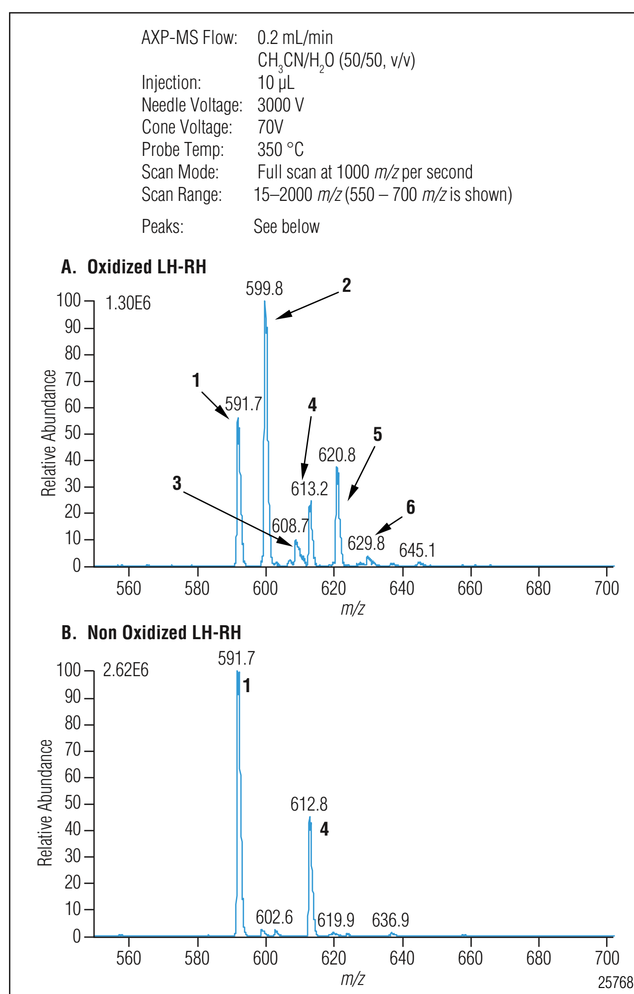

Figure 2. Mass spectra of (A) oxidized LH-RH and (B) non-oxidized LH-RH.

two peaks were identical (data not shown). The source of the two impurity peaks observed on the HIC-10 column is uncertain, as the supplier reports the product is 97% pure by reversed-phase chromatography.

The Trp in LH-RH was forcibly oxidized with DMSO and HCl. Figure 1B shows four additional peaks, or two oxidation products for each retained peak in the non-oxidized LH-RH chromatogram. The presence of peaks 5 and 6, with reduced peak areas in the oxidized LH-RH indicates incomplete oxidation. All six peaks were well resolved using the HIC-10 column. Figure 3 shows a variety of Trp oxidation products described by E.L. Finley et al.[7] The expected products were: hydroxy-tryptophan (HTRP), N-formylkyurenine (NFK), kynurenine (KYN), and 3-hydroxykyurenine (3OH-KYN). The authors were able to identify two of the products using MS detection.

Figure 2B shows the presence of four additional major ions, three of which were identified. The following assignments were made based on mass/charge ratios.

Peak 1 LH-RH, $[M+2H]^{2+}$

Peak 2 Hydroxy-tryptophan (HTRP), oxidized product of LH-RH, $[(M+16)+2H]^{2+}$

Peak 3 N-formylkyurenine (NFK), oxidized product of LH-RH, $[(M+32)+2H]^{2+}$

Peak 4 Acetonitrile adduct of LH-RH, $[LH\text{-}RH+CH_3CN+2H]^{2+}$

Peak 5 Acetonitrile adduct of Hydroxy-tryptophan (HTRP), oxidized product of LH-RH, $[HTRP+CH_3CN+2H]^{2+}$

Peak 6 Acetonitrile adduct of N-formylkuyrenine, oxidized product of LH-RH, $[NFK+CH_3CN+2H]^{2+}$

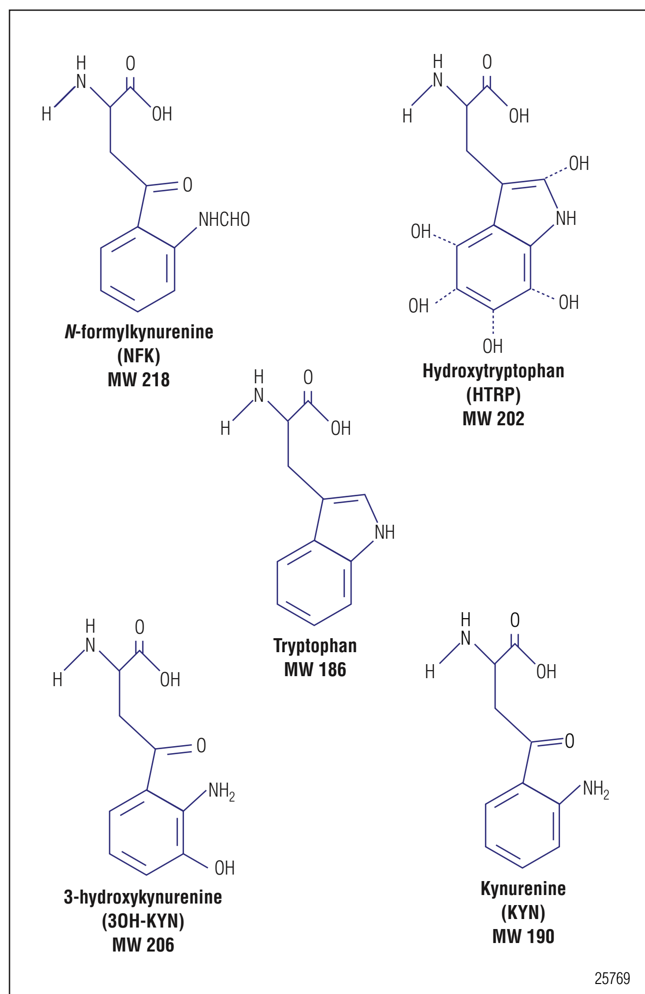

Figure 3. The chemical structure of tryptophan and its major oxidation products.[7]

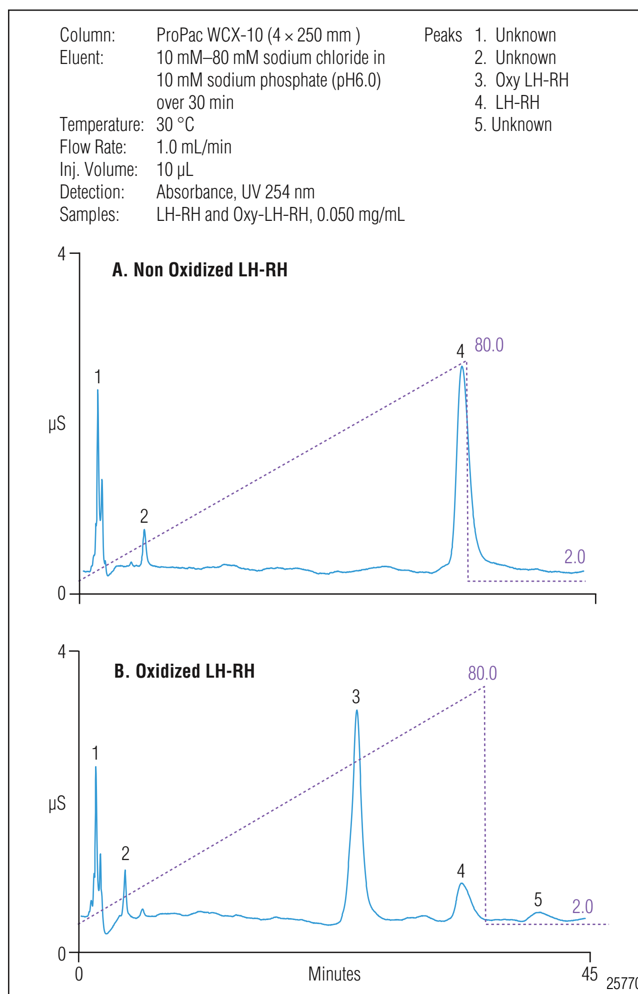

Figure 4. Separation of (A) non-oxidized LH-RH and (B) oxidized LH-RH using the ProPac WCX-10 column.

The mass spectroscopy results support our interpretation of the chromatography results for the oxidation of LH-RH.

Figure 4A shows the elution of non-oxidized LH-RH at 22 min on the ProPac WCX-10 column at 254 nm. These results show a single non-oxidized LH-RH at all wavelengths (214, 254, and 280 nm) used in this study, confirming the data previously published in AN129. The ProPac WCX-10 did not resolve the two forms of this peptide observed on the HIC column. After tryptophan oxidation, oxidized LH-RH isoforms eluted earlier at 16–17 min, as a single peak (Figure 4B). As observed with the HIC column, the presence of a small non-oxidized LH-RH component (peak 4, with a retention time of 22 min) remaining in the oxidized LH-RH sample suggested that LH-RH was not completely oxidized.

Figure 5A shows non-oxidized α-MSH (peak 1) at 26 min on the HIC-10 column. After forced oxidation of the methionine in α-MSH, a new peak for the oxidized α-MSH (peak 2) was observed at 20 min (Figure 4B). The presence of a remaining non-oxidized MSH peak at 26 min indicated that α-MSH was not completely oxidized. Although α-MSH also contains tryptophan residue, forcible oxidation was not attempted. These results show an orthogonal method to AN 129, which uses the ProPac WCX-10 column for separation of methionine oxidation variants.

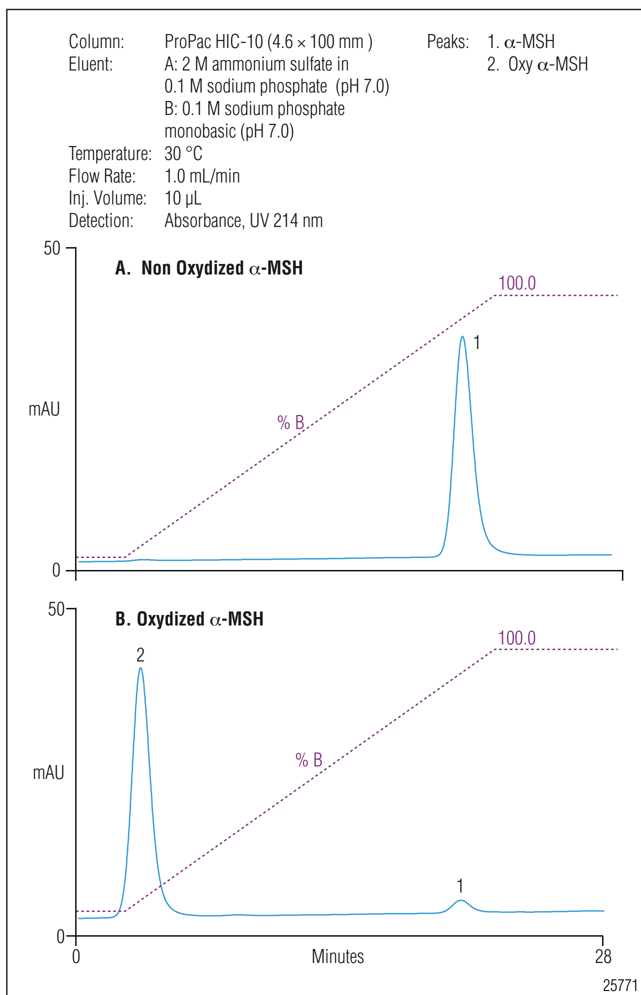

Figure 5. Separation of (A) non-oxidized α-MSH and (B) oxidized α-MSH using the ProPac HIC-10 column.

PRECAUTIONS

LH-RH and α-MSH are bioactive peptides. Observe all safety precautions when handling these materials. Review the material safety data sheets (MSDS) for these materials prior to handling, use, and disposal.

SUMMARY

The method described herein demonstrates separation of peptides with differences as small as the oxidation of a single amino acid residue using the ProPac HIC-10 column. The separations occur in a non-denaturing environment, and further demonstrate that HIC is a viable alternative to reversed-phase HPLC for separation of peptide variants. This technique is also an alternative to weak cation-exchange chromatography for separation of peptide variants as shown in AN 129, and may provide improved sensitivity and selectivity over that method.

SUPPLIERS

Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103, Tel: 800-521-8956, www.sigmaaldrich.com

Mallinckrodt Baker, Inc., 222 Red School Lane, Phillipsburg, NJ 08865, Tel: 908-859-2151, www.solvitcenter.com

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