

Fluorescence  
Sensitivity  
Enhancements for  
Proteins and Peptides  
Using AQC  
Derivatization with  
Capillary Liquid  
Chromatography

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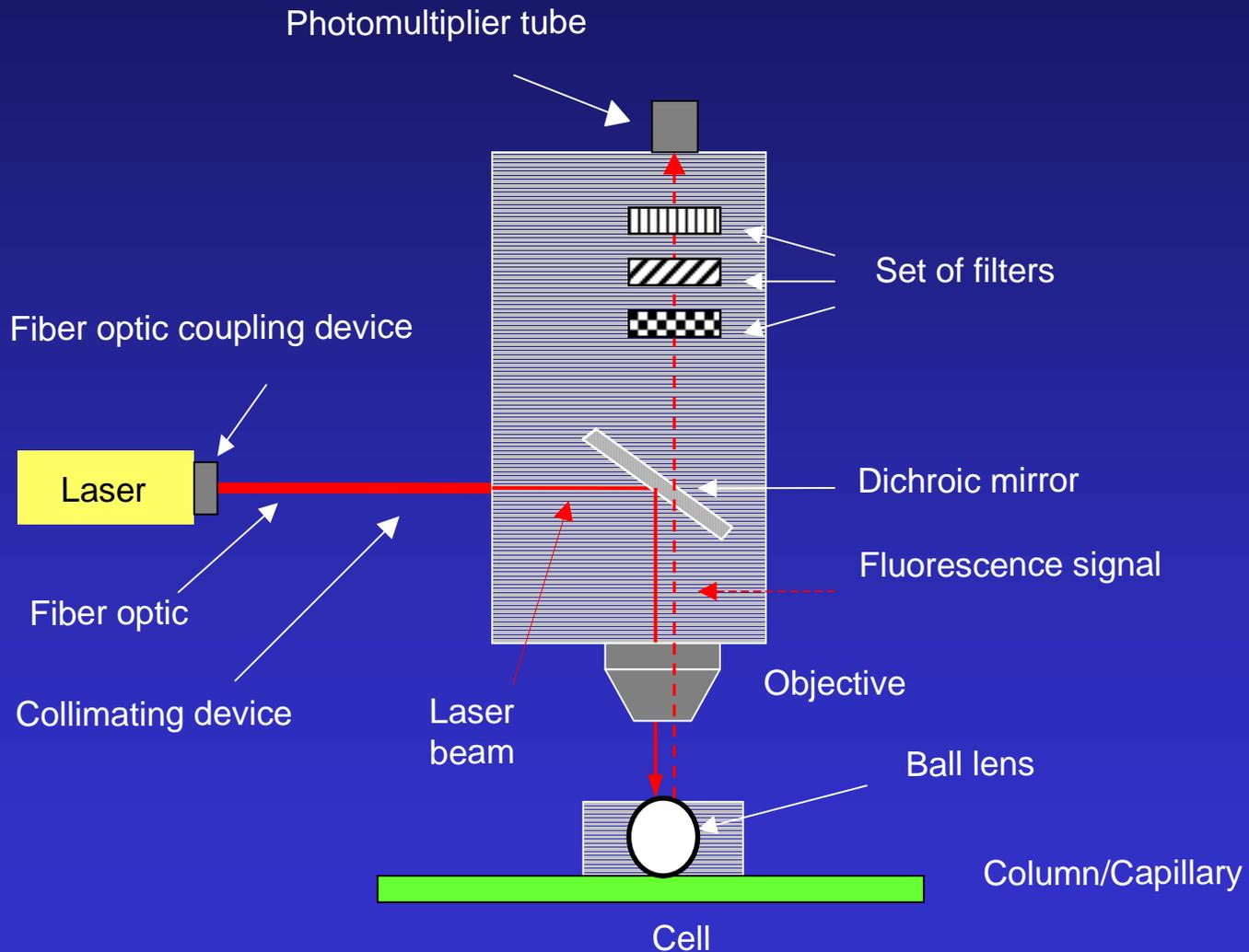
# Why Switch to Capillary LC?

- Analysis Sensitivity
  - Microanalytical
  - Microprep
- Sample Limited
- Excellent MS Compatibility, Especially ESI
- Small Fraction Volume

# Overview

- Applications with Native Fluorescent Molecules
- Derivatization of Proteins and Peptides with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC)

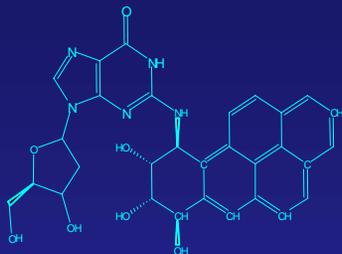
# LIF Detector Optical Path



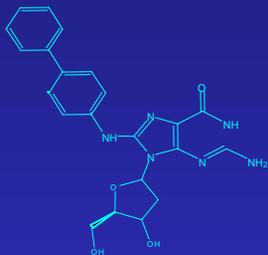
# Conditions for Small Molecule Study

- Waters CapLC System, Liconix Helium Cadmium Laser, Picometrics Zetalif Fluorescence Detector
  - 15 cm x 0.32 mm Symmetry C18 (100 Å, 5 μm)
  - 10 μl/min, A: 0.01% TFA, B: 100% MeOH
  - 30 to 70% B in 20 min, 50°C Column Temperature
  - 1 μl Injection
  - PDA from 220 to 400 nm
  - Excitation at 325 nm, Emission Detection at 395 nm
  - Total Run Time: 30 min
- **Samples**
  - Nucleotide adducts and precursor analog: deoxyguanosyl-amino-biphenyl (dG-8-ABP), tetrol and benzo[a]pyrene diol deoxyguanosine (BPdG), samples courtesy of Dr. Radoslav Goldman, NIH)

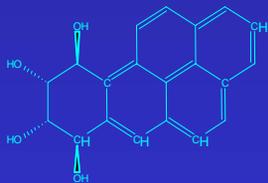
# UV Spectra of Small Molecules



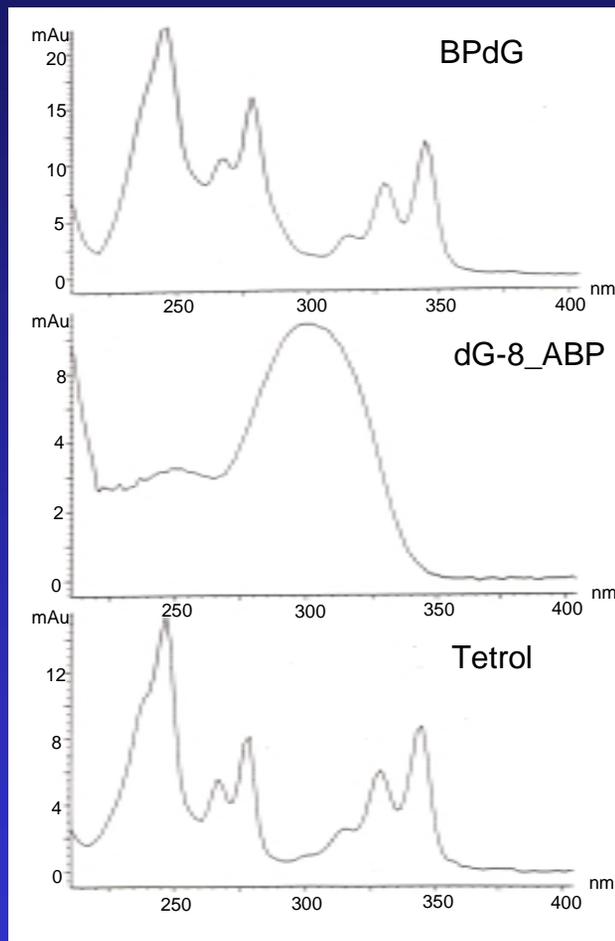
BPdG



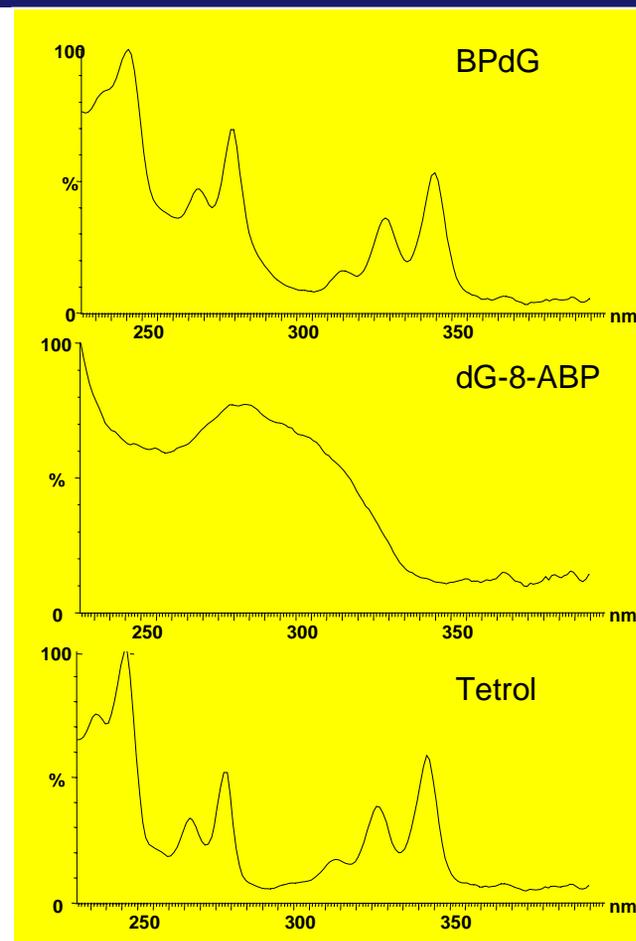
dG-8-ABP



Tetrol



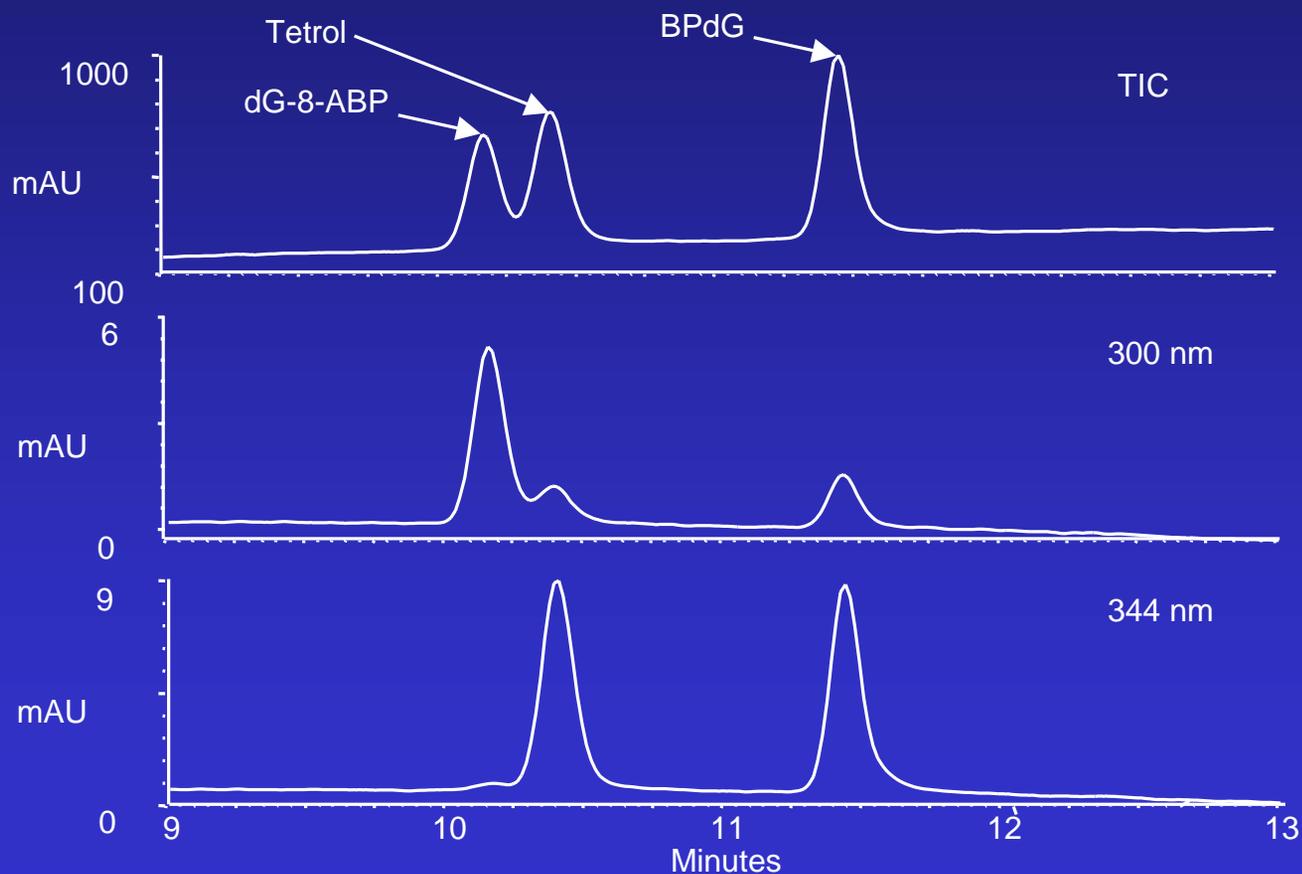
Scanning UV Detector



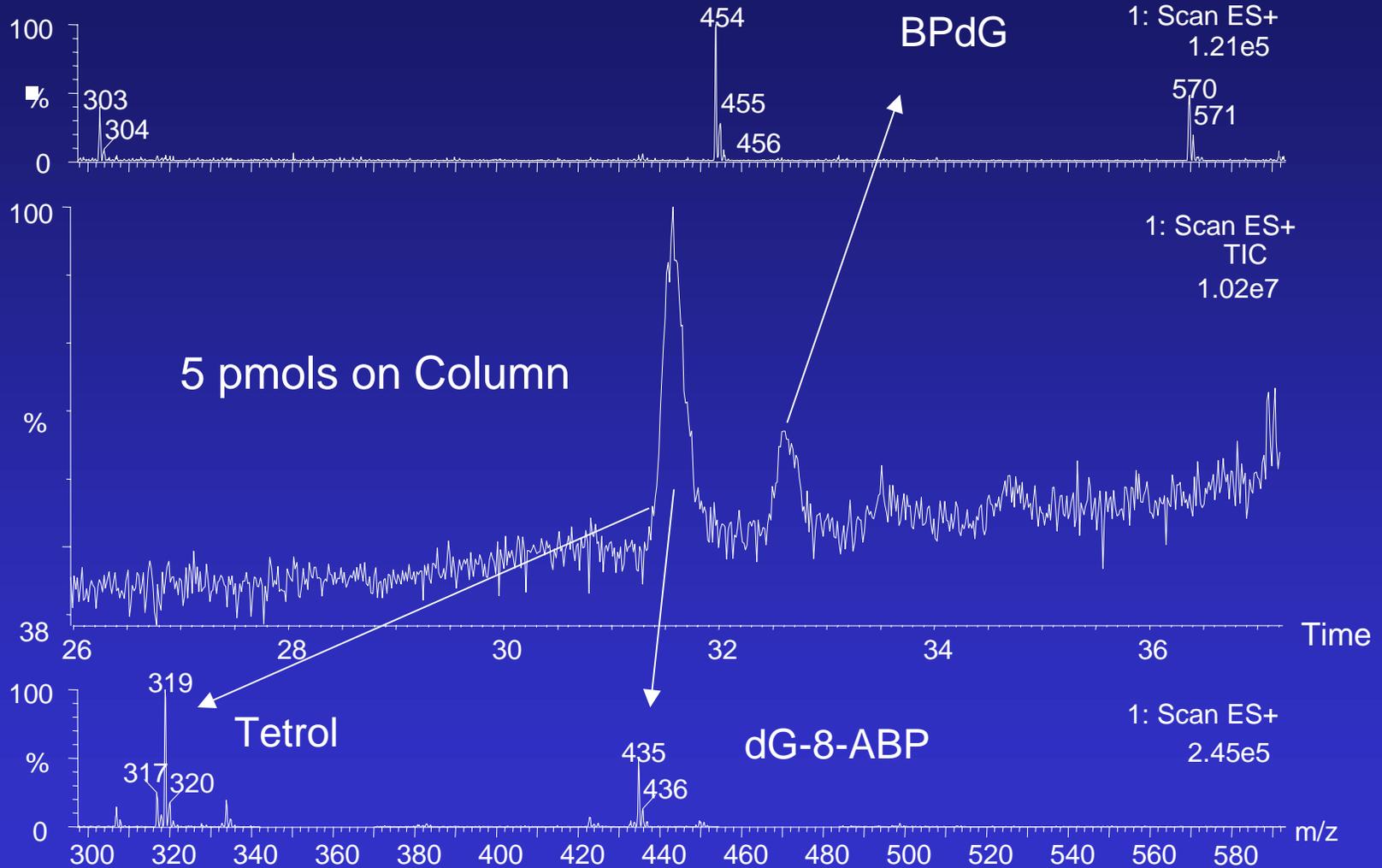
Waters CapLC PDA

# UV Detection for Small Molecule Mixture

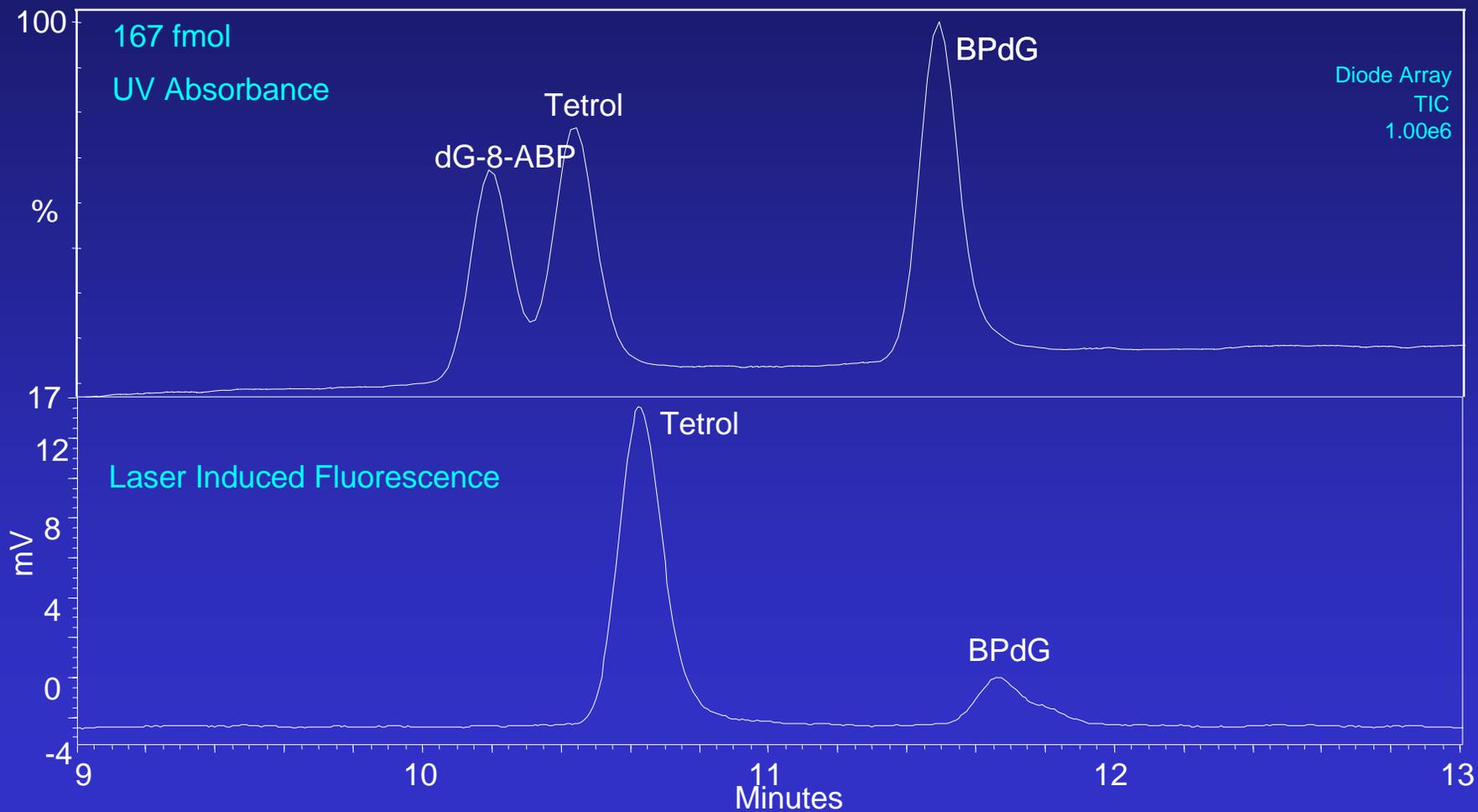
167 fmol Sample  
Full Spectrum and Extracted Wavelength  
Chromatograms



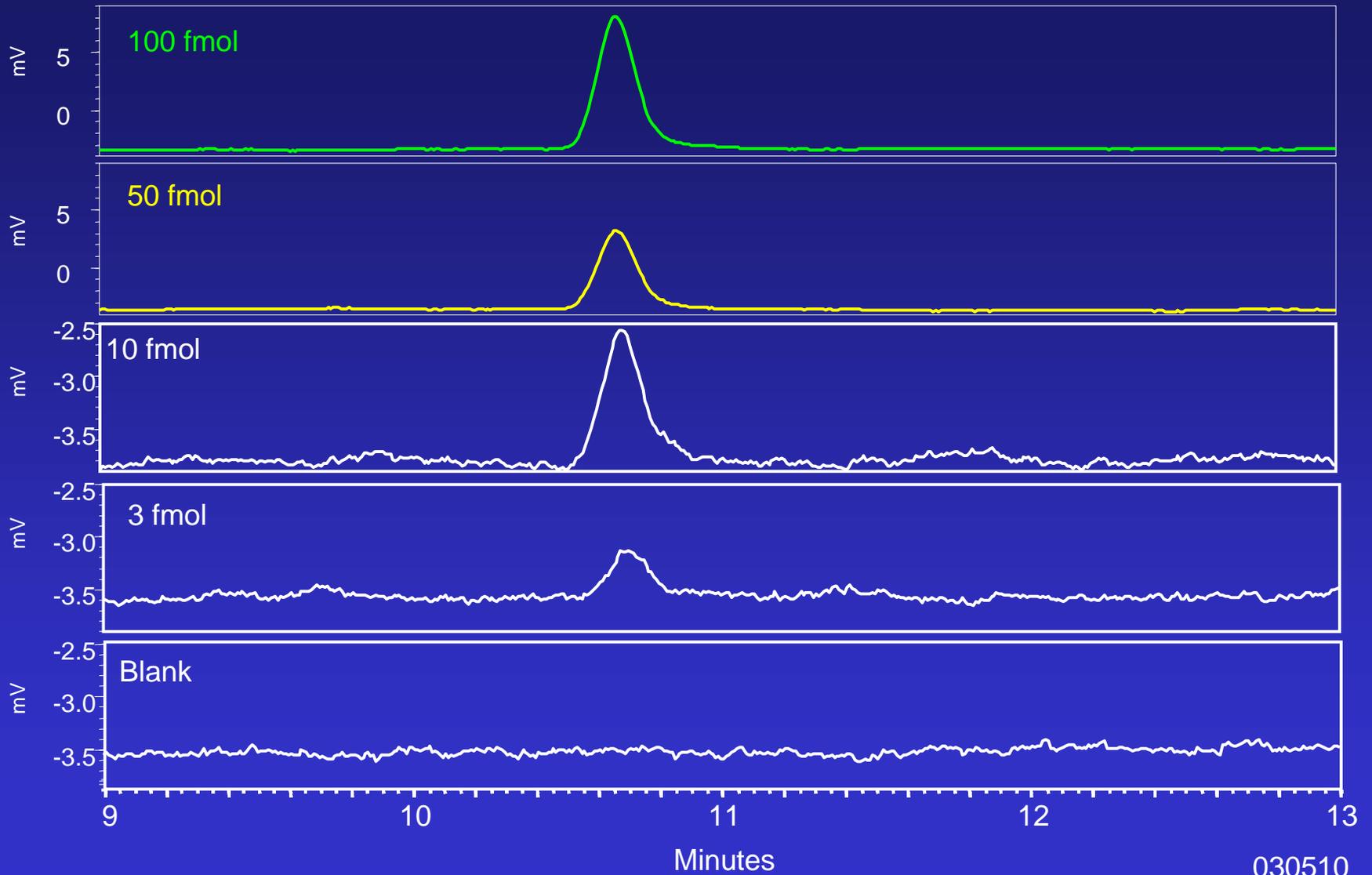
# MS Scan of Compounds 1, 2 and 3



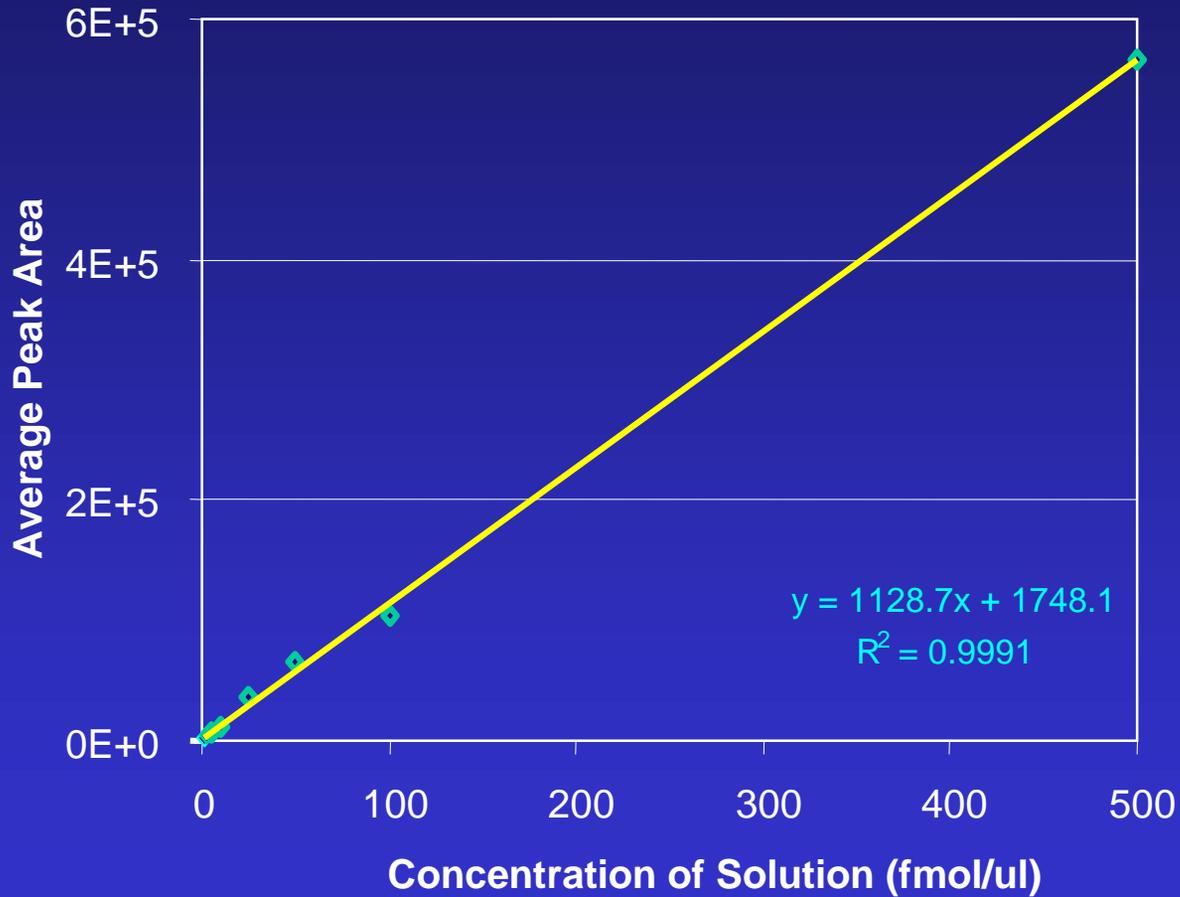
# Comparison of UV and LIF Detection for Small Molecule Mixture



# LIF Response to Tetrol



# Response Linearity for Tetrol



# Experimental Objectives

- Show improvements in sensitivity possible with laser-induced fluorescence (LIF) detection
- Compare detection modes (UV, LIF and MS) for derivatized and underivatized peptide mixtures
- Explore potential advantages of peptide derivatization with 6-aminoquinolyl-N-hydroxysuccinimide carbamate (AQC)
  - Sensitivity
  - Detection of weakly retained underivatized components
  - Orthogonal chromatographic mode for peptide mixtures
- Use LC/MS of derivatized peptides to study derivatization chemistry

# Experimental Protocols

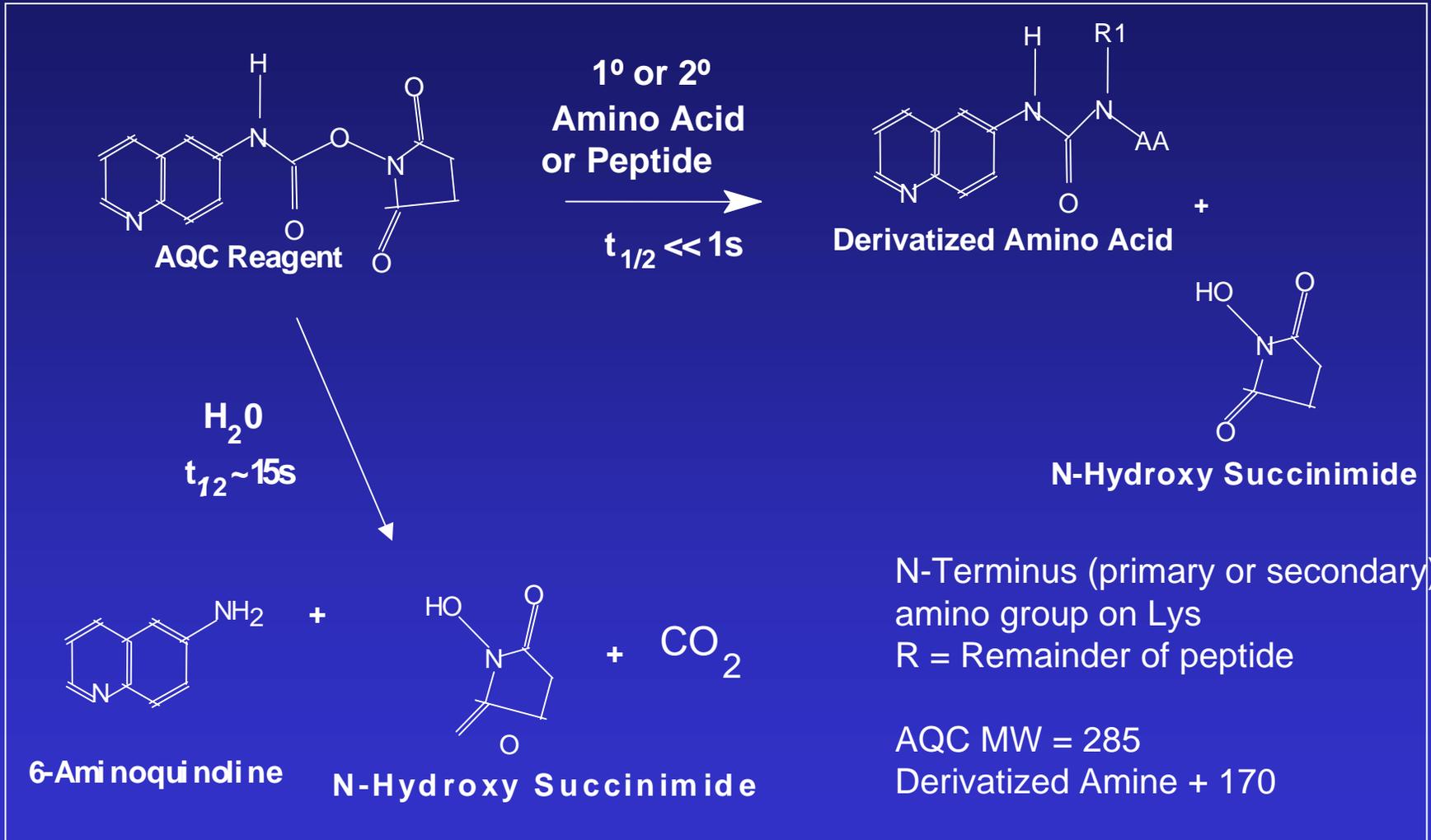
- **Chromatographic System**

- Waters CapLC™ System
- Waters ZMD Mass Spectrometer
- Picometrics Zetalif LIF Detector emission at > 395nm
- Liconix HeCd Laser operating at 325 nm
- Waters Symmetry® C18 columns (0.32 x 150 mm)

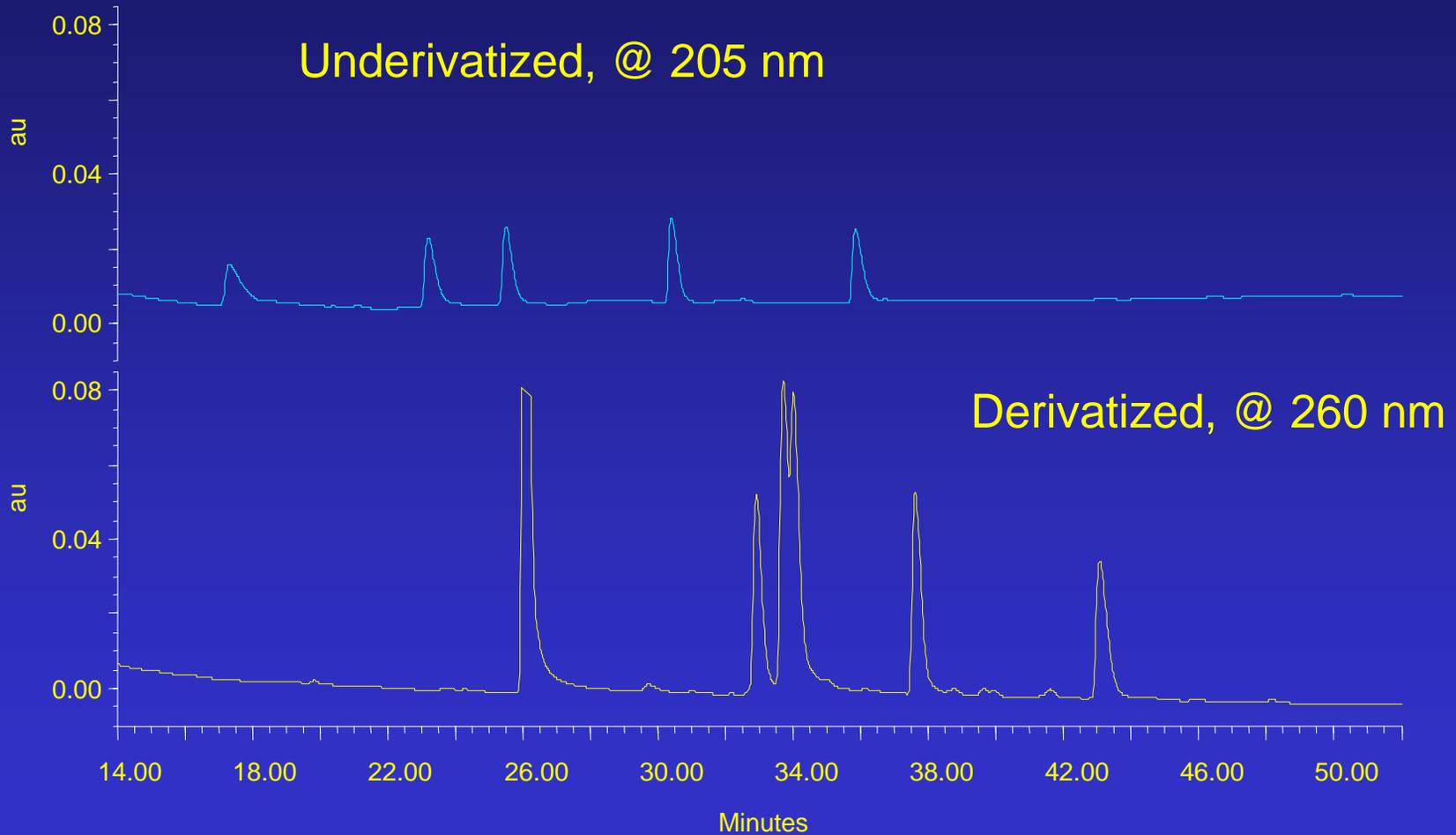
- **Peptide Derivatization**

- **Digest Preparation:** Bovine Cytochrome c was digested with 1 percent Trypsin + CaCL<sub>2</sub> in NaHCO<sub>3</sub> (pH 8.5).
- Sample was incubated at 37 C for 24 hours.
- **Derivatization:** The digest was diluted 10x with water before derivatization (0.1 mg/ml).
- The diluted sample (20 ul) was mixed with 60 ul of borate buffer (0.2 M, pH 8.8) and 20 ul of AQC.
- The final concentration in the derivative was 20 ug/ml (1.6 pmol/ul)

# Derivatization of Peptides with AQC



# Analysis of Peptide Mix at 750 fmol



# Conditions for Peptide Separations

## Chromatographic Conditions

Solvent A: 50 mM NH<sub>4</sub>Ac, pH 6.90.  
Solvent B: 60/40 acetonitrile/water .  
Flow rate: 5 ul/min.  
Gradient: 15 - 50%B in 50 min

Column: Symmetry C18 (100A, 5 um),  
0.32x 150 mm

UV Detection: PDA, 248 nm channel

Fluorescence Detection:  
Excitation 325nm  
Emission > 370nm

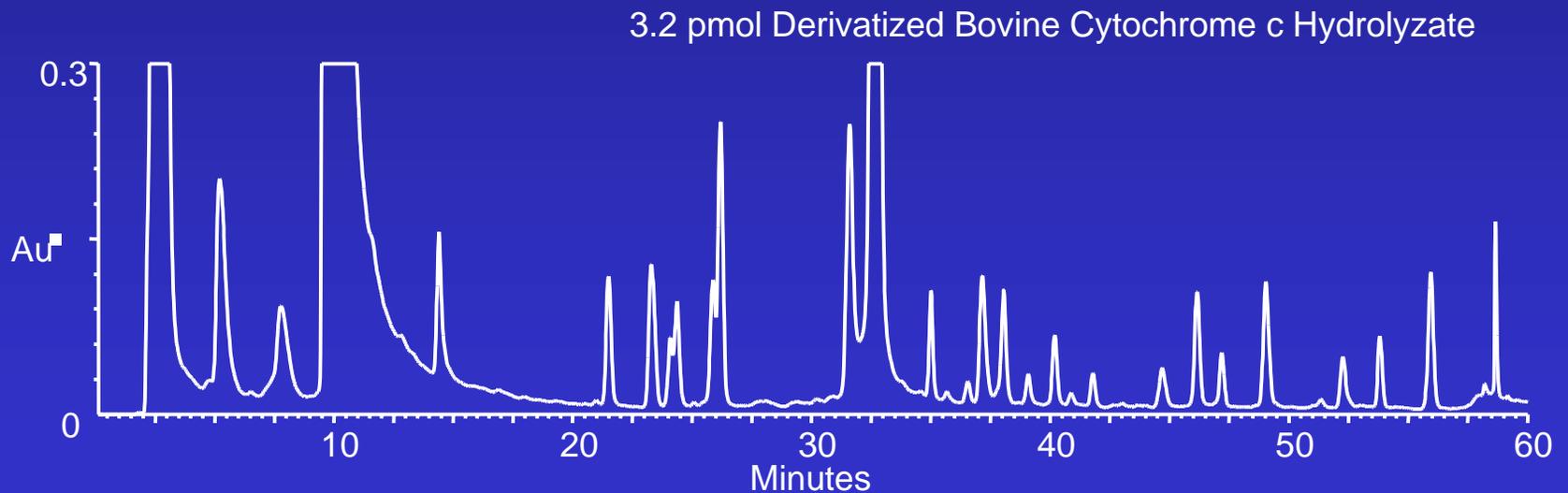
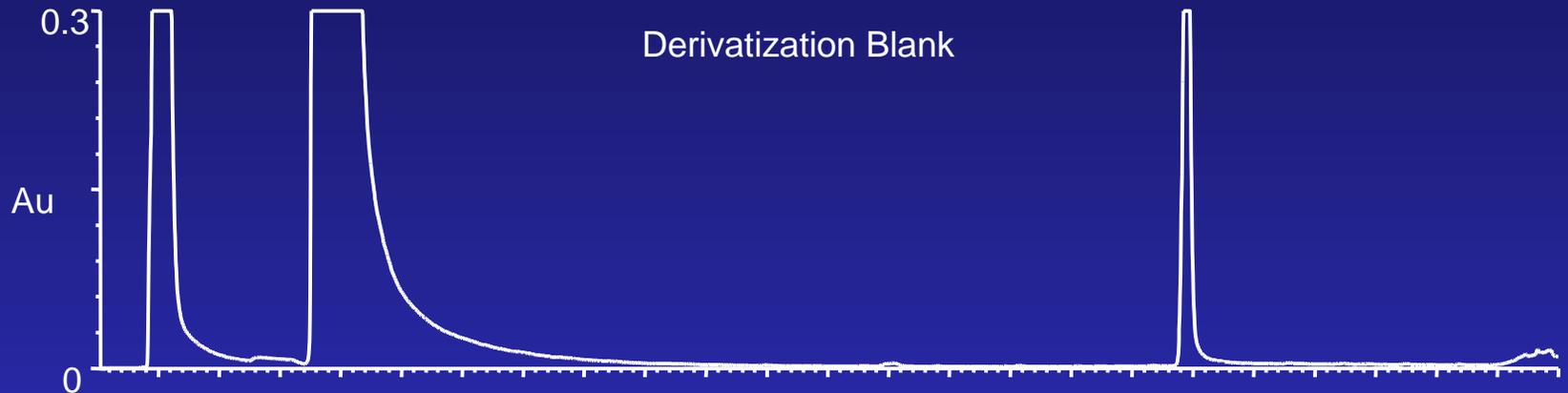
## MS

Ionization Mode: ES+  
Data Type: Compressed centroid  
Mass Range: 500 to 2500

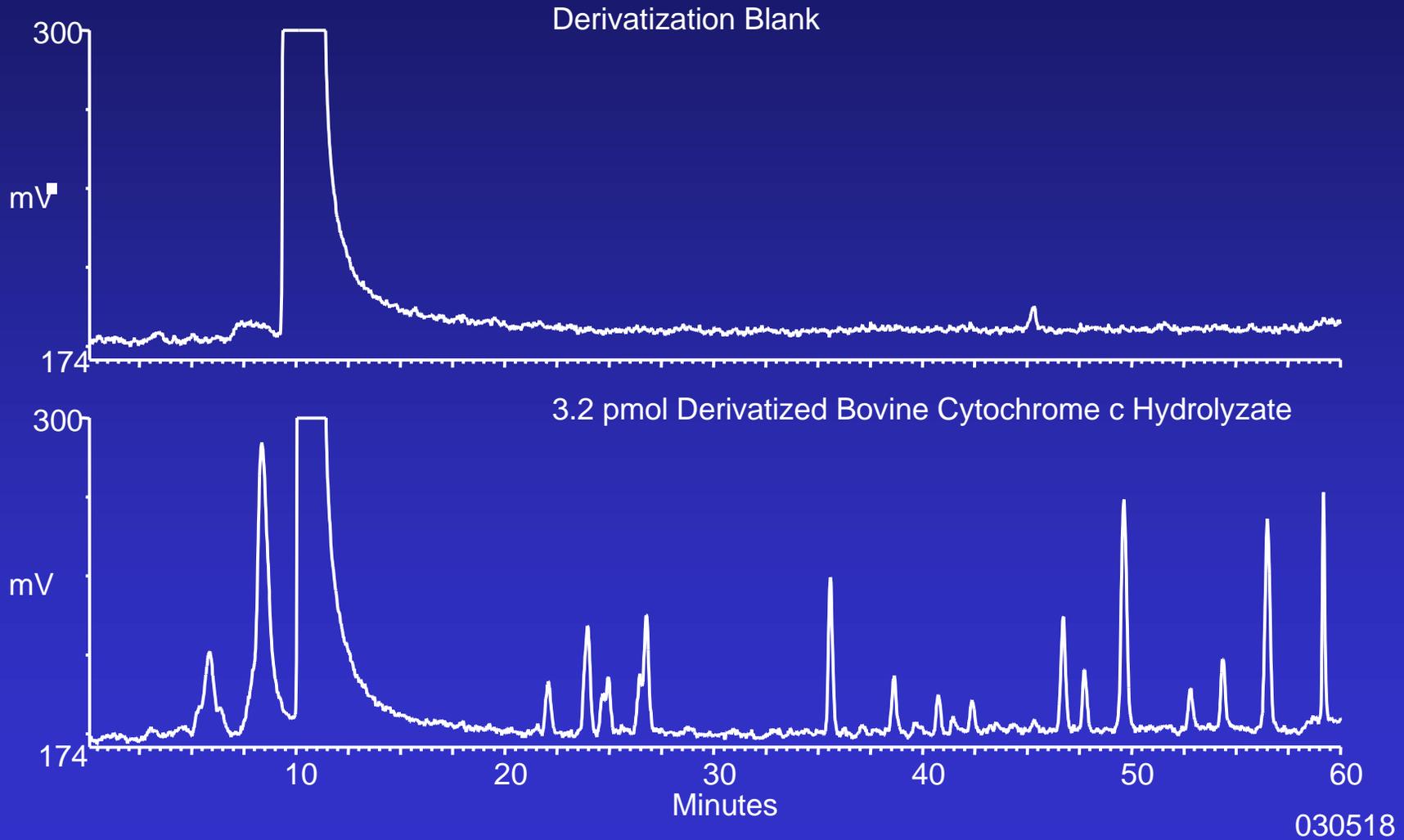
## Tuning Parameters: ES+

Source Page (ESI)  
Capillary: 3.2 kV  
Cone: 50 V  
Extractor: 4 V

# UV Detection for the Derivatized Peptide Mixture

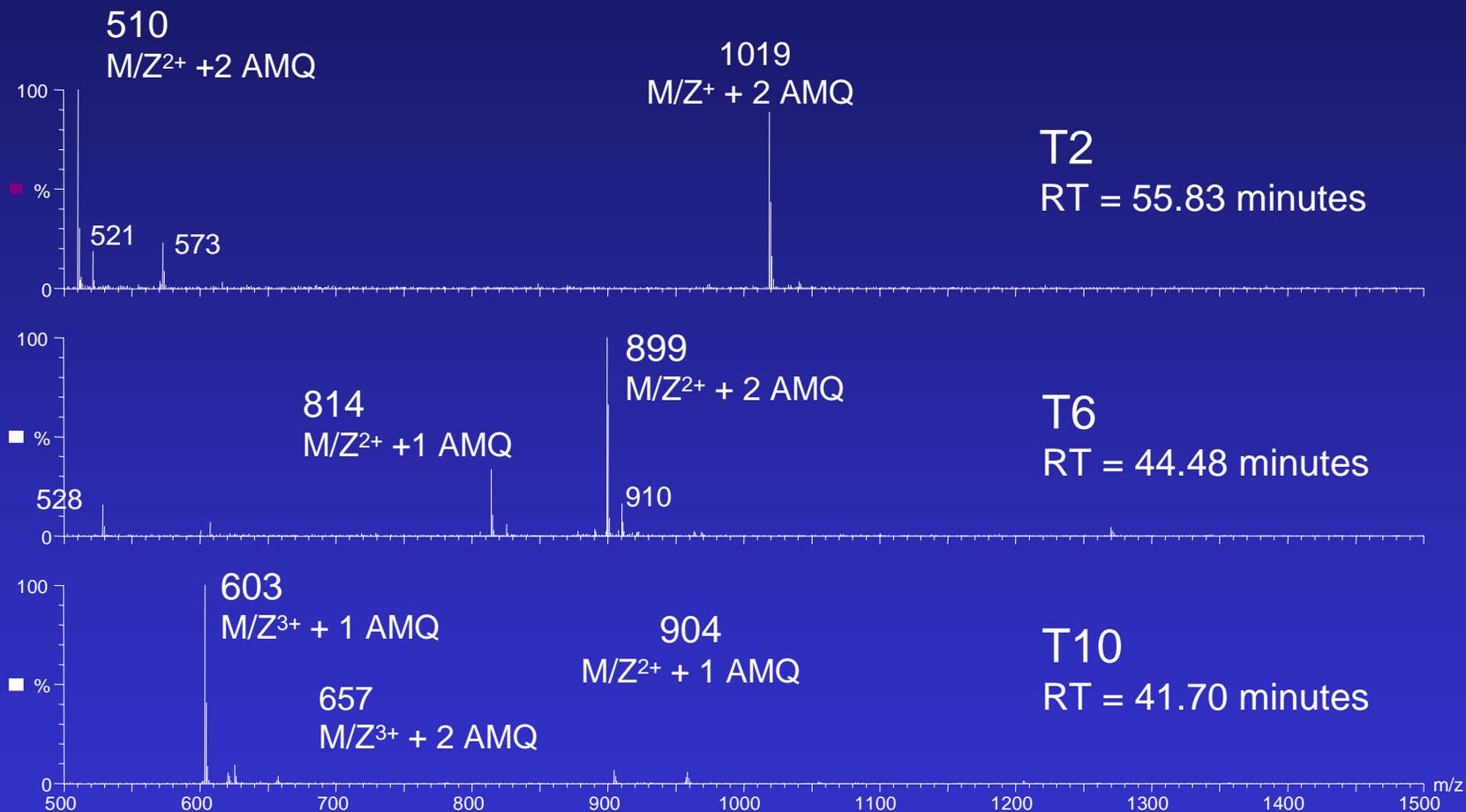


# LIF Detection for Derivatized Peptides





# Mass Spectra for Derivatized Peptides

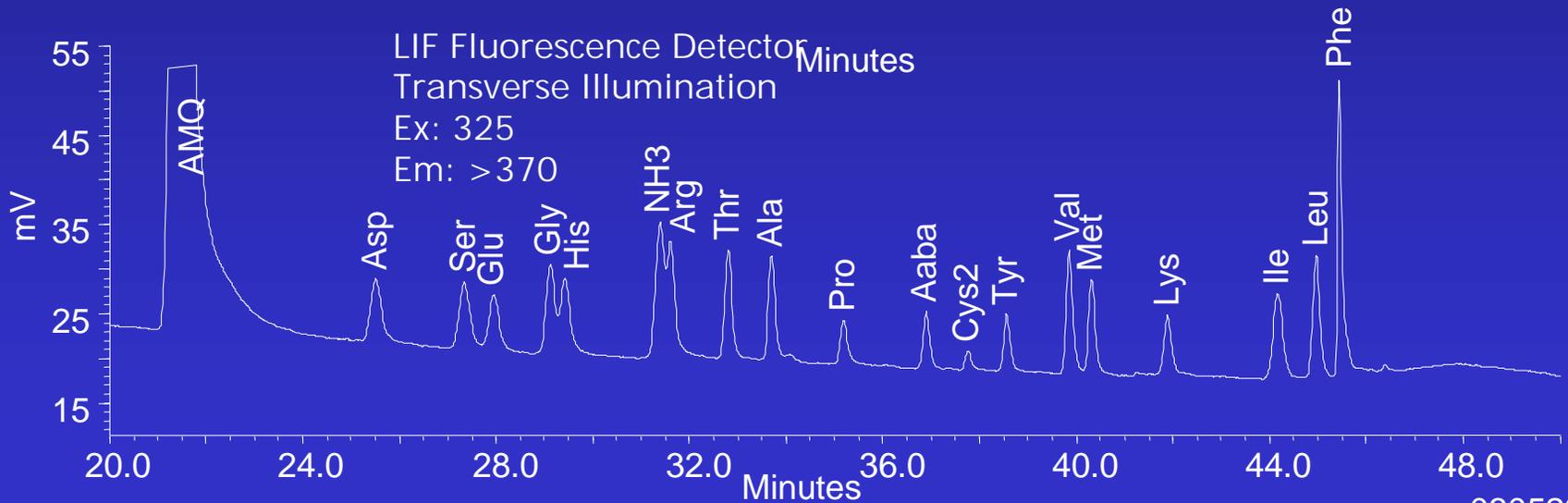
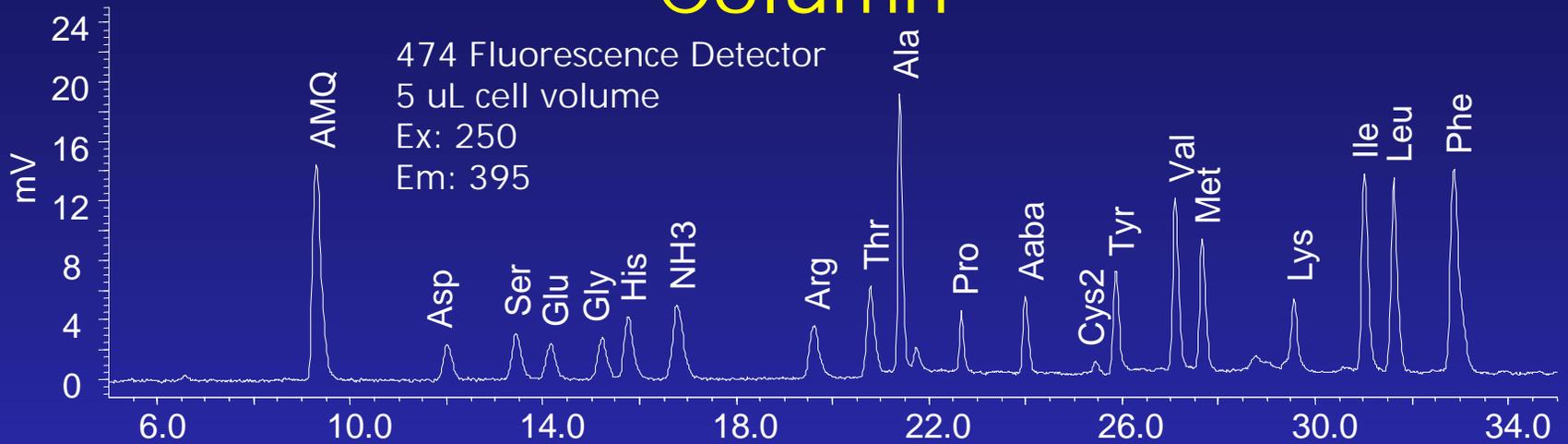


# Details for MS Analysis of Derivatized Peptides

Worksheet for Tagged Peptides

Peak	AA Sequence	#AA	Other	#Tag	Expected			Retention Time with NH4Ac	Observed MW
					MW	MW/2	MW/3		
2	YIPGTK	6		2	1018.7	509.4	339.6	55.83	510, 1019
				1	848.6	424.3	282.9		
6	TGQAPGFSYTDANK	14		2	1797.0	898.5	599.0	44.48	729, 814, 899
				1	1626.9	813.4	542.3		
10	CAQCHTVEK	9	heme	2	1803.0	901.5	601.0	41.7	603, 657, 904
				1	1973.0	986.5	657.7		

# Future Analysis: AQC Derivatized Amino Acids with LIF - 2 pmols on Column



# Summary and Conclusions

- Capillary LC analysis can provide low or even sub-femtomole detection limits for a variety of analytes
- LIF detection is highly suitable for capillary LC and is one of the most sensitive detection methods for molecules with fluorescence at available laser wavelengths
- Derivatization of peptide mixtures with AQC offers a highly sensitive alternative to analysis of underivatized samples
- MS results demonstrate successful production of fully tagged peptides with little evidence of partial derivatization
- Derivatization of tryptic digests provides retention for hydrophilic small peptides and simplifies LC and LC/MS analysis of these components
- Derivatization can provide improved sequence coverage of the protein by analysis of the hydrophilic peptides

# Acknowledgments:

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