Novel Improvements On The Analytical Chemistry Of Polycyclic Aromatic Hydrocarbons And Their Metabolites

2010

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NOVEL IMPROVEMENTS ON THE ANALYTICAL CHEMISTRY OF POLYCYCLIC AROMATIC HYDROCARBONS AND THEIR METABOLITES

by

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A dissertation submitted in partial fulfillment required for the degree of Doctor of Philosophy in the Department of Chemistry in the College of Sciences at the University of Central Florida Orlando, Florida

Summer Term
2010

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAH) are important environmental pollutants originating from a wide variety of natural and anthropogenic sources. Because many of them are highly suspect as etiological agents in human cancer, chemical analysis of PAH is of great environmental and toxicological importance. Current methodology for PAH follows the classical pattern of sample preparation and chromatographic analysis. Sample preparation pre-concentrates PAH, simplifies matrix composition, and facilitates analytical resolution in the chromatographic column. Among the several approaches that exist to pre-concentrate PAH from water samples, the Environmental Protection Agency (EPA) recommends the use of solid-phase extraction (SPE). High-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) are the basis for standard PAH identification and determination. Ultraviolet (UV) absorption and room temperature fluorescence detection are both widely used in HPLC, but the specificity of these detectors is modest. Since PAH identification is solely based on retention times, unambiguous PAH identification requires complete chromatographic resolution of sample components. When HPLC is applied to “unfamiliar” samples, the EPA recommends that a supporting analytical technique such as GC-MS be applied to verify compound identification and to check peak-purity HPLC fractions. Independent of the volume of extracted water, the approximate time required to separate and determine the sixteen “priority pollutants” (EPA-PAH) via HPLC is approximately 60min. If additional GC-MS analysis is required for unambiguous PAH determination, the total analysis time will reach 2-3 hours per sample. If the concentrations of target species are found to lie outside the detector’s response range, the sample must be diluted and the process repeated. These are important considerations
when routine analysis of numerous samples is contemplated.

Parent PAH are relatively inert and need metabolic activation to express their carcinogenicity. By virtue of the rich heterogeneous distribution of metabolic products they produce, PAH provide a full spectrum of the complexity associated with understanding the initial phase of carcinogenesis. PAH metabolites include a variety of products such as expoxides, hydroxyl aromatics, quinines, dihydriodols, dioepoxides, tetrols and water soluble conjugates. During the past decades tremendous efforts have been made to develop bio-analytical techniques that possess the selectivity and sensitivity for the problem at hand. Depending on the complexity of the sample and the relative concentrations of the targeted metabolites, a combination of sample preparation techniques is often necessary to reach the limits of detection of the instrumental method of analysis. The numerous preparation steps open ample opportunity to metabolite loss and collection of inaccurate data. Separation of metabolites has been accomplished via HPLC, capillary electrophoresis (CE) and GC-MS. Unfortunately, the existence of chemically related metabolic products with virtually identical fragmentation patterns often challenges the specificity of these techniques.

This dissertation presents significant improvements in various fronts. Its first original component – which we have named solid-phase nano-extraction (SPNE) - deals with the use of gold nanoparticles (Au NPs) as extracting material for PAH. The advantages of SPNE are demonstrated for the analysis of PAH in water samples via both HPLC\(^1\) and Laser-Excited Time-Resolved Shpol’skii Spectroscopy (LETRSS).\(^2\) The same concept is then extended to the analysis of monohydroxy-PAH in urine samples via SPE- HPLC\(^3\) and In-Capillary SPNE-CE.\(^4\) The second original component of this dissertation describes the application of Shpol’skii Spectroscopy to the analysis of polar PAH metabolites. The outstanding selectivity and
sensitivity for the direct analysis of PAH at trace concentration levels has made Shpol’skii spectroscopy a leading technique in environmental analysis. Unfortunately, the requirement of a specific guest-host combination - typically a non-polar PAH dissolved in an n-alkane - has hindered its widespread application to the field of analytical chemistry. This dissertation takes the first steps in removing this limitation demonstrating its feasibility for the analysis of polar benzo[a]pyrene metabolites in alcohol matrixes.
ACKNOWLEDGMENTS

Foremost, I would like to express my sincere gratitude to my advisor Prof. Andres D. Campiglia for his continuous and enormous support for my Ph.D. study and research with his patience, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D. study. I would have been lost without him.

Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Kevin D. Belfield Dr. Robert Igarashi, Dr. Shengli Zou, Dr. Xun Gong. It’s such a privilege to have them on my dissertation committee.

I would also like to thank for the valuable help from my lab mates, Dr. Shenjiang Yu, Dr. Matthew Rex, Korina Calimag, Krishnaveni Appalaneni, Gaston Knobel, Walter Brent Wilson and Anthony Moore. The friendship, laughs, and yummy food in the lab will never be forgotten.

My deepest gratitude goes to my wife, Pin Wang, for her unflinching love and support throughout my life; this dissertation is simply impossible without her. Last but not least, I would like to thank my parents, for giving me life in the first place, unconditional support, and encouragement to pursue my interests.
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CHAPTER 1  POLYCYCLIC AROMATIC HYDROCARBONS AND THEIR METABOLIC PRODUCTS

1.1 Environmental and Toxicological Relevance of Polycyclic Aromatic Hydrocarbons

Polycyclic Aromatic hydrocarbons (PAH) are important environmental pollutants originating from a wide variety of natural and anthropogenic sources. PAH are generally formed during incomplete combustion of organic matter containing carbon and hydrogen.\textsuperscript{7-17} Due to the carcinogenic nature of some PAH, their chemical analysis is of great environmental and toxicological importance. Among the hundreds of PAH present in the environment, the EPA lists the following sixteen as "Consent Decree" priority pollutants: benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, indeno[1,2,3-cd]pyrene, naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, and benzo[g,h,i]perylene. Their molecular structures are shown in Figure 1.1. According to the EPA, these PAH should be routinely monitored to avoid human exposure to contaminated sites.

Human exposure to PAH derives from a variety of sources such as air pollution from automobile, diesel and industrial or incinerator emissions, which may modulate a spectrum of their other combustion, dietary and occupational sources. The primary source of PAH exposure in humans is through food consumption.\textsuperscript{18,19} Cereal and vegetables are the major dietary sources of PAH, except when there is a high consumption of meat cooked over an open flame.\textsuperscript{20} More than 150 PAH have been found in tobacco smoke,\textsuperscript{21} which also contributes significantly to exposure to PAH\textsuperscript{22} and to adverse health effects.\textsuperscript{23}
Figure 1.1 Molecular structures of 16 EPA PAH.
A less pervasive source of air pollution leading to PAH exposure involves biomass burning from forest fires and peat bogs resulting from incidental incineration, or from deliberate forest cleaning strategies that expend beyond planned boundaries. Wood combustion as an energy source for heating and/or cooking also generates a source of indoor PAH pollution leading to health problems. Occupational exposures may result from daily contact with intensive combustion processes such as iron and steel production, coke production, waste incinerators, transportation maintenance, mining exhaust exposure and aluminum smelters, or from tobacco smoke in commercial venues such as bars, restaurants and nightclubs. More intensive PAH exposures in occupational environments – relative to the general population – have resulted in reports of lung, genitourinary and skin cancers.

1.2 Solid-Phase Extraction of PAH

In addition to inhalation and dermal absorption in occupational environments, a primary route of human exposure to PAH is the ingestion of contaminated water. Current methodologies for the analysis of EPA-PAH in public water supplies follows the classical pattern of sample preparation and chromatographic analysis. Sample preparation pre-concentrates PAH, simplifies matrix composition, and facilitates analytical resolution in the chromatographic column. Among the several approaches that exist to pre-concentrate PAH from water samples – which include liquid-liquid extraction, solid-phase micro-extraction, open tubular trap, stir bar sorptive extraction and enzyme-linked immunosorbent assays (ELISA) – EPA recommends the use of solid-phase extraction (SPE). Typically, 1L of water is processed through the extraction membrane in approximately 1 h. This volume of water is recommended to reach detectable concentrations by chromatographic techniques, namely high performance liquid
chromatography (HPLC) and gas chromatography – mass spectroscopy (GC-MS).

SPE of PAH is usually carried out with the aid of non-polar sorbents such as octadecyl-
silica particles. The effectiveness of octadecyl-silica sorbents is based on the same sorption-
desorption phenomena as in reversed-phase liquid chromatography. The main mechanism of
PAH isolation is a non-polar interaction, i.e. Van der Waals forces. PAH will pass into the solid
surface, and given time, the equilibrium will be established between the two phases. The
equilibrium for each PAH is described by its partition coefficient, which is simply the ratio of
PAH concentrations in the two phases. The driving force for the partition mechanism results
from the difference in the chemical potential of the PAH in the solid (sorbent) and the liquid
phase (aqueous sample). The chemical potential equation of any given PAH (PAH$_i$) in any given
phase (p) is given by

$$\mu_i^p = (\mu^0)_i^p + RT \ln C_i$$  \hspace{1cm} (1.1)

where $\mu_i^p$ is the chemical potential of PAH$_i$ in phase “p”, $(\mu^0)_i^p$ is the standard chemical potential
of PAH$_i$ in phase “p”, and $C_i$ is the PAH concentration in phase “p”. When equilibrium is
established, the chemical potential of PAH$_i$ in the sorbent (s) and aqueous (a) phases reaches the
same value, i.e.:

$$\mu_i^s = \mu_i^a$$  \hspace{1cm} (1.2)

The concentration of PAH$_i$ in each phase depends on its partition coefficient. The larger the
partition coefficient the better the retention of PAH$_i$ will be in the solid phase. The partition
coefficient (K) can be represented by

$$\frac{C_i^s}{C_i^a} = \exp\left[ -\frac{\Delta \mu^0_i}{RT} \right]$$  \hspace{1cm} (1.3)
\[ K = \frac{C_i^s}{C_i^a} \] (1.4)

SPE materials are packed in two formats, discs and cartridges. A SPE cartridge consists of a small column (generally an open syringe barrel) containing a sorbent with an average particle size of about 40 packed between plastic frits. The sorbent bed occupies about one-third of the syringe barrel volume. The remaining volume is used as sample reservoir. SPE discs are particle-loaded membranes in the form of flexible discs of various diameters and 0.5-1.0 mm of thickness. They are packed in a way that the sorbent is enmeshed into a web of some other inert polymer, e.g., Teflon, or is trapped in a glass fiber or paper filter. SPE discs are preferred over cartridges because their larger cross-sectional area and shorter bed depth allow higher flow rates and shorter analysis time.

The experimental procedure for SPE consists of four steps: conditioning, retention, rinsing and drying. The solid sorbent must undergo proper conditioning to wet the packing material before the sample passes through the SPE device. In its dry form, the octadecyl (C\(_{18}\)) bonded-phase material is randomly oriented on the surface. If the sorbent is placed in contact with an aqueous sample, the environment surrounding the bonded organic moiety would be highly polar and entirely incompatible with a C\(_{18}\) bonded-phase. The bonded groups try to minimize the exposure to the high polarity medium by forming clusters among them that are close to each other. In such a configuration, the organic surface that is exposed to the solute is very small. This arrangement of the bonded organic groups diminishes the extraction efficiency of SPE. The conditioning step “activates” the sorbent by treatment with an organic solvent, typically methanol. Under these conditions, the bonded-organic moiety is more open and available for interaction with the solute. This process can be determined as one of the most important steps for a successful SPE. After conditioning, the sample is loaded to the SPE device.
by gravity feed, pumping, or vacuum. Depending on the type of sample, micro-liters to liters of liquid sample can be applied through the sorbent phase. During this step, PAH are extracted and pre-concentrated in the solid sorbent. Some of the matrix concomitants may also be retained along with PAH, making matrix composition more challenging for further PAH analysis. A rinsing step is then used to remove potential interferences from the SPE material. For aqueous samples, a water-organic-solvent mixture is usually used as the rinsing solvent. The final step is the elution of PAH from the sorbent with an appropriate solvent specifically chosen to disrupt the PAH-sorbent interaction. The elution solvent should remove as little as possible of the other substances sorbed on the column.

For efficient SPE of PAH, one would desire the capacity factor to be very large in aqueous solutions. Similarly, the capacity factor should approach zero in 100% organic eluting solvent unless non-reversed-phase interactions were taking place. Unfortunately, non-reversed-phase interactions in C\textsubscript{18} sorbent materials result from the existence of residual silanol groups on the surface of silica particles. These groups, which interact with PAH through both hydrogen bonding and dipole-dipole interactions, make difficult PAH desorption for subsequent chromatographic analysis.\textsuperscript{40} Table 1.1 summarizes the overall recoveries of EPA-PAH obtained from water samples via HPLC\textsuperscript{41-46} and GC-MS analyses\textsuperscript{47-51}. The large variations observed for the recoveries of individual PAH can be partially attributed to the difficulty of PAH desorption from C\textsubscript{18} sorbents. Although previously reported data on the standard deviations of average recoveries of individual PAH is scarce and incomplete, the reported values\textsuperscript{44,49} show relatively high standard deviations that affect both the precision and the accuracy of analysis. It is within this context that development of new extraction approaches and instrumental methods become relevant to the analysis of PAH in water samples.
Table 1.1 EPA-PAH Recoveries from Water Samples via SPE-HPLC and SPE-GC-MS.

<table>
<thead>
<tr>
<th>PAH</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC</td>
</tr>
<tr>
<td>naphthalene</td>
<td>(65.5±1.1)</td>
</tr>
<tr>
<td></td>
<td>65, 646</td>
</tr>
<tr>
<td>acenaphthylene</td>
<td>81, 3943, 4045, 6746</td>
</tr>
<tr>
<td>acenaphthene</td>
<td>(65.5±1.8)</td>
</tr>
<tr>
<td></td>
<td>79, 2543, 9143, 6847</td>
</tr>
<tr>
<td>fluorene</td>
<td>(58.5±0.3)</td>
</tr>
<tr>
<td></td>
<td>82, 5243, 8147, 6847</td>
</tr>
<tr>
<td>phenanthrene</td>
<td>(57.0±0.5)</td>
</tr>
<tr>
<td></td>
<td>95, 6943, 9345, 5646</td>
</tr>
<tr>
<td>anthracene</td>
<td>(67.6±1.6)</td>
</tr>
<tr>
<td></td>
<td>97, 7743, 10245, 7146</td>
</tr>
<tr>
<td>fluoranthene</td>
<td>(65.6±1.2)</td>
</tr>
<tr>
<td></td>
<td>101, 8343, 6445, 7546</td>
</tr>
<tr>
<td>pyrene</td>
<td>(67.7±1.7)</td>
</tr>
<tr>
<td></td>
<td>91, 9443, 9843, 10745</td>
</tr>
<tr>
<td>benzo[a]anthracene</td>
<td>(70.6±0.5)</td>
</tr>
<tr>
<td></td>
<td>92, 9843, 11547, 6248</td>
</tr>
<tr>
<td>chrysene</td>
<td>(68.3±0.3)</td>
</tr>
<tr>
<td></td>
<td>89, 9643, 8547, 2748</td>
</tr>
<tr>
<td>benzo[b]fluoranthene</td>
<td>(68.3±0.3)</td>
</tr>
<tr>
<td></td>
<td>89, 9643, 8547, 2748</td>
</tr>
<tr>
<td>benzo[k]fluoranthene</td>
<td>(68.3±0.3)</td>
</tr>
<tr>
<td></td>
<td>89, 9643, 8547, 2748</td>
</tr>
<tr>
<td>benzo[a]pyrene</td>
<td>(95±13)</td>
</tr>
<tr>
<td></td>
<td>97, (86±13), 8545, 4046</td>
</tr>
<tr>
<td>dibenz[a,h]anthracene</td>
<td>(95±13)</td>
</tr>
<tr>
<td></td>
<td>95, 7545, 3646, 8547</td>
</tr>
<tr>
<td>benzo[g,h,i]pyrene</td>
<td>(8842, 9743,(83±11), 7145, 8247, 78.1, 53, 53, 76.19)</td>
</tr>
<tr>
<td></td>
<td>3846</td>
</tr>
<tr>
<td>indeno[1,2,3-cd]pyrene</td>
<td>(8742, 9543,(75±15), 9145, 11647, 71.1, (61±9), 57, 56)</td>
</tr>
<tr>
<td></td>
<td>4446</td>
</tr>
</tbody>
</table>
1.3 Chromatographic Analysis of EPA-PAH

HPLC and GC-MS are the basis for standard PAH identification and determination. UV absorption and room temperature fluorescence detection are both widely used in HPLC, but the specificity of these detectors is modest. Since PAH identification is solely based on retention times, unambiguous PAH identification requires complete chromatographic resolution of sample components. When HPLC is applied to “unfamiliar” samples, EPA recommends that a supporting analytical technique such as GC-MS be applied to verify compound identification and to check peak-purity HPLC fractions.

On drinking waters, the EPA recommends the routine monitoring of benzo[a]pyrene. This is the most toxic PAH in the EPA list, and its concentration alone is often used as a measure of risk. According to the EPA, its maximum contaminant level (MCL) should not exceed 200ng.L⁻¹. In addition to benzo[a]pyrene, the European Union and the World Health Organization have regulated fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[g,h,i]perylene, and indeno[1,2,3-cd]pyrene, MCL values were set at 10ng.L⁻¹ for the highly toxic benzo[a]pyrene and 200ng.L⁻¹ for the remaining PAH.⁵²-⁵⁴ Table 1.2 summarizes the limits of detection (LOD) of EPA-PAH obtained via SPE-HPLC⁴¹-⁴⁶ and SPE-GC-MS⁴⁹,⁵⁵-⁵⁷ analyses. Although a straightforward comparison of LOD is difficult as different mathematical and experimental approaches have been used for their calculation, most reported values fall within the parts-per-trillion concentration range. With one exception, ⁴⁵ the LOD for the regulated PAH are below the stipulated MCL values. The volume of water processed through the SPE device varied from 10 to 1000mL.⁴¹-⁵¹,⁵⁵-⁵⁷ The possibility to adjust the volume of extracted water to reach the low MCL values is one of the main advantages of interfacing SPE with chromatographic methods. Independent of the volume of extracted water, the approximate time required to separate and
determine the 16 EPA-PAH via HPLC is approximately 60min. If additional GC-MS analysis is required for unambiguous PAH determination, the total analysis time will reach 2-3 hours per sample. If the concentrations of target species are found to lie outside the detector’s response range, the sample must be diluted and the process repeated. These are important considerations when routine analysis of numerous samples is contemplated. Our research addresses these disadvantages providing PAH monitoring via a simple, cost-effective, and direct method of analysis.

Table 1.2 Limits of detection of EPA-PAH via SPE-HPLC and SPE-GC-MS.

<table>
<thead>
<tr>
<th>PAH</th>
<th>LOD (ng.L⁻¹)</th>
<th>GC-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC</td>
<td>GC-MS</td>
</tr>
<tr>
<td>naphthalene</td>
<td>46^(41), 36^(45), 200^(45), 0.58^(46)</td>
<td>41^(55), 0.67^(49), 2^(56), 800^(57)</td>
</tr>
<tr>
<td>acenaphthylene</td>
<td>107^(41), 17^(43), 400^(45), 0.67^(46)</td>
<td>11^(55), 0.33^(49), 9^(56), 2800^(57)</td>
</tr>
<tr>
<td>acenaphthene</td>
<td>7^(43), 300^(45), 0.52^(46)</td>
<td>4^(55), 0.44^(49), 4^(56), 4100^(57)</td>
</tr>
<tr>
<td>fluorene</td>
<td>19^(41), 0.4^(43), 500^(45), 0.58^(46)</td>
<td>12^(55), 0.32^(49), 2^(56), 1600^(57)</td>
</tr>
<tr>
<td>acenaphthenne</td>
<td>5^(41), 0.6^(45), 300^(45), 0.52^(46)</td>
<td>5^(55), 0.80^(49), 1^(56), 3400^(57)</td>
</tr>
<tr>
<td>phenanthrene</td>
<td>9^(41), 0.1^(43), 300^(45), 0.68^(46)</td>
<td>1^(55), 0.75^(49), 7^(56), 4200^(57)</td>
</tr>
<tr>
<td>anthracene</td>
<td>54^(41), 9.68^(44), 900^(45), 0.78^(46)</td>
<td>2^(55), 0.52^(49), 3^(56), 3900^(57)</td>
</tr>
<tr>
<td>fluoranthene</td>
<td>41^(41), 3^(43), 700^(44), 0.65^(46)</td>
<td>3^(55), 0.50^(49), 3^(56), 5100^(57)</td>
</tr>
<tr>
<td>pyrene</td>
<td>24^(41), 0.43^(43), 300^(45), 0.92^(46)</td>
<td>4^(55), 0.21^(49), 30^(56), 5800^(57)</td>
</tr>
<tr>
<td>benzo[a]anthracene</td>
<td>0.4^(43), 300^(45), 0.73^(46)</td>
<td>14^(55), 0.26^(49), 10^(56), 4900^(57)</td>
</tr>
<tr>
<td>chrysene</td>
<td>17^(41), 0.4^(43), 0.84^(44), 800^(45), 0.78^(46)</td>
<td>18^(55), 0.31^(49), 60^(56), 3500^(57)</td>
</tr>
<tr>
<td>benzo[b]fluoranthene</td>
<td>33^(41), 0.03^(43), 0.81^(44), 1200^(45), 0.76^(46)</td>
<td>39^(55), 0.21^(49), 30^(56), 3600^(57)</td>
</tr>
<tr>
<td>benzo[k]fluoranthene</td>
<td>0.1^(43), 0.68^(44), 1500^(45), 0.79^(46)</td>
<td>7^(55), 0.30^(49), 110^(56), 5600^(57)</td>
</tr>
<tr>
<td>benzo[a]pyrene</td>
<td>0.2^(43), 600^(45), 0.98^(46)</td>
<td>3^(55), 0.16^(49), 150^(56), 36000^(57)</td>
</tr>
<tr>
<td>dibenz[a,h]anthracene</td>
<td>0.7^(43), 0.78^(44), 2500^(45), 0.92^(46)</td>
<td>20^(55), 0.12^(49), 120^(56), 7600^(57)</td>
</tr>
<tr>
<td>benzo[g,h,i]perylene</td>
<td>0.6^(43), 1500^(45), 1.2^(46)</td>
<td>7^(55), 0.14^(49), 60^(56), 7900^(57)</td>
</tr>
<tr>
<td>indeno[1,2,3-cd]pyrene</td>
<td>30^(41), 5.58^(44), 2500^(45), 1.2^(46)</td>
<td>7^(55), 0.14^(49), 60^(56), 7900^(57)</td>
</tr>
</tbody>
</table>
1.4 High Molecular Weight PAH

Recent toxicological studies attribute a significant portion of the biological activity of PAH contaminated samples to the presence of high molecular weight (HMW) PAH, i.e. PAH with MW $\geq 300$. The first group of HMW PAH, i.e. the MW 300 and MW 302 isomers, has been identified in combustion related samples with high PAH content such as coal tar, carbon black, and fuel combustion exhaust. Their presence has also been confirmed in environmental samples such as air particulate matter, mussels, sediments and soils. Coronene is generally the only MW 300 isomer measured, even though a number of other isomers are possible. However, only a limited number of studies have reported the quantification of individual isomers. The majority of the reports have only been able to make a tentative identification of individual isomers or had to report concentrations in terms of a total fraction composed of several isomers. Because the carcinogenic properties of HMW PAH 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 their less toxic isomers.

The need for unambiguous isomer identification is further intensified by recent findings showing that some HMW PAH can exhibit far higher carcinogenic properties than EPA-PAH. A crucial example is Dibenzo[a,l]pyrene (DB[a,l]P). This compound is the most potent carcinogenic PAH known to date. Its carcinogenic potency is approximately 100 times higher than that of B[a]P. There are several more isomers of dybenzopyrene - including dibenzo[a,e]pyrene (DB[a,e]P), dibenzo[a,h]pyrene (DB[a,h]P), dibenzo[a,i]pyrene (DB[a,i]P) and dibenzo[e,l]pyrene (DB[e,l]P) – which are also carcinogenic, but not to the extent DB[a,l]P is. Thus, unambiguous identification of DB[a,l]P in environmental samples is highly relevant even if the other isomers are present at 10 to 100 times higher concentrations. Although DB[a,l]P
is the most potent carcinogenic PAH known to man, there are only limited data on its formation and presence in the environment. Early carcinogenicity studies mistakenly used the relatively weaker carcinogen dibenzo[a,e]fluoranthene. As a matter of fact, only recently the U.S. Department of Health and Human Services listed DB[a,l]P, DB[a,e]P, DB[a,h]P and DB[a,i]P as potential carcinogens to humans.81

Unfortunately, EPA methodology cannot always meet the challenge of specifically analyzing structural isomers at the low concentration levels of environmental samples. The main problems that confront EPA methodology arise from the relatively low concentration levels and the large number of HMW PAH isomers with very similar elution times and similar, possibly even virtually identical, fragmentation patterns. An important component of our research, then, focuses on developing methods with the ability to efficiently and reliably detect the presence and determine the amounts of the most toxic HMW PAH, even if their less toxic isomers are present in far higher concentrations.

1.5 Metabolic Products of PAH

Parent PAH are relatively inert and need metabolic activation to express their carcinogenicity. Covalent binding to DNA is believed to be the first critical step in the initiation of the tumor formation process. Complex heterogeneity makes the identification of the type(s) of chemical lesion(s) an extremely challenging problem. For instance, a given metabolite may bind to different bases or different nucleophilic centers of a base and exist in distinctly different adduct-DNA conformations. Metabolism may also lead to a distribution of stereoisomeric metabolites and, as a consequence, stereo specific additions to DNA. The
Complexity of the problem is well illustrated in figure 1.2. In the initial step of the metabolic activation of B[a]P, cytochrome P450s A1, 1A2, 1B1, 3A4, and 2C catalyze the formation of arene oxides and phenols.\textsuperscript{87-90} The arene oxides may arrange spontaneously to phenols or undergo hydration catalyzed by epoxide hydrolase, leading dihydrodiols. 7,8-Dihydro-7,8-dihydroxybenzo[a]pyrene is further oxidized to \textit{anti-} and \textit{syn}-7,8-dihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) in a reaction catalyzed by P450s 1A1, 1A2, and 1B1, as well as other enzymes.\textsuperscript{91-93} Among the four BPDE enantiomers produced in these reactions, the (7R,8S,9S,10R)-enantiomer of anti-BPDE is generally formed to the greatest extent and shows the highest carcinogenic activity.\textsuperscript{91-94} This diol epoxide reacts with DNA in vitro and in vivo, producing a major adduct in which the exocyclic amino group of deoxyguanosine undergoes \textit{trans} addition to carbon 10 of BPDE. Convincing evidence clearly supports the hypothesis that the formation of this and related minor DNA adducts is the major metabolic activation pathway of B[a]P.\textsuperscript{91-96} Other pathways of B[a]P metabolism lead predominantly to detoxification. These include phenol formation, glutathione conjugation of arene oxides and diol epoxides involving GSTM1, GSTA1, and GSTP1, and glucuronidation of dihydrodiols.
By virtue of the rich heterogeneous distribution of metabolic products they produce, PAH provide a full spectrum of the complexity associated with understanding the initial phase of carcinogenesis. During the past decades tremendous efforts have been made to develop bio-analytical techniques that possess the selectivity and sensitivity for the problem at hand. The general scheme consists on sample clean-up and pre-concentration prior to metabolites separation and determination. Depending on the complexity of the sample and the relative concentrations of the targeted metabolites, a combination of preparation techniques is often

Figure 1.2 Overview of B[a]P metabolism.

EH, epoxide hydrolase; DHD, dihydrodiol dehydrogenase. Adapted from ref. [91].
necessary to reach the limits of detection of the instrumental method of analysis. The numerous preparation steps open ample opportunity to metabolite loss and collection of inaccurate data. Separation of metabolites has been accomplished via HPLC, capillary electrophoresis (CE) and GC. Ultraviolet –visible absorption and room temperature fluorescence detection have been widely used with both HPLC and CE.

Although the strong fluorescence of metabolic products provides these methods with excellent LOD, the broad nature of room-temperature fluorescence spectra drastically reduces their qualitative potential for unambiguous determination of co-eluted metabolites. Since the identification of metabolic products is solely based on retention times, the unambiguous determination of closely related metabolites requires complete resolution in the chromatographic column. This is not a trivial task as many metabolites often present similar chromatographic behaviors. The ultimate specificity belongs to high-resolution MS either coupled to gas chromatography (GC-MS) or HPLC (HPLC-MS). However, the existence of chemically related metabolic products with virtually identical fragmentation patterns often challenges the specificity of these techniques. The issue of selectivity becomes even more crucial when facing a distribution of stereo-isomeric metabolites and, therefore, stereo-specific additions to DNA. The ability to directly determine the presence and the amounts of targeted metabolites without the need of extensive sample preparation and/or chromatographic separation, even if closely related stereo-isomers are present in far higher concentrations than their most carcinogenic counterparts, would be a huge breakthrough. Our ultimate goal, then, is to fulfill this gap.
1.6 **Original Components of this Dissertation**

The research presented here has led to considerable improvements in various fronts. The first original component of this dissertation – which we have named solid-phase nano-extraction (SPNE) - deals with the use of gold nanoparticles (Au NPs) as extracting material for PAH. The advantages of SPNE are demonstrated for the analysis of PAH in water samples via both HPLC and Laser-Excited Time-Resolved Shpol’skii Spectroscopy (LETRSS). The same approach is then extended to the analysis of monohydroxy-PAH in urine samples via SPE- HPLC and In-Capillary SPNE-CE.

The second original component of this dissertation describes the application of Shpol’skii Spectroscopy to the analysis of polar PAH metabolites. The outstanding selectivity and sensitivity for the direct analysis of PAH at trace concentration levels has made Shpol’skii spectroscopy a leading technique in environmental analysis. Unfortunately, the requirement of a specific guest-host combination - typically a non-polar PAH dissolved in an n-alkane - has hindered its widespread application to the field of analytical chemistry. This dissertation takes the first steps in removing this limitation demonstrating its feasibility for the analysis of polar benzo[\(a\)]pyrene metabolites in alcohol matrixes.
CHAPTER 2  HIGH RESOLUTION MOLECULAR SPECTROSCOPY IN LOW-TEMPERATURE CRYSTALLINE MATRICES


Absorption and luminescence spectroscopy of organic molecules in liquid solutions and solid matrices are important tools for compound identification and quantitation, structure elucidation and for studying reaction chemistry. The simplicity of experimental procedures makes room-temperature analyses the most popular approaches. Unfortunately, absorption and luminescence (fluorescence and phosphorescence) spectra at room temperature are generally very broad and diffuse, with spectral bandwidths ranging from a few hundred to a few thousand wave-numbers. As a result, the information content of room-temperature spectra is rather limited. This limitation has prompted numerous researches to devote significant efforts on finding conditions that would enable one to observe vibrational structure in condensed-phase electronic spectra. The most important techniques currently available for high-resolution spectra are fluorescence line-narrowing spectroscopy, spectral hole burning spectroscopy and Shpol’skii spectroscopy. Their fields of applications range from organic molecules in crystals, polymers and glasses, to very complex biological systems, including proteins and nucleic acids containing chromophores, as well as reaction-center complexes of photosynthetic units.\textsuperscript{100,114}

Closely related to this dissertation is Shpol’skii spectroscopy. The name Shpol’skii was introduced in honour of the Russian scientist E.V. Shpol’skii, who published an important experimental discovery in 1952\textsuperscript{115} and initiated worldwide research in this field. He found that
upon cooling PAH to 77 K in polycrystalline $n$-alkane solvents the otherwise broad fluorescence and absorption spectra showed much narrower spectral bands – denoted as ‘quasi-lines’. Although Shpol'skii spectrometry has been recognized as a powerful technique for the environmental analysis of PAH, its widespread use has been hampered by several reasons. These include inconvenient sample freezing procedures, questions about signal reproducibility for calibration purposes, lack of reference databases, and most importantly, lack of selectivity for the unambiguous determination of targeted PAH in highly complex samples. In this chapter, we review basic principles on luminescence phenomena, homogeneous and inhomogeneous band-broadening and state-of-the-art research that eliminates most of the limitations of Shpol'skii Spectroscopy.

2.1 Basic Concepts on Luminescence Phenomena

A partial Jablonski diagram, depicting the various activation and deactivation processes for an aromatic molecule, is shown in Figure 2.1. An absorption, or excitation, process (A) can occur into the first or higher excited singlet state. Excitations into the first and second singlet states ($S_1$ and $S_2$) are shown. This process is relatively fast and occurs on the order of $10^{-15}$ s. Following excitation, the molecule typically undergoes vibrational relaxation (VR) into the ground vibrational level of the electronic excited state. The excess of vibrational energy is converted to thermal or translational energy. VR occurs on the order of $10^{-11}$ to $10^{-10}$ s. If the molecule resides in an upper excited singlet state ($S_2$), internal conversion (IC) into the $S_1$ electronic level can occur. This process is efficient if the ground vibrational level of the $S_2$ state has the same energy as an upper vibrational level of the $S_1$ state. The internal conversion process
occurs on the order of $10^{-12}$ s. Following IC, VR occurs into the ground vibrational level of the $S_1$ state.

![Jablonski diagram](image)

**Figure 2.1 Jablonski diagram.**

A is the absorption, F is the fluorescence, P is the phosphorescence, VR is the vibrational relaxation, IC is the internal conversion, and ISC is the intersystem crossing.

From the $S_1$ state, deactivation of the molecule may happen through various processes. External conversion (EC) is a non-radiative deactivation process in which the excited molecule transfers its excess electronic energy to surrounding molecules or solvent molecules through collisions. From the lowest vibrational level of $S_1$, the molecule may also release the excess of electronic energy in the form of a photon. This process is known as fluorescence (F) and it occurs on the order of $10^{-10}$ to $10^{-6}$ s. Since IC and VR are comparatively rapid process,
fluorescence most often occurs from the ground vibrational lever of the first excited single state and is of a longer wavelength than absorption. Fluorescence from the S₂ state is rare and will only occur if the energy gap between the S₁ and S₂ states is large enough that it is not favored.

The S₁ state may also be deactivated by a non-radiative process called inter-system crossing (ISC). This process involves a crossover of electronic states similar to IC but it occurs between states of different spin multiplicity. Population of the upper vibrational levels of the triplet state manifold is followed by VR and IC into the ground vibrational level of the first triplet excited state (T₁). Deactivation of T₁ via the emission of a photon is called phosphorescence (P) and it occurs on the order of 10⁻³ to 10³ s. Since phosphorescence occurs on a relatively long time scale, T₁ is particularly susceptible to collisional deactivations.¹¹⁷ Non-radiative deactivation of T₁, or quenching, can be a particular problem in the presence of oxygen. Molecular oxygen resides naturally in a ground triplet configuration possessing two low-lying singlet states with excitation energies of approximately 23 and 38 kcal/mol. Any species having excitation energy as low as 23 kcal/mol may be quenched by oxygen via energy transfer. Excited singlet states may also be affected by oxygen, but since oxygen quenching is diffusion controlled, a high oxygen concentration is necessary to quench the relatively short-lived excited singlet state. The removal of oxygen is often accomplished through de-oxygenation of sample solution or purging the sample compartment with inert gases. Similar to phosphorescence, fluorescence is always in competition with the various non-radiative deactivation processes of the excited state. As a result, fluorescence and phosphorescence intensities intrinsically depend on the relative efficiencies of all competing processes affecting their respective excited states. The efficiencies of fluorescence and phosphorescence are often expressed in terms of quantum yield (ϕ).

The quantum yield for fluorescence, ϕₖ, is the ratio of the rate of fluorescence with the
rate of absorption, $\Phi_F/\Phi_A$, where $\Phi_F = k_F n_{S1} V$ and $\Phi_A = k_A n_{S0} V$. $V$ is the volume of sample illuminated; $n_{Sx}$ is the number of molecules occupying the given electronic state $x$; and $k_F$ and $k_A$ are the rate of fluorescence and absorption, respectively. Under steady state conditions, we can assume that $n_{S1} = n_{S0} k_A / (k_F + k_{nr})$, where $k_{nr}$ is the sum of rates for the non-radiative processes (external conversion, $k_{ec}$; internal conversion, $k_{ic}$; and inter-system crossing, $k_{isc}$). Using these relationships, the fluorescence quantum yield in terms of the rates of the various activation and deactivation processes is given by equation (2.1):

$$\phi_F = \frac{k_F}{k_F + k_{nr}} \quad (2.1)$$

which shows that, in order to improve the fluorescence quantum yield, and hence the fluorescence intensity, one needs to minimize the rate contributions from non-radiative processes. A similar situation arises for phosphorescence. The phosphorescence quantum yield ($\phi_P$) can be expressed as:

$$\phi_P = \frac{k_{isc}}{k_{isc} + k_F + k_{nr}} \times \frac{k_p}{k_p + k_{nr}} \quad (2.2)$$

Note that the first term in equation (2.2) takes into account the efficiency of inter-system crossing. Therefore, to maximize the phosphorescence efficiency, hence the phosphorescence intensity, minimization of both non-radiative processes and fluorescence relative to inter-system crossing becomes evident.

Because the excited states ($S_1$ and $T_1$) are often deactivated by first-order processes, the decay of either fluorescence or phosphorescence can be described by equation (2.3):

$$I_t = I^0 e^{-t/\tau_L} \quad (2.3)$$
Where $I_0$ is the luminescence intensity at time zero and $\tau_L$ is the luminescence lifetime. The lifetime is defined as the time it takes for the luminescence to decay to $1/e$ of its initial value. The lifetimes for fluorescence and phosphorescence are related to the rate constant for deactivation by equations (2.4) and (2.5):

$$\tau_F = \left( k_F + k_{nr} \right)^{-1} \quad (2.4)$$

$$\tau_P = \left( k_p + k_{nr} \right)^{-1} \quad (2.5)$$

Equation (2.1), (2.2), (2.4) and (2.5) indicate that non-radiative decay processes decrease the fluorescence and phosphorescence intensities and lifetimes by the same factor. In other words, the lifetime and the quantum efficiency are proportional to $(k_L + k_{nr})^{-1}$.

### 2.2 Achieving High-Resolution Spectra under Shpol'skii Conditions

Apart from possible instrumental contributions, the spectral line-width in condensed-phase electronic spectra is determined by inhomogeneous and homogeneous broadening effects. Inhomogeneous broadening is related to the fact that in a sample we are usually studying a large ensemble of molecules that – although chemically identical – may still show slightly different spectra. That is mainly the result of differences in the structure of the solvation shell surrounding the chromophore molecules: each individual molecule will experience a slightly different interaction with the solvent, resulting in a Gaussian distribution of transition energies (as illustrated in Figure 2.2a). In flexible molecules the occurrence of different conformations may also play a role. However, when solute molecules adopt the same conformation and experience exactly the same environment, like in a theoretically perfect crystalline matrix, their transition
energies would coincide exactly, as in Figure 2.2b.

![Figure 2.2 Cartoon of analyte in amorphous and crystalline matrix and resulting spectral bandwidth.](image)

In practice, the inhomogeneous broadening can be strongly reduced but not completely removed. Typical bandwidths in Shpol’skii experiments carried out in \( n \)-alkane matrices at temperatures of 20 K or lower are 1-3 cm\(^{-1} \). That is sufficient to obtain vibrational resolved spectra, but in low-temperature single-molecule experiments thousand-fold narrower transitions have been observed.\(^\text{118} \) Homogeneous broadening factors affect each individual molecule to the same extent. In condensed-phase spectroscopy it originates mostly from electron-phonon interactions. A phonon is a quantized lattice vibration of the matrix. During an electronic transition one or several lattice vibrations can be simultaneously excited.

The homogeneous spectrum will therefore consist of a superposition of a narrow zero-phonon line (ZPL) and a broad phonon side band or phonon wing (PW) as can be seen in Figure
2.3. In emission the PW appears as a broad continuum at the low-energy side of the ZPL and in absorption at the high-energy side. In a low-temperature environment this phonon sideband is often well distinguishable from the ZPL, as shown in Figure 2.3. For most applications one would like to obtain strong ZPLs, which means that the temperature should be as low as practically possible and solute-matrix interactions should be weak. Both requirements are usually met when working under cryogenic conditions with non-polar compounds in an $n$-alkane matrix, but other solute-matrix combinations have also shown Shpol’skii-type spectral narrowing.\textsuperscript{119}

The intensity $I$ of the profile in 3 may be described in the form:

$$I(\nu, T) = I_{ZPL}(\nu, T) + I_{PW}(\nu, T) \quad (2.6)$$

The relative intensity of the ZPL can be characterized by the Debye-Waller factor $\alpha(T)$,

$$\alpha(T) = \frac{I_{ZPL}}{I_{ZPL} + I_{PW}} = \exp[-S(T)] \quad (2.7)$$

where $S$, the so-called Huang-Rhys parameter, is a dimensionless quantity that indicates the strength of the electron-phonon coupling as a function of temperature.\textsuperscript{119,120}
Figure 2.3 Electron phonon coupling in emission.

On the left side three of the many possible phonons are shown, present in a number of overtones. The zero phonon transitions for all phonons coincide, resulting in a sharp zero-phonon line (ZPL). The phonons and their overtones do not overlap exactly and show up as a broad phonon side band (PSB), red-shifted in emission. In practice, excitation into a PSB results in additional broad red shifted emission from an inhomogeneous distribution of ZPLs.

Ultimately, the remaining source of homogeneous broadening is mainly determined by the lifetimes of the different states involved in the transitions. The shorter the lifetime, the less precise its energy and the broader the corresponding spectral lines will be. This so-called lifetime broadening is a quantum mechanical effect related to Heisenberg’s uncertainty principle,\textsuperscript{121} and is defined as follows:

\[ \tau \cdot \delta E \approx \hbar/2 \] (2.8)
where $h$ is Planck’s constant, $\delta E$ is the uncertainty in energy and $\tau$ is the lifetime of the state involved. When higher excited states are involved, and vibrational relaxation and internal conversion are fast, the corresponding spectral broadening is readily observed. In contrast, from relatively long-living states such as the first excited state $S_1$ the broadening effect is often negligible. Thus, the higher-energy transitions observed in Shpol’skii excitation or absorption spectra tend to be broader than $S_0-S_1$ transitions.

In order to obtain a Shpol’skii spectrum the solvent should form a poly-crystalline matrix at low temperature. In practice, mostly $n$-alkane solvents are used. Finding the most appropriate solvent is not always evident and may involve some trial and error. Secondly, the analyte should fit well into this matrix. In this regard, the three-dimensional shape, polarity, rigidity and the number of substituents of the fluorophore are very important. Ideally, the analyte molecule displaces a distinct number of solvent molecules in the lattice and will only fit in a single, well-defined manner in the available space. In that case, the molecules will experience exactly the same interaction with the crystalline matrix, which means that the $S_0-S_1$ energy differences will be identical. However, sometimes there are two or several ways in which the molecule fits in the matrix; the molecule is said to occupy different sites (see Figure 2.4). For each site the $S_0-S_1$ energy difference is likely to be different. As a result, a multiplet will appear in the spectrum, both in absorption and emission and for every vibronic transition. In other words, two or more identical spectra are observed, but shifted over a small amount of energy. The intensity ratio, which reflects the relative populations of each site, is constant for every multiplet. By using a tuneable laser, one of these subpopulations can be selectively excited in the 0-0 transition or in a vibration of the first excited state. Only this site will then appear in the emission spectrum.
A laser is not strictly needed to obtain high resolution spectra in Shpol’skii spectroscopy. Literature reports show that high quality Shpol’skii spectra can be recorded with a standard spectrofluorimeter equipped with a broad-band, continuous wave xenon arc lamp. The main pre-requisite in Shpol’skii spectroscopy is that the solvent forms a regular poly-crystalline matrix upon cooling. In the ideal situation the analyte molecule displaces a distinct number of solvent molecules in a lattice and fits in a well-defined manner in the available space thus provided. Under excellent host-guest compatibility conditions, all solute molecules experience the same interactions with the environment and the broadening due to matrix inhomogeneity is reduced to about 1cm⁻¹. In the absence of instrumental contributions, that is often also the total spectral bandwidth observed which – as shown in Figure 2.5 – it results in vibrational spectral information for “fingerprint” compound identification.
Figure 2.5 Jablonski diagram illustrating the concept of Shpol'skii spectroscopy (A) and the resulting spectrum (B).
Only five vibrations are shown; in the absence of reorientation during the fluorescence lifetime.

Significant extra broadening can be observed if one of the states involved in excitation or emission has a lifetime shorter than approximately one picosecond. The origin of this so-called lifetime broadening is the time-energy uncertainty relationship, i.e. when the time of the transition can be determined accurately its energy cannot and vice versa. From the broadened spectral lines the lifetime can be calculated according to the following equation:

$$\tau = \frac{1}{2 \cdot \pi \cdot \Delta \nu \cdot c}$$  \hspace{1cm} (2.9)

where $\tau$ is the lifetime of the short-living state, $\Delta \nu$ the homogeneous broadening (in cm$^{-1}$) and c
the speed of light (in cm/s). Usually, in Shpol’skii spectroscopy these effects are only observed in excitation when higher electronic states (S2, S1, ...) are involved, since subsequent vibrational relaxation and/or internal conversion is fast. As a result, the bands observed in the excitation and/or absorption spectrum tend to be broader at shorter wavelengths.\textsuperscript{122,125} That is the main reason selective excitation is best accomplished within the S\textsubscript{1} band.

2.3 Sample Freezing Procedures

The classic sample preparation procedure for 77 K measurements consists of immersing a solution-filled small-diameter tube into an optical dewar filled with liquid nitrogen. The fragility of the dewar flask, bubbling in the cryogen at irregular intervals from small ice particles that act as nucleation sites, and condensation (frosting) on the outside of the Dewar are nuisances. But scattering at each of the air/glass, glass/liquid nitrogen or glass/matrix interfaces can also be a severe problem, as stray light usually determines the spectral background and the limit of detection. Options to reach temperatures of 5-20 K include using closed-cycle helium refrigerators or Joule-Thomson miniature refrigerators. The trade-offs include higher cost and reduced sample throughput. Depending on the cooling device, sample size and final temperature, the procedure can take between 40 and 100 min. Ariese et al. developed a sample holder that strongly improved the user friendliness of the closed-cycle helium refrigerator. It enables the simultaneous cooling down of four 10-\textmu l samples in shallow depressions in a gold-plated copper sample holder, covered with sapphire windows. These materials guarantee high thermal conductivity and therefore fast sample solidification, which is a main condition to obtain good-quality Shpol’skii spectra.\textsuperscript{126}
Our approach to low-temperature analysis eliminates the need for an optical dewar and/or helium cryostats. It consists of a bifurcated fiber-optic probe (FOP) that delivers the excitation light directly into the frozen sample. The FOP can simply be lowered into the liquid cryogen for fast and reproducible freezing. All interfaces that could scatter exciting light into the detection system have been removed from the optical path, providing high quality spectra and excellent reproducibility of measurements. A schematic diagram of the FOP is shown in Figure 2.6. The probe typically incorporates one delivery and six silica-clad silica collection fibers. A section of copper tubing provides mechanical support for lowering the probe into the liquid cryogen. At the sample end, the excitation and emission fibers are bundled in a six-around-one configuration with vacuum epoxy and fed into a metal sleeve for mechanical support. A sample vial can be screwed onto the probe head, assuring a fixed distance from the sample to the tip of the fibers. At the instrument end, the collection fibers are bundled with vacuum epoxy in a slit configuration and aligned with the entrance slit of the spectrometer.
Figure 2.6 Cryogenic fiber optic probes for 77K (A) and 4.2K (B) measurements.

After the sample is introduced into the sample vial, it is screwed onto the probe head. The tip of the FOP is positioned either above or below the solution surface, and the sample tube is lowered into a container filled with liquid cryogen. The cell is allowed to cool for 90 s prior to fluorescence measurement to ensure complete sample freezing. If the probe comes into physical contact with the sample, the approximately one-minute probe clean up procedure involves removing the probe head from the liquid cryogen, melting the frozen matrix and warming the resulting solution to approximately room temperature with a heat gun, rinsing the fiber tip with clean solvent, and drying it with warm air from the heat gun.
2.4 Instrumentation for Emission Measurements in Shpol'skii Matrixes

The transition wavelengths for many PAH often require frequency doubling or mixing techniques to shift the visible laser light into the ultraviolet range. The linewidths of most Shpol'skii samples at 77 K are on the order of 20-40 cm$^{-1}$; even at liquid helium temperature, linewidths of less than 1 cm$^{-1}$ are comparatively rare. Therefore, the 0.1 cm$^{-1}$ linewidth of high performance commercial dye lasers or OPO (optical parametric oscillator) systems is not a critical factor, neither is their ability to generate hundreds of mJ in the visible and tens of mJ in the ultraviolet. In our lab, we have acquired high quality 77 K and 4.2 K spectra with a very compact tuneable dye laser with specifications that are well matched for Shpol'skii spectroscopy. Its scanning capability (0.01 nm/step) and its narrow excitation bandwidth (<0.03 nm) allow one to record high-resolution excitation spectra or site-selective excitation. Its excitation energy (5 mJ at the peak of Rhodamine 6G) usually induces sufficient fluorescence so that spectra can be recorded with optimum spectrograph slit widths and maximum spectral resolution.

As for detection, high-resolution Shpol'skii spectra have been recorded with single- and multi-channel detection systems.\textsuperscript{70,127-132} Multi-channel detectors such as a diode arrays\textsuperscript{131} or charge-coupled devices (CCD)\textsuperscript{70} can acquire emission spectra much faster than is possible with a scanning monochromator and a single photomultiplier detector, as was commonly done in earlier studies. Collecting the entire emission spectrum at once also avoids problems associated with pulse-to-pulse fluctuation, laser intensity drift, and photodecomposition. Pulsed excitation sources offer prospects for time-resolved detection in order to improve signal-to-background ratios and reduce spectral interference.\textsuperscript{70,128-132} Early reports on laser-excited Shpols'kii spectrometry (LESS) implemented time-resolution at fixed delay time intervals using laboratory-constructed gated integrators or commercial boxcar averages.\textsuperscript{127-131} More recently, commercially
available delay generators and gated intensified linear photodiode arrays have come into use.\textsuperscript{70}

Our group has developed instrumentation to efficiently collect multidimensional data formats during the lifetime decays of fluorescence (nanoseconds to microseconds) or phosphorescence (milliseconds to seconds).\textsuperscript{133-135} Figure 2.7 shows a schematic diagram of the multidimensional luminescence instrument, which integrates a pulsed tuneable dye laser, a delay generator, a spectrograph and an intensifier-charged coupled device (ICCD). By setting the ICCD gate step parameter, this system automatically increments the time interval between successive scans to collect emission spectra at different delay times between the laser firing and the opening of the intensifier gate. The series of spectra can be assembled into a wavelength-time matrix (WTM), analogous to an excitation-emission matrix (EEM). The tuning of the dye-laser is computer controlled, which facilitates the collection of EEM and time-resolved excitation-emission matrices (TREEM) in short analysis time. A mechanical shutter facilitates phosphorescence spectra and lifetime acquisition over much longer time scales. The shutter is controlled either manually (always open or always closed) or programmed through the digital delay generator. The entire system is computer controlled with home-written LabView\textsuperscript{TM}-based acquisition software.
Figure 2.7 Instrumentation for multidimensional luminescence spectroscopy.

FOP: fiber optic probe. ICCD: intensified charge coupled device. Fluorescence measurements are performed with the shutter in the open position. The shutter facilitates acquisition of phosphorescence spectra and phosphorescence lifetimes.

Multidimensional formats - such as wavelength-time matrices (WTM) and time-resolved excitation-emission matrices (TREEM) - combine spectral and lifetime information with tremendous potential for the direct analysis of target compounds in complex matrixes. The raw data of such formats provide at least five sets of qualitative parameters for compound determination, namely excitation, fluorescence and phosphorescence wavelengths and two lifetimes. Lifetime analysis from WTM$s provides information on spectral peak purity for accurate quantitative determination. TREEM$s give the analyst the opportunity to select the best time window for minimum overlap in highly complex spectra. Applications of this approach, which we have named Laser-Excited Time-Resolved Shpol’skii Spectroscopy (LETRSS), have focused on the direct analysis of PAH in water and soil samples.
2.5 Instrumentation for Absorption Measurements in Shpol’skii Matrixes

Recording absorption spectra via transmittance through frozen matrixes is a challenging task. The main reason is the difficulty to overcome the strong scattering light reaching the detector. This is particularly true when thick samples are necessary for recording absorption spectra of weak oscillators. In the case of strongly fluorescent (and/or phosphorescent compounds), additional errors in absorbance measurements arise from the luminescence reaching the detector, which might have an intensity comparable to that of the transmitted light. Commercial double-beam spectrophotometers – which afford a very low stray light level – meet the requirement for accurate transmittance measurements through highly scattering media but fall short with regard to high spectral resolution.

Nakhimovsky et al. designed the first scanning spectrophotometer for high-resolution absorption measurements of PAH and some hetero-PAHs in highly scattering frozen matrices. Their instrument consisted of a double-beam spectrometer using two Czerny-Turner monochromators and a continuous-wave xenon lamp as the excitation source. The light selected by the first monochromator was alternately sent by rotating mirrors toward the sample and the reference. Subsequently the beams were focussed on the second monochromator. Both monochromators -which were equipped with 1200 lines/mm gratings- were set at the same wavelength and scanned synchronously. To minimize the effect of a possible drift of the wavelength drive, a larger bandwidth was used for the first monochromator. A photomultiplier was used for sample (IS) and reference (IR) signal detection. Both signals were integrated over a certain number of mirror cycles, digitally converted and sent to a computer that calculated the absorbance (log(IR/IS)) as a function of excitation wavelength. The inherent disadvantage of this system was the time it needed to record a spectrum, which was typically 30 minutes.
In 2003 Banasiewicz et al. reported high-resolution Shpol’skii absorption spectra of pentacene using a single-beam absorption setup.\textsuperscript{136} A tungsten lamp was used as the light source, which was transmitted alternatively through the solid sample and the solid blank solution. The authors used a homemade sample cuvette comprising two quartz windows separated by a 1.5-mm Teflon ring. A scanning monochromator and photomultiplier operating in the photon-counting mode was used for detection. In 2008 de Klerk et al. developed an easy-to-use homemade absorption spectrometer set-up based on transmission measurement that can handle highly scattering solid solutions.\textsuperscript{137} As light source a D\textsubscript{2}-lamp and white 1 Watt LED was used, together covering the whole UV/visible range. This white light – without applying wavelength selection – was focussed on the sample, consisting of two cylindrical cells with a 7-mm diameter and 1-mm or 5-mm optical path length and sapphire windows on both sides. They were used for the sample and the solvent blank (reference), respectively. The samples were cooled by a closed-cycle helium refrigerator to 14 K. Detection was performed with a 30-cm monochromator and a CCD camera. The monochromator is equipped with 300, 1200 and 2400 l/mm gratings. The spectra were corrected for lamp profile and background scatter (we are dealing with strong scatter in the case of frozen polycrystalline solvents) by consecutive measurements of the sample and the blank. The advantage of this setup is that an absorption spectrum can be measured at once without monochromator scanning; the time to record a full spectrum is less than 1 minute. Its feasibility was demonstrated for a number of PAH in polycrystalline $n$-alkane matrixes at 14 K.
Figure 2.8 (A) Representation of optical phenomena occurring at the irradiated surface of the frozen matrix; (B) Schematic diagram of the tip of the cryogenic fiber optic probe. Where \( I_0 \) = incident radiation; \( I_T \) = transmitted radiation; \( I_S \) = scattered radiation; \( I_{PL} \) = photoluminescence (fluorescence and/or phosphorescence); \( I_{RFL} \) = reflected radiation.

Our approach to low-temperature measurements is fundamentally different as the sought for information is the intensity of the laser excitation returning from the frozen sample to the detector device\(^{125}\). The basis of our approach is illustrated in Figure 2.8. Laser excitation returning from the sample is collected with the emission fibers of the cryogenic probe located above the surface (~1 cm) of the frozen matrix. Equation (2.10) is the basis for generating absorption spectra in the backscattering mode:

\[
I_0 (\lambda_0) = I_A (\lambda_0) + I_{RR} (\lambda_0)
\]

(2.10)

where \( I_0 \), \( I_A \) and \( I_{RR} \) represent the radiant powers of laser excitation, absorption and returning intensities at the wavelength of excitation (\( \lambda_0 \)), respectively. The experimental procedure is straightforward. \( I_{RR} \) is monitored as a function of excitation wavelength for both blank \( (I_{RR}^B) \) and analyte \( (I_{RR}^{An+B}) \) solutions. The absorption spectrum of the analyte is then obtained from the graph \( I_{RR}^B - I_{RR}^{An+B} \) versus \( \lambda_0 \).
Figure 2.9 Experimental data generated at 4.2 K with the FOP and the instrument.

Data was generated from 10μg.mL⁻¹ pyrene solution in n-octane. Curves I and II represent the intensities of the excitation energies monitored from the blank \([I_{RR}^{B}(\lambda_0)]\) and analyte \([I_{RR}^{An+B}(\lambda_0)]\) solutions. Curve III represents the net PAH signal. Each data point in the graphs corresponds to the accumulation of 100 laser pulses. Delay and gate times were 0 and 10ns, respectively. Slit width of the spectrograph, 40μm.

Figure 2.9 illustrates the type of data generated with the cryogenic probe and the laser system in Figure 2.7. Curves I and II represent the intensities of excitation energies returning from n-octane \((I_{RR}^{B})\) and PAH/n-octane \((I_{RR}^{An+B})\), respectively. As expected, the relatively lower absorption of n-octane provides a higher signal than the PAH. Curve III represents the net PAH signal, i.e. \(I_{RR}^{B} - I_{RR}^{An+B}\).
Figure 2.10 shows the 4.2 K absorption spectra of naphthalene in n-pentane and benzo[a]pyrene in n-octane. These PAH/n-alkane combinations are well known examples of single-site and multisite systems, respectively. Both spectra compare well to previously reported spectra at 5 K. A peculiar feature of these spectra is the presence of featureless absorption bands underlying the quasi-line absorption spectra. The broad absorption bands have been attributed to the presence of disordered groups of solute molecules or pre-aggregates in the frozen matrix.100

2.6 Applications of the Fiber Optic Probe and the Multidimensional Luminescence System to the Environmental Analysis of PAH

The fiber optic probe shown in Figure 2.6 and Figure 2.7 provides a simple way to combine sample extraction and spectroscopic analysis. We have taken advantage of this feature interfacing LETRSS to SPE and solid-liquid extraction for the analysis of liquid and solid
samples, respectively.\textsuperscript{134,138-151} Figure 2.11 depicts the procedure for SPE-LETRSS analysis of liquid samples, including SPE extracts of water samples and HPLC fractions. The experimental procedure is straightforward. After passing the water sample through the SPE cartridge, the extraction membrane is placed in the sample vial and micro-liters of Shpol’skii solvent are spiked on its surface. A fraction of non-polar analytes such as PAH will partition into the $n$-alkane solvent. The sample is allowed to equilibrate at room-temperature for approximately 5 min, then the vial is attached to the FOP and the fiber assembly is lowered into the liquid cryogen for LETRSS measurements. PAH fitting the polycrystalline matrix will provide quasi-line Shpol’skii spectra for direct fingerprint determination. Peak assignment is carried out via spectral comparison to standards in the pure Shpol’skii solvent. Spectral purity of target peaks is tested via WTM collection. A single exponential decay indicates the presence of a single component. Further comparison of the fluorescence lifetime to the lifetime of the standard provides additional information for unambiguous determination. Figure 2.12 shows the direct spectral identification (without prior HPLC separation) of 15 EPA-PAH listed as priority pollutants from a contaminated water sample at the parts-per-trillion level. The analysis was done at 4.2K using only one Shpol’skii solvent (n-hexane).
Figure 2.11 Experimental steps of SPE-LETRSS of liquid samples (drawing is not to scale).
Figure 2.12 Laser Excited Shpol’skii spectrum of polluted surface water sample showing specific peaks of 15 PAH.

I = Fluorene; II = Benzo[k]fluoranthene; III = Anthracene; IV = Indeno[1,2,3-cd]pyrene; V = Benzo[a]pyrene; VI = Dibenz[a,h]anthracene; VII = Acenaphthene; VIII = Benzo[b]fluoranthene; IX = Fluoranthene; X = Benzo[a]anthracene; XI = Phenanthrene; XII = Chrysene; XIII = Benzo[g,h,i]perylene; XIV = Naphthalene; XV = Pyrene.

Quantitative analysis can be done either by the calibration curve or the multiple standard additions method. There is a linear relationship between the PAH concentration in the Shpol’skii layer and its concentration in the liquid sample that follows the relationship below:\textsuperscript{134,135}

\[
C_{\text{H}_2\text{O}} = \left(\frac{V_{\text{SS}}}{f_i} V_{\text{H}_2\text{O}} + K_d V_{\text{SLE}}/f_i V_{\text{H}_2\text{O}}\right) C_{\text{SS}},
\]  
\text{(2.11)}
where $f_i$ is the isomer fraction extracted from the water sample, $K_d$ is the distribution coefficient between the membrane and the Shpol’skii solvent, and $V_{SS}$, $V_{SLE}$, and $V_{H2O}$ are the volumes of Shpol’skii solvent, extraction membrane, and water, respectively. Equation (2.11) predicts a linear relationship between $C_{H2O}$ and $C_{SS}$ upon constant $f_i$, $K_d$, $V_{SS}$, $V_{H2O}$, and $V_{SLE}$. The analyst sets constant values for $V_{SS}$, $V_{H2O}$, $V_{SLE}$, and $f_i$. In our case, $V_{SS} = 100$ μL, $V_{H2O} = 10$ mL, and $V_{SLE} = 23.8$ μL. $V_{SLE}$ is calculated using the standard formula of a cylinder $V = \pi r^2 h$, where $r$ is the average radius of a disk (0.275 cm) and $h$ is its average thickness (0.1 cm).

The main advantages of SPE-LETRSS include short analysis time (~ 10 min per sample), reduced solvent consumption (100 μL per sample) and lower limits of detection (LOD). Depending on the compound, the LETRSS method was one to two orders of magnitude better than HPLC with fluorescence detection.\textsuperscript{144,148}

We developed a similar approach to screen PAH in soil samples.\textsuperscript{144} A known amount of soil sample (0.0025-0.05 g) is mixed with 0.25 mL of Shpol’skii solvent in the sample vial and the mixture is sonicated at room temperature for 30 min in a sonication bath. After 5 min of settling time, the vial is coupled to the copper tubing of the FOP, keeping the tip of the fiber assembly at a fixed distance above the surface of the liquid layer. LETRSS measurements are then performed after lowering the copper tubing into the liquid cryogen. Figure 2.13 shows examples of Shpol’skii spectra generated with the screening method. The 15 EPA-PAH are clearly identified in both types of soils with no apparent matrix interference. PAH partition into the layer of extracting solvent to provide highly resolved spectra and distinct lifetime values for unambiguous compound determination.
Figure 2.13 4.2K fluorescence spectra obtained from extracts of reference standard materials CRM 104-100 (Panel A) and CRM 105-100 (Panel B).

Identified PAH are the following: I = fluorene, II = naphthalene, III = acenaphthene, IV = phenanthrene, V = chrysene, VI = pyrene, VII = anthracene, VIII = benzo[a]anthracene, IX = dibenz[a,h]anthracene, X = benzo[b]fluoranthene, XI = benzo[a]pyrene, XII = fluoranthene, XIII = benzo[g,h,i]perylene, XIV = benzo[k]fluoranthene, XV = indeno[1,2,3-cd]pyrene. Portions of spectra within dashed vertical lines represent magnifications of entire fluorescence spectrum. Compounds II and III were determined using delay/gate times equal to 200/1000ns and 0/40ns, respectively.
PAH recoveries are reported in Table 2.1. As individual PAH are present in soils at the ng.g\(^{-1}\) level, the direct determination of nanogram of PAH in mg of soil sample – i.e. sub-ng.g\(^{-1}\) to pg.g\(^{-1}\) - with no need for sample pre-concentration, clean up steps and previous PAH separation demonstrates the remarkable potential of the screening method. Using the same solvent (n-octane) for PAH extraction and LETRSS analysis leads to acceptable PAH recoveries with minimum sample handling. The entire LETRSS analysis takes less than 10 min per sample and it consumes 250 µL of organic solvent. These facts make this approach environmentally friendly and cost-effective for routine analysis of numerous samples.
Table 2.1 PAH recoveries obtained with the screening method.

<table>
<thead>
<tr>
<th>PAH</th>
<th>CRM 104-100</th>
<th>CRM 105-100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recoverya (%)</td>
<td>PAH Massb (ng)</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>44.01 ± 5.07</td>
<td>16.9 ± 2.0</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>40.23 ± 2.35</td>
<td>15.5 ± 0.9</td>
</tr>
<tr>
<td>Fluorene</td>
<td>48.27 ± 1.25</td>
<td>15.9 ± 0.4</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>41.90 ± 9.46</td>
<td>121.3 ± 27.4</td>
</tr>
<tr>
<td>Anthracene</td>
<td>42.08 ± 6.76</td>
<td>30.3 ± 4.9</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>21.52 ± 1.51</td>
<td>264.7 ± 18.6</td>
</tr>
<tr>
<td>Pyrene</td>
<td>30.79 ± 1.89</td>
<td>230.9 ± 14.2</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>30.93 ± 3.04</td>
<td>123.4 ± 12.1</td>
</tr>
<tr>
<td>Chrysene</td>
<td>51.84 ± 3.67</td>
<td>222.9 ± 15.8</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>52.94 ± 8.85</td>
<td>256.5 ± 42.9</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>41.75±4.86</td>
<td>106.5 ± 12.4</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>48.88 ± 1.91</td>
<td>124.4 ± 4.9</td>
</tr>
<tr>
<td>Indeno[I,2,3-cd]pyrene</td>
<td>42.11 ± 3.08</td>
<td>93.9 ± 6.9</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>48.39 ± 3.77</td>
<td>37.5 ± 2.9</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>32.36 ± 2.57</td>
<td>57.9 ± 4.6</td>
</tr>
</tbody>
</table>

a PAH recoveries were calculated according to the equation: Recovery (%) = [mass of PAH in extract / mass of PAH in soil sample] x 100; where mass of PAH in extract (ng) = C PAH in EXTRACT (ng/mL) x 0.25mL and mass of PAH in soil sample (ng) = C PAH in SOIL (ng/g) x weight of soil sample (g). The PAH concentration in the soil extract (C PAH in EXTRACT) was determined via multiple standard additions. The PAH concentration in soil (C PAH in SOIL) is the certified PAH concentration of the reference standard. Reported values are an average of three soil extractions. b Mass of PAH in soil extract, calculated by multiplying the PAH concentration in the soil extract by the extract volume (0.25mL).
As previously noted, the carcinogenic potency of DB[a,l]P is approximately 100 times higher than that of B[a]P. Several of its isomers - including DB[a,e]P, DB[a,h]P, DB[a,i]P and DB[e,l]P - are also carcinogenic, but to a lesser extent than DB[a,l]P. Thus, isomer-specific determination of DB[a,l]P in environmental samples is highly relevant, even when present at relatively low concentrations. Until recently, analysis was hampered by chromatographic interference and the large number of isomers. Using a Shpol’skii approach, however, Kozin and coworkers were able to identify and quantitate DB[a,l]P in river sediment extracts. In an n-octane matrix the compound occurs in two distinct sites, which could each be selectively excited with a laser for its unambiguous identification. For these experiments the use of time-resolved detection with a relatively long delay was also crucial to reduce interference from other compounds with shorter fluorescence lifetimes. Although its concentration was typically two orders of magnitude lower than that of B[a]P in the samples analyzed, combined with its 100 times higher potency it is nevertheless highly relevant. Although in recent years there has been some effort trying to improve analytical methodology for DB[a,l]P, there are still only very limited data on its formation and presence in the environment.

Our group developed a method for determining the five DBP isomers in water samples. The method is based on SPE-LETRSS and follows the experimental procedure shown in Figure 2.11. Once extracted on the membrane, DBP isomers partition into the spiked layer of Shpol’skii solvent (n-octane) to provide highly resolved spectra and distinct lifetime values for unambiguous isomer determination. Figure 2.14 shows the fluorescence spectra of five synthetic mixtures containing the DBP isomers. Each mixture was prepared with the target isomer at a concentration 100 × lower than the concentrations of the other isomers. Each spectrum corresponds to the first spectrum of a WTM. Each WTM was collected at the
maximum excitation wavelength of the target isomer using its optimum delay and gate times. Spectral comparison to individual standards provided a minimum of two peaks per isomer with no spectral overlapping from concomitants. The fluorescence decays — also stripped from the WTM at the target fluorescence wavelength of each isomer — were single-exponential decays showing spectral purity at target wavelengths.
Figure 2.14 4.2 K fluorescence spectra from synthetic mixtures with the five DP isomers.

Each mixture contained the target isomer at 0.1 µg·mL⁻¹ concentration and the other four isomers at 10 µg·mL⁻¹ concentration. In each mixture, the target isomer was the following: (A) DB[a,l]P; (B) DB[a,e]P; (C) DB[a,h]P; (D) DB[a,i]P; (E) DB[e,l]P. Experimental parameters were the following: (A) $\lambda_{\text{exc}} = 323$ nm, $t_d/t_g = 10/250$ ns; (B) $\lambda_{\text{exc}} = 310$ nm, $t_d/t_g = 10/150$ ns; (C) $\lambda_{\text{exc}} = 316.5$ nm, $t_d/t_g = 0/15$ ns; (D) $\lambda_{\text{exc}} = 304$ nm, $t_d/t_g = 10/650$ ns; (E) $\lambda_{\text{exc}} = 288$ nm, $t_d/t_g = 10/150$ ns. In all cases, spectrograph slits were 40 µm. All spectra correspond to the accumulation of 10 laser pulses.
Table 2.2 compares fluorescence intensities and lifetimes of standards and mixtures. The intensity ratios recorded from standards ($I_S$) and mixtures ($I_M$) are close to unity showing that no inner filter effects deteriorate the accuracy of analysis at these concentration levels (data not shown). The statistical equivalence of fluorescence lifetimes ($N_1 = N_2 = 6; \alpha =0.05$) shows no synergistic effects and confirms unambiguous isomer determination without previous separation.

<table>
<thead>
<tr>
<th>PAH</th>
<th>Lifetime of standards (ns)</th>
<th>Lifetimes from mixtures (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB[a,l]P</td>
<td>78.0±2.9</td>
<td>77.1±1.6</td>
</tr>
<tr>
<td>DB[a,e]P</td>
<td>55.6±1.4</td>
<td>53.8±1.2</td>
</tr>
<tr>
<td>DB[a,h]P</td>
<td>5.9±0.2</td>
<td>6.0±0.2</td>
</tr>
<tr>
<td>DB[a,i]P</td>
<td>214.2±6.9</td>
<td>220.1±5.0</td>
</tr>
<tr>
<td>DB[e,l]P</td>
<td>53.2±1.2</td>
<td>50.5±2.7</td>
</tr>
</tbody>
</table>

*a Lifetimes and standard deviations are based on six replicate measurements from different frozen aliquots.
CHAPTER 3 INSTRUMENTATION

This chapter presents the instrumentation used to collect the experimental data that is common to all subsequent chapters of this dissertation.

3.1 Room-Temperature Absorption Measurements with Commercial Instrumentation

Absorbance measurements were carried out with a single-beam spectrophotometer (model Cary 50, Varian) equipped with a 75-W pulsed xenon lamp, 2-nm fixed bandpass, ±0.1 nm wavelength precision, and 24 000 nm.min\(^{-1}\) maximum scan rate. All measurements were made by pouring liquid solutions into 1 cm × 1 cm quartz cuvettes.

3.2 Room-Temperature and 77 K Fluorescence Measurements with Commercial Instrumentation

Fluorescence excitation and emission spectra were acquired with a spectrofluorometer from Photon Technology International. The excitation source was a continuous wave 75-W xenon lamp with broadband illumination from 200 to 2000 nm. Detection was made with a photomultiplier tube (model 1527) with spectral response from 185 to 650 nm. The excitation and emission monochromators had the same reciprocal linear dispersion (4 nm.mm\(^{-1}\)) and accuracy (±1 nm with 0.25-nm resolution). Their 1200 grooves/nm gratings were blazed at 300 and 400 nm, respectively. Wavelength reproducibility was ~±2 nm. The instrument was computer controlled using commercial software (Felix32) specifically designed for the system. Excitation and emission spectra were corrected for wavelength dependence of excitation light source and detector sensitivity, respectively. Correction was made in the post acquisition mode.
using the radiometric correction factors included in Felix32 software. Room-temperature measurements were made by pouring liquid solutions into 1 cm×1 cm quartz cuvettes. The 77 K measurements followed the classic procedure of immersing the sample solution in a quartz tube into a nitrogen filled Dewar flask.

### 3.3 Fluorescence and Absorption Measurements at 4.2K

Measurements at 4.2K were carried out with the aid of the FOP and the MLS shown in Figure 2.6 and 2.7, respectively. The complete description of the MLS and its capabilities with regard to fluorescence and phosphorescence measurements have been reported previously. Here we only provide a brief description of the hardware for absorption and fluorescence measurements. Excitation energy is generated by directing the output of a Northern Lights tunable dye laser (Dakota Technologies, Inc.) through a KDP frequency-doubling crystal. When pumped with ~30 mJ from the second harmonic generator of a Nd:YAG Q-switched laser (Quanta Ray), it produces more than 5 mJ at peak of rhodamine 6G in a spectral bandwidth less than 0.03 nm. The minimum wavelength step of the tunable dye laser is 0.1 nm. The multichannel detector consists of a front illuminated ICCD (Andor Technology). The intensifier in front of the CCD chip acts as a superfast shutter, capable of operating on nanosecond time scale. The minimum gate time (full width at half-maximum) is 2 ns. The CCD has the following specifications: active area, 690×256 pixels (26 μm² pixel size at photocathode); dark current, 0.002 electrons pixel⁻¹ s⁻¹; and readout noise, 4 electrons at 20 kHz. The ICCD is mounted at the exit focal plane of a spectrograph (SPEX 270M) equipped with a 1200 grooves/mm grating blazed at 500 nm. The distance between collection probe and the entrance slit of the spectrograph
was optimized to completely illuminate the diffraction grating area. The system is operated in the external trigger mode. A trigger signal from the Nd:YAG laser prepulse trigger is sent to the delay generator (Stanford Research System DG535) 140 ns before the laser fires to account for inherent delays in the electrical cables and components.

Data acquisition parameters (gate delay and gate width) are entered on the control computer with the Andor software and the appropriate control signals are sent via a GPIB interface to the pulse generator. Once triggered by the laser, the pulse generator uses this information to determine when the image intensifier in the detector head is gated on (gate delay) and for how long it is gated on (gate width). When the intensifier is gated off, the acquired data are transferred from the detector head to the controller card (32-bit Intelligent Bus-Mastering PCI card) in the computer. Complete instrument control was carried out with LabView (National Instruments, version 6.0) based software developed in our laboratory.

All spectra were recorded by tuning the grating of the spectrograph to the central wavelength of the spectral range of interest. For each position of the grating, the spectrograph covered a 40-nm spectral range. If a larger spectral range were of interest, the spectrograph would have to be tuned to the central wavelength of the new spectral range and the process repeated. Unless otherwise noted, each spectrum corresponds to the accumulation of 100 laser pulses. A 0-ns delay and a 10-ns gate allowed the CCD to acquire laser scatter during the duration of the pulse (full width at half-maximum (fwhm) ~5 ns). The 10-ns gate width provided satisfactory signal-to-noise ratios with minimum fluorescence interference. Fluorescence spectra were recorded using a minimum delay of 10 ns, which was sufficient to avoid the need to consider convolution of the laser pulse with the analyte signal. The gate width varied with the fluorescence lifetime of the PAH. Gates equivalent to ~ 4× or 5× the lifetime of the PAH were
sufficient to collect most of the PAH fluorescence and still avoid instrumental noise.

For absorption measurements, the limiting resolution of the instrumental system was determined by the laser scanning rate, i.e., 0.1 nm/data point. For fluorescence measurements, the limiting resolution was determined by the multichannel detection system. The limiting resolution of the spectrograph/ICCD was determined with the Hg lamp placed at the analysis end of the fiber-optic probe. The experimental value (0.42 nm) of the fwhm for the Hg line at ~313 nm was within the theoretical value calculated from the spectral range of an individual pixel in the CCD array. Considering the reciprocal linear dispersion ($R_L = 3.1$ nm/mm) of the spectrograph, the number of active pixels ($690 \times 256$) in the array, and the dimensions ($18 \times 6.7$ mm) of the active area of the CCD chip, the spectral range of 1 pixel should give a 0.08-nm limiting resolution. Because of the detector cross talk and the intensifier, the factual limiting resolution corresponds to 4-5 pixels (5), i.e., 0.32-0.40 nm.

3.4 HPLC Analysis

PAH were analyzed using a computer controlled HPLC system from Hitachi (San Jose, CA) equipped with the following basic components: a pump (L-7100), a UV detector (L-7400 UV), a fluorescence detector (L-7485), and an online degasser (L-761). Separation was carried out on a Supelco (Bellefonte, PA) Supelcosil TM LC-PAH column with the following characteristics: 15-cm length, 4-mm diameter, and 5-$\mu$m average particle diameters. Laboratory reagent blanks were run in conjunction with each series of samples using identical conditions of glassware, equipment, solvents, and analysis to ensure absence of interfering contamination. Complete chromatographic separation of EPA-PAH was first monitored via ultraviolet-visible (UV-VIS)
absorption spectroscopy. Satisfactory resolution of the 16 PAH was achieved with a methanol/water mobile-phase gradient. The settings and conditions of the analysis included 1.5 mL/min flow rate, isocratic elution with 60/40 methanol/water for 5 min, and then linear gradient to 99% methanol over 20 min. The total separation time was ~50 min. All sample injections were held constant at 20 μL using a fixed volume injection loop.

Analytical figures of merit (AFOM) in the fluorescence detection mode were obtained using the following time-programmed excitation/emission wavelengths: 0 min, 240/330 nm; 11 min, 234/320 nm; 16.5 min, 252/320 nm; 18 min, 252/370 nm; 20.5 min, 252/402 nm; 22.5 min, 252/402 nm; 23.6 min, 238/398 nm; 26.0 min, 278/395 nm; 31 min, 268/398 nm; 39 min, 304/421 nm; 45 min, 300/466 nm; and 50 min, 300/466 nm. In cases where contiguous PAH elution was too fast for individual wavelength optimization, a compromise was made among the maximum excitation/emission wavelengths of the contiguous PAH. Acenaphthylene was the only PAH to present no RTF under the experimental conditions of this study. Its determination via UV-VIS absorption detection was not attempted.

3.5 Capillary Electrophoresis (CE)

CE was carried out with a GPA100 system purchased from Groton Biosystems. Its detection unit consisted of a variable, single wavelength absorption spectrometer (Model VUV 9022-0000; JMST Systems) equipped with a deuterium lamp and a silicon photodiode detector. The capillary consisted of a single fused-silica tube, 82 cm in length and 50 mm id acquired from Polymicro Technologies. Its polyamide coating was removed with a window maker (MicroSolv-CE) to provide an UV transparent detection window of approximately 2 mm long. Complete instrument
control was performed via PC with customized software (WinPrinCE, PrinCE CE systems). Sample injection was accomplished via hydrodynamic pressure.

Electrophoretic measurements were made with the aid of an 82cm ×50μm I.D. (375μm O.D.) fused-silica capillary (Polymicro Technologies). The polyamide coating was removed with a window maker (MicroSolv-CE) to provide an UV transparent detection window with an approximate length of 2 mm. The center of the optical window was located at 58cm from the injection port. Each day the capillary was rinsed for 30min with 1M NaOH followed by a 5min rinse with buffer to the beginning of data collection. The capillary was rinsed for 30min at the end of each day with Nanopure water to remove any impurities left in the capillary.
CHAPTER 4  DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN DRINKING WATER SAMPLES BY SOLID-PHASE NANO-EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY


4.1 Introduction

In this chapter, we present a novel alternative for the extraction and pre-concentration of PAH. Our approach, which we have named solid-phase nano-extraction (SPNE), takes advantage of the physico-chemical affinity that exists between PAH and gold nanoparticles (Au NPs). Carefully optimization of experimental parameters has led to an SPNE-HPLC method with excellent analytical figures of merit (AFOM). Its most significant figure correlates to the small volume of water sample one needs for complete PAH analyses. 500 μL of water sample are sufficient to obtain outstanding precision of measurements at the parts-per-trillion to parts-per-billion concentration level. The limits of detection (LOD) are excellent as well and range from 0.9 ng.L⁻¹ (anthracene) to 58ng.L⁻¹ (fluorene). The relative standard deviations at medium calibration concentrations varied from 3.2% (acenaphthene) to 9.1% (naphthalene). The analytical recoveries of the six regulated WHO-PAH varied from 83.3 ± 2.4% (benzo[k]fluoranthene) to 90.8 ± 1.2% (benzo[g,h,i]perylene). The outstanding relative standard deviations of the recoveries demonstrate the superior performance of Au NPs over C₁₈ extraction membranes currently used for PAH extraction from water samples.
4.2 Experimental Section

4.2.1 Chemicals

HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). Analytical grade 1-pentanethiol and n-octane were from Acros Organics (Atlanta, GA, USA). Otherwise specified, Nanopure water from a Barnstead Nanopure Infinity water system (Massachusetts, USA) was used throughout. Au NPs were purchased from Ted Pella, Inc. (Redding, CA, USA). All PAH standards were acquired from Sigma-Aldrich (Milwaukee, WI, USA) at their highest available purity (>98%) and used with no further purification.

Note: use extreme cautions when handling PAH that are known to be extremely toxic.

4.2.2 Solution Preparation

Stock PAH solutions were prepared in pure methanol and kept in the dark at 4°C. Possible PAH degradation was monitored via RTF spectroscopy. PAH working solutions were prepared by serial dilution with the appropriate solvent. Commercial solutions of Au NPs were kept in the dark at 4°C. The physical integrity of colloids was monitored via UV-VIS absorption spectroscopy. Working solutions of Au NPs were prepared by diluting commercial solutions with nanopure water. An AccuStandard PAH mixture (Product M-610A) was used as a reference standard for HPLC analysis of 16 EPA-PAH. For PAH identification, a working standard was prepared by diluting 1.00 ml of the AccuStandard mix to a final volume of 10.00 ml in acetonitrile.
4.2.3 Sample Mixing and Centrifugation for PAH Extraction with Gold Colloids

Sample mixing was carried out with a Maxi Mix III Rotary Shaker (Type M65800, Barnstead-Thermolyne) equipped with a PT500X6A Vortex Mixer accessory. Centrifugation was performed with a MiniSpin centrifuge (Eppendorf) with maximum rotational speed of 13,400 rotations per minute (rpm).

4.2.4 UV-VIS Absorption Spectroscopy

See section 3.1 for instrumental details.

4.2.5 RTF Spectroscopy

See section 3.2 for instrumental details.

4.2.6 HPLC Analysis

See section 3.4 for instrumental and experimental details.
4.3 Results and Discussion

4.3.1 Preliminary Studies on the Interaction of PAH with Au NPs

The commercial Au NPs solutions used in these studies were synthesized through a citric acid reduction reaction. Trace amounts of residual citrate ions coexist on the surface of Au NPs and act as electrostatic stabilizing agents. Electrostatic stabilization arises from the mutual repulsion between neighboring NPs carrying citrate layers with negative surface charges. The possibility to extract PAH with Au NPs was first investigated in the absence of citrate ions. Au NPs were generated in our laboratory via laser ablation. This is a well-known process that creates Au NPs without the presence of counterions or surfactants in the final solution. A 10 mm × 10 mm Au foil substrate (purity >99.99%) was placed at the bottom of a quartz cuvette partially filled with 1 mL of Nanopure water. The output of the second harmonic (520 nm) of a 10-Hz Nd:YAG Q-switched solid-state laser (Big Sky Laser Technologies) was directed toward the metallic surface. After 5 min of sample irradiation, the appearance of a dark red color in the supernatant indicated the presence of Au NPs. Absorption spectra recorded from the supernatants showed the characteristic Plasmon resonance structure at ~520 nm, confirming the presence of Au NPs. PAH extraction was immediately tested using benzo[a]pyrene as the model compound. The 990 μL of the Au NPs solution was mixed with 10 μL of a 1 μg ·mL⁻¹ benzo[a]pyrene methanol solution. After sample shaking for 5 min and centrifugation for additional 20 min, the precipitate was separated from the supernatant with a micropipet. RTF spectra of the liquid solution confirmed the absence of benzo[a]pyrene in the supernatant and, therefore, its adsorption to the surface of Au NPs. Complete extraction of benzo[a]pyrene was also observed with commercial solutions of Au NPs. Because the presence of citrate ions does not prevent the extraction of PAH, all further studies were carried out with commercial colloid solutions.
4.3.2 **Centrifugation Time for Au NPs Precipitation**

Quantitative extraction of PAH requires an appropriate centrifugation time for complete collection of Au NPs precipitate. This parameter was investigated via UV-VIS absorption spectroscopy. Au NPs solutions with average particle diameters of 5, 10, 20, 40, 60, 80 and 100 nm were prepared in 1% methanol-water (v/v). This methanol volume was added to facilitate the eventual dissolution of trace levels of PAH. Centrifugation was carried out in 2 mL micro-plain glass tubes at the maximum rotational speed (13,400 rpm) of our centrifuge. Centrifuged solutions of Au NPs with particle diameters equal to or larger than 20 nm presented a clear aqueous phase (supernatant) and a reddish solid phase due to the precipitation of Au NPs. Au NPs with smaller diameters than 20 nm showed no apparent precipitation at the maximum speed of our centrifuge as evidenced by a reddish aqueous phase.

Absorption spectra were recorded after removing the supernatant from the Microplain tube with a glass micro-pipette. Signal intensities were monitored at the maximum absorption wavelengths of the Au surface plasmon resonance bands, which varied between 520.3 nm (20 nm Au NPs diameter) and 566.7 nm (100 nm Au NPs diameter). Figure 4.1 summaries the behavior of the absorption intensity of the supernatant as a function of centrifugation time for 20-, 40- and 60-nm Au NPs diameters. The expected correlation between precipitation time and particle size is clearly observed as the larger (or heavier) Au NPs required shorter centrifugation times for complete precipitation. Further studies were then carried out with 20 min of centrifugation time to assure the complete precipitation of 20-100 nm Au NPs.
Figure 4.1 Absorbance intensity of Au NPs as a function of centrifugation time.

Average diameter of Au NPs was as follows: (●) 20nm, (▲) 40nm and (■) 60nm. The insert shows the absorption spectra of 20 nm particles diameter after 0 (a), 1 (b), 2 (c), 5 (d), 7 (e) and 15 (f) min of centrifugation time.

4.3.3 PAH Precipitation in the Absence of Au NPs

The possibility of PAH adhesion to the walls of the centrifugation vessels or PAH precipitation in the absence of Au NPs - i.e. solely due to centrifugation - were investigated via
RTF spectroscopy. Working solutions were prepared in 1% methanol-water (v/v). Centrifugation was carried out in 2mL Microplain glass tubes for 20 min at 13,400 rpm. Fluorescence intensities were monitored before ($I_{NC}$) and after centrifugation ($I_{C}$) at the maximum excitation and emission wavelengths of each PAH. A complete set of RT excitation and fluorescence spectra of EPA PAH is presented in Appendix A. The statistical comparisons of $I_{NC}$ and $I_{C}$ were based on their average values from triplicate measurements of three individually centrifuged aliquots, i.e. $N_{NC} = N_{C} = 9$. Working solutions that had statistically equivalent $I_{NC}$ and $I_{C}$ were considered free from PAH precipitation. Table 4.1 summaries the maximum concentrations free from PAH precipitation along with their statistical comparison. All maximum values are well above PAH concentrations usually encountered in drinking water samples.
Table 4.1 Maximum PAH concentration free from precipitation.

<table>
<thead>
<tr>
<th>PAH (^a)</th>
<th>Maximum Ex /Em (nm)</th>
<th>PAH concentration (^b) (ng.mL(^{-1}))</th>
<th>(t_{exp}) (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>278/322</td>
<td>30</td>
<td>2.38</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>292/322</td>
<td>50</td>
<td>1.76</td>
</tr>
<tr>
<td>Fluorene</td>
<td>279/306</td>
<td>30</td>
<td>1.02</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>294/347</td>
<td>200</td>
<td>2.53</td>
</tr>
<tr>
<td>Anthracene</td>
<td>251/378</td>
<td>40</td>
<td>2.66</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>288/450</td>
<td>200</td>
<td>0.68</td>
</tr>
<tr>
<td>Pyrene</td>
<td>334/371</td>
<td>75</td>
<td>0.85</td>
</tr>
<tr>
<td>Benzo[(a)]anthracene</td>
<td>287/386</td>
<td>20</td>
<td>2.54</td>
</tr>
<tr>
<td>Chrysene</td>
<td>269/361</td>
<td>20</td>
<td>1.08</td>
</tr>
<tr>
<td>Benzo[(b)]fluoranthene</td>
<td>300/445</td>
<td>10</td>
<td>2.14</td>
</tr>
<tr>
<td>Benzo[(k)]fluoranthene</td>
<td>307/413</td>
<td>30</td>
<td>1.29</td>
</tr>
<tr>
<td>Benzo[(a)]pyrene</td>
<td>298/404</td>
<td>30</td>
<td>1.83</td>
</tr>
<tr>
<td>Dibenz[(a,h)]anthracene</td>
<td>300/469</td>
<td>50</td>
<td>1.20</td>
</tr>
<tr>
<td>Benzo[(g,h,i)]perylene</td>
<td>305/420</td>
<td>10</td>
<td>2.60</td>
</tr>
<tr>
<td>Indeno[(1,2,3-cd)]pyrene</td>
<td>302/510</td>
<td>30</td>
<td>0.96</td>
</tr>
</tbody>
</table>

\(^a\) PAH solutions were made in 1% methanol-water (v/v). \(^b\) Maximum concentration free from PAH precipitation. \(^c\) \(t\)-test of experimental average before (\(I_{NC}\)) and after (\(I_C\)) centrifugation. \(T_{critical} = 3.18\) (\(a = 0.05\); \(N_C = 9\)).
4.3.4 Efficiency of PAH Extraction as a Function of Particle Diameter

Four model PAH, namely phenanthrene, chrysene, benzo[α]pyrene and indeno[1,2,3-cd]pyrene were selected as representative cases of EPA-PAH containing 3, 4, 5 and 6 rings, respectively. All PAH were extracted at the 10ng.mL$^{-1}$ concentration from standard solutions with the same solvent composition, i.e. 1% methanol/water (v/v). All extracting solutions were prepared by diluting an appropriate volume of commercial colloid solution with appropriate volumes of water and methanol to provide a 1% methanol/water (v/v) final solvent composition. The final concentration of Au NPs was the same ($5.54 \times 10^9$ particles per milliliter) for all particle diameters and so it was their final solvent composition (1% methanol/water (v/v)). Extracting solutions for each particle diameter were prepared from three different commercial batches to account for possible batch-to-batch variations. The extraction procedure consisted of shaking 0.95 mL of PAH solution with 0.05 mL of NPs solution for 5min followed by 20 min of mixture centrifugation. The shaking time was found to be long enough to achieve maximum PAH extraction at 1400rpm mixing speed. Table 4.2 summaries the extraction efficiencies as a function of average particle size diameter calculated from RTF signals of PAH standards before and after centrifugation. The signal intensities from the supernatants, i.e. PAH solutions after centrifugation, were measured in the absence of Au NPs to avoid potential fluorescence quenching. As expected, the number of PAH molecules extracted per particle increased with particle diameter and total surface area of Au. More interesting to note, however, was the inverse trend we observed from both the number of extracted molecules per gram of Au and the number of extracted molecules per nm$^2$ of surface area of Au. Because the smaller particles appear to be more efficient than the larger particles, our further studies focused on 20 nm Au NPs.
### Table 4.2 PAH extraction as a function of Au nanoparticle diameter and total surface area of Au.

<table>
<thead>
<tr>
<th>D nm</th>
<th>TSA&lt;sup&gt;b&lt;/sup&gt; (10&lt;sup&gt;14&lt;/sup&gt;nm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Phenanthrene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chrysene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Benzo[a]pyrene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Indeno[1,2,3-cd]pyrene&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>molec&lt;sup&gt;c&lt;/sup&gt; 10&lt;sup&gt;20&lt;/sup&gt; molec&lt;sup&gt;d&lt;/sup&gt;</td>
<td>molec&lt;sup&gt;c&lt;/sup&gt; g</td>
<td>molec&lt;sup&gt;c&lt;/sup&gt; 10&lt;sup&gt;20&lt;/sup&gt; molec&lt;sup&gt;d&lt;/sup&gt;</td>
<td>molec&lt;sup&gt;c&lt;/sup&gt; g</td>
<td>molec&lt;sup&gt;c&lt;/sup&gt; 10&lt;sup&gt;20&lt;/sup&gt; molec&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>20</td>
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<td>698</td>
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<td>0.627</td>
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<td>2,658</td>
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<td>7.7</td>
<td>0.198</td>
<td>3,309</td>
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<td>100</td>
<td>1.742</td>
<td>5,368</td>
<td>5.3</td>
<td>0.171</td>
<td>4,832</td>
</tr>
</tbody>
</table>

<sup>a</sup> Initial PAH concentration was 10ppb. All working solutions were prepared in 1% methanol/water (v/v). <sup>b</sup> Surface area of one particle was calculated according to the formula 4πr<sup>2</sup>, where r is the radius of the particle. Total surface area of Au in each working solution was calculated according to the formula: [No. of Au NPs in working solution] × [surface area of one particle].<sup>c</sup> Average number of extracted molecules per Au particle = [number of PAH molecules in 1mL of working solution] × [extraction efficiency] / 5.544 x 10<sup>9</sup> particles. Number of PAH molecules in 1mL of working solution were calculated according to the formula: [10x10<sup>-9</sup>g.mL<sup>-1</sup>x1mL / formula weight of PAH (g/mol)] x [6.02x10<sup>23</sup> molecules/mol].<sup>d</sup> Average number of extracted molecules per gram of Au = [ average molecules per particle] / [ mass of one particle in gram]. The density of Au is 19.3g/mL.<sup>e</sup> Average number of extracted molecules per nm<sup>2</sup> of surface area of Au = [average molecules per particle] / [surface area of one particle in nm<sup>2</sup>].
4.3.5 Maximum Amount of Extractable PAH and its Correlation to the Volume of Commercial Solution for Complete PAH Extraction

The extraction procedure was the same as the one previously described. The concentration of 20nm Au NPs was kept constant in all extracting solutions \((3.5 \times 10^{10} \text{ particles per milliliter})\) while PAH concentrations were varied within their respective linear dynamic ranges. PAH extraction was monitored via RTF spectroscopy by comparing the RTF intensities of supernatants to those recorded from “control” solutions, i.e. PAH standard solutions submitted to the same experimental procedure but in the absence of Au NPs.

Figure 4.2 shows the experimental results obtained for the model PAH. All linear fittings were made via the least squares method. The straight lines correlating the data points labeled as (▲) represent the fluorescence intensities of PAH standard solutions in the absence of Au NPs. The straight line segments representing data points labeled as (●) and (■) correspond to the fluorescence intensities of supernatants after centrifugation with Au NPs. Statistical comparison showed that the fluorescence intensities within the (●) segments were statistically equivalent to the blank signals \((\alpha = 0.05, N_1 = N_2 = 3)\). This fact accounts for the absence of PAH in the supernatants and complete PAH extraction within the concentration ranges represented as (●). Fluorescence intensities within the (■) segments were statistically different from blank signals and increased linearly with PAH concentrations. The signal intensities in the (■) segments were lower than the intensities in the (▲) segments as a result of the lower PAH concentrations in the supernatants. The lower concentrations reflect the amount of PAH extracted with Au NPs. The intercepts of (●) and (▲) segments provide an estimate of the maximum concentration of extracted PAH. A complete set of “titration” curve for EPA PAH is presented in Appendix B.
Figure 4.2 Fluorescence intensity as a function of PAH concentration (ng.mL\(^{-1}\)): (A) = phenanthrene; (B) = chrysene; (C) = benzo[a]pyrene; (D) = indeno[1,2,3-cd]pyrene.

Signal intensities were recorded from PAH standard solutions prepared in 1% methanol/water (v/v) (▲) and from supernatants of PAH standards previously extracted with 5% 20nm Au NPs solutions (●/■). Best linear fittings were calculated via the least squares method. (cps = counts per second)
Table 4.3 lists the maximum PAH concentration that a 5% 20 nm Au NP solution can extract from individual PAH standard solutions. The maximum extractable mass of PAH per Au NP (MEM<sub>PAH</sub>) was calculated dividing the maximum extracted concentration by the number of Au NPs in the extracting solution (3.5x10<sup>10</sup>). The minimum number of Au NPs (mN<sub>Au NPs</sub>) necessary to completely extract a certain mass of PAH in a given volume of water sample (V<sub>H2O</sub>) can then be estimated with the following equation:

\[
mN_{Au NPs} = \frac{[PAH] \times V_{H2O}}{MEM_{PAH}}
\]  

Table 4.3 Maximum amount of PAH extracted with 5% (v/v) 20 nm Au NPs.

<table>
<thead>
<tr>
<th>PAH</th>
<th>Max. conc. b (ng.mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>MEM&lt;sub&gt;PAH&lt;/sub&gt; c (10&lt;sup&gt;-10&lt;/sup&gt; ng/particle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>4.54 ± 0.11</td>
<td>1.30 ± 0.03</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>6.08 ± 0.15</td>
<td>1.74 ± 0.04</td>
</tr>
<tr>
<td>Fluorene</td>
<td>6.09 ± 0.13</td>
<td>1.74 ± 0.04</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>6.98 ± 0.28</td>
<td>1.99 ± 0.08</td>
</tr>
<tr>
<td>Anthracene</td>
<td>5.71 ± 0.13</td>
<td>1.63 ± 0.04</td>
</tr>
<tr>
<td>Fluoranthen</td>
<td>6.93 ± 0.29</td>
<td>1.98 ± 0.08</td>
</tr>
<tr>
<td>Pyrene</td>
<td>5.67 ± 0.03</td>
<td>1.62 ± 0.01</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>6.61 ± 0.23</td>
<td>1.89 ± 0.07</td>
</tr>
<tr>
<td>Chrysene</td>
<td>6.50 ± 0.06</td>
<td>1.86 ± 0.02</td>
</tr>
<tr>
<td>Benzo[b]fluoranthen</td>
<td>7.48 ± 0.27</td>
<td>2.14 ± 0.08</td>
</tr>
<tr>
<td>Benzo[k]fluoranthen</td>
<td>6.36 ± 0.32</td>
<td>1.82 ± 0.09</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>7.46 ± 0.61</td>
<td>2.13 ± 0.17</td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene</td>
<td>6.12 ± 0.13</td>
<td>1.75 ± 0.04</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>6.55 ± 0.08</td>
<td>1.87 ± 0.02</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd] pyrene</td>
<td>6.58 ± 0.03</td>
<td>1.88 ± 0.01</td>
</tr>
</tbody>
</table>

a All working solutions were prepared in 1% methanol/water (v/v). Reported values are the averages of 3 independent extractions from the same PAH solution.

b Maximum concentration of extracted PAH; it was calculated as shown in figure 2.

c MEM<sub>PAH</sub> = Maximum mass of extracted PAH per Au NP unit; it was calculated according to the formula: [Max. conc.] / 3.5 x 10<sup>10</sup> particles / mL.
where \([PAH]\) refers to the PAH concentration (ng.mL\(^{-1}\)) in the water sample. Based on this expression, the co-extraction of a multi-PAH mixture should lead to the following total minimum number of Au NPs (\(T_{mN\text{ Au NPs}}\)):

\[
T_{mN\text{ Au NPs}} = \sum_{i=1}^{n} [PAH]_i \frac{V_{H2O}}{MEM_{PAHi}}
\] (4.2)

where \(i\) refers to each type of PAH in the mixture. The volume of commercial solution (\(V_{CS}\)) with known concentration of Au NPs (\(C_{CS}\)) can be adjusted to match the total minimum number of Au NPs, i.e. \(T_{mN\text{ Au NPs}} = C_{CS} \times V_{CS}\). As such, the minimum volume of commercial solution (\(mV_{CS}\)) necessary to completely extract a multi-PAH mixture can then be estimated as follows:

\[
mV_{CS} = \sum_{i=1}^{n} [PAH]_i \frac{V_{H2O}}{C_{CS} \times MEM_{PAHi}} = \left(\sum_{i=1}^{n} \frac{[PAH]_i}{MEM_{PAHi}}\right) \frac{V_{H2O}}{C_{CS}}
\] (4.3)

Because Eq. (4.1-4.3) do not account for the potential co-extraction of sample concomitants and the resulting up-take of particle surface, the analyst should always use a larger extracting volume than its minimum value, i.e. \(V_{CS} > mV_{CS}\). Considering that PAH contamination in drinking water usually occurs at the sub-nanogram.mL\(^{-1}\) concentration levels, we arbitrarily set \(V_{H2O} = 0.5\text{mL}\) and \(V_{CS} = 0.95\text{mL}\) for all further experiments. Based on Eq. (4.3), 0.95mL of a 20nm Au NPs solution with \(C_{CS} = 7.0 \times 10^{11}\) particles.mL\(^{-1}\) should account for the complete extraction of the 15 EPA-PAH at the individual concentration level of \(\sim 1.6 \times 10^4\) ng.L\(^{-1}\). This concentration is well above PAH concentrations usually encountered in drinking water samples.

4.3.6 Releasing PAH for HPLC Analysis

The forethought strategy to release PAH molecules from the surface of Au NPs was to
use an organic solvent with strong binding affinity for Au colloids which, upon its binding to the metallic surface, would release PAH to the surrounding medium. Several solvents were attempted for this purpose including alcohols (methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol and 1-octanol), alkanes (hexane, heptane and octane) and alkane thiols (1-pentanethiol, 1-hexanethiol, 1-heptanethiol and 1-octanethiol).

PAH extractions were carried out from 500μL aliquots of Nanopure water previously spiked with 15 EPA-PAH at the $1 \times 10^3 \text{ng.L}^{-1}$ concentration level. This concentration ($C_{\text{PAH}}$) is five times higher than the highest MCL (200ng.L$^{-1}$) of regulated PAH. After sample centrifugation, the supernatant (supernatant A) was removed from the vial and saved for HPLC analysis. Micro-litter volumes of releasing solvent were added to the precipitate and mixed with 48μL of n-octane to favor PAH partitioning into a non-polar environment. The new mixture was shaken for 5min at 1400 rpm and centrifuged for 20min at 13,400rpm. Its supernatant (supernatant B) was separated from the precipitate and saved for HPLC analysis.

PAH concentrations in supernatants A ($[\text{PAH}]_A$) and B ($[\text{PAH}]_B$) were determined via the calibration curve method. Their values were used in Eq. 4.4 and 4.5 to calculate the overall recovery of SPNE-HPLC (OR) analysis and the extraction efficiency (E) of SPNE:

\[
\text{OR} = 100 \frac{[\text{PAH}]_B \times V_B}{C_{\text{PAH}} \times V_W} \quad (4.4)
\]
\[
\text{E} = 100\frac{(C_{\text{PAH}} \times V_W - [\text{PAH}]_A \times V_A)}{C_{\text{PAH}} \times V_W} \quad (4.5)
\]

where $V_W$ refers to the volume of extracted water and $V_A$ and $V_B$ to the volumes of supernatants A and B, respectively. The releasing efficiencies (RE) of the studied solvents were calculated with the following equation:
\[ \text{RE} = 100\left(\frac{[\text{PAH}]_B \times V_B}{C_{\text{PAH}} \times V_W - [\text{PAH}]_A}\right) \quad (4.6) \]

The best releasing efficiencies were obtained with alkane thiols, more specifically with 2 to 4 \( \mu \)L of 1-pentanethiol. As shown in Figure 4.3, RE varied from ~ 50 (fluorene) to ~ 90% (anthracene, benzo[b]fluoranthene, dibenz[a,h]anthracene and benzo[g,h,i]perylene). The observed differences among PAH reflect the relative affinity of PAH toward the surface of Au NPs and their surroundings. Because no much difference was observed within this volume range, all further studies were performed with 2 \( \mu \)L of 1-pentanethiol. Table 4.4 summarizes the OR, E and RE values obtained with this solvent volume of 1-pentanethiol.
Figure 4.3 Effect of 1-pentanethiol on the releasing efficiency of extracted PAH with 20nm Au NPs.

Extracts made from 500mL of Nanopure water spiked with the 15 EPA-PAH at the 1000ng.L⁻¹ individual concentration.
Table 4.4 Recoveries for the three-step SPNE procedure.

<table>
<thead>
<tr>
<th>PAH a</th>
<th>OR (%) b</th>
<th>E (%) c</th>
<th>RE (%) d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>64.7 ± 3.3</td>
<td>82.2 ± 0.8</td>
<td>78.7 ± 4.1</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>71.6 ± 2.1</td>
<td>96.3 ± 2.3</td>
<td>74.4 ± 2.8</td>
</tr>
<tr>
<td>Fluorene</td>
<td>55.7 ± 3.1</td>
<td>98.1 ± 1.2</td>
<td>56.8 ± 3.2</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>88.4 ± 1.8</td>
<td>98.8 ± 0.4</td>
<td>89.5 ± 1.9</td>
</tr>
<tr>
<td>Anthracene</td>
<td>94.6 ± 1.6</td>
<td>99.3 ± 0.4</td>
<td>95.3 ± 1.7</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>85.0 ± 3.0</td>
<td>97.1 ± 0.3</td>
<td>87.5 ± 3.1</td>
</tr>
<tr>
<td>Pyrene</td>
<td>85.8 ± 2.5</td>
<td>95.6 ± 0.3</td>
<td>89.7 ± 2.6</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>68.4 ± 2.8</td>
<td>98.6 ± 1.5</td>
<td>69.4 ± 3.0</td>
</tr>
<tr>
<td>Chrysene</td>
<td>74.9 ± 2.1</td>
<td>97.1 ± 0.3</td>
<td>77.1 ± 2.2</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>88.5 ± 1.4</td>
<td>98.3 ± 1.1</td>
<td>90.0 ± 1.7</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>83.3 ± 2.4</td>
<td>94.1 ± 0.8</td>
<td>88.5 ± 2.7</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>88.6 ± 2.3</td>
<td>96.3 ± 0.6</td>
<td>92.0 ± 2.5</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>87.9 ± 0.8</td>
<td>89.0 ± 1.0</td>
<td>98.8 ± 1.4</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>83.1 ± 1.8</td>
<td>86.8 ± 2.8</td>
<td>95.7 ± 4.1</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>85.0 ± 2.5</td>
<td>97.5 ± 0.4</td>
<td>86.7 ± 2.6</td>
</tr>
</tbody>
</table>

a All PAH were prepared in 1% (v/v) methanol. b Overall recovery. PAH were extracted by 20nm gold nanoparticles from water solution and released by 2 µL 1-pentanethiol in 48µL octane. c Extraction efficiency of 20nm gold nanoparticles. d Release efficiency is the percent of PAH released from the surface of 20nm nanoparticles by 1-pentanethiol. They were calculated by the equation: OR(%) = E(%) x RE(%).
4.3.7 Analytical Figures of Merit

Table 4.5 summaries the AFOM obtained via SPNE-HPLC analysis. Aqueous standards were prepared by spiking milliliters of Nanopure water with micro-liters of PAH solutions in 1% methanol-water (v/v). SPNE was carried out with 500μL water aliquots as previously described. All linear dynamic ranges are based on the average intensities of at least five PAH concentrations. The average intensities correspond to a minimum of nine HPLC measurements made from triplicate aliquots collected from three complete experimental trials. No efforts were made to reach the upper concentration limits of the calibration curves. The lowest linear concentrations correspond to the limits of quantitation, which were calculated as 10S_B/m, where S_B is the standard deviation of 16 blank determinations at the peak base and m is the slope of the calibration curve. The excellent correlation coefficients (R) demonstrate the existence of linear relationships in all cases. The limits of detection – which were calculated as 3S_B/m - varied from 0.9 (phenanthrene) to 58ng/L (fluorene). Although there is a wide range of values – which probably reflects the differences in both overall recoveries and fluorescence intensities of the studied PAH - all LOD are well below the MCL of regulated PAH. The relative standard deviations (RSD) demonstrate excellent precision of measurements at the parts-per-trillion concentration level.
Table 4.5 Analytical figures of merit via SPNE-HPLC.

<table>
<thead>
<tr>
<th>PAH a</th>
<th>Retention (min)</th>
<th>Ex/Em b (nm)</th>
<th>LDR c (ng.L⁻¹)</th>
<th>R²</th>
<th>LOD d (ng.L⁻¹)</th>
<th>RSD e (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>9.11</td>
<td>240/330</td>
<td>160 -1000</td>
<td>0.9921</td>
<td>48</td>
<td>9.1</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>15.07</td>
<td>234/320</td>
<td>7 -1000</td>
<td>0.9972</td>
<td>2.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Fluorene</td>
<td>16.98</td>
<td>252/320</td>
<td>193 -1000</td>
<td>0.9764</td>
<td>58</td>
<td>5.6</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>18.79</td>
<td>252/370</td>
<td>3 -1000</td>
<td>0.9983</td>
<td>0.9</td>
<td>7.0</td>
</tr>
<tr>
<td>Anthracene</td>
<td>20.99</td>
<td>252/402</td>
<td>22 -1000</td>
<td>0.9965</td>
<td>6.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>22.83</td>
<td>284/464</td>
<td>126 -1000</td>
<td>0.9869</td>
<td>38</td>
<td>3.9</td>
</tr>
<tr>
<td>Pyrene</td>
<td>23.78</td>
<td>238/398</td>
<td>6 -1000</td>
<td>0.992</td>
<td>1.6</td>
<td>5.7</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>29.11</td>
<td>278/395</td>
<td>5 -1000</td>
<td>0.9916</td>
<td>1.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Chrysene</td>
<td>30.29</td>
<td>278/395</td>
<td>14 -1000</td>
<td>0.9893</td>
<td>4.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>33.13</td>
<td>268/398</td>
<td>37 -1000</td>
<td>0.9958</td>
<td>11</td>
<td>5.3</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>35.35</td>
<td>268/398</td>
<td>25 -1000</td>
<td>0.9937</td>
<td>7.5</td>
<td>6.1</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>36.45</td>
<td>268/398</td>
<td>4 -1000</td>
<td>0.996</td>
<td>1.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene</td>
<td>41.51</td>
<td>304/421</td>
<td>94 -1000</td>
<td>0.9943</td>
<td>28</td>
<td>3.7</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>42.53</td>
<td>304/421</td>
<td>8 -1000</td>
<td>0.9905</td>
<td>2.3</td>
<td>4.9</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>45.96</td>
<td>300/466</td>
<td>87-1000</td>
<td>0.9974</td>
<td>26</td>
<td>4.3</td>
</tr>
</tbody>
</table>

a All working solutions were prepared in 1% methanol/water (v/v). b Excitation and emission set for the HPLC fluorescence detector. c Limit of quantitation (LOQ) is calculated by equation: 10 S b / m. d Limit of detection (LOD) estimated as 3 S b / m. e Relative standard deviation (RSD) is calculated according to the medium concentration of the linear dynamic range (LDR).

4.3.8 PAH Determination in Tap Water

The feasibility to monitor PAH in tap water was investigated with three water samples of unknown matrix composition. Each water sample was collected at a different location within the city of Orlando, FL, and examined in triplicate. Milliliter volumes of tap water were spiked with microliter volumes of PAH standard solutions to provide final concentrations at the MCL of the regulated PAH. SPNE-HPLC analysis was carried out with 500μL aliquot samples. Table 4.6 summarizes the average recoveries and their standard deviations for the three tested samples. The RSD of the recoveries were lower than 25% and, therefore, meeting the RSD criteria (RSD =
25%) for regulated PAH.\textsuperscript{54} Comparison to overall recoveries in Table 4.4 shows no effects from the matrix composition of the unknown drinking water samples.

Table 4.6 PAH recoveries from drinking water samples.

<table>
<thead>
<tr>
<th>PAH</th>
<th>Recovery (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sample 1</td>
<td>sample 2</td>
<td>sample 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rec.</td>
<td>RSD</td>
<td>rec.</td>
<td>RSD</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>55.6 ± 6.26</td>
<td>11.3</td>
<td>53.1 ± 2.66</td>
<td>5.01</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>77.3 ± 3.81</td>
<td>4.93</td>
<td>71.6 ± 4.16</td>
<td>5.81</td>
</tr>
<tr>
<td>Fluorene</td>
<td>45.4 ± 3.18</td>
<td>7.00</td>
<td>48.6 ± 5.25</td>
<td>10.8</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>90.1 ± 2.72</td>
<td>3.02</td>
<td>91.2 ± 3.53</td>
<td>3.87</td>
</tr>
<tr>
<td>Anthracene</td>
<td>93.2 ± 3.61</td>
<td>3.87</td>
<td>86.2 ± 4.07</td>
<td>4.72</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>78.9 ± 6.44</td>
<td>8.16</td>
<td>72.9 ± 4.38</td>
<td>6.01</td>
</tr>
<tr>
<td>Pyrene</td>
<td>83.5 ± 3.06</td>
<td>3.66</td>
<td>78.6 ± 4.32</td>
<td>5.50</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>65.9 ± 3.34</td>
<td>5.07</td>
<td>60.1 ± 1.56</td>
<td>2.60</td>
</tr>
<tr>
<td>Chrysene</td>
<td>67.5 ± 4.39</td>
<td>6.50</td>
<td>61.5 ± 2.48</td>
<td>4.03</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>85.3 ± 3.25</td>
<td>3.81</td>
<td>80.9 ± 5.44</td>
<td>6.72</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>85.8 ± 3.62</td>
<td>4.22</td>
<td>89.8 ± 2.63</td>
<td>2.93</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>87.8 ± 4.26</td>
<td>4.85</td>
<td>81.8 ± 3.13</td>
<td>3.83</td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene</td>
<td>88.1 ± 5.05</td>
<td>5.73</td>
<td>81.2 ± 3.52</td>
<td>4.33</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>86.9 ± 4.68</td>
<td>5.39</td>
<td>79.7 ± 3.62</td>
<td>4.54</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>82.4 ± 4.96</td>
<td>6.02</td>
<td>75.6 ± 2.57</td>
<td>3.40</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All working solutions were prepared in 1% methanol/water (v/v). With the exception of benzo[a]pyrene, which was spiked at the 10 ng · L\textsuperscript{-1} concentration, all remaining PAH were spiked at the 200 ng · L\textsuperscript{-1} concentration. \textsuperscript{b} Relative standard deviation.
4.4 Conclusions

SPNE is still in its infancy, and further studies are certainly needed to fully understand the adsorption of PAH on Au colloids. But the facts presented in this chapter make this approach extremely attractive for the problem at hand. Within the 20-100-nm particle diameter range, the 20-nm Au NPs showed the best PAH extraction efficiencies. Independent of the size of the studied PAH, i.e., 3-6 rings, the 20-nm particles extracted more PAH molecules per gram of Au than their larger counterparts.

Although a straightforward LOD comparison to those reported in the literature via HPLC is difficult as different instrumental setups, experimental and mathematical approaches have been used for their calculation,\textsuperscript{151,158-164} our LOD compare favorably. This comparison becomes relevant mainly if one considers the small water volume (500 $\mu$L) one needs for complete PAH analysis. To the extent of our literature search, there are no methods capable to detect these levels of PAH in microliters of water sample. This feature makes SPNE well suited for routine analysis of numerous samples as the small sample volume facilitates the implementation of simultaneous sample extraction. The entire extraction procedure consumes less than 100 $\mu$L of organic solvents per sample, which makes it environmentally friendly. The small volume of extracting solution - i.e., 0.95 mL of 20-nm Au NPs per water sample - adds US $\sim$2.25 to the total analysis cost, which makes SPNE a relatively inexpensive extraction approach.
CHAPTER 5  SOLID-PHASE NANO-EXTRACTION AND LASER-EXCITED TIME-RESOLVED SHPOL’SKII SPECTROSCOPY FOR THE ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS IN DRINKING WATER SAMPLES


5.1 Introduction

New instrumental developments for chromatography\textsuperscript{165-169} and spectroscopy,\textsuperscript{138,170-172} as well as sensors,\textsuperscript{173-177} have made PAH analysis more selective and sensitive. Despite the sophisticated arsenal of analytical tools, PAH monitoring via simple and cost effective methods of analyses still remains an analytical challenge.\textsuperscript{151,158-160,25} The basis for standard PAH identification and determination.\textsuperscript{164} Particularly challenging is the analysis of PAH in drinking water samples.\textsuperscript{52-54} The ideal approach should provide fast analysis time via a selective and sensitive method for individual PAH determination. Additional desirable features should include low analysis cost and rather low usage of organic solvents via an environmentally friendly experimental procedure.

This chapter presents methodology with significant advances on all fronts. We combine SPNE to LETRSS for the direct analysis of 15 EPA-PAH without previous chromatographic separation. The analytical performance of SPNE-LET RSS is demonstrated with the analysis of three tap water samples of unknown composition. The entire experimental procedure – including SPNE and LETRSS measurements – takes less than 40min per sample. The method is well suited for routine analysis of numerous samples as the small volumes of water sample (500\textmu L) and organic solvents (50\textmu L) facilitate the implementation of simultaneous sample extraction via an
environmentally friendly experimental procedure. Limits of detection are at the parts-per-trillion level. Analytical recoveries are similar to those obtained via HPLC. The simplicity of the experimental procedure, use of micro-liters of organic solvent, short analysis time, selectivity, and excellent analytical figures of merit demonstrate the advantages of this approach for routine analysis of numerous samples.

5.2 Experimental Section

5.2.1 Reagents

HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). Analytical grade 1-pentanethiol and n-octane were from Acros Organics (Atlanta, GA, USA). Nanopure water from a Barnstead Nanopure Infinity water system (Massachusetts, USA) was used throughout. Gold nanoparticles (Au NPs) were purchased from Ted Pella, Inc. (Redding, CA, USA). All PAH standards were acquired from Sigma-Aldrich (Milwaukee, WI, USA) at their highest available purity (>98%) and used with no further purification.

Note: use extreme cautions when handling PAH that are known to be extremely toxic.

5.2.2 Solution Preparation

Stock PAH solutions were prepared in pure methanol and kept in the dark at 4°C. Possible PAH degradation was monitored via room temperature fluorescence spectroscopy. PAH working solutions were prepared by serial dilution with the appropriate solvent. Commercial solutions of Au NPs were kept in the dark at 4°C. The physical integrity of the Au colloids was
monitored via ultraviolet (UV)-visible absorption spectroscopy. Working solutions of Au NPs were prepared by diluting commercial solutions with nanopure water. An AccuStandard PAH mixture (Product M-610A) was used as a reference standard for HPLC analysis of 16 EPA-PAH. For PAH identification, a working standard was prepared by diluting 1 ml of the AccuStandard mix to a final volume of 10 ml in acetonitrile.

5.2.3 SPNE of PAH from Water Samples

Figure 5.1 shows a schematic diagram of the SPNE procedure. The shaking and centrifugation times were optimized for maximum PAH extraction and minimum analysis time. Sample mixing was carried out at 1400-rpm mixing speed with a Maxi Mix III Rotary Shaker (Type M65800, Barnstead-Thermolyne) equipped with a PT500X6A Vortex Mixer accessory. Centrifugation was performed in 2 mL micro-plain glass tubes at the maximum rotational speed of our centrifuge, which consisted of a MiniSpin centrifuge (Eppendorf) with maximum rotational speed of 13,400 rotations per minute (rpm). The volumes of 1-pentanethiol and n-octane were optimized to provide maximum PAH release into the supernatant with the minimum possible usage of organic solvents. PAH determination in the final supernatant was carried out either via HPLC or LETRSS analysis.

5.2.4 HPLC Analysis

See section 3.4 for instrumental and experimental details.
Figure 5.1 Schematic diagram with the experimental steps of the SPNE procedure for the analysis of PAH in drinking water samples.

V_{H2O} refers to the volume of extracted water sample (500μl), and V_{CS} and C_{CS} refer to the volume (0.95ml) and concentration (7×10^{11} particle.ml^{-1}) of Au NPs in the extracting solution, respectively.

5.2.5  *Survey of 77K-Excitation and Fluorescence Spectra*

See section 3.2 for instrumental and experimental details.

5.2.6  *Instrumentation for LETRSS Analysis*

See section 3.3 for instrumental details.

5.2.7  *Spectral Acquisition with the MLS*

See section 3.3 for experimental details.
5.2.8 Lifetime Acquisition with the MLS

Fluorescence lifetimes were measured via the WTM procedure, which consists of the following three steps: (i) full sample and background WTM collection; (ii) background decay curve subtraction from the fluorescence decay curve at a wavelength of maximum emission for each PAH, and (iii) fitting of the background corrected data to single exponential decays. In cases of unknown sample composition where the formulation of a correct blank for lifetime background correction was not possible, the fluorescence decay at the base of the target peak was used for background subtraction at the target wavelength. All fluorescence lifetimes were recorded using 5ns minimum delay. This delay was sufficient to avoid the need to consider convolution of the dye laser pulse with the analytical signal. The accuracy of this procedure has been confirmed previously.148 Origin software (version 7.5, Micronal Software) was used for curve fitting of fluorescence decays. Fluorescence lifetimes were obtained from decay curves fitted to $y = y_0 + A_1 e^{-(x-x_0)/t_1}$ were obtained by fixing $x_0$ and $y_0$ at a value of zero.

5.2.9 77K and 4.2K Sample Procedures

77K measurements with the spectrofluorimeter followed the classic procedure of immersing the sample solution in a quartz tube into a nitrogen filled Dewar flask. 4.2 K measurements were carried out as follows. After transferring a known volume (typically 100–200 μl) of undegassed sample solution with a pipette into the sample vial of the cryogenic probe, the tip of the fiber-optic bundle was positioned and held constant with the screw cap above the solution surface. Sample freezing was accomplished by lowering the copper tubing into the liquid helium, which was held in a Dewar flask with 60 L storage capacity. The liquid helium would typically last 3 weeks with daily use, averaging 15 to 20 samples per day. Complete
sample freezing took less than 90 s per sample. Replacing the frozen sample involved removing the sample vial from the cryogen container and melting the frozen sample with a heat gun. Because no physical contact between the tip of the fiber-optic bundle and the sample ever occurred during measurements, probe cleanup between measurements was not necessary. The entire freeze, thaw, and sample replacement cycle took no longer than 5 min.

5.3 Results and Discussion

5.3.1 HPLC Analysis of EPA PAH

For the purpose of comparison, Table 5.1 combines the information previously presented in Table 4.4 and 4.6 for the analysis of EPA-PAH in Nanopure and drinking water via SPNE-HPLC, respectively. Comparison among the recoveries from Nanopure and tap water samples shows no clear trend that indicates possible matrix interference from the tap water samples. In both cases, the observed differences among overall recoveries probably reflect the relative affinity of PAH towards the surface of Au NPs and their surroundings, which include water, 1-pentanethiol and n-octane (see experimental procedure in figure 5.1).
Table 5.1 Overall recoveries for EPA-PAH via SPNE-HPLC of three water sample tested.

<table>
<thead>
<tr>
<th>PAH</th>
<th>Retention time (min)</th>
<th>Recovery (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Nanopure water&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Tap water&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>9.11 ± 0.08</td>
<td>64.7 ± 3.3</td>
<td>54.3 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>15.07 ± 0.05</td>
<td>71.6 ± 2.1</td>
<td>74.5 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Fluorene</td>
<td>16.98 ± 0.06</td>
<td>55.7 ± 3.1</td>
<td>44.6 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>18.79 ± 0.09</td>
<td>88.4 ± 1.8</td>
<td>89.3 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Anthracene</td>
<td>20.99 ± 0.08</td>
<td>94.6 ± 1.6</td>
<td>90.5 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>22.83 ± 0.11</td>
<td>85.0 ± 3.0</td>
<td>76.1 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Pyrene</td>
<td>23.78 ± 0.07</td>
<td>85.8 ± 2.5</td>
<td>80.0 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>29.11 ± 0.03</td>
<td>68.4 ± 2.8</td>
<td>60.6 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>Chrysene</td>
<td>30.29 ± 0.06</td>
<td>74.9 ± 2.1</td>
<td>65.4 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>33.13 ± 0.07</td>
<td>88.5 ± 1.4</td>
<td>83.2 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>35.35 ± 0.12</td>
<td>83.3 ± 2.4</td>
<td>86.9 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>36.45 ± 0.09</td>
<td>88.6 ± 2.3</td>
<td>85.1 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene</td>
<td>41.51 ± 0.02</td>
<td>87.9 ± 0.8</td>
<td>85.2 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>Benzo[g,h,i]pyrene</td>
<td>42.53 ± 0.05</td>
<td>83.1 ± 1.8</td>
<td>84.4 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>45.96 ± 0.11</td>
<td>85.0 ± 2.5</td>
<td>78.9 ± 3.4</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> All work solution were made in 1% (v/v) methanol. <sup>b</sup> Overall recovery. PAH were extracted by 20nm gold nanoparticles from water solution and released by 2 µL 1-pentanethiol in 48µL octane. <sup>c</sup> Overall recoveries obtained with nanopure water samples. <sup>d</sup> Overall recoveries obtained with three tap water samples.

Figure 5.2 compares the fluorescence intensities of two chromatograms recorded from the same water sample via HPLC and SPNE-HPLC. EPA-PAH concentrations were adjusted to provide a spiked sample with final concentrations at the parts-per-trillion level. Clearly, the pre-concentration afforded by SPNE makes possible PAH analysis at the concentration levels below the maximum contaminant levels of regulated PAH.
Figure 5.2 Chromatograms of 15 EPA PAH obtained by SPNE-HPLC (A) and HPLC (B).

I) naphthalene 250 ng.L⁻¹; II) acenaphthene 50 ng.L⁻¹; III) fluorene 250 ng.L⁻¹; IV) phenanthrene 50 ng.L⁻¹; V) anthracene 100 ng.L⁻¹; VI) fluoranthene 250 ng.L⁻¹; VII) pyrene 50 ng.L⁻¹; VIII) benzo[a]anthracene 50 ng.L⁻¹; IX) chrysene 25 ng.L⁻¹; X) benzo[b]fluoranthene 50 ng.L⁻¹; XI) benzo[k]fluoranthene 40 ng.L⁻¹; XII) benzo[a]pyrene 20 ng.L⁻¹; XIII) dibenz[a,h]anthracene 100 ng.L⁻¹; XIV) benzo[g,h,i]perylene 25 ng.L⁻¹; XV) indeno[1,2,3-cd]pyrene 100 ng.L⁻¹.
5.3.2 4.2K LETRSS of EPA-PAH

For the specific case of EPA-PAH, the solvent matching criterion for Shpol’skii Spectroscopy leads to one of the following five n-alkanes: n-pentane (naphthalene, acenaphthene, and acenaphthylene), n-hexane (phenanthrene and pyrene), n-heptane (fluorene, fluoranthene, benzo[g,h,i]perylene, benzo[b]fluoranthene and anthracene), n-octane (benzo[a]pyrene, dibenz[a,h]anthracene, chrysene and benz[a]anthracene), and n-nonane (indeno[1,2,3-cd]pyrene and benzo[k]fluoranthene). Considering the use of five organic solvents per sample practically unattractive, previous work in our lab demonstrated the feasibility to determine the 15 EPA-PAH under generic Shpol’skii conditions, this is with only one n-alkane (either n-hexane or n-octane) and one excitation wavelength (283.2nm). Using a generic excitation wavelength simplify instrumental manipulation because it allows the analyst to operate the tunable dye laser with only one organic dye.

Table 5.2 summarizes the 4.2K excitation and fluorescence wavelengths and 4.2K fluorescence lifetimes recorded from undegassed PAH solutions in n-octane. Appendix C compiles a complete set of 77K excitation and fluorescence of the 15 EPA-PAH in n-octane. Appendix D presents a complete set of 4.2K fluorescence spectra and lifetimes for the 15 EPA-PAH in n-octane. Excitation spectra were first recorded at 77K with the commercial spectrofluorimeter and the maximum wavelengths for each excitation peak were then confirmed at 4.2K with the MLS and the fiber optic probe. With the exception of acenaphthylene, all the other PAH showed 4.2K fluorescence emission in n-octane. The lack of fluorescence from acenaphthylene persisted after sample de-oxygenation (15min of nitrogen bubbling prior to fluorescence measurements) or analyte freezing in n-pentane, n-hexane, n-heptane and n-nonane.
Currently, we have no explanation foracenaphthylene behavior. The remaining PAH showed well-resolved Shpol’skii spectra with several excitation and fluorescence peaks between 200 and 500nm.
Table 5.2 Excitation and emission of 15 PAH in n-octane at 4.2K.

<table>
<thead>
<tr>
<th>PAH</th>
<th>$\lambda_{ex}$ (nm) b</th>
<th>$\lambda_{em}$ (nm) c</th>
<th>$\tau$ (ns) d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>225, 269, 280, 288, 291</td>
<td>315.3, 320.4, 322.8, 324.8, 330.8, 338.3, 340.2, 347.3, 351.6</td>
<td>200.9 ± 1.7</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>230, 283, 295</td>
<td>320.3, 325.6, 331.0, 335.8, 342.5, 347.1, 352.2</td>
<td>44.8 ± 0.7</td>
</tr>
<tr>
<td>Fluorene</td>
<td>266, 275, 281, 291, 295, 302</td>
<td>302.7, 304.5, 309.3, 316.7</td>
<td>6.9 ± 0.2</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>259, 270, 285, 293, 296</td>
<td>346.5, 351.5, 356.8, 363.4, 364.5, 366.5, 383.1</td>
<td>46.8 ± 0.4</td>
</tr>
<tr>
<td>Anthracene</td>
<td>256, 327, 343, 362, 365</td>
<td>378.6, 406.2</td>
<td></td>
</tr>
<tr>
<td>Fluorantheine</td>
<td>279, 285, 290, 325, 344, 363</td>
<td>407.8, 415.4, 434.6, 436.8, 443.6, 466.4</td>
<td>55.4 ± 1.1</td>
</tr>
<tr>
<td>Pyrene</td>
<td>243, 256, 277, 309, 322, 334, 338</td>
<td>372.0, 372.8, 377.5, 378.5, 382.0, 383.4, 384.3, 387.6, 388.7, 392.5, 393.6</td>
<td>516.2 ± 4.4</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>272, 282, 293, 316, 331, 347, 392</td>
<td>383.6, 396.4, 405.3</td>
<td>52.5 ± 1.6</td>
</tr>
<tr>
<td>Chrysene</td>
<td>262, 273, 287, 298, 310, 323</td>
<td>360.8, 372.6, 380.7, 392.5, 401.1</td>
<td>57.8 ± 2.3</td>
</tr>
<tr>
<td>Benzo[b]fluorantheine</td>
<td>292, 304, 351, 385</td>
<td>397.5, 405.0, 414.8, 424.1, 430.1, 449.3</td>
<td>43.8 ± 1.1</td>
</tr>
<tr>
<td>Benzo[k]fluorantheine</td>
<td>254, 287, 299, 310, 327, 362, 374, 382, 396</td>
<td>404.1, 405.3, 407.6, 413.0, 421.9, 426.4, 429.3</td>
<td>8.9 ± 0.3</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>269, 288, 298, 348, 370</td>
<td>402.8, 404.6, 408.1, 416.8, 423.6, 426.2, 430.2</td>
<td>38.6 ± 0.3</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>290, 301, 324, 335, 352</td>
<td>393.9, 395.3, 396.5, 404.5, 414.5, 416.2, 419.2</td>
<td>41.9 ± 0.5</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>293, 305, 330, 350, 368, 390</td>
<td>407.1, 413.2, 415.4, 416.2, 419.7, 422.5, 429.0, 445.3</td>
<td>127.2 ± 1.5</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>254, 281, 306, 320, 367, 382, 390</td>
<td>463.0, 466.7, 472.2, 473.4, 474.5, 481.5, 502.3</td>
<td>10.4 ± 0.1</td>
</tr>
</tbody>
</table>

Underlined wavelength indicates the maximum excitation wavelength. a All PAH solutions were prepared in n-octane. b The excitation were measured at 77K. c The emission were measured at 4.2K at the excitation of 283nm. d Lifetime were measured at 4.2K in octane. e Target wavelength
Careful examination of the excitation ranges of the 15 EPA-PAH confirms the possibility to using 283.2nm as a common excitation wavelength. The worst fluorescence overlapping was observed between naphthalene and acenaphthene. All the other PAH showed at least one fluorescent peak with no spectral overlapping from the other EPA-PAH. Their maximum wavelengths, which are reported under target wavelengths in Table 5.2, provide accurate determination of each EPA-PAH at the trace concentration level (ng.L⁻¹).

Spectral purities at target wavelengths were confirmed via lifetime measurements taken from synthetic mixtures with the 15 EPA-PAH at concentrations 100x higher than the target PAH. Fluorescence lifetimes were obtained via the WTM procedure. Figure 5.3 shows an example of a 4.2 K WTM for benzo[a]pyrene. For the 13 PAH with no spectral interference, the lifetime from the mixtures at the target wavelengths were single exponential decays. For a confidence interval of 95% (α = 0.05) and six determinations (n = 6),¹⁵⁷ their lifetime values matched the standard lifetimes shown in Table 5.2. In all cases, the agreement between the calculated and observed points over the first two lifetimes of the decays agreed to within 1% and the residuals showed no systematic trends. The lifetime measurements at the target peaks of naphthalene and acenaphthene gave biexponential decays. The short and long components of the biexponential decays statistically matched (α = 0.05, n = 6)¹⁵⁷ the fluorescence lifetimes of acenaphthene (44.8 ± 0.7 ns) and naphthalene (200.9 ± 1.7 ns), respectively. Their spectral discrimination was accomplished on the basis of time resolution with appropriate gate times, that is, 40 ns for acenaphthene and 1000 ns for naphthalene.
Figure 5.3 (A) 4.2 K WTM recorded with the fiber-optic probe and the MLS. (B) Fluorescence spectrum extracted from WTM at 5ns delay. (C) Fluorescence decay obtained from fitting the intensity of fluorescence emission at 404 nm as a function of time.

Instrumental parameters: excitation wavelength = 283.2 nm, initial gate delay = 5ns, gate width = 1000ns, gate step = 5ns, 100 laser pulses per fluorescence spectrum, and slit of spectrograph = 40μm.
5.3.3 4.2K LETRSS Analytical Figures of Merit

Figure 5.4 shows typical examples of calibration curves obtained via SPNE-LETRSS analysis. The complete set of calibration curves obtained for the 15 EPA-PAH is shown in Appendix E. Table 5.3 summarizes its analytical figures of merit for the 15 EPA-PAH. Aqueous standards were prepared by spiking milliliters of nanopure water with microliters of PAH solutions in 1% methanol-water (v/v). SPNE was carried out with 500μL water aliquots as previously described in Figure 5.1. All linear dynamic ranges (LDRs) were based on the average intensities of at least five PAH concentrations. The average intensities correspond to a minimum of nine LETRSS measurements made from triplicate aliquots collected from three complete experimental trials. No efforts were made to reach the upper concentration limits of the calibration curves. The lowest linear concentrations correspond to the limits of quantitation, which were calculated as 10S_B/m, where S_B is the standard deviation of 16 blank determinations at the peak base and m is the slope of the calibration curve. The excellent correlation coefficients demonstrate the existence of linear relationships in all cases. The limits of detection (LODs) – which were calculated as 3S_B/m - varied from 0.8ng/L (benzo[a]pyrene) to 88ng/L (fluorene). Although there is a wide range of values – probably reflecting the differences in both overall recoveries and fluorescence intensities of the studied PAH - all LODs are well below the maximum concentration level of regulated PAH. The relative standard deviations demonstrate excellent precision of measurements at the parts-per-trillion concentration level.
Figure 5.4 Low concentration ranges of calibration curves obtained via SPNE-LETSS.

(A) anthracene, (B) pyrene, (C) benzo[a]pyrene and (D) benzo[g,h,i]perylene. Excitation = 283nm; delay/gate times = 5/1000 ns.
Table 5.3 Analytical figure of merit of SPNE-LETRSS.

<table>
<thead>
<tr>
<th>PAH</th>
<th>$\lambda_{em}$ b (nm)</th>
<th>LDR c (ng.L$^{-1}$)</th>
<th>$R^2$</th>
<th>LOD d (ng.L$^{-1}$)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>320.4</td>
<td>66 – 1000</td>
<td>0.9901</td>
<td>20</td>
<td>5.2</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>320.3</td>
<td>100 – 1000</td>
<td>0.9965</td>
<td>29</td>
<td>4.3</td>
</tr>
<tr>
<td>Fluorene</td>
<td>309.3</td>
<td>200 – 1000</td>
<td>0.9836</td>
<td>88</td>
<td>0.9</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>346.5</td>
<td>10 – 1000</td>
<td>0.9749</td>
<td>2.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Anthracene</td>
<td>378.6</td>
<td>15 – 1000</td>
<td>0.9941</td>
<td>4.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>407.8</td>
<td>30 – 1000</td>
<td>0.9776</td>
<td>8.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Pyrene</td>
<td>372.0</td>
<td>11 – 1000</td>
<td>0.9944</td>
<td>3.4</td>
<td>4.2</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>383.6</td>
<td>9 – 1000</td>
<td>0.9968</td>
<td>2.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Chrysene</td>
<td>360.8</td>
<td>22 – 1000</td>
<td>0.9968</td>
<td>6.6</td>
<td>3.5</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>397.5</td>
<td>29 – 1000</td>
<td>0.9849</td>
<td>8.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>426.4</td>
<td>6 – 1000</td>
<td>0.9574</td>
<td>1.7</td>
<td>6.3</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>402.8</td>
<td>3 – 1000</td>
<td>0.9978</td>
<td>0.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>393.9</td>
<td>53 – 1000</td>
<td>0.9755</td>
<td>16</td>
<td>2.8</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>419.7</td>
<td>8 – 1000</td>
<td>0.9927</td>
<td>2.3</td>
<td>4.2</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>463.0</td>
<td>24 – 1000</td>
<td>0.9717</td>
<td>7.1</td>
<td>3.8</td>
</tr>
</tbody>
</table>

a All work solution were made in 1% (v/v) methanol. b Targeted emission wavelength upon excitation of naphthalene (200/1000 ns) and acenaphthene (0/40 ns). All PAHs were determined using delay/gate times = 0/1000 ns. c Linear dynamic range (LDR) are calculated from limit of quantitation (LOQ). LOQ is calculated by equation: $10 \, S_b / m$. d Limits of detection (LOD) were calculated on the basis of the equation LOD = $3 S_b / m$. 

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5.3.4 **PAH Recoveries from Tap Water Samples via SPNE-LETRSS Analysis**

The feasibility of monitoring PAH via SPNE–LETRSS in tap water was investigated with the three water samples previously analyzed via SPNE–HPLC (see Table 5.1 for PAH recoveries). Table 5.4 summarizes the average recoveries and their standard deviations obtained via SPNE–LETRSS. In all cases, the relative standard deviations of the recoveries were lower than 25%, which meets the criterion for regulated PAH (≤25%).

Comparison with values in Table 5.1 reveals no particular trend in the ability of SPNE–LETRSS to provide either lower or higher recoveries than SPNE–HPLC. All recoveries are in good agreement and provide PAH concentrations within the confidence of the standards. Figure 5.5 shows an example of a two-dimensional 4.2 K Shpol’skii spectrum recorded at a time window - delay = 5 ns and gate = 1000 ns - that captures the fluorescence emission of the 15 EPA–PAH. All targeted PAH are clearly identified in the water sample with no apparent matrix interference. WTM analyses yielded single exponential decays and matching lifetimes to those from the standards, confirming peak purity for accurate quantitation in all cases.
Table 5.4 Overall recoveries for EPA-PAH via SPNE-LETRSS of three water sample tested.

<table>
<thead>
<tr>
<th>PAH</th>
<th>Recovery (%)</th>
<th>Nanopure water</th>
<th>Tap water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>73.1 ± 5.3</td>
<td>61.9 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>68.3 ± 1.9</td>
<td>72.0 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Fluorene</td>
<td>58.6 ± 2.7</td>
<td>53.5 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>90.4 ± 2.3</td>
<td>90.5 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Anthracene</td>
<td>93.5 ± 2.1</td>
<td>92.6 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>85.8 ± 3.2</td>
<td>87.5 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Pyrene</td>
<td>88.1 ± 3.6</td>
<td>84.1 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>71.2 ± 2.7</td>
<td>66.3 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Chrysene</td>
<td>72.9 ± 1.8</td>
<td>74.6 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>90.3 ± 3.4</td>
<td>87.2 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>85.0 ± 3.0</td>
<td>84.3 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>89.9 ± 3.5</td>
<td>90.5 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene</td>
<td>87.3 ± 2.8</td>
<td>88.5 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>83.4 ± 2.3</td>
<td>85.6 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>91.1 ± 1.6</td>
<td>88.3 ± 3.0</td>
<td></td>
</tr>
</tbody>
</table>

*a All work solution were made in 1% (v/v) methanol.  
*b Overall recovery. PAH were extracted by 20nm gold nanoparticles from water solution and released by 2 µL 1-pentanethiol in 48µL octane.  
*c Overall recoveries obtained with nanopure water samples.  
*d The average recoveries obtained with three tap water samples.
Figure 5.5 4.2 K fluorescence spectrum of PAH spiked drinking water sample submitted to SPNE–LETRSS analysis.

Peak assignments and PAH final concentrations are as follows: (I) 200 ng.L\(^{-1}\) flourene; (II) 200 ng.L\(^{-1}\) naphthalene; (III) 50 ng.L\(^{-1}\)acenaphthene; (IV) 50 ng.L\(^{-1}\) phenanthrene; (V) 25 ng.L\(^{-1}\) chrysene; (VI) 50 ng.L\(^{-1}\) pyrene; (VII) 100 ng.L\(^{-1}\) anthracene; (VIII) 50 ng.L\(^{-1}\) benzo[a]anthracene; (IX) 100 ng.L\(^{-1}\) dibenz[a,h]anthracene; (X) 50 ng.L\(^{-1}\) benzo[b]fluoranthene; (XI) 20 ng.L\(^{-1}\) benzo[a]pyrene; (XII) 200 ng.L\(^{-1}\) fluoranthene; (XIII) 25 ng.L\(^{-1}\) benzo[g,h,i]perylene; (XIV) 40 ng.L\(^{-1}\) benzo[k]fluoranthene; (XV) 100 ng.L\(^{-1}\) indeno[1,2,3-cd]pyrene. Compounds II and III were determined using delay/gate times equal to 200/1000 and 5/40 ns, respectively. The remaining PAHs were determined using delay/gate times equal to 5/1000 ns.
5.4 Conclusion

We have presented the first successful application of SPNE and LETRSS to the analysis of PAH in drinking water samples. The new approach presents several desirable features for routine monitoring of numerous samples. The precision of measurements at the parts-per-billion concentration level is outstanding; relative standard deviations varied from 0.9% (fluorene) to 6.3% (benzo[k]fluoranthene). The LODs—which varied from 0.8 ng.L⁻¹ (benzo[a]pyrene) to 88 ng.L⁻¹ (fluorene)—are excellent as well and compare favorably with those reported previously with other methods.¹⁵⁸-¹⁶⁴ The analytical recoveries of the 15 EPA–PAH are in good agreement with those obtained via SPNE–HPLC. The wide recovery range probably reflects the relative affinity of PAH for the surface of Au NPs and the surrounding solution. For the six regulated WHO–PAH, the recovery values varied from 84.3 ± 3.8% (benzo[k]fluoranthene) to 90.5 ± 3.6% (benzo[a]pyrene). Their relative standard deviations were lower than 25% and, therefore, met the criterion for regulated PAHs (≤25%).⁵³,⁵⁴ Comparison with higher recoveries reported previously with established solid-phase extraction methods¹⁵¹,¹⁵⁸,¹⁵⁹ should take into consideration the early stage of SPNE. Future studies in our group will optimize the polarity of the surrounding solution to achieve higher analytical recoveries.

Similar to SPNE–HPLC,¹ complete PAH analysis is possible with only 500 μl of water aliquots. This small volume facilitates the implementation of SPNE for simultaneous sample extraction of numerous samples. In comparison with HPLC, LETRSS eliminates the need for PAH separation. The direct determination of pictograms of PAHs in microliters of water sample with no need for previous separation is a tremendous asset for routine analysis of numerous samples. This feature reduces both analysis time and the use of organic solvents. Complete PAH determination takes less than 10 min per sample via an environmentally friendly procedure that
consumes only 50 μl of organic solvent. The facts that 60 L of liquid helium typically lasts for 3 weeks of daily use—averaging 15 to 20 samples per day—and that the extracting solution adds only approximately US $2.25 to the total analysis cost make SPNE–LETRSS a cost-effective approach.
6.1 Introduction

Because the first step in the metabolic pathway of PAH forms hydroxy-PAH (OH-PAH), urine analysis of OH-PAH is recognized as an accurate assessment of human exposure to PAH.\textsuperscript{178,179} The general approach for monitoring OH-PAH in urine samples follows the sequence of urine hydrolysis, sample clean-up and pre-concentration, chromatographic separation and determination. The hydrolysis step – which can be either enzymatic or acidic - is included to dissociate OH-PAH from their glucuronide and/or sulfate conjugates. As previously mentioned, popular approaches for sample clean up and pre-concentration are liquid-liquid extraction,\textsuperscript{180,181} SPE\textsuperscript{182} and solid-phase micro-extraction.\textsuperscript{183} Chromatographic analysis is usually carried out either with HPLC - RTF\textsuperscript{183-185} or GC – MS.\textsuperscript{186-188}

Research in our lab has been focused on screening methods for monitoring OH-PAH in numerous urine samples.\textsuperscript{189} The development of easy-to-use and cost-effective techniques with high sample throughput is becoming increasingly relevant to investigate the uptake of PAH by large human populations.\textsuperscript{187,188} During the course of our experiments we realized that one of the main sources of metabolite loss in SPE-HPLC methodology is the evaporation of the eluting solvent prior to chromatographic determination. Recoveries as low as 45-48% have been reported for 3-hydroxybenzo[\textit{a}]pyrene, one of the metabolites monitored for exposure to
In a typical SPE-HPLC procedure, OH-PAH are eluted from C18 cartridges with milliliter volumes of methanol and pre-concentrated to micro-liters for HPLC determination. Eliminating the evaporation step carries with it the obvious deterioration of LOD, which need to be at the sub-parts-per-billion (ng.mL\(^{-1}\)) concentration levels. This chapter presents an original solution for the problem at hand. We substitute the evaporation step with a pre-concentration approach that extracts OH-PAH with Au NPs. The analytical potential of SPNE is evaluated with the following six metabolites: 9-hydroxyphenanthrene, 2-hydroxyfluorene, 1-hydroxypyrene, 6-hydroxychrysene, 3-hydroxybenzo[\(a\)]pyrene and 4-hydroxybenzo[\(a\)]pyrene. We demonstrate that the substitution of the evaporation step with the Au NPs procedure improves the overall recoveries, the relative standard deviations of the average recoveries and the LOD of SPE-HPLC analysis. The overall recoveries of the studied metabolites varied from 59.7 ± 3.6% (2-hydroxyfluorene) to 92.3 ± 2.5% (6-hydroxychrysene). The relative standard deviations of the average recoveries were than 6%. The LOD were at the parts-per-trillion levels and varied from ~2pg.mL\(^{-1}\) (6-hydroxychrysene) to ~18 pg.mL\(^{-1}\) (2-hydroxyfluorene).

### 6.2 Experimental Section

#### 6.2.1 Chemicals

HPLC grade methanol, hydrochloric acid, potassium biphthalate sodium hydroxide buffer, and synthetic urine were purchased from Fisher Scientific (Pittsburgh, PA). Analytical grade 1-pentanethiol was from Acros Organics (Atlanta, GA). Au NP were purchased from Ted Pella, Inc. (Redding, CA). Nanopure water from a Barnstead Nanopure Infinity water system was used
throughout. All OH-PAH were purchased at their highest available purity (>98%) and used with no further purification. 9-hydroxyphenanthrene (9-OHphe) and 2-hydroxyfluorene (2-OHflu) were acquired from Sigma-Aldrich (Milwaukee, WI). 1-hydroxypyrene (1-OHpyr) and 6-hydroxychrysene (6-OHchr) were purchased from Accustandard (New Haven, CT). 3-hydroxybenzo[a]pyrene (3-OHb(a)p) and 4-hydroxybenzo[a]pyrene (4-OHb(a)p) were obtained from Midwest Research Institute (Missouri, 64110).

Note: use extreme caution when handling OH-PAH that are known to be extremely toxic.

6.2.2 Solution Preparation

Stock solutions of OH-PAH were prepared in pure methanol. Their working solutions were prepared by serial dilution with 5% methanol (v/v). Possible metabolite degradation was monitored daily via fluorescence spectroscopy. Working solutions of Au NPs were prepared by diluting their commercial solutions with Nanopure water. The physical integrity of Au NPs was monitored daily via absorption spectroscopy. All solutions were kept stored in the dark at 4°C.

6.2.3 Hydrolysis of Urine Samples

Prior to hydrolysis, urine samples were spiked with micro-litters of metabolite stock solutions of appropriate concentration and allowed to equilibrate overnight. Acidic hydrolysis (pH = 5.0) of the spiked samples was then carried out by mixing and shaking (30 min, 1400 rpm) 8mL of urine sample, 500 µL of 0.1 M HCl and 500 µL of 0.05 M potassium biphthalate sodium hydroxide buffer.
6.2.4 Solid-Phase Extraction of Urine Samples

SPE was carried out via an experimental procedure previously optimized in our lab. Urine samples were processed through a Sep-Pak® C-18 (Waters, Milford, MA) cartridge preconditioned with 15 mL of methanol and 5 mL of water. All solutions were processed through the cartridge with the aid of a 10 mL syringe attached to the cartridge. The cartridge was sequentially rinsed with 10 mL of water and 10 mL of 20% methanol/water (v/v). OH-PAH were eluted with 3 mL of 100% methanol. The eluate was concentrated almost to dryness under a gentle stream of nitrogen and then dissolved to 1 mL with methanol for HPLC analysis.

6.2.5 Extraction of OH-PAH with Au NPs

A 500 µL aliquot of the methanol eluate were mixed with 1 mL of 20-nm Au NPs. The mixture was shaken for 5 min at 1400 rpm and centrifuged for 20 min at 13,400 rpm. The supernatant was separated from the precipitate with a micro-pipette. 2 µL of 1-pentanethiol followed by 48 µL of methanol were added to the precipitate. The new mixture was shaken for 5 min at 1400 rpm and then centrifuged for another 20 min at 13,400 rpm. All mixing and shaking times were optimized for best metabolite recovery.

6.2.6 Sample Mixing and Centrifugation for Metabolite Extraction with Au NPs

Sample mixing was carried out with a Maxi Mix III Rotary Shaker (type M65800, Barnstead-Thermolyne) equipped with a PT500X6A Vortex Mixer accessory. Centrifugation was performed with a MiniSpin centrifuge (Eppendorf) with maximum rotational speed of 13,400
6.2.7 **pH Measurements**

pH of solutions was measured with the help of an AR-15 pH/mV/°C meter and a glass body standard size combination electrodes from Fisher Scientific (Pittsburgh, PA).

6.2.8 **Absorption Spectroscopy**

See section 3.1 for instrumental and experimental details.

6.2.9 **RTF Spectroscopy**

See section 3.2 for instrumental and experimental details.

6.2.10 **HPLC Analysis**

See section 3.4 for instrumental details. OH-PAH separation was carried out on a Supelco (Bellefonte, PA) Supelcosil TM LC-OH-PAH column with the following characteristics: 15-cm length, 4-mm diameter, and 5-µm average particle diameters. All sample injections were held constant at 20 µL using a fixed-volume injection loop. Blank measurements were made in conjunction with each series of samples using identical conditions of glassware, equipment, solvents, and analysis to ensure prevention of interfering contamination.
6.3 Results and Discussion

6.3.1 Optimization of HPLC Analysis

The strategy we used to evaluate the pre-concentration of OH-PAH with Au NPs included the four most common features of current SPE-HPLC methodology, namely: (a) extraction of OH-PAH from hydrolyzed urine samples with C-18 silica cartridges; (b) elution of metabolites from the solid sorbent with methanol; (c) evaporation of the eluting solvent with a gentle N₂ gas stream; and (d) re-dissolution of the residual solid with methanol prior to chromatographic determination. Metabolite recoveries were monitored via HPLC analysis. Table 6.1 summarizes the settings and conditions for the complete chromatographic separation of the studied metabolites. Their separation was achieved with a linear water–methanol gradient varying from 70% to 90% methanol (v/v) and flow rates increasing from 0.5 to 1.5mL.min⁻¹. Metabolite detection was carried out in the fluorescence mode using the following time-programmed excitation/emission wavelengths: 0min, 273/326nm (2-OHflu); 8.9 min, 252/381nm(9-OHphe); 17.0 min, 345/386nm(1-OHpyr); 21.0 min, 270/381nm (6-OHchry); 26.0 min, 372/419nm (4-OHb(a)p); and 33.0 min, 381/432nm(3-OHb(a)p). The measurement wavelengths were programmed to match the maximum excitation and emission wavelengths of the studied OH-PAH. Figure 6.1 compares their excitation and fluorescence spectra in methanol–water mixtures with relative volume/volume (v/v) compositions of 30–70% and 10–90%. Because the spectral features of the studied metabolites did not vary significantly with the composition of the mobile phase, the accurate monitoring of their analytical concentrations was possible with a single set of excitation and fluorescence wavelengths.
Table 6.1 HPLC parameters for the separation of OH-PAH.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Water (%)</th>
<th>Methanol (%)</th>
<th>Flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>30</td>
<td>70</td>
<td>0.5</td>
</tr>
<tr>
<td>15.0</td>
<td>30</td>
<td>70</td>
<td>0.8</td>
</tr>
<tr>
<td>20.0</td>
<td>20</td>
<td>80</td>
<td>1.0</td>
</tr>
<tr>
<td>25.0</td>
<td>20</td>
<td>80</td>
<td>1.0</td>
</tr>
<tr>
<td>30.0</td>
<td>10</td>
<td>90</td>
<td>1.5</td>
</tr>
<tr>
<td>41.0</td>
<td>10</td>
<td>90</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Figure 6.1 Excitation and fluorescence spectra in methanol-water 30-70(v/v) (solid line) and 10-90(v/v) (dotted line). (A) 2-OHflu; (B) 9-OHphe; (C) 1-OHpyr; (D) 6-OHchr; (E) 4-OHb(a)p; (F) 3-OHb(a)p.
Figure 6.2 shows a typical chromatogram with the complete separation of the six metabolites. Three chromatographic runs of the same standard mixture recorded from three independent injections of 20μL aliquots provided the following average retention times (min): 8.49±0.13 (2-OHflu), 9.46±0.09 (9-OHphe), 19.00±0.06 (1-OHpyr), 22.50±0.15 (6-OHchr), 29.99±0.20 (4-OHb(a)p) and 37.80±0.10 (3-OHb(a)p). The distinct values of the experimental averages show the possibility to identify the six metabolites from their retention times. Comparison of spectral features in Figure 6.1 shows the potential for unambiguous identification of closely related metabolites – such as 3-OHb(a)p and 4-OH-b(a)p – on the bases of excitation and fluorescence spectra of HPLC fractions.
6.3.2 **OH-PAH Precipitation in the Absence of Au NPs**

Quantitative extraction of OH-PAH via the SPNE procedure requires an appropriate centrifugation time for the complete collection of Au NPs precipitate. Previous studies in our lab have investigated this parameter as a function of average particle diameter.\(^1\) For 20 nm Au NPs, i.e. the average particle diameter arbitrarily chosen for the present studies, 20 min of centrifugation time was sufficient to completely precipitate the colloidal particles. The possibility of OH-PAH adhesion to the walls of the centrifugation vessels or metabolite precipitation in the absence of NPs – i.e. solely due to metabolite precipitation – was then investigated up to 20 min of centrifugation time. Standard solutions of OH-PAH were prepared in pure methanol, i.e. the
eluting solvent of our SPE procedure. Centrifugation was carried out at 13,400rpm in 2mL polypropylene graduated tubes equipped with locking lids. OH-PAH concentrations were monitored via RTF spectroscopy. Fluorescence intensities were measured before ($I_{NC}$) and after centrifugation ($I_C$) at the maximum fluorescence excitation and emission maxima of each metabolite. Working solutions that had statistically equivalent $I_{NC}$ and $I_C$ were considered free from OH-PAH precipitation. The statistical comparisons were based on average values taken from three triplicate measurements of three individually centrifuged aliquots, i.e. $N_{NC} = N_C = 9$. Table 6.2 summarizes the concentration ranges of OH-PAH free from precipitation along with the statistical comparison for the highest tested concentration. Because concentration levels of OH-PAH in urine samples are usually below 30 ng.mL$^{-1}$, no efforts were made to investigate higher concentration values.

Table 6.2 Concentrations of OH-PAH free from precipitation.

<table>
<thead>
<tr>
<th>OH-PAH $^a$</th>
<th>$\lambda_{exc}$ (nm)$^b$</th>
<th>$\lambda_{em}$ (nm)$^b$</th>
<th>OH-PAH concentration $^c$ (ng.mL$^{-1}$)</th>
<th>$t_{exp}$ $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-OHflu</td>
<td>273, 302</td>
<td>300, 326</td>
<td>30</td>
<td>2.23</td>
</tr>
<tr>
<td>9-OHphe</td>
<td>252, 274, 298</td>
<td>371, 381</td>
<td>30</td>
<td>1.78</td>
</tr>
<tr>
<td>1-OHpyr</td>
<td>242, 279, 333, 345, 363s</td>
<td>386, 406, 427s</td>
<td>30</td>
<td>0.51</td>
</tr>
<tr>
<td>6-OHchr</td>
<td>270, 336</td>
<td>381, 398</td>
<td>30</td>
<td>2.52</td>
</tr>
<tr>
<td>4-OHb(a)p</td>
<td>266, 301, 372</td>
<td>419, 441s</td>
<td>30</td>
<td>0.22</td>
</tr>
<tr>
<td>3-OHb(a)p</td>
<td>267, 293, 362, 381, 400</td>
<td>432, 453s</td>
<td>30</td>
<td>1.16</td>
</tr>
</tbody>
</table>

$^a$ All metabolite standard solutions were prepared in methanol. $^b$ Excitation ($\lambda_{exc}$) and emission ($\lambda_{em}$) wavelength of OH-PAH at room temperature. Underlined wavelengths indicate the maximum excitation and emission wavelength; s = shoulder. $^c$ OH-PAH concentrations range free from precipitation due to centrifugation. $^d$ $t$-test of fluorescence intensity before and after centrifugation for [OH-PAH] = 30ng.mL$^{-1}$. $t_{critical} = 2.78$ ($a = 0.05$; $N_c = 6$).
6.3.3 Complete Metabolite Extraction

The minimum volume of Au NPs solution (mVCS) that one needs to completely extract a multi-component mixture can be estimated with the following equation: \(^1,^2\)

\[
mVCS = \sum \frac{[\text{COM}]_i}{\text{MEM}_{\text{COMi}}} \left( \frac{V_S}{C_{CS}} \right)
\]  

(6.1)

where \([\text{COM}]_i\) is the concentration (mass/volume) of any given component in the sample, \(V_S\) is the volume of extracted sample, \(C_{CS}\) is the concentration of Au NPs in the extracting solution (particles/volume), and \(\text{MEM}_{\text{COMi}}\) is the maximum extracted mass of any given component of the mixture per Au NP. The \(\text{MEM}_{\text{COMi}}\) values were experimentally obtained via an original method developed in our lab.\(^1\) OH-PAH were individually extracted from methanol standard solutions with 100µL of 20 nm colloidal solutions containing 7.0×10\(^{11}\) particles mL\(^{-1}\). The SPNE procedure consisted of mixing 500µL of metabolite standard solution, 900µL of water and 100µL of Au NPs solution in a 2 mL polypropylene tube followed by 5 min of mechanical shaken at 1400rpm and 20 min of centrifugation at 13,400 rpm. Metabolite extractions were monitored via RTF spectroscopy by comparing the fluorescence intensities of the supernatants to those recorded from pure standards submitted to the same experimental procedure in the absence of Au NPs. All extractions were carried out within the linear dynamic ranges of the RTF calibration curves.

Figure 6.3 shows the experimental results obtained for four of the studied metabolites. All linear fittings were calculated via the least-squares method. The straight lines correlating the data points labeled as (▼) represent the fluorescence intensities of metabolites standard solutions in the absence of Au NPs. The straight line segments correlating data points labeled as (●) and (■)
represent the fluorescence intensities of the supernatants after centrifugation with Au NPs. Statistical comparison showed that the fluorescence intensities within the (●) segments were statistically equivalent to the blank signals ($\alpha = 0.05$, $N_1 = N_2 = 3$). This fact accounts for the absence of OH-PAH and complete metabolite extraction within the concentration ranges represented as (●). Fluorescence intensities within the (■) segments were statistically different from blank signals and increased linearly with metabolite concentrations. The signal intensities in the (■) segments were lower than the intensities in the (▼) segments as a result of the lower metabolite concentrations in the supernatants. The lower concentrations reflect the amount of OH-PAH extracted with Au NPs. The intercepts of (●) and (▼) segments provide the MEMCOMi values reported in Table 6.3.
Figure 6.3 Fluorescence intensity as a function of OH-PAH concentration (ng.mL^{-1}): (A) 2-OHflu; (B) 1-OHpyr; (C) 6-OHchry; (D) 3-OHb(a)p.

Single intensity were recorded from OH-PAH standard solutions prepared in methanol (▼) and from supernatant of OH-PAH standards previously extracted with 10% 20-nm Au NPs solution (●/■). Best linear fittings were calculated via the least-squares method.
Table 6.3 Maximum amount of extracted OH-PAH with 100μL commercial 20-nm Au NPs.

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>Max conc (ng·mL⁻¹)</th>
<th>MEMCOMi (10⁻¹⁰ ng/particle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-OHflu</td>
<td>8.98 ± 0.35</td>
<td>1.92 ± 0.07</td>
</tr>
<tr>
<td>9-OHphe</td>
<td>8.78 ± 0.26</td>
<td>1.88 ± 0.06</td>
</tr>
<tr>
<td>1-OHpyr</td>
<td>8.76 ± 0.41</td>
<td>1.88 ± 0.09</td>
</tr>
<tr>
<td>6-OHchr</td>
<td>9.59 ± 0.24</td>
<td>2.06 ± 0.05</td>
</tr>
<tr>
<td>4-OHb(a)p</td>
<td>9.01 ± 0.22</td>
<td>1.93 ± 0.05</td>
</tr>
<tr>
<td>3-OHb(a)p</td>
<td>8.69 ± 0.23</td>
<td>1.86 ± 0.05</td>
</tr>
</tbody>
</table>

a All metabolite standard solutions were prepared in methanol. b Maximum concentration of extracted OH-PAH with 100μL of 20-nm Au NPs (7.0 x 10¹¹ particles.mL⁻¹). c MEMCOMi: maximum mass of extracted OH-PAH per particle unit; it was calculated according to the formula: [max. conc.]/7×10¹⁰ particles/mL.

According to Eq. (6.1), 1mL of a 20 nm Au NPs solution with C_{CS} =7.0×10^{11} particles.mL⁻¹ should account for the complete extraction of the six metabolites at individual concentration levels of ~4.5×10⁴ ng L⁻¹. This concentration level is much higher than the OH-PAH concentration levels usually encountered in urine samples.¹²,¹⁸²-¹⁹¹ Because Eq. (6.1) does not account for the potential co-extraction of concomitants in the sample and the resulting uptake of particle surface, we kept V_{CS} at 1mL for all further experiments.

6.3.4 Releasing Metabolites from Au NPs

Previous studies in our lab investigated the efficiency of alcohols, alkanes and alkanethiols to release PAH adsorbed to the surface of Au NPs.¹² The best recoveries were obtained with 1-pentanethiol. This solvent was then used here to release OH-PAH from Au colloids. Metabolites were individually extracted from 500μL of 10ng.mL⁻¹ standard solutions.
previously mixed with 1000μL of a 20 nm Au NPs commercial solution (7.0×10^{11} particles). After sample centrifugation, the supernatant (supernatant A) was separated from the precipitate and saved for further analysis via RTF spectroscopy. 2μL of 1-pentanethiol were added to the precipitate and mixed with 500μL of methanol to favor OH-PAH partitioning into the liquid solution. The new mixture was shaken for 5 min at 1400rpm and centrifuged for 20 min at 13,400 rpm. Its supernatant (supernatant B) was separated from the precipitate and saved for further analysis via RTF spectroscopy.

The concentrations of metabolites in supernatants A ([OHPAH]_A) and B ([OH-PAH]_B) were determined with the calibration curve method. Their values were substituted in Eqs. (6.2)–(6.4) to calculate the extraction efficiency (E), the releasing efficiency (RE) and the overall recovery (OR) of each metabolite:

\[
E = 100 \left( \frac{C_{OH-PAH} V_M - [OH-PAH]_A V_A}{C_{OH-PAH} V_M} \right) 
\]

(6.2)

\[
RE = 100 \left( \frac{[OH-PAH]_B V_B}{C_{OH-PAH} V_M - [OH-PAH]_A} \right) 
\]

(6.3)

\[
OR = 100 \left( \frac{[OH-PAH]_B V_B}{C_{OH-PAH} V_M} \right) 
\]

(6.4)

where \( C_{OH-PAH} \) refers to the metabolite concentration in the original sample, \( V_M \) to the extracted volume of methanol, and \( V_A \) and \( V_B \) to the volumes of supernatants A and B, respectively.

Figure 6.4 shows the RE values as a function of micro-liter volumes of 1-pentanethiol. Larger volumes than 2μL caused fluorescence quenching in all cases. Within a confidence interval of 95%, the RE values obtained for each metabolite with 1 and 2μL of 1-pentanethiol
were statistically equivalent ($N_1 = N_2 = 3$). Table 6.4 summarizes the $E$, RE and OR values obtained for the six metabolites. With the exception of 2-OHflu, all the other OH-PAH were extracted from the eluting solvent (methanol) with an efficiency statistically equivalent to 100% ($\alpha = 0.05, N=3$). As a result, the OR variations predominantly reflect the observed differences among RE values. The different RE values can be attributed to the physical–chemical affinity OH-PAH have towards the surface of Au NPs and their surroundings.

Figure 6.4 Effect of 1-pentanethiol on the releasing efficiency of extracted OH-PAH with 20-nm Au NPs; from left to right: 0, 0.2, 0.5, 1, 2μL. Extractions were made from 0.5 mL of methanol solution containing 20ng•mL$^{-1}$ individual concentrations of OH-PAH.
Table 6.4 Overall recovery of SPNE.

<table>
<thead>
<tr>
<th>OH-PAH (a)</th>
<th>Ex/Em (b)</th>
<th>E (%) (c)</th>
<th>RE (%) (d)</th>
<th>OR (%) (e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-OHflu</td>
<td>273/321</td>
<td>90.9 ± 0.8</td>
<td>94.7 ± 2.4</td>
<td>86.1 ± 2.0</td>
</tr>
<tr>
<td>9-OHphe</td>
<td>253/381</td>
<td>98.9 ± 1.9</td>
<td>88.0 ± 3.1</td>
<td>87.0 ± 2.5</td>
</tr>
<tr>
<td>1-OHpyr</td>
<td>347/384</td>
<td>98.4 ± 0.5</td>
<td>97.6 ± 1.9</td>
<td>96.0 ± 1.8</td>
</tr>
<tr>
<td>6-OHchr</td>
<td>270/378</td>
<td>97.6 ± 2.1</td>
<td>98.3 ± 2.3</td>
<td>95.9 ± 0.9</td>
</tr>
<tr>
<td>4-OHb(a)p</td>
<td>371/419</td>
<td>98.8 ± 2.0</td>
<td>94.7 ± 2.3</td>
<td>93.6 ± 1.2</td>
</tr>
<tr>
<td>3-OHb(a)p</td>
<td>308/430</td>
<td>99.6 ± 1.1</td>
<td>92.4 ± 2.7</td>
<td>92.0 ± 2.3</td>
</tr>
</tbody>
</table>

(a) Standard solutions were prepared in methanol. (b) Fluorescence excitation and emission wavelengths at which intensity measurements were made. All calculations are based on three independent extractions made with 1000 µL of a 20-nm Au NPs commercial solution (7.0 x 10¹¹ particles). (c) Extraction efficiency of OH-PAH. (d) Releasing efficiency of OH-PAH with 2µL of 1-pentanethiol. (e) Overall recovery of OH-PAH in 0.5 mL methanol.

6.3.5 Comparison of the Evaporation Step to the Au NPs Procedure

Table 6.5 summarizes the metabolites recoveries obtained via the SPE procedure and compares the OR of the SPE – evaporation procedure to those obtained with the SPE – Au NPs procedure. Standard mixtures of the six metabolites were prepared in pure methanol and spiked in 8mL of synthetic urine sample to provide final individual concentrations of 2 ng.mL⁻¹. The metabolites concentrations in the SPE eluting solvent (methanol) and the final extract of the SPE–evaporation and SPE–Au NPs procedures were monitored via HPLC analysis using calibration curves built with a minimum of five standard solutions. The average intensities plotted in the calibration graphs corresponded to a minimum of nine HPLC measurements made from triplicate aliquots collected from three complete experimental trials. Although a general improvement of the OR values is observed with the SPE–Au NPs procedure, their statistical comparison to the OR values of the SPE–evaporation procedure (α = 0.05, N₁ = N₂ = 9) shows
significant improvements only for five of the six studied OH-PAH. 2-OHflu showed statistically
equivalent recoveries because the OR improvement via the SPE–Au NPs procedure was not
enough to overcome the rather large standard deviation of the OR obtained via the SPE–
evaporation procedure. Keeping in mind that the SPE step was the same for both types of
procedures, our data clearly shows the considerable improvement of reproducibility of
measurements that one can achieve with the Au NPs step.

Table 6.5 Metabolites recoveries from synthetic urine samples via SPE-HPLC, SPE-evaporation-HPLC and
SPE-Au NPs-HPLC analysis.

<table>
<thead>
<tr>
<th>OH-PAH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SPE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SPE-evaporation</th>
<th>SPE-Au NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rec (%)</td>
<td>RSD(%)</td>
<td>OR (%)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-OHflu</td>
<td>65.3 ± 4.2</td>
<td>6.43</td>
<td>44.5 ± 11.5</td>
</tr>
<tr>
<td>9-Ohphe</td>
<td>86.9 ± 3.5</td>
<td>4.03</td>
<td>62.0 ± 9.1</td>
</tr>
<tr>
<td>1-OHpyr</td>
<td>83.2 ± 2.6</td>
<td>3.12</td>
<td>63.1 ± 6.7</td>
</tr>
<tr>
<td>6-OHchr</td>
<td>93.5 ± 3.0</td>
<td>3.21</td>
<td>67.7 ± 10.5</td>
</tr>
<tr>
<td>4-OHb(a)p</td>
<td>71.3 ± 3.7</td>
<td>5.19</td>
<td>57.5 ± 7.1</td>
</tr>
<tr>
<td>3-OHb(a)p</td>
<td>68.5 ± 4.0</td>
<td>5.84</td>
<td>53.2 ± 6.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mixture of six OH-PAH spiked into 8 mL of synthetic urine sample to reach final
individual concentration equal to 2 ng.mL<sup>-1</sup>. <sup>b</sup> Percent of recovery obtained via SPE. HPLC
analysis was made in the SPE eluting solvent (methanol) prior to its pre-concentration step or
the Au NPs step. <sup>c</sup> Percent of overall recovery (OR) obtained via SPE and evaporation (SPE-
evaporation) or SPE and the Au NPs step (SPE-Au NPs). HPLC analysis was carried out
after pre-concentration the SPE eluting solvent via the evaporation or the Au NPs step. <sup>d</sup>
Relative standard deviation based on average values obtained from triplicate measurements
of three independent extractions.
Figure 6.5 – which compares the fluorescence intensities of three chromatographs recorded from individual aliquots of the same urine sample – confirms the excellent reproducibility of measurements of the SPE–Au NPs procedure at the low parts-per-billion concentration levels.

Table 6.6 summarizes the AFOM of the two approaches obtained from synthetic urine samples containing the six studied metabolites. Standard OH-PAH mixtures were prepared in pure methanol and spiked in synthetic urine samples. The excellent variances ($R^2$) from the linear fittings calculated via the least-squares method demonstrate the existence of linear relationships
in all cases. No efforts were made to reach the upper concentration limits of the calibration curves. The lowest linear concentrations correspond to the limits of quantitation (LOQ), which were calculated as $10 \frac{S_B}{m}$. The LOD were calculated as $3 \frac{S_B}{m}$. In both cases, $S_B$ was the standard deviation of 16 blank determinations at the peak base and $m$ was the slope of the calibration curve. A head-to-head comparison of our data in Table 6.6 shows the clear advantage of substituting the evaporation with the Au NPs step. LOD improvements varied from $5.7 \times$ (2-OHflu) to $14.05 \times$ (3-OHb(a)p). The observed range of LOD most likely reflects the differences in both overall recoveries and fluorescence intensities of the studied metabolites.
Table 6.6 Analytical figures of merit of HPLC analysis with previous sample concentration via SPE-evaporation and SPE-Au NPs procedure.

| OH-PAH | Retention Time (min) | Ex/Em (nm) | SPE-evaporation | SPE-Au NPs | | | |
|--------|----------------------|-------------|-----------------|------------|----------------|-------------|
|        |                      |             | LDR (ng.mL\(^{-1}\)) | LOD (ng.mL\(^{-1}\)) | R\(^2\) | LDR (ng.mL\(^{-1}\)) | LOD (ng.mL\(^{-1}\)) | R\(^2\) |
| 2-OHflu | 8.49 ± 0.13 | 227/330 | 0.343-10 | 0.103 | 0.9893 | 0.059-2 | 0.0178 | 0.9859 |
| 9-OHphe | 9.46 ± 0.09 | 244/370 | 0.153-10 | 0.046 | 0.9967 | 0.023-2 | 0.0070 | 0.9771 |
| 1-OHpyr | 19.00 ± 0.06 | 242/388 | 0.083-10 | 0.025 | 0.9993 | 0.0073-2 | 0.0022 | 0.9990 |
| 6-OHchr | 22.50 ± 0.15 | 269/378 | 0.070-10 | 0.021 | 0.9884 | 0.0060-2 | 0.0018 | 0.9998 |
| 4-OHb(a)p | 29.99 ± 0.20 | 248/433 | 0.720-10 | 0.216 | 0.9991 | 0.049-2 | 0.0147 | 0.9918 |
| 3-OHb(a)p | 37.80 ± 0.10 | 248/433 | 0.543-10 | 0.163 | 0.9903 | 0.038-2 | 0.0116 | 0.9894 |

\( a \) All working solutions were synthetic urine samples previously spiked with micro-litters of metabolite standard solutions of appropriate concentrations. \( b \) Fluorescence excitation and emission wavelengths for HPLC analysis. \( c \) SPE followed by evaporation of eluting solvent. \( d \) SPE followed by SPNE. \( e \) Linear dynamic range (LDR). \( f \) Limits of detection (LOD) were calculated by the equation: LOD = 3 S\(_B\) /m, where S\(_B\) is the standard deviation of 16 blank determinations and m is the slope of the calibration curve.
6.4 Conclusions

Our studies have devised a unique approach to improve the AFOM of SPE-HPLC for the analysis of OH-PAH in urine samples. The use of Au NPs to pre-concentrate metabolites from the SPE eluting solvent provided OR improvements (\( \text{OR}_{\text{Au NPs}} - \text{OR}_{\text{Evaporation}} \)) varying from \(~10.6\%\) (4-OHb(a)p) to \(~24.6\%\) (6-OHchr). The significant improvements observed in the standard deviations of the OR values via the SPE–Au NPs procedure provided relative standard deviations lower than 6%. Significant improvements were also observed in the LOD. Because the Au NPs step improved the LOD by at least one order of magnitude, the minimum detection levels of all the studied metabolites were at parts-per-trillion (pg.mL\(^{-1}\)) level.

Numerous SPE-HPLC methods have been published for the analysis of OH-PAH metabolites in human urine samples. A wide range of OR and relative standard deviations have been reported for the metabolites investigated in this chapter. These include 41±5% (12.2%) for 3-OHb(a)p, 53±13% (24.5%) for 2-OHflu, 67±1% (1.5%) for 9-OHphen, and 57±10.3% (18%) for 1-OHpyr. The same is true for the reported LOD, which include 0.72 ng.mL\(^{-1}\) for 2-OHflu, 0.16 ng.mL\(^{-1}\) for 9-OHphe, 0.004 ng.mL\(^{-1}\), 0.005 ng.mL\(^{-1}\) and 40 ng.mL\(^{-1}\) for 1-OHpy and 0.006 ng.mL\(^{-1}\), 0.121ng.mL\(^{-1}\) and 0.161 ng.mL\(^{-1}\) for 3-OHb(a)p.

A straightforward comparison to our data is rather arguable because our experiments were carried out with synthetic urine samples. However, the fact that our SPE-HPLC results from synthetic urine samples are consistent with previously reported data on human urine samples provides a general prospective of the excellent potential of the new approach for the analysis of OH-PAH. The determination of 6-OHchr and 4-OHb(a)p via SPE-HPLC provides a
new tool to obtain additional information on human exposure to chrysene and benzo[a]pyrene.
7.1 Introduction

Capillary Electrophoresis (CE) is a separation technique based on the differential migration rate of charged species in a dc electric field applied across a capillary tube. A particular strength of CE is its unique ability to separate a large variety of charged species, including peptides and proteins, nucleic acids (DNA and RNA), lipids, low-molecular weight drugs, and even inorganic ions. This versatility makes CE an extremely attractive technique in biochemical, biological and biomedical research as well as in the biotechnology industry. Similar to high-performance liquid chromatography (HPLC), the simplicity and wide applicability of ultraviolet and visible absorption spectroscopy (UV-VIS) makes this technique the most common detection mode in CE. The main disadvantage of CE-UV-VIS in bio-analysis is its rather poor concentration sensitivity. The unsatisfactory limits of detection (LOD) mainly result from the short optical path length of on-capillary absorption measurements and the small sample injection volumes (10 – 100nL). Several strategies exist to improve the LOD of CE. These include measuring absorption through longer optical path lengths with the aid of Z- or U-shaped detection cells or bubble cells, using more sensitive detectors, which could be based on laser induced fluorescence spectroscopy or mass spectroscopy, or performing sample pre-concentration prior to CE.

Known pre-concentration strategies for CE include both chromatographic-based and
Chromatographic pre-concentration using solid-phase extraction (SPE) appears to be widely accepted due to its general applicability and relatively high pre-concentration factors. The simplest way to combine SPE and CE is to perform the two steps separately via an off-line approach. At-line approaches use a robotic or a similar system to transfer the samples from SPE to the CE system. In both off-line and at-line systems there is no direct stream of liquid between SPE and CE. The integration of the two steps into a single system provides the advantages of minimum sample handling and possible automation. Two types of integrated SPE-CE systems exist. On-line coupling requires a physical connection for continuous flow from the SPE column to the separation capillary. Valves, flow injection analysis systems and T-splits have been used for this purpose with various degrees of success. Ideal coupling is achieved with in-line SPE-CE systems, were the SPE column is placed inside the separation capillary. Pre-concentration and separation within the same capillary requires the use of an eluting solvent chemically compatible with the electrophoretic mobile phase. For those cases where the eluting solvent is chemically incompatible with the separation medium, a new procedure was recently developed with the name of in-capillary micro-extraction. The new method is easy to implement and does not require micro-valves, external connectors or T-splits. In-capillary micro-extraction CE uses two different capillaries, one for sample pre-concentration and the other for electrophoretic separation. Both steps are carried out in the CE system with minimum sample handling. Pre-concentration is made with the aid of a chemically modified capillary that is also used for sample elution. The eluted sample is collected into the injection vial for electrophoretic separation, which is then carried out with a second silica capillary. This approach was successfully applied to the analysis of several carbamate pesticides and their degradation products in water samples. Pre-concentration took place on a monolithic
polymeric sorbent synthesized in a silica capillary. Using a polydivinylbenzene monolith of 16cm length, an average pre-concentration factor of 60 was obtained for the studied pesticides.

Only a few reports exist on the analyses of PAH metabolites via CE. Table 7.1 correlates the name of the metabolite to the method of analysis used for its determination. Smith and co-workers\textsuperscript{206} reported the separation of twelve OH-PAH via cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) using a buffer consisting of 40mM $\gamma$-CD, 60mM sodium dodecyl sulfate (SDS) and 30mM borate. Metabolite detection was carried out via laser induced fluorescence (LIF). A UV-transmitting fiber optic cable was used to couple the 325nm laser light from a He-Cd to the detection window in the separation capillary. The inner diameter of the capillary tube provided a detection window with optical path-length (effective path-length) equal to 50$\mu$m. Fluorescence was collected into a commercial spectrofluorimeter for intensity and spectra recording. LOD at the sub-parts-per-million level (sub-$\mu$g/mL) were obtained for all the studied metabolites. Xu and Hurtubise\textsuperscript{236,237} demonstrated the successful separation of nine PAH metabolites via capillary zone electrophoresis (CZE) using a 100mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer solution (pH 10.4) with 40\% (v/v) methanol. The studied metabolites - which included tetrols, benzo[a]pyrene diols and OH-PAH – were detected via UV absorption spectroscopy. This detection approach was useful for the optimization of electrophoretic parameters but not sensitive enough for metabolite detection at trace concentration levels. Kuijt and co-workers\textsuperscript{110} separated 5 OH-PAH via CD-MECK using a 30mM borate buffer (pH 9.0) containing 60 mM SDS and 12.5mM $\gamma$-CD. Their quantitation was carried out via both conventional fluorescence and LIF. The best LOD (low ng/mL concentration range) were obtained with LIF home-made set-up. Open path sample excitation was carried out with a pulsed Nd-YAG laser at 266nm. Fluorescence from the separation capillary (detection
window with a 75 μm effective path-length) was detected with single channel system consisting of a band-pass filter and a photomultiplier tube.
<table>
<thead>
<tr>
<th>PAH Metabolites</th>
<th>CE</th>
<th>Electrophoretic Medium</th>
<th>Detection Mode</th>
<th>LOD</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-OHnaphthalene</td>
<td>CD-MEKC</td>
<td>γ-CD/SDS/borate buffer</td>
<td>CF/LIF</td>
<td>40/4 ng/ml</td>
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<td>7/2 ng/ml</td>
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<td>γ-CD/SDS/borate buffer</td>
<td>LIF</td>
<td>~ 270ng/ml</td>
<td>[206]</td>
</tr>
<tr>
<td>3-OHbenzo[a]pyrene</td>
<td>CD-MEKC</td>
<td>γ-CD/SDS/Na$_2$B$_4$O$_7$/H$_3$BO$_3$</td>
<td>UV</td>
<td>/</td>
<td>[238]</td>
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<td></td>
<td>CD-MEKC</td>
<td>γ-CD/SDS/borate buffer</td>
<td>LIF</td>
<td>~ 270ng/ml</td>
<td>[206]</td>
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<tr>
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<td>/</td>
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<td>/</td>
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<td>γ-CD/SDS/borate buffer</td>
<td>LIF</td>
<td>~ 270ng/ml</td>
<td>[206]</td>
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<td>CAPS/MeOH/H$_2$O (40% v/v)</td>
<td>UV</td>
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<td>[236]</td>
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<tr>
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<td>γ-CD/SDS/borate buffer</td>
<td>LIF</td>
<td>~ 270ng/ml</td>
<td>[206]</td>
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<td></td>
<td>CZE</td>
<td>CAPS/MeOH/H$_2$O (40% v/v)</td>
<td>UV</td>
<td>/</td>
<td>[236]</td>
</tr>
<tr>
<td>12-OHbenzo[a]pyrene</td>
<td>CZE</td>
<td>CAPS/MeOH/H$_2$O (40% v/v)</td>
<td>UV</td>
<td>/</td>
<td>[236]</td>
</tr>
<tr>
<td>1-OHbenzo[a]anthracene</td>
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<td>γ-CD/SDS/borate buffer</td>
<td>LIF</td>
<td>~ 250ng/ml</td>
<td>[206]</td>
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<tr>
<td>3-OHbenzo[a]anthracene</td>
<td>CD-MEKC</td>
<td>γ-CD/SDS/borate buffer</td>
<td>LIF</td>
<td>~ 250ng/ml</td>
<td>[206]</td>
</tr>
<tr>
<td></td>
<td>CZE</td>
<td>CAPS/MeOH/H$_2$O (40% v/v)</td>
<td>UV</td>
<td>/</td>
<td>[236]</td>
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<tr>
<td>8-OHbenzo[a]anthracene</td>
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<td>γ-CD/SDS/Na$_2$B$_4$O$_7$/H$_3$BO$_3$</td>
<td>UV</td>
<td>/</td>
<td>[238]</td>
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<td>/</td>
<td>[238]</td>
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<td>/</td>
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<td>γ-CD/SDS/borate buffer</td>
<td>CF/LIF</td>
<td>1/2 ng/ml</td>
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Table 7.1 Overview of the CE analysis of PAH metabolites (continue).

<table>
<thead>
<tr>
<th>PAH Metabolites</th>
<th>CE</th>
<th>Electrophoretic Medium</th>
<th>Detection Mode</th>
<th>LOD</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-OHbenzo([b])fluoranthene</td>
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<tr>
<td>12-OHbenzo([b])fluoranthene</td>
<td>CD-MEKC</td>
<td>γ-CD/SDS/Na(_2)B(_4)O(_7)/H(_3)BO(_3)</td>
<td>UV</td>
<td>/</td>
<td>[238]</td>
</tr>
<tr>
<td>2-OHindeno([1,2,3-cd])pyrene</td>
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<td>γ-CD/SDS/borate buffer</td>
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<td>~ 300ng/ml</td>
<td>[206]</td>
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<td>benzo([a])pyrene-(trans-4,5)-dihydrodiol</td>
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<td>UV</td>
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<tr>
<td>benzo([a])pyrene-(trans-7,8)-dihydrodiol</td>
<td>CZE</td>
<td>CAPS/MeOH/H(_2)O (40% v/v)</td>
<td>UV</td>
<td>/</td>
<td>[236]</td>
</tr>
<tr>
<td>benzo([a])pyrene-(trans-9,10)-dihydrodiol</td>
<td>CZE</td>
<td>CAPS/MeOH/H(_2)O (40% v/v)</td>
<td>UV</td>
<td>/</td>
<td>[236]</td>
</tr>
<tr>
<td>benzo([a])pyrene-(r)-(7-trans-8,9)-c(1)s-10-tetrahydrotetrol</td>
<td>CZE</td>
<td>CAPS/MeOH/H(_2)O (40% v/v)</td>
<td>UV</td>
<td>/</td>
<td>[236]</td>
</tr>
</tbody>
</table>

Abbreviation: CD-MEKC: cyclodextrin modified micellar electrokinetic capillary chromatography; CZE: capillary zone electrophoresis; LIF: laser induced fluorescence; CF: conventional fluorescence. SDS: sodium dodecyl sulfate; MeOH: methanol.
In this chapter, we present the first application of gold nanoparticles (Au NPs) deposited capillaries as sample pre-concentration devices for in-capillary micro-extraction electrophoresis and their use for the analysis of OH-PAH in synthetic urine samples. We demonstrate the feasibility to obtain competitive UV absorption LOD (S/N = 3) with commercial instrumentation. 1-hydroxypyrene (1-OHpyr), 9-hydroxyphenanthrene (9-OHphe), 3-hydroxybenzo[a]pyrene (3-OHbap), 4-hydroxybenzo[a]pyrene (4-OHbap) and 5-hydroxybenzo[a]pyrene (5-OHbap) were separated via CZE using a 100mM CAPS buffer solution (pH 10.4) 40% (v/v) methanol. Sample pre-concentration with Au NPs deposited capillaries provided enrichment factors ranging from 87 (9-OHphen) to 100 (3-OHbap) and made possible to reach UV absorption LOD from 9ng.mL⁻¹ (9-OHphen and 3-OHbap) to 14ng.mL⁻¹ (4-OHbap).

7.2 Experimental Section

7.2.1 Chemicals and Materials

HPLC grade methanol, hydrochloric acid, potassium biphthalate sodium hydroxide buffer and synthetic urine were purchased from Fisher Scientific (Pittsburgh, PA). CAPS and 3-mercaptopropyl-trimethoxysilane (MPTMS) were purchased from Sigma–Aldrich (Milwaukee, WI). Analytical grade 1-pentanethiol was from Acros Organics (Atlanta, GA). Au NPs were purchased from Ted Pella, Inc. (Redding, CA). Nanopure water from a Barnstead Nanopure Infinity water system was used throughout. All OH-PAH were purchased at their highest available purity (>98%) and used with no further purification. 9-OHphe was acquired from Sigma–Aldrich (Milwaukee, WI). 1-OHpyr was purchased from Accustandard (New Haven, CT). 3-OHbap, 4-OHbap and 5-OHbap were obtained from Midwest Research-Institute (Missouri,
Fused-silica capillary with 50μm internal diameter and 375μm outer diameter were purchased from Polymicro Technologies. The polyamide coating was removed with a window maker (MicroSolv-CE) to provide an UV transparent detection window with an approximate length of 2 mm. The center of the optical window was located at 58cm from the injection port of the CE instrument.

Note: use extreme caution when handling OH-PAH that are known to be extremely toxic.

7.2.2 Solution preparation

Stock solutions of OH-PAH were prepared in pure methanol. Working metabolite solutions were prepared by serial dilution with 5% methanol (v/v). Possible metabolite degradation was monitored daily via fluorescence spectroscopy. All solutions were stored in the dark at 4 °C. The buffer solution for OH-PAH separation was prepared daily by adding 1.107g of CAPS to 60mL of Nanopure water (18.1MΩ) and 40mL of HPLC grade methanol. Its final pH was adjusted to 10.4 using a 1M NaOH solution. The 10mM MPTMS we used to coat the inner walls of the pre-concentration capillary tube was prepared in ethanol.

7.2.3 Instrumentation

Solution mixing was carried out with a Maxi Mix III Rotary Shaker (Type M65800, Barnstead-Thermolyne) equipped with a PT500X6A Vortex Mixer accessory. pH measurements were made with an AR-15 pH/mV/C meter and a glass body standard size combination electrodes from Fisher Scientific (Pittsburgh, PA). Images of Au NPs deposited capillaries were
recorded with a Zeiss scanning electron microscope (Ultra 55, Carl Zeiss SMT AG, Germany). Prior to imaging, capillaries were sputter coated with a uniform layer of Au-Pd (5nm thick) using an SPI bench-top module spotter coater (Structure Probe, West Chester, PA, USA). Instrumentation for absorption, fluorescence and CE measurements was previously described in sections 3.1, 3.2 and 3.5, respectively.

7.2.4 Fabrication of Au NPs deposited capillaries

Fabrication of Au NPs deposited capillaries followed the method published in the literatures\textsuperscript{239-246} All reagents were pumped through the capillaries with the aid of our CE instrument. All coated capillaries had the same length (100cm) and square internal channel (74μm x 74μm) dimensions. With the aim of exposing the surface silanol groups on their inner walls, capillaries were first etched by pressure rinsing (1000mbar) with 1M NaOH (30min), de-ionized water (10min) and 0.1 M HCl (30min). Upon rinsing with DI water again, the capillary was placed in an oven and dried at 100°C for 1h to remove all moisture. The organosilane derivatization step was carried out with a 10mM MPTMS solution. The coupling agent was introduced into the capillaries by pressure (1000mbar) for a period of 2h and left it to stand overnight. The modified capillary was then rinsed with ethanol and annealed at 110°C in the oven for 10 min. After rinsing the capillaries with de-ionized water, 2mL of a Au NPs aqueous solution (20nm particles at a $7 \times 10^{11}$ particle/mL concentration) was pressure introduced into the capillaries at 500mbar. The excess of Au NPs was removed by pressure rinsing the capillaries with de-ionized water. All Au NPs deposited capillaries were stored in Nanopure water until their use.
7.2.5 In-capillary micro-extraction procedure

Metabolites were retained in the coated capillary by pressuring through 1mL of sample solution at 1000mbar. The same CE pressure was used to release the metabolites with micro-litters of 1-pentanethiol. The eluting solution was collected in 300μL glass vials (Analytical Sales and Services Inc.; Pompton Plains, NJ) for subsequent electrophoretic separation.

7.2.6 Analysis of Urine Samples

Prior to hydrolysis, urine samples were spiked with micro-litters of metabolite stock solutions of appropriate concentration and allowed to equilibrate overnight. Acidic hydrolysis (pH = 5.0) of the spiked samples was then carried out by mixing and shaking (30 min, 1400 rpm) 8mL of urine sample, 500 μL of 0.1 M HCl and 500 μL of 0.05 M potassium biphthalate sodium hydroxide buffer. SPE was carried out via an experimental procedure previously optimized in our lab.¹⁸⁹ Urine samples were processed through a Sep-Pak® C-18 (Waters, Milford, MA) cartridge pre-conditioned with 15 mL of methanol and 5 mL of water. All solutions were processed through the cartridge with the aid of a 10 mL syringe attached to the cartridge. The cartridge was sequentially rinsed with 10 mL of water and 10 mL of 20% methanol/water (v/v). OH-PAH were eluted with 3 mL of 100% methanol and submitted to CE analysis.

7.3 Results and discussion

7.3.1 CZE separation of the studied metabolites

The buffer system we used in these studies was originally optimized for the compound-
class separation of nine PAH metabolites, including tetrols, benzo[a]pyrene diols, 3-OHbap and 1-OHpyr.\textsuperscript{236,237} To the extent of our literature search, the CZE separation of 9-OHphen, 4-OHbap and 5-OHbap has not been reported yet. The HPLC analysis of 9-OHphen in urine samples has been already published\textsuperscript{182,247} but the chromatographic separation of 4-OHbap and 5 OH-bap has not been reported yet. HPLC studies carried out in our lab have shown the difficulty to achieve a single set of optimum chromatographic conditions for the separation of 3-OHbap, 4-OHbap and 5-OHbap.\textsuperscript{248} Table 7.2 lists the five OH-PAH in increasing order of migration time using a 100mM CAPES buffer solution (pH 10.4) 40% (v/v) methanol. The number of theoretical plates, N, was calculated from the equation $N = 16(t_r/w)^2$ where $t_r$ denotes the migration time and w the width at the base peak.\textsuperscript{249} Under these buffer conditions, the separation efficiency of CZE produces baseline resolution of the five studied metabolites.
Table 7.2 CE separation parameters for the 5 OH-PAH.

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>Retention time (min) (^a)</th>
<th>Theoretical Plates (^b)</th>
<th>Resolution (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-OHphe</td>
<td>31.16 ± 0.27</td>
<td>33,269</td>
<td>/</td>
</tr>
<tr>
<td>5-OHBap</td>
<td>33.72 ± 0.31</td>
<td>83,537</td>
<td>4.45</td>
</tr>
<tr>
<td>4-OHBap</td>
<td>34.37 ± 0.35</td>
<td>75,603</td>
<td>1.34</td>
</tr>
<tr>
<td>3-OHBap</td>
<td>38.87 ± 0.58</td>
<td>96,696</td>
<td>9.01</td>
</tr>
<tr>
<td>1-OHpyr</td>
<td>39.87 ± 0.44</td>
<td>108,872</td>
<td>2.03</td>
</tr>
</tbody>
</table>

\(^a\) CE analysis conditions for the 5 OH-PAH: buffer, 100mM CAPS with 40% (v/v) methanol; apparent pH 10.1; voltage, 26kV; temperature, 25ºC. \(^b\) The number of theoretical plates were calculated from the equation \(N = 16(t_r/w)^2\) where \(t_r\) denotes the migration time and \(w\) the width at the base peak. \(^c\) The resolution of two metabolites (A and B) were calculated from the equation \(R = 2(t_{rA} - t_{rB})/(w_A + w_B)\).

7.3.2 Characterization of Au NPs deposited capillaries

Figure 7.1a compares the UV-Vis absorption spectra of a 20nm Au NPs solution that were recorded before and after coating of the inner walls of a capillary tube. Both spectra show the characteristic Plasmon resonance structure of Au NPs at \(\sim 525\) nm. The intensity drop observed in the spectrum after the capillary coating correlates to the immobilization of Au NPs in the inner surface of capillary tube and the lower concentration of Au NPs in solution. Figures 7.1b – d compare the scanning electron microscopy (SEM) images recorded from a 50mm un-coated capillary segment (7.1b) to those recorded from 50mm Au NPs deposited capillaries segments (7.1c and 7.1d). Similar images recorded from different segments of numerous capillary tubes confirm the reproducible deposition of Au NPs within the entire length of the derivatized capillaries.
Figure 7.1 UV absorption spectra of Au NPs solution before and after coating to the capillary and SEM images of the inner surface of capillary.
(a) UV absorption of 20nm Au NP commercial solutions before and after deposition. (b) untreated capillary; (c) and (d) coated with 20nm Au NP.

7.3.3 Sample flow rate for in-capillary pre-concentration of OH-PAH

The retention capacity of OH-PAH metabolites increased with the length of the pre-concentration capillary tube. An inverse correlation was observed between the flow rate of the
sample solution and the length of the capillary tube. The resistance to the passage of fluids increased with the length of the capillary tube and slowed down the sample flow rate. Because the use of rather short capillary tubes and relatively high sample flow rates prevented the adequate retention of OH-PAH, we optimized the sample flow rate for capillaries 100cm in length. Metabolite retention was monitored via fluorescence spectroscopy, a technique sensitive enough to monitor OH-PAH at trace concentration levels.\(^3\) Fluorescence intensities were measured at the maximum fluorescence excitation and emission maxima of each metabolite. All measured intensities were within the linear dynamic ranges of the calibration curves. All calibration curves were built with a minimum of five OH-PAH linear concentrations. Each point in the calibration graphs corresponded to the average value of at least three intensity measurements. The best straight lines were obtained via the least squares method. All extractions were made by pressuring 1mL OH-PAH solution through the pre-concentration capillary tube. All calculations were based on average values taken from triplicate measurements of three independent metabolite extractions carried out with three coated capillaries. The percent of extracted metabolite (\(\%E\)) was calculated according to equation (7.1):

\[
\%E = 100 \left( C_{\text{OH-PAH}} - [\text{OH-PAH}]_A \right) / C_{\text{OH-PAH}} \quad (7.1)
\]

Where \(C_{\text{OH-PAH}}\) corresponds to the original metabolite concentration in the sample and \([\text{OH-PAH}]_A\) is the concentration of OH-PAH remaining in the extracted solution. The sample flow rate (\(F\)) was controlled by varying the pressure of the CE injection pump from 200 to 2000mbar. \(F\) was calculated according to the equation \(F = 1,000 \mu\text{L}/\text{t}\), where \(t\) was the time it took 1,000\(\mu\text{L}\) to flow through the capillary tube. Figure 7.2 correlates the extraction efficiency of 3-OHbap to the pump pressure and the sample flow rate. As the pump pressure was varied from 200 to 1,000mbar and the sample flow rate increased from 2.2\(\mu\text{L}/\text{min}\) (200mbar) to approximately
7.8μL/min (1,000mbar), the extraction efficiency of 3-OHbap remained statistically equivalent 
(N₁ = N₂ = N₃ = 3; P = 0.05). Considerable drops of the %E values were observed at 1,500 and 
2,000mbar pump pressures. The same trend was observed for the remaining OH-PAH. Because 
the pre-concentration time is inversely proportional to the pump pressure, we adopted 1,000mbar 
for all remaining studies.

Figure 7.2 Effect of CE pump pressure on the flow rate and extraction efficiency of 3-OHbap.

7.3.4 Releasing OH-PAH from Au NPs deposited capillaries

Previous studies in our lab investigated the efficiency of alcohols, alkanes and 
alkanethiols to release PAH₁² and OH-PAH³ adsorbed on the surface of Au NPs. The best
recoveries for OH-PAH were obtained with a 4% 1-pentanethiol-methanol (v/v) solution. This mixture was then used here to release OH-PAH from Au NPs deposited capillaries. Metabolites were individually pre-concentrated from 1,000μL aliquots of 20ng.mL⁻¹ OH-PAH standard solutions. This metabolite concentration (C_{OH-PAH}) is within the concentration range of OH-PAH in urine samples. All extractions were made by pressuring 1mL OH-PAH solution through the pre-concentration capillary tube at 1,000mbar. The releasing solvent was then pumped through the coated capillary under the same sample pressure and the eluted solution was collected for further dilution with methanol. The dilution step provided appropriate aliquot volumes (500μL) for the smallest cuvettes available in our lab. Metabolite concentrations were then determined via fluorescence spectroscopy and the calibration curve method. The percent of extracted metabolite (%E) was calculated according to equation (7.1). The overall recovery of OH –PAH (%OR) was calculated according to equation (7.2):

\[
\%OR = 100\frac{[OH-PAH]_B V_B}{C_{OH-PAH} V_S} \quad (7.2)
\]

Where \([OH-PAH]_B\) corresponds to the metabolite concentration released from the pre-concentration capillary, \(V_B = 500μL\), \(C_{OH-PAH}\) is the metabolite concentration in the sample and \(V_S = 1mL\). The percent of released metabolite (%RE) was calculated with the following equation:

\[
\%RE = 100\frac{[OH-PAH]_B V_B}{(C_{OH-PAH} V_S - [OH-PAH]_A V_A)} \quad (7.3)
\]

Several volumes of releasing solvent (4, 6, 8, 10 and 15μL) were investigated for best releasing efficiencies and overall recoveries. The best percentages were obtained with 8 and 10μL of 4% 1-pentanethiol-methanol (v/v). Table 7.3 summarizes the %E, %RE and %OR values obtained for the five OH-PAH with 8μL of releasing solvent. The statistical comparison of the tabulated values \((N_1 = N_2 = 3; P = 0.05)\) shows no difference on the extraction efficiencies of the studied metabolites. The same is true for the releasing efficiencies and the overall recoveries. This is an
indication that possible differences among the physical-chemical affinities OH-PAH might have towards the inner surfaces of coated capillaries and their surroundings are within the precision of our measurements.

Table 7.3 Overall recovery of in-capillary micro-extraction.

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>Ex/Em</th>
<th>E (%)</th>
<th>RE (%)</th>
<th>OR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-OHphe</td>
<td>253/381</td>
<td>76.5 ± 5.9</td>
<td>91.2 ± 5.1</td>
<td>69.8 ± 5.5</td>
</tr>
<tr>
<td>5-OHbap</td>
<td>305/429</td>
<td>89.4 ± 5.0</td>
<td>84.7 ± 6.3</td>
<td>75.7 ± 6.2</td>
</tr>
<tr>
<td>4-OHbap</td>
<td>371/419</td>
<td>84.5 ± 4.5</td>
<td>85.6 ± 4.3</td>
<td>72.4 ± 3.2</td>
</tr>
<tr>
<td>3-OHbap</td>
<td>308/430</td>
<td>87.1 ± 4.1</td>
<td>92.5 ± 3.7</td>
<td>80.6 ± 5.3</td>
</tr>
<tr>
<td>1-OHpyr</td>
<td>347/384</td>
<td>85.8 ± 3.5</td>
<td>88.9 ± 4.9</td>
<td>76.3 ± 4.8</td>
</tr>
</tbody>
</table>

| a Standard solutions were prepared in methanol. b Fluorescence excitation and emission wavelengths at which intensity measurements were made. All calculations are based on three independent extractions. c Extraction efficiency of OH-PAH. d Releasing efficiency of OH-PAH with 4% (v/v) of 1-pentanethiol/methanol. e Overall recovery of OH-PAH |

7.3.5 **Analytical figures of merit (AFOM)**

The AFOM of in-capillary micro-extraction CZE were compared to those obtained via CZE. Calibration curves were built with standard mixtures containing known concentrations of the five metabolites in methanol. Sample pre-concentration via in-capillary micro-extraction was carried out processing 1mL of the standard mixture at 1,000mbar pressure. Metabolites release was accomplished passing 8μL of a 4% 1-pentanethiol – methanol solution (v/v) through the pre-concentration capillary at 1,000mbar pressure. CZE was carried out with 8μL of sample using 100mM CAPS buffer solution (pH 10.4) in 40% methanol (v/v). Table 7.4 compares the AFOM of the two approaches. All linear dynamic ranges were based on the average intensities of at least five OH-PAH concentrations. The average intensities plotted in the calibration graphs
correspond to a minimum of three measurements made from triplicate aliquots submitted to three complete experimental trials. No efforts were made to reach the upper concentration limits of the calibration curves. The excellent correlation coefficients (R) demonstrate the existence of linear relationships in all cases. The main significant difference between the AFOM of the two approaches resides on the LOD. Their values – which were calculated according to S/N = 3 – clearly show the advantage of using Au NPs deposited capillaries for pre-concentration in-capillary micro-extraction CZE. Concentration factors of approximately two orders of magnitude provided UV absorption LOD considerably lower or of the same order of magnitude as those previously reported via LIF.\textsuperscript{110,206}
Table 7.4 Comparison of the analytical figures of merit of the CE method and in-capillary micro-extraction CE method.

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>Retention time (min)</th>
<th>CE</th>
<th>In-capillary Micro-extraction CE</th>
<th>C-Factor&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LDR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>LOD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>9-OHphe</td>
<td>31.16 ± 0.27</td>
<td>1 - 10</td>
<td>0.9938</td>
<td>0.61</td>
</tr>
<tr>
<td>5-OHbap</td>
<td>33.72 ± 0.31</td>
<td>1 - 10</td>
<td>0.9981</td>
<td>0.86</td>
</tr>
<tr>
<td>4-OHbap</td>
<td>34.37 ± 0.35</td>
<td>1 - 10</td>
<td>0.9939</td>
<td>1.08</td>
</tr>
<tr>
<td>3-OHbap</td>
<td>38.87 ± 0.58</td>
<td>1 - 10</td>
<td>0.9894</td>
<td>0.75</td>
</tr>
<tr>
<td>1-OHpyr</td>
<td>39.87 ± 0.44</td>
<td>1 - 10</td>
<td>0.9898</td>
<td>0.94</td>
</tr>
</tbody>
</table>

<sup>a</sup> LDR: linear dynamic range; <sup>b</sup> LOD were calculated according to S/N = 3, where S is the intensity of the signal and N is intensity of noise; <sup>c</sup> Concentration factor were calculated according to the equation: C-factor = (Analyte desorption efficiency × Analyte volume loaded)/In-capillary micro-extraction desorption volume.
7.3.6 Urine Analysis

Table 7.5 lists the metabolites absorption intensities recorded from electropherograms of three spiked urine samples and three methanol standard mixtures. For comparison purposes, all samples had the metabolites at the same parts-per-billion concentration level. Because urine matrix constituents showed to degrade free Au NPs in aqueous solutions, we decided to first extract the metabolites from the spiked urine samples and then perform their in-capillary micro-extraction from the methanol extract. SPE was carried out via an experimental procedure previously developed in our lab. SPE methanol extracts and methanol standard mixtures (No SPE) were submitted to in-capillary micro-extraction using one Au NPs deposited capillary per sample. In-capillary micro-extraction was carried out by pressuring through the capillary 1mL of solution at 1,000mbar. Comparison of SPE to No SPE values show lower absorption intensities for all the samples previously submitted to SPE. This was expected as the SPE procedure provides overall recoveries lower than 100% for all the studied metabolites. The comparison of the relative standard deviations (RSD) consistently shows better precision of measurements for the No SPE samples. The No SPE RSD values correspond to the precision of measurements obtained via the in-capillary micro-extraction procedure. Because their averages were calculated from three electrophoretic runs using three different capillaries, the reported values also reflect the excellent reproducibility of Au NPs deposited capillaries.
Table 7.5 Reproducibility of In-Capillary Micro-Extraction CZE for the Analysis of OH-PAH (Absorption intensities ×10^-4).

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>Capillary 1 and 2</th>
<th>Capillary 3 and 4</th>
<th>Capillary 5 and 6</th>
<th>Average (RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SPE</td>
<td>No SPE</td>
<td>SPE</td>
<td>No SPE</td>
</tr>
<tr>
<td>9-OHphe</td>
<td>2.70</td>
<td>3.44</td>
<td>2.34</td>
<td>3.23</td>
</tr>
<tr>
<td>5-OHbap</td>
<td>2.35</td>
<td>2.94</td>
<td>1.87</td>
<td>3.05</td>
</tr>
<tr>
<td>4-OHbap</td>
<td>1.27</td>
<td>1.59</td>
<td>1.12</td>
<td>1.48</td>
</tr>
<tr>
<td>3-OHbap</td>
<td>2.88</td>
<td>3.75</td>
<td>2.36</td>
<td>3.72</td>
</tr>
<tr>
<td>1-OHpyr</td>
<td>1.33</td>
<td>1.89</td>
<td>1.19</td>
<td>1.74</td>
</tr>
</tbody>
</table>
Figure 7.3 displays the electropherograms of three standard mixtures recorded under the same CZE conditions. Comparison of electropherograms 7.3a and 7.3b show the advantage of using in-capillary micro-extraction to pre-concentrate the sample prior to CZE analysis. Although the concentration of individual metabolites in the pre-concentrated sample (7.3b) is 50x lower than their concentration in the no pre-concentrated sample (7.3a), the signal intensities in 7.3b are approximately 2x higher than those in 7.3a. Furthermore, the presence of 1-pentanethiol in the separation buffers - which appears as peak I in 7.3b - shows no effect on the migration times of the studied OH-PAH. Comparison of electropherograms 7.3b and 7.3c demonstrates no effect of the possible presence of urine matrix components in the separation buffer. Because the migration times of the studied metabolites and their peak widths remain approximately the same, we can state that the use of Au NPs deposited capillaries as pre-concentration devices for urine SPE extracts does not affect the ability of CZE to separate OH-PAH.
Figure 7.3 Electropherogram of a mixture of 5 OH-PAH and 1-pentanethiol.

a) 1.5 μg/mL standard solution; b) 0.03μg/mL OH-PAH solution after in-capillary micro-extraction; c) 0.01μg/mL OH-PAH urine sample after SPE and in-capillary micro-extraction. I) EOF marker (Methanol); II) 1-pentanethiol; III) 9-OHphen; IV) 5-OHbap; V) 4-OHbap; VI) 3-OHbap; VII) 1-OHpyr. CE analysis conditions for the 5 OH-PAH: buffer, 100mM CAPS with 40% (v/v) methanol; apparent pH 10.1; voltage, 26kV; temperature, 25°C.
7.4 Conclusion

We have developed a novel and sensitive method for the analysis of OH-PAH via CZE. It is possible now to obtain UV absorption LOD at the low parts-per-billion level with commercial CE instrumentation. Our LOD are comparable to those previously reported via CE-LIF.\textsuperscript{110,206}

The main original component of our method is the use of Au NPs deposited capillaries for the pre-concentration of samples prior to CE analysis. Their application to the pre-concentration of OH-PAH provides concentration factors of approximately two orders of magnitude. The reproducibility of measurements is excellent as well. The second original component demonstrates the CZE separation of 3-OHbap, 4-OHbap and 5OH-bap using a 100mM CAPES buffer solution (pH 10.4) with 40% (v/v) methanol. The pre-concentration of OH-PAH with Au NPs deposited capillaries requires the use of 1-pentanethiol for subsequent metabolite release. Our studies show that this releasing solvent has no effect on the successful CZE separation of the studied compounds.
8.1 Introduction

Previous articles have shown that room-temperature excitation and fluorescence spectra of PAH metabolites and PAH-DNA adducts do not have sufficient resolution to distinguish among closely related species. Similar findings have been reported at low-temperature, which have shown very broad spectra with bandwidths of several hundreds of reciprocal centimeters. The nature of band broadening is well known and thoroughly discussed in chapter 2 of this dissertation. For amorphous matrixes in the condensed phase, characterized by their lack of long-range order, the local conditions (microenvironments) that influence the transition energies of the solute molecules (predominantly through electron-electron interactions) differ from one solute molecule to another. The resulting differences in electronic transition energies lead to a Gaussian broadening of excitation and emission spectra. This type of band broadening is called inhomogeneous band broadening. Homogeneous broadening affects all molecules to the same extent. It arises from vibronic coupling to the rapidly fluctuating surrounding matrix and from the limited lifetimes of the states involved in the electronic transition. In an ideal situation, i.e. when all solute molecules experience exactly the same environment, the only remaining source of band broadening (apart from instrumental contributions) is homogeneous.

In addition to Shpol’skii spectroscopy, another well-known technique to reduce
inhomogeneous band broadening is fluorescence line narrowing spectroscopy. Similar to Shpol’skii spectroscopy, fluorescence line narrowing spectroscopy deals with cryogenic temperatures where samples are frozen to 77K or below. In fluorescence line narrowing spectroscopy, inhomogeneous broadening is suppressed with the aid of a narrow-band tunable laser that excites a small selection of analyte molecules in the frozen sample. Excitation can be accomplished within the 0-0 transition wavelength region or into the vibronic region of the first excited state \( S_1-S_0 \). When the conformations of the analyte molecules and the orientations of the surrounding matrix molecules do not change substantially during the lifetime of the excited state, only the small sub-selection (isochromat) of analyte molecules is excited and their fluorescence line-narrowed spectrum is observed. The freedom in solvent choice has prompted the application of fluorescence line narrowing spectroscopy to a variety of problems. In particular, for chemical identification, conformational analysis, and/or probing the micro-environment of DNA (or protein) - adducts.\(^{99,253}\) Unfortunately, fluorescence line narrowing spectroscopy is distinctly less sensitive than Shpol’skii spectroscopy because the excited isochromat only comprises a small fraction of the total number of analyte molecules in the frozen matrix.\(^{253}\) On the other end, the requirement of a specific analyte-solvent combination has confined Shpol’skii spectroscopy to the analysis of parent PAH.\(^{254}\) The research presented here has the potential to remove this limitation and extend the applicability of Shpol’skii Spectroscopy to the analysis of polar PAH metabolites.

The determination of PAH metabolites prior to DNA damage fills an important niche to prevent extreme body burdens and minimize cancer risk. One would certainly prefer an early warning parameter over a toxicological endpoint – such as DNA-adducts - that indicates the damage has already been done. Publications describing the use of Shpol’skii spectroscopy for
PAH metabolites are relatively rare. Reviewing the early Russian literature, Nurmukhametov states that “Attempts to obtain an effectively resolved structure for the electronic bands of aromatic alcohols...in n-hydrocarbon solution did not give positive results”. Nevertheless, a few years later Khesina et al. observed quasi-line spectra for two phenolic benzo[a]pyrene (B[a]P) metabolites, 3-hydroxy-B[a]P (3-OHB[a]P) and 6-OHB[a]P, in frozen n-octane. Garrigues and Edwald reported quasi-line spectra for 9-OHB[a]P. Among the hydroxyl-PAH included in Karcher’s spectral atlas, a Shpol’skii spectrum was included only for 3-OHB[a]P. Ariese and co-workers noted that the sensitivity of the Shpol’skii method for monohydroxy (phenolic) metabolites of B[a]P is more than one order of magnitude poorer in comparison with the parent compound B[a]P, although at room temperature the fluorescence quantum yields of the two compounds are similar. In the case of 1-hydroxypyrene the difference in sensitivity is even larger. Experimental evidence suggests that this phenomenon is caused by the limited guest-host compatibility due to the polar hydroxyl group, the later having a stronger influence in the case of the five ring B[a]P derivative. In principle two effects can occur, resulting from either the poorer solubility in n-alkanes or the poorer compatibility with the n-alkane crystal lattice. The first is the formation of aggregates during the cooling procedure, leading to precipitation and reduced quantum yields. The second concerns freezing out of the analyte molecules, at the point of matrix solidification, from the crystalline phase being formed into the amorphous phase, resulting in broad-band emission at the expense of the intensity of the Shpol’skii lines. As a result, the direct Shpol’skii analysis of phenolic metabolites can only be applied if trace-level sensitivity is not required.

As a tentative means of improving the compatibility of PAH metabolites with typical n-alkane Shpol’skii matrixes, Weeks and co-workers first described a procedure to transform
monohydroxybenz[a]anthracenes into less polar methoxy derivatives, which could subsequently be analyzed by means of Laser-Excited Shpol’skii Spectroscopy. The methylation procedure was later extended to several other types of B[a]P metabolites, including monohydroxy-B[a]P derivatives, B[a]P-dihydrodiols, B[a]P-dihydrodiolepoxide, and B[a]P-tertahydrotetrol.258,261 The approach was found to be suitable only for phenolic metabolites. The fluorescence intensity of 3-methoxy-B[a]P and 1-methoxy-B[a]P - i.e. the derivatives of 3-OHB[a]P and 1-OHB[a]P, respectively – in n-octane was found to be comparable to that of B[a]P.258 Good results were also obtained for phenolic metabolites when other derivatizing agents were used; narrow Shpol’skii spectra and good detection limits were obtained for acetylated 6-OHB[a]P and for octyl derivatives of 3-OHB[a]P, 6-OHB[a]P, and 9-OHB[a]P.261 Unfortunately, derivatization of polyhydroxylated metabolites proved to be much more complicated. In the case of methylation of B[a]P dihydrodiol metabolites a mixture of products was obtained.258 For instance, B[a]P 9,10-dihydrodiol yielded 9-methoxy-B[a]P, 10-methoxy-B[a]P, as well as the expected dimethylated product 9,10-dihydrodimethoxy-B[a]P. Their relative concentration ratios depended critically on the derivatization conditions, which made the reproducibility of measurements poor. Other problems were related to the limited sensitivity of Shpol’skii spectra of methylated polyhydroxy metabolites. The detection limits found for permethylated B[a]P diols, B[a]P diolepoxide, and B[a]P tetroxid were many orders of magnitude poorer than those observed for methylated phenolic B[a]P metabolites, although the same instrumental set up was used. In summary, Shpol’skii spectroscopy, after derivatization, is a well suited approach for monohydroxy metabolites but falls short for polyhydroxylated metabolites for reasons of both reproducibility and sensitivity.
8.2 Experimental Section

8.2.1 Chemicals

HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). N-Alkanes and alcohols were acquired from Acros Organics (Atlanta, GA, USA). Otherwise specified, Nanopure water from a Barnstead Nanopure Infinity water system (Massachusetts, USA) was used throughout. Commercial standards of the PAH metabolites were acquired from the Midwest Research Institute (Kansas City, Missouri) at their highest purity available and used with no further purification.

8.2.2 Solution Preparation

Stock metabolite solutions were prepared daily and kept in the dark at 4°C. Possible metabolite degradation was monitored via RTF spectroscopy. Metabolite working solutions were prepared by serial dilution with the appropriate solvent.

8.2.3 UV-VIS Absorption Spectroscopy

See section 3.1 for instrumental details.

8.2.4 RTF Spectroscopy

See section 3.2 for instrumental details.

8.2.5 Instrumentation for LETRSS Analysis

See section 3.3 for instrumental details.
8.2.6 **Spectral Acquisition with the MLS**

See section 3.3 for experimental details.

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8.2.7 **Lifetime Acquisition with the MLS**

Fluorescence lifetimes were measured via the WTM procedure, which consists of the following three steps: (i) full sample and background WTM collection; (ii) background decay curve subtraction from the fluorescence decay curve at a wavelength of maximum emission for each PAH, and (iii) fitting of the background corrected data to single exponential decays. In cases of unknown sample composition where the formulation of a correct blank for lifetime background correction was not possible, the fluorescence decay at the base of the target peak was used for background subtraction at the target wavelength. All fluorescence lifetimes were recorded using 5ns minimum delay. This delay was sufficient to avoid the need to consider convolution of the dye laser pulse with the analytical signal. The accuracy of this procedure has been confirmed previously.\(^{148}\) Origin software (version 7.5, Micronal Software) was used for curve fitting of fluorescence decays. Fluorescence lifetimes were obtained from decay curves fitted to \( y = y_0 + A_1e^{-(x-x_0)t} \) were obtaining by fixing \( x_0 \) and \( y_0 \) at a value of zero.

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8.2.8 **77K and 4.2K Sample Procedures**

77K measurements with the spectrofluorimeter followed the classic procedure of immersing the sample solution in a quartz tube into a nitrogen filled Dewar flask. 4.2 K measurements were carried out as follows. After transferring a known volume (typically 100–
200 μl) of undegassed sample solution with a pipette into the sample vial of the cryogenic probe, the tip of the fiber-optic bundle was positioned and held constant with the screw cap above the solution surface. Sample freezing was accomplished by lowering the copper tubing into the liquid helium, which was held in a Dewar flask with 60 L storage capacity. The liquid helium would typically last 3 weeks with daily use, averaging 15 to 20 samples per day. Complete sample freezing took less than 90 s per sample. Replacing the frozen sample involved removing the sample vial from the cryogen container and melting the frozen sample with a heat gun. Because no physical contact between the tip of the fiber-optic bundle and the sample ever occurred during measurements, probe cleanup between measurements was not necessary. The entire freeze, thaw, and sample replacement cycle took no longer than 5 min.

8.3 Results and Discussion

Preliminary studies in our lab support a rather simple solution for the problem at hand. As a way of improving the solubility of PAH metabolites in frozen matrixes, we tested a series of primary alcohols (RCH₂OH) as solvent hosts. By optimizing the length of the alkyl group (R), our expectation was to minimize the number of crystallographic sites occupied by metabolite molecules in the crystal lattice of the frozen alcohol. We thought that the combination of these two effects should lead to spectral narrowing and strong fluorescence emission for trace determination of PAH metabolites.

A criterion often employed for solvent selection in Shpol’skii Spectroscopy is to match the length of the n-alkane molecule to the effective length of the parent PAH.¹⁰⁰ The same criterion was then adopted to guide the study of PAH metabolites in primary alcohols (RCH₂OH).
We selected 1-hexanol for 1-hydroxypyrene, 2-hydroxyfluorene and 9-hydroxyphenanthrene and 1-octanol for 3-OHB[a]P. Table 1 compares their 77K-fluorescence intensities to those obtained in their corresponding n-alkane solutions. Fluorescence enhancements in the presence of the primary alcohols were observed in all cases.

Table 8.1 77K Fluorescence Intensities of PAH Metabolites in Alcohol and n-Alkane Matrixes.

<table>
<thead>
<tr>
<th>Monohydroxy-PAH</th>
<th>I&lt;sub&gt;Alcohol&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>I&lt;sub&gt;Alkane&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>I&lt;sub&gt;Alcohol&lt;/sub&gt; / I&lt;sub&gt;Alkane&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-hydroxypyrene</td>
<td>(1.80 ± 0.08) × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>(6.74 ± 0.06) × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.67 ± 0.12</td>
</tr>
<tr>
<td>2-hydroxyfluorene</td>
<td>(3.71 ± 0.06) × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>(1.08 ± 0.03) × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3.44 ± 0.11</td>
</tr>
<tr>
<td>3-OHB[a]P</td>
<td>(6.97 ± 0.08) × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>(3.83 ± 0.05) × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.82 ± 0.03</td>
</tr>
<tr>
<td>9-hydroxyphenanthrene</td>
<td>(7.49 ± 0.05) × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>(2.36 ± 0.10) × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>3.17 ± 0.13</td>
</tr>
</tbody>
</table>

<sup>a</sup> All metabolite concentrations were 1ng.mL<sup>-1</sup>. <sup>b</sup> I<sub>Alcohol</sub> and I<sub>Alkane</sub> = fluorescence intensities in the frozen alcohol and n-alkane matrixes, respectively: 1-hexanol and n-hexane (1-hydroxypyrene, 2-hydroxyfluorene and 9-hydroxyphenanthrene) and 1-octanol and n-octane (3-OHB[a]P). All fluorescence intensities represent an average of three independent measurements made at the maximum excitation (λ<sub>exc</sub>) and emission (λ<sub>em</sub>) wavelengths of each metabolite.

Although the Shpol’skii effect is significant at liquid nitrogen temperature, fluorescence excitation and emission spectra only become extremely sharp at temperatures below 20 K. Figure 8.1 compares the 4.2K fluorescence spectra of 1-hydroxypyrene and 3-OHB[a]P in primary alcohols with various alkyl chain lengths. A drastic improvement of spectral narrowing is observed for both metabolites as the length of the RCH<sub>2</sub>- group approaches the length of the n-alkane that best fits the linear dimensions of the parent PAH, i.e. n-hexane (pyrene) and n-octane (B[a]P).
Figure 8.1 4.2K fluorescence emission spectra recorded upon laser excitation at 347.9nm (1-OHpyrene,) 311.2nm (3-OHB[a]P).
10ns delay and 1000ns gate times, and spectral band-pass of 0.32nm. Both spectra represent the accumulation of 100 laser pulses per data point. Metabolites concentrations were 10ng.mL⁻¹.
The spectra of 1-OHpyrene in 1-hexanol and 3-OHB[a]P in 1-octanol show the quasi-line structure expected from Shpol'skii systems. Another peculiar feature of these spectra is the presence of featureless fluorescence bands underlying the quasi-line fluorescence spectra. Broad emission bands in the spectra of PAH/n-alkane systems have been attributed to the presence of disordered groups of solute molecules or pre-aggregates in the frozen matrix. We should mention that we did not attempt site-selective excitation or sample excitation near the 0-0 transition region of PAHs metabolites. Bearing in mind that both approaches usually improve spectral resolution in Shpol’skii matrixes, it is reasonable to expect even narrower spectra from PAHs metabolites.

The effect of site-selective excitation is clearly noted in Figure 8.2. Upon fast cooling to 4.2K in 1-octanol, B[a]P-trans-9,10-dihydrodiol occupies predominantly two crystallographic sites that yield two distinctive fluorescence spectra slightly shifted by small wavelength differences. Under multi-site excitation conditions ($\lambda_{\text{exc}} = 347.6$nm), the maximum wavelengths of the doublet emissions from the 0-0 transitions appear at 399.6nm (site 1) and 402.6nm (site 2). Site-selective excitation of “site 1” at 346.9nm removes the fluorescence contribution of site 2 and considerably narrows the emission spectrum of the metabolite.
Figure 8.2 4.2K fluorescence spectrum of 10ng.mL-1 B[a]P-trans-9,10-dihydrodiol in 1-octanol.

Both spectra represent the accumulation of 100 laser pulses per data point. Other parameters are: 10ns delay and 1000ns gate times, and spectral band-pass of 0.32nm.

Table 8.2 summaries the 77K and 4.2K fluorescence quantum yields of B[a]P-r-7,8-dihydrodiol-c-9,10-poxide(±), (syn) and B[a]P-r-7,8-dihydrodiol-t-9,10-poxide(±), (anti) in n-octane and 1-octanol. In comparison to 1-octanol, lowering the temperature to 4.2K caused relatively small improvements in the fluorescence quantum yields. Similar results were observed for 3-OHB[a]P, 4-OHB[a]P, 5-OHB[a]P, B[a]P-trans-9,10-dihydrodiol and B[a]P-trans-7,8-dihydrodiol (±). As such, the strong fluorescence emission observed at 77K and 4.2K can be mainly attributed to 1-octanol.
Table 8.2 Fluorescence Quantum Yields a of anti-BPDE (±) and syn-BPDE (±).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>77K</th>
<th>4.2K</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-BPDE (±)</td>
<td>n-octane</td>
<td>0.046 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>1-octanol</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>syn-BPDE (±)</td>
<td>n-octane</td>
<td>0.075 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>1-octanol</td>
<td>0.23 ± 0.06</td>
</tr>
</tbody>
</table>

a All values represent an average of three independent measurements; anti-BPDE (±) = B[a]P-r-7,t-8-dihydrodiol-t-9,10-poxide(±), (anti); syn-BPDE (±) = B[a]P-r-7,t-8-dihydrodiol-c-9,10-poxide(±), (syn).

Table 8.3 summaries the analytical figures of merit for B[a]P metabolites in this solvent. The LOD were calculated using the formula $3s_B / m$; where $s_B$ is the standard deviation of 16 blank (1-octanol) and m is the slope of the LDR of the calibration curve. The relative standard deviations of three fluorescence measurements at medium linear concentrations varied between 3% (4-OHBaP) and 6% (B[a]P-trans-7,8-dihydrodiol (±)). Clearly, using 1-octanol as the Shpol’skii matrix makes the quantitative analysis of B[a]P metabolites at trace concentration levels possible.

<table>
<thead>
<tr>
<th>B[a]P metabolite</th>
<th>$\lambda_{exc} / \lambda_{em}$ (nm)</th>
<th>LDR (ng.mL⁻¹)</th>
<th>R</th>
<th>LOD (ng.mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-OHB[a]P</td>
<td>311.5 / 433.5</td>
<td>2.4 - 100</td>
<td>0.9980</td>
<td>0.8</td>
</tr>
<tr>
<td>4-OHB[a]P</td>
<td>309.1 / 418.6</td>
<td>0.6 - 100</td>
<td>0.9978</td>
<td>0.2</td>
</tr>
<tr>
<td>5-OHB[a]P</td>
<td>309.1 / 422.3</td>
<td>1.8 - 100</td>
<td>0.9956</td>
<td>0.6</td>
</tr>
<tr>
<td>B[a]P-trans-7,8-dihydrodiol (±)</td>
<td>347.7 / 394.5</td>
<td>0.9 - 100</td>
<td>0.9993</td>
<td>0.3</td>
</tr>
<tr>
<td>B[a]P-trans-9,10-dihydrodiol</td>
<td>347.7 / 402.6</td>
<td>0.6 - 100</td>
<td>0.9974</td>
<td>0.2</td>
</tr>
<tr>
<td>anti-BPDE (±)</td>
<td>348.3 / 379.6</td>
<td>0.3 - 100</td>
<td>0.9997</td>
<td>0.1</td>
</tr>
<tr>
<td>syn-BPDE (±)</td>
<td>347.9 / 377.4</td>
<td>0.9 - 100</td>
<td>0.9990</td>
<td>0.3</td>
</tr>
</tbody>
</table>

a Lower limit of LDR corresponds to the limit of quantitation, i.e. 3 x LOD. No efforts were made to experimentally determine the upper linear concentration. R = correlation coefficient of LDR.
When frozen in 1-octanol, the 4.2K fluorescence spectra of anti- and syn-BPDE (±) present the predominant contribution of four and two crystallographic sites, respectively. Figure 8.3 shows the effect of wavelength excitation on the spectral features of both racemic mixtures. Sample excitation was within the wavelength range of site selective excitation of anti-BPDE (±).
Figure 8.3 4.2K fluorescence emission spectra of anti-BPDE (±) and syn-BPDE (±) in 1-octanol.

Sample excitation (ex) was within the wavelength range of site selective excitation of anti-BPDE (±). All spectra represent the accumulation of 100 laser pulses per data point. Other parameters are: 10ns delay and 1000ns gate times, and spectral band-pass of 0.32nm.
Table 8.4 summarizes the fluorescence lifetimes of the racemic mixtures measured at the maximum emission wavelength of each crystallographic site. Our observations are consistent with the well known sensitivity of fluorescence lifetimes to the microenvironment of the fluorophor. It is generally accepted that the multiplet structure of fluorescence spectra arise from the different orientations of guest molecules in the Shpol’skii matrix. The different orientations lead to nonequivalent crystal field effects and hence wavelength shifts. Most likely, the field effects are also sensed by the fluorescence lifetimes of the metabolites. We should mention that we were the first to report the dependence of fluorescence lifetimes on the crystallographic sites of parent PAHs.$^{134}$ The comparison of values in Table 8.4 shows that the lifetime difference from site to site is larger than their wavelength difference. This fact enhances the specificity of site-selective excitation because it facilitates time-resolution of partially overlapped spectra. Time-resolution makes it possible to select appropriate delay and gate times to either minimize or enhance the spectral contribution of one site over the contribution of the remaining sites.

<table>
<thead>
<tr>
<th></th>
<th>376.4nm</th>
<th>377.4nm</th>
<th>377.9nm</th>
<th>379.0nm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>anti-BPDE (±)</strong></td>
<td>142.7 ± 3.3</td>
<td>189.9 ± 3.4</td>
<td>177.1 ± 2.3</td>
<td>132.7 ± 5.2</td>
</tr>
<tr>
<td><strong>syn-BPDE (±)</strong></td>
<td>224.6 ± 2.5</td>
<td>201.8 ± 1.3</td>
<td>No site</td>
<td>No site</td>
</tr>
</tbody>
</table>

*a Fluorescence lifetimes are in nanoseconds. Reported values represent the averages of three individual measurements from three frozen aliquots.*
Figure 8.4 overlaps the fluorescence spectra of the two racemic mixtures recorded upon sample excitation at 347.5nm, i.e. an excitation wavelength that promotes multi-site excitation of the two mixtures. A delay time of 200ns and a gate time of 500ns reduce the fluorescence intensity of $anti$-BPDE ($\pm$) to approximately 25% of its original value. If the direct determination of $syn$-BPDE ($\pm$) were of interest, the contribution of the residual fluorescence of $anti$-BPDE ($\pm$) could be eliminated by tuning the excitation wavelength to an excitation wavelength that mainly promotes the fluorescence emission of $syn$-BPDE ($\pm$). Similarly, if the direct determination of $anti$-BPDE ($\pm$) were of interest, it could be achieved via site selective excitation of its site at 379.5nm. The combination of multiple fluorescence sites with narrow peaks and different fluorescence lifetimes associated to site-selective excitation holds tremendous potential for the direct determination of closely related BPDE ($\pm$) metabolites.
Figure 8.4 Effect of delay (200ns) and gate (500ns) times on the 4.2K fluorescence spectra of syn-BPDE (±) (---) and anti-BPDE (±) (—) in 1-octanol. Sample excitation (ex) was within the wavelength range of site selective excitation of anti-BPDE (±). All spectra represent the accumulation of 100 laser pulses per data point. Other parameters are: 10ns delay and 1000ns gate times, and spectral band-pass of 0.32nm.

8.4 Conclusion and Future Studies

The outstanding selectivity and sensitivity for the direct analysis of PAHs at trace concentration levels has made Shpol’skii spectroscopy a leading technique in environmental analysis. Unfortunately, the requirement of a specific guest-host combination - typically a non-polar PAH dissolved in an n-alkane - has hindered its widespread application to the field of
analytical chemistry. The research presented in this chapter takes the first steps in removing this limitation with the direct analysis of polar PAH metabolites in alcohol matrixes.

Finding the most appropriate Shpol’skii matrix for the fluorophor under investigation is not always evident and requires some trial and error. The properties of the fluorophor play an important role, in particular the three-dimensional shape of the molecule, its rigidity, and the number (and positions) of polar substituent groups. In general, the difficulty in achieving Shpol’skii spectra increases with the number of polar groups present in the fluorophor since they reduce the solubility in n-alkanes and tend to promote the formation of aggregates and clusters during cooling. It has been reported that in a 10K n-octane Shpol’skii matrix, addition of a hydrogen bonding impurity like water, methanol or diethyl ether to the sample does not affect the high resolution of 3-hydroxyflavone. The reason for this is that 3-hydroxyflavone fits tightly into the matrix so there is no space available for an additional hydrogen-bonding molecule; upon cooling the added polar solvent is simply frozen out and a phase separation is obtained. If, on the contrary, a hydrogen bonding impurity is added that can replace a particular octane molecule of the crystalline surrounding of a 3-hydroxyflavone molecule, a different effect is observed. Adding a minor amount of 2-octanol to the octane matrix of 3-hydroxyflavone resulted in the appearance of a new site at 507.2nm. Addition of a much larger amount of 3-octanol results in a new site further shifted to the blue, i.e., at 504.7nm. On the other end, the addition 1-octanol or 4-octanol showed no effect.

Under this perspective, we expect the position of the hydroxyl group in the alcohol molecule to affect both the spectral resolution of PAH metabolites and the number of sites in the Shpol’skii matrix. We also expect the position of the hydroxyl group in the alcohol molecule to be somehow related to the length of the alcohol molecule that best fits the molecular dimensions.
of the metabolite. We will investigate these possibilities monitoring the spectral properties of PAH metabolites in several types of solvent systems. For the particular case of B[a]P, we will investigate (a) 1-heptanol, 2-heptanol, 3-heptanol and 4-heptanol; (b) 1-octanol, 2-octanol, 3-octanol and 4-octanol; and (c) 1-nonanol, 2-nonanol, 3-nonanol, 4-nonanol and 5-nonanol. We will also investigate the titration of a metabolite in an n-alkane solution with small amounts of an alcohol that best matches the molecular length of both the n-alkane and the metabolite. The best choice will be made on the bases of spectral narrowing, signal intensities and number of crystallographic sites.

Analytical methods to elucidate the initial phase of carcinogenesis are extremely relevant to our society. Covalent binding of PAHs metabolites to DNA appears to be the first critical step in the initiation of the tumor formation process. Under this prospective, the determination of PAH metabolites prior to DNA damage fills an important niche to prevent extreme body burdens and minimize cancer risk. A plethora of analytical techniques are currently needed to determine its metabolites in biological matrixes. We propose a single but powerful approach to track down trace concentration levels of all PAH major metabolites. The ability to directly determine the presence and the amounts of optical isomers even if closely related stereo-isomers are present in far higher concentrations would be a huge breakthrough. The long term aim of our research is to fulfill this gap.
APPENDIX A: EXCITATION AND FLUORESCENCE SPECTRA OF 15 EPA PAH AT ROOM TEMPERATURE
Figure A-1 Excitation and fluorescence spectra of naphthalene 20ppb in 1% methanol/water at RT.

Figure A-2 Excitation and fluorescence spectra of acenaphthene 10ppb in 1% methanol/water at RT.
Figure A-3 Excitation and fluorescence spectra of fluorene 10ppb in 1% methanol/water at RT.

Figure A-4 Excitation and fluorescence spectra of phenanthrene 10ppb in 1% methanol/water at RT.
Figure A-5 Excitation and fluorescence spectra of anthracene 10ppb in 1% methanol/water at RT.

Figure A-6 Excitation and fluorescence spectra of fluoranthene 20ppb in 1% methanol/water at RT.
Figure A-7 Excitation and fluorescence spectra of pyrene 10ppb in 1% methanol/water at RT.

Figure A-8 Excitation and fluorescence spectra of benzo[a]anthracene 10ppb in 1% methanol/water at RT.
Figure A-9 Excitation and fluorescence spectra of chrysene 10ppb in 1% methanol/water at RT.

Figure A-10 Excitation and fluorescence spectra of benzo[b]fluoranthene 20ppb in 1% methanol/water at RT.
Figure A-11 Excitation and fluorescence spectra of benzo[k]fluoranthene 10ppb in 1% methanol/water at RT.

Figure A-12 Excitation and fluorescence spectra of benzo[a]pyrene 10ppb in 1% methanol/water at RT.
Figure A-13 Excitation and fluorescence spectra of dibenz[a,h]anthracene 10ppb in 1% methanol/water at RT.

Figure A-14 Excitation and fluorescence spectra of benzo[g,h,i]perylene 20ppb in 1% methanol/water at RT.
Figure A-15 Excitation and fluorescence spectra of indeno[1,2,3-cd]pyrene 30ppb in 1% methanol/water at RT.
APPENDIX B: “TITRATION” CURVE FOR 15 EPA PAH WITH 5% 20-NM AU NPS
Figure B-1 Fluorescence intensity as a function of naphthalene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 285/320nm)

Figure B-2 Fluorescence intensity as a function of acenaphthene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 292/322nm)
Figure B-3 Fluorescence intensity as a function of fluorene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 279/306nm)

Figure B-4 Fluorescence intensity as a function of phenanthrene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 294/347nm)
Figure B-5 Fluorescence intensity as a function of anthracene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 252/380nm)

Figure B-6 Fluorescence intensity as a function of fluoranthene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 288/450nm)
Figure B-7 Fluorescence intensity as a function of pyrene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 336/373nm)

Figure B-8 Fluorescence intensity as a function of benzo[a]anthracene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 289/387nm)
Figure B-9 Fluorescence intensity as a function of chrysene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 269/363nm)

Figure B-10 Fluorescence intensity as a function of benzo[b]fluoranthene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 305/426nm)
Figure B-11 Fluorescence intensity as a function of benzo[k]fluoranthene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 307/412nm)

Figure B-12 Fluorescence intensity as a function of benzo[a]pyrene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 297/405nm)
Figure B-13 Fluorescence intensity as a function of dibenz[a,h]anthracene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 304/396nm)

Figure B-14 Fluorescence intensity as a function of benzo[g,h,i]perylene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 305/420nm)
Figure B-15 Fluorescence intensity as a function of indeno[1,2,3-cd]pyrene concentration after extracted by 5% 20-nm Au NPs. (Ex/Em = 3055/510nm)

$y = 0.0341x + 0.8808$

$R^2 = 0.7292$

$y = 0.369x - 1.3108$

$R^2 = 0.9884$

$y = 0.4329x + 0.7654$

$R^2 = 0.9931$
APPENDIX C: EXCITATION AND FLUORESCENCE SPECTRA OF 15 EPA PAH IN N-OCTANE AT 77K
Figure C-1 Excitation and fluorescence spectra of naphthalene 0.5 ppm in n-octane at 77K.

Figure C-2 Excitation and fluorescence spectra of acenaphthene 0.5 ppm in n-octane at 77K.
Figure C-3 Excitation and fluorescence spectra of fluorene 0.5ppm in n-octane at 77K.

Figure C-4 Excitation and fluorescence spectra of phenanthrene 0.5ppm in n-octane at 77K.
Figure C-5 Excitation and fluorescence spectra of anthracene 0.5ppm in n-octane at 77K.

Figure C-6 Excitation and fluorescence spectra of fluoranthene 0.5ppm in n-octane at 77K.
Figure C-7 Excitation and fluorescence spectra of pyrene 0.5ppm in n-octane at 77K.

Figure C-8 Excitation and fluorescence spectra of benzo[a]anthracene 0.5ppm in n-octane at 77K.
Figure C-9 Excitation and fluorescence spectra of chrysene 0.5ppm in n-octane at 77K.

Figure C-10 Excitation and fluorescence spectra of benzo[b]fluoranthene 0.5ppm in n-octane at 77K.
Figure C-11 Excitation and fluorescence spectra of benzo[k]fluoranthene 0.5ppm in n-octane at 77K.

Figure C-12 Excitation and fluorescence spectra of benzo[a]pyrene 0.5ppm in n-octane at 77K.
Figure C-13 Excitation and fluorescence spectra of dibenz[a,h]anthracene 0.5ppm in n-octane at 77K.

Figure C-14 Excitation and fluorescence spectra of benzo[g,h,i]perylene 0.5ppm in n-octane at 77K.
Figure C-15 Excitation and fluorescence spectra of indeno[1,2,3-cd]pyrene 0.5ppm in n-octane at 77K.
APPENDIX D: FLUORESCENCE SPECTRA AND LIFETIME DECAY OF 15 EPA PAH IN N-OCTANE AT 4.2K
Figure D-1 Fluorescence spectra of naphthalene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-2 Fluorescence decay of naphthalene in n-octane at 4.2K. Ex/Em = 283/318.5nm; \( t_d = 5\text{ns}; t_g = 1000\text{ns}; \) step = 30ns.
Figure D-3 Fluorescence spectra of acenaphthene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-4 Fluorescence decay of acenaphthene in n-octane at 4.2K. Ex/Em = 283/320.1nm; \( t_d = 5\) ns; \( t_g = 1000\) ns; step = 5ns.
Figure D-5 Fluorescence spectra of fluorene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-6 Fluorescence decay of fluorene in n-octane at 4.2K. Ex/Em = 283/301.8nm; t_d = 5ns; t_g = 1000ns; step = 1ns.
Figure D-7 Fluorescence spectra of phenanthrene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-8 Fluorescence decay of phenanthrene in n-octane at 4.2K. Ex/Em = 283/363.4nm; t_d = 5ns; t_g = 1000ns; step = 10ns.
Figure D-9 Fluorescence spectra of anthracene 0.5ppm in n-octane at 4.2K. Ex = 343nm

Figure D-10 Fluorescence decay of anthracene in n-octane at 4.2K. Ex/Em = 343/384.4nm; t₀ = 5ns; t₉ = 1000ns; step = 1ns.
Figure D-11 Fluorescence spectra of fluoranthene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-12 Fluorescence decay of fluoranthene in n-octane at 4.2K. Ex/Em = 283/436.8nm; \( t_d = 5\)ns; \( t_g = 1000\)ns; step = 5ns.
Figure D-13 Fluorescence spectra of pyrene 0.5ppm in n-octane at 4.2K. Ex = 283nm

![Fluorescence spectra of pyrene 0.5ppm in n-octane at 4.2K. Ex = 283nm](image)

lifetime = 516.2ns

Figure D-14 Fluorescence decay of pyrene in n-octane at 4.2K. Ex/Em = 283/372.0nm; t_d = 5ns; t_g = 1000ns; step = 50ns.

![Fluorescence decay of pyrene in n-octane at 4.2K. Ex/Em = 283/372.0nm](image)
Figure D-15 Fluorescence spectra of benzo[a]anthracene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-16 Fluorescence decay of benzo[a]anthracene in n-octane at 4.2K. Ex/Em = 283/383.6nm; \( t_d = 5\)ns; \( t_g = 1000\)ns; step = 5ns.
Figure D-17 Fluorescence spectra of chrysene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-18 Fluorescence decay of chrysene in n-octane at 4.2K. Ex/Em = 283/380.7nm; t_d = 5ns; t_g = 1000ns; step = 10ns.
Figure D-19 Fluorescence spectra of benzo[b]fluoranthene 0.5ppm in n-octane at 4.2K. Ex = 283nm.

Figure D-20 Fluorescence decay of benzo[b]fluoranthene in n-octane at 4.2K. Ex/Em = 283/405.0nm; t_d = 5ns; t_g = 1000ns; step = 5ns.
Figure D-21 Fluorescence spectra of benzo[k]fluoranthene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-22 Fluorescence decay of benzo[k]fluoranthene in n-octane at 4.2K. Ex/Em = 283/404.1nm; t_d = 5ns; t_g = 1000ns; step = 2ns.
Figure D-23 Fluorescence spectra of benzo[a]pyrene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-24 Fluorescence decay of benzo[a]pyrene in n-octane at 4.2K. Ex/Em = 283/402.8nm; t_d = 5ns; t_g = 1000ns; step = 5ns.
Figure D-25 Fluorescence spectra of dibenz[a,h]anthracene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-26 Fluorescence decay of dibenz[a,h]anthracene in n-octane at 4.2K. Ex/Em = 283/393.9nm; t_d = 5ns; t_g = 1000ns; step = 5ns.
Figure D-27 Fluorescence spectra of benzo[g,h,i]perylene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-28 Fluorescence decay of benzo[g,h,i]perylene in n-octane at 4.2K. Ex = 283/419.7nm; \( t_d = 5\)ns; \( t_g = 1000\)ns; step = 20ns.
Figure D-29 Fluorescence spectra of indeno[1,2,3-cd]pyrene 0.5ppm in n-octane at 4.2K. Ex = 283nm

Figure D-30 Fluorescence decay of indeno[1,2,3-cd]pyrene in n-octane at 4.2K. Ex/Em = 283/463.0nm; td = 5ns; tg = 1000ns; step = 2ns.
APPENDIX E: LOW CONCENTRATION RANGES OF CALIBRATION CURVES OBTAINED VIA SPNE-LETRSS FOR 15 EPA PAH
Figure E-1 Calibration curve obtained via SPNE-LETRSS for naphthalene (Ex = 283nm).

Figure E-2 Calibration curve obtained via SPNE-LETRSS for acenaphthene (Ex = 283nm).
Figure E-3 Calibration curve obtained via SPNE-LETRSS for fluorene (Ex = 283nm).

Figure E-4 Calibration curve obtained via SPNE-LETRSS for phenanthrene (Ex = 283nm).
Figure E-5 Calibration curve obtained via SPNE-LETRSS for anthracene (Ex = 283nm).

Figure E-6 Calibration curve obtained via SPNE-LETRSS for fluoranthene (Ex = 283nm).
Figure E-7 Calibration curve obtained via SPNE-LETRSS for pyrene (Ex = 283nm).

Figure E-8 Calibration curve obtained via SPNE-LETRSS for benzo[a]anthracene (Ex = 283nm).
Figure E-9 Calibration curve obtained via SPNE-LETRSS for chrysene (Ex = 283nm).

\[ y = 67.02703x + 39854 \]
\[ R = 0.9826 \]

Figure E-10 Calibration curve obtained via SPNE-LETRSS for benzo[b]fluoranthene (Ex = 283nm).

\[ y = 51.4462x + 72999 \]
\[ R = 0.9849 \]
Figure E-11 Calibration curve obtained via SPNE-LETRSS for benzo[\(k\)]fluoranthene (Ex = 283nm).

\[ y = 164.6618x + 84459 \]
\[ R = 0.9574 \]

Figure E-12 Calibration curve obtained via SPNE-LETRSS for benzo[\(a\)]pyrene (Ex = 283nm).

\[ y = 454.21344x + 66402 \]
\[ R = 0.9968 \]
Figure E-13 Calibration curve obtained via SPNE-LETRSS for dibenz[a,h]anthracene (Ex = 283nm).

\[ y = 43.32046x + 53472 \]
\[ R = 0.9755 \]

Figure E-14 Calibration curve obtained via SPNE-LETRSS for benzo[g,h,i]perylene (Ex = 283nm).

\[ y = 423.54581x + 11443 \]
\[ R = 0.9927 \]
Figure E-15 Calibration curve obtained via SPNE-LETRSS for indeno[1,2,3-cd]pyrene (Ex = 283nm).
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