Smaller is Better: Femtomole Analysis of Peptides with Capillary HPLC and UV, Fluorescence and Mass Spectrometry Detection

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Abstract

Peptide analysis is now commonly being performed with extremely small amounts of sample. Consequently, biochemists are turning to capillary separation methods to solve serious issues with detection sensitivity. Capillary liquid chromatography is especially well-suited for these problems and is readily employed in conjunction with high sensitivity detector such as a mass spectrometer (MS) or laser induced fluorescence (LIF) detector. We have used an integrated approach to optimizing a capillary HPLC system for performance and sensitivity. Using columns ranging in size from 0.18 – 0.5 mm ID, UV sensitivity is maximized using a light guiding long pathlength (5mm) flow cell incorporated into a photodiode array detector. With this design, detection limits for underivatized peptides are approximately 100 fmol. Derivatization with the aminoquinolyl tag lowers this to 30 – 50 fmol on-column via UV detection at 248 nm. Using an LIF detector with a helium-cadmium lamp and excitation at 325 nm, limits of detection for labeled peptides are decreased to the low femtomole range, and newer laser technology promises to reduce this to attomole levels. Both derivatized and underivatized peptides are amenable to MS detection. LC/MS analysis of peptide mixtures with the capillary system and a Q-Tof MS provides capability for low femtomole analysis of protein spots excised from 2D gels. The MS analysis provides accurate peptide molecular weight determination as well as sequence information via MS/MS analysis.

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-hydroxysuccinimiyl 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

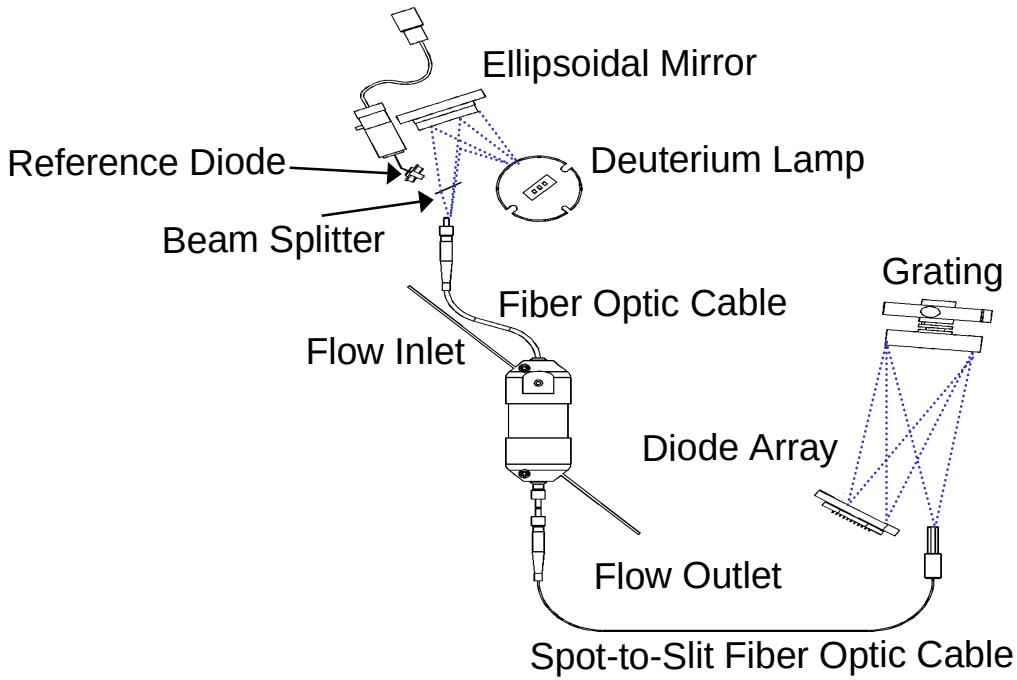
Experimental Protocols

- **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**: Cytochrome c (Sigma, 400ul,10 mg/ml) was mixed with 1 ml of NaHCO3 (pH 8.5) and 400 ul of 0.1 M CaCl2. Trypsin (40 ul, 1 mg/ml, Worthington) and 2160 ml of water were then added. The 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)

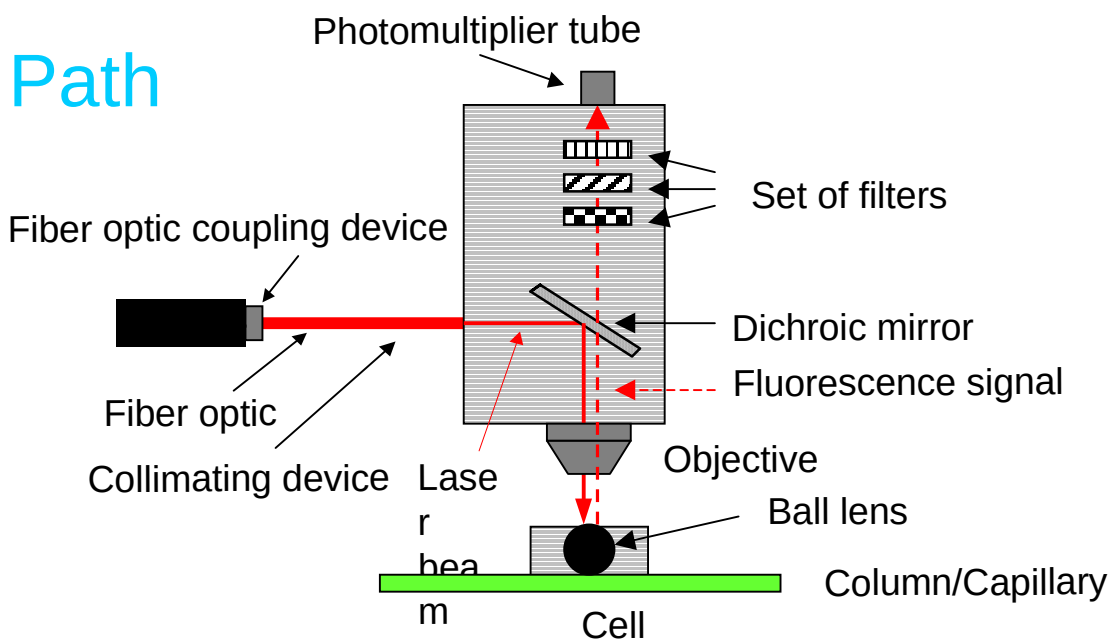
Photodiode Array Detector Optical Path

The PDA detector uses fiber optics and patented light-guiding technology to introduce the light into a long (5mm) pathlength flow cell. The flow cell is made of Teflon(R) AF, a polymer with very low refractive index, that allows for total internal reflection of the light. The results is nearly quantitative light throughput with excellent sensitivity and linearity.



LIF Detector Optical Path

The LIF detector also uses fiber optics to transmit light from the source to the flow cell. A ball-shaped lens focuses the beam on a capillary cell (transverse illumination) and the emitted light is captured 180 degrees to the incident light. The emitted energy (at higher wavelength than the laser light) passes through the dichroic mirror and is detected by the photomultiplier tube.



Experimental Conditions for LC/UV/LIF Experiments

Instrumentation

Waters CapLC System, Liconix Helium Cadmium Laser and Picometrics Zetalif Fluorescence Detector

Chromatographic Conditions

- Column: 15 cm x 0.32 mm Symmetry® C18, 100 Å, 5 µm
- Flow Rate: 10 µl/min
- Mobile Phase: A: 0.01% TFA in MilliQ Water, B: 100% MeOH
- Gradient Slope: 30-70% B in 20 minutes
- Column Temperature: 50°C
- Injection Volume: 1 µl
- Total Run Time: 30 minutes

Detection Parameters

- PDA Detection from 220 to 400 nm
- Fluorescence Excitation at 325 nm, Emission Detection at 395 nm

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)

Experimental Conditions for LC/MS Experiments

Initial MS Scan of Compounds of Three Test Compoinds

Ionization mode:	ES+
Mass Range	200 to 600
Inter Scan delay	0.08
Scan Duration (Secs)	0.8

Ion Optics:

Cone	30 V
Capillary	3.2 KV
Extractor	5 V
RF Lens	0.5 V
Source Block Temp	100°C
Desolvation Temp	100°C

Single Ion Recording (SIR) Conditions for ABG

Mass = 435 (M+1)

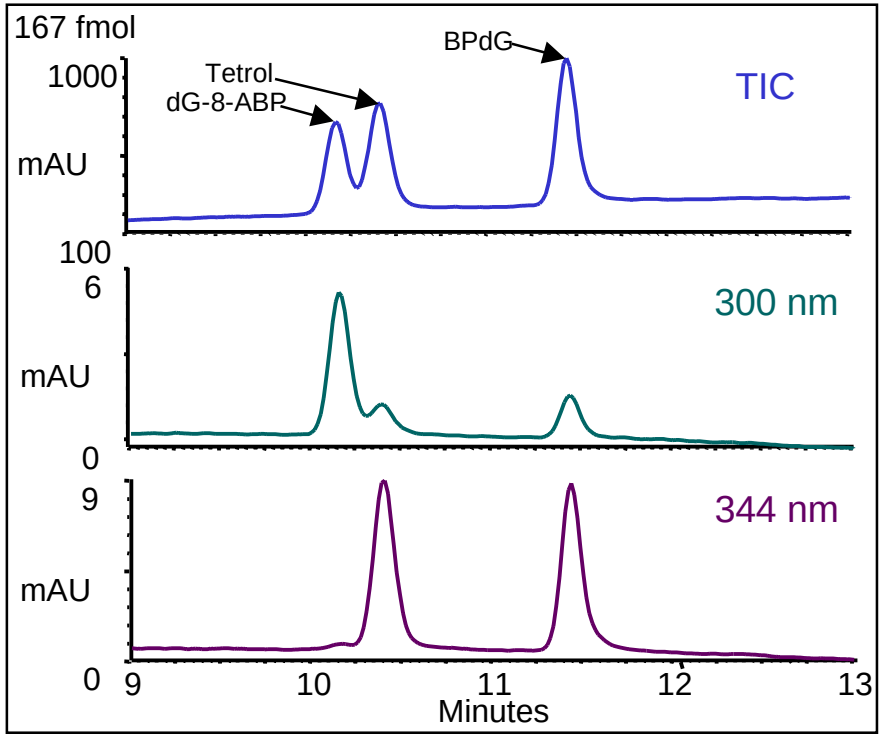
Inter Channel delay	0.02
Span (Daltons)	0.10
Start Time (Mins)	0.00
End Time	30.00
Repeats	1
Dwell (Secs)	0.30

Ion Optics:

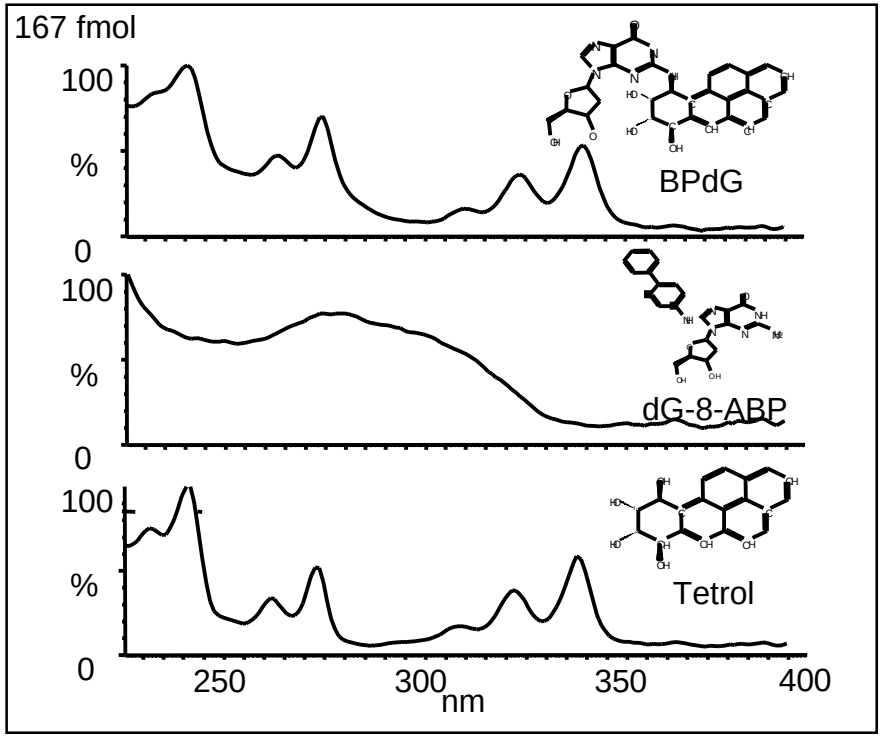
Cone	30 V
Capillary	3.2 KV
Extractor	5 V
RF Lens	0.20 V
Source Block Temp	100°C
Desolvation Temp	100°C

UV and LIF for Small Molecule Mixture

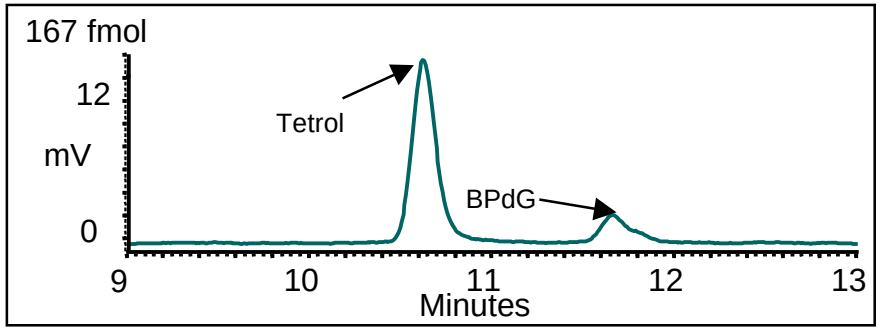
Full Spectrum and
Extracted Wavelength Chromatograms



Absorbance Spectra for
Components of Mixture

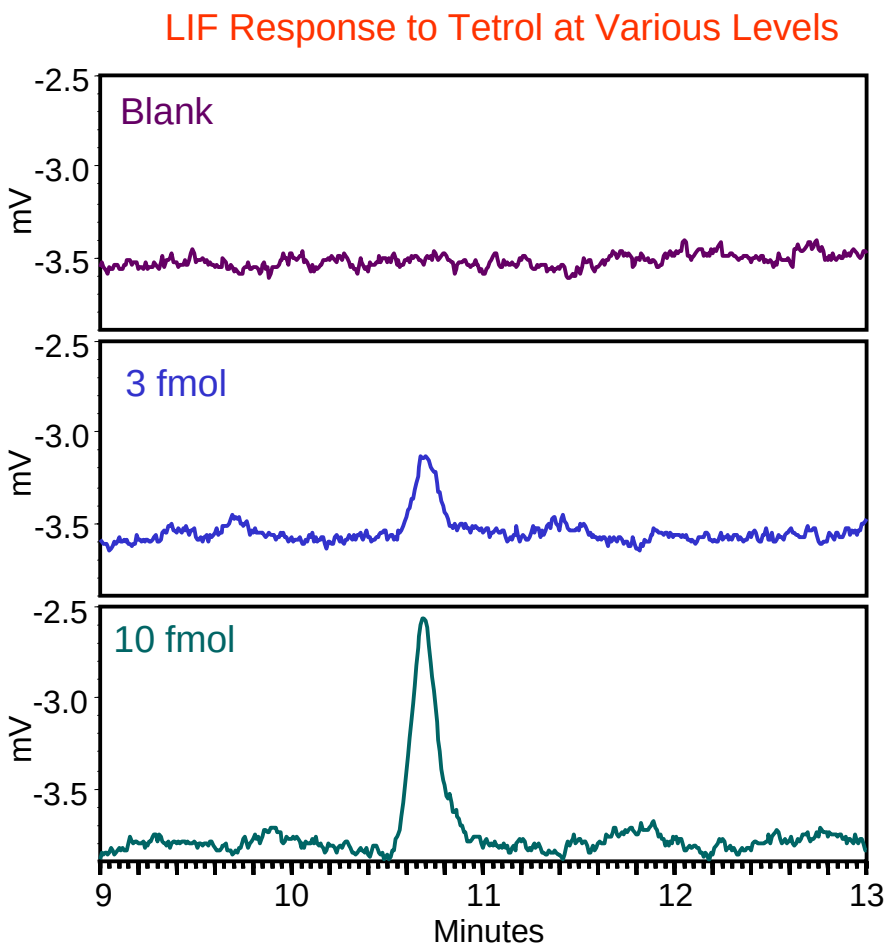


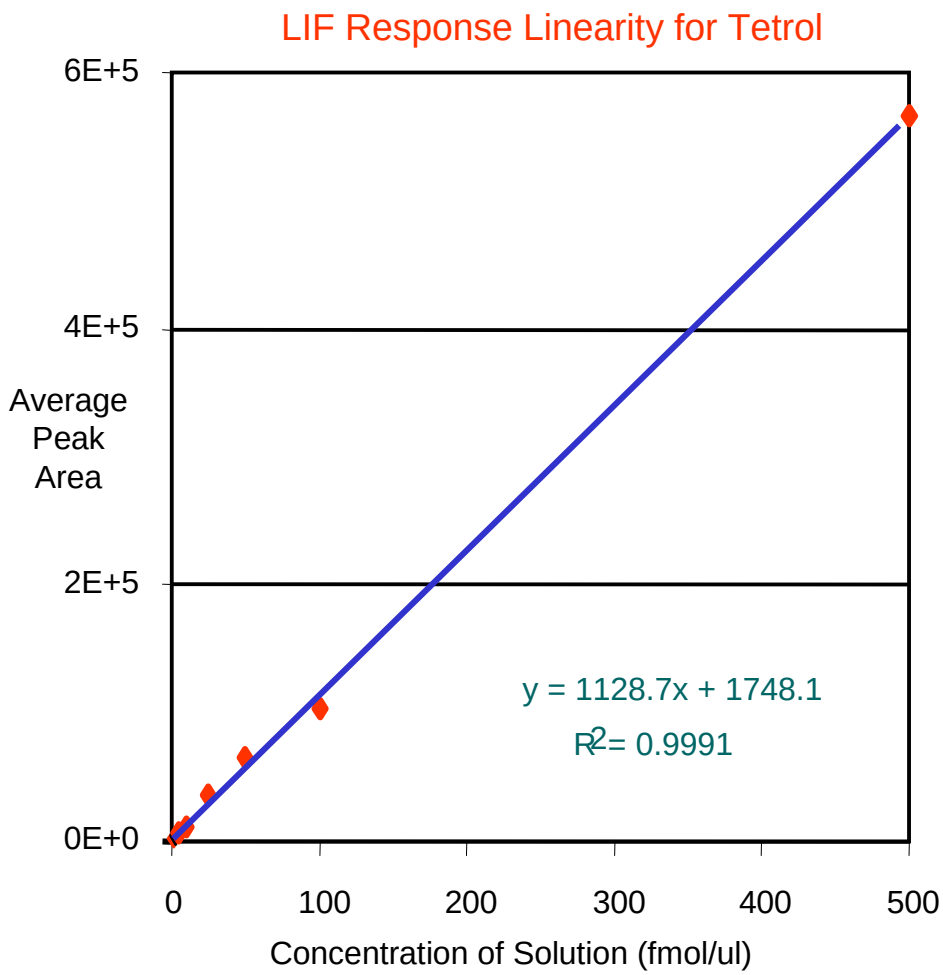
Fluorescence Chromatogram



Ultra-violet detection of the small molecule mixture allows low detection limits for the 3 components--down to 10 fmol for tetrol. It also affords the ability to collect absorbance spectra for each component and the opportunity to extract wavelengths of interest. However, for fluorescent molecules like tetrol, the limits of detection may be greatly improved by incorporating laser induced fluorescence into the detection scheme.

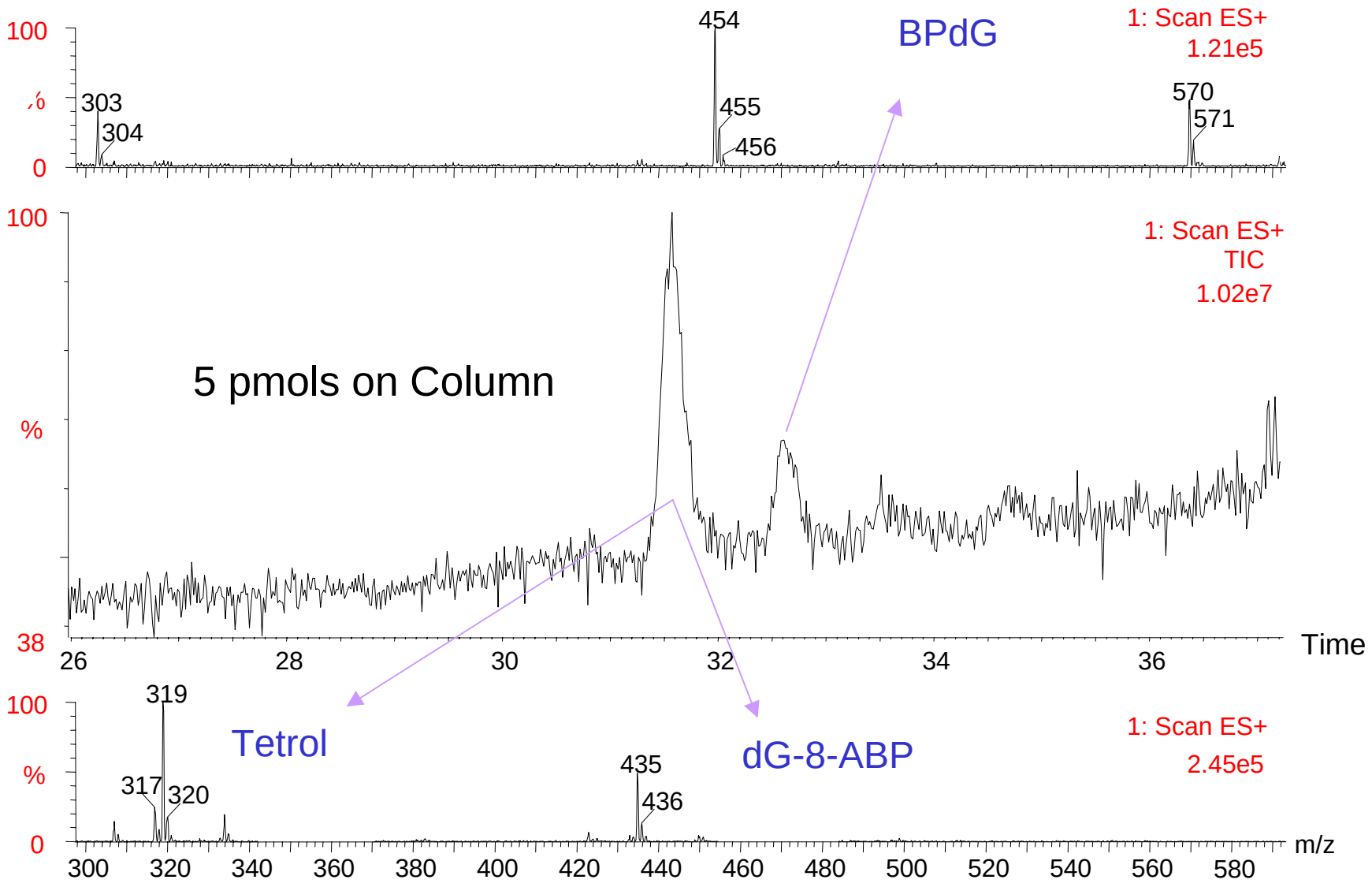
LIF Response to Tetrol



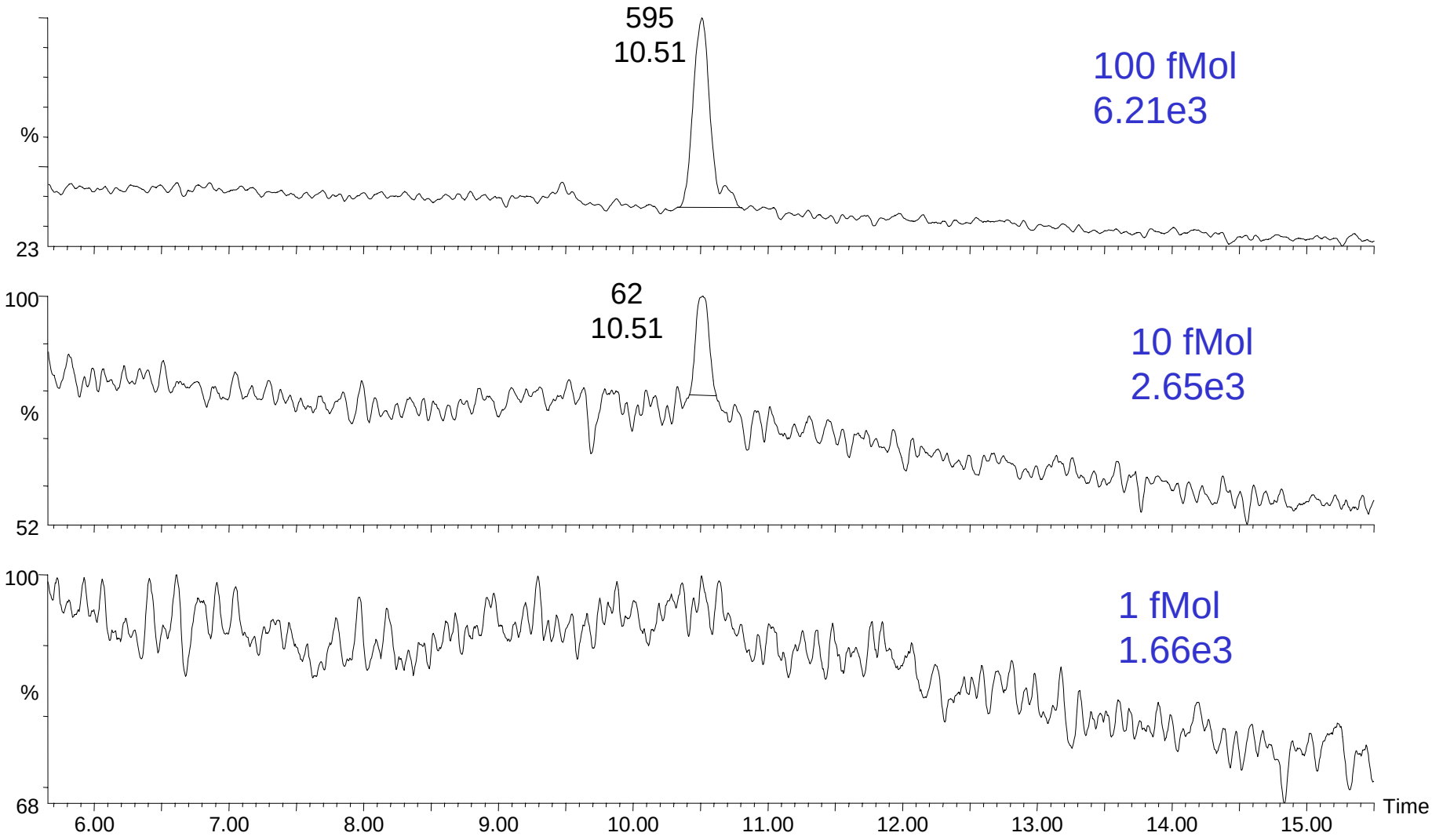


Laser induced fluorescence detection of tetrol yields limits of detection down to 1 fmol, a 10-fold improvement over UV detection. The LIF response to tetrol was linear from 3 fmol/ul to 500 fmol/ul.

MS Scan of Compounds 1, 2 and 3



SIR Compound dG-8-ABP, Level of Detection



Conditions for Peptide Separations

Chromatographic Conditions

Solvent A: 50 mM NH_4Ac, pH 6.90.
Solvent B: 60/40 acetonitrile/water .
Flow rate: 5 ul/min.
Column: Symmetry C18 (100A, 5 um), 0.32x 150 mm
Gradient: 15 - 50%B in 50 min
Injection Volume: 1 - 5 ul
UV Detection: PDA, 248 nm channel
Fluorescence Detection:
    Excitation 325nm
    Emission > 395nm

MS

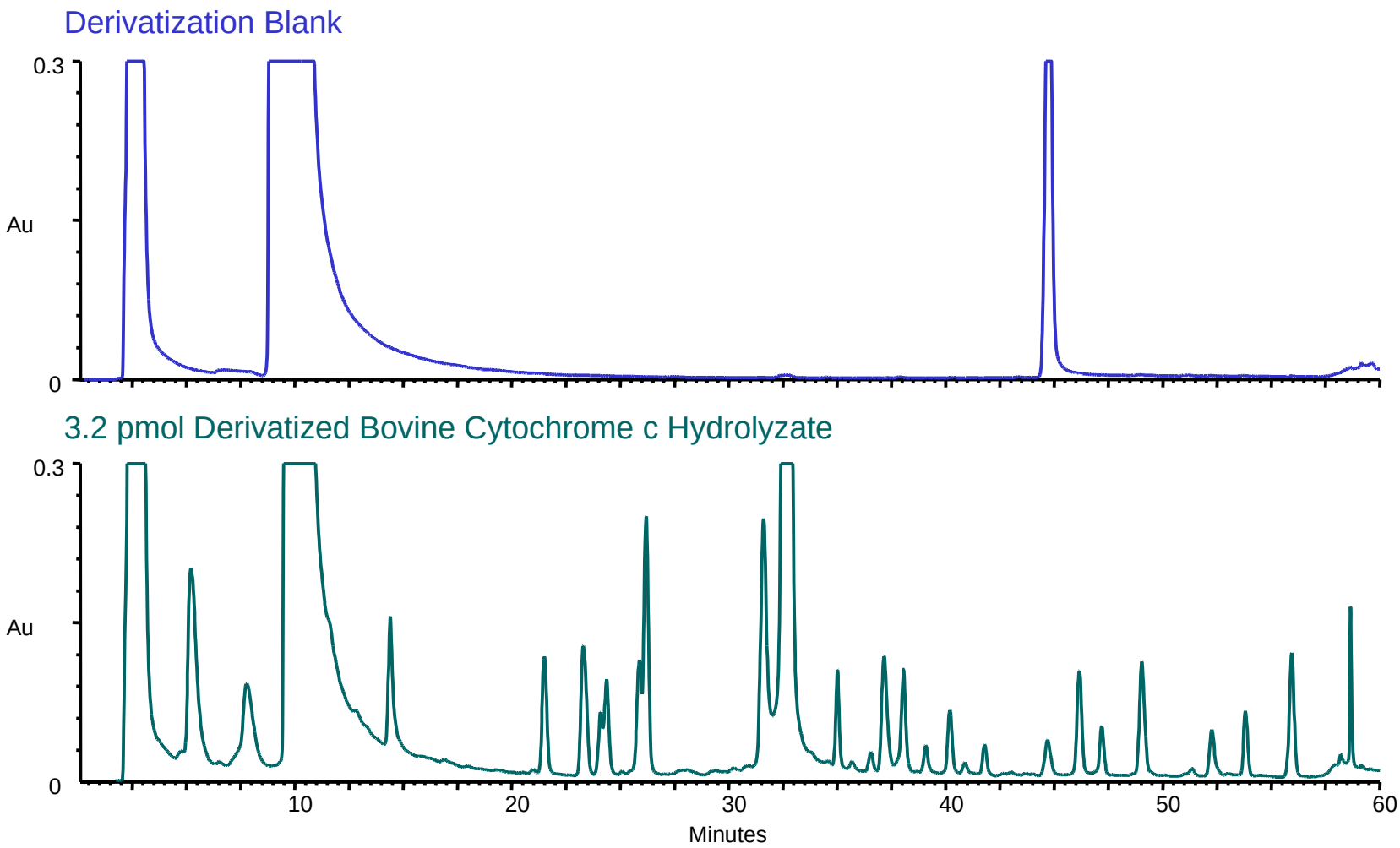
Scan duration (secs):	1.99
Interscan delay (secs):	0.09
Retention window (mins):	0.000 to 65.000
Ionization mode:	ES+
Data type:	Compressed centroid
Function type:	Scan
Mass range:	500 to 2500
Cone Voltage:	52

Tuning Parameters: ES+

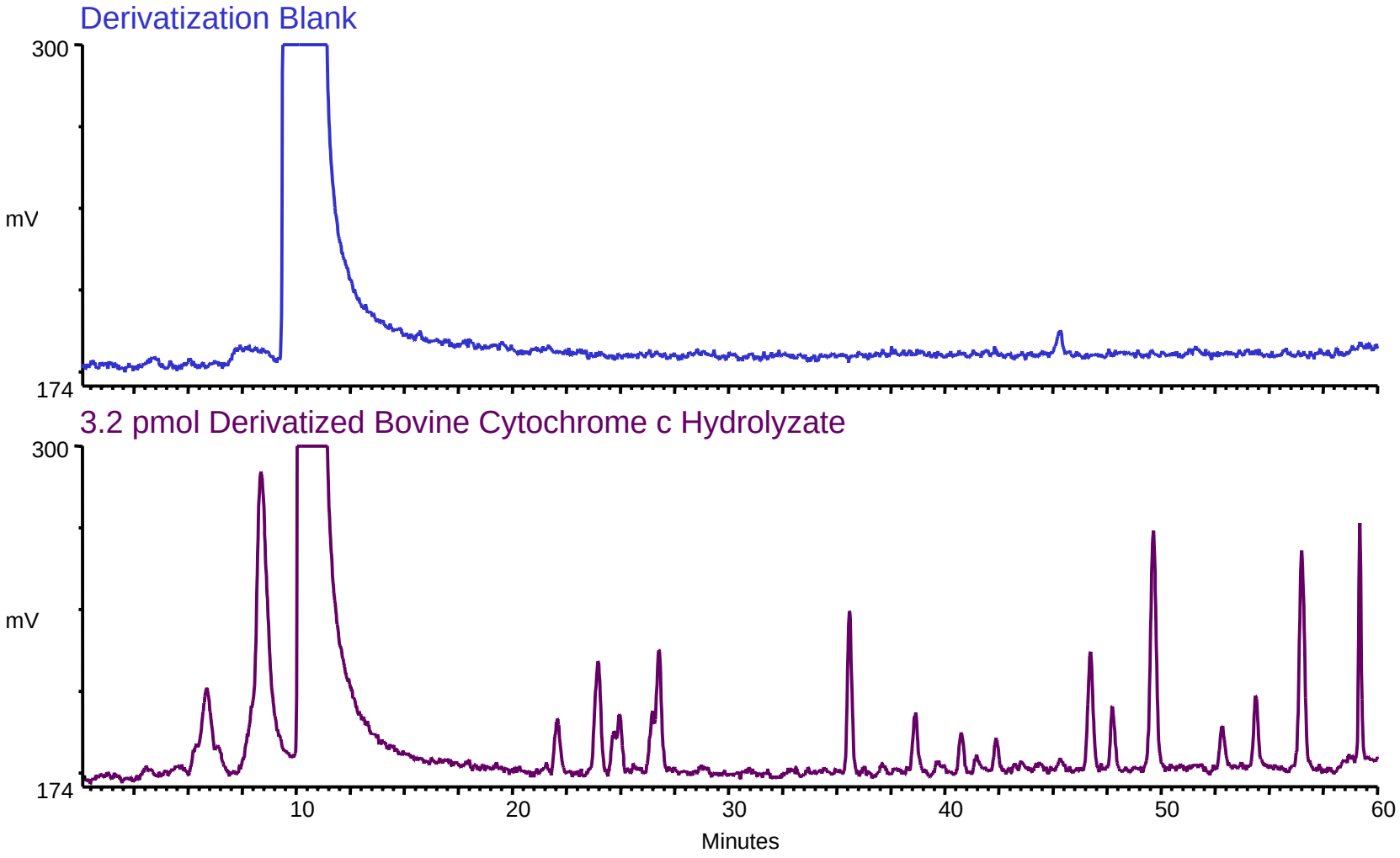
Source Page (ESI)

Capillary:	3.20	kVolts
Cone:	50	Volts
Extractor:	4	Volts
RF Lens:	0.20	Volts
Source Block Temp.:	100 degrees C	
Desolvation Temp.:	100 degrees	
Ion Energy:	0.2	Volts
Ion Energy Ramp:	0.0	Volt
LM Resolution:	12.0	
HM Resolution:	12.0	
Multiplier:	650	Volts

UV Detection for the Derivatized Peptide Mixture

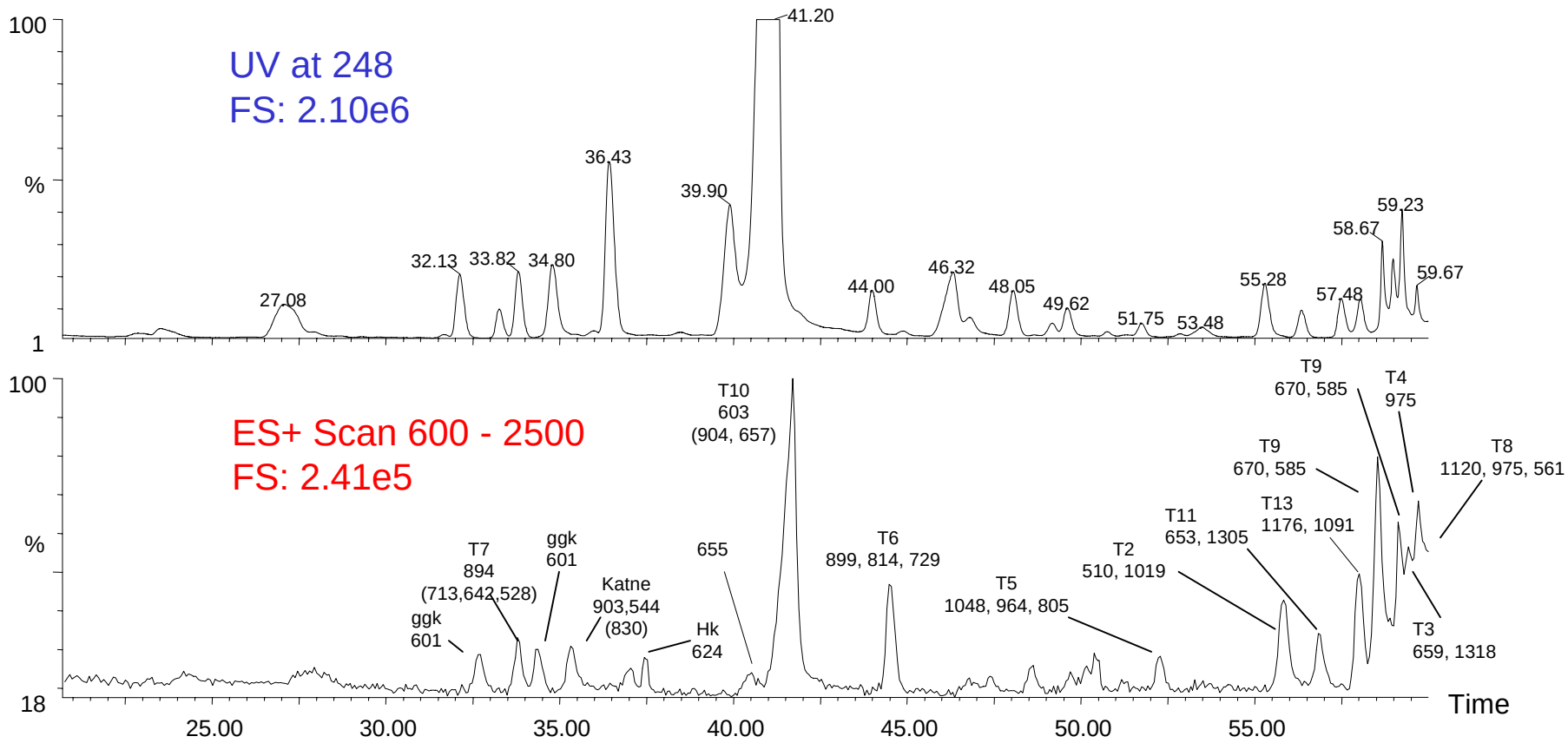


LIF Detection for Derivatized Peptides

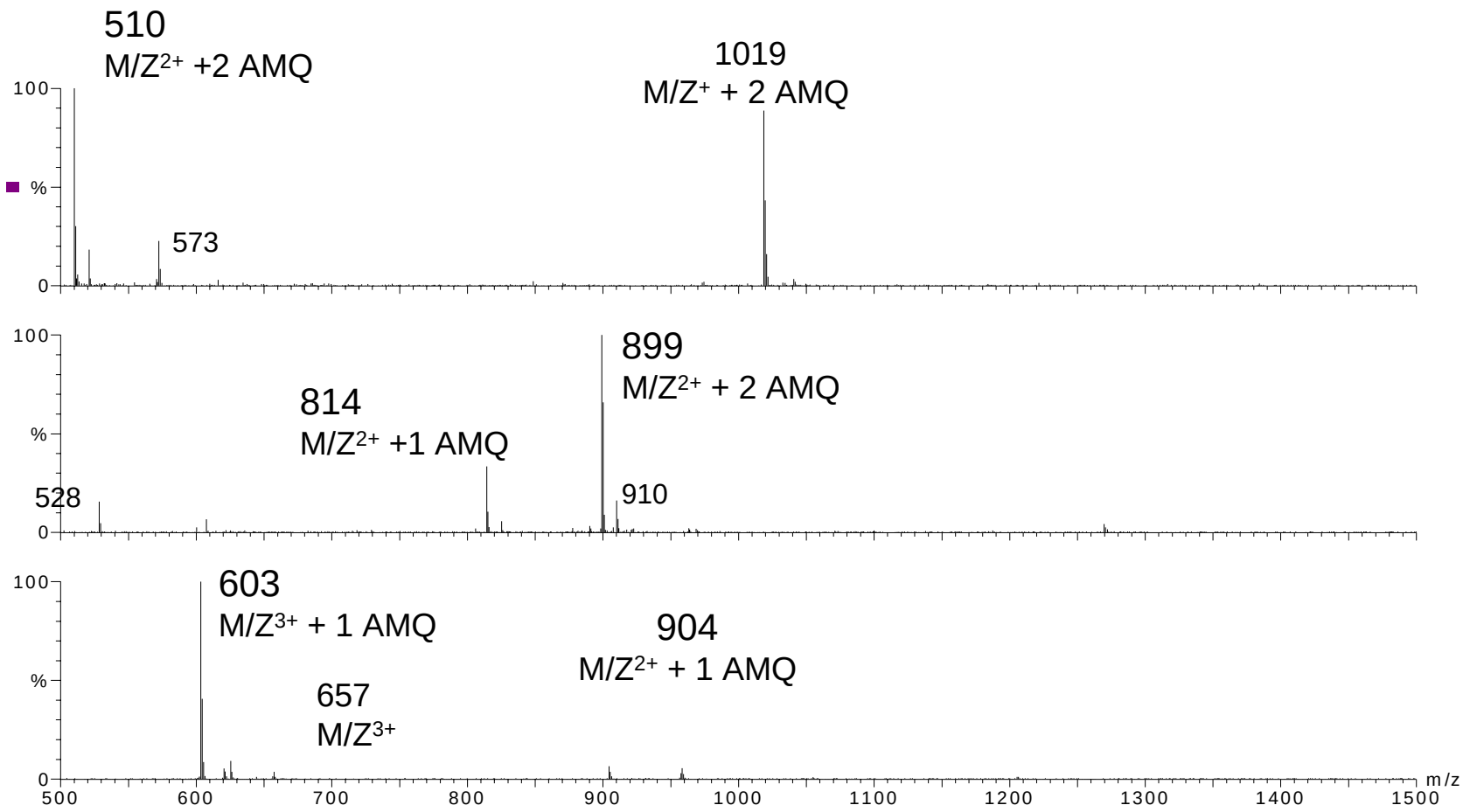


LC/MS Analysis of Derivatized Digest

1.6 pmols Bovine Cytochrome c



Results from an LC/UV/MS analysis are shown above. In the MS TIC trace peaks are numbered according to their relative retention as underivatized peptides using a 0.1% TFA mobile phase system. Note the dramatic change in retention order, due to both the change in eluent pH and the strong influence of the number of tags on a particular peptide. Peaks are also labeled with the most prominent ions observed in their mass spectra. See the table for more details.


510
M/Z2+ +2 AMQ
1019
M/Z+ + 2 AMQ
573
899
M/Z2+ + 2 AMQ
814
M/Z2+ +1 AMQ
528
910
603
M/Z3+ + 1 AMQ
904
M/Z2+ + 1 AMQ
657
M/Z3+
100
%
0
500
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z