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93-2-36

# **Protein Society '89**

## Poster Presentation

### Photodiode Array Spectral Identification of Modified Amino Acids in Synthetic Peptides Following High Resolution HPLC Separation

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Photodiode array spectral identification of modified amino acids in synthetic peptides following high resolution HPLC separation. Patricia M. Young<sup>\*</sup>, Peter Coassin<sup>+</sup> and Robert F. Burgoyne<sup>\*</sup>, <sup>\*</sup>Waters Chromatography Division, Millipore Corp., Maple St., Milford, MA 01757 and <sup>+</sup>Milligen/Biosearch Division, Millipore Corp., Burlington, MA, 01803.

#### ABSTRACT

<u>Objective.</u> To separate synthetic peptides containing blocked amino acid (AA) residues by HPLC and identify the blocking group by photodiode array detection.

<u>Content.</u> Purification by HPLC combined with high sensitivity spectral analysis by photodiode array detection provides information on amino acid composition of peptides and can be used to identify peptides containing amino acids with side chain modifications. During solid-phase synthesis of peptides, labile portions of certain amino acids are conjugated with protecting groups. Many of these protecting groups contain distinctive structural features that can be characterized by spectroscopy. Although the synthesis protocol provides for removal of these protecting groups, it is possible that these protecting groups may not be completely removed. Since their presence may adversely effect the biological activity of the synthesized peptide, detection is essential.

<u>Conclusion</u>. Spectral analysis using photodiode array detection over a wide wavelength range in conjunction with high resolution HPLC separation can be used to identify these protected amino acid residues in low levels of synthetic peptides.

#### MATERIALS AND METHODS

Peptides were synthesized by continuous flow automated FMOC-solid phase protocol using the Milligen PepSynthesizer<sup>TM</sup> (model 9050). Following release, peptides were chromatographed on the Waters Peptide Analyzer using photodiode array detection (model 990+). The peptides were purified by reverse phase HPLC using a Waters DeltaPak<sup>TM</sup> C18, 5µm, 300Å (2mm x 15cm). The eluents were: A=H20/0.1% TFA, B=ACN/0.1% TFA with a linear gradient of 3%B/min to 100%B.

#### **Data Analysis Formats in Photodiode Array Detection**

The Waters model 990+ Photodiode Array (PDA) Detector collects and stores a three-dimensional (3-D) data set: time, absorbance, and wavelength. While such data is displayed as a 3-D plot, other data presentation formats are also used. A contour plot is a view from the top, showing time vs. wavelength with concentric circles of varying colors to indicate increasing absorbance. Another data format is the chromatogram plot showing absorbance as a function of time at a specific wavelength. Additionally, the absorbance spectrum at a given time of the data set may be obtained. The PDA computer has menu-driven software enabling the user to perform mathematical calculations such as addition, subtraction, and derivative calculation.





Figure 1a: Chromatogram of 5  $\mu$ g synthetic peptide (Ala-Asn-Lys-Gly-Phe-Glu-Glu-Val). During synthesis the amide of the asparginine (Asn) residue was protected by a dimethoxybenzhydral (MBH) group. Upon release from the resin, the MBH group should be removed; however, two peaks were obtained.



Figure 1b: Contour plot of the chromatogram in figure 1a. Both peptides absorb in the 200-220 nm range due to the peptide bond. The first peptide (RT=18.61 min) has absorbance at 260 nm consistent with the presence of a Phe residue. The peptide at 21.9 min has an absorbance maximum at approximately 275 nm due to the MBH group. As expected, the presence of the MBH group increases the hydrophobicity of the peptide resulting in greater retention by the DeltaPak<sup>TM</sup> C18 solid phase.



Figure 1c: Spectrum Analysis derived from the chromatogram in figure 1a indicates the two peptides have distinctly different spectra.



Figure 2a: Chromatogram of 30 µg synthetic peptide (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) with N-terminal blocked by 9-Fluorenylmethoxycarbonyl (FMOC) group. Chromatographic data displyed at 214 nm does not reveal the presence of the FMOC group.



Figure 2b: Spectrum Analysis indicates the presence of the FMOC group by the absorbance maxima at 250 nm and 305 nm.



Figure 3: A wide range of blocking groups may be used in peptide synthesis. For example, the presence of a tosyl (p-toluenesulfonyl) group on the imidazole of histidine (His) is shown in Spectrum Analysis. The spectrum for unblocked his (green line) has no absorbance above 235 nm. His-tosyl has an additional absorbance maxima at 234 nm.



Figure 4a: The indole of trytophan (Trp) may be protected with a formyl group. A Spectrum Analysis plot of BOC-trp (N-butyloxycarbonyl-trp, red line), N-acetyl-trytophanamide (green line), and N<sup>in</sup>-formyl-trp (N-butyloxycarbonyl-N<sup>in</sup>-(formyl)-trp, yellow line) indicates that N<sup>in</sup>-formyl-trp has a markedly different spectrum than BOC-trp and N-acetyl-trp. BOC-trp and N-acetyl-trp have absorbance maxima at 220 nm and 280 nm, while N<sup>in</sup>-formyl-trp has maxima at 238 nm and 294 nm.



Figure 4b: Calculation of the second derivatives of spectra in figure 4a. The second derivative calculation of the spectrum for BOC-trp (red line) has minima at 281 nm and 290 nm and a maximum at 285 nm. The second derivative spectrum for N<sup>in</sup>-formyl-trp has minima at 293 nm and 304 nm and a maximum at 295 nm. These discrete features facilitate structural confirmation.

#### CONCLUSIONS

1. Reverse phase chromatography is an effective tool for defining the complexities of products from peptide synthesis and guiding purification strategies.

2. The use of a photodiode array detector facillitates the analysis of synthetic peptide separations by collecting the spectra properties of each peptide and providing information regarding peak identity and homogeneity.

3. The Waters 990+ photodiode array detector can readily identify the presence of many blocking groups used in peptide synthesis. Since elution order and peak size are not reliable indicators of product purity, spectral information greatly simplifies interpretation of the chromatographic data.

4. Closely related spectra can be more readily distinguished through calculation of second derivatives. This mathematic procedure is a simple, menu-driven feature of the 990+.