HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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• High Performance Liquid Chromatography (HPLC) is one of the most widely used techniques for identification, quantification and purification of mixtures of organic compounds.

• In HPLC, as in all chromatographic methods, components of a mixture are partitioned between an adsorbent (the stationary phase) and a solvent (the mobile phase).

• The stationary phase is made up of very small particles contained in a steel column. Due to the small particle size (3-5 um), pressure is required to force the mobile phase through the stationary phase.

• There are a wide variety of stationary phases available for HPLC. In this lab we will use a normal phase (Silica gel), although reverse phase (silica gel in which a 18 carbon hydrocarbon is covalently bound to the surface of the silica) columns are currently one of the most commonly used HPLC stationary phases.
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC System

http://www.chemistry.nmsu.edu/Instrumentation/Waters_HPLC_MS_TitlePg.html
High-speed isocratic separation. Column dimensions: 4 cm length, 0.4 cm i.d.; Packing: 3-μm Sperisorb; Mobile phase: 4.1% ethyl acetate in n-hexane. Compounds: (1) p-xylene, (2) anisole, (3) benzyl acetate, (4) dioctyl phthalate, (5) dipentyl phthalate, (6) dibutyl phthalate, (7) dipropyl phthalate, (8) diethyl phthalate. (From R. P. W. Scott, *Small Bore Liquid Chromatography Columns: Their Properties and Uses*, p. 156. New York: Wiley 1984.)
Figure 26-3  Two-component chromatograms illustrating two methods of improving separators: (a) original chromatogram with overlapping peaks; improvements brought about by (b) an increase in band separation, and (c) a decrease in band spread.

Schematic Presentation of a Chromatogram

$t_0$ = the time required by a component to migrate through the chromatographic system without interacting with the stationary phase, (also called the air or gas peak, or the “dead time”)

$t_{R1}$ = the time required for component $R_1$ to migrate through the system

$t_{R2}$ = the time required for component $R_2$ to migrate through the system

$t_{R1}' = t_{R1} - t_0 = \text{the net retention time or the difference between the total retention time and the dead time } t_0.$
Resolution ($R_S$) of a column provides a quantitative measure of its ability to separate two analytes

$$R_S = \frac{\Delta Z}{1/2(W_A + W_B)}$$

$$R_S = \frac{2 \Delta Z}{W_A + W_B} = \frac{2[(t_R)_A - (t_R)_B]}{W_A + W_B}$$

The relative retention does not provide any information on the quality of a separation, since for equal values of a two very broad peaks may overlap, (as shown in trace a), or may be completely resolved (as in trace b), if they are correspondingly narrow.

For this reason the peak width at half height ($w_{1/2}$) is taken into account when considering the resolution $R$ according to:

$$R_S = \frac{(t'_2 - t'_1)}{(W_{1/2})_2 + (W_{1/2})_1}$$
HPLC - Resolution

\[ R_s = \frac{(t'_2 - t'_1)}{(W_{1/2})_2 + (W_{1/2})_1} \]

Figure 26-11 Separations at three resolutions. Here, \( R_i = 2\Delta Z/(W_A + W_B) \).

**Capacity Factor** \((k')\): Also called retention factor. Is a measure for the position of a sample peak in the chromatogram. 
\[
k' = \frac{(t_{R1} - t_0)}{t_0}
\]
- specific for a given compound and constant under constant conditions
- A function of column and mobile phase chemistry
- Primarily applicable under isocratic conditions
- In general, a change in the \(k'\) of one peak will move all peaks in the same direction.

**Selectivity Factor** \((\alpha)\): Also called separation or selectivity coefficient is defined as
\[
\alpha = \frac{k_2'}{k_1'} = \frac{(t_{R2} - t_0)}{(t_{R1} - t_0)}
\]
- A function of column and mobile phase chemistry
- Primarily applicable under isocratic conditions
- Changes in selectivity will affect different compounds in different ways.

HPLC - Resolution

Theoretical Plates (N): The number of theoretical plates characterizes the quality or efficiency of a column.

\[ N = 5.54 \left[ \frac{(t_R)}{w_{1/2}} \right]^2 \]

\(N = 16 \left( \frac{t_R}{W} \right)^2\)

Plate Height (H): The height equivalent to a theoretical plate (HEPT = H)

\[ H = \frac{L}{N} \]

Resolution (Rs) depends on the number of theoretical plates:

\[ R_s = \frac{(t_R)_B - (t_R)_A}{(t_R)_B} \times \frac{\sqrt{N}}{4} \]

\[ R_s = \frac{k'_B - k'_A}{1 + k'_B} \times \frac{\sqrt{N}}{4} \]

\[ R_s = \frac{(t'_{1/2} - t'_{1})}{(W_{1/2})^2 + (W_{1/2})_1} \]

<table>
<thead>
<tr>
<th>Name</th>
<th>Calculation of Derived Quantities</th>
<th>Relationship to Other Quantities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear mobile-phase velocity</td>
<td>$u = L/t_M$</td>
<td></td>
</tr>
<tr>
<td>Volume of mobile phase</td>
<td>$V_M = t_M F$</td>
<td></td>
</tr>
<tr>
<td>Retention factor</td>
<td>$k' = (t_R - t_M)/t_M$</td>
<td>$k' = \frac{KV_S}{V_M}$</td>
</tr>
<tr>
<td>Distribution constant</td>
<td>$K = \frac{k'V_M}{V_S}$</td>
<td>$K = \frac{c_S}{c_M}$</td>
</tr>
<tr>
<td>Selectivity factor</td>
<td>$\alpha = \frac{(t_R)_B - t_M}{(t_R)_A - t_M}$</td>
<td>$\alpha = \frac{k'_B}{k'_A} = \frac{K_B}{K_A}$</td>
</tr>
<tr>
<td>Resolution</td>
<td>$R_s = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$</td>
<td>$R_s = \frac{\sqrt{N}}{4} \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k'_B}{1 + k'_B} \right)$</td>
</tr>
<tr>
<td>Number of plates</td>
<td>$N = 16 \left( \frac{t_R}{W} \right)^2$</td>
<td>$N = 16R_s^2 \left( \frac{\alpha}{\alpha - 1} \right)^2 \left( \frac{1 + k'_B}{k'_B} \right)^2$</td>
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<tr>
<td>Plate height</td>
<td>$H = L/N$</td>
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<tr>
<td>Retention time</td>
<td>$(t_R)_B = \frac{16R_s^2H}{u} \left( \frac{\alpha}{\alpha - 1} \right)^2 \left( 1 + k'_B \right)^3 \left( k'_B \right)^2$</td>
<td></td>
</tr>
</tbody>
</table>

Figure 26-14  Illustration of the general elution problem in chromatography.
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
(TLC vs Normal Phase and Reverse Phase HPLC)

Normal Phase (SiO₂) TLC

Reverse Phase (C18)

Time

0

0
28D-1 Columns for Bonded-Phase Chromatography

The supports for the majority of bonded-phase packings for partition chromatography are prepared from rigid silica, or silica-based, compositions. These solids are formed as uniform, porous, mechanically sturdy particles commonly having diameters of 3, 5, or 10 μm. The surface of fully hydrolyzed silica (hydrolyzed by heating with 0.1 M HCl for a day or two) is made up of chemically reactive silanol groups. That is,

```
  Si    O    O    O    O
  \    /    \    \    \
   OH  OH  OH  OH
```

Typical silica surfaces contain about 8 μmol/m² of OH groups.

The most useful bonded-phase coatings are siloxanes formed by reaction of the hydrolyzed surface with an organochlorosilane. For example,

```
  Si-OH + ClSi-R → Si-O-Si-R
  \    /    \    \    
   CH₃  CH₃  CH₃
```

where R is an alkyl group or a substituted alkyl group.

Surface coverage by silanization is limited to 4 μmol/m² or less because of steric effects. The unreacted SiOH groups, unfortunately, impart an undesirable polarity to the surface, which may lead to tailing of chromatographic peaks, particularly for basic solutes. To lessen this effect, siloxane packings are frequently capped by further reaction with chlorotrimethylsilane that, because of its smaller size, can bond many of the unreacted silanol groups.

Normal Phase vs. Reverse Phase HPLC

Figure 28-14  The relationship between polarity and elution times for normal-phase and reversed-phase chromatography.

Solute polarities: $A > B > C$

**RP-HPLC – Stationary Phase**

Peak identification:
1. Uracil
2. Phenol
3. Acetophenone
4. Nitrobenzene
5. Methyl benzoate
6. Toluene

---

**Figure 28-15** Effect of chain length on performance of reversed-phase siloxane columns packed with 5-μm particles. Mobile phase: 50/50 methanol/water. Flow rate: 1.0 mL/min.

_Skoog and Leary: Principals of Instrumental Analysis, 5th ed. Saunders, 1998_
**Figure 28-16** Systematic approach to the separation of six steroids. The use of water to adjust $k'$ is shown in (a) and (b). The effects of varying $\alpha$ at constant $k'$ are shown in (b), (c), (d), and (e). Column: 0.4 x 150 mm packed with 5 μm C₈ bonded, reversed-phase particles. Temperature: 50°C. Flow rate: 3.0 cm³/min. Detector: UV 254 nm. THF = tetrahydrofuran. CH₃CN = acetonitrile. Compounds: (1) prednisone, (2) cortisone, (3) hydrocortisone, (4) dexamethasone, (5) corticosterone; (6) cortoexolone. (Courtesy of DuPont Instrument Systems, Wilmington, DE.)
**RP-HPLC - Optimization**

### Anilines

1. Aniline
2. Dimethylaniline
3. Diethylaniline

**Column:** Prevail™ Amide, 3μm, 150 x 4.6mm  
(Part No. 88675)

**Mobile Phase:** 20mM K₂HPO₄, pH 7.0:CH₃CN (50:50)

**Flowrate:** 1.0mL/min

**Detector:** UV at 210nm

### Analgesics

1. Aspirin
2. Acetaminophen
3. Naproxen
4. Fenprofen

**Column:** Prevail™ Amide, 5μm, 150 x 4.6mm  
(Part No. 88660)

**Mobile Phase:** 20mM K₂HPO₄, pH 7.0:CH₃CN (75:25)

**Flowrate:** 1.0mL/min

**Detector:** UV at 210nm

*Alltech Chromatography Sourcebook, 2004-04 catalog*
**RP-HPLC – Gradient Elution**

1. Phenol
2. 4-Nitrophenol
3. 2,4-Dinitrophenol
4. o-Chlorophenol
5. 2-Nitrophenol
6. 2,4-Dimethylphenol
7. 4-Chloro-3-methylphenol
8. 2,4-Dichlorophenol
9. 2,4,6-Trichlorophenol
10. Pentachlorophenol

**Column:** Econosphere™ C8, 5μm, 150 x 4.6mm

**Mobile Phase:**
- A: Water + 1% Acetic Acid
- B: Methanol + 1% Acetic Acid

**Gradient:**

<table>
<thead>
<tr>
<th>Time</th>
<th>%B</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
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</tbody>
</table>

**Flowrate:** 1.5mL/min

**Detector:** UV at 280nm
### Packing Characteristics Reference Guide

The table below lists the physical characteristics of the most common [Alltech HPLC packings](mailto:tech_service@alltechemail.com) to help you choose an appropriate column for your application. In the US, our technical specialists can be reached by phone at 1-800-33SOLVE Monday through Friday from 7:30am to 5:30pm (central time zone). The USP L-code listing lets you know that these columns meet the specifications set forth in USP Volume 24.

### Alltech HPLC Column Packing Characteristics

<table>
<thead>
<tr>
<th>Adsorbosil®</th>
<th>Adsorbosphere®</th>
<th>Adsorbosphere® XL</th>
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<tr>
<td><strong>Packing</strong></td>
<td><strong>Base Material</strong></td>
<td><strong>Particle Shape</strong></td>
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<td>Silica</td>
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<td>C2</td>
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<td>Spherical</td>
</tr>
<tr>
<td>SCX</td>
<td>Silica</td>
<td>Spherical</td>
</tr>
</tbody>
</table>
RP-HPLC – Variables

1. Phenol
2. 4-Nitrophenol
3. 2,4-Dinitrophenol
4. o-Chlorophenol
5. 2-Nitrophenol
6. 2,4-Dimethylphenol
7. 4-Chloro-3-methylphenol
8. 2,4-Dichlorophenol
9. 2,4,6-Trichlorophenol
10. Pentachlorophenol

Column: Econosphere™ C8, 5μm, 150 x 4.6mm
Mobile Phase: A: Water + 1% Acetic Acid
              B: Methanol + 1% Acetic Acid
Gradient:     Time: | 0 | 15 |
              %B:   | 40 | 100|
Flowrate:     1.5mL/min
Detector:     UV at 280nm
HPLC OF ANALGESICS - UV Detection

Standard Analgesics

<table>
<thead>
<tr>
<th>Analgesic</th>
<th>Retention Time</th>
</tr>
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<tbody>
<tr>
<td>Acetaminophen</td>
<td>2.82</td>
</tr>
<tr>
<td>Aspirin</td>
<td>1.48</td>
</tr>
<tr>
<td>Caffeine</td>
<td>7.11</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>1.35</td>
</tr>
</tbody>
</table>

Gradient =
0 min: 100% EtOAC (+ 0.2% HOAc)
3 min: 100% EtOAC (+ 0.2% HOAc)
5 min: 15% MeOH, 85% EtOAc (+ 0.2% HOAc)
8 min: 15% MeOH, 85% EtOAc (+ 0.2% HOAc)
10 min: 100% EtOAC (+ 0.2% HOAc)

SiO₂
Flow Rate = 1 mL/min
UV detector set at 240 nm
**Question**

The peak areas of aspirin and acetaminophen are very different, even though they are present in equal amounts (250mg/tablet) in Excedrin ES.

Caffeine is present at ~ ¼ the concentration of aspirin (65 mg/tablet vs. 250 mg/tablet), but its peak area is greater than the peak area of aspirin.

**WHY?** UV Absorbance of analgesics vs UV setting of detector
**HPLC: Peak Area vs Detector setting**

**UV Max**
- Aspirin: 225, 296 nm
- Acetaminophen: 248 nm
- Caffeine: 272 nm

**Area %**

**Detector set at 240 nm**
- Aspirin: 19.5%
- Acetaminophen: 50.0%
- Caffeine: 20.5%

**Detector set at 254 nm**
- Aspirin: 7.3%
- Acetaminophen: 81.9%
- Caffeine: 10.8%

**Detector set at 280 nm**
- Aspirin: 24.8%
- Acetaminophen: 39.3%
- Caffeine: 35.9%
Figure 2. HPLC (SiO2) of crude turmeric extract.

Gradient
0-2 min, 4% EtOAc/Hexane;
2-9 min 4 to 80% EtOAc;
9-11 min, 80% EtOAc/hexane;
11-13 min, 80 to 4% EtOAc/hex,
13-15 min, 4% EtOAc/hexane.

(A) Detector set at 420 nm.
(B) Detector set at 254 nm.
(C) Detector set at 254 nm (0-3.5 min), 420 nm 3.5-15 min.
Method Development for Biomonitoring of Polycyclic Aromatic Hydrocarbons

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PAH Exposure

Urinary/biliary Excretion

PAH Adducts
PAH Metabolites

Carcinogenic / Genotoxic

Metabolism

T_{1/2} = hours
Biotransformation of PAHs
Phase 1 Transformation

- increased 3,4-oxidation in smokers due to induced cytochrome P4501A2 enzymes
- multiple metabolites for each parent PAH
- comprehensive methodology will measure all known metabolites
Biotransformation of PAHs
Phase 2 Transformation - Conjugation

- most hydroxylated metabolites are further conjugated to aid urinary excretion
Biotransformation of nitro-PAHs

• nitro-PAHs undergo reductive biotransformation to detoxify and aid excretion
• can form reactive intermediates through biotransformation

-3nitro-benzanthrone

\[
\text{NO}_2
\]

Cytosolic / P450 reductase

Phase II Metabolism

N-O-acetyltransferases (NATs)
Sulfotransferases (SULTs)

• leads to formation of reactive esters
• potential DNA adduct formation

-3amino-benzanthrone

\[
\text{NH}_2
\]

CYP P450

Urinary Excretion
-3Aminobenzanthrone – Urinary Biomarker for Diesel Exhaust

- 3Nitrobenzanthrone has been isolated from diesel exhaust extracts and has been shown to be a direct mutagen.
- 3Aminobenzanthrone has been used as the diesel-specific urinary biomarker of exposure to 3-nitrobenzanthrone.
- Instability problem has been observed on 3-aminobenzanthrone standards; it is necessary to re-make standards frequently.
Amino-PAHs

1. -1amino-naphthalene
   -2amino-biphenyl
2. -2amino-naphthalene
3. -4amino-biphenyl
4. -1amino-fluorene
5. -2amino-fluorene
6. -9amino-phenanthrene
7. -1amino-anthracene
8. -2amino-anthracene
9. -3amino-fluoranthe
10. -1amino-pyrene
11. -6amino-chrysene
12. -3amino-benzanthrone

Time (min)
Group A PAH-OHs

1. -1OH-naphthalene
2. -2OH-naphthalene
3. -3OH-fluorene
4. -2OH-fluorene
5. -9OH-fluorene
6. -9OH-phenanthrene
7. -3OH-phenanthrene
8. -2OH-phenanthrene
9. -1OH-phenanthrene
10. -4OH-phenanthrene
11. -1OH-benzo(c)phenanthrene
12. -3OH-fluoranthene
13. -1OH-pyrene
14. -2OH-benzo(c)phenanthrene
15. -1OH-benz(a)anthracene
16. -4OH-chrysene
17. -6OH-chrysene
18. -3OH-benzo(c)phenanthrene
19. -3OH-chrysene
20. -1OH-chrysene
21. -3OH-benz(a)anthracene
22. -2OH-chrysene
1. -8OH-benzo(b)fluoranthene
2. -7OH-benzo(b)fluoranthene
3. -1OH-benzo(b)fluoranthene
   -9OH-benzo(b)fluoranthene
4. -2OH-benzo(b)fluoranthene
   -12OH-benzo(b)fluoranthene
   -8OH-benzo(b)fluoranthene
5. -9OH-benzo(e)pyrene
6. -3OH-benzo(b)fluoranthene
7. -12OH-benzo(a)pyrene
8. -5OH-benzo(a)pyrene
9. -1OH-benzo(b)fluoranthene
10. -6OH-benzo(b)fluoranthene
11. -3OH-benzo(k)fluoranthene
12. -4OH-benzo(e)pyrene
13. -10OH-benzo(b)fluoranthene
14. -9OH-benzo(k)fluoranthene
15. -7OH-benzo(a)pyrene
16. -1OH-benzo(e)pyrene
17. -3OH-benzo(e)pyrene
18. -3OH-benzo(a)pyrene
19. -2OH-benzo(e)pyrene
20. -2OH-indeno-[1,2,3-c,d]-pyrene
21. -8OH-indeno-[1,2,3-c,d]-pyrene
22. -3OH-dibenzo[a,h]anthracene
Current PAH-OH Methodology

Sample Preparation

- aliquot 3 mL of urine
- spike with 9 labeled ($^{13}$C$_6$) internal quantification standards (10 µL, 100 pg/µL):
  1. naphthalene
  2. fluorene
  3. phenanthrene
  1. pyrene
  3. fluoranthene
  3. chrysene
  6. chrysene
  1. benzo[a]anthracene
  3. benzo[c]phenanthrene

- dilute with sodium acetate buffer (0.1 M, 5 mL, pH=5.5)

De-conjugation of PAHm by enzymatic hydrolysis

Solid Phase Extraction (SPE)

Derivatization

GC/HRMS analysis
Future PAH-OH Methodology

Sample Preparation

De-conjugation of PAHm by enzymatic hydrolysis

Solid Phase Extraction (SPE)

Derivatization

GC/HRMS analysis

- adding additional group A PAH-OHs
- increasing number of labeled \(^{13}\text{C}_6\) internal quantification standards
- add Group B PAH-OHs to methodology
- automated sample preparation
Sample Preparation

De-conjugation of PAHm by enzymatic hydrolysis

- add β-glucuronidase / arylsulfatase (5 µL)
- incubate 3 hours @ 37ºC

Solid Phase Extraction (SPE)

Derivatization

GC/HRMS analysis
Current PAH-OH Methodology

Sample Preparation
- EnvirElut-PAH cartridges (6 mL, 1 gram)
- 12 port vacuum manifold
- condition with methanol (5 mL), then water (5 mL)
- add samples
- wash with water (5 mL), then water/methanol (1:1, 5 mL)
- dry
- elute with acetonitrile:methanol (1:1, 5 mL)
- dry extract over anhydrous sodium sulfate (2.5 g), additional rinse acetonitrile:methanol (1:1, 2 mL)
- evaporate to dryness with nitrogen (55°C)

De-conjugation of PAHm by enzymatic hydrolysis

Solid Phase Extraction (SPE)

Derivatization

GC/HRMS analysis
**Future PAH-OH Methodology**

**Sample Preparation**

- De-conjugation of PAHm by enzymatic hydrolysis
- **Solid Phase Extraction (SPE)**
  - convert to automated SPE system for high throughput
    - increased sample potential (100 per day versus 24 in current method)
    - unattended operation
    - can collect fractions
  - high efficiency polymeric sorbent for better extraction and cleaner extracts
    - tested 5 polymeric sorbents
  - samples evaporated using vacuum instead of nitrogen blowdown
Solid Phase Extraction Method Development

- Five different sorbents tested for both NH$_2$- and PAH-OHs
  - OASIS, FOCUS, STRATA-X, STRATA-XC, C-18
- FOCUS and STRATA-XC gave better recoveries for PAH-OHs
- OASIS and STRATA-X gave better results for NH$_2$-PAHs
- C-18 was used in existing method for PAH-OH; however, it gave very bad results on NH$_2$-PAHs
- Further method development using OASIS, FOCUS, and STRATA-X underway
Further experiments underway to optimize extraction and purification.
Evaporation

• PAH metabolites have both volatility and thermal stability issues
• Evaporation must remove solvent quickly (stability) and gently (volatility)
• SPE eluant changed to mostly DCM
• Rapidvap uses vacuum instead of nitrogen
Evaporation

- Extracts were evaporated to dryness; re-constituted in toluene and derivatized with MSTFA
- During evaporation, analyte loss can reach 80%
- Adding keeper (5 µL dodecane) improved recoveries
Current PAH-OH Methodology

Sample Preparation

De-conjugation of PAHm by enzymatic hydrolysis

Solid Phase Extraction (SPE)

Derivatization

• reconstitute with toluene (20 µL)
• transfer to injection vial
• add MSTFA (5 µL)
• add argon and crimp seal vial
• incubate 1 hour @ 60°C

GC/HRMS analysis
Future PAH-OH Methodology

Sample Preparation

De-conjugation of PAHm by enzymatic hydrolysis

Solid Phase Extraction (SPE)

Derivatization

GC/HRMS analysis

- testing different derivatization techniques
  - MSTFA, PFBC, PFPA, HFBA
  - work underway optimizing each reaction

- adding electron capture functionality
  - increased AMU for cleaner mass spectra
  - potential ECNI detection for more sensitive analysis
  - potential MSD analysis using ECNI detection
Derivatization Reactions

MSTFA = N-Methyl-N-(trimethylsilyl)trifluoroacetamide ; PFPA = Pentafluoropropionic anhydride
PCBC = pentachlorobenzoylchloride ; HFBA = Heptafluorobutyric anhydride

-2hydroxy-naphthalene
Exact Mass: 144.05752

MSTFA
Mass: 216.09704

HFBA
Mass: 340.03343

PFPA
Mass: 290.03662

PFBC
Mass: 338.03662
Optimizing Derivatization
MSTFA for Amino-PAHs

Derivatization needed to cap functionalities and improve peak detection.

Ideally – need quantitative conversion for maximum sensitivity

- Amount of derivatizing reagent
- Catalyst for reaction
- Temperature of reaction
- Time for reaction
Amino-PAHs require 15 µL of MSTFA

Default condition: 20 µL standard solution, 5 µL MSTFA, 5 µL pyridine, 60 °C, 1 hour
Amino-PAHs Derivatized by MSTFA (Catalyst)

- Amino-PAHs require 5 µL of pyridine for maximum recovery

**Default condition**: 20 µL standard solution, 5 µL MSTFA, 5 µL pyridine, 60 °C, 1 hour
Amino-PAHs Derivatized by MSTFA

- Reaction Temperature

- Default condition: 20 µL standard solution, 5 µL MSTFA, 5 µL pyridine, 60 °C, 1 hour

- Amino-PAHs require 60 minute reaction time

Conversion rate (%) vs. Temperature

- 100% conversion at 60 °C for most PAHs
- 20 °C shows lower conversion rates for most PAHs
- 110 °C shows higher conversion rates for most PAHs
Amino-PAHs Derivatized by MSTFA

Reaction Time

- Amino-PAHs require a 30 minute reaction time

**Default condition:** 20 µL standard solution, 5 µL MSTFA, 5 µL pyridine, 60 °C, 1 hour
Current PAH-OH Methodology

Sample Preparation

De-conjugation of PAHm by enzymatic hydrolysis

Solid Phase Extraction (SPE)

Derivatization

GC/HRMS analysis

- Finnigan MAT95XP HRMS
- 1 µL injection
- DB-5MS GC column
- Injection Temperature: 290°C
- 1 mL/min He flow / 2 min. purge
- Transfer line: 290°C
- Source Temperature: 260°C
- Temperature Program:
  - 100°C – hold 2 min
  - 15°C/min to 160°C
  - 10°C/min to 295°C
  - hold 3 min
- Total run time: **23 minutes**
- Calibration – 6 points – 10 – 10,000 ppt
- Processed standards in water
Future PAH-OH Methodology

Sample Preparation

De-conjugation of PAHm by enzymatic hydrolysis

Solid Phase Extraction (SPE)

Derivatization

GC/HRMS analysis

• Decreased GC run time to 15 min.
• Now using external calibration standards
  • Improved linearity and stability of calibration curve
  • lower instrument quantification limits (IQLs) set at 5x S/N
• Increase analytes to 23 Group A and potentially 27 Group B
### Instrument Quantification Limits for PAH-OHs (Group A Metabolites)

<table>
<thead>
<tr>
<th>#</th>
<th>PAH-OH</th>
<th>IQL</th>
<th>#</th>
<th>PAH-OH</th>
<th>IQL</th>
</tr>
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<tr>
<td>1</td>
<td>1-hydroxynaphthalene</td>
<td>60 ppq</td>
<td>13</td>
<td>1-hydroxybenzo(c)phenanthrene</td>
<td>20 ppt</td>
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<td>2</td>
<td>2-hydroxynaphthalene</td>
<td>90 ppq</td>
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<td>3</td>
<td>2-hydroxyfluorene</td>
<td>2 ppt</td>
<td>15</td>
<td>3-hydroxybenzo(c)phenanthrene</td>
<td>10 ppt</td>
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<tr>
<td>4</td>
<td>3-hydroxyfluorene</td>
<td>2 ppt</td>
<td>16</td>
<td>1-hydroxychrysene</td>
<td>n.a.</td>
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<tr>
<td>5</td>
<td>9-hydroxyfluorene</td>
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<td>17</td>
<td>2-hydroxychrysene</td>
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<tr>
<td>6</td>
<td>1-hydroxyphenanthrene</td>
<td>8 ppt</td>
<td>18</td>
<td>3-hydroxychrysene</td>
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</tr>
<tr>
<td>7</td>
<td>2-hydroxyphenanthrene</td>
<td>10 ppt</td>
<td>19</td>
<td>4-hydroxychrysene</td>
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<tr>
<td>8</td>
<td>3-hydroxyphenanthrene</td>
<td>8 ppt</td>
<td>20</td>
<td>6-hydroxychrysene</td>
<td>30 ppt</td>
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<td>10 ppt</td>
<td>21</td>
<td>1-hydroxybenz(a)anthracene</td>
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<td>3-hydroxybenz(a)anthracene</td>
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<td>1-hydroxypyrene</td>
<td>6 ppt</td>
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</tbody>
</table>

**Abbreviations:**
- **ppt** – parts per trillion
- **ppq** – parts per quadrillion
- **n.a.** – not available at this time