Analysis of Polycyclic Aromatic Hydrocarbons in Tobacco Related Samples via High Performance Liquid Chromatography and Laser Excited Time Resolved Shpol' Skii Spectroscopy

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ABSTRACT

Human exposure to polycyclic aromatic hydrocarbons (PAHs) due to cigarette smoke is well known. It is also known that PAHs are not inherent components of tobacco. Their presence in cigarette smoke results from the curing process of tobacco leaves used for the fabrication of cigarettes and the incomplete combustion of tobacco during the smoking process. The two chromatographic versions of EPA method 610 are popular approaches for the analysis of PAHs in a wide variety of samples. This method, which is based either on High-Performance Liquid-Chromatography (HPLC) or Gas-Chromatography (GC) has shown the presence of Benzo[a]pyrene (B[a]P) in tobacco samples and cigarette smoke. B[a]P is the most toxic PAH in the U.S Environmental Protection Agency (EPA) priority pollutant list.

Herein, we apply the HPLC Method 610 to the analysis of ten PAHs of MM 302 Da. In the presence of the 15 EPA-PAHs, co-elution was observed between Dibenzo[a,h]anthracene/Naphtho[2,3-k]fluoranthene and Benzo[a]pyrene/Dibenzo[a,l]pyrene. The extent of our literature search reveals no publications on the capabilities of Method 610 to measure Dibenzo[a,l]pyrene (DB[a,l]P) in tobacco samples and cigarette smoke. This PAH is approximately one hundred times more toxic than B[a]P. Our studies demonstrate the feasibility to directly determine the four co-eluting PAHs in chromatographic fractions without further separation. Their unambiguous determination is based on spectral and lifetime information with a two-step experimental procedure consisting of the evaporation of the chromatographic fraction followed by the dissolution of the residue with microliters of n-octane. Limits of detection at the parts-per-billion concentration level (ng.mL^{-1}) were obtained from microliter sample volumes via Laser Excited Time Resolved Shpol’skii Spectroscopy.
This dissertation is dedicated to

My husband

Alejandro

For his constant, unconditional support and for inspiring me to never give up

My mother

Tania

For her eternal love, compassion, and dedication

My sister

Laura

For being the best sister in the world
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# TABLE OF CONTENTS

LIST OF FIGURES ....................................................................................................................... ix

LIST OF TABLES ........................................................................................................................ xvi

CHAPTER ONE: INTRODUCTION ............................................................................................. 1
  1.1 Polycyclic Aromatic Hydrocarbons ...................................................................................... 1
  1.2 Polycyclic Aromatic Hydrocarbons in the Environmental Protection Agency List .......... 4
  1.3 High-Molecular Weight Polycyclic Aromatic Hydrocarbons .............................................. 7
  1.4 References ............................................................................................................................. 9

CHAPTER TWO: PAH ANALYSIS IN TOBACCO PRODUCTS - A REVIEW ..................... 15
  2.1 Background ......................................................................................................................... 15
  2.2 Analysis of PAHs in Sidestream and Mainstream Smoke .................................................. 16
  2.3 Analysis of PAHs in Smoke Condensate ............................................................................ 18
  2.4 Analysis of PAHs in Cigarette Filters ................................................................................. 19
  2.5 Analysis of PAHs in Smokeless Tobacco ........................................................................... 20
  2.6 Analysis of PAHs in Tobacco Leaves ................................................................................ 21
  2.7 Conclusions ......................................................................................................................... 21
  2.8 Research Objectives ............................................................................................................ 22
  2.9 References ........................................................................................................................... 23

CHAPTER THREE: INSTRUMENTATION .............................................................................. 27
  3.1 UV-Vis Absorption Spectroscopy ...................................................................................... 27
  3.2 Fluorescence Spectroscopy ................................................................................................. 27
  3.3 Cryogenic Fiber Optic Probe .............................................................................................. 28
  3.4 Laser Excited Time Resolved Shpol’ Skii Spectroscopy ................................................... 29
APPENDIX D: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FLUORESCENCE (HPLC-FL) DETECTION CALIBRATION CURVES OF HMW-PAHs .......................... 100
APPENDIX E: CALCULATIONS RELATED TO THE MASS OF BaP................................ 111
APPENDIX F: COPYRIGHT PERMISSIONS ........................................................................ 114
LIST OF FIGURES

Figure 1: Examples of well-known PAHs showing their different structure arrangements.......... 1

Figure 2: UV absorption spectrum of Benzo[b]perylene and Dibenzo[b,k]fluoranthene........... 2

Figure 3: Room temperature excitation and emission spectra of Benzo[a]perylene and Dibenzo[b,k]fluoranthene. .............................................................................................................. 3

Figure 4: Molecular structures, names, and molecular weights of the 16 EPA-PAHs. ............... 5

Figure 5: Molecular structure for benzo[a]pyrene and its bay region..................................... 6

Figure 6: Metabolic activation of B[a]P via bay region dihydrodiol epoxide pathway. .......... 7

Figure 7: Fjord region of DB[a,l]P. .......................................................................................... 9

Figure 8: Molecular structures of PAH isomers with MM 302 Da. ..................................... 32

Figure 9: HPLC chromatogram of a typical synthetic mixture containing the 25 PAHs selected for these studies. Fluorescence detection at the maximum excitation and emission wavelengths was used in all cases. PAH concentrations were as follows: (1) 100 ng mL⁻¹ Naphthalene, (2) 25 ng mL⁻¹ Acenaphthene, (3) 30 ng mL⁻¹ Fluorene, (4) 25 ng mL⁻¹ Phenanthrene, (5) 25 ng mL⁻¹ Anthracene, (6) 200 ng mL⁻¹ Fluoranthene, (7) 25 ng mL⁻¹ Pyrene, (8) 25 ng mL⁻¹ Benzo[a]anthracene, (9) 50 ng mL⁻¹ Chrysene, (10) 100 ng mL⁻¹ Benzo[b]fluoranthene, (11) 100 ng mL⁻¹ Benzo[k]fluoranthene, (12) 250 ng mL⁻¹ BaP, (13) 100 ng mL⁻¹ DBaP, (14) 100 ng mL⁻¹ Dibenzo[a,h]anthracene, (15) 100 ng mL⁻¹ Benzo[g,h,i]perylene, (16) 1500 ng mL⁻¹ Indeno[1,2,3-cd]pyrene, (17) 1000 ng mL⁻¹ Naphtho[2,3-e]pyrene, (18) 500 ng mL⁻¹ Dibenzo[a,e]pyrene, (19) 200 ng mL⁻¹ Naphtho[1,2-k]fluoranthene, (20) 250 ng mL⁻¹ Benzo[b]pyrene, (21) 100 ng mL⁻¹ Dibenzo[b,k]fluoranthene, (22) 2000 ng mL⁻¹ Dibenzo[a,i]pyrene, (23) 100 ng mL⁻¹ Naphtho[2,3-a]pyrene, (24) 1000 ng mL⁻¹ Naphtho[2,3-k]fluoranthene, and (25) 100 ng mL⁻¹ Dibenzo[a,h]pyrene. .............................................................................................................. 39
Figure 10: 77 K WTM for BaP and DBalP. WTM were recorded at the maximum excitation wavelength of each PAH. ............................................................................................................. 40

Figure 11: Excitation and Emission Spectra of BaP and DBalP ............................................................................................................................... 41

Figure 12: Fluorescence Time Decays of BaP and DBalP. Fluorescence decays were recorded at the maximum excitation and emission wavelength of each PAH. A 10 μm spectrograph slit was used for all measurements. The time delay (td) and the gate delay (tg) were as follows: BaP (td = 6ns and tg = 150ns) and DBalP (td = 6ns and tg = 240ns). ............................................................................................................. 42

Figure 13: Comparison of 77 K LETRSS fluorescence spectra and lifetimes recorded from: (A) 10 ng.mL⁻¹ pure standard of BaP recorded at the maximum excitation and emission wavelength of BaP (l_{exc}/l_{em} = 388.5 nm / 403 nm); (B) 1:1 synthetic mixture of BaP and DBalP recorded at the maximum excitation and emission wavelength of BaP (l_{exc}/l_{em} = 388.5 nm / 403 nm). All spectra were recorded from octane solutions with a 10 mm spectrograph slit. Delay and gate times (td/tg) were as follows: (A) and (B) td/tg = 6 ns / 150 ns. ............................................................................................................. 44

Figure 14: Comparison of 77 K LETRSS fluorescence spectra and lifetimes recorded from: (B) 10 ng.mL⁻¹ pure standard of DBalP recorded at the maximum excitation and emission wavelength of DBalP (l_{exc}/l_{em} = 321.5 nm / 419 nm); (D) 1:1 synthetic mixture of BaP and DBalP recorded at the maximum excitation and emission wavelength of DBalP (l_{exc}/l_{em} =321.5 nm / 419 nm); (E) 10:1 synthetic mixture of BaP and DBalP recorded at the maximum excitation and emission wavelength of DBalP (l_{exc}/l_{em} =321.5 nm / 419 nm). All spectra were recorded from octane solutions with a 10 mm spectrograph slit. Delay and gate times (td/tg) were as follows: (B), (D) and (E) td/tg = 6 ns / 240 ns. ............................................................................................................. 45

Figure 15: 77K fluorescence spectrum and fluorescence lifetime of an HPLC fraction of tobacco extract recorded under optimum excitation (λ_{exc} = 388.5 nm) and emission (λ_{em} = 403 nm)
wavelengths, and delay (td = 6 ns) and gate (tg = 150 ns) times of the LETRSS determination of BaP. τ_{HPLC} = fluorescence lifetime of HPLC fraction; τ_{std} = fluorescence lifetime of BaP; T_{crit} = t critical (α = 0.05; N = 3) and T_{exp} = t experimental (α = 0.05; N = 3).

**Figure 16:** Calibration plot for the MSA determination of BaP in an HPLC fraction of a tobacco Extract.

**Figure 17:** LETRSS analysis of an HPLC fraction of a tobacco extract. (A) Fluorescence spectrum of the un-spiked HPLC fraction; (B) Fluorescence spectrum of the HPLC fraction spiked with DBalP; and (C) Fluorescence decay of the HPLC fraction spiked with DBalP. Fluorescence spectra and fluorescence decays were recorded under optimum instrumental parameters for the determination of DBalP. T_{crit} = t critical (α = 0.05; N = 3) and T_{exp} = t experimental (α = 0.05; N= 3).

**Figure 18:** (A) 77K LETRSS spectra of an HPLC fraction of tobacco extract and octane; (B) 77K LETRSS spectra of 1.5 ppb DBalP in octane and of an HPLC fraction of tobacco extract. All spectra were recorded at l_{exc}/l_{em} =321.5 nm / 419 nm and t_{d}/t_{g} = 6 ns / 240 ns.

**Figure 19:** HPLC-FL chromatogram of 100 ng mL^{-1} naphthalene pure standard prepared with acetonitrile and using excitation (240 nm) and emission (330 nm). Sample volume = 20 µL. Flow rate = 2 mL/min^{-1}.

**Figure 20:** HPLC-FL chromatogram of 25 ng mL^{-1} acenaphthene pure standard prepared with acetonitrile and using excitation (240 nm) and emission (330 nm). Sample volume = 20 µL. Flow rate = 2 mL/min^{-1}.

**Figure 21:** HPLC-FL chromatogram of 30 ng mL^{-1} acenaphthene pure standard prepared with acetonitrile and using excitation (252 nm) and emission (320 nm). Sample volume = 20 µL. Flow rate = 2 mL/min^{-1}.
Figure 22: HPLC-FL chromatogram of 25 ng mL⁻¹ phenanthrene pure standard prepared with acetonitrile and using excitation (252 nm) and emission (370 nm). Sample volume = 20 μL. Flow rate = 2 mL/min⁻¹. .......................................................... 61

Figure 23: HPLC-FL chromatogram of 25 ng mL⁻¹ anthracene pure standard prepared with acetonitrile and using excitation (252 nm) and emission (402 nm). Sample volume = 20 μL. Flow rate = 2 mL/min⁻¹. ............................................................................................................................... 62

Figure 24: HPLC-FL chromatogram of 50 ng mL⁻¹ anthracene pure standard prepared with acetonitrile and using excitation (284 nm) and emission (464 nm). Sample volume = 20 μL. Flow rate = 2 mL/min⁻¹. ............................................................................................................................... 63

Figure 25: HPLC-FL chromatogram of 25 ng mL⁻¹ pyrene pure standard prepared with acetonitrile and using excitation (238 nm) and emission (398 nm). Sample volume = 20 μL. Flow rate = 2 mL/min⁻¹. ............................................................................................................................... 64

Figure 26: HPLC-FL chromatogram of 25 ng mL⁻¹ benzo[a]anthracene pure standard prepared with acetonitrile and using excitation (278 nm) and emission (395 nm). Sample volume = 20 μL. Flow rate = 2 mL/min⁻¹. ............................................................................................................................... 65

Figure 27: HPLC-FL chromatogram of 50 ng mL⁻¹ chrysene pure standard prepared with acetonitrile and using excitation (278 nm) and emission (395 nm). Sample volume = 20 μL. Flow rate = 2 mL/min⁻¹. ............................................................................................................................... 66

Figure 28: HPLC-FL chromatogram of 100 ng mL⁻¹ benzo[b]fluoranthene pure standard prepared with acetonitrile and using excitation (268 nm) and emission (398 nm). Sample volume = 20 μL. Flow rate = 2 mL/min⁻¹. ............................................................................................................................... 67
Figure 29: HPLC-FL chromatogram of 100 ng mL⁻¹ benzo[k]fluoranthene pure standard prepared with acetonitrile and using excitation (268 nm) and emission (398 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹................................................................. 68

Figure 30: HPLC-FL chromatogram of 500 ng mL⁻¹ benzo[a]pyrene pure standard prepared with acetonitrile and using excitation (380 nm) and emission (405 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹................................................................. 69

Figure 31: HPLC-FL chromatogram of 100 ng mL⁻¹ dibenzo[a,h]anthracene pure standard prepared with acetonitrile and using excitation (304 nm) and emission (421 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹................................................................. 70

Figure 32: HPLC-FL chromatogram of 100 ng mL⁻¹ benzo[g,h,i]perylene pure standard prepared with acetonitrile and using excitation (304 nm) and emission (421 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹................................................................. 71

Figure 33: HPLC-FL chromatogram of 100 ng mL⁻¹ indeno[1,2,3-cd]pyrene pure standard prepared with acetonitrile and using excitation (300 nm) and emission (466 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹................................................................. 72

Figure 34: HPLC-FL detection calibration curve of naphthalene. Measurements were made using excitation (240 nm) and emission (330 nm) wavelengths.................................................. 74

Figure 35: HPLC-FL detection calibration curve of acenaphthene. Measurements were made using excitation (234 nm) and emission (320 nm) wavelengths.................................................. 75

Figure 36: HPLC-FL detection calibration curve of fluorene. Measurements were made using excitation (252 nm) and emission (320 nm) wavelengths.................................................. 76

Figure 37: HPLC-FL detection calibration curve of phenanthrene. Measurements were made using excitation (252 nm) and emission (370 nm) wavelengths.................................................. 77
Figure 38: HPLC-FL detection calibration curve of anthracene. Measurements were made using excitation (252 nm) and emission (402 nm) wavelengths. ........................................................... 78

Figure 39: HPLC-FL detection calibration curve of fluoranthene. Measurements were made using excitation (284 nm) and emission (464 nm) wavelengths. ........................................................... 79

Figure 40: HPLC-FL detection calibration curve of pyrene. Measurements were made using excitation (238 nm) and emission (398 nm) wavelengths. ........................................................... 80

Figure 41: HPLC-FL detection calibration curve of benzo[a]anthracene. Measurements were made using excitation (278 nm) and emission (395 nm) wavelengths. ........................................................... 81

Figure 42: HPLC-FL detection calibration curve of chrysene. Measurements were made using excitation (278 nm) and emission (395 nm) wavelengths. ........................................................... 82

Figure 43: HPLC-FL detection calibration curve of benzo[b]fluoranthene. Measurements were made using excitation (268 nm) and emission (398 nm) wavelengths. ........................................................... 83

Figure 44: HPLC-FL detection calibration curve of benzo[k]fluoranthene. Measurements were made using excitation (268 nm) and emission (398 nm) wavelengths. ........................................................... 84

Figure 45: HPLC-FL detection calibration curve of benzo[a]pyrene. Measurements were made using excitation (380 nm) and emission (405 nm) wavelengths. ........................................................... 85

Figure 46: HPLC-FL detection calibration curve of dibenzo[a,h]anthracene. Measurements were made using excitation (304 nm) and emission (421 nm) wavelengths. ........................................................... 86

Figure 47: HPLC-FL detection calibration curve of benzo[g,h,i]perylene. Measurements were made using excitation (304 nm) and emission (421 nm) wavelengths. ........................................................... 87

Figure 48: HPLC-FL detection calibration curve of indeno[1,2,3-cd]pyrene. Measurements were made using excitation (300 nm) and emission (466 nm) wavelengths. ........................................................... 88
**Figure 49:** HPLC-FL chromatogram of 100 ng mL$^{-1}$ dibenzo[a,l]pyrene pure standard prepared with acetonitrile and using excitation (316 nm) and emission (420 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 50:** HPLC-FL chromatogram of 300 ng mL$^{-1}$ naphtho[2,3-e]pyrene pure standard prepared with acetonitrile and using excitation (320 nm) and emission (406 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 51:** HPLC-FL chromatogram of 100 ng mL$^{-1}$ dibenzo[a,e]pyrene pure standard prepared with acetonitrile and using excitation (305 nm) and emission (495 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 52:** HPLC-FL chromatogram of 100 ng mL$^{-1}$ naphtho[1,2-k]fluoranthene pure standard prepared with acetonitrile and using excitation (326 nm) and emission (440 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 53:** HPLC-FL chromatogram of 50 ng mL$^{-1}$ benzo[b]perylene pure standard prepared with acetonitrile and using excitation (406 nm) and emission (440 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 54:** HPLC-FL chromatogram of 100 ng mL$^{-1}$ dibenzo[b,k]fluoranthene pure standard prepared with acetonitrile and using excitation (412 nm) and emission (409 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 55:** HPLC-FL chromatogram of 2000 ng mL$^{-1}$ dibenzo[a,i]pyrene pure standard prepared with acetonitrile and using excitation (393 nm) and emission (433 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

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**Figure 49:** HPLC-FL chromatogram of 100 ng mL$^{-1}$ dibenzo[a,l]pyrene pure standard prepared with acetonitrile and using excitation (316 nm) and emission (420 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 50:** HPLC-FL chromatogram of 300 ng mL$^{-1}$ naphtho[2,3-e]pyrene pure standard prepared with acetonitrile and using excitation (320 nm) and emission (406 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 51:** HPLC-FL chromatogram of 100 ng mL$^{-1}$ dibenzo[a,e]pyrene pure standard prepared with acetonitrile and using excitation (305 nm) and emission (495 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 52:** HPLC-FL chromatogram of 100 ng mL$^{-1}$ naphtho[1,2-k]fluoranthene pure standard prepared with acetonitrile and using excitation (326 nm) and emission (440 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 53:** HPLC-FL chromatogram of 50 ng mL$^{-1}$ benzo[b]perylene pure standard prepared with acetonitrile and using excitation (406 nm) and emission (440 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 54:** HPLC-FL chromatogram of 100 ng mL$^{-1}$ dibenzo[b,k]fluoranthene pure standard prepared with acetonitrile and using excitation (412 nm) and emission (409 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

**Figure 55:** HPLC-FL chromatogram of 2000 ng mL$^{-1}$ dibenzo[a,i]pyrene pure standard prepared with acetonitrile and using excitation (393 nm) and emission (433 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$.
Figure 56: HPLC-FL chromatogram of 100 ng mL⁻¹ naphtho[2,3-a]pyrene pure standard prepared with acetonitrile and using excitation (333 nm) and emission (462 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹. ................................................................................................. 97

Figure 57: HPLC-FL chromatogram of 1000 ng mL⁻¹ naphtho[2,3-k]fluoranthene pure standard prepared with acetonitrile and using excitation (333 nm) and emission (455 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹. ................................................................................................. 98

Figure 58: HPLC-FL chromatogram of 100 ng mL⁻¹ dibenzo[a,h]pyrene pure standard prepared with acetonitrile and using excitation (311 nm) and emission (450 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹. ................................................................................................................ 99

Figure 59: HPLC-FL detection calibration curve of dibenzo[a,l]pyrene. Measurements were made using excitation (316 nm) and emission (420 nm) wavelengths. ................................................. 101

Figure 60: HPLC-FL detection calibration curve of naphtho[2,3-e]pyrene. Measurements were made using excitation (320 nm) and emission (406 nm) wavelengths. .................................................. 102

Figure 61: HPLC-FL detection calibration curve of dibenzo[a,e]pyrene. Measurements were made using excitation (305 nm) and emission (395 nm) wavelengths. ................................................. 103

Figure 62: HPLC-FL detection calibration curve of naphtho[1,2-k]fluoranthene. Measurements were made using excitation (326 nm) and emission (440 nm) wavelengths. ......................... 104

Figure 63: HPLC-FL detection calibration curve of benzo[b]perylene. Measurements were made using excitation (406 nm) and emission (440 nm) wavelengths. ................................. 105

Figure 64: HPLC-FL detection calibration curve of dibenzo[b,k]fluoranthene. Measurements were made using excitation (312 nm) and emission (409 nm) wavelengths. ......................... 106

Figure 65: HPLC-FL detection calibration curve of dibenzo[a,i]pyrene. Measurements were made using excitation (393 nm) and emission (433 nm) wavelengths. ................................. 107
**Figure 66:** HPLC-FL detection calibration curve of naphtho[2,3-a]pyrene. Measurements were made using excitation (333 nm) and emission (462 nm) wavelengths. .......................................... 108

**Figure 67:** HPLC-FL detection calibration curve of naphtho[2,3-k]fluoranthene. Measurements were made using excitation (333 nm) and emission (455 nm) wavelengths. .............................. 109

**Figure 68:** HPLC-FL detection calibration curve of dibenzo[a,h]pyrene. Measurements were made using excitation (311 nm) and emission (450 nm) wavelengths. ............................................. 110
LIST OF TABLES

Table 1: Modifications Made to EPA Method 610 for the Separation of EPA-PAHs ....................... 35

Table 2: Analytical Figures of Merit of EPA- under EPA Method 610 ...................................... 36

Table 3: Analytical Figures of Merit of MM 302 Da PAHs under EPA Method 610 ...................... 38

Table 4: 77 K Analytical Figures of Merit of DBalP and BaP obtained via LETRSS ................. 42

Table 5: Analyte Recoveries of Sample Procedure Prior to HPLC Analysis ............................. 46

Table 6: Analyte Recoveries for LETRSS Analysis of HPLC Fractions .................................. 46
CHAPTER ONE: INTRODUCTION

1.1 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons or PAHs comprise a complex class of condensed multi-ring benzenoid chemicals containing only carbon and hydrogen\textsuperscript{1-3}. PAHs are solid compounds that can vary in colors. They are mainly found as colorless, white, or yellow pale. PAHs are comprised of two or more benzene rings that are bonded in linear, cluster or angular arrangements. The three different types of arrangements for PAHs are illustrated in Figure 1.

<table>
<thead>
<tr>
<th>Types of Arrangements</th>
<th>Examples</th>
<th>Structure</th>
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<tbody>
<tr>
<td>Linear</td>
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<td><img src="image" alt="Anthracene" /></td>
</tr>
<tr>
<td>Cluster</td>
<td>Dibenz(a,h)anthracene</td>
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</tr>
<tr>
<td>Angular</td>
<td>Pyrene</td>
<td><img src="image" alt="Pyrene" /></td>
</tr>
</tbody>
</table>

**Figure 1:** Examples of well-known PAHs showing their different structure arrangements.

PAHs are classified as “small” PAHs and “large” PAHs depending on the number of aromatic rings present in their structure. For example, PAHs containing up to six fused aromatic rings are often known as “small” PAHs, and those containing more than six aromatic rings are referred as “large” PAHs. Due to the availability of samples, most of the research conducted on PAHs has been on small PAHs.

PAH characteristics are high melting and boiling points, low vapor pressure and not very soluble in water, but soluble in organic solvents. Their solubility decreases as the molecular weight
Another characteristic of PAHs is their UV absorbance spectra. The UV absorbance spectra of each PAH is mostly unique, and each isomer also has its own spectra. Figure 2 shows the difference in UV absorption between two well-known PAHs (Benzo[b]perylene and Dibenzo[b,k]fluoranthene) showing different wavelengths.

**Figure 2**: UV absorption spectrum of Benzo[b]perylene and Dibenzo[b,k]fluoranthene.

Figure 2 shows a maximum absorption band at 230 nm for Benzo[b]perylene and a maximum absorption band of 217 nm for Dibenzo[b,k]fluoranthene.

Most PAHs are also fluorescent and emit characteristic wavelengths when they are excited. Their fluorescence spectra are also unique and varies from PAH to PAH.
Figure 3: Room temperature excitation and emission spectra of Benzo[a]perylene and Dibenzo[b,k]fluoranthene.

Figure 3 shows characteristic excitation and emission maximums for both PAHs. In the case of Benzo[b]perylene its maximum excitation is at 407 nm and its maximum excitation is at 436 nm. In contrast, Dibenzo[b,k]fluoranthene shows an excitation maximum at 312 nm and an emission maximum at 400 nm.

PAHs originate either from natural or anthropogenic sources. The natural introduction of PAHs into the environment can occur by the burning of forest and brush fires, volcanoes, petroleum seeps and erosion of sedimentary rocks containing petroleum hydrocarbons. Anthropogenic sources can include industrial processes, automotive emissions, oil spills, smoke from wood burning stoves, airplane exhausts, barbecues, and tobacco smoke. Independent of the source of origin, PAHs result from the incomplete combustion of organic matter at high temperatures, which typically range from 100 °C to 800 °C.

When incomplete combustion of organic matter occurs, PAHs are mainly dispersed to the atmosphere and because of this the atmosphere receives a high environmental load of PAHs.
resulting in PAHs being abundant in the environment. Higher PAH concentrations are usually found around urban areas rather than rural areas because most of the sources of PAHs are found in or near urban areas.

Due to their hydrophobic properties, PAHs adsorb onto atmospheric particles and deposit into soils and plants. Once deposited into soils, PAHs can migrate into groundwater supplies and enter the food chain. Other sources of human contact with PAHs include tobacco and agricultural products, pharmaceuticals, thermosetting plastics, lubricating materials, and chemical industries in general. Some examples of the use of PAHs in industry are in the manufacture of pigments and dies, wood preservatives, pesticides, and resins.

1.2 Polycyclic Aromatic Hydrocarbons in the Environmental Protection Agency List

PAH properties have been well studied over the past few decades. One of the main concerns for the presence of PAHs in the environment is their toxicity, mutagenicity, and even carcinogenicity to living organisms, including humans. To prevent human contamination from PAHs, the U. S. Environmental Protection Agency recommends the environmental monitoring of sixteen PAHs included in its priority pollutants list. Figure 4 shows the molecular structures of the sixteen PAHs included in the EPA priority pollutants list (EPA-PAHs).
Figure 4: Molecular structures, names, and molecular weights of the 16 EPA-PAHs.
Some PAHs contain a region in their structure called the “bay region”. Bay regions in polycyclic aromatic compounds refers to space between the aromatic rings of the PAH molecule. Figure 5 shows an example of the bay region of B[a]P$^{31}$. The bay region of B[a]P is between carbon 10 and carbon 11. Of the 16 EPA-PAHs, 10 have bay regions in their respective molecular structures.

![Bay region](image)

**Figure 5**: Molecular structure for benzo[a]pyrene and its bay region.

Metabolic studies have demonstrated that the bay regions of PAHs lead to the formation of diol-epoxides $^{32}$. B[a]P is the most carcinogenic PAH in the EPA list, and it is often used as a model compound to investigate the toxic effects of PAHs$^{5}$. Its high toxicity is attributed to the presence of such bay region in its molecular structure$^{33}$. 


For PAHs to have carcinogenic properties they must enter the body and be metabolized. The bay region dyhydrodiol epoxide pathway is a metabolic activation pathway of carcinogenic PAHs. This metabolic pathway is very well understood and established. During this metabolic pathway, PAHs such as B[a]P undergo three different reactions that are enzyme mediated. The first reaction that occurs during this metabolic process is the oxidation of a double bond that is catalyzed by P450 enzymes (CYPs) to form unstable arene oxides. Second, microsomal epoxide hydrolase or (EH) hydrolyses the unstable arene epoxide to form trans dihydrodiols. Finally, a second CYP-catalyzed oxidation at the double bond adjacent to the diol generates a diol-epoxide. This metabolic pathway can lead to sterically hindered bay region diol-epoxides. These diol-epoxides act as electrophiles and can then bind to DNA bases. An overview of B[a]P metabolism is illustrated in Figure 6.

**Figure 6**: Metabolic activation of B[a]P via bay region dihydrodiol epoxide pathway.

### 1.3 High-Molecular Weight Polycyclic Aromatic Hydrocarbons

Among the hundreds of PAHs present in the environment, the EPA lists only sixteen as "Consent Decree" priority pollutants. Unfortunately, there are other PAHs that exhibit equally or
far stronger carcinogenic properties than B[a]P. Of particular concern is the omission of PAHs with molecular mass (MW) greater than 300 g mol\(^{-1}\). Biological activities of high molecular weight PAHs (HMW-PAHs) have revealed positive mutagenic response when isolated from environmental and combustion-related samples\(^{36}\). An example of utmost importance is DB[a,l]P. Its relative potency factor is approximately 100 times higher than B[a]P’s factor. There are several more PAH isomers with the same molecular weight (≈ 302 g.mol\(^{-1}\)) that are also carcinogenic, but not to the extent of DB[a,l]P. Since the carcinogenic properties of HMW-PAHs differ significantly from isomer to isomer, it is of paramount importance to determine the most toxic isomers even if they are present at much lower concentrations than other PAHs. The metabolic pathway of DB[a,l]P is very similar of that of B[a]P; where diol-epoxides are formed. Diol-epoxide formation during metabolism of DB[a,l]P does not occur at the bay region but rather at the fjord region. The fjord regions are basically a bay region that have either an additional fused ring or a methyl substituent on the inside aromatic carbon\(^{37-38}\). Figure 7 shows the fjord region of DB[a,l]P. PAHs that have fjord regions undergo the same metabolic pathway as PAHs with bay regions, where diol-epoxides that act as electrophiles bind to DNA bases.
Figure 7: Fjord region of DB[a,l]P.

1.4 References


CHAPTER TWO: PAH ANALYSIS IN TOBACCO PRODUCTS - A REVIEW

2.1 Background

Humans have consumed tobacco for a very long time. Tobacco was first introduced in Europe when colonizers brought it back from the new world. When Europeans arrived in the Americas, they witnessed native Americans smoking something new they have never seen. Native Americans used cane pipes to smoke tobacco, and they called these pipes “tabaco”. When Europeans heard the word “tabaco”, they mistakenly named it tobacco.

Tobacco is a plant grown from its leaves. The leaves are grown, fermented, aged, and before going into tobacco products, the leaves go under a curing process. Curing is essential for its consumption because in its raw form, the tobacco leaf is too wet to ignite and be smoked. Another reason why tobacco leaves are cured is because the curing process allows the oxidation and degradation of carotenoids in the tobacco leaves. This helps increase the nicotine levels, improve the taste and smoothness of the tobacco.

The “smoldering” process is a curing process of a slow and flameless form of wood combustion where the tobacco leaves cure over time while they hung in large barns. This “smoldering” process produces tobacco low in sugars and very high in nicotine which is the addictive component of tobacco products.

Tobacco products can be separated into three classes:

1. Those in which tobacco is rolled, combusted, and smoked (e.g., cigarettes, bidis, kreteks)
2. Those in which tobacco is heated but not combusted (e.g., water pipes, hookah, nargile)
3. Those in which tobacco is not heated or combusted, (i.e., “smokeless tobacco,” e.g., snuff); these are chewed, but some are used nasally.
It is well known that PAHs are present in tobacco. It is important to note that PAHs do not naturally occur in the tobacco plant but are formed primarily by incomplete combustion of tobacco and other organic components during smoking. PAHs are present in unburned tobacco products, particularly those containing fire-cured tobacco varieties. During fire curing, PAHs in combustion fumes generated by smoldering wood are deposited on the tobacco leaves.

Nicotine is one of the most addictive substances known to man and its addictive nature contributes tremendously to the consumption of tobacco products which exposes humans to carcinogenic PAHs. The exposure to PAHs can contribute to development of cancer. Cancer is a major public health problem in the United States and many other parts of the world. One in four deaths in the United States is due to cancer. Although there are various types of cancers, lung cancers are more frequent than other types of cancers. An estimated 156,900 lung cancers occurred in the United States alone in 2011. It is well documented that not only cancers, but a variety of respiratory and cardiovascular diseases could develop from the consumption of tobacco products. The International Agency for Research on Cancer reported that 10 carcinogenic PAHs along with 53 other known carcinogens are present in tobacco products. Due to the great amount of cancer cases related to tobacco products, it is imperative to accurately analyze carcinogenic PAHs in such products.

PAHs have been analyzed in sidestream smoke, mainstream smoke, smoke condensate, cigarette filters, smokeless tobacco, and tobacco leaves. Sections 2.2-2.6 of this chapter provides information on previously used techniques for the analysis of PAHs in tobacco products.

2.2 Analysis of PAHs in Sidestream and Mainstream Smoke

The term sidestream smoke refers to the smoke that is released from the end of a burning cigarette, a cigar, or a pipe. In contrast, the term mainstream smoke is referred to as the smoke that
is inhaled by a smoker and then exhaled into the environment. It is important to understand that second-hand smoking is the term used to describe both sidestream smoke, and mainstream smoke. Eighty-five percent of second-hand smoke comes from sidestream smoke and sidestream smoke contains ten times higher amounts of carcinogenic PAHs than mainstream smoke.

Gas-chromatography mass-spectrometry (GC/MS) for the analysis of EPA-PAHs in sidestream smoke from cigarettes have been employed previously. In an experiment conducted by H. Lee et al., samples were generated with a home-made smoking machine and collected on glass fiber filters. The difference in mass of the filters was subtracted to account for the net weight of PAHs. The PAHs on the filters were then extracted with methanol using an ultrasonic bath. The extracts were then vacuum dried and re-constituted in methanol followed by GC/MS analysis. In this study, the authors determined the concentration of 22 PAHs including the EPA-PAHs. Five PAHs of the 22 PAHs were not detected because they were below the limit of detection. The undetected PAHs were: Benzo[b]fluoranthene, Perylene, Dibenz[a,h]anthracene, Coronene, and Dibenzo[a,e]pyrene. The concentration of Benzo[a]pyrene found per cigarette was 144 nanograms.

Another study by E. Sepetdjian et al. analyzed 16 EPA-PAHs in mainstream smoke of narghile waterpipe tobacco by Gas-chromatography mass-spectrometry coupled with selected ion current profile (GC-MS-SICP). Herein, a digital waterpipe smoking machine was used for generating the smoke. Ten grams of narghile flavored tobacco were loaded on the smoking machine. The smoke was passed through a silica cartridge and eluted with hexane. The eluent was then preconcentrated and reconstituted in acetonitrile and analyzed by GC-MS-SICP. This study found that the chromatograms were heavily populated with overlapping peaks. The overlapping peaks corresponded to co-eluting pairs Chrysene/Benzo[a]anthracene and
Benzo[k]fluoranthene/Benzo[b]fluoranthene. Naphthalene was not quantified because it was below the limit of detection (LOD).

2.3 Analysis of PAHs in Smoke Condensate

PAHs have not only been found in sidestream smoke and mainstream smoke but also in smoke condensate. Smoke condensate refers to mainstream smoke that has been condensed to liquid form with the aid of a filter pad and a solvent\textsuperscript{13}. Methods for the measurement of PAHs in smoke condensate have employed chromatographic techniques such as HPLC and GC-MS. Current studies that employ these chromatographic methods analyze the EPA-PAHs in smoke condensate.

EPA-PAHs were analyzed previously by high-performance liquid-chromatography coupled with ultra-violet absorption and fluorescence detection in aqueous phase of smoke condensate\textsuperscript{14}. In this study, 10 grams of the liquid condensate and 100 μL of internal standard solution in 2-propanol were combined in a 250 ml round bottom flask. To this mixture, 3.2 g solid potassium hydroxide and 32 mL of methanol were added. The solution was refluxed for 30 min. The sample was extracted with 25 mL of cyclohexane by liquid/liquid partitioning. The organic phases were combined in a round-bottom flask and dried with anhydrous sodium sulphate. After evaporation of the solvent under reduced pressure, the sample was reconstituted in 1 mL of cyclohexane. For removal of residual interferences, the extract was passed through a silica gel solid phase extraction tube and eluted with 7 ml cyclohexane. After evaporation of the eluate and reconstitution of the residue with 1 mL of acetonitrile; the extract was analyzed by HPLC-UV/FLD. For the separation a Pinacle II reverse phase column for PAHs, 250 x 2.1 mm and 5 microns was used. The mobile phase consisted of a gradient of acetonitrile/water at 0.3 mL/min.
This study presented co-elution between compounds Benzo[\textit{a}]anthracene and Cyclopenta\textit{cd}pyrene.

The analysis of 16 EPA-PAHs in smoke condensate has also been performed by GC/MS\textsuperscript{15}. In this study, smoke condensate collection was achieved using a DIN ISO 3402 smoking machine followed by a sample pre-treatment with methanol and ultrasonic bath. Solid-phase extraction using a C\textsubscript{18} cartridge was employed before analysis by GC/MS. All 16 EPA-PAHs were quantified in this study ranging from Benzo\textit{k}fluoranthene (0.2 ng/cigarette) to Naphthalene (7.8 ng/cigarette).

2.4 Analysis of PAHs in Cigarette Filters

PAHs have been studied in smoked cigarette filters after release to the environment\textsuperscript{16} and after smoking in a controlled laboratory setting\textsuperscript{11}. The study based on cigarette filters released into the environment focuses on the leaching of PAHs into groundwater sources and potential contamination of these groundwater supplies. In this study cigarette filters were collected from urban areas of Essen and Mulheim an der Ruhr in Germany to study 16 EPA-PAHs. After the samples were collected, the filters were opened and placed in a vial containing cyclohexane. The samples were shaken and placed in an ultrasonic cleaner for 6 hours followed by GC-MS analysis. All 16 EPA-PAHs were found in this study. PAH concentrations ranged from 0.640 micrograms/g (Phenanthrene) to 12.8 micrograms/g (Naphthalene)\textsuperscript{16}.

The literature also reveals the analysis of 9 EPA PAHs in cigarette filters\textsuperscript{11}. The 9 PAHs were as followed: Anthracene, Benzo[\textit{a}]anthracene, Benzo[\textit{b}]fluoranthene, Benzo[\textit{k}]fluoranthene, Benzo[\textit{ghi}]perylene, Benzo[\textit{a}]pyrene, Dibenzo[\textit{a,\textit{h}}]anthracene, Fluoranthene, and Pyrene. These cigarette filters were not collected from the environment but instead, the cigarettes were smoked and the PAHs on the filters were analyzed via GC/MS. After cigarettes were smoked and filters
were cut into portions, all portions were placed into 260 mL bottles and extracted with 40 mL of dichloromethane for 25 min by ultrasonic cleaner. The extract was concentrated to 2 mL using a rotary evaporator and filtered using Whatman paper. The extract was then concentrated to 0.1 mL under nitrogen gas and reconstituted to 2 mL with hexane for analysis by GC/MS. Only 5 PAHs out of the 9 PAHs studied were found in the cigarette filters. The concentrations of the found PAHs were: 42.4 ng/g (Benzo[a]anthracene), 56.6 ng/g (Benzo[b]fluoranthene), 101 ng/g (Anthracene), 119 ng/g (Pyrene), and 135 ng/g (Fluoranthene)\(^\text{11}\).

### 2.5 Analysis of PAHs in Smokeless Tobacco

Smokeless tobacco is a tobacco product that is used by means other than smoking. Smokeless tobacco products can be chewed, sniffed, or placed between the gum and the cheek or lip\(^\text{17}\). Smokeless tobacco products contain the highly addictive substance nicotine, just like any other tobacco containing product. Quitting smokeless tobacco use can be as challenging as quitting cigarettes or other tobacco related products\(^\text{18}\) and because of this, the analysis of PAHs in these types of tobacco product cannot be disregarded.

Most of the papers that analyze PAHs in smokeless tobacco products in the literature are mainly based in chromatographic techniques such as HPLC and GC\(^\text{19-23}\). A study by M. Carradus et al., analyzed Benzo[a]pyrene in a blend of smokeless tobacco products by HPLC-FL. The samples were hydrated and extracted with hexane and acetone and subjected to adsorption chromatography using based-modified silica to remove co-extracted substances. The extract was then reconstituted in acetonitrile and analyzed. The concentration of Benzo[a]pyrene in the samples varied from 0.38 ng g\(^{-1}\) to 150 ng g\(^{-1}\) depending on the tobacco blend.

Benzo[a]pyrene was also analyzed by J. W. Flora et al., in a variety of smokeless tobacco products by GC/MS. Tobacco samples were placed in glass vials and 10 mL of methanol was
Samples were agitated for 30 minutes at 350 rpm. Solids were allowed to settle to the bottom of the vial for 15 minutes and the clear sample was decanted into a syringe that contained a 0.45-micron filter. The solution was then passed through solid-phase extraction. Next, the samples were reconstituted in 300 μL of 50/50 toluene:iso-octane before GC/MS analysis. The concentration of Benzo[a]pyrene in the different samples ranged from 0.83 ng/g to 58.70 ng/g.

Besides Benzo[a]pyrene, other EPA-PAHs have also been analyzed previously in smokeless tobacco products. In a study conducted by I. Stepanov et al., twenty-three PAHs including the EPA-PAHs were analyzed by GC/MS following a similar extraction procedure to the previously mentioned methods in this chapter. The limits of quantitation for different PAHs varied between 0.3 and 11 ng/g tobacco.

2.6 Analysis of PAHs in Tobacco Leaves

To the extent of our literature search we were able to find only one paper that analyzed PAHs in tobacco leaves from cigarettes. In this paper 9 PAHs were analyzed by GC/MS. The PAHs were as followed: Anthracene, Benzo[a]anthracene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[ghi]perylene, Benzo[a]pyrene, Dibenzo[ah]anthracene, Fluoranthene, and Pyrene. In this study it was found that PAHs in the leaves ranged from 206 to 2400 ng/g.

2.7 Conclusions

The current methodology for the analysis of PAHs in tobacco products employs mainly chromatographic techniques such as HPLC and GC. These methodologies have been mainly applied to lower molecular weight PAHs such as the EPA-PAHs but to the extent of our literature
search there is no research done on high molecular weight PAHs. It is imperative to analyze high molecular weight PAHs such as Dibenzo[a,l]pyrene, the most carcinogenic PAH.

Although these chromatographic techniques have been applied widely to this type of analysis for many years, they present challenges. High-performance liquid chromatography with fluorescence detection is often used for the analysis of PAHs, however; since fluorescence chromatograms are usually recorded at a single set of excitation and emission wavelengths, qualitative parameters for PAH identification are restricted to characteristic retention times which it becomes problematic when PAHs exhibit similar retention times. Another limitation of room temperature fluorescence technique is the broadness of excitation spectra. This in terms limits the selectivity potential towards targeted compounds in complex matrices. The spectral band broadening phenomenon is well known and is determined by inhomogeneous and homogeneous band broadening effects. When analyte molecules are exposed to different microenvironments within the same matrix; inhomogeneous band broadening occurs. These differences in microenvironments result in Gaussian distribution of electronic transition energies, and band broadening happens. In contrast, homogeneous broadening arises from electron-photon coupling and from the limited lifetimes of the states involved in the electronic transition. This type of broadening affects all the analyte molecules to the same extent. GC-MS also presents drawbacks when analyzing PAHs in tobacco samples. The combination of similar retention times and virtually identical fragmentation patterns makes it almost impossible to analyze PAH isomers by GC-MS. Another drawback of using these techniques is the ability to detect PAHs at low concentrations.

2.8 Research Objectives

This dissertation is focused on the analysis of EPA-PAHs and HMW-PAHs in tobacco extracts using the HPLC portion of EPA method 610 (see chapter 4) combined with laser excited
time resolved Shpol’skii spectroscopy. Since inhomogeneous band broadening is not ideal because it affects the selectivity potential towards compounds such as PAHs, Shpol’skii spectroscopy comes in handy. Under Shpol’skii conditions, PAH molecules are frozen in a solvent host (usually an n-alkane) to produce ordered polycrystalline matrixes at 77 K or below. Under these conditions, molecules are isolated from each other and rigidly fixed in the solvent, resulting in the appearance of atomic-like quasi-line spectra. Matrix isolation of PAH molecules at cryogenic temperatures reduces inhomogeneous band broadening to produce vibrational fluorescence spectra with fingerprint information. Our approach to Shpol’skii spectroscopy which we have named laser excited time-resolved Shpol’skii spectroscopy (LETRSS) - combines spectral and lifetime information in multidimensional data formats known as wavelength time matrices (WTMs). Recording WTMs during the fluorescence decay of the sample makes possible to identify PAHs on the bases of spectral and lifetime information. Fluorescence lifetimes also report on spectral peak purity25-26. The developed method improves limits of detection, achieves accurate analysis of co-eluting PAHs as well as reduces analysis time and analysis cost compared to current methodology.

2.9 References


3. “Tobacco Barn Retrofit”. The University of Georgia.


doi:10.1021/acs.chemrestox.5b00190.


13. Han, Sung Gu, Kamala Pant, Shannon W. Bruce, and C. Gary Gairola. "Bhas 42 cell transformation activity of cigarette smoke condensate is modulated by selenium and arsenic." Environmental and Molecular Mutagenesis 57, no. 3 (2016): 220-228.


CHAPTER THREE: INSTRUMENTATION

The four main instruments used to perform the experiments discussed in this dissertation were an ultraviolet-visible (UV-VIS) absorption spectrometer, a commercial spectrofluorometer, a high-performance liquid chromatography (HPLC) system coupled to a single-channel fluorescence detector, and a Radiant 355 LD UV tunable laser system. A brief description of each instrument is provided in the following sections of this chapter. In addition, a fiber optic probe for cryogenic measurements is presented that improved the signal to noise ratio of experiments at liquid nitrogen (77 K) temperatures.

3.1 UV-Vis Absorption Spectroscopy

UV-VIS absorption measurements were made with the aid of a single beam spectrophotometer (model Cary 50, Varian) equipped with a 75W pulsed xenon lamp emitting broadband radiation from 190 nm to 1100 nm. Wavelength selection and spectra collection was accomplished with a dual beam, Czerny-Turner monochromator with a 1.5 nm spectral bandwidth. Radiation intensities were detected with two Si photodiode detectors, one of which was used to record the reference signal from the excitation source. Although the maximum scanning rate of the instrument is 24,000 nm.min-1, all the absorption spectra presented in these studies were recorded with a 600 nm.min-1 scanning speed. This scanning speed provided spectral resolution appropriate for the purposes of our studies.

3.2 Fluorescence Spectroscopy

Steady state excitation and fluorescence spectra were recorded with a commercial spectrofluorimeter (FluoroMax-P from Horiba Jobin-Yvon) equipped with a 150W continuous xenon lamp with broadband illumination from 200 to 1100 nm. The excitation and emission
monochromators had the same reciprocal linear dispersion (4.2 nm mm\(^{-1}\)) and accuracy (±0.5 nm with 0.3 nm resolution). Both diffraction gratings had the same number of grooves per unit length (1200 grooves mm\(^{-1}\)) and were blazed at 330 nm (excitation) and 500 nm (emission). A photomultiplier tube (Hamamatsu, model R928) with spectral response from 185 to 650 nm was used for fluorescence detection operating at room temperature in the photon-counting mode. Commercial software (DataMax) was used to computer-control the instrument. Room temperature measurements were made by pouring undegassed liquid solutions into micro-quartz cuvettes (1 cm path length x 2 mm width) that held a maximum volume of 400 µL. Fluorescence emission was collected at 90 degrees from excitation using appropriate cutoff filters to reject straight-light and second order emission.

### 3.3 Cryogenic Fiber Optic Probe

The FOP consisted of one excitation fiber and six emission fibers. All fibers were 2 m long and 500 µm core diameter, silica-clad silica with polyimide buffer coating (Polymicro Technologies, Inc.). The fibers were fed into 1.2 m long sections of copper tubing that provided mechanical support for lowering the probe into the liquid cryogen. At the sample end, the fibers were arranged in a six-around-one configuration with the excitation fiber in the center. At the instrument end, the six emission fibers were position in a “slit” (vertical line) configuration. Vacuum epoxy was used to hold the fibers in place, which were then fed into metal sleeves for mechanical support. At the sample end, the copper tubing section was flared stopping at a phenolic screw cap threaded for a 0.75 mL propylene sample vial.
3.4 Laser Excited Time Resolved Shpol' Skii Spectroscopy

LETRSS measurements were performed with an instrumental set-up built in-house. A Radiant 355 LD UV tunable laser system from OPOTek Inc. with tuning capability from 210 to 2500 nm was used as the excitation source. The laser power was controlled with a half-wave plate, a polarizer cube, and a neutral density filter. The polarized cube was set at a fixed position from the tunable laser to allow for maximum transmittance of the excitation light. By rotating the halfwave plate, it was possible to control the intensity of radiation that passed through the polarizer cube. The fraction of laser light reflected by the polarizer was directed towards a StarLite power meter (Ophir). The neutral density filter transmitted 10% of the laser intensity at the desired excitation wavelengths and blocked the residuals of the laser output at 1064 nm, 532 nm, and 355 nm. After passing through a digitally controlled shutter, the remaining portion of the laser light was focused into the excitation fiber of a cryogenic fiber optic probe (FOP).

The excitation fiber of the FOP was set on a translation stage that allowed fine movements to achieve horizontal (X) and vertical (Y) alignment with the laser beam. A focusing lens reduced the spot size of the laser beam to the inner diameter of the fiber and helped to maximize optical throughput into the sample. The six-emission fiber optic probe bundle of the FOP was mounted in a XY translational stage that had an additional mode of rotation to allow for alignment into the entrance slit of the spectrograph (Shamrock; Andor). The spectrograph was equipped with a diffractive grating (1200 grooves.mm-1) blazed at 500 nm and attached to an iStar ICCD (Andor). The ICCD had an active area of 690 x 256 pixels, pixel size of 26 mm, and a well depth of 500,000 electrons. Data acquisition and instrument control was made with the aid of LABVIEW software developed in-house. Unless otherwise noticed, all spectra were recorded using a 10 μm spectrograph slit width.
3.5 High Performance Liquid Chromatography

Chromatographic analysis was conducted with a Hitachi HPLC system (San Jose, CA) equipped with a model L7100 gradient pump, an L-7400 UV detector, an L-7485 fluorescence detector, an L-761 online degasser and a D-7000 control interface. The HPLC operation was computer controlled with Hitachi software. Chromatographic separation was performed with an Eclipse PAH column with the following characteristics: 250 mm length, 4.6 mm inner diameter, and 5 μm particle size. The mobile phase consisted of isocratic elution for five minutes using 40% acetonitrile and 60% water, and then linear gradient elution to 100% with acetonitrile over 25 minutes. The mobile phase flow rate was 1.5 mL min⁻¹ and the column temperature was approximately 25 °C. Sample injection (20 μL) was made with a fixed-volume injection loop. RPLC fractions were collected in 7.0 mL amber vials.

3.6 References

4.1 Introduction

A classic example for the analysis of EPA-PAHs is the EPA Method 610\(^1\), which provides two chromatographic options for the purpose at hand. Originally developed for the analysis of municipal and industrial wastewater samples, Method 610 determines PAHs via HPLC or GC. With minor modifications, the HPLC option of Method 610 has proven useful for the determination of EPA-PAHs in a wide variety of samples. These include hair\(^2\); food\(^3-5\); soil and sediment\(^6-9\); water\(^10-12\); tissue\(^13-14\); drugs\(^15\); oil\(^16-17\); air\(^18\); saliva\(^19\); and tobacco products\(^20-24\).

Herein, we apply the HPLC Method 610 to the analysis of HMW-PAHs. Although the MM 302 Da PAHs represent the most investigated PAHs within the class denoted as high molecular weight PAHs\(^25-26\), the extent of our literature search reveals no publications on the capabilities of Method 610 to measure PAH isomers of MM 302. Figure 8 shows the molecular structures of the MM 302 Da isomers selected for these studies.

*Adapted from Analytical Methods, A. Comas, A. Santana, A. D. Campiglia, On the co-elution of Benzo[a]pyrene and Dibenzo[a,l]pyrene in chromatographic fractions and their unambiguous determination in tobacco extracts via Laser-Excited Time Resolved Shpol’skii Spectroscopy. Copyright 2023, no permission required from Analytical Methods.
Their HPLC analytical figures of merit (HPLC-AFOMs) were obtained under EPA-Method 610 along with 15 EPA-PAHs. Since strong co-elution was observed between BaP and DBaLP, their unambiguous determination in HPLC fractions was obtained via laser-excited time resolved Shpol’skii spectroscopy (LETRSS). The developed methodology was then applied to the analysis of BaP and DBaLP in tobacco samples.

4.2 Materials and Methods

4.2.1 Chemicals

All reagents were purchased at the highest available purity. Analytical standards of naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, Benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-cd]pyrene, Dibenzo[a,l]pyrene,
dibenzo[a,e]pyrene, and naphtho[2,3-a]pyrene were purchased from Sigma Aldrich. Naphto[1,2-k]fluoranthene, dibenzo[b,k]fluoranthene, and naphtho[2,3-k]fluoranthene were purchased from Chiron. Naphtho[2,3-e]pyrene, benzo[b]pyrene, dibenzo[a,i]pyrene, dibenzo[a,h]pyrene, and naphtho[2,3-a]pyrene were purchased from TRC. Nanopure water was used in all experiments. 100% (HPLC grade) acetonitrile and methanol were purchased from Fisher Scientific. N-octane was of 99% purity and purchased from Acros Organics. Marlboro and Camel Menthol Crush commercial cigarettes were purchased at local stores.

4.2.2 Measurement Procedures

Measurements with the FOP were made as follows: (1) 100 μL of un-degassed sample solution were pipetted into the sample vial; (2) the sample vial was secured to the sample end of the copper tubing; and (3) the tip of the FOP was positioned at a constant depth below the solution surface; and (4) sample freezing was accomplished by lowering the sample vial into the liquid nitrogen. The probe clean-up procedure involved removing the sample vial from the cryogen container, melting the frozen matrix, and warming the resulting solution to approximately room temperature with a heat gun, rinsing the probe with organic solvent, and drying it with warm air from the heat gun. The entire freeze, thaw, and clean up cycle took less than 5 min per sample.

4.2.3 Fluorescence Lifetimes

Fluorescence lifetimes were calculated from emission decays recorded via a three-step procedure that included (1) recording WTMś from individual standards and n-octane (background decay) at the maximum excitation wavelength of each PAH; (2) subtracting the background decay from the fluorescence decay at the maximum fluorescence wavelength of the PAH; and (3) fitting the background corrected data to single exponential decays30.
4.2.4 HPLC-LETRSS Analysis of Tobacco Samples

Five cigarettes from the same commercial brand were cut open with a scalpel and the filters were discarded. The tobacco was placed in a 100 mL Erlenmeyer flask and 50 mL of methanol was added to the Erlenmeyer flask. The flasks were stoppered and sonicated for 1 hour in a Powersonic sonicator (Crest Ultrasonics, New Jersey, USA). Ice chips were periodically added to the sonicator to keep the temperature of the water from rising. The sample extracts were vacuum filtered through Whatman paper twice. 5 mL of sample extract was evaporated to dryness and reconstituted in 1 mL of acetonitrile. 20 μL of the re-constituted tobacco extract were injected into the HPLC system. HPLC fractions were collected within the 29-32 min time window, evaporated to dryness, and reconstituted in 1mL of octane for LETRSS analysis.

4.3 Results and Discussion

4.3.1 HPLC Analysis under EPA-Method 610

Table 1 compares the chromatographic conditions recommended by Method 610 to those employed in these studies. The main differences were the internal diameter of our column and the flow rate of the mobile phase. Our flow rate (2mL/min) was adjusted according to EPA guidelines, which recommend a constant linear mobile phase velocity equal to 2 mm/sec.\textsuperscript{5}
Table 1: Modifications Made to EPA Method 610 for the Separation of EPA-PAHs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EPA Method 610</th>
<th>Modified EPA Method 610</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Type</td>
<td>Reverse Phase HC-ODS Sil-X</td>
<td>Reverse Phase Eclipse PAH Column</td>
</tr>
<tr>
<td>Particle Size</td>
<td>5-micron</td>
<td>5-micron</td>
</tr>
<tr>
<td>Column Dimensions</td>
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<td>250 mm x 4.6 mm</td>
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<tr>
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<td>Ambient</td>
<td>Ambient</td>
</tr>
<tr>
<td>Mobile Phase</td>
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<td>Acetonitrile/Water</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1.5 mL/min</td>
<td>2.0 mL/min</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>20 microliters</td>
<td>20 microliters</td>
</tr>
<tr>
<td>Elution</td>
<td>Gradient</td>
<td>Gradient</td>
</tr>
</tbody>
</table>

1\text{Volume flow rate} = [\text{cross sectional area of chromatographic column}] \times [\text{linear velocity}] = [\pi r^2] \times [2\text{mm/sec}] = [\pi(2.3\text{mm})^2] \times [2\text{mm/sec}] = 33.238 \text{ mm}^3/\text{sec}; \text{Flow rate in mm}^3/\text{min} = [33.328 \text{ mm}^3/\text{sec}] \times 60 \text{ sec/min} = 1994.28 \text{ mm}^3/\text{min}; \text{Flow rate in mL/min} = 1994.28 \text{ mm}^3/\text{min} \times 1 \text{ mL/1000 mm}^3 = 2 \text{ mL/min}.

Table 2 summarizes the HPLC analytical figures of merit (AFOMs) of 15 EPA-PAHs obtained with a tunable fluorescence detector. All measurements were made with pure standard solutions injecting 20 μL of sample volume per chromatographic run. Acenaphthylene is not included in the table due to its lack of fluorescence emission at room temperature. No attempts were made to monitor acenaphthylene or any other PAH via ultraviolet-visible detection. Appendixes A and B show the chromatographic separations and calibration curves of the 15 EPA-PAHs.
Table 2: Analytical Figures of Merit of EPA- under EPA Method 610

<table>
<thead>
<tr>
<th>PAH</th>
<th>$\lambda_{exc}/\lambda_{em}$ (nm)</th>
<th>tr</th>
<th>LDR (ng mL$^{-1}$)</th>
<th>$R^2$</th>
<th>LOD (ng mL$^{-1}$)</th>
<th>LOQ (ng mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>240/330</td>
<td>9.7 ± 0.2</td>
<td>78.0-100</td>
<td>0.9704</td>
<td>23.3</td>
<td>78.0</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>234/320</td>
<td>13.6 ± 0.3</td>
<td>1.8-25</td>
<td>0.9967</td>
<td>0.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Fluorene</td>
<td>252/320</td>
<td>14.6 ± 0.2</td>
<td>3.7-30</td>
<td>0.9973</td>
<td>1.1</td>
<td>3.7</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>252/370</td>
<td>16.4 ± 0.1</td>
<td>1.5-25</td>
<td>1.0000</td>
<td>0.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Anthracene</td>
<td>252/402</td>
<td>17.9 ± 0.3</td>
<td>0.4-25</td>
<td>0.9983</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>284/464</td>
<td>19.2 ±0.03</td>
<td>1.7-50</td>
<td>0.9973</td>
<td>0.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Pyrene</td>
<td>238/398</td>
<td>20.6 ± 0.2</td>
<td>1.0-25</td>
<td>1.0000</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>278/395</td>
<td>24.3 ± 0.3</td>
<td>1.3-25</td>
<td>0.9994</td>
<td>0.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Chrysene</td>
<td>278/395</td>
<td>25.4 ± 0.3</td>
<td>16.0-50</td>
<td>0.9845</td>
<td>4.7</td>
<td>16.0</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>268/398</td>
<td>28.4 ± 0.3</td>
<td>7.4-100</td>
<td>0.9994</td>
<td>2.2</td>
<td>7.4</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>268/398</td>
<td>29.8 ± 0.2</td>
<td>0.4-100</td>
<td>0.9994</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>380/405</td>
<td>30.9 ± 0.2</td>
<td>16.0-500</td>
<td>0.9898</td>
<td>4.7</td>
<td>16.0</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>304/421</td>
<td>33.4 ± 0.2</td>
<td>2.0-100</td>
<td>0.9991</td>
<td>0.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Benzo[g,h,i]pyrene</td>
<td>304/421</td>
<td>34.3 ± 0.2</td>
<td>3.0-100</td>
<td>0.9926</td>
<td>0.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>300/466</td>
<td>36.1 ± 1.3</td>
<td>8.1-100</td>
<td>0.9991</td>
<td>2.4</td>
<td>8.1</td>
</tr>
</tbody>
</table>

$^{a}$ Working solutions were prepared by diluting pure standards with acetonitrile.

$^{b}$ Maximum excitation ($\lambda_{exc}$) and emission ($\lambda_{em}$) wavelengths used for HPLC analysis.

$^{c}$ Retention times.

$^{d}$ LDR, linear dynamic range.

$^{e}$ R, correlation coefficient.

$^{f}$ Limits of detection (LOD) were calculated as $3 \times N_{p-p}/m$; where $N_{p-p}$ is the baseline noise and m is the slope of the calibration curve.

$^{g}$ Limits of quantitation (LOQ) were calculated as $10 \times N_{p-p}/m$.

The excitation and emission wavelengths for fluorescence detection were optimized by monitoring the signal-to-noise ratio (S/Np-p) of each PAH. Signal intensities (S) were measured at the retention time (tr) of each compound and averaged over three independent chromatographic runs. All measurements were made with linear PAH concentrations. The noise (Np-p) was
estimated from peak-to-peak variations of the baseline for 90 sec prior to the elution window of each PAH.

The retention times reflect the expected trend for the chromatographic separation of EPA-PAHs under reversed phase conditions\textsuperscript{25}. Heavier PAHs with larger number of aromatic rings tend to elute later than lighter PAHs as a result of stronger affinities for the octadecyl stationary phase of the column. The linear dynamic range (LDR) of each PAH was based on the average fluorescence intensities of at least three standard concentrations. No efforts were made to experimentally determine the upper linear concentrations of the calibration curves. The correlation coefficients (R) and the slopes (m) of the calibration curves were calculated with the least squares method\textsuperscript{31}. In all cases, the R\textsuperscript{2} values were close in unity demonstrating linear correlations between fluorescence intensities and PAH concentrations. The lowest concentrations of the LDRs correspond to the limits of quantitation (LOQs). The LOQs and the limits of detection (LODs) were calculated as 10xNp-p/m and 3xNp-p/m, respectively. With the exception of naphthalene, the LODs of all the other PAHs were at the low parts-per-billion level and varied from 0.1 ng.mL\textsuperscript{-1} (anthracene and benzo[k]fluoranthene) to 4.7 ng.mL\textsuperscript{-1} (chrysene and benzo[a]pyrene). The relatively high LOD of naphthalene (23.3 ng.mL\textsuperscript{-1}) is due to the peak-to-peak noise of the baseline, which was approximately one order of magnitude higher than the baseline noise of the other PAHs.

Table 3 summarizes the HPLC-AFOMs of the 10 HMW-PAHs obtained under the same parameters as those in Table 2. Appendixes C and D show the chromatographic separations of each HMW-PAH and their calibration curves.
Table 3: Analytical Figures of Merit of MM 302 Da PAHs under EPA Method 610.

| PAH\(^a\)                  | \(\lambda_{\text{exc}}/\lambda_{\text{em}}\)^b (nm) | \(t_R\)^c | LDR\(^d\) (ng mL\(^{-1}\)) | \(R^2\)\(^e\)   | LOD\(^f\) (ng mL\(^{-1}\)) | LOQ\(^g\) (ng mL\(^{-1}\)) |
|----------------------------|--------------------------------------------------|----------|----------------|----------------|----------------|----------------|----------------|
| Dibenzo[a,l]pyrene         | 316/420                                          | 31.8 ± 0.2 | 2.5-100        | 0.996          | 0.8            | 2.5            |
| Naphtho[2,3-e]pyrene       | 320/406                                          | 36.7 ± 0.2 | 17.0-300       | 0.9997         | 5.1            | 17.0           |
| Dibenzo[a,e]pyrene         | 305/395                                          | 38.2 ± 0.5 | 1.6-100        | 0.9991         | 0.5            | 1.6            |
| Naphtho[1,2-k]fluoranthene | 326/440                                          | 39.3 ± 0.7 | 30.0-100       | 0.9962         | 9.0            | 30.0           |
| Benzo[b]perylene           | 406/440                                          | 39.8 ± 0.02 | 1.6-50       | 0.9978         | 0.5            | 1.6            |
| Dibenzo[b,k]fluoranthene   | 312/409                                          | 45.9 ± 0.7 | 0.6-100        | 0.9751         | 0.2            | 0.6            |
| Dibenzo[a,i]pyrene         | 393/433                                          | 67.0 ± 1.2 | 33.0-2000      | 0.9993         | 9.9            | 33.0           |
| Naphtho[2,3-a]pyrene       | 333/462                                          | 71.9 ± 1.2 | 4.4-100        | 0.9934         | 1.3            | 4.4            |
| Naphtho[2,3-k]fluoranthene | 333/455                                          | 80.8 ± 0.6 | 28.0-1000      | 0.9861         | 8.5            | 28.0           |
| Dibenzo[a,h]pyrene         | 311/450                                          | 82.8 ± 0.1 | 1.9-100        | 0.9949         | 0.6            | 1.9            |

\(^a\) Working solutions were prepared by diluting pure standards with acetonitrile.

\(^b\) Maximum excitation (\(\lambda_{\text{exc}}\)) and emission (\(\lambda_{\text{em}}\)) wavelengths used for HPLC analysis.

\(^c\) Retention times.

\(^d\) LDR, linear dynamic range.

\(^e\) R, correlation coefficient.

\(^f\) Limits of detection (LOD) were calculated as 3 \(\times N_{p-p}/m\); where \(N_{p-p}\) is the baseline noise and \(m\) is the slope of the calibration curve.

\(^g\) Limits of quantitation (LOQ) were calculated as 10 \(\times N_{p-p}/m\).

All measurements were done with pure standard solutions in acetonitrile. Similar to EPA-PAHs, the LODs and the LOQs of the studied 302 Da isomers were at the low parts-per-billion concentration levels. As expected, most of these isomers eluted at longer retention times than the EPA-PAHs. The only exception was DBalP (\(t_R = 31.8 ± 0.2\) min), which showed a similar retention time to BaP (30.9 ± 0.2 min) and, therefore, eluted earlier than dibenzo[a,h]anthracene,
benzo[g,h,i]perylene, and indeno[1,2,3-cd]pyrene. A typical chromatogram of a synthetic mixture containing the 15 EPA-PAHs and the 10 PAH isomers with MM 302 Da is shown in Figure 9. Except for BaP/DBaP and N23kF/DaP, all the other PAHs were separated satisfactorily.

**Figure 9**: HPLC chromatogram of a typical synthetic mixture containing the 25 PAHs selected for these studies. Fluorescence detection at the maximum excitation and emission wavelengths was used in all cases. PAH concentrations were as follows: (1) 100 ng mL$^{-1}$ Naphthalene, (2) 25 ng mL$^{-1}$ Acenaphthene, (3) 30 ng mL$^{-1}$ Fluorene, (4) 25 ng mL$^{-1}$ Phenanthrene, (5) 25 ng mL$^{-1}$ Anthracene, (6) 200 ng mL$^{-1}$ Fluoranthene, (7) 25 ng mL$^{-1}$ Pyrene, (8) 25 ng mL$^{-1}$ Benzo[a]anthracene, (9) 50 ng mL$^{-1}$ Chrysene, (10) 100 ng mL$^{-1}$ Benzo[b]fluoranthene, (11) 100 ng mL$^{-1}$ Benzo[k]fluoranthene, (12) 250 ng mL$^{-1}$ BaP, (13) 100 ng mL$^{-1}$ DBaP, (14) 100 ng mL$^{-1}$ Dibenzo[a,h]anthracene, (15) 100 ng mL$^{-1}$ Benzo[g,h,i]perylene, (16) 1500 ng mL$^{-1}$ Indeno[1,2,3-cd]pyrene, (17) 1000 ng mL$^{-1}$ Naphtho[2,3-e]pyrene, (18) 500 ng mL$^{-1}$ Dibenzo[a,e]pyrene, (19) 200 ng mL$^{-1}$ Naphtho[1,2-k]fluoranthene, (20) 250 ng mL$^{-1}$ Benzo[b]pyrene, (21) 100 ng mL$^{-1}$ Dibenzo[b,k]fluoranthene, (22) 2000 ng mL$^{-1}$ Dibenzo[a,i]pyrene, (23) 100 ng mL$^{-1}$ Naphtho[2,3-a]pyrene, (24) 1000 ng mL$^{-1}$ Naphtho[2,3-k]fluoranthene, and (25) 100 ng mL$^{-1}$ Dibenzo[a,h]pyrene.
4.3.2 Laser Excited Time-Resolved Shpol'skii Spectroscopy (LETRSS)

Since the LETRSS analysis of N23kF and DBahP in co-eluted HPLC fractions was already reported from our lab\(^{29}\). Spectral resolution of N23kF and DahP was achieved by freezing the sample at liquid helium temperature (4.2 K). The present studies will focus on BaP and DBalP and their LETRSS analysis at 77 K. Although better spectral resolution and lower limits of detection are usually obtained at 4.2 K, the analysis of samples at liquid nitrogen temperature (77 K) present the advantage of lower analysis costs. Figure 10 shows 77 K WTM s of BaP and DBalP recorded from pure standard solutions in n-octane, an n-alkane known to provide adequate vibrational resolution for both PAHs\(^{32-33}\).

![Figure 10: 77 K WTM s for BaP and DBalP. WTM s were recorded at the maximum excitation wavelength of each PAH.](image)

Each WTM was recorded at the maximum excitation wavelength of the PAH. The delay (\(t_d\)) and gate (\(t_g\)) widths were optimized to collect most of the fluorescence from the PAH and still avoid instrumental noise. The gate steps were optimized to record a sufficient number of data
points within the lifetime decay of each PAH. The resulting WTMss consisted of a total of 25 fluorescence spectra. Each fluorescence spectrum was averaged over 100 laser pulses. Figure 11 compares the 77 K excitation and emission spectra of the two PAHs in n-octane.

![Figure 11: Excitation and Emission Spectra of BaP and DBaP.](image)

The emission spectra correspond to the first fluorescence spectrum of the WTMss. The excitation spectra were obtained by stepping the laser at 0.5 nm intervals and recording the emission at the maximum fluorescence wavelength of each PAH.

Figure 12 shows the fluorescence decays obtained from the WTMss by plotting the fluorescence intensity of each PAH at its maximum wavelength as a function of delay time. Single exponential decays were obtained in both cases with no systematic errors.
Figure 12: Fluorescence Time Decays of BaP and DBaLP. Fluorescence decays were recorded at the maximum excitation and emission wavelength of each PAH. A 10 μm spectrograph slit was used for all measurements. The time delay (td) and the gate delay (tg) were as follows: BaP (td = 6ns and tg = 150ns) and DBaLP (td = 6ns and tg = 240ns).

Table 4 summarizes the 77 K LETRSS AFOMs of BaP and DBaLP in n-octane. All fluorescence measurements were made at the maximum excitation and emission wavelength of each PAH using the delay and gate times previously optimized for WTM collection.

Table 4: 77 K Analytical Figures of Merit of DBaLP and BaP obtained via LETRSS

<table>
<thead>
<tr>
<th>PAH</th>
<th>λ_ex/λ_em</th>
<th>Delay/Gate</th>
<th>LOD</th>
<th>LOQ</th>
<th>LDR</th>
<th>R²</th>
<th>RSD(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBaLP</td>
<td>321.5/419</td>
<td>6/250</td>
<td>0.009</td>
<td>0.031</td>
<td>0.031-6</td>
<td>0.9939</td>
<td>4.52</td>
</tr>
<tr>
<td>BaP</td>
<td>388.5/403</td>
<td>6/150</td>
<td>0.081</td>
<td>0.271</td>
<td>0.271-12</td>
<td>0.9799</td>
<td>2.03</td>
</tr>
</tbody>
</table>

aAll working solutions were prepared in n-octane.
bExcitation and emission wavelengths.
cLimit of detection calculated as 3 x S_B/m; where S_B is the standard deviation of 16 blank measurements and m is the slope of the calibration curve.
dLimit of quantitation as 10 x S_B/m; where S_B is the standard deviation of 16 blank measurements and m is the slope of the calibration curve.
eLinear dynamic range (LDR); lower concentration limit = limit of quantitation.
R² = correlation coefficient.
Relativestandard deviation (RSD) calculated from medium concentrations of linear dynamic ranges.
Calibration curves were built with at least three standard solutions of each PAH. No attempts were made to reach the upper concentration limit of the linear dynamic range (LDR). The lowest linear concentrations of the LDRs correspond to the LOQs. Their values were calculated as $10 \times s_B/m$; where $s_B$ is the standard deviation of 16 blank measurements and $m$ is the slope of the calibration curve. The LODs were calculated as $3 \times s_B/m$. In all cases, the blank signals were measured from frozen octane solutions at the maximum excitation and emission wavelength of each PAH. The LODs for both compounds were at the parts-per-trillion concentration levels. The relative standard deviations (RSD), which are based on triplicate measurements of signal intensities recorded from three frozen aliquots, indicate excellent precision of measurements at the parts per billion concentration levels.

4.3.3 LETRSS Analysis of Binary Mixtures of BaP and DBalP

The possibility to identify BaP and DBalP in co-eluted HPLC fractions was investigated with binary mixtures of the two PAHs. Figure 13 A and B compares the 77 K fluorescence spectrum and lifetime of BaP to those recorded from a 1:1 binary mixture with DBalP.
Figure 13: Comparison of 77 K LETRSS fluorescence spectra and lifetimes recorded from: (A) 10 ng.mL\(^{-1}\) pure standard of BaP recorded at the maximum excitation and emission wavelength of BaP \((λ_{exc}/λ_{em} = 388.5 \text{ nm} / 403 \text{ nm})\); (B) 1:1 synthetic mixture of BaP and DBaP recorded at the maximum excitation and emission wavelength of BaP \((λ_{exc}/λ_{em} = 388.5 \text{ nm} / 403 \text{ nm})\). All spectra were recorded from octane solutions with a 10 mm spectrograph slit. Delay and gate times \((t_d/t_g)\) were as follows: (A) and (B) \(t_d/t_g = 6 \text{ ns} / 150 \text{ ns}\).

Both spectra and lifetimes were recorded in n-octane at the maximum excitation and emission wavelengths of BaP \((λ_{exc}/λ_{em} = 388.5 \text{ nm} / 403 \text{ nm})\). By selecting the appropriate delay \((t_d = 6 \text{ ns})\) and gate \((t_d = 150 \text{ ns})\), the spectral contribution of DBaP is completely removed from the fluorescence spectrum of the mixture. The single exponential time decays obtained from both solutions indicate a single fluorescence emitter at the maximum wavelength of BaP \((λ_{em} = 403 \text{ nm})\). The statistical equivalence of the fluorescence lifetimes \((P = 95\%; n_1 = n_2 = 3)\) recorded from both solutions confirms the lack of spectral overlapping from DBaP at 403 nm. Figure 14B, D,
and E show a similar outcome for the detection of DBalP in the presence of BaP, even when DBalP is present at a 10 x lower concentration than BaP.

**Figure 14:** Comparison of 77 K LETRSS fluorescence spectra and lifetimes recorded from: (B) 10 ng.mL⁻¹ pure standard of DBalP recorded at the maximum excitation and emission wavelength of DBalP (λ<sub>exc/lem</sub> = 321.5 nm / 419 nm); (D) 1:1 synthetic mixture of BaP and DBalP recorded at the maximum excitation and emission wavelength of DBalP (λ<sub>exc/lem</sub> =321.5 nm / 419 nm); (E) 10:1 synthetic mixture of BaP and DBalP recorded at the maximum excitation and emission wavelength of DBalP (λ<sub>exc/lem</sub> =321.5 nm / 419 nm). All spectra were recorded from octane solutions with a 10 mm spectrograph slit. Delay and gate times (t<sub>d</sub>/t<sub>g</sub>) were as follows: (B), (D) and (E) t<sub>d</sub>/t<sub>g</sub> = 6 ns / 240 ns.

### 4.3.4 HPLC-LETRSS Analysis of Tobacco Samples

Analyte recoveries of the sample preparation procedure for HPLC analysis was investigated by comparing the chromatographic responses of PAH standards directly injected into the HPLC system to those from PAH standards submitted to the entire procedure. Table 5 shows the obtained results.
Table 5: Analyte Recoveries of Sample Procedure Prior to HPLC Analysis

<table>
<thead>
<tr>
<th>PAH</th>
<th>$A_0$ ($\mu$V*s)$^a$</th>
<th>$A_1$ ($\mu$V*s)$^b$</th>
<th>Recovery (%)$^c$</th>
<th>t-test$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP</td>
<td>55937 ± 417</td>
<td>57755 ± 2179</td>
<td>103 ± 4</td>
<td>1.29</td>
</tr>
<tr>
<td>DBalP</td>
<td>147383 ± 1607</td>
<td>150878 ± 996</td>
<td>102 ± 1.0</td>
<td>3.46</td>
</tr>
</tbody>
</table>

$^a$A$_0$ = Average chromatographic areas of PAH standards directly injected into the HPLC system. A$_0$ is based on three sample injections of the same analyte concentration. Injection volume = 20 µL.

$^b$A$_1$ = Average chromatographic areas of PAH standards submitted to the entire sample preparation procedure prior to HPLC analysis. A$_1$ is based on three sample injections of the same analyte concentration. Injection volume = 20 µL.

$^c$Recoveries were calculated with the formula 100*($A_1 / A_0$).

$^d$t-test comparing experimental recoveries to 100%; t critical ($\alpha = 0.05$; N = 3) = 4.3.

Since statistical equivalence to 100% was achieved in both cases ($\alpha = 0.05$; N = 3), PAH loss during sample clean up and preconcentration procedure of tobacco extracts was negligible. Possible PAH losses during the LETRSS analysis of HPLC fractions were calculated via the multiple standard additions (MSA) method. Standard additions were made to chromatographic fractions collected from HPLC injections of pure standard solutions. The obtained results are shown in Table 6. At the 95% confidence level, the only PAH to show a recovery lower than 100% was BaP (89 ± 2.8%).

Table 6: Analyte Recoveries for LETRSS Analysis of HPLC Fractions.

<table>
<thead>
<tr>
<th>PAH</th>
<th>Recovery (%)$^a$</th>
<th>t-test$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP</td>
<td>89 ± 2.8</td>
<td>6.35</td>
</tr>
<tr>
<td>DBalP</td>
<td>103 ± 3.5</td>
<td>1.48</td>
</tr>
</tbody>
</table>

$^a$Recoveries were calculated with the formula 100*($M_1 / M_0$); where $M_1$ is the mass of PAH in the HPLC fraction obtained via MSA and $M_0$ is the theoretical mass of PAH in the HPLC fraction assuming no analyte loss; i.e., 100% recovery. $M_1$ values were based on five data points in the calibration graphs. Each data point was the average of three signal measurements. All the signals plotted in the graph were subtracted from blank signals recorded from n-octane at the maximum emission wavelength of each PAH.

$^b$t-test comparing experimental recoveries to 100%; t critical ($\alpha = 0.05$; N = 3) = 4.3.
Figure 15 shows the 77K fluorescence spectra and fluorescence lifetimes of an HPLC fraction of tobacco extract recorded under optimum LETRSS parameters for BaP determination. The statistical equivalence ($\alpha = 0.05; N_1 = N_2 = 3$) observed in the fluorescence lifetimes of BaP confirms its peak assignment at 403 nm. The single exponential decay recorded from the HPLC fraction at 403 nm rules out possible spectral interference at the target wavelength.

<table>
<thead>
<tr>
<th>Target Compound</th>
<th>$\tau_{HPLC}$</th>
<th>$\tau_{std}$</th>
<th>$T_{exp}$</th>
<th>$T_{crit}$</th>
<th>Statistically Equivalent?</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP</td>
<td>41.22 ± 1.29</td>
<td>42.15 ± 2.51</td>
<td>0.782</td>
<td>3.182</td>
<td>Y</td>
</tr>
</tbody>
</table>

**Figure 15**: 77K fluorescence spectrum and fluorescence lifetime of an HPLC fraction of tobacco extract recorded under optimum excitation ($\lambda_{exc} = 388.5$ nm) and emission ($\lambda_{em} = 403$ nm) wavelengths, and delay ($t_d = 6$ ns) and gate ($t_g = 150$ ns) times of the LETRSS determination of BaP. $\tau_{HPLC}$ = fluorescence lifetime of HPLC fraction; $\tau_{std}$ = fluorescence lifetime of BaP; $T_{crit}$ = $t$ critical ($\alpha = 0.05; N = 3$) and $T_{exp}$ = $t$ experimental ($\alpha = 0.05; N = 3$).

Figure 16 shows the calibration plot obtained from three sample extracts. All the signals plotted in the graph were subtracted from blank signals recorded from n-octane submitted to the entire extraction procedure. The three data points plotted in the graph represent the average signals of three determinations of the same aliquot recorded from original sample (zero standard addition) and from sample aliquots spiked with increasing volumes of a BaP standard. The mass
of BaP per gram of tobacco was 68.94 ± 10.01 ng/g. This mass of BaP was calculated by taking into consideration the recovery of the method (89 %) and all the dilutions made in the entire HPLC-LETRSS procedure described in 4.2.7. Step by step calculations are provided in appendix E.

Figure 16: Calibration plot for the MSA determination of BaP in an HPLC fraction of a tobacco Extract.

While the presence of DBaLp was not detected in the tobacco extract, the feasibility of identifying it at the parts-per-billion concentration level was investigated by spiking a tobacco sample extract with a 2-ppb pure analyte standard solution. The obtained results are shown in Figure 17. Figure 18A compares the LETRSS background of octane to the typical LETRSS background of an HPLC fraction. Both spectra were recorded at the maximum excitation and emission wavelengths of DBaLp using its optimum delay and gate (td/tg = 6 ns / 240 ns). As expected, the background signal of the tobacco extract was higher than the background of octane.
Based on repetitive measurements of numerous HPLC fractions and octane aliquots, the difference between their average signals at the maximum fluorescence wavelength of DBalP is approximately 25,000 counts. Figure 18B compares the spectrum of the HPLC fraction to the LETRSS spectrum of a 1.5 ppb standard solution of DBalP in octane. Although the background of the HPLC fraction is higher than the background signal of octane, the HPLC-LETRSS determination of DBalP would still be possible at the parts per billion level. Detection of lower concentrations of DBalP in tobacco samples would require a more concentrated extract, which could be easily accomplished by increasing the mass of the tobacco sample.

<table>
<thead>
<tr>
<th>HPLC fraction</th>
<th>$\tau_{\text{HPLC}}$</th>
<th>$\tau_{\text{std}}$</th>
<th>$\tau_{\text{exp}}$</th>
<th>$\tau_{\text{crit}}$</th>
<th>Statistically Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Spike</td>
<td>-</td>
<td>74.70 ± 3.21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spiked with DBalP</td>
<td>71.3 ± 2.15</td>
<td>74.70 ± 3.21</td>
<td>1.52</td>
<td>2.776</td>
<td>Y</td>
</tr>
</tbody>
</table>

**Figure 17**: LETRSS analysis of an HPLC fraction of a tobacco extract. (A) Fluorescence spectrum of the un-spiked HPLC fraction; (B) Fluorescence spectrum of the HPLC fraction spiked with DBalP; and (C) Fluorescence decay of the HPLC fraction spiked with DBalP. Fluorescence spectra and fluorescence decays were recorded under optimum instrumental parameters for the determination of DBalP. $T_{\text{crit}} = t$ critical ($\alpha = 0.05; N = 3$) and $T_{\text{exp}} = t$ experimental ($\alpha = 0.05; N = 3$).
Figure 18: (A) 77K LETRSS spectra of an HPLC fraction of tobacco extract and octane; (B) 77K LETRSS spectra of 1.5 ppb DBaP in octane and of an HPLC fraction of tobacco extract. All spectra were recorded at \( \lambda_{\text{exc}}/\lambda_{\text{em}} = 321.5 \text{ nm} / 419 \text{ nm} \) and \( t_d/t_g = 6 \text{ ns} / 240 \text{ ns} \).

4.4 Conclusions and Future Work

The analysis of PAHs in tobacco related products is of extreme importance due to the toxicological properties of PAHs. For many years the most used methodology for the analysis of PAHs in tobacco related products has been based on HPLC and GC methods. Unfortunately, the presence of PAHs with similar retention times challenges the selectivity of chromatographic methodology. Relevant to this dissertation is the case of B[\( \alpha \)]P and DB[\( a,l \)]P. B[\( \alpha \)]P is the most toxic PAH in the EPA priority pollutants list and DB[\( a,l \)]P is approximately 100 x more toxic than B[\( \alpha \)]P. Although B[\( \alpha \)]P has been found in a variety of tobacco products and/or cigarette smoke, there are no reports on the presence of DB[\( a,l \)]P via HPLC method 610. This is rather intriguing, particularly if one considers the incomplete combustion of tobacco during the smoking process. In most cases, the identification of B[\( \alpha \)]P has been based on HPLC retention times obtained HPLC with no further confirmation via GC.

Our studies show that B[\( \alpha \)]P and DB[\( a,l \)]P co-elute under HPLC conditions. Therefore, their unambiguous identification requires further analysis of HPLC fractions via an alternative
analytical method. One possibility is to perform the analysis via GC-MS. Although this technique would be able to differentiate B[a]P from DB[a,l]P, it would not be able to distinguish DB[a,l]P from other PAH isomers with the same molecular mass (302 Da), similar GC retention times, and virtually identical mass fragmentation patterns.

The high-resolution fluorescence method we propose provides their unambiguous determination on the basis of spectral and lifetime information with a two-step experimental procedure consisting of the evaporation of the chromatographic fraction followed by the dissolution of the residue with microliters of n-octane. Low temperature analysis is easily performed with the aid of a cryogenic fiber optic probe that freezes the sample in a matter of seconds. Limits of detection at the parts-per-billion concentration level (ng.mL⁻¹) are obtained with no need of further separation of HPLC fractions.

Although we did not find DB[a,l]P in the one tobacco sample tested in this dissertation, we demonstrated that it can be unequivocally identified at very low concentration levels with the LETRSS method. Future studies will apply LETRSS to a variety of commercially available tobacco products as well as cigarette filters and cigarette smoke to provide an accurate answer to the pertinent question raised in this dissertation.

4.5 References


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APPENDIX A: CHROMATOGRAPHIC SEPARATIONS OF 15 EPA-PAHs USING FLUORESCENCE DETECTION
Figure 19: HPLC-FL chromatogram of 100 ng mL$^{-1}$ naphthalene pure standard prepared with acetonitrile and using excitation (240 nm) and emission (330 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 
Figure 20: HPLC-FL chromatogram of 25 ng mL⁻¹ acenaphthene pure standard prepared with acetonitrile and using excitation (240 nm) and emission (330 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹.
Figure 21: HPLC-FL chromatogram of 30 ng mL$^{-1}$ acenaphthene pure standard prepared with acetonitrile and using excitation (252 nm) and emission (320 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 
Figure 22: HPLC-FL chromatogram of 25 ng mL⁻¹ phenanthrene pure standard prepared with acetonitrile and using excitation (252 nm) and emission (370 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹.
Figure 23: HPLC-FL chromatogram of 25 ng mL\(^{-1}\) anthracene pure standard prepared with acetonitrile and using excitation (252 nm) and emission (402 nm). Sample volume = 20 µL. Flow rate = 2 mL/min\(^{-1}\).
Figure 24: HPLC-FL chromatogram of 50 ng mL\textsuperscript{-1} anthracene pure standard prepared with acetonitrile and using excitation (284 nm) and emission (464 nm). Sample volume = 20 µL. Flow rate = 2 mL/min\textsuperscript{-1}.
**Figure 25:** HPLC-FL chromatogram of 25 ng mL$^{-1}$ pyrene pure standard prepared with acetonitrile and using excitation (238 nm) and emission (398 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 
Figure 26: HPLC-FL chromatogram of 25 ng mL\(^{-1}\) benzo[a]anthracene pure standard prepared with acetonitrile and using excitation (278 nm) and emission (395 nm). Sample volume = 20 µL. Flow rate = 2 mL/min\(^{-1}\).
Figure 27: HPLC-FL chromatogram of 50 ng mL⁻¹ chrysene pure standard prepared with acetonitrile and using excitation (278 nm) and emission (395 nm). Sample volume = 20 μL. Flow rate = 2 mL/min⁻¹.
**Figure 28:** HPLC-FL chromatogram of 100 ng mL$^{-1}$ benzo[b]fluoranthene pure standard prepared with acetonitrile and using excitation (268 nm) and emission (398 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 
Figure 29: HPLC-FL chromatogram of 100 ng mL$^{-1}$ benzo[k]fluoranthene pure standard prepared with acetonitrile and using excitation (268 nm) and emission (398 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$.
Figure 30: HPLC-FL chromatogram of 500 ng mL⁻¹ benzo[a]pyrene pure standard prepared with acetonitrile and using excitation (380 nm) and emission (405 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹.
Figure 31: HPLC-FL chromatogram of 100 ng mL$^{-1}$ dibenzo[a,h]anthracene pure standard prepared with acetonitrile and using excitation (304 nm) and emission (421 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 
Figure 32: HPLC-FL chromatogram of 100 ng mL\(^{-1}\) benzo[g,h,i]perylene pure standard prepared with acetonitrile and using excitation (304 nm) and emission (421 nm). Sample volume = 20 µL. Flow rate = 2 mL/min\(^{-1}\).
Figure 33: HPLC-FL chromatogram of 100 ng mL$^{-1}$ indeno[1,2,3-cd]pyrene pure standard prepared with acetonitrile and using excitation (300 nm) and emission (466 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 
Figure 34: HPLC-FL detection calibration curve of naphthalene. Measurements were made using excitation (240 nm) and emission (330 nm) wavelengths.
Figure 35: HPLC-FL detection calibration curve of acenaphthene. Measurements were made using excitation (234 nm) and emission (320 nm) wavelengths.

\[ y = 38.213x + 30.635 \]

\[ R^2 = 0.9967 \]
Figure 36: HPLC-FL detection calibration curve of fluorene. Measurements were made using excitation (252 nm) and emission (320 nm) wavelengths.

The equation for the curve is:

\[ y = 32.217x + 33.072 \]

\[ R^2 = 0.9973 \]
Figure 37: HPLC-FL detection calibration curve of phenanthrene. Measurements were made using excitation (252 nm) and emission (370 nm) wavelengths.

\[ y = 16.401x + 1.918 \]

\[ R^2 = 1 \]
Figure 38: HPLC-FL detection calibration curve of anthracene. Measurements were made using excitation (252 nm) and emission (402 nm) wavelengths.
Figure 39: HPLC-FL detection calibration curve of fluoranthene. Measurements were made using excitation (284 nm) and emission (464 nm) wavelengths.

\[ y = 2.8002x + 1.7958 \]
\[ R^2 = 0.9973 \]
Figure 40: HPLC-FL detection calibration curve of pyrene. Measurements were made using excitation (238 nm) and emission (398 nm) wavelengths.

\[ y = 16.098x + 1.1175 \]
\[ R^2 = 1 \]
Figure 41: HPLC-FL detection calibration curve of benzo[a]anthracene. Measurements were made using excitation (278 nm) and emission (395 nm) wavelengths.

\[ y = 13.52x + 0.4875 \]

\[ R^2 = 0.9994 \]
Figure 42: HPLC-FL detection calibration curve of chrysene. Measurements were made using excitation (278 nm) and emission (395 nm) wavelengths.
Figure 43: HPLC-FL detection calibration curve of benzo[b]fluoranthene. Measurements were made using excitation (268 nm) and emission (398 nm) wavelengths.

\[
y = 2.4709x + 2.926 \\
R^2 = 0.9994
\]
Figure 44: HPLC-FL detection calibration curve of benzo[k]fluoranthene. Measurements were made using excitation (268 nm) and emission (398 nm) wavelengths.
Figure 45: HPLC-FL detection calibration curve of benzo[a]pyrene. Measurements were made using excitation (380 nm) and emission (405 nm) wavelengths.

\[ y = 1.8701x + 14.436 \]
\[ R^2 = 0.9898 \]
Figure 46: HPLC-FL detection calibration curve of dibenzo[a,h]anthracene. Measurements were made using excitation (304 nm) and emission (421 nm) wavelengths.
Figure 47: HPLC-FL detection calibration curve of benzo[g,h,i]perylene. Measurements were made using excitation (304 nm) and emission (421 nm) wavelengths.

\[ y = 1.0956x + 2.19 \]

\[ R^2 = 0.9926 \]
Figure 48: HPLC-FL detection calibration curve of indeno[1,2,3-cd]pyrene. Measurements were made using excitation (300 nm) and emission (466 nm) wavelengths.

\[ y = 0.2292x + 0.3867 \]
\[ R^2 = 0.9991 \]
APPENDIX C: CHROMATOGRAPHIC SEPARATIONS OF HMW-PAHs USING FLUORESCENCE DETECTION
Figure 49: HPLC-FL chromatogram of 100 ng mL⁻¹ dibenzo[a,l]pyrene pure standard prepared with acetonitrile and using excitation (316 nm) and emission (420 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹.
Figure 50: HPLC-FL chromatogram of 300 ng mL⁻¹ naphtho[2,3-e]pyrene pure standard prepared with acetonitrile and using excitation (320 nm) and emission (406 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹.
**Figure 51:** HPLC-FL chromatogram of 100 ng mL$^{-1}$ dibenzo[a,e]pyrene pure standard prepared with acetonitrile and using excitation (305 nm) and emission (495 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 
Figure 52: HPLC-FL chromatogram of 100 ng mL$^{-1}$ naphtho[1,2-k]fluoranthene pure standard prepared with acetonitrile and using excitation (326 nm) and emission (440 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 

![HPLC-FL chromatogram of 100 ng mL$^{-1}$ naphtho[1,2-k]fluoranthene pure standard prepared with acetonitrile and using excitation (326 nm) and emission (440 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$.

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93
Figure 53: HPLC-FL chromatogram of 50 ng mL$^{-1}$ benzo[b]perylene pure standard prepared with acetonitrile and using excitation (406 nm) and emission (440 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 
Figure 54: HPLC-FL chromatogram of 100 ng mL$^{-1}$ dibenzo[b,k]fluoranthene pure standard prepared with acetonitrile and using excitation (412 nm) and emission (409 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 
Figure 55: HPLC-FL chromatogram of 2000 ng mL\(^{-1}\) dibenzo[a,i]pyrene pure standard prepared with acetonitrile and using excitation (393 nm) and emission (433 nm). Sample volume = 20 µL. Flow rate = 2 mL/min\(^{-1}\).
Figure 56: HPLC-FL chromatogram of 100 ng mL⁻¹ naphtho[2,3-a]pyrene pure standard prepared with acetonitrile and using excitation (333 nm) and emission (462 nm). Sample volume = 20 µL. Flow rate = 2 mL/min⁻¹.
Figure 57: HPLC-FL chromatogram of 1000 ng mL\(^{-1}\) naphtho[2,3-k]fluoranthene pure standard prepared with acetonitrile and using excitation (333 nm) and emission (455 nm). Sample volume = 20 µL. Flow rate = 2 mL/min\(^{-1}\).
Figure 58: HPLC-FL chromatogram of 100 ng mL$^{-1}$ dibenzo[a,h]pyrene pure standard prepared with acetonitrile and using excitation (311 nm) and emission (450 nm). Sample volume = 20 µL. Flow rate = 2 mL/min$^{-1}$. 
APPENDIX D: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FLUORESCENCE (HPLC-FL) DETECTION CALIBRATION CURVES OF HMW-PAHs
Figure 59: HPLC-FL detection calibration curve of dibenzo[a,l]pyrene. Measurements were made using excitation (316 nm) and emission (420 nm) wavelengths.

\[ y = 1.7875x + 0.98 \]

\[ R^2 = 0.9960 \]
**Figure 60:** HPLC-FL detection calibration curve of naphtho[2,3-e]pyrene. Measurements were made using excitation (320 nm) and emission (406 nm) wavelengths.
Figure 61: HPLC-FL detection calibration curve of dibenzo[a,e]pyrene. Measurements were made using excitation (305 nm) and emission (395 nm) wavelengths.
Figure 62: HPLC-FL detection calibration curve of naphtho[1,2-k]fluoranthene. Measurements were made using excitation (326 nm) and emission (440 nm) wavelengths.

\[
y = 4.2536x + 1.58 \\
R^2 = 0.9962
\]
Figure 63: HPLC-FL detection calibration curve of benzo[b]perylene. Measurements were made using excitation (406 nm) and emission (440 nm) wavelengths.

\[ y = 3.1196x + 0.2527 \]

\[ R^2 = 0.9978 \]
Figure 64: HPLC-FL detection calibration curve of dibenzo[b,k]fluoranthene. Measurements were made using excitation (312 nm) and emission (409 nm) wavelengths.

\[ y = 6.7928x + 39.836 \]

\[ R^2 = 0.9751 \]
Figure 65: HPLC-FL detection calibration curve of dibenzo[a,i]pyrene. Measurements were made using excitation (393 nm) and emission (433 nm) wavelengths.

\[ y = 0.2503x + 6.746 \]

\[ R^2 = 0.9993 \]
Figure 66: HPLC-FL detection calibration curve of naphtho[2,3-a]pyrene. Measurements were made using excitation (333 nm) and emission (462 nm) wavelengths.
Figure 67: HPLC-FL detection calibration curve of naphtho[2,3-k]fluoranthene. Measurements were made using excitation (333 nm) and emission (455 nm) wavelengths.

\[
y = 1.4509x + 59.886 \\
R^2 = 0.9861
\]
Figure 68: HPLC-FL detection calibration curve of dibenzo[a,h]pyrene. Measurements were made using excitation (311 nm) and emission (450 nm) wavelengths.

\[ y = 2.7081x + 7.062 \]
\[ R^2 = 0.9949 \]
APPENDIX E: CALCULATIONS RELATED TO THE MASS OF BaP
The mass of BaP found by the MSA procedure was 0.09 ng ± 0.01 ng. The standard deviation was calculated according to the equation:

$$s_c = \frac{s_{y/x}}{b} \left[ \frac{1}{N} + \frac{\bar{y}}{b^2 \sum_{i}(x_i - \bar{x})^2} \right]^{1/2}$$

Where $s_c$ is the standard deviation of the concentration, $s_{y/x}$ is the standard deviation of the regression, $m$ is the slope from the MSA linear fit, $\bar{y}$ and $\bar{x}$ are the averages of the $y$ and $x$ values, respectively, from the MSA graph, and $x_i$ is the $i^{th}$ x value. Note: The mass of BaP = 0.09 ng ± 0.01 ng is equivalent to 68.94 ng ± 10.01 ng of BaP per gram of tobacco sample. This value was calculated by taking into consideration the recovery of the method (89 %) and all the dilutions made in the entire HPLC-LETRSS procedure described in section 2.7. Step-by-step calculations are as follows:

Step 1: 5 mL aliquot from 50 mL tobacco extract was used for HPLC analysis.

\[
\text{Mass of BaP in 5 mL aliquot} = \text{Total mass of BaP in tobacco sample} \times \frac{5 \text{ mL}}{50 \text{ mL}}
\]

Step 2: 5 mL aliquot was evaporated to dryness and re-constituted in 1 mL of acetonitrile.

Step 3: 20 mL of the 1 mL solution prepared in step 2 was injected in the HPLC system.

\[
\text{Mass of BaP injected in the HPLC system} = 20 \text{ mL} / 1000 \text{ mL} \times \{\text{Total mass of BaP in tobacco sample} \times \frac{5 \text{ mL}}{50 \text{ mL}}\}
\]

Step 4: HPLC fraction is evaporated to dryness and reconstituted in 1 mL of octane.

Step 5: 90 mL aliquot of the solution in step 4 was used for the MSA procedure:

\[
90 \text{ mL} / 1000 \text{ mL} \times 20 \text{ mL} / 1000 \text{ mL} \times \{\text{Total mass of BaP in tobacco sample} \times \frac{5 \text{ mL}}{50 \text{ mL}}\}.
\]

Therefore: 0.09 ng ± 0.01 ng = 90 mL / 1000 mL x 20 mL / 1000 mL x {Total
mass of BaP in tobacco sample $\times [5 \text{ mL} / 50 \text{ mL}]$. Total mass of BaP in tobacco sample $= 503 \text{ ng} \pm 53.3 \text{ ng}$. 

Taking into consideration the recovery of the HPLC-LETRSS procedure: Total mass of BaP in tobacco sample $= 503 \text{ ng} \pm 53.3 \text{ ng} / 0.89 = 558 \text{ ng} \pm 81 \text{ ng}$. Since the total mass of tobacco was $8.094 \text{ g}$, the mass of BaP per gram of tobacco was: $558 \text{ ng} \pm 81 \text{ ng} / 8.094 \text{ g} = 68.94 \pm 10.01 \text{ ng}$. 


APPENDIX F: COPYRIGHT PERMISSIONS
On the co-elution of Benzo[a]pyrene and Dibenzo[a,l]pyrene in chromatographic fractions and their unambiguous determination in tobacco extracts via Laser-Excited Time Resolved Shpol'skii Spectroscopy


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