Femtomole and Subfemtomole Analysis by Capillary HPLC

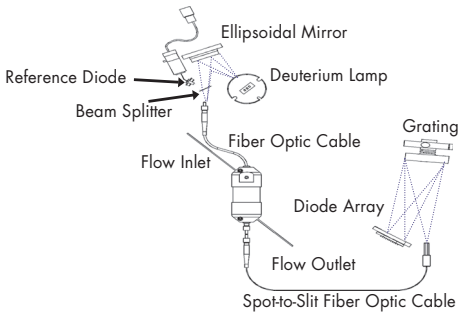
Steven A. Cohen and Cozette M. Cuppett
Waters Corporation, 34 Maple Street, Milford, MA, 01757

Experimental Objectives

- Study sensitivity limits in capillary chromatography
- Compare UV, laser-induced fluorescence (LIF) and mass spectrometry detection
- Explore the applicability of derivatization for enhancing amino acid and peptide detection in capillary chromatography

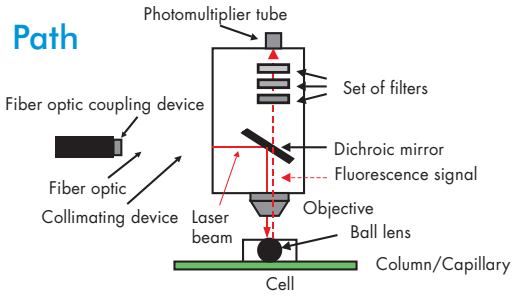
Photodiode Array Detector Optical Path

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® AF, a polymer with very low refractive index, that allows for total internal reflection of the light. The result 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.



Instrumental Setup

System 1: LC/UV/LIF Studies

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

- DNA adducts
- Derivatized peptides
- Derivatized amino acids

System 2: LC/UV/MS Studies

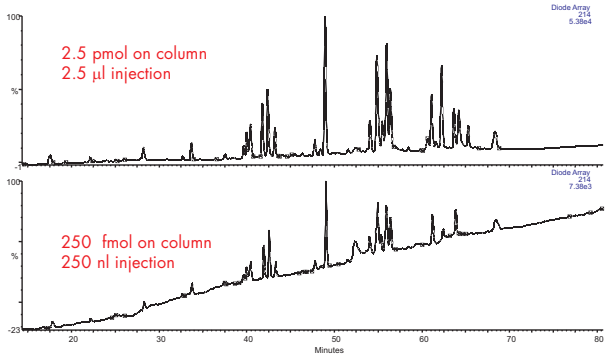
Waters CapLC System plus Waters/Micromass ZMD Mass Spectrometer

- DNA adducts
- Derivatized peptides

Femtomole and Subfemtomole Analysis by Capillary HPLC

Steven A. Cohen and Cozette M. Cuppett
Waters Corporation, 34 Maple Street, Milford, MA, 01757

LC/UV of Peptides



This separation of a complex peptide digest shows the excellent resolution and high sensitivity possible with an optimized capillary system design with light-guiding flow cell technology (US Patent 5,184,192).

Conditions for High Sensitivity UV Peptide Detection

Chromatographic Conditions

- Column: 0.32x150 mm Symmetry C_{18}, 300 Å, 5 µm
- Eluent A: 0.02% TFA in H2O
- Eluent B: 0.017% TFA in MeCN
- Flow Rate: 5 µl/min
- Gradient: Hold at 2% B for 2 min, 2-40% B in 70 min, 40-60% B in 25 min, 60-100% B in 10 min, hold at 100% B for 10 min
- Total Run Time: 140 min

Detection Parameters

- Photodiode Array Detector with light guiding flow cell, 200 - 300 nm

Sample

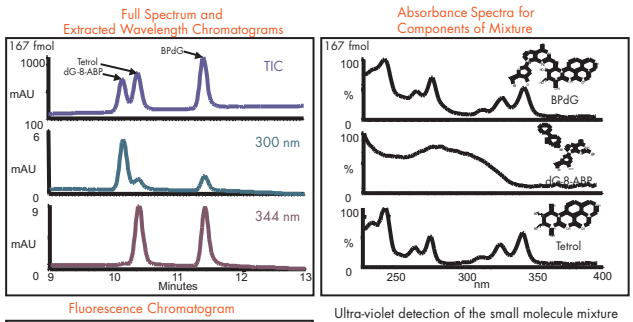
- Tryptic digest of an 80kD protein

Femtomole and Subfemtomole Analysis by Capillary HPLC

Steven A. Cohen and Cozette M. Cuppett
Waters Corporation, 34 Maple Street, Milford, MA, 01757

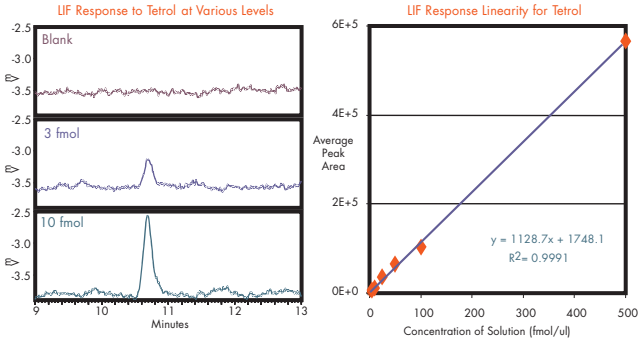
LC/UV/LIF of DNA-Type Adducts

UV and LIF for Small Molecule Mixture



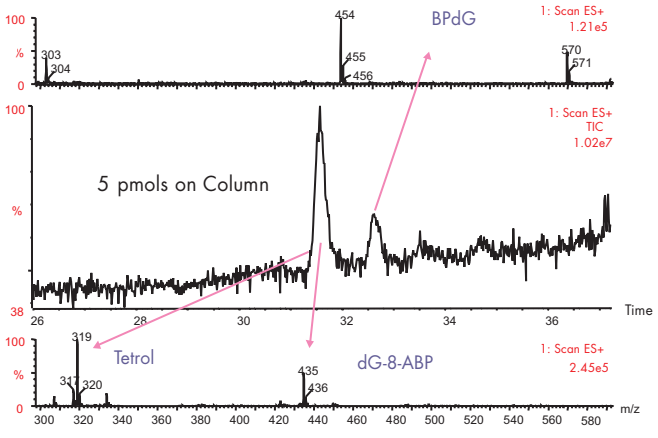
Ultra-violet detection of the small molecule mixture allows low detection limits for the 3 components–down to 10 fmol for tetrol. It also affords the ability to collect absorbance spectra for each component and the opportunity to extract wavelengths of interest. However, for fluorescent molecules like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

LIF Response to Tetrol

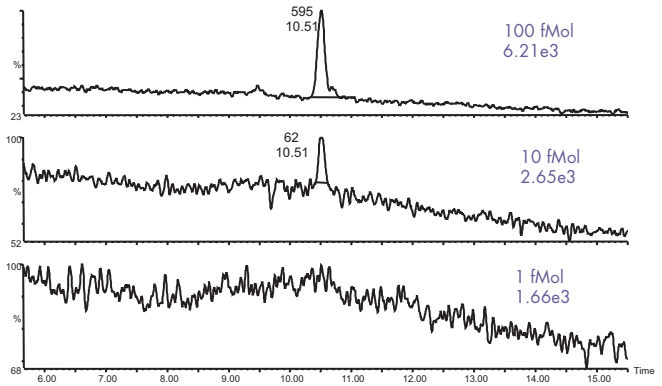


Laser induced fluorescence detection of tetrol yields limits of detection down to 1 fmol, a 10-fold improvement over UV detection. The LIF response to tetrol was linear from 3 fmol/ul to 500 fmol/ul.

MS Scan of Compounds 1, 2 and 3



SIR Compound dG-8-ABP, Level of Detection



Experimental Conditions for LC/UV/LIF Study of DNA-Type Adducts

Chromatographic Conditions

- Column: 0.32 x 15 mm Symmetry® C18, 100 Å, 5µm
- Flow Rate: 10 µL/min
- Mobile Phase: A: 0.01% TFA in MilliQ Water, B: 100% MeOH
- Gradient: 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 > 360 nm

Samples

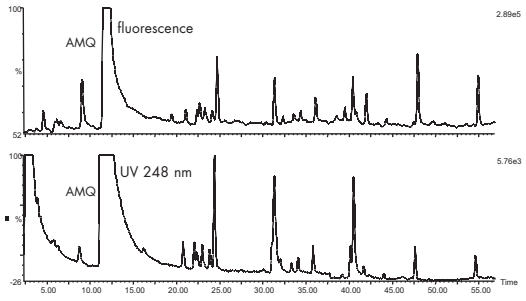
- Nucleotide adducts and precursor analog: deoxyguanosyl-amino-biphenyl (dG-8-ABP), tetrol and benzo[a]pyrene diol deoxyguanosine (BPdG), samples courtesy of Dr. Radoslav Goldman, NIH)

Femtomole and Subfemtomole Analysis by Capillary HPLC

Steven A. Cohen and Cozette M. Cuppett
Waters Corporation, 34 Maple Street, Milford, MA, 01757

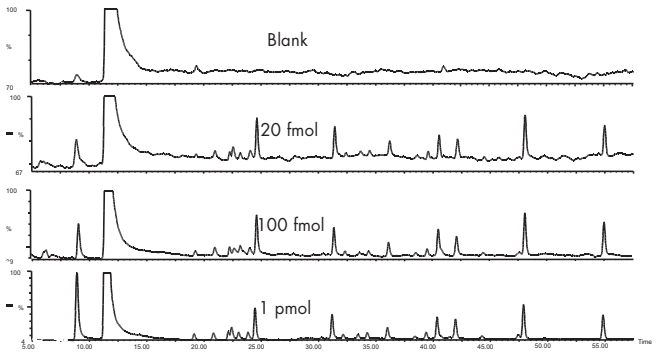
LC/UV/LIF and LC/UV/MS of Derivatized Peptides

CapLC-UV/LIF Chromatograms of 40 fmol Derivatized Peptide Digest

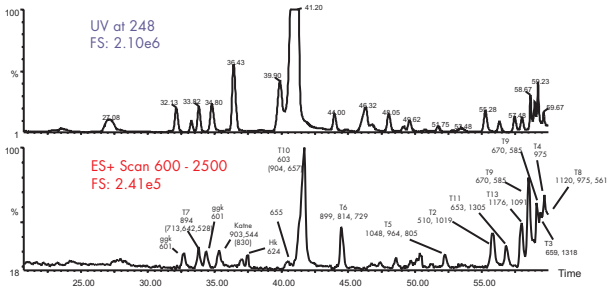


Detection of derivatized peptides by both UV and LIF provides excellent sensitivity with low femtomole limits. The large reagent peak (AMQ) is somewhat reduced in magnitude with the current detection system using a cutoff filter; a narrow bandpass filter(ca. 395nm) would greatly reduce the reagent peak size.

CapLC-LIF of Derivatized Cytochrome C Digest

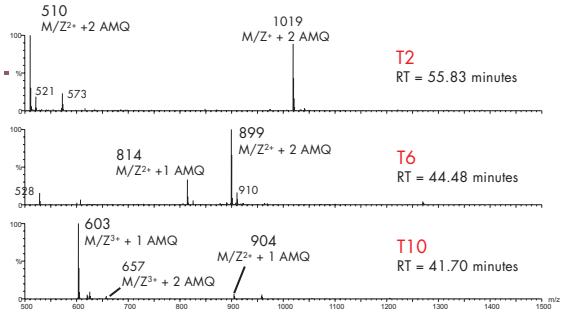


LC/MS Analysis of Derivatized Digest
1.6 pmols Bovine Cytochrome c



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.

Selected Mass Spectra for Derivatized Peptides



Spectral data reveal several characteristics for the AQC-derivatized peptides. (1) In general, the doubly charged ions predominate. (2) With the conditions used (cone voltage = 50V), many peptides show fragmentation to lose one or more of the quinolyl tags. These m/z values are the same as those expected for partially tagged products, but they only appear in peaks containing the fully tagged peptides. Even with only appooximately1.6 pmol of sample, the spectra show excellent signal-to-noise.

Experimental Conditions for Peptide Analysis

Chromatographic Conditions

- Column: 0.32 x 150 mm Symmetry C18, 100 Å, 5 µm
- Eluent A: 50 mM ammonium acetate, pH 6.90; eluent B: 60% acetonitrile in water
- Gradient: 15%B to 60%B in 50 mins.
- Column temperature: 50 C.
- Samples injected: Sample 2 (20, 40, 100 fmol); Sample 1 (1 pmol

Detection

- PDA Detection from 220 to 400 nm
- LIF: Fluorescence Excitation at 325 nm, Emission Detection > 360 nm

Samples

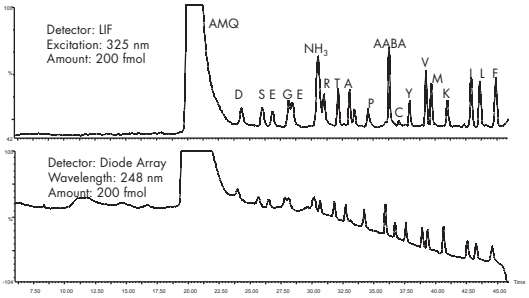
- Tryptic digestion of 1.25 mg/ml bovine cytochrome C with 12.5 ug/ml trypsin in 25 mM borate and 5 mM $CaCl_2$
- Dilute the digest 50 times with 25 mM borate
- Derivatized 160 ul of the dilute digest with 40 ul of 10 mM AQC (Sample 1)
- Dilute 20 times Sample 1 with 20% acetonitrile (Sample 2)

Femtomole and Subfemtomole Analysis by Capillary HPLC

Steven A. Cohen and Cozette M. Cuppett
Waters Corporation, 34 Maple Street, Milford, MA, 01757

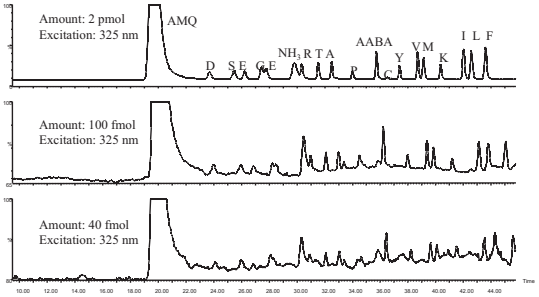
LC/UV/LIF of Derivatized Amino Acids

Comparison of Capillary LC Separation of 18 Derivatized Amino Acids with LIF Detection and Photodiode Array Detection



Both LIF and UV detection provide femtomole level detection for the AQC derivatized amino acids. Because a cutoff filter rather than a narrow bandpass filter was employed, the reagent hydrolysis peak (AMQ) is larger than normally observed.

Capillary LC Separation of 18 Derivatized Amino Acids with LIF Detection



The AQC derivatized amino acids are highly fluorescent. Under the conditions used here, detection limits are in the low femtomole range. However, the excitation maximum for the derivatives is approximately 248 nm, not the 325 nm provided by the HeCd laser. We estimate that detection limits would improve by an order of magnitude with newer deep UV lasers that may be available within the next year.

Experimental Conditions for Capillary LC Separation of Derivatized Amino Acids

Chromatographic Conditions:

- Column: 0.32 x 150 mm Symmetry C18, 100 Å, 5 µm
- Flow rate: 5 µl/min
- Eluents: Eluent A: 140 mM sodium acetate, 17 mM triethylamine (TEA), pH 5.05 containing 1 mM Calcium disodium EDTA; Eluent B: 60% ACN/40% water
- Gradient: 0 min=0%B; 0.5 min = 2% B; 15 min = 7%; 19 min = 10% B; 32 min= 33%B; 38 min = 100%B; 43 min= 0% B
- Column temperature: 37° C

Detection parameters

- LIF: Excitation power ~5 mW, Excitation: 325 nm, Emission cutoff filter > 365 nm
- PDA: 248 nm channel

Sample Preparation:

- Place 10 µl of dilute amino acids standard in a vial
- Add 70 µ l of borate buffer; mix
- Add 20 µ l of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent (3mg/ml in MeCN)
- Heat the vial in a reaction block or oven for 10 min at 55° C
- Inject 1 µl = 10 pmol

Summary and Conclusions

- Capillary chromatography can routinely provide low to mid-femtomole level detection with common available detection methods
- With suitable derivatization techniques, LIF can enable low femtomole analysis or peptides and amino acids
- MS confirms single products for the reaction of AQC with peptides
- The next generation of lasers may permit sub-femtomole analysis of AQC derivatized compounds

Acknowledgements

- Amino acid separations — Dr. Wensheng Xu, Waters
- Peptide separations — Hongji Liu, Waters
- DNA Adducts — Dr. Radoslav Goldman, NIH