New Developments On High-resolution Luminescence Spectroscopy And Their Application To The Direct Analysis Of Organic Pollutants

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NEW DEVELOPMENTS ON HIGH-RESOLUTION LUMINESCENCE SPECTROSCOPY
AND THEIR APPLICATION TO THE DIRECT ANALYSIS OF ORGANIC POLLUTANTS
IN ENVIRONMENTAL SAMPLES

by

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B.S. Fudan University of China, 2001

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ABSTRACT

Polycyclic aromatic compounds (PACs), which comprise a complex class of condensed multi-ring benzenoid compounds, are important environmental pollutants originating from a wide variety of natural and anthropogenic sources. PACs are generally formed during incomplete combustion of pyrolysis of organic matter containing carbon and hydrogen. Because combustion of organic materials is involved in countless natural processes or human activities, PACs are omnipresent and abundant pollutants in air, soil, and water. Chemical analysis of PACs is of great environmental and toxicological importance. Many of them are highly suspect as etiological agents in human cancer. Because PACs carcinogenic properties strongly depend on molecular structure and 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. Gas chromatography (GC), high-resolution GC, and high-performance liquid chromatography (HPLC) are the basis for standard PACs identification and determination. Many cases exist where GC, HPLC, and even HR-GC have not been capable to provide unambiguous isomer identification. The lack of reliable analytical data has lead to serious errors in environmental and toxicological studies.

This dissertation deals with the development of novel instrumentation and analytical methods for the analysis of PACs in environmental samples. The developed methodology is based on two well-known high-resolution luminescence techniques, namely Shpol’skii Spectroscopy (SS) and Fluorescence Line Narrowing Spectroscopy (FLNS). Although these two techniques have long been recognized for their capability in providing direct determination of
target PACs in complex environmental samples, several reasons have hampered their widespread use for the problem at hand. These include inconvenient sample freezing procedures; questions about signal reproducibility; lengthy spectral acquisition, which might cause severe sample degradation due to prolonged excitation; broadband fluorescence background that degrades quality of spectra, precision of measurements and detection limits; solvent constrains imposed by the need of optically transparent media; and, most importantly, the lack of selectivity and sensitivity for unambiguous determination of closely related PACs metabolites. This dissertation presents significant advances on all fronts.

The analytical methodology is then extended to the analysis of fluoroquinolones (FQs) in aqueous samples. FQs are one of the most powerful classes of antibiotics currently used for the treatment of urinary tract infections. Their widespread use in both human and animal medicine has prompted their appearance in aquatic systems. The search for a universal method capable to face this new environmental challenge has been centered on HPLC. Depending on the FQ and its concentration level, successful determination has been accomplished with mass spectrometry, room-temperature fluorescence (RTF) or UV absorption spectrometry. Unfortunately, no single detection mode has shown the ability to detect all FQ at the concentration ratios found in environmental waters. We provide a feasible alternative based on FLNS.

On the instrumentation side, we present a single instrument with the capability to collect multidimensional data formats in both the fluorescence and the phosphorescence time domains. We demonstrate the ability to perform luminescence measurements in highly scattering media by comparing the precision of measurements in optically transparent solvents (Shpol’skii solvents) to those obtained in “snow-like” matrixes and solid samples. For decades, conventional low-temperature methodology has been restricted to optically transparent media. This restriction has
limited its application to organic solvents that freeze into a glass. In this dissertation, we remove this limitation with the use of cryogenic fiber-optic probes.

Our final efforts deal with low-temperature absorption measurements. 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 compounds, additional errors in absorbance measurements arise from the emission reaching the detector, which might have comparable intensity to that of the transmitted light. We present a fundamentally different approach to low-temperature absorption measurements as the sought-for-information is the intensity of laser excitation returning from the frozen sample to the intensified-charge coupled device (ICCD). Laser excitation is collected with the aid of a cryogenic fiber optic probe. The feasibility of our approach is demonstrated with single-site and multiple-site Shpol’skii systems. 4.2K absorption spectra show excellent agreement to their literature counterparts recorded via transmittance with closed cycle cryogenators. Fluorescence quantum yields measured at room-temperature compare well to experimental data acquired in our lab via classical methodology. Similar agreement is observed between 77K fluorescence quantum yields and previously reported data acquired with classical methodology. We then extend our approach to generate original data on fluorescence quantum yields at 4.2K.
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1 CHAPTER: INTRODUCTION

1.1 General Features of Fluorescence and Phosphorescence

A partial Jablonski diagram, depicting the various activation and deactivation processes for an aromatic molecule, is shown in Figure 1.1. An absorption, or excitation, process (A) can occur into the first or higher excited singlet state. Excitations into the first and second singlet states (S₁ and S₂) are shown. This process is relatively fast and occurs on the order of $10^{-15}$ s. Flowing 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. Vibrational relaxation (vr) occurs on the order of $10^{-11}$ to $10^{-10}$ s. If the molecule resides in an upper excited singlet state (S₂), internal conversion (ic) into the S₁ electronic level can occur. This process is efficient if the ground vibrational level of the S₂ state has the same energy as an upper vibrational level of the S₁ state. The internal conversion process occurs on the order of $10^{-12}$ s. Following internal conversion (ic), vibrational relaxation (vr) occurs into the ground vibrational level of the S₁ state.

From the S₁ 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. Since this deactivation is a collisional process, isolating or cooling the sample will most often reduce external conversion (ec) contributions. The molecule may also release the excess electronic energy in the form of a photon. If the originating electronic state is the S₁ state, this process is called fluorescence (F). Fluorescence occurs on the order of $10^{-10}$ to $10^{-6}$ s. Since
internal conversion (ic) and vibrational relaxation (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 internal conversion (ic) is not favored.

The S₁ state may also be deactivated by a non-radiative process called inter-system crossing (isc). Inter-system crossing (isc) involves a crossover of electronic states similar to internal conversion (ic) with the exception that inter-system crossing (isc) occurs between states of differing spin multiplicity. Population of the upper vibrational levels of the triplet state is followed by vibrational relaxation into the ground vibrational level of the excited triplet state. Deactivation of the triplet state via emission of a photon is called phosphorescence (P) and occurs on the order of 10⁻³ to 10³ s because it is spin-forbidden process. Since phosphorescence occurs on a relatively long time scale, the triplet state is particularly susceptible to collisional deactivations, or external conversion (ec), and is often seen only when the sample is cooled or isolated by other means.²
Non-radiative deactivation of the triplet state, 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 about 23 kcal/mol and 38 kcal/mole. Any species having excitation energies as low as 23 kcal/mole 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 deoxygenation of sample solution or purging the sample compartment with inert gasses.

The radiative deactivation processes (fluorescence and phosphorescence) are always in competition with the various non-radiative deactivation processes. The intensity of fluorescence and phosphorescence will then depend upon the relative efficiencies of all competing processes. The efficiencies of fluorescence and phosphorescence are often expressed in terms of quantum
yield, $\phi$. The quantum yield for fluorescence, $\phi_F$, 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):

$$\phi_F = \frac{k_F}{k_F + k_{nr}}$$  \hspace{1cm} (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:

$$\phi_P = \frac{k_{isc}}{k_{isc} + k_F + k_{nr}} \times \frac{k_P}{k_P + k_{nr}}$$  \hspace{1cm} (2.2)

Note that the first term in equation (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):
\[ I_t = I_0 e^{-t/\tau_L} \]  

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):

\[ \tau_F = (k_F + k_{nr})^{-1} \]  
\[ \tau_P = (k_P + k_{nr})^{-1} \]

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 \( (k_L + k_{nr})^{-1} \).

1.2 High Resolution Luminescence Spectroscopy

The multidimensional nature of photoluminescence provides fluorescence and phosphorescence techniques with unique potential for the direct analysis of target compounds in complex matrixes. The combination of excitation and emission spectra to lifetime information within the fluorescence or phosphorescence time domain gives at least three qualitative parameters for compound determination, namely, one excitation and emission wavelength and one lifetime. The simplicity of the experimental procedure makes room-temperature fluorescence the most popular approach. Fluorescence is readily observed from liquid solutions with no need for sample deoxygenation. Room-temperature phosphorescence is rarely observed from liquid solutions but it can be enhanced with the use of solid substrates, micelle-stabilized aqueous
solutions, or heavy-atom salts. In all three phosphorescence approaches, sample deoxygenation is crucial to avoid quenching of the triplet excited state.

The main limitation of room-temperature fluorescence and phosphorescence techniques toward the selectivity of analysis is the broad nature of excitation and luminescence (fluorescence and phosphorescence) spectra. Similar to room-temperature absorption techniques, the diffuse character of such spectra limits their information content and drastically reduces the selective potential for the analysis of target compounds in complex matrixes. Several strategies exist to improve the specificity of luminescence techniques, including reducing the sample temperature to enhance vibronic spectral resolution, collection of multidimensional data formats, and time-resolved spectroscopy (lifetime measurements).

Reducing the sample temperature offers several advantages. Luminescence quantum yields often increase, and the complications of oxygen quenching and energy transfer are eliminated. But the temperature effects on luminescence are specially pronounced in the so-called high-resolution techniques, namely, Shpol'skii Spectroscopy (SS) and Fluorescence Line Narrowing Spectroscopy (FLNS). In these techniques, the sharp spectra with vibrational information result from homogeneous and inhomogeneous band-broadening reduction.

Shpol’skii matrix spectrometry has been recognized as approach for analysis of polycyclic aromatic hydrocarbons (PAH) in complex samples. The term Shpol’skii matrix generically refers to a dilute solution of a guest molecule frozen to 77K or below in a solvent host, wherein in three conditions are met; (1) the solvent freezes to an order polycrystalline matrix, not a random glass; (2) the guest-host interactions are weak; (3) the guest and host molecular dimensions match up well enough that the guest molecules occupy a small number of crystallographic sites (ideally just one) in the host lattice. The combination of reduced thermal
and inhomogeneous broadening leads to much “sharper” absorption and luminescence spectra than is the case for alkane solvent hosts that freeze to a glass (e.g., 3-methylpentane) or polar glass-forming solvents such as ethyl ether: isopentane: ethyl alcohol (5:5:2). The so-called sharp-line or quasi-linear fine structure facilitates spectral identification and quantization without prior separation steps.

When compare to SS, FLNS has wider applicability because the requirements between analyte and sample matrix are greatly relaxed. At sufficiently low temperatures, narrow band laser excitation of an inhomogeneously broadened absorption band provides excitation of only those molecules with absorptive transitions which exactly match the wavelength of the laser light. If the emission originates form the same excited electronic state as the absorption, the resulting emission spectra have narrow linewidths and provide information on the vibrational energies of the excited electronic state.

Several reasons have hampered the widespread use of SS and FLNS for routine analysis. These include inconvenient sample freezing procedures; questions about signal reproducibility; lengthy spectral acquisition, which might cause severe sample degradation due to prolonged excitation; broadband fluorescence background that degrades quality of spectra, precision of measurements and detection limits; contamination of excitation stray light on FLN spectra; solvent constrains imposed by the need of optically transparent media; and, most importantly, the lack of selectivity and sensitivity for unambiguous determination of closely related PACs. This dissertation presents significant advances on all fronts.
1.3 Our Approach to High-Resolution Luminescence Spectroscopy

This dissertation builds upon significant improvements we have introduced to measurements in Shpol'skii matrices at 77 K and 4.2 K.\textsuperscript{25,26} Individual elements of our approach have been anticipated and implemented previously, including low-temperature studies with tunable laser excitation,\textsuperscript{21} a fiber-optic cell for low-temperature measurements,\textsuperscript{27} multidimensional data formats,\textsuperscript{28,29} and the use of fluorescence decays as a mathematically well-behaved way to distinguish emitting components.\textsuperscript{17,30,31,32,33,34,35,36} However, the optimal combination of these features has not been realized and the techniques have generally not been practical enough for straightforward implementation.

The main significant aspect of our approach is the practical, smooth way we are integrating all these features together. The complications of traditional low-temperature methodology have been removed by using a cryogenic fiber optic probe (FOP) with the distal end frozen directly into the sample matrix. We can now routinely perform measurements at liquid nitrogen and helium temperatures; frozen samples are prepared in a matter of seconds. Time-resolved fluorescence analysis has been achieved with a pulsed tunable dye laser for sample excitation, a spectrograph and an intensifier-charged coupled device (ICCD). Because of the spectrograph and the ICCD, wavelength time matrices (WTMs) are collected in short analysis time.\textsuperscript{25,26}

1.3.1 Cryogenic Fiber Optic Probes

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 on the outside of the dewar are nuisances. But scattering at each of the interfaces encountered by the excitation light on its way to the sample is a much more severe problem, as stray light in the emission monochromator degrades the limit of detection. The classic approach typically involves three air/glass interfaces, two liquid nitrogen/glass interfaces, and one matrix/glass interface. Options that can reduce the number of scattering interfaces and the temperature to below 20 K include using closed-cycle helium refrigerators or Joule-Thomson miniature refrigerators. The trade-offs include higher cost, reduced sample throughput, and possibly less efficient or slower cooling of the sample. Depending on the solvent and the final temperature, freezing times can take between 20 and 40 min per sample.

Our approach eliminates the need for an optical dewar and/or helium cryostats. Even at 4.2 K, the FOP retains the simplicity of dunking the sample 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. The probes typically incorporate one delivery and six silica-clad silica collection fibers. At the sample end the fibers are arranged in a conventional six-around-one configuration with the delivery fiber in the center. At the collection end the excitation and collection fibers are separated and vertically aligned with the spectrograph entrance slit. Figure 1.2 shows an example of the 4.2 K probe.

After the sample is introduced into the sample tube, the tip of the FOP is positioned below the solution surface as 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. The approximately one-minute probe clean up procedure involves removing the test tube from the liquid cryogen, melting the frozen matrix and warming the
resulting solution to approximately room temperature with a heat gun, rinsing the probe with the sample solvent, and drying it with warm air from the heat gun. Preparing frozen samples for luminescence measurements at 77K and 4.2K is now a routine technique. Samples are frozen in a matter of seconds.
The excitation and collection fibers are fed into a section of copper tubing that provides mechanical support for lowering the probe into the liquid helium. At the analysis end, the excitation and emission fibers are bundled with vacuum epoxy and fed into a metal sleeve for mechanical support. The copper tubing is flared stopping a swage nut tapped to allow for the threading of the sample vial. At the instrument end, the emission fibers are bundled with vacuum epoxy in a slit configuration, fed into a metal sleeve and aligned with the entrance slit of the spectrometer.
1.3.2 **Instrumental System**

Pulsed excitation sources offer prospects for time-resolving the emission to improve signal-to-background ratios and reduce spectral interference. Early reports on laser excited Shpol'skii spectrometry implemented time resolution at fixed delay time intervals by using laboratory-constructed gated integrators or commercial boxcar averagers\textsuperscript{14,37,38,39}. More recently, advantage has been taken of commercially available delay generators and intensified linear photodiode arrays\textsuperscript{40}. Multi-channel detectors can acquire emission spectra at good signal-to-noise ratio much faster than is possible with a scanning monochromator. Collecting the entire emission spectrum at once avoids problems associated with pulse-to-pulse fluctuation, laser intensity drift, and photodecomposition that could degrade analytical figures of merit. We have introduced an ICCD, which allows the multi-channel detection and time-resolved (lifetime) determination.\textsuperscript{21,26} As an excitation source, we have used a compact frequency doubled pulsed tunable dye laser whose bandwidth (< 0.03nm) is well matched for selective excitation of narrow Shpol'skii excitation spectra. The ICCD is coupled to a spectrograph to rapidly collect a series of emission spectra at different delay times between the laser firing and the opening of the gate on the ICCD. The series of spectra can be assembled into a WTM, analogous to an excitation-emission matrix (EEM). Because of the spectrograph and the ICCD, EEMs are collected in relatively short analysis time. Tuning the excitation wavelength also allows the rapid collection of time-resolved excitation-emission matrices (TREEMs). Figure 1.3 shows a schematic diagram of the multi-channel system.
The tunable dye laser is operated through a KDP frequency-doubling crystal. When pumped with approximately 30 mJ of the second harmonic generator of a Nd:YAG Q-switched laser, the dye laser produces more than 5 mJ at peak of Rhodamine 6G in a spectral bandwidth less than 0.03 nm. Fluorescence spectra are recorded with a spectrometer operating either in the scanning or spectrograph mode. The spectrometer is equipped with a flat, 1800 grooves/mm grating and has a reciprocal linear dispersion of 0.8 nm/mm.
2  CHAPTER: SHPOL’S KII S ELECTROSCOPY AT THE INTERFACE OF TWO NON-POLAR MICROENVIRONMENTS: A NOVEL APPROACH FOR THE ANALYSIS OF POLYCYLIC AROMATIC COMPOUNDS

2.1  Introduction

Several polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PDBFs) are part of the Environmental Protection Agency (EPA) priority pollutant list and routinely monitored in environmental samples. This concern results from their toxicity to humans and their resistance to degradation under environmental conditions. Because one of the main sources of human contamination is the ingestion of contaminated water, the EPA enforces their routine monitoring in public water supplies.

Classical methodology for the determination of PAHs, PCBs and PDBFs follows the general pattern of sample extraction and chromatographic analysis. Extraction techniques provide sample pre-concentration, simplify matrix composition and facilitate analytical resolution in the chromatographic column. Solid-liquid extraction (SLE), the recommended method for sample pre-treatment, concentrates the organic pollutants from aqueous samples by sorption onto a C_{18} organic phase chemically bonded to silica particles\(^4\).

Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are the basis for PCBs, PDBFs and PAHs monitoring. Packed-column gas chromatography-electron capture detection (GC-ECD) or HPLC are used to provide data on “total PCBs” or “total PDBFs” contents in samples. If congener-specific determination is required, high-resolution GC-mass spectrometry (HR-GCMS) is the technique of choice. PAH screening is mainly done by
HPLC and gas chromatography-mass spectrometry (GC-MS) is used for compound confirmation and peak-purity check of HPLC fractions.

The work presented in this chapter deals with a single technique for monitoring PAHs, PCBs and PDBFs in aqueous samples and HPLC fractions. It combines solid liquid extraction (SLE) to laser-excited low temperature phosphorimetry (LELTP) in Shpol'skii matrixes. The pollutants are extracted from the aqueous sample with an octadecyl silica membrane using a syringe kit for rapid SLE,42,43,44. The substrate is spiked with 100 μl of Shpol'skii solvent and LELTP is directly performed on the surface of the membrane. Because PAHs, PCBs and PDBFs partition into the Shpol'skii solvent highly resolved spectra are obtained. The quantitative aspects of the spiking procedure are thoroughly investigated with a set of five model compounds and the analytical figures of merit for 4-chlorobiphenyl, 3,3’,4,4’-tetrachlorobiphenyl, 2,3’,4,4’,5-pentachlorobiphenyl, 2-chlorodibenzofuran, 2,8-dichlorodibenzofuran and 2,3,7,8-tetrachlorodibenzofuran are presented.

2.2 Experimental

2.2.1 Chemicals

All chemicals were analytical-reagent grade and were used without further purification. Nanopure water was employed throughout. SLEC® octadecyl membranes were obtained from Ansys Diagnostics, Inc. Their composition consisted of bonded silica particles enmeshed in a glass fiber support. The average size and mean pore size of the silica particles were 30 μm and 7 nm, respectively. The mean thickness and diameter of the membranes were 1000 μm and 47 mm, respectively. Biphenyl; 4-chlorobiphenyl; 2,3’,4,4’,5-pentachlorobiphenyl; 4,4’,3,3’,4,4’-
tetrachlorobiphenyl; dibenzofuran; 2-chlorodibenzofuran; 2,8-dichlorodibenzofuran; 2,3,7,8-
tetrachlorodibenzofuran were obtained from Accustandard in isooctane at 100 μg ml⁻¹.
Phenanthrene, chrysene and fluorene were purchased in the solid form from Aldrich at their
highest purity available. Methanol, n-pentane, n-hexane, n-heptane and n-octane were acquired
from EM Science at 99.8% purity.

2.2.2 Instrumentation

Solvent optimization and initial survey of excitation and phosphorescence spectra was
done at 77 K with a Fluorolog-3 spectrofluorimeter (ISA, Jobin Yvon-Spex, model FL3-11). A
pulsed-lamp phosphorimeter attachment (Spex 1934) provides signal-gating circuitry so that only
a selected window of sample emission (gate time), after excitation (delay time), is allowed to
reach the detector. The pulsed source consists of a UV–vis Xenon lamp with adjustable pulse
rate between 0.05 and 33 flashes s⁻¹. The full-width at half maximum (FWHM) of each pulse is
3 μs. Two single-grating (1200 grooves mm⁻¹) spectrometers are used for wavelength selection.
Their excitation and emission blaze wavelengths are 250 and 500 nm, respectively. The
reciprocal linear dispersion of both monochromators is 4.2 nm mm⁻¹. The detector is a
photomultiplier tube (Hamamatsu, model R928) operating at room temperature in a photon-
counting mode. dM 3000 software from Spex Industries, Inc. is used for automated scanning and
luminescence data acquisition. Sample signals and luminescence spectra were collected at 90°.
Luminescence spectra were corrected for instrumental response using the radiometric correction
factors included in the XCORRECT and MCORRECT files of the spectrofluorimeter's software.
When needed, long pass filters discriminated against second order emission.
A complete description of the instrumental system used for 4.2 K LELTP analysis has been published recently\textsuperscript{45}. A tunable dye laser (Dakota Technologies Inc.) is used for sample excitation. When pumped with 30 mJ of the second-harmonic generator of the Nd:YAG laser, the dye laser produces more than 5 mJ at peak of rhodamine 6G in a spectral bandwidth less than 0.03 nm. The output of the dye laser is frequency doubled by a KDP crystal (Inrad). A single focusing lens allows for the dye laser's output to be launched into the fiber optic probe. The electronic shutter located prior to the launch fiber provides precise control of the excitation beam on/off time. Shutter control is automated with an Oriel controller. A digital delay generator (DG 535 Stanford Research Systems) is used as the data acquisition trigger and system wide time base. The minimum 2 $\mu$s step between data points, which is due to computer software and hardware limitations, was not a problem because of the long phosphorescence lifetimes. Phosphorescence from the fiber optic probe is directed into an HR-320 (ISA) monochromator equipped with an 1800 grooves mm$^{-1}$ grating blazed at 260 nm. The reciprocal linear dispersion of the monochromator is 1.2 nm mm$^{-1}$. The distance between the emission fibers and the entrance slit of the monochromator is optimized to completely illuminate the diffraction grating area. The best resolution at 313.1 nm is 0.013 nm (Hg lamp). The grating is controlled with a stepping motor via an automated motor control system (The Motion Group). Serial RS-232 communication is used for stepper motor control. Phosphorescence is detected with a photomultiplier tube (R928 PMT, Hamamatsu) in a chilled housing (Hamamatsu). A low pass electronic filter is placed inline with the signal to enhance signal to noise ratio in spectral acquisition. The low pass filter was not used in any lifetime determination to avoid decay distortions due to the capacitor. Data acquisition is made via photon counter (Stanford Research) with a DC 300 MHz amplifier (Stanford Research). All communications between the controlling
computer and the photon counter are made via general-purpose interface bus (GPIB). A LabVIEW (National Instruments) based in-house program is used for instrument control, lifetime and spectra acquisition, mathematical filtering, data storage device, and data interpreter. The delay and gate of the photon counter were set to 10 and 80 ms respectively. The delay time negated fluorescence detection and the gate time ensured entire data acquisition between successive laser pulses. Figure 2.1 shows a schematic diagram of the instrumental system. The fiber optic probe is the same as mentioned in Chapter 1.

![Diagram](image)

Figure 2.1 Fiber optic phosphorescence system for low-temperature measurements.

The electronic shutter provides precise temporal control of initial excitation time, total excitation time, and initial time for data acquisition. The LABVIEW program is designed to have its electronic filtering as a modular unit only for spectra collection. As any commercial instrument, the electronic filtering improves the signal-to-background ratio. The filtering unit in our system can be removed for complete decay lifetime acquisition. This is not an option in commercial instrumentation, which would distort the phosphorescence decay if it were collected in its entirety.45
2.2.3 Sample Procedure

Measurements with the spectrofluorimeter were carried out following the classic 77 K sample freezing procedure, which consists of immersing the un-degassed sample solution in a quartz tube into a nitrogen-filled Dewar flask. A cork borer was used to dissect 47 mm SLE disks into 5.5 mm diameter disks. The disk was placed into a stainless steel filter syringe kit (Alltech, IL) and attached to a 10 ml syringe (Hamilton, NE). Positive pressure was used to force the liquid solutions through the membrane. Prior to sample application, the disk was conditioned with the appropriate solvent. Analyte solutions were pushed through the membrane at about 30 ml min flow rates. Following sample extraction, void water was mechanically removed with a 100 ml syringe forcing three 100 ml volumes of air through the disk. The extraction disk was placed in the sample vial of the cell for low temperature measurements. In some cases, the membrane had been previously spiked with 100 μl of Shpol'skii solvent using a microliter syringe (Hamilton, NE)(see Figure 2.2). The distance between the fiber optic probe and the substrate was adjusted to irradiate the entire surface of the membrane. Because there was no physical contact between the probe and the membrane, probe clean up between membrane measurements was not necessary. Measurements from liquid matrixes were made with the tip of the fiber optic probe approximately 1 cm below the solution surface. In all cases, the position of the probe was held constant with the screw cap of the sample cell. Sample freezing was accomplished by lowering the copper tubing and the cell into the liquid cryogen. The liquid nitrogen and liquid helium were held in two separate Dewars with 5 and 60 L storage capacity, respectively. The 60 L liquid helium typically lasted three weeks of daily use, averaging 15–20 samples per day. At both temperatures, complete sample freezing took less than 90 s. The
approximately 1 min probe clean up procedure involved removing the sample vial from the
cryogen container, melting the frozen matrix and warming the resulting solution to
approximately room temperature with a heat gun, rinsing the probe with $n$-alkane, and drying it
with warm air from the heat gun. The entire freeze, thaw, and cleanup cycle took no longer than
5 min.

![Diagram of SLE procedure]

**Figure 2.2 SLE procedure**

### 2.3 Results and Discussion

Our research group recently presented an approach based on SLE-laser-excited time-
resolved Shpol'skii spectroscopy (LETRSS) for the direct analysis PAH in water samples. The
assay consisted of a three-step sample procedure and took less than 10 min per sample. The
PAHs were extracted from the water sample with a 13 mm disk, eluted with 5 ml of $n$-hexane
and directly determined via fluorescence emission at 77 K. Because the phosphorescence of
PCBs and PDBFs is more intense than their fluorescence, and the low-temperature
phosphorescence of PAHs contributes significantly to their total luminescence spectrum at temperatures below 20 K\textsuperscript{45}, we decided to investigate the analytical potential of SLE-LELTP at 4.2 K. The experimental procedure was the same as the one for SLE-LETRSS but the solvent was \textit{n}-heptane. Unfortunately, our early investigations revealed a rather large sample-to-sample variation of the amount of PCB or PDBF eluted from the extraction membrane. The worst-case scenario was observed with 4-chlorobiphenyl, which showed zero percent recovery from the extraction membrane.

2.3.1 \textit{Phosphorescence Spectra and Lifetimes on Spiked Membranes}

The feasibility to record Shpol'skii spectra from spiked membranes was first observed with SLE-LETRSS. The fluorescence spectra of four PAHs, namely anthracene, chrysene, benzo[\textit{g,\textit{h,i}}]perylene and benzo[a]pyrene matched those recorded from standard solutions in \textit{n}-hexane\textsuperscript{47}. Their fluorescence decays on spiked membranes were single exponential decays indicating homogeneous microenvironments for PAH molecules. Because their lifetimes matched those from \textit{n}-hexane, PAH partitioning into the layer of Shpol'skii solvent was postulated as the main reason for the observed phenomena.

The work presented here expands the spiking method to SLE-LELTP analysis and provides a thorough investigation of its potential for quantitative determination of PAHs, PCBs and PDBFs. A set of five compounds known to emit phosphorescence at 77 K in Shpol'skii matrices\textsuperscript{45} was selected to investigate quantitative parameters. These included three PAHs (phenanthrene, fluorene and chrysene), biphenyl and dibenzofuran, which are the molecular moieties of PCBs and PDBFs, respectively.
The Shpol'skii solvent for each PAH was selected from previous optimization experiments that showed the narrowest FWHM in the presence of hexane (phenanthrene), heptane (fluorene) and octane (chrysene).\textsuperscript{26} Solvent selection for biphenyl and dibenzofuran was made among the three \textit{n}-alkanes that best matched their molecular lengths, namely \textit{n}-hexane, \textit{n}-heptane and \textit{n}-octane. The FWHM of their 77 K phosphorescence spectra were recorded with the spectrophosphorimeter using slit-widths for optimum spectral resolution. Because the narrowest FWHM were obtained with heptane, this solvent was used for all further studies.

4.2 K phosphorescence spectra and lifetimes ($\tau_p$) were collected with the laser system and the cryogenic probe. Figure 2.3 shows phenanthrene spectra recorded from a non-spiked membrane (Figure 2.3A), \textit{n}-hexane (Figure 2.3B) and a spiked membrane (Figure 2.3C). All spectra were recorded using slit-widths for optimum spectral resolution but site selective excitation was not attempted. The extraction membranes were spiked with 100 $\mu$l of Shpol'skii solvent. This volume covered the entire surface of the membrane providing a thin film between the substrate and the fiber optic probe. There was no physical contact between the probe and the organic solvent and the distance between them assured complete irradiation of the film surface. The spectrum from the spiked membrane shows the quasi-line structure typical from phenanthrene in \textit{n}-hexane. The spectral narrowing gained by spiking the Shpol'skii solvent on the extraction membrane is clearly noticed comparing Figure 2.3A and Figure 2.3C. The same types of results were obtained for the remaining compounds. Similar to our previous observations from fluorescence spectra of PAHs, the highly resolved phosphorescence spectra on spiked membranes suggest Shpol'skii microenvironments for analyte molecules on extraction membranes.
Figure 2.3 4.2 K phosphorescence spectra of phenanthrene on (A) SLE membrane; (B) n-hexane and (C) SLE membrane spiked with 100 μl of n-hexane.

10 ml of a 10 ng ml⁻¹ solution (80% water/methanol) were used for SLE. A 100 ng ml⁻¹ standard solution was used for spectrum B. All measurements were made using excitation at 284 nm, slit widths of 100 μm and monochromator speed of 1.05 nm s⁻¹. All the spectra were blank subtracted.

Table 2.1 shows the phosphorescence lifetimes (τₚ) in the three types of matrixes. For a confidence level of 95% (α=0.05; N₁=N₂=48)⁴⁸, the τₚ in alkane solutions and extraction membranes were slightly statistically different showing that phosphorescence lifetimes are sufficiently sensitive to probe these two types of microenvironments. The fact that all τₚ from non-spiked membranes were single exponential decays shows that the PTFE mesh, which is used to support the silica particles in the membrane, does not contribute significantly to the measured lifetime. This observation is consistent with the fact that most analyte molecules are retained within the octadecyl chains of the silica particle. The single exponential decays also suggest that only one type of microenvironment surrounds analyte molecules. Apparently, the residual silanol and siloxane groups of octadecyl silica particles do not contribute to the measured lifetime.
Table 2.1 4.2 K Phosphorescence lifetimesa of organic pollutants in Shpol'skii solventsb (SS), non-spiked membranes (NSM) and spiked membranes (SM)

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Phenanthrene</th>
<th>Chrysene</th>
<th>Fluorene</th>
<th>Dibenzofuran</th>
<th>Bipheny</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>3.87±0.16</td>
<td>2.49±0.03</td>
<td>6.25±0.07</td>
<td>5.70±0.21</td>
<td>3.64±0.04</td>
</tr>
<tr>
<td>NSMc</td>
<td>3.64±0.09</td>
<td>2.10±0.02</td>
<td>5.78±0.06</td>
<td>4.39±0.11</td>
<td>3.57±0.17</td>
</tr>
<tr>
<td>SM</td>
<td>3.47±0.07</td>
<td>2.44±0.05</td>
<td>5.99±0.08</td>
<td>5.46±0.15</td>
<td>3.65±0.06</td>
</tr>
</tbody>
</table>

a Each lifetime (S) was average of 16 measurements. Measurements were done at phosphorescence wavelength of maximum intensity. Excitation wavelength was at 284 nm and slit widths were 100 µm. b Shpol’skii solvents were: n-hexane for phenanthrene, n-octane for chrysene and n-heptane for fluorine, biphenyl and dibenzofuran. c 10ml of 100 ng ml⁻¹ aqueous standard was employed for SLE.

2.3.2 Relationship Between the Analyte Concentration in The Water Sample and Its Concentration in the Shpol'skii Layer of the Spiked Membrane

The spectral and lifetime data provides sufficient foundation to attribute most of the phosphorescence signal to the analyte molecules partitioning into the Shpol'skii layer of the spiked membrane. Because the relationship between the analyte concentration in the water sample \( C_{H2O} \) and its concentration in the Shpol'skii solvent \( C_{SS} \) is important for quantitative analysis, we first derive a mathematical expression and then test it experimentally. Our mathematical approach assumes no analyte losses and no chemical and/or spectral interference. We also assume that all conditioning solvents except for the void water have been mechanically removed from the membrane before spiking the Shpol'skii solvent. Because of the low solubility of the model compounds in water, the void water remaining in the membrane is disregarded and a binary system is considered for analyte partition.

The analyte mass \( m_{H2O} \) in the water sample is given by Equation 2.1

\[
m_{H2O} = C_{H2O} \times V_{H2O}
\]  

(2.1)

where \( C_{H2O} \) is the analyte concentration in the water sample and \( V_{H2O} \) is the volume of extracted water. The analyte mass extracted with the membrane \( m_o \) is then given by:
\[ m_w = f_1 \times m_{H2O} \quad (2.2) \]

where \( f_1 \) is the analyte fraction extracted from the water sample. Since the total analyte mass remains constant after adding Shpol'skii solvent to the membrane:

\[ m_a = m_{SS} + m_{SLE} \quad (2.3) \]

where \( m_{SS} \) and \( m_{SLE} \) are the analyte masses in the organic layer and the extraction membrane, respectively.

From equation (2.2) and (2.3) we know that:

\[ m_{H2O} = \frac{1}{f_1} \times (m_{SS} + m_{SLE}) \quad (2.4) \]

The analyte mass in the Shpol'skii solvent is given by:

\[ m_{SS} = C_{SS} \times V_{SS} \quad (2.5) \]

where \( C_{SS} \) is the analyte concentration in the organic layer of volume \( V_{SS} \). Similarly, the analyte mass in the extraction membrane is given by:

\[ m_{SLE} = C_{SLE} \times V_{SLE} \quad (2.6) \]

where \( C_{SLE} \) is the analyte concentration in the SLE membrane of volume \( V_{SLE} \). Substitution of equation (2.5) and equation (2.6), in equation (2.4) gives:

\[ m_{H2O} = \frac{1}{f_1} \times (C_{SS} \times V_{SS} + C_{SLE} \times V_{SLE}) \quad (2.7) \]

Substitution of equation (2.2), in equation (2.7) gives:

\[ C_{H2O} \times V_{H2O} = \frac{1}{f_1} \times (C_{SS} \times V_{SS} + C_{SLE} \times V_{SLE}) \quad (2.8) \]

The distribution coefficient (Kd) between the membrane and the Shpol'skii solvent can be defined as follows\(^49\):

\[ K_d = \frac{C_{SLE}}{C_{SS}} \quad (2.9) \]

Substitution of \( C_{SLE} = C_{SS} \times K_d \) in Equation (2.8) gives:

\[ C_{H2O} = \frac{V_{SS}}{f_1} \times \frac{V_{H2O}}{f_1} \times (\frac{C_{SS}}{f_1} \times V_{H2O} + K_d \times V_{SLE} \times \frac{C_{SLE}}{f_1} \times V_{H2O}) \times \frac{C_{SS}}{f_1} \]

\[ (2.10) \]
Equation (2.10) predicts a linear relationship between $C_{\text{H2O}}$ and $C_{\text{SS}}$ upon constant $V_{\text{SS}}$, $V_{\text{H2O}}$, $V_{\text{SLE}}$, $f_1$ and $K_d$. Obviously, the analyst sets constant values for $V_{\text{SS}}$, $V_{\text{H2O}}$ and $V_{\text{SLE}}$. Careful optimization of experimental parameters provides constant extraction efficiencies ($f_i$). Therefore, the only parameter that needs further investigation is the distribution ratio ($K_d$).

2.3.3 **Measuring Distribution Ratios on Spiked Membranes**

Because the distribution ratio should be constant under equilibrium conditions, the first step to determining $K_d$ was to investigate partitioning time profiles on spiked membranes. This was done by monitoring the phosphorescence intensity ($I_p$) of the spiked membrane as a function of elapsed time ($t$) between membrane spiking and freezing. During elapsed time, the spiked membrane was kept at room temperature in the sample vial of the fiber optic probe. The vial was closed to minimize continuous solvent evaporation. Figure 2.4 shows the $I_p$ vs. $t$ plots for the five model compounds. Each point plotted in the graph represents the average of three individual measurements taken from three spiked membranes. For chrysene and phenanthrene, $I_p$ reached a maximum value at 5 min and then remained constant ($\alpha=0.05; N_1=N_2=3$). For fluorene, dibenzofuran and biphenyl the maximum $I_p$ was reached at 2 min. Defining equilibrium time as the time after which the amount of analyte partitioning into the Shpol'skii solvent remains constant—within the limits of experimental error—and corresponds to the maximum amount extracted at infinite time—30 min—our experiments show 5 min equilibrium times for biphenyl, dibenzofuran and fluorene, and 2 min for chrysene and phenanthrene.
Figure 2.4 Time dependence of dibenzofuran (▲), chrysene (○), phenanthrene (+), biphenyl (●) and fluorene (*\(^\)\) partitioning into the Shpol'skii solvent of the spiked membrane.

10 ml of a 50 ng ml\(^{-1}\) solution (80% water/methanol) were used for SLE. 100 μl of organic solvent were spiked on the SLE membrane. Each point in the curve is the average of individual measurements taken from three SLE membranes. Excitation wavelength was at 284 nm and slit widths were 100 μm.

Going back to equation (2.9), the second step for determining \( K_d \) was to measure \( C_{SS} \) and \( C_{SLE} \) from a series of experiments where \( V_{H2O} \), \( V_{SLE} \) and \( V_{SS} \) were held constant. This was performed as follows: 10 ml of aqueous solution with known \( C_{H2O} \) were extracted and the membrane was placed into a Teflon sample vial. The membrane was spiked with 100 μl of Shpol'skii solvent and the vial was closed. After 5 min of equilibration time, the layer of Shpol'skii solvent was drawn from the vessel with a micropipette and dispensed into the sample cell of the fiber optic probe. \( C_{SS} \) and \( C_{SLE} \) were determined via the single standard addition method. For \( C_{SS} \), the standard addition was made directly to \( V_{SS} \) at room temperature. For \( C_{SLE} \), the standard addition was made to the membrane via SLE with an aqueous standard solution.

Figure 2.5 shows the \( C_{SS} \) vs. \( C_{SLE} \) plots for the five compounds. Each point in the plot represents the average of three \( I_p \) measurements made from individual extractions. Table 2.2
summaries the variance and the slope of the linear regression plot. The slope of the isotherm corresponds to $K_d$. The correlation coefficient, the slope and the standard deviation of the slope were calculated via the bivariate least-squares regression method, which takes into account the uncertainties in both axes. The variance close to unity shows the linear relationship between $C_{SS}$ and $C_{SLE}$ and the constant slope demonstrates that $K_d$ remains the same within the studied concentration range.

Figure 2.5 $C_{SLE}$ vs. $C_{SS}$ plots of dibenzofuran (A), chrysene (B), phenanthrene (C), biphenyl (D) and fluorene (E). 10 ml of aqueous solution (80% water/methanol) were used for SLE.

Each point represents the average of three $I_p$ measurements from individual extractions. The relative standard deviation of measurements was less than 10%. Excitation wavelength was at 284 nm and slits widths were 100 μm.
Table 2.2 Distribution coefficienta between SLE membrane and Shpol'skii solvent

<table>
<thead>
<tr>
<th>Compound</th>
<th>R^b</th>
<th>K_d</th>
<th>S_{Kd}^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td>0.9970</td>
<td>3.30</td>
<td>0.41</td>
</tr>
<tr>
<td>Chrysene</td>
<td>0.9820</td>
<td>10.80</td>
<td>2.50</td>
</tr>
<tr>
<td>Fluorene</td>
<td>0.9915</td>
<td>8.00</td>
<td>1.40</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>0.9999</td>
<td>9.78</td>
<td>0.14</td>
</tr>
<tr>
<td>Dibenzofuran</td>
<td>0.9997</td>
<td>4.00</td>
<td>0.11</td>
</tr>
</tbody>
</table>

^a K_d, distribution coefficients obtained from bivariate least squares regressions of plots in Figure 2.5.  
^b R, correlation coefficient of the linear plot.  
^c S_{Kd} is the standard deviation of K_d.

Because K_d is constant, equation (2.10) should hold and the relationship between C_{SS} and C_{H2O} should be linear. Figure 2.6 shows the C_{H2O} vs. C_{SS} plots for the five compounds. Table 2.3 summarizes the linear fits and the correlation coefficients of the experimental data and compares them to the theoretical slopes obtained with equation (2.10) using the K_d values in Table 2.2. The other parameters in equation (2.10) were V_{H2O}=10 ml, V_{SS}=100 μl and V_{SLE}=23.8 μl. V_{SLE} was calculated using the standard formula for the volume of a cylinder V=πr^2h, where r was the radius of the disk (0.275 cm) and h is its thickness (0.1 cm). The agreement between the experimental and theoretical slopes is satisfactory; mainly if one considers that the experimental values were obtained via the ordinary least-squares method, i.e. assuming no experimental errors in the abscissa axis (C_{H2O}).
30

Figure 2.6 C_{SLE} vs. C_{SS} plots of dibenzofuran (A), chrysene (B), phenanthrene (C), biphenyl (D) and fluorene (E). 10 ml of aqueous solution (80% water/methanol) were used for SLE.

Each point represents the average of three Ip measurements from individual extractions. Excitation wavelength was at 284 nm and slit widths were 100 μm.

Table 2.3 Comparison of the theoretical slope and the linear regression of C_{SS}×C_{H2O} plots

<table>
<thead>
<tr>
<th>Compound</th>
<th>Theoretical slope a</th>
<th>Linear fit (C_{SS} × C_{H2O})</th>
<th>R c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td>56.10</td>
<td>Y = 57.76X – 091</td>
<td>0.9994</td>
</tr>
<tr>
<td>Chrysene</td>
<td>27.65</td>
<td>Y = 24.48X + 0.22</td>
<td>0.9897</td>
</tr>
<tr>
<td>Fluorene</td>
<td>34.02</td>
<td>Y = 32.71X + 0.33</td>
<td>0.9962</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>29.75</td>
<td>Y = 30.87X – 0.67</td>
<td>0.9998</td>
</tr>
<tr>
<td>Dibenzofuran</td>
<td>50.71</td>
<td>Y = 53.54X – 0.62</td>
<td>0.9983</td>
</tr>
</tbody>
</table>

a Theoretical slope calculated from equation (2.10) using experimental Kd in Table 2.2. b Least square linear regression from C_{SS} vs. C_{H2O} plots. c R, correlation coefficients of linear plots.

2.3.4 Analytical Figures of Merit

The existence of a linear relationship between C_{H2O} and the analyte signal (I_p) is demonstrated in Table 2.4. Within the linear dynamic ranges (LDR) of the calibration curves, the relative standard deviations at medium concentrations were lower than 10%. The limits of detection (LODs) with the spiking method were compared to those obtained with the eluting
procedure. LOD values were calculated as $3S_B/m$, where $S_B$ is the standard deviation from 16 blank determinations and $m$ is the slope of the calibration curve. The blank signals for the spiking procedure were measured from 16 disks extracted with 10 ml of blank aqueous solution and spiked with 100 μl of Shpol'skii solvent. The blank signals for the eluting procedure were measured from 16 aliquots of Shpol'skii solvent. In both cases, the concentrations for the limits of detection represent the concentrations in the aqueous sample prior to SLE, i.e. $C_{H2O}$.

Table 2.4 K Analytical figures of merit of organic pollutants on SLE membranes spiked with Shpol'skii solvent

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ex}/\lambda_{em}$ (nm)</th>
<th>$R^c$</th>
<th>LDR$^d$ (ng ml$^{-1}$)</th>
<th>Spiking$^e$ LOD (ng ml$^{-1}$)</th>
<th>Eluting$^f$ LOD (ng ml$^{-1}$)</th>
<th>RSD$^g$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysene</td>
<td>284/501</td>
<td>0.9984</td>
<td>0.98-200</td>
<td>0.98</td>
<td>12.91</td>
<td>9.6</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>284/496</td>
<td>0.9988</td>
<td>0.48-200</td>
<td>0.48</td>
<td>15.89</td>
<td>6.3</td>
</tr>
<tr>
<td>Fluorene</td>
<td>284/420</td>
<td>0.9987</td>
<td>0.72-170</td>
<td>0.72</td>
<td>30.07</td>
<td>5.5</td>
</tr>
<tr>
<td>Dibenzofuran</td>
<td>284/402</td>
<td>0.9991</td>
<td>0.57-300</td>
<td>0.57</td>
<td>315.03</td>
<td>3.8</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>284/440</td>
<td>0.9997</td>
<td>5.20-200</td>
<td>5.20</td>
<td>67.74</td>
<td>3.8</td>
</tr>
</tbody>
</table>

$^a$ Analytical figures of merit were obtained with 10 ml of aqueous standard solution.$^b$ Excitation ($\lambda_{ex}$) and phosphorescence ($\lambda_{em}$) wavelengths.$^c$ R, correlation coefficient of the calibration curve.$^d$ LDR, linear dynamic range estimated from the LOD to the upper linear concentration. $^e$ LOD, limit of detection calculated based on the equation $LOD = 3S_B/m$; $S_B$ is the standard deviation of the blank based on 16 measurements and $m$ is the slope of the calibration curve based on five concentrations within the LDR.$^f$ LOD, limit of detection calculated considering the pre-concentration factor with the elution procedure, from the equation $LOD = f_1/f_2 \times V_{SS}/V_{H2O} \times LOD_{SS}$; $f_1$ is the extraction efficiency from water sample, $f_2$ is the elution efficiency from extracting membrane, $V_{SS}$ is the Shpol’skii solvent volume spiked in the SLE membrane, $V_{H2O}$ is the water volume used in the extraction and $LOD_{SS}$ is the LOD on the Shpol’skii solvent.$^g$ RSD, relative standard deviation from three sample measurements at medium concentrations within LDR.

The spiking method improved the LODs by at least one order of magnitude. The significant improvement can be mainly attributed to the larger sample pre-concentration achieved with the spiking method. The pre-concentration factors with the spiking method are the same as the slopes of the $C_{H2O}$ vs. $C_{SS}$ plots. They vary between 25 (chrysene) and 58 (phenanthrene). The pre-concentration factor with the eluting procedure is given by
\( \frac{f_2}{f_1} \times \frac{V_{H2O}}{V_{SS}} \), where \( f_2 \) is the eluting efficiency from the extracting membrane and \( V_{SS} \) is the volume of eluting (Shpol'skii) solvent\(^{42,43,44} \). Therefore, the best pre-concentration factor that one can obtain for \( V_{H2O} = 10 \) ml and \( V_{SS} = 5 \) ml is 2 \( (f_1 = f_2 = 1) \). For many PAH, including fluorene and phenanthrene, \( f_2 \) is lower than 0.6 and the pre-concentration factor is lower than 2.

2.3.5 Analysis of PCB and PCDF on Spiked Membranes

The fact that \( V_{H2O} \) can be adjusted to reach a measurable concentration in the analytical sample still makes the eluting method a valuable approach for the analysis of PAH with low eluting efficiencies. The true limitation of the eluting method is observed for the analysis of PCBs and PDBFs. The amount of PCB or PDBF that is eluted from the extraction membrane behaves randomly and makes the method unsuited for analytical use. In other cases, the amount of PCB or PDBF eluted is very low. The worst-case scenario was observed with 4-chlorobiphenyl, which had zero recovery from the extraction membrane.

Table 2.5 shows the distribution ratios for three PCBs and PDBFs measured after 5 min of equilibration time. The \( K_d \) values demonstrate the highest affinity of 4-chlorobiphenyl for the extraction membrane, which correlates well to the zero percent recovery obtained with the eluting procedure. It is also interesting to note that the distribution ratio of biphenyl \( (9.78) \) is lower than the \( K_d \) values for PCBs. The same is true for dibenzofuran \( (K_d=4.08) \) and PDBFs. The higher affinity of the chlorinated pollutants for the membrane can be attributed to non-reversed-phase interactions (hydrogen bonding and dipole–dipole interactions) with the unreacted (residual) silanol and siloxane groups on the surface of octadecyl silica phases. Although hydrogen bonding and dipole–dipole interactions might also occur with biphenyl and dibenzofuran, the presence of chlorine atoms possibly enhances their effect.
Table 2.5 Distribution coefficient\(^a\) of organic pollutants between SLE membrane and Shpol'skii solvent

<table>
<thead>
<tr>
<th>Compound</th>
<th>(R^b)</th>
<th>(K_d)</th>
<th>(s_{Kd}^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-chlorobiphenyl</td>
<td>0.9933</td>
<td>133.84</td>
<td>16.32</td>
</tr>
<tr>
<td>3,3’,4,4’-Tetrachlorobiphenyl</td>
<td>0.9859</td>
<td>13.42</td>
<td>2.21</td>
</tr>
<tr>
<td>2,3’,4,4’,5-Pentaclorobiphenyl</td>
<td>0.9851</td>
<td>24.58</td>
<td>3.11</td>
</tr>
<tr>
<td>2-Chlorodibenzofuran</td>
<td>0.9989</td>
<td>7.34</td>
<td>0.55</td>
</tr>
<tr>
<td>2,8-Dichlorodibenzofuran</td>
<td>0.9871</td>
<td>24.75</td>
<td>3.16</td>
</tr>
<tr>
<td>2,3,7,8-Tetrachlorodibenzofuran</td>
<td>0.9998</td>
<td>9.33</td>
<td>0.10</td>
</tr>
</tbody>
</table>

\(^a\) \(K_d\) is the distribution coefficient defined as \(K_d = \frac{C_{SLE}}{C_{SS}}\). Where \(C_{SLE}\) is the analyte concentration in the SLE membrane and \(C_{SS}\) is the analyte concentration in the organic solvent. \(K_d\) and \(s_{Kd}\) were obtained from linear regression plots. \(^b\) \(R\), correlation coefficient of the linear plot.

Despite the rather large \(K_d\), the analysis of PCBs and PCDFs with the spiking procedure is still possible. Table 2.6 summarises the analytical figures of merit obtained with 10 ml of water and 100 \(\mu\)l of heptane. The correlation coefficients of the calibration curves are close to unity, the LDR extend over two orders of magnitude and the LODs are at the parts-per-billion level.

The RSD measured at medium concentrations within the LDR are lower than 10\%, which is excellent for measurements at helium liquid temperature. It is important to note that the RSD represent the precision of the method and include the random errors propagated from water extraction to signal measurement. It is also important to note that the spiking method makes the analysis of 4-chlorobiphenyl feasible.
Table 2.6 K Analytical figures of merit of PCBs and PDBFs on SLE spiked with Shpol'skii solvent

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{exc}}$/ $\lambda_{\text{em}}$ (nm)</th>
<th>$R^d$</th>
<th>Spiking LOD (ng ml$^{-1}$)</th>
<th>Eluting LOD (ng ml$^{-1}$)</th>
<th>RSD$^g$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-chlorobiphenyl</td>
<td>284/482</td>
<td>0.9996</td>
<td>12.8-2000</td>
<td>7.7</td>
<td>7.2</td>
</tr>
<tr>
<td>3,3',4,4'-Tetrachlorobiphenyl</td>
<td>284/494</td>
<td>0.9996</td>
<td>5.3-1000</td>
<td>5.3</td>
<td>8.8</td>
</tr>
<tr>
<td>2,3',4,4',5-Pentaclorobiphenyl</td>
<td>284/491</td>
<td>0.9998</td>
<td>5.5-2000</td>
<td>5.5</td>
<td>6.4</td>
</tr>
<tr>
<td>2-Chlorodibenzofuran</td>
<td>284/440</td>
<td>0.9984</td>
<td>0.27-150</td>
<td>0.27</td>
<td>9.1</td>
</tr>
<tr>
<td>2,8-Dichlorodibenzofuran</td>
<td>284/434</td>
<td>0.9998</td>
<td>0.85-200</td>
<td>0.85</td>
<td>8.4</td>
</tr>
<tr>
<td>2,3,7,8-Tetrachlorodibenzofuran</td>
<td>284/440</td>
<td>0.9987</td>
<td>0.76-200</td>
<td>0.76</td>
<td>7.7</td>
</tr>
</tbody>
</table>

$^a$ Analytical figures of merit were obtained with 10 ml of aqueous standard solution.$^b$ 100 ml of n-heptane as Shpol'skii solvent were used to spike the SLE membrane.$^c$ Excitation ($\lambda_{\text{exc}}$) and phosphorescence ($\lambda_{\text{em}}$) wavelengths.$^d$ $R$, correlation coefficient of the calibration curve.$^e$ LDR, linear dynamic range estimated from the limit of detection to the upper linear concentration.$^f$ LOD, limit of detection calculated from equation $\text{LOD}=3s_B/m$; $s_B$ is the standard deviation of the blank based on 16 measurements and $m$ is the slope of the calibration curve based on five concentrations within the LDR.$^g$ RSD, relative standard deviation from three sample measurements at medium concentration within LDR.

2.4 Conclusions

The comparison of phosphorescence spectra and lifetimes recorded from spiked membranes and Shpol'skii solvents matches our previous observations from fluorescence data and supports the hypothesis of analyte partition into the organic layer of the spiked membrane. In some cases, phosphorescence lifetimes on spiked membranes are statistically different ($\alpha=0.05$) than those in Shpol'skii standard solutions. The differences can be attributed to substrate perturbation of the Shpol'skii environment. Because the perturbation does not cause significant loss in spectral resolution and the phosphorescence decays on spiked membranes follow “well-behaved” single exponential decays, the spiking method still holds its potential for multidimensional analysis, i.e. the identification of target compounds based on spectral and lifetime analysis.

The accuracy and precision needed for quantitative analysis in aqueous samples was demonstrated with the thorough investigation of quantitative parameters. The analyte
concentration in the layer of Shpol'skii solvent follows a linear relationship with the analyte concentration in the water sample and the same is observed for the phosphorescence signal of the cryogenic probe.

In comparison to the eluting method,\textsuperscript{26,53} the spiking method presents several advantages. Because the eluting step is eliminated from the experimental procedure, the consumption of Shpol'skii solvent is significantly reduced (50\times). Keeping in mind that Shpol'skii solvents are highly pure (HPLC grade), costly and expensive to discard, reducing solvent consumption is a valid consideration for screening numerous samples. In addition, the spiking method provides better sample pre-concentration, which leads to lower LODs. The values reported here were at the parts-per-trillion level, but it is always possible to increase the volume of extracted sample to reach lower concentration levels.

The main advantage of the spiking method is making the analysis of PCBs and PDBFs possible. Under equilibrium conditions, analyte partitioning into the solvent layer is reproducible leading to acceptable precision of measurements for quantitative analysis. Even for those compounds with relatively high affinity for the extraction membrane, trace level determination is feasible due to the small volume of Shpol'skii solvent spiked on the membrane. In comparison to liquid–liquid extraction and Shpol'skii spectrometry of HPLC fractions,\textsuperscript{54} the spiking method improves the pre-concentration of the fraction.
3 CHAPTER: LASER-EXCITED TIME-RESOLVED SHPOL’SKII SPECTROSCOPY FOR THE DIRECT ANALYSIS OF DIBENZOPYRENE ISOMERS IN LIQUID CHROMATOGRAPHY

3.1 Introduction

This chapter builds upon a novel laser-induced fluorescence technique that can rapidly detect and analyze polycyclic aromatic hydrocarbons (PAHs) in water and other complex environmental samples. The basis of the technique, which we have named laser-excited time-resolved Shpol’skii spectrometry (LETRSS), has been described in recent publications.25,55, The analytical potential of our approach has been successfully compared to classical chromatographic techniques for the analysis of PAHs in the U.S. Environmental Protection Agency (EPA) priority pollutant’s list.26,54,56 The main advantages of LETRSS included shorter analysis time (~ 15min per sample), reduced solvent consumption (100μL per sample) and lower limits of detection (LOD). Depending on the PAH, LETRSS-LOD were one to two order of magnitude better than HPLC-LOD.

But this chapter tackles a different aspect as it focuses on PAHs for which satisfactory analytical techniques are completely lacking at present. Recent toxicological studies correlate a significant portion of the biological activity of contaminated samples to PAHs not included in the EPA priority pollutants list.57 Dibenzo[a,l]-pyrene (DB[a,l]P) is a crucial example. This compound is considered the most potent carcinogenic of all PAHs ever tested. DB[a,l]P shows 1-2 orders of magnitude higher tumorigenicity than benzo[a]pyrene in mouse experiments.58,59,60 Its four isomers, which include dibenzo[a,e]pyrene (DB[a,e]P), dibenzo[a,h]pyrene (DB[a,h]P),
dibenz[a,i]pyrene (DB[a,i]P), and dibenzo[e,l]-pyrene (DB[e,l]P), are also carcinogenic but not to the extent DB[a,l]P.

PAHs’ standard methodology does not meet the challenge of specifically analyzing these structural isomers at low concentration levels.\textsuperscript{61} HPLC with UV absorption and room-temperature fluorescence detection is not specific enough, particularly in the case of coeluting isomers. The limitations confronted by high-resolution GC/MS include very similar elution times and similar, virtually identical, fragmentation patterns.\textsuperscript{62,63} Until recently, GC/MS identification of DB[a,l]P in complex environmental samples had not been reported even though its identification in cigarette smoke condensate, diesel exhaust, and coal conversion and combustion had been attempted.\textsuperscript{64,65,66,67} Those studies had only been able to make a tentative identification of DB[a,l]P or had to report concentration in terms of a total fraction composed of several dibenzopyrene isomers. The problem that confronts GC-MS is that there are 33 possible PAH isomers, including the four dibenzopyrenes, of molecular weight 302. Many will have very similar elution times and similar, possibly even virtually identical, fragmentation patterns.

In this chapter, we present LETRSS methodology to directly determine DB[a,l]P and its four dibenzopyrene isomers in HPLC fractions. PAH determination is accomplished through WTM formats, which give simultaneous access to spectral and lifetime information. Prior to LETRSS analysis, HPLC fractions are pre-concentrated by liquid–liquid extraction (LLE) or SLE. Both steps are performed at the tip of the fiber-optic probe with microliters of Shpol’skii solvent. The combination of LLE or SLE with LETRSS provides accurate and precise determination at the parts-per-billion level. The best limits of detection are obtained with SLE-LETRSS analysis, which provides detection of DB[a,l]P at the femtomole level.
3.2 Experimental

3.2.1 High-Performance Liquid Chromatography Analysis

HPLC analysis was performed using a Hitachi HPLC system equipped with a model L-7100 gradient pump, an L-7400 UV detector, an L-7485 fluorescence detector, and a D-7000 control interface. HPLC operation was computer controlled with Hitachi software. A Supelco Supercosil LC-PAH column (15 cm length, 4 mm inner diameter, and 5 mm particle sizes) was used for PAH separation. All mixtures and standard injections were at a volume of 20 µL using a fixed-volume injection loop. HPLC fractions were collected in 1.5 mL sample vials with the aid of a Gilson fraction collector (modelFC 20313).

All solvents were Aldrich HPLC grade. Nanopure water was used throughout. A Supelco PAH mixture (EPA610) in methanol/methylene chloride (1:1 v/v) was used as a reference standard for the 16 EPA-PAHs. DB[a,i]P, DB[a,l]P, DB[a,h]P, and DB[a,e]P were purchased from Accustandard at their highest available purity. DB[e,l]P was acquired from Dr. Ehrenstorfer (Augsburg, Germany) at its highest available purity.

Working solutions of PAH mixtures and individual standards were prepared in methanol. The column was allowed to re-equilibrate for a minimum of 15 min at initial conditions between chromatographic runs.

3.2.2 Steady-State Excitation and Fluorescence Spectra.

Steady-state excitation and fluorescence spectra were recorded with a commercial spectrofluorimeter (Photon Technology International). The excitation source was a continuous wave 75 W Xenon lamp. The excitation and the emission monochromator had diffraction gratings blazed at 300 and 400 nm, respectively. Its reciprocal linear dispersion was 4.2 nm
Detection was made with a photomultiplier tube (PMT, Model 1527). In the photon counting mode, the maximum count rate was 4 MHz, rise time was 20 ns, and fall time was 100 ns, with a 220 ns pulse width. The instrument was computer controlled using commercial software (Felix32t) specifically designed for the system. Room-temperature measurements were made from un-degassed solutions with standard quartz cuvettes (1 cm × 1 cm). Excitation at 77 K and fluorescence spectra were acquired with the classic procedure of immersing the un-degassed sample solution in a quartz tube into a nitrogen-filled Dewar flask. PAH standard solutions were prepared with HPLC grade n-hexane, n-heptane, and n-octane.

3.2.3 Ultraviolet and Visible Spectroscopy

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 band-pass, and 24 000 nm min⁻¹ maximum scan rate. The instrument was computer controlled with Varian commercial software (Cary WinUV).

3.2.4 Laser-Excited Time-Resolved Shpol’skii Spectroscopy Analysis

LETRSS measurements were carried out with the FOP and the instrument shown in Figures 1.2 and 1.3, respectively.

3.2.5 Freezing Procedure

Samples were frozen with the following procedure. After 0.6 mL of un-degassed sample solution was pipetted into the sample vial, the tip of the fiber-optic probe was positioned and held constant with the screw cap below the solution surface. The dimensions of the vial were as follows: 30 mm length, 5.5 mm inner diameter, and 7 mm outer diameter. Sample freezing was
accomplished by lowering the copper tubing into the liquid cryogen. The liquid nitrogen and liquid helium were held in two separate Dewars with 5 L and 60L storage capacity, respectively. The 60 L liquid helium would typically last for three weeks of daily use, averaging 15–20 samples per day. At both temperatures, complete sample freezing takes less than 90 s. The approximately one-minute probe clean-up procedure involved removing the sample vial from the cryogen container, melting the frozen matrix, and warming the resulting solution to approximately room temperature with a heat gun, rinsing the probe with n-alkane, and drying it with warm air from the heat gun. The entire freeze, thaw, and cleanup cycle took no longer than 5 minutes.

3.2.6 Lifetime Analysis

Fluorescence lifetimes were determined via a three-step procedure: (1) full sample and background WTM were collected; (2) the background decay curve was subtracted from the fluorescence decay curve at the wavelength of maximum fluorescence for each PAH; and (3) the background corrected data were fit to single exponentials. In cases where the sample composition was not exactly known and the formulation of a correct blank for lifetime background subtraction was not possible, the fluorescence decay at the base of the target peak was used for background subtraction at the target wavelength. The accuracy of this procedure has been confirmed previously.\textsuperscript{26} Origin software (version 5, Microcal Software, Inc.) was used for curve fitting of fluorescence lifetimes. Fitted decay curves (\(y = y_0 + A_1 \exp^{-(x - x_0)y_1}\)) were obtained by fixing \(y_0\) and \(x_0\) at a value of zero.
3.2.7 Laser-Excited Time-Resolved Shpol’skii Spectroscopy Analysis of High Performance Liquid Chromatography Fractions.

The LLE-LETRSS analysis was performed as follows: for each fraction tested, 50 mL of the mobile phase was pipetted into the sample vial followed by 200 µL of Shpol’skii solvent. In both cases, the aliquot volumes were arbitrarily chosen. The sample vial was vigorously shaken for about 2 min and allowed to stand for about 1 min to separate the two liquid layers. The sample vial was then attached to the fiber-optic probe and the fiber was vertically adjusted so that the tip was about 1 cm below the surface of the Shpol’skii layer. SLE of HPLC fractions was carried out with a syringe kit procedure previously described.

Commercial extraction membranes (47 mm disks) were dissected into 5.5 mm diameter disks to fit in the vessel of the FOP. The 5.5 mm disk was placed into a stainless steel filter syringe kit (Alltech, IL) and attached to a 10 mL syringe (Hamilton, NV). Positive pressure was used to force the liquid solutions through the membrane. Prior to sample application, the disk was conditioned with 1 mL of methanol and 1 mL of nanopure water. The HPLC fraction was then pushed through the membrane at 30 mL min⁻¹ flow rate. Following extraction, void aqueous phase was mechanically removed with a 100 mL syringe by forcing three 100 mL volumes of air through the disk. The membrane was placed in the vessel of the fiber-optic probe and spiked with 200 mL of octane. The distance between the fiber-optic probe and the membrane was adjusted to irradiate the entire surface of the disk. The position of the probe was held constant with the screw cap of the sample cell.
3.3 Results and Discussion

3.3.1 High-Performance Liquid Chromatography Analysis

Figure 3.1 shows a typical UV chromatogram of a synthetic mixture with the 16 EPA-PAHs and the 5 dibenzopyrene isomers. Chromatographic conditions were as stipulated by Supelco for the separation of the 16 EPA priority pollutants; i.e., 1.5 mL/min flow rate, isocratic elution with water/methanol (40:60 v/v) for 5 min, and then linear gradient to 100% methanol over 30 min. With the exception of DB[a,l]P and DB[a,h]P, all the remaining compounds were chromatographically resolved. Considering that most PAHs exhibit vibronic structure in their room-temperature excitation and fluorescence spectra and fluorescence detection is often employed to enhance the selectivity of HPLC analysis, we investigated the selective determination of DB[a,l]P and DB[a,h]P via room-temperature fluorescence spectroscopy. Their excitation and fluorescence spectra were then recorded from standard solutions in methanol, the mobile phase of their HPLC fraction. Because DB[a,h]P does not fluoresce upon excitation at 363 nm or 383 nm, it is possible to use fluorescence detection to spectrally resolve DB[a,l]P in the HPLC fraction. This possibility is not available in the absorption detection mode because the absorbance spectra of the two isomers strongly overlap within their entire absorption range (200–320 nm). On the other hand, spectral resolution of DB[a,h]P by room-temperature fluorescence is not possible due to complete overlapping of excitation and fluorescence spectra.
Figure 3.1 Ultraviolet chromatogram of a synthetic mixture containing the 16 EPA priority pollutants and the 5 dibenzopyrene isomers.

Components are (1) 100 mg mL\(^{-1}\) naphthalene, (2) 200 mg mL\(^{-1}\) acenaphthylene, (3) 100 mg mL\(^{-1}\) acenaphthene, (4) 20 mg mL\(^{-1}\) fluorene, (5) 10 mg mL\(^{-1}\) phenanthrene, (6) 10 mg mL\(^{-1}\) anthracene, (7) 20 mg mL\(^{-1}\) fluoranthene, (8) 10 mg mL\(^{-1}\) pyrene, (9) 10 mg mL\(^{-1}\) benzo[a]anthracene, (10) 10 mg mL\(^{-1}\) chrysene, (11) 20 mg mL\(^{-1}\) benzo[b]fluoranthene, (12) 10 mg mL\(^{-1}\) benzo[k]fluoranthene, (13) 10 mg mL\(^{-1}\) benzo[a]pyrene, (14) 10 mg mL\(^{-1}\) dibenzo[a,i]pyrene, (15) 10 mg mL\(^{-1}\) dibenzo[a,l]pyrene, (16) 10 mg mL\(^{-1}\) dibenzo[a,h]pyrene, (17) 20 mg mL\(^{-1}\) dibenzo[a,h]anthracene, (18) 20 mg mL\(^{-1}\) benzo[g,h,i]perylene, (19) 10 mg mL\(^{-1}\) indeno[1,2,3-cd]pyrene, (20) 10 mg mL\(^{-1}\) dibenzo[a,e]pyrene, and (21) 10 mg mL\(^{-1}\) dibenzo[e,l]pyrene. Sample volume = 20 µL.
3.3.2 77K and 4.2K Spectral Characteristics of Dibenzopyrenes isomers in Shpol’skii Solvents.

The Shpol’skii solvent for DB[a,l]P was selected among the three n-alkanes that best match its molecular length, namely n-hexane, n-heptane, and n-octane. Fluorescence spectra at 77 K were recorded with a commercial spectrofluorimeter using slit-widths (42 mm) for optimum spectral resolution (about 0.18 nm band-pass). Because the narrowest FWHM was obtained with octane, this solvent was then used for all further studies.

Figure 3.2 shows the 77 K excitation and fluorescence spectra of the five dibenzopyrene isomers in octane. All spectra were collected using 0.18 nm excitation and emission band-pass. No attempts were made to correct spectra for instrumental response. Site selective excitation was not attempted. The pairs of excitation/emission wavelengths for each compound are shown in the figure caption. All spectra show the quasi-line structure expected from Shpol’skii systems. Moreover, the spectral features in emission appear significantly narrower than in excitation, even though excitation and emission were recorded with the same band-pass. The primary reason is that the displayed portions of the excitation spectra correspond to the excitation to singlet states higher than S₁ and are subject to uncertainty broadening from rapid S₀–S₁ internal conversion.
Figure 3.2 77K excitation and emission spectra of DB[a,e]P, DB[a,l]P, DB[a,i]P, DB[a,h]P and DB[e,l]P.

(A) DB[a,e]P ($\lambda_{\text{exc}}$=310 nm, $\lambda_{\text{em}}$=396 nm); (B) DB[a,l]P ($\lambda_{\text{exc}}$=323 nm, $\lambda_{\text{em}}$=420 nm); (C) DB[a,i]P ($\lambda_{\text{exc}}$=400 nm, $\lambda_{\text{em}}$=433 nm); (D) DB[a,h]P ($\lambda_{\text{exc}}$=316 nm, $\lambda_{\text{em}}$=449 nm); and (E) DB[e,l]P ($\lambda_{\text{exc}}$=288 nm, $\lambda_{\text{em}}$=385 nm). All solutions were prepared in n-octane at 1 mg mL$^{-1}$. All spectra were recorded with a commercial spectrofluorimeter. $\lambda_{\text{exc}}$ and $\lambda_{\text{em}}$ refer to the excitation and the emission wavelengths used to record emission and excitation spectra, respectively.
Figure 3.3A shows the 77 K and 4.2 K fluorescence spectra of DB[a,l]P recorded with the laser system and the fiber-optic probe. All spectra were recorded at the limiting resolution of the system (about 0.13 nm bandpass). The 4.2 K FWHM of the 0–0 transition (maximum wavelength at 418.1 nm) was approximately 23 narrower than the 77 K value. The gain in spectral resolution revealed the presence of two principal sites, which explains the small shoulder observed at 77 K in the high-energy side of the 0–0 transition peak. Tuning the dye laser from 323 nm to 324.6 nm changes the relative intensities of the two peaks and confirms the presence of two populations with different orientations in the frozen matrix. Figure 3.3 B shows the 4.2 K fluorescence spectrum of DB[a,h]P comparison to II or III in Figure 3.3 A shows partial fluorescence overlapping with DB[a,l]P around 450 nm. Because the gate width (1000 ns) and the gate delay (10 ns) of the ICCD were set to collect most of the fluorescence decay from each compound, time resolution did not have much impact on the spectral features. Comparison of their 77 K excitation and fluorescence spectra in Figure 3.2 also shows partial overlapping between the excitation spectrum of DB[a,h]P and the emission spectrum of DB[a,l]P. Depending on the relative concentrations of the two compounds in the fraction, significant spectral overlapping may occur and lead to inaccurate results.
Figure 3.3 DB[a,l]P and DB[a,h]P 4.2K fluorescence spectra

(A) 77 K (I) and 4.2 K (II and III) fluorescence spectra of DB[a,l]P recorded with the fiber-optic probe, tunable dye laser, spectrograph, and ICCD camera. Spectra were recorded from a 1 mg mL⁻¹ standard solution in octane upon sample excitation at 323 nm (I and II) and at 324.6 nm (III). (B) 4.2 K fluorescence spectrum of DB[a,h]P collected from 1 mg mL⁻¹ standard solutions in octane upon excitation at 316.5 nm. In all cases, spectra are the accumulation of 100 laser pulses. Delay and gate times were 10 ns and 1000 ns, respectively. Spectrograph slit was 42 mm.
Fortunately, their fluorescence lifetimes are remarkably different and time discrimination of fluorescence should minimize, or even eliminate, overlapping of emission spectra. Because DB[α,l]P lifetime (78.1 ns) is longer than DB[α,h]P lifetime (6.0 ns), a relatively short gate time should enhance DB[α,h]P fluorescence over DB[α,l]P emission. Figure 3.4A shows the spectra of three binary mixtures (DB[α,h]P/DB[α,l]P) at the 1:1, 1:10, and 1:100 concentration ratios. Spectra were recorded with a zero ns delay and a 15 ns gate, i.e., approximately 23 the lifetime of DB[α,h]P. Sample excitation was set at 316.5 nm, the maximum excitation wavelength of DB[α,h]P. Their comparison shows no contribution from DB[α,l]P. A single exponential decay at 449.2 nm and a fluorescence lifetime matching the one in Table I confirm spectral purity and peak assignment solely to DB[α,h]P. Although sample excitation at 316.5 nm promotes strong fluorescence from DB[α,l]P, its intensity is reduced to a negligible value by shortening the gate of the ICCD (see Figure 3.4B).
Figure 3.4 4.2 K fluorescence spectra from three DB[a,h]P and DB[a,l]P mixtures and DB[a,l]P standards. 

(A) three DB[a,h]P and DB[a,l]P mixtures at the following concentration ratios: (I) 1:1, (II) 1:10, and (III) 1:100. In all mixtures, DB[a,h]P concentration was 0.1 mg mL\(^{-1}\). Gate and delay times were 0 and 15 ns, respectively. (B) 10 mg mL\(^{-1}\) DB[a,l]P using 15 ns (---) and 150 ns (-----) gate times. All spectra were recorded with the fiber-optic probe, tunable dye laser, spectrograph, and ICCD camera from octane solutions upon excitation at 316.5 nm. Spectrograph slits were 42 mm.
The selective determination of DB$_{a,l}$P in the presence of DB$_{a,h}$P was attempted in a similar way. Figure 3.5 overlaps the fluorescence spectra of three binary mixtures (DB$_{a,l}$P/DB$_{a,h}$P) at 1:1, 1:10, and 1:100 concentration ratios. Spectra were collected upon excitation at the maximum wavelength (323 nm) of DB$_{a,l}$P. The delay (10 ns) and gate (150 ns) were selected to enhance its signal over the emission of DB$_{a,h}$P. For a confidence interval of 95\% ($a = 0.05$) and six determinations ($N = 6$), the signal of the mixtures at 418.1 nm (0–0 band of DB$_{a,l}$P) were statistically equivalent to the one recorded from the standard solution. Similarly, no significant difference was observed between the fluorescence decays collected from the standard and the mixture or between their lifetime values. In addition to the lack of interference due to fluorescence overlapping, our results show that—at these concentration levels—the overlapping between the DB$_{a,h}$P excitation spectrum and the DB$_{a,l}$P fluorescence emission do not affect the accuracy of analysis.
Figure 3.5 4.2 K fluorescence spectra of 0.1 mg mL\(^{-1}\) DB\([a,l]\)P solutions containing DB\([a,h]\)P in n-octane. The concentration of DB\([a,h]\)P in each solution was: (I) 0.1 mg mL\(^{-1}\); (II) 1 mg mL\(^{-1}\); and (III) 10 mg mL\(^{-1}\). All spectra were collected upon excitation at 323 nm, with a 10 ns delay and 150 ns gate time, and a 42 mm spectrograph slit-width.

Table 3.1 relates the 4.2 K LETRSS parameters for the identification of the five isomers in HPLC fractions. In complex fractions with many co-eluted PAHs, the tabulated excitation wavelengths, which correspond to maximum excitation peaks, might not be the best wavelengths to selectively excite the isomer of interest. The analyst should always collect a WTM to check for peak spectral purity via lifetime analysis. If multi-exponential decays are observed or lifetime
analysis of single exponential decays do not provide statistically matching lifetimes to those from pure standards in n-octane, fluorescence overlapping from concomitants can be avoided by tuning the excitation wavelength within the excitation ranges shown in Figure 3.2.

Table 3.1 4.2K LETRSS parameters for monitoring dibenzopyrenes in HPLC fractions

<table>
<thead>
<tr>
<th>PAHa</th>
<th>Retention timeb (min)</th>
<th>λexc (nm)c</th>
<th>λem(nm)d</th>
<th>td/tg e (ns)</th>
<th>f τ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibenzo[a,l]pyrene</td>
<td>37.2±0.5</td>
<td>323</td>
<td>418.1, 424.8, 443.2</td>
<td>10/250</td>
<td>78.1±3.1</td>
</tr>
<tr>
<td>Dibenzo[a,e]pyrene</td>
<td>50.4±0.4</td>
<td>310</td>
<td>395.1, 396.0, 401.8</td>
<td>10/150</td>
<td>55.8±1.1</td>
</tr>
<tr>
<td>Dibenzo[a,h]pyrene</td>
<td>37.2±0.2</td>
<td>316.5</td>
<td>449.2, 454.6, 479.0</td>
<td>10/150</td>
<td>53.0±1.0</td>
</tr>
<tr>
<td>Dibenzo[a,i]pyrene</td>
<td>36.2±0.1</td>
<td>304</td>
<td>432.5, 447.4, 459.6</td>
<td>10/650</td>
<td>219.9±7.7</td>
</tr>
<tr>
<td>Dibenzo[e,l]pyrene</td>
<td>54.0±0.6</td>
<td>288</td>
<td>383.7, 388.6, 403.4, 409.4</td>
<td>10/150</td>
<td>53.0±1.0</td>
</tr>
</tbody>
</table>

a Standard solutions were prepared in n-octane at 0.1 mm mL⁻¹. b Average retention times of three chromatographic runs. c λexc = excitation wavelength corresponding to the maximum excitation wavelength of the compound. d Emission wavelengths; maximum emission wavelength is underlined. e td = delay time; tg = gate time. f Average fluorescence lifetime of three measurements taken from three frozen aliquots. All compounds showed single exponential decays at their maximum excitation and emission wavelengths. The residuals between the calculated and the observed points were less than 15 within the first two lifetimes of the decays and showed no systematic errors.

3.3.3 Quantitative Analysis of HPLC Fractions

The feasibility to perform LLE-LETRSS analysis in HPLC fractions has been demonstrated for several EPA-PAHs. 54,68 Quantitative analysis was performed via the multiple standard addition method. Small aliquots of standard solution were spiked into the vessel of the fiber-optic probe to determine the concentration of target compounds. Later, we developed SLE-LETRSS for the analysis of polycyclic aromatic compounds (EPA-PAHs, polychlorinated biphenyls, and polychlorinated dibenzofurans) in aqueous samples. A mathematical expression
for quantitative analysis via the calibration curve was demonstrated with a thorough experimental investigation of quantitative parameters.

A similar approach is used here to derive a mathematical expression for LLE-LETRSS analysis via the calibration curve method. Considering a binary system between the mobile phase and the Shpol’skii solvent, the distribution coefficient ($K_d$) of a PAH between the Shpol’skii solvent and the mobile phase can be defined as follows:

$$K_d = \frac{C_{SS}}{C_{MP}}$$ \hspace{1cm} (3.1)

where $C_{SS}$ and $C_{MP}$ are the PAH concentrations in the Shpol’skii solvent and the mobile phase, respectively. Assuming that a linear relationship exists between the fluorescence signal ($I_f$) and $C_{SS}$, and considering that our measurements are made under equilibrium conditions, $K_d$ should be constant and the calibration curve method should provide a direct way to determine PAH isomers via LLE-LETRSS. This assumption is demonstrated in Table 3.2 which summarizes the analytical figures of merit (AFOM) obtained by HPLC-LLE-LETRSS. Each calibration curve was built with at least five standard solutions prepared in methanol. Twenty microliters of standard solution were injected into the HPLC system and each signal plotted in the calibration graph was the average of at least three chromatographic runs. No efforts were made to reach the upper linear concentration of the linear dynamic range. The correlation coefficients of the linear regression plots close to unity demonstrate the correctness of our assumption.
Table 3.2 HPLC-LLE-LETRSS analytical figures of merit for the five dibenzopyrenes

<table>
<thead>
<tr>
<th>PAH(\text{a})</th>
<th>(\lambda_{\text{exc}}/\lambda_{\text{em}}) (nm)</th>
<th>(t_d/t_g) (ns)</th>
<th>LDR(\text{d}) (ng/mL)</th>
<th>(\text{R}^e)</th>
<th>RSD(\text{f}) (%)</th>
<th>LOD(\text{g}) (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB([a,f])P</td>
<td>323/418.1</td>
<td>10/250</td>
<td>5-5000</td>
<td>0.9994</td>
<td>3.6</td>
<td>5</td>
</tr>
<tr>
<td>DB([a,e])P</td>
<td>310/396</td>
<td>10/150</td>
<td>6-5000</td>
<td>0.9991</td>
<td>7.6</td>
<td>6</td>
</tr>
<tr>
<td>DB([a,h])P</td>
<td>316.5/449.2</td>
<td>0/15</td>
<td>6-5000</td>
<td>0.9998</td>
<td>6.3</td>
<td>6</td>
</tr>
<tr>
<td>DB([a,i])P</td>
<td>304/432.5</td>
<td>10/650</td>
<td>11-5000</td>
<td>0.9997</td>
<td>3.0</td>
<td>11</td>
</tr>
<tr>
<td>DB([e,f])P</td>
<td>288/388.6</td>
<td>10/150</td>
<td>19-5000</td>
<td>0.9991</td>
<td>2.8</td>
<td>17</td>
</tr>
</tbody>
</table>

\(\text{a}\) Standard solutions were prepared in n-octane at 0.1 mm mL\(^{-1}\). \(\text{b}\) Average retention times of three chromatographic runs. \(\text{c}\) \(\lambda_{\text{exc}}\) = excitation wavelength corresponding to the maximum excitation wavelength of the compound. \(\text{d}\) Emission wavelengths; maximum emission wavelength is underlined. \(\text{e}\) \(t_d\) = delay time; \(t_g\) = gate time. \(\text{f}\) Average fluorescence lifetime of three measurements taken from three frozen aliquots. All compounds showed single exponential decays at their maximum excitation and emission wavelengths. The residuals between the calculated and the observed points were less than 15 within the first two lifetimes of the decays and showed no systematic errors.

The mathematical expression correlating \(I_f\) and \(C_{\text{MP}}\) for HPLC-SLE-LETRSS is similar to the original equation reported for water analysis:

\[
I_f = K' x (f \times V_{\text{MP}}) (1/V_{\text{SS}} + K_d' \times V_{\text{SLE}}) \times C_{\text{MP}}
\]

(3.2)

where \(K'\) is a proportionality constant predicting the direct proportionality between \(I_f\) and \(C_{\text{MP}}\), \(f\) is the extraction efficiency of SLE, \(K_d\) is the PAH partition coefficient between the Shpol’skii solvent and the extraction membrane, and \(V_{\text{SLE}}\) is the volume of the extraction membrane. Table 3.3 reports the AFOM obtained by HPLC SLE-LETRSS. The calibration curves were built in the same manner as those in Table 3.2. \(V_{\text{MP}}\) was 4.5 mL, \(V_{\text{SS}}\) was 200 \(\mu\)L, and \(V_{\text{SLE}}\) was 23.8 \(\mu\)L. \(V_{\text{SLE}}\) was calculated using the standard formula for the volume of a cylinder \(V = \pi r^2 h\), where \(r\) is the radius of the disk (0.2715 cm) and \(h\) is its thickness (0.1 cm). The extraction efficiencies (\(f\)) were obtained from fluorescence measurements made in mobile-phase fractions before and after SLE. Their values were lower than unity but constant throughout the entire range of the calibration curves. Considering the capacity of C\(_{18}\) sorbents (1–10%, w/w)\(^{59}\) and the average
weight of the disk (0.0132 g), it is unlikely to surpass the breakthrough volume of the extraction system processing 4.5 mL of PAH solutions at the parts-per-million (5000 ng mL\(^{-1}\)) level. PAH loss could have been prevented by adjusting the polarity of the mobile phase. For EPA-PAHs, a 20% water/methanol ratio (v/v) provides extraction efficiencies close to 100\%\(^{42}\). Similar to HPLC-LLE-LETTRSS, the correlation coefficients of the linear regression plots close to unity demonstrate a linear relationship between I\(_f\) and C\(_{\text{MP}}\).

Table 3.3 HPLC-SLE-LETTRSS analytical figures of merit for the five dibenzopyrenes.

<table>
<thead>
<tr>
<th>PAH(^{a})</th>
<th>I(^{b})</th>
<th>(\lambda_{\text{exc}}/\lambda_{\text{em}}) (^{c}) (nm)</th>
<th>(t_d/t_g) (^{d}) (ns)</th>
<th>LDR(^{e}) (ng/mL)</th>
<th>R(^f)</th>
<th>RSD(^g) (%)</th>
<th>LOD(^h) (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB[(a,1)]P</td>
<td>93.4±0.6</td>
<td>323/418.1</td>
<td>10/250</td>
<td>0.04-100</td>
<td>0.9918</td>
<td>4.7</td>
<td>0.04</td>
</tr>
<tr>
<td>DB[(a,e)]P</td>
<td>91.1±1.4</td>
<td>310/396.0</td>
<td>10/150</td>
<td>0.1-200</td>
<td>0.9942</td>
<td>5.2</td>
<td>0.1</td>
</tr>
<tr>
<td>DB[(a,h)]P</td>
<td>89.9±2.0</td>
<td>316.5/449.2</td>
<td>0/15</td>
<td>0.07-200</td>
<td>0.9901</td>
<td>2.5</td>
<td>0.07</td>
</tr>
<tr>
<td>DB[(a,i)]P</td>
<td>89.5±1.0</td>
<td>304/432.5</td>
<td>10/650</td>
<td>0.15-200</td>
<td>0.9952</td>
<td>1.9</td>
<td>0.15</td>
</tr>
<tr>
<td>DB[(e,l)]P</td>
<td>94.3±1.3</td>
<td>288/388.6</td>
<td>10/150</td>
<td>0.2-200</td>
<td>0.9923</td>
<td>7.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\(^{a}\)PAH standard solutions were prepared in methanol. Sample volume was 20 mL. PAHs were SLE from 4.5 mL of mobile phase. SLE membrane was spiked with 200 mL of n-octane. \(^{b}\) Extraction efficiency of SLE procedure. \(^{c}\) Excitation (\(\lambda_{\text{exc}}\)) and emission (\(\lambda_{\text{em}}\)) wavelengths. \(^{d}\) Delay (\(t_d\)) and gate (\(t_g\)) times. \(^{e}\) Linear dynamic ranges. \(^{f}\) Correlation coefficients. \(^{g}\) Relative standard deviations from six measurements of six mobile-phase fractions with PAH at medium concentrations within their respective linear dynamic ranges. \(^{h}\) Limits of detection. LOD were calculated on the basis of the equation CL = 3SB/m, where SB is the standard deviation of 16 blank measurements and m is the slope of the calibration curve. Absolute limit of detection calculated as 20 \(\mu\)L \(\times\) LOD.

Table 3.4 shows the limits of detection (LOD) obtained by conventional HPLC analysis. The LOD were obtained in the same manner as those in Tables II and III. Clearly, both HPLC-LLE-LETTRSS and HPLC-SLE-LETTRSS provide better LOD than conventional HPLC. The improvement observed with HPLC-LLE-LETTRSS can be partially attributed to reducing the sample temperature to 4.2 K. This parameter often increases the quantum yield of PAHs and eliminates the complications of oxygen quenching and energy transfer. Considering that 50 mL of mobile phase was LLE with 200 \(\mu\)L of Shpol’skii solvent, sample pre-concentration did not
Table 3.4 Limits of detection for the five dibenzopyrenes obtained by conventional HPLC analysis.

<table>
<thead>
<tr>
<th>PAHa</th>
<th>UV-Vis detection</th>
<th>Fluorescence detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ_{abs}b (nm)</td>
<td>LODc (ng mL^{-1})</td>
</tr>
<tr>
<td>DB[a,l]P</td>
<td>270</td>
<td>550</td>
</tr>
<tr>
<td>DB[a,e]P</td>
<td>302</td>
<td>600</td>
</tr>
<tr>
<td>DB[a,h]P</td>
<td>310</td>
<td>750</td>
</tr>
<tr>
<td>DB[a,i]P</td>
<td>241</td>
<td>1500</td>
</tr>
<tr>
<td>DB[e,l]P</td>
<td>273</td>
<td>1200</td>
</tr>
</tbody>
</table>

a PAH standard solutions were prepared in methanol. Sample volume was 20 mL. b Absorption wavelength for UV-vis detection. λ_{abs} corresponds to maximum absorption wavelength. c Limits of detection. LOD were calculated on the basis of the equation CL = 3SB/m, where SB is the standard deviation of 16 blank measurements and m is the slope of the calibration curve. d Excitation (λ_{exc}) and emission (λ_{em}) wavelengths for fluorescence detection. λ_{exc} and λ_{em} correspond to the maximum room-temperature excitation and fluorescence wavelengths, respectively.

3.3.4 HPLC Solid-Liquid Extraction Laser-Excited Time-Resolved Shpol’Skii Spectroscopy

Analysis of DB[a,l]P in Water Samples

The potential of our approach was put to the test for the analysis of DB[a,l]P in a heavily contaminated water sample. The sample was prepared in the lab by mixing equal volumes of Red River (North Dakota) water and water collected from the output water stream of the American Petroleum Institute (API) separator at a North Dakota refinery. Water from the Red River presents a high content of dissolved organic matter, but based on GC-MS analysis, no indication of PAH content has been reported. API water is used to desalt and scrub crude oil and oil
fractions in the refinery process and shows a visible surface petroleum layer at the separator trough. Previous HPLC-LETRSS analysis of this water has shown heavy PAH contamination.

Figure 3.6 A shows a chromatogram of the water sample spiked with DB[a,l]P to provide a 1 ng mL⁻¹ final concentration. The 4.2 K WTM of the HPLC fraction collected from 34 to 38 min is shown in Figure 3.6B. The intense doublet at the 0–0 transition provides strong evidence of DB[a,l]P presence. The fluorescence decay—stripped from the WTM at the target wavelength (418.1 nm)—was a single exponential decay, indicating the spectral purity of the target wavelength. The fluorescence lifetime (77.8 ± 2.8 ns) was statistically equivalent (P = 95%, N₁ = N₂ = 3) to the one from the standard (78.1 ± 3.2 ns), confirming the unambiguous determination of DB[a,l]P. Three individual determinations via the calibration curve method provided a 0.92 ± 0.04 ng mL⁻¹ DB[a,l]P concentration in the HPLC fraction. Accounting for the extraction efficiency of the method (93.4 ± 0.6%), the concentration in the water sample was 0.985 ± 0.049 ng mL⁻¹. The good agreement with the concentration spiked in the sample is a strong indication of the accuracy of the method. One should keep in mind, however, that the absence of DB[a,l]P in the original sample was never confirmed. The relative standard deviation (5%) demonstrates excellent precision at the parts-per-billion level.
Figure 3.6 (A) HPLC chromatogram retention time of peak I is 37.38 min. (B) 4.2 K WTM of HPLC fraction eluted from 34 to 38 min.

Initial delay was 10 ns, gate-width was 150 ns, and delay step was 4 ns. Dashed vertical lines indicate HPLC fraction further analyzed by SLE-LETRSS.
3.4 Conclusions

We have presented a unique tool for the analysis of DB[a,l]P and its four dibenzopyrene isomers in HPLC fractions. Monitoring DB[a,l]P in water samples is now possible via conventional HPLC methodology. In comparison to GC-MS, our approach determines PAH content directly from the HPLC fraction with no further separation. The entire procedure, from HPLC fraction collection to LETRSS analysis, takes less than 10 min per sample. Including HPLC analysis, the entire procedure takes approximately 45 min. Unambiguous isomer determination is based on WTM formats, which combine fluorescence decays and spectral data. Both LLE-LETRSS and SLELETRSS provide determination of the five isomers at the parts-per-billion level. Their analytical figures of merit demonstrate the feasibility of performing quantitative analysis of mobile-phase fractions via the calibration curve method. Under equilibrium conditions, PAH partitioning into the layer of Shpol’skii solvent is reproducible and leads to excellent accuracy and precision of measurements. Because of sample pre-concentration, SLE LETRSS provides the best LOD, which varies from 40 pg mL\(^{-1}\) (DB[a,l]P) to 0.2 ng mL\(^{-1}\) (DB[e,l]P). Our approach not only enhances the selectivity of conventional HPLC but also lowers its LOD by at least three orders of magnitude.
4 CHAPTER: DIRECT DETERMINATION OF DIBENZO[a,l]PYRENE AND ITS FOUR DIBENZOPYRENE ISOMERS IN WATER SAMPLES BY SOLID-LIQUID EXTRACTION AND LASER-EXCITED TIME-RESOLVED SHPOL’SKII SPECTROMETRY

4.1 Introduction

This chapter deals with the direct determination of benzopyrene isomers in heavily contaminated water samples. In analytical spectroscopy it is common to analyze mixtures with two, three, four, etc. components. Although such studies are useful for testing the potential of new approaches, it generally falls well short of realism for applications. In cases where the PACs mixtures are inherently so complex chemically, it is very difficult to "synthesize" a realistic mixture. It is important to keep in mind that even the most extensive GC or HPLC analyses only identify a fraction of the components. This is the main reason why – in the previous chapter - we proposed LETRSS for the analysis of DB[a,l]P and its four isomers in HPLC fractions. However, these facts did not prevent us from testing real-world samples to see if we could directly identify the target isomers without previous separation from the complex matrix. Can we find the needle in the haystack directly without chromatographic elution?

4.2 Experimental

4.2.1 Chemicals

All solvents were Aldrich HPLC grade. Unless otherwise noted, Nanopure water was used throughout. All chemicals were analytical reagent grade and used without further purification. DB[a,i]P, DB[a,l]P, DB[a,h]P, and DB[a,e]P were purchased from Accustandard at
their highest purity available. DB[e,l]P was acquired from Dr.Ehrenstorfer (Augsburg, Germany) at its highest purity available.

4.2.2 *Solid-Liquid Extraction*

SLE was performed according to procedure described in section 2.2.3 and illustrated in Figure 2.2.

4.2.3 *Solution Preparation*

Standard solutions for calibration curves were prepared from stock solutions in methanol by mixing 1 mL of stock solution of appropriate concentration with 80 mL of Nanopure water and 19 mL of methanol (final methanol-water volume ratio of 20:80 v/v). Synthetic DP isomer mixtures were also prepared in methanol-water 20:80 v/v. Each mixture contained the target isomer at a concentration 100 × lower than the concentrations of the other four isomers. SLE-LETRSS analysis was performed as previously described using 100 mL of water for sample extraction and 100μL of n-octane for membrane spiking.

4.2.4 *Recovery Studies in Contaminated Water*

Recovery studies were performed with Red River water and the output water stream of the American Petroleum Institute (API) separator at a North Dakota refinery. Red River water of the North Basin (Minnesota, North Dakota, and South Dakota) was collected at different locations within the Fargo-Moorhead area. The API water sample was collected from a sampling valve on the output pipe of the API separator. Both types of samples were collected in glass bottles with PTFE-lined caps and stored at 4 °C. Analytical samples were prepared by mixing 1 mL of a 10 μg mL⁻¹ stock solution of DP isomer with 80 mL of Red River or API water and 19
mL of methanol. SLE-LETRSS analysis was performed as previously described using 100 mL of water for sample extraction and 100 μL of n-octane for membrane spiking.

4.2.5 Sample Freezing

Sample freezing was carried out following procedure described in section 3.2.5.

4.2.6 Laser-Excited Time-Resolved Shpol’skii Spectroscopy

LETRSS measurements were carried out with the FOP and the instrumentation shown in Figures 1.2 and 1.3, respectively.

4.2.7 Lifetime Analysis

4.2K Fluorescence lifetimes were measured as described in section 3.2.6.

4.3 Results and Discussion

4.3.1 Shpol’skii Spectroscopy of DP Isomers on Spiked Membranes

An initial survey of 77 K excitation and fluorescence spectra of the five DP isomers was conducted with a commercial spectrofluorometer. Three n-alkanes, namely, hexane, heptane, and octane, were tested for best spectral resolution. For the five isomers, the narrowest fwhm was observed in n-octane. This solvent was then used for all further studies. Figure 4.1 compares the 4.2 K fluorescence spectra and decay of DB[a,]P recorded from an octane standard solution and a spiked membrane. Both spectra were recorded with the fiber-optic probe and the laser system using the narrowest spectrograph slit widths (42 μm). The spectrum from the spiked membrane shows the quasi-line structure typical from DB[a,]P in n-octane. Moreover, the FWHM of the 0-0 transition (maximum wavelength at 418.1 nm) was approximately the same, indicating that a similar microenvironment surrounds DB[a,]P in the standard and on the spiked membrane.
Because no site-selective excitation was attempted, both spectra show the contribution of two crystallographic sites with maximum emission wavelengths at 417.4 and 418.1 nm. The proximity of the membrane to the organic layer has no effect on the relative intensities of the two maximum peaks. This is a strong indication that the presence of the membrane has no measurable effect on the distribution of molecules between the two sites. The single-exponential decay in the presence of the membrane also suggests that only one type of microenvironment surrounds DB[\textit{a,\textit{l}}]P molecules. Quasi-line fluorescence spectra and single exponential decays were also observed for the other DP isomers. In all cases, their fluorescence lifetime values on spiked membranes were practically the same as those recorded from standard solutions. Within a confidence interval of 95% $\alpha = 0.05$), the experimental averages of six replicate measurements from each type of sample ($N_1 = N_2 = 6$) were statistically the same ($t_{\text{exp}} < \text{critical value of } t = 2.57$).\textsuperscript{48} These facts confirm previous observations with EPA PAHs\textsuperscript{47,71} and support the hypothesis of analyte partitioning into the organic layer of the spiked membrane. Because the presence of the membrane does not deteriorate spectral resolution and the fluorescence decays on spiked membranes follow “well-behaved” single-exponential decays with remarkably different lifetime values, the direct determination of the five DP isomers on the bases of spectral and lifetime analysis is possible.
Figure 4.1 4.2 K fluorescence spectra and decay of DB[a,l]P recorded from (A) octane and (B) membrane spiked with octane.

Spectra were recorded upon excitation at 323 nm. Delay and gate times were 10 and 150 ns, respectively. The following acquisition parameters were used for decay collection: 323-nm excitation and 418.1-nm emission wavelengths, total gate time 160 ns, and gate step 4 ns.
4.3.2 Quantitative analysis of DP isomers on spiked membranes

The relationship between the analyte concentration in the Shpol’skii layer (C_{SS}) and its concentration in the water sample (C_{H2O}) is given by the following equation:

\[ C_{H2O} = \frac{V_{SS}/f_1 V_{H2O} + K_d V_{SLE}/f_1 V_{H2O}}{} C_{SS} \]

(4.1)

where \( f_1 \) is the analyte 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 4.1 predicts a linear relationship between \( C_{H2O} \) and \( C_{SS} \) upon constant \( f_1, K_d, V_{SS}, V_{H2O}, \) and \( V_{SLE} \). The analyst sets constant values for \( V_{SS}, V_{H2O}, V_{SLE}, \) and \( f_1 \). 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 work presented here expands SLE-LETRSS to the analysis of DP and provides a thorough investigation of pertinent analytical parameters for their determination in water samples. Table 4.1 shows the extraction fractions for the five DP isomers, which under careful control of extraction parameters remain constant throughout the entire working concentration range (0.1-10 ng·mL^{-1}). Therefore, the only parameter that needs further investigation is the distribution ratio \( (K_d = C_{SLE}/C_{SS}) \). Because reproducible \( K_d \) values should be best obtained under equilibrium conditions, the first step was to investigate the equilibrium time of each DP isomer in the biphasic system. Figure 4.2 shows the partitioning time profiles of the five isomers on spiked membranes. The five isotherms were obtained by monitoring the 4.2 K fluorescence intensity of the spiked membrane (I_t) as a function of elapsed time (t) between membrane spiking and freezing. During the elapsed time, the spiked membrane was kept at room temperature in the sample vial of the probe. The vial was closed to minimize solvent evaporation. Each point plotted in the graph
represents the average of three individual measurements taken from three spiked membranes. For the five compounds, Iᵣ reaches a maximum value at about 5 min and then remains constant within experimental error (α = 0.05; N₁ = N₂ = 3). Our experiments, therefore, show 5-min equilibrium time for the five isomers.

Table 4.1 Experimental and theoretical parameters concerning SLE-LETRESS analysis of DP isomers in water

<table>
<thead>
<tr>
<th>PAHᵃ</th>
<th>Extractionᵇ [%]</th>
<th>Rᶜ</th>
<th>Kᵈ</th>
<th>Sₖᵈ</th>
<th>Theoretical Slope</th>
<th>Linear fit (Cₛₛ × Cₛₑ)⁸</th>
<th>Rʰ</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB[a,l]P</td>
<td>95.3±0.9</td>
<td>0.9992</td>
<td>11.01</td>
<td>0.41</td>
<td>26.16</td>
<td>Y=23.19X-6.69</td>
<td>0.9998</td>
</tr>
<tr>
<td>DB[a,e]P</td>
<td>92.5±0.5</td>
<td>0.9997</td>
<td>10.14</td>
<td>0.72</td>
<td>27.34</td>
<td>Y=26.75X+4.27</td>
<td>0.9999</td>
</tr>
<tr>
<td>DB[a,h]P</td>
<td>93.2±1.1</td>
<td>0.9989</td>
<td>11.84</td>
<td>0.52</td>
<td>24.56</td>
<td>Y=21.72X+1.67</td>
<td>0.9975</td>
</tr>
<tr>
<td>DB[a,i]P</td>
<td>91.7±0.7</td>
<td>0.9981</td>
<td>11.64</td>
<td>0.66</td>
<td>24.47</td>
<td>Y=25.84X+5.60</td>
<td>0.9947</td>
</tr>
<tr>
<td>DB[e,l]P</td>
<td>94.9±1.2</td>
<td>0.981</td>
<td>12.62</td>
<td>0.88</td>
<td>23.85</td>
<td>Y=24.93X-4.34</td>
<td>0.9977</td>
</tr>
</tbody>
</table>

ᵃ 10-mL aliquot of 100 ng/mL standard solution (20:80 v/v methanol-water) was employed.ᵇ Extraction efficiency represents the average of individual fluorescence measurements taken from six membranes at the maximum excitation and emission wavelength of each isomer.ᶜ R, correlation coefficient of the experimental linear plot Cₛₛ × Cₛₑ.ᵈ Kᵈ, distribution coefficients obtained from bivariate least squares regressions Cₛₛ × Cₛₑ plots.ᵉ Sₖᵈ, standard deviation of Kᵈ.ᶠ Theoretical slope calculated from equation 4.1 using experimental Kᵈ values.ᵍ Least-squares linear regression from Cₛₛ vs Cₛₑ plots.ʰ Rʰ, correlation coefficients of linear fit (Cₛₛ × Cₛₑ).
Figure 4.2 Time dependence of DB[a/l]P (*), DB[a,h]P (▲), DB[a,e]P (■), DB[a,i]P (+) and DB[e,l]P (○) partitioning into the Shpol’skii solvent of the spiked membrane.

10 ml of 50 ng ml⁻¹ solutions (20% methanol/water) were used for SLE. 100 μL of organic solvent were spiked on the SLE membrane. Each point in the curve is the average of individual measurements taken from three SLE membranes.

The second step was to measure $C_{SS}$ and $C_{SLE}$ from a series of experiments where $V_{SS}$, $V_{H2O}$, and $V_{SLE}$ were held constant. The experiments were performed as follows: 10 mL of aqueous solution with known $C_{H2O}$ was extracted, and the membrane was placed into a Teflon sample vial. The membrane was spiked with 100 μL of Shpol’skii solvent, and the vial was closed. After 5 min of equilibration time, the layer of Shpol’skii solvent was drawn from the vessel with a micropipet and dispensed into the sample cell of the fiber-optic probe. $C_{SS}$ and $C_{SLE}$ were determined via the single standard addition method. For $C_{SS}$, the standard addition was
made directly to \( V_{SS} \) at room temperature. For \( C_{SLE} \), the standard addition was made to the membrane via SLE with an aqueous standard solution.

Table 4.1 summaries the variances and the slopes of the \( C_{SS} \) versus \( C_{SLE} \) linear regression plots. Each point in the plot represents the average of three \( I_f \) measurements made from individual extractions. The slope of the isotherm corresponds to \( K_d \). The correlation coefficient, the slope, and its standard deviation were calculated via the bivariate least-squares regression method, which takes into account the uncertainties in both axes.\(^5\) The correlation coefficient close to unity shows the linear relationship between \( C_{SS} \) and \( C_{SLE} \), and the constant slope demonstrates that \( K_d \) remains the same within the studied concentration range of each isomer.

Because \( K_d \) is constant, the relationship between \( C_{SS} \) and \( C_{H2O} \) should be linear (see equation 4.1). Table 4.1 summaries the linear fits and the correlation coefficients of the experimental plots (\( C_{H2O} \) vs \( C_{SS} \)) for the five isomers. It also compares the experimental slopes to the theoretical slopes obtained with equation 1 using \( K_d \) and \( f_i \) values in Table 1, \( V_{H2O} = 10 \) \( \mu \)L, \( V_{SS} = 100 \) mL, \( V_{SLE} = 28.3 \) \( \mu \)L. The agreement between the experimental and theoretical slopes is satisfactory; mainly if one considers that the experimental values were obtained via the ordinary least-squares method, i.e., assuming no experimental errors in the abscissa axis (\( C_{H2O} \)).

### 4.3.3 Analytical Figures of Merit

Table 4.2 summaries the SLE-LETRSS analytical figures of merit obtained for the five DP isomers. It also compares LODs to those obtained by LETRSS in octane. The excellent correlation coefficients demonstrate the existence of a linear relationship between \( C_{H2O} \) and the fluorescence signal from the probe. Similarly, the reproducibility of measurements at the parts-per-billion level is excellent. No efforts were made to reach the upper concentration limits of the
calibration curve and their linear dynamic ranges are below the breakthrough volumes of the extraction system. The LOD values were calculated as $3s_B/m$, where $s_B$ is the standard deviation from 16 blank determinations and $m$ is the slope of the calibration curve. The blank signals for SLE-LETRSS were measured from 16 disks extracted with 100 mL of blank aqueous solution and spiked with 100 µL of octane. The blank signals for LETRSS were measured from 16 aliquots of Shpol’skii solvent. Clearly, SLE improved LOD by 2 orders of magnitude. However, further LOD improvement is possible by optimizing $V_{H2O}$ and $V_{SS}$ (see equation 4.1).

Table 4.2 4.2K SLE-LETRSS analytical figures of merit of DP isomers

<table>
<thead>
<tr>
<th>PAH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$\lambda_{exc}/\lambda_{em}$ (nm)</th>
<th>$t_d/t_g$&lt;sup&gt;c&lt;/sup&gt; (ns)</th>
<th>LDR&lt;sup&gt;d&lt;/sup&gt; (ng/mL)</th>
<th>R&lt;sup&gt;e&lt;/sup&gt;</th>
<th>LOD&lt;sup&gt;f&lt;/sup&gt; (ng/mL)</th>
<th>SLE</th>
<th>In octane</th>
<th>RSD&lt;sup&gt;g&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB[α,l]P</td>
<td>323/418.1</td>
<td>10/250</td>
<td>0.003-10</td>
<td>0.9999</td>
<td>0.003</td>
<td>0.3</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>DB[α,e]P</td>
<td>310/396.0</td>
<td>10/150</td>
<td>0.007-10</td>
<td>0.9989</td>
<td>0.007</td>
<td>0.8</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>DB[α,h]P</td>
<td>316.5/449.2</td>
<td>0/15</td>
<td>0.006-10</td>
<td>0.9955</td>
<td>0.006</td>
<td>0.5</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>DB[α,i]P</td>
<td>304/432.5</td>
<td>10/650</td>
<td>0.011-10</td>
<td>0.9954</td>
<td>0.011</td>
<td>1.0</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>DB[ε,l]P</td>
<td>288/388.6</td>
<td>10/150</td>
<td>0.015-10</td>
<td>0.9996</td>
<td>0.015</td>
<td>1.2</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Solutions for SLE were prepared mixing PAH stock solutions in methanol with Nanopure water (20:80 v/v methanol-water). Working solutions in octane were prepared by appropriate dilution of stock solutions in octane. <sup>b</sup> Excitation ($\lambda_{exc}$) and emission ($\lambda_{em}$) wavelengths. <sup>c</sup> Optimum gate delay ($t_d$) and gate width ($t_g$). <sup>d</sup> LDR, linear dynamic range estimated from the limit of detection to the upper linear concentration. <sup>e</sup> R, correlation coefficient of LDR. <sup>f</sup> LOD, limit of detection; see text for calculation. SLE was performed with 100 mL of sample. <sup>g</sup> Relative standard deviation based upon six replicate measurements of medium concentration of LDR.

### 4.3.4 Direct Analysis of DP Isomers in Synthetic Mixtures

The feasibility to determine each DP in the presence of the other four isomers was tested with five synthetic mixtures prepared in Nanopure water. Each mixture was prepared with the target isomer at a concentration 100 × lower than the concentrations of the other isomers. Figure 4.3 shows the fluorescence spectra of the five mixtures. Each spectrum corresponds to the first spectrum of the 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 4.3 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) λ_{exc} = 323 nm, t_{d}/t_{g} = 10/250 ns; (B) λ_{exc} = 310 nm, t_{d}/t_{g} = 10/150 ns; (C) λ_{exc} = 316.5 nm, t_{d}/t_{g} = 0/15 ns; (D) λ_{exc} = 304 nm, t_{d}/t_{g} = 10/650 ns; (E) λ_{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 4.3 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; α = 0.05) shows no synergistic effects and confirms unambiguous isomer determination without previous separation.

Table 4.3 Lifetime analysis of DB isomers on extraction membranes spiked with 100 µL of octane

<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>

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

### 4.3.5 Direct analysis of DP Isomers in Contaminated Water Samples

The feasibility to monitor heavily contaminated waters was demonstrated with two sample matrixes of unknown composition, namely, Red River water and the output water stream of the API separator at a North Dakota refinery. Red River water of the North Basin (Minnesota, North Dakota, and South Dakota) was collected at different locations within the Fargo-Moorhead area. Previous GC/MS analysis of this water showed a high content of dissolved organic matter. The API water was collected from a sampling valve on the output pipe of the API separator. API water is used to desalt and scrub crude oil and oil fractions in the refinery process and shows a visible surface petroleum layer at the separator trough. Previous HPLC-LETRSS analysis of this water has shown heavy contamination with fluorescent pollutants, including several EPA PAHs. The complexity of this sample is illustrated in Figure 4.4A, which shows a 4.2 K excitation-emission matrix (EEM) collected within the wavelength ranges of DB[a,l]P.
The delay (10 ns) and the gate (1000 ns) were selected to collect a substantial portion of the total fluorescence of the sample. The low-intensity features throughout most of the EEM indicate extensive fluorescence background from several concomitants in the sample. Figure 4.4B shows the 4.2 K EEM of API water spiked with DB[a,l]P at the 0.5 ng·mL⁻¹ level. Because the delay (10 ns) and the gate (250 ns) were optimized to enhance the spectral features of DB[a,l]P, the fluorescence background disappears and the presence of DB[a,l]P is clearly noted. Similar EEM were observed for the other isomers.
Figure 4.4 EEM of API water and DB[a,l]P spiked API water from the membrane

(A) 4.2 K EEM of API water recorded from extraction membrane spiked with 100 µL of octane. Delay and gate times were 10 and 1000 ns, respectively. (B) 4.2 K EEM of API water spiked with 5 ng·mL⁻¹ DB[a,l]P prior to SLE. EEM was recorded from extraction membrane spiked with 100 µL of octane. Delay and gate times were 10 and 150 ns, respectively. In both cases, spectra were accumulated over 100 laser shots at each excitation wavelength using increments of 0.2 nm. Spectrograph slits were 40 µm.
Table 4.4 summaries the recoveries obtained for the five DP isomers spiked in the two sample matrixes (Red River and API water). Spectral purity for each isomer was confirmed via fluorescence decay analysis at its target fluorescence peak. Single exponential decays with fluorescence lifetime values equivalent to those in Table 3 were obtained in all cases. Quantitative analysis was made via the calibration curve method. All the recoveries were statistically equivalent to 100% (N = 3, \( \alpha = 3 \))\(^5\), which demonstrates satisfactory accuracy of analysis. It is important to note that the calculated recoveries account for the extraction efficiency of the method (see Table 4.1). Relative standard deviations were lower than 3%, which shows excellent precision of measurements at the sub-parts-per-billion concentration level (0.1 ng·mL\(^{-1}\)).

Table 4.4 Recovery of DP isomers from API water

<table>
<thead>
<tr>
<th>PAH(^a)</th>
<th>Recovery(^b) (%)</th>
<th>(t_{\text{exp}})(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB([a, l])P</td>
<td>103.0±2.0</td>
<td>2.59</td>
</tr>
<tr>
<td>DB([a, e])P</td>
<td>96.0±2.7</td>
<td>2.56</td>
</tr>
<tr>
<td>DB([a, h])P</td>
<td>98.0±1.6</td>
<td>2.17</td>
</tr>
<tr>
<td>DB([a, i])P</td>
<td>103.4±2.1</td>
<td>2.80</td>
</tr>
<tr>
<td>DB([e, l])P</td>
<td>104.8±2.8</td>
<td>2.97</td>
</tr>
</tbody>
</table>

\(^{a}\) Measurements done using instrumental parameters (\(\lambda_{\text{exc}}/\lambda_{\text{em}}\); \(t_d/t_g\)) shown in Table 4.2. \(^{b}\) Recoveries were obtained through the calibration curve method. Standard deviations (sx0) include propagation of errors within the linear dynamic range of the calibration curve. The formulas employed for their calculation are as follows:\(^4\) \(sx_0 = (sx_0/b)(1 + 1/n + (y^0 - y)^2/b^2\sum(x_i - x_i^2)^{1/2}\), where \(b\) is the slope of the calibration curve, \(x\) is the mean value of the standard concentrations (\(x_i\)), \(y\) is the mean value of the standard intensities (\(y_i\)), \(y^0\) is the intensity of the unknown, \(n\) is the number of data points (\(x_i\), \(y_i\)) used in the calibration graph, and \(sy/x = \{\sum(y_i - y)^2/(n - 2)\}^{1/2}\); where \(y_i\) are the intensity values corresponding to the \(x_i\) concentrations calculated through the regression line. \(^{c}\) \(t_{\text{exp}}\) was calculated as \(t = (x - \mu)n^{1/2}/s\); where \(t\) corresponds to the statistical comparison between the experimental recovery (\(x_0\)) and the concentration of isomer (\(i\)) spiked in the water sample. \(s\) is the standard deviation of the Gaussian population. Critical \(t\) value is 3.18 (\(n = 3, \alpha = 0.05\))\(^4\).
4.4 Conclusions

We have developed a direct method of analysis for DB[a,l]P and its four DP isomers in water samples. The pollutants are determined on the surface of a solid-liquid extraction membrane with no further separation from the sample matrix. Once extracted on the membrane, DP isomers partition into the layer of spiked octane to provide highly resolved spectra and distinct lifetime values for unambiguous isomer determination. Highly reproducible spectra and lifetime values are easily obtained with that aid of the fiber-optic probe. The analytical figures of merit demonstrate the existence of a linear relationship between the isomer concentration in the water sample and the fluorescence signal from the probe. The slope of the calibration curve depends on experimental parameters that can be easily adjusted to reach appropriate LOD. The values reported here are at the parts-per-trillion level, but no attempts were made for their optimization. The accuracy of analysis via the calibration curve method was tested with two real-world samples of unknown but complex composition. The recovery values were excellent, showing the quantitative potential of our approach. The entire procedures from water extraction to LETRSS analysis takes less than 15 min/sample and it consumes only 100 µL of organic solvent. This fact makes our approach environmentally friendly and cost-effective.
5 CHAPTER: SONICAION-LASER EXCITED TIME-RESOLVED SHPOL ’SKII SPECTROMETRY: A FACILE METHOD FOR THE DIRECT DETERMINATION OF FIFTEEN PRIORIT POLLUTANTS IN SOIL SAMPLES

5.1 Introduction

Particularly challenging is the analysis of PAHs in soil samples. Soil is one of the most important reservoirs for PAH, which are deposited in the gaseous state or associated to particles even at sites far from industry. Extraction methods include methanolic saponification, sonication, Soxhlet extraction, supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE). Pre-concentration and clean-up steps are necessary for all these techniques except for SFE. Its ability to accommodate on-line clean up steps reduces contamination risks and/or PAH loss. SFE also presents the advantages of low solvent consumption (~10mL per sample) and short extraction time (~ 1.2hr per sample). The same is true for ASE. Unfortunately, instrumental and operational costs are quite high for both techniques.

It is within this context that screening methodology becomes relevant. Immunoassay and similar screening techniques provide semi-quantitative information on total PAH content but lack the necessary specificity and sensitivity for individual components. This is a major drawback if one considers that carcinogenic properties strongly depend on molecular structure and differ significantly from PAH to PAH. It is important to determine the most toxic PAH even if they are present at much lower concentrations than their less toxic concomitants.
This chapter presents screening methodology with significant advances in all fronts. Our approach is based on LETRSS and its well-known capability to directly determine PAH in complex environmental extracts. The complications of fluorescence methodology at liquid helium temperature (4.2K) are avoided with the aid of a cryogenic FOP. Unambiguous PAH determination is accomplished via WTM data formats which combine spectral and lifetime information. Soil samples are sonicated into the vial of the FOP with 250μL of n-octane. This solvent serves the dual purpose of PAH extraction and Shpol’skii matrix. PAH partition into the layer of extracting solvent to provide highly resolved spectra and characteristic fluorescence lifetimes. The sensitivity and selectivity of LETRSS eliminate the need for sample pre-concentration and clean up steps, as well as the need for PAH separation previous to detection. Because sample handling is limited to weighing the soil sample into the probe vial contamination risks and/or PAH loss is kept to a minimum. The entire experimental procedure – including sonication (~ 30min) and LETRSS measurements (~ 8min) - takes less than 40min per sample. The method is well-suited for routine analysis of numerous samples as the small volumes of the sample vessel (0.75mL) and the organic solvent (0.25mL) facilitate the implementation of simultaneous sample extraction. The analytical performance of the screening method is here compared to methanolic saponification, sonication and HPLC for the analysis of two reference materials.

5.2 Experimental

5.2.1 Chemicals

All solvents were HPLC grade and purchased from Fisher. Unless otherwise noted, Nanopure water - from a Barnstead Nanopure Infinity water system - was used throughout. All
chemicals – including those used for PAH extraction - were analytical reagent grade and used without further purification. Soil samples of known composition – Natural Matrix Reference Material CRM 104-100 and CRM 105-100 – were acquired from Resource Technology Corporation, Laramie, WY. A Supelco PAH mixture (EPA 610) in methanol:methyl chloride (1:1 v/v) from Supelco was used as the reference standard for HPLC analysis. PAH standards for LETRSS analysis were purchased from Aldrich at their highest available purity. Rhodamine 6G was acquired from Exciton and used with the tunable dye laser according to specifications.

5.2.2 PAH Analysis via Screening Method

A complete description of the instrumentation for 4.2K LETRSS analysis has been reported previously. Figure 5.1 illustrates the extraction steps of the screening method. 0.0025g (CRM 105-100) or 0.05g (CRM 104-100) of soil sample was mixed with 250μL of n-octane in the vessel of the FOP. The mixture was sonicated at room-temperature for 30min in a sonication bath (Branson, Model 3210). After 5 minutes of settling time, the sample vial was coupled to the copper tubing of the FOP keeping the tip of the fiber assembly ~ 0.5cm above the surface of the octane layer. Sample freezing was carried out by lowering the copper tubing into the liquid helium held in a Dewar flask with 60-L storage capacity. 60L of liquid helium would typically last for 3 weeks of daily use, averaging 15-20 samples per day. Complete sample freezing took less than 90s. Because no physical contact was made between the probe and the sample, probe clean up between measurements was not necessary.
5.2.3 *PAH Extraction via Established Methodology*

Methanolic Saponification was performed as follows: 20g (CRM 104-100) or 0.5g (CRM 105-100) of dried soil were mixed with 100mL of 2M KOH/CH₃OH and heated at 70°C for 2h. After cooling, the mixture was filtered with a 9.0 cm GF/C glass micro-fiber filter (Whatman International, UK) and the methanol phase extracted three times with 50mL of hexane. The non-polar phase was concentrated to approximately 1-2mL and submitted to sample clean up.⁸⁴

Sonication was performed as follows: 20g (CRM 104-100) or 0.5g (CRM 105-100) of dried soil were mixed in an Erlenmeyer flask with 100mL of hexane:acetone:toluene (10:5:1 v/v/v). The mixture was sonicated at room-temperature in a sonication bath (Branson, Model
3210) for 30min with occasional swirling to prevent sticking on the bottom of the flask. The mixture was filtered with a 9.0 cm GF/C glass micro-fiber filter (Whatman International, UK), concentrated to approximately 1-2mL and submitted to sample clean up.

Prior to loading the extracts onto the solid-phase extraction silica gel cartridges (Supelco 6mL LC-18 SLE cartridges), small amounts of Na$_2$SO$_4$ were added to the cartridges to protect the silica gel surface. Each cartridge was preconditioned twice with 5mL of hexane and the extracts were loaded by means of a Pasteur pipette. PAH elution was carried out with a total hexane volume of 10mL.

5.2.4 HPLC Analysis

It was performed using a computer driven HPLC system from Hitachi equipped with the following basic components: a pump model L-7100, a UV (model L-7400 UV) and a fluorescence (model L-7485) detector, and an online degasser (model L-761). A Supelco Supelcosil TM LC-PAH column (15cm length, 4mm diameter and 5μm particle size) provided adequate resolution of the 16 EPA-PAH. The mobile phase was a mixture of methanol/water. Column conditions include a 1.5mL/min flow rate, isocratic elution with 40/60 water methanol for 5min and then linear gradient to 99% methanol over 20min. The total separation time was approximately 40min. All sample injections were at a volume of 20μL using a fixed volume injection loop. Laboratory reagent blanks were run in conjunction with each series of samples using identical conditions of glassware, equipment, solvents and analysis to ensure the absence of interfering contamination. PAH detection was carried out in the fluorescence mode using time programmed excitation/emission wavelengths: 0min, 220/330nm (naphthalene); 7 min, 225/315nm (acenaphthene and fluorene); 10.1min, 244/370nm (phenanthrene and anthracene); 13min,
237/460nm (fluoranthene); 14.3min, 237/385nm (pyrene); 16min, 277/376nm (benzo[a]anthracene and chrysene); 21.2min, 255/420nm (benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[a]pyrene); 25.5min, 300/415nm (dibenzo[a,h]anthracene and benzo[g,h,i]perylene; 27.5min, 250/495nm (indeno[1,2,3-cd]pyrene). For the detection of individual PAH, the selected wavelengths corresponded to the maximum excitation and emission wavelengths. In cases were PAH elution was too fast for individual wavelength optimization, the selected wavelengths corresponded to a compromise among the maximum excitation and emission wavelengths of the eluted PAH.

5.3 Results and Discussion

Our choice for methanolic saponification as one of the established methods of extraction was based on its relatively high PAH extraction efficiency. Saponification under methanolic conditions breaks down polymeric structures of organic matter – which is frequently present in soil samples and chemically associated to PAH - and increases the accessibility of the solvent for PAH extraction. The sonication method was selected because of its short extraction time and easy implementation. We should mention, however, that even after careful optimization of extraction parameters (solvent composition and extraction time), PAH recoveries with sonication tend to be lower than with other extraction techniques. The solvent mixture we chose - hexane:acetone:toluene (10:5:1 v/v/v) – was reported to yield comparatively high extraction efficiencies. Soil extracts were then analyzed via established HPLC methodology and 4.2K LETRSS. The obtained data was then used to evaluate the analytical performance of the screening method.
5.3.1 PAH Recoveries via HPLC Analysis

The concentration ranges of EPA-PAH in the two reference materials varied from 0.66mg/Kg to 24.6mg/Kg (CRM 104-100) and from 15.7mg/Kg to 1,410mg/Kg (CRM 105-100). Assuming 100% PAH extraction efficiencies via sonication and methanolic saponification, the weighed mass of soil would yield analytical samples with EPA-PAH concentrations at the parts-per-million level (μg.mL⁻¹). Table 5.1 correlates the HPLC parameters for their determination via fluorescence detection. With the exception of acenaphthylene, all the other PAH showed strong fluorescence at room temperature. Their LOD are at the parts-per-billion level (ng.mL⁻¹) and, therefore, well below the EPA-PAH concentration ranges of the soil samples. Figure 5.2 depicts typical chromatograms of the two types of soil samples. Clearly, the selectivity of the method is appropriate for the problem at hand as complete resolution of 15 EPA-PAH is achieved with both types of soil.
Table 5.1 Chromatographic parameters for the determination of EPA-PAH via HPLC analysis

<table>
<thead>
<tr>
<th>PAH</th>
<th>Retention time (min)</th>
<th>$\lambda_{\text{exc}}/\lambda_{\text{em}}$ (nm)</th>
<th>LOD (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>7.5</td>
<td>240/330</td>
<td>42</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>14.8</td>
<td>296/325</td>
<td>27</td>
</tr>
<tr>
<td>Fluorene</td>
<td>15.5</td>
<td>258/404</td>
<td>52</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>17.0</td>
<td>258/404</td>
<td>54</td>
</tr>
<tr>
<td>Anthracene</td>
<td>19.4</td>
<td>258/404</td>
<td>99</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>20.8</td>
<td>278/395</td>
<td>87</td>
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<tr>
<td>Pyrene</td>
<td>22.5</td>
<td>278/395</td>
<td>6</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>26.8</td>
<td>278/395</td>
<td>48</td>
</tr>
<tr>
<td>Chrysene</td>
<td>27.9</td>
<td>278/395</td>
<td>7</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>32.1</td>
<td>363/412</td>
<td>20</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>32.9</td>
<td>363/412</td>
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<td>49</td>
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<td>Benzo[g,h,i]perylene</td>
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<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>38.2</td>
<td>308/467</td>
<td>36</td>
</tr>
</tbody>
</table>

$^a$ Sample volume is 20 $\mu$L. $^b$ Average retention time of three chromatographic runs. Column conditions include a 1.5mL/min flow rate, isocratic elution with 60:40 water methanol for 5min and then linear gradient to 99% methanol over 20min. $^c$ Excitation and emission wavelength for fluorescence detection. $^d$ Limits of detection (LOD) are based on signal-to-noise ratios of 3.
Figure 5.2 Chromatograms obtained from extracts of reference standard materials CRM 104-100 (Panel A) and CRM 105-100 (Panel B).

Identified PAH are the following: (1) naphthalene, (2) acenaphthene, (3) fluorene, (4) phenanthrene, (5) anthracene, (6) fluoranthene, (7) pyrene, benzo[a]anthracene (8), chrysene (9), benzo[b]fluoranthene (10), benzo[k]fluoranthene (11), benzo[a]pyrene (12), dibenzo[a,h]anthracene (13), benzo[g,h,i]perylene (14) and indeno[1,2,3-cd]pyrene.
Error! Reference source not found. reports the recoveries obtained via methanolic saponification and sonication. As expected, the highest recoveries were obtained with methanolic saponification. Interesting to note are the following: (i) all recoveries were lower than 100%; (ii) comparison between soil samples reveals cases with different recoveries for the same PAH and method; and (iii) relative standard deviations appear to depend not only on the soil sample but also on the PAH. These facts can be attributed to several factors, including inefficient PAH extraction, matrix interference and uncontrolled PAH loss during sample preparation.

Table 5.2 PAH recoveries obtained with methanolic saponification, sonication and HPLC analysis

<table>
<thead>
<tr>
<th>PAH</th>
<th>CRM 104-100</th>
<th></th>
<th>CRM 105-100</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saponification (%)</td>
<td>Sonication (%)</td>
<td>Saponification (%)</td>
<td>Sonication (%)</td>
</tr>
<tr>
<td>Napthalene</td>
<td>80.23 ± 5.23</td>
<td>44.39 ± 5.56</td>
<td>70.45 ± 2.65</td>
<td>64.58 ± 4.25</td>
</tr>
<tr>
<td>Acenapthene</td>
<td>78.58 ± 2.56</td>
<td>37.89 ± 2.54</td>
<td>75.29 ± 2.60</td>
<td>66.32 ± 5.38</td>
</tr>
<tr>
<td>Fluorene</td>
<td>70.12 ± 6.28</td>
<td>35.24 ± 4.71</td>
<td>72.49 ± 7.87</td>
<td>20.23 ± 1.25</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>80.75 ± 3.05</td>
<td>52.49 ± 5.62</td>
<td>68.78 ± 2.36</td>
<td>53.25 ± 1.21</td>
</tr>
<tr>
<td>Anthracene</td>
<td>72.69 ± 3.58</td>
<td>35.68 ± 3.25</td>
<td>60.25 ± 6.25</td>
<td>50.23 ± 2.53</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>64.35 ± 1.57</td>
<td>24.12 ± 1.17</td>
<td>71.46 ± 9.04</td>
<td>40.28 ± 6.21</td>
</tr>
<tr>
<td>Pyrene</td>
<td>74.85 ± 3.56</td>
<td>31.20 ± 2.46</td>
<td>68.42 ± 0.73</td>
<td>40.23 ± 5.26</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>68.42 ± 5.45</td>
<td>31.63 ± 0.74</td>
<td>73.43 ± 7.87</td>
<td>40.88 ± 1.25</td>
</tr>
<tr>
<td>Chrysene</td>
<td>65.02 ± 3.65</td>
<td>45.90 ± 0.68</td>
<td>83.57 ± 3.15</td>
<td>52.32 ± 5.02</td>
</tr>
<tr>
<td>Benzo[bc]fluoranthene</td>
<td>83.73 ± 3.61</td>
<td>49.81 ± 2.51</td>
<td>79.93 ± 5.36</td>
<td>50.55 ± 4.25</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>75.02 ± 4.03</td>
<td>59.55 ± 2.45</td>
<td>76.42 ± 2.38</td>
<td>57.00 ± 2.36</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>89.53 ± 3.43</td>
<td>69.38 ± 3.48</td>
<td>79.29 ± 1.89</td>
<td>59.25 ± 5.27</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>64.25 ± 5.23</td>
<td>44.25 ± 3.33</td>
<td>62.58 ± 4.25</td>
<td>41.95 ± 5.23</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>75.85 ± 6.19</td>
<td>56.71 ± 2.47</td>
<td>70.25 ± 1.75</td>
<td>52.14 ± 3.65</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>52.51 ± 2.49</td>
<td>46.41 ± 5.05</td>
<td>66.43 ± 5.12</td>
<td>45.87 ± 4.65</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.
5.3.2 **PAH Recoveries via 4.2K LETRSS Analysis**

A criteria often employed for solvent selection in Shpol’skii Spectroscopy matches the length of the solvent molecule to the effective length of the PAH\(^{19}\). One of five organic solvents best fits the linear dimensions of EPA-PAH: 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, benzo[\(a\)]anthracene), and n-nonane (indeno[\(1,2,3-cd\)]pyrene and benzo[\(k\)]fluoranthene). Determining each compound under optimum Shpol’skii conditions would require performing soil extraction with five organic solvents per sample or carrying out five extraction steps after each sample extraction. Considering the two options unsuited for the purpose at hand, we carried out the determination of the 16 pollutants in n-octane. Our preference for n-octane was based on preliminary studies showing better PAH extraction efficiencies than those obtained with the other four solvents.

Table 5.3 lists the 4.2K LETRSS parameters for the determination of the 16 EPA-PAH in n-octane. With the exception of acenaphthylene, all the other PAH showed 4.2K fluorescence emission in n-octane. At present, we have no explanation for acenaphthylene behavior. The lack of fluorescence persisted after sample de-oxygenation (15 min of nitrogen bubbling prior to fluorescence measurements). Similarly, no fluorescence was observed by freezing the analyte in n-pentane, n-hexane, n-heptane or n-nonane. Each PAH showed well resolved Shpol’skii spectra with several excitation and fluorescence peaks between 200 and 500 nm. Careful examination of maximum excitation and emission wavelengths (\(\lambda_{\text{exc}}/\lambda_{\text{em}}\)) shows no spectral overlapping among maximum excitation and emission peaks. Selective excitation of each PAH should then provide the best LOD for the method. However, sample excitation from 291.4nm (naphthalene) to
362.6nm (anthracene) requires operating the tunable dye laser with more than one laser dye. Considering simplicity and speed of analysis two of the main objectives of this work, we attempted the determination of the 15EPA-PAH with only one excitation wavelength (283.2nm). The worst fluorescence overlapping was observed for naphthalene and acenaphthene. The remaining PAH emitted at least one fluorescence peak with no spectral interference from the other priority pollutants. Their wavelengths are reported in Table 5.2 under target wavelengths (λ\text{target}). Determination of naphthalene and acenaphthene was performed at their maximum fluorescence peaks as no spectral overlapping from the remaining PAH occurs. Their spectral discrimination was accomplished on the bases of time resolution with appropriate delay and gate times (see Figure 5.3). In all cases, peak purity check was carried out via WTM analysis. Single exponential decays and matching lifetimes to those of the standards guaranteed unambiguous PAH determination at each target wavelength.
Table 5.3 PAH recoveries obtained with methanolic saponification, sonication and HPLC analysis

<table>
<thead>
<tr>
<th>PAH</th>
<th>CRM 104-100</th>
<th></th>
<th>CRM 105-100</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saponification (%)</td>
<td>Sonication (%)</td>
<td>Saponification (%)</td>
<td>Sonication (%)</td>
</tr>
<tr>
<td>Napthalene</td>
<td>80.23 ± 5.23</td>
<td>44.39 ± 5.56</td>
<td>70.45 ± 2.65</td>
<td>64.58 ± 4.25</td>
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<tr>
<td>Acenapthene</td>
<td>78.58 ± 2.56</td>
<td>37.89 ± 2.54</td>
<td>75.29 ± 2.60</td>
<td>66.32 ± 5.38</td>
</tr>
<tr>
<td>Fluorene</td>
<td>70.12 ± 6.28</td>
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<td>72.49 ± 7.87</td>
<td>20.23 ± 1.25</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>80.75 ± 3.05</td>
<td>52.49 ± 5.62</td>
<td>68.78 ± 2.36</td>
<td>53.25 ± 1.21</td>
</tr>
<tr>
<td>Anthracene</td>
<td>72.69 ± 3.58</td>
<td>35.68 ± 3.25</td>
<td>60.25 ± 6.25</td>
<td>50.23 ± 2.53</td>
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<tr>
<td>Fluoranthene</td>
<td>64.35 ± 1.57</td>
<td>24.12 ± 1.17</td>
<td>71.46 ± 9.04</td>
<td>40.28 ± 6.21</td>
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<tr>
<td>Pyrene</td>
<td>74.85 ± 3.56</td>
<td>31.20 ± 2.46</td>
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<td>40.23 ± 5.26</td>
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<td>Benzo[a]anthracene</td>
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<td>31.63 ± 0.74</td>
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<tr>
<td>Chrysene</td>
<td>65.02 ± 3.65</td>
<td>45.90 ± 0.68</td>
<td>83.57 ± 3.15</td>
<td>52.32 ± 5.02</td>
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<tr>
<td>Benzo[b]fluoranthene</td>
<td>83.73 ± 3.61</td>
<td>49.81 ± 2.51</td>
<td>79.93 ± 5.36</td>
<td>50.55 ± 4.25</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>75.02 ± 4.03</td>
<td>59.55 ± 2.45</td>
<td>76.42 ± 2.38</td>
<td>57.00 ± 2.36</td>
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<tr>
<td>Benzo[a]pyrene</td>
<td>89.53 ± 3.43</td>
<td>69.38 ± 3.48</td>
<td>79.29 ± 1.89</td>
<td>59.25 ± 5.27</td>
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<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>64.25 ± 5.23</td>
<td>44.25 ± 3.33</td>
<td>62.58 ± 4.25</td>
<td>41.95 ± 5.23</td>
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<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>75.85 ± 6.19</td>
<td>56.71 ± 2.47</td>
<td>70.25 ± 1.75</td>
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<tr>
<td>Benzo[g,h,i]perylene</td>
<td>52.51 ± 2.49</td>
<td>46.41 ± 5.05</td>
<td>66.43 ± 5.12</td>
<td>45.87 ± 4.65</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.
Figure 5.3 4.2 K fluorescence spectrum recorded from 250 µL of a synthetic EPA-PAH mixture (Supelco, EPA 610).

The analytical sample was prepared by diluting the original sample 100x with n-octane. EPA-PAH concentrations in the analytical sample were at the low mg.mL⁻¹ concentration level. 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

Instrumental parameters for LETRSS analysis are shown in Error! Reference source not found.. Portions of spectra within dashed vertical lines represent magnifications of original spectrum. Compounds II and III were determined using delay/gate times equal to 200/1000ns and 0/40ns, respectively.

The LOD with LETRSS are approximately one order of magnitude better than those obtained with HPLC. Although fluorescence detection was carried out with different solvents
and instrumental set ups, comparatively better LOD were expected with LETRSS as lowering the temperature usually increases PAH fluorescence quantum yields. This is a significant advantage for the screening method as no pre-concentration steps are performed. Depending on the original PAH concentration in the soil sample, the screening extraction procedure yields analytical samples with PAH concentrations at the sub-parts-per-million level. These concentrations are measurable via HPLC but their relatively low signal intensities provide unacceptable precisions for analytical work.

Table 5.4 reports the recoveries obtained via methanolic saponification and sonication. Keeping in mind that each analytical sample was analyzed by both HPLC and LETRSS, comparison of Error! Reference source not found. and Table 5.4 reveals no particular trend in their ability to provide either lower or higher recoveries. The obtained values are in good agreement and provide PAH concentrations within the confidence interval of the standards.
Table 5.4 PAH recoveries\textsuperscript{a} obtained with methanolic saponification, sonication and 4.2K LETRSS analysis\textsuperscript{b}

<table>
<thead>
<tr>
<th>PAH</th>
<th>CRM 104-100</th>
<th>CRM 105-100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saponification (%)</td>
<td>Sonication (%)</td>
</tr>
<tr>
<td>Napthalene</td>
<td>90.28 ± 6.08</td>
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<tr>
<td>Acenaphthene</td>
<td>81.16 ± 7.33</td>
<td>55.26 ± 6.25</td>
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<tr>
<td>Fluorene</td>
<td>74.35 ± 8.07</td>
<td>42.15 ± 3.75</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>78.01 ± 8.10</td>
<td>39.41 ± 3.25</td>
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<td>Anthracene</td>
<td>73.25 ± 5.23</td>
<td>48.68 ± 8.51</td>
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<td>Fluoranthe</td>
<td>44.81 ± 2.60</td>
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<td>Pyrene</td>
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<td>31.28 ± 2.01</td>
</tr>
<tr>
<td>Chrysene</td>
<td>61.94 ± 4.71</td>
<td>43.92 ± 5.81</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>78.23 ± 4.24</td>
<td>50.77 ± 3.32</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>69.03 ± 1.11</td>
<td>61.13 ± 1.04</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>80.45 ± 3.51</td>
<td>59.98 ± 0.98</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>48.40 ± 4.47</td>
<td>44.28 ± 2.53</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>82.19 ± 3.58</td>
<td>57.94 ± 7.00</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>51.84 ± 3.29</td>
<td>37.68 ± 3.00</td>
</tr>
</tbody>
</table>

\textsuperscript{a} PAH recoveries were calculated according to the equation: Recovery (%) = \left(\frac{\text{mass of PAH in extract}}{\text{mass of PAH in soil sample}}\right) \times 100; where mass of PAH in extract (ng) = C_{PAH\ in\ EXTRACT} (ng/mL) \times 0.25mL and mass of PAH in soil sample (ng) = C_{PAH\ in\ SOIL} (ng/g) \times \text{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. \textsuperscript{b} LETRSS parameters for PAH determinations are shown in Table 5.2.

5.3.3 \textit{PAH Recoveries via the screening Method}

Figure 5.4 shows examples of Shpol’kskii spectra generated with the screening method. The 15 EPA-PAH are clearly identified in both types of soils with no apparent matrix interference. Peak purity was confirmed via WTM analysis, which yielded single exponential decays and matching fluorescence lifetimes to those of the standards. PAH recoveries are reported in Table 5.5. Their values are consistent with the sonication values reported in Error!
The compromise we made for simplicity of analysis did not affect PAH determination. Using the same solvent (n-octane) for PAH extraction and LETRSS analysis leads to acceptable PAH recoveries with minimum sample handling. PAH partition into the layer of extracting solvent to provide highly resolved spectra and distinct lifetime values for unambiguous compound determination. Highly reproducible spectra and lifetime values are easily obtained from the layer of extracting solvent with the aid of the FOP. The entire LETRSS analysis takes less than 10min per sample and it consumes 250μL of organic solvent. These facts make our approach environmentally friendly and cost-effective for routine analysis of numerous samples. As individual PAH are present in soils at the ng.g⁻¹ level, the direct determination of ng 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.
Figure 5.4 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]fluoranthen, XI = benzo[a]pyrene, XII = fluoreanthene, XIII = benzo[g,h,i]perylene, XIV = benzo[k]fluorenthene, XV = indeno[1,2,3-cd]pyrene.

Instrumental parameters for LETRSS analysis are shown in Table 2. 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.
Table 5.5 PAH recoveries\(^a\) 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>Recovery (%)</td>
<td>PAH Mass(^b) (ng)</td>
</tr>
<tr>
<td>Napthalene</td>
<td>44.01 ± 5.07</td>
<td>16.9 ± 2.0</td>
</tr>
<tr>
<td>Acenapthene</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[(1,2,3-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 (%) = \(\frac{\text{mass of PAH in extract} \times 100}{\text{mass of PAH in soil sample}}\), where mass of PAH in extract (ng) = \(C_{\text{PAH in extract}} \times \frac{0.25\text{mL}}{0.25\text{mL}}\), and mass of PAH in soil sample (ng) = \(C_{\text{PAH in soil}} \times \frac{\text{weight of soil sample (g)}}{\text{weight of soil sample (g)}}\). The PAH concentration in the soil extract \(C_{\text{PAH in extract}}\) was determined via multiple standard additions. The PAH concentration in soil \(C_{\text{PAH in soil}}\) is the certified PAH concentration of the reference standard. Reported values are an average of three soil extractions. LETRSS parameters for PAH determinations are shown in Table 5.2.\(^b\) Mass of PAH in soil extract. It was calculated multiplying the PAH concentration in the soil extract by the extract volume (0.25mL). PAH concentration was determined according to multiple standard additions.

5.4 Conclusions

A unique method for monitoring PAH in soil samples is reported. Milligrams of soil are sonicated into the vial of a cryogenic fiber optic probe with 250 µL of n-octane. Pollutants partition into the layer of extracting solvent and the cryogenic probe is then used for fluorescence measurements at liquid helium temperature (4.2K). N-octane provides PAH with the appropriate Shpol’skii matrix to record highly resolved spectra and characteristic fluorescence lifetimes.
Unambiguous PAH determination is directly performed from the layer of extracting solvent with no need for previous PAH separation. The total analysis time from the extraction to PAH identification is approximately 35 min per sample. Limits of detection are at the sub-parts per billion levels. The simplicity of the experimental procedure, the short analysis time, the selectivity and the excellent analytical figures of merit demonstrate the advantages of this approach for routine analysis of soil samples.
6 CHAPTER: SOLID-LIQUID EXTRACTION FLUORESCENCE LINE NARROWING SLECTROSCOPY: A DEIRECT APPROACH TO THE ANALYSIS OF FLUOROQUINOLONES IN WATER SAMPLES

6.1 Introduction

An emerging interest exists among the scientific community on investigating the impact the environmental presence of antibiotics may have on human health. Although maximum residue levels for antibiotics in environmental waters have not been established yet, both the European Agency for the Evaluation of Medicinal Products (EMEA) and the Food and Drug Administration (FDA) of the United States require environmental risk assessment. The way in which antibiotics reach the environment depends on the specific use of each compound. In the case of antibiotics used to treat human diseases, their spread to surface waters appears to occur as a result of inappropriate treatment of waste waters. In fact, it has been reported that the actual treatment procedures are not able to completely remove antibiotics from waste waters. The main source of contamination related to antibiotics in veterinarian medicine has been reported to be the use of animal excretions for manure. Apparently, antibiotics are directly spread to the soil, and after rainfall, migrate to ground and surface water. Fluoroquinolones (FQ) are one of the most powerful classes of antibiotics currently used for the treatment of urinary tract infections. Their widespread use in both human and animal medicine has prompted their appearance in aquatic systems. Their presence has been reported in the output of a treatment water plant, hospital waste water, and surface waters.

The search for a universal method capable to face this new environmental challenge has been centered on HPLC. SLE has been the method of choice for sample clean-up and pre-
concentration. Depending on the FQ and its concentration level, successful determination has been accomplished with mass spectrometry, RTF or UV absorption spectrometry. Unfortunately, no single detection mode has shown the ability to detect all FQ at the concentration ratios found in environmental waters. A similar statement can be made with regards to the composition of the mobile phase, which might require individual optimization of ionic strength, acidity and modifiers for complete resolution of chromatographic peaks. The second most popular approach is capillary electrophoresis (CE). Careful optimization of buffer pH and composition, flow rate and temperature provides adequate separation and determination of various FQ. Particularly challenging to this technique is the separation of norfloxacin, ciprofloxacin and sarafloxacin. Their separation usually causes overlapping with other FQ present in the sample, as it is the case with sarafloxacin and marbofloxacin.

The strong fluorescence of FQ provides RTF spectrometry with appropriate sensitivity for the problem at hand. Concentration levels of FQ in environmental waters are rather low (~ng.L⁻¹). The main disadvantage of this technique is the lack of selectivity. RTF spectra lack fingerprint information for FQ identification. The broad nature of excitation and fluorescence spectra forces complete compound separation prior to detection. In this article, we present a solution to the problem based on FLNS. FLNS deals with cryogenic temperatures where samples are frozen to 10K or below. 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 S₁-S₀. 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 fluoresce line-narrowed spectrum is observed. Because of its matrix versatility and its high selectivity, FLNS has been used as a stand alone technique or coupled to separation techniques such as thin-layer chromatography, HPLC and CE. In this work, we present the first application of FLNS towards the analysis of FQ. We provide a novel method with excellent selectivity and sensitivity via a simple experimental procedure with no need for previous FQ separation.

6.2 Experimental

6.2.1 Chemicals

All chemicals were analytical-reagent grade and used without further purification. Nanopure water was used throughout. Enoxacin (ENO), ofloxacine (OFL), norfloxacin (NOR), oxolinic acid (OXO), cinoxacin (CIN) and sarafloxacin (SAR) were purchased from Sigma. Ciprofloxacin (CIP) was purchased from MP Biomedicals, LLC. HPLC grade methanol and ethanol were obtained from Fisher Scientific.

6.2.2 4.2K Fluorescence Line Narrowing Spectroscopy Analysis

Measurements were made with the aid of an instrumental setup previously described. The dye laser was operated on Exalite 376 (369 -381nm), Stilbene 420 (415-435nm) or Coumarin 460 (443 – 485nm) laser dyes (Exciton), and it was pumped with the third harmonic of a 10-Hz Hd:YAG Q-switched solid-state laser (Big Sky Laser Technologies)
6.2.3 Solution Preparation

Standard solutions for calibration curves were prepared from stock solutions in methanol. SLE was performed with standard solutions (methanol/water 20:80 v/v) prepared by mixing 1mL of stock solution of appropriate concentration with 70mL of Nanopure water, 10mL of buffer solution (Fisher) and 19mL of methanol. FQ mixtures were also prepared in methanol-water 20:80 v/v. With the exception of CIN, which showed best extraction efficiency at pH = 6, all the other FQ were extracted at pH = 4. Otherwise noted, all extractions were made with 100mL of aqueous samples. All measurements were performed from extraction membranes previously spiked with 100μL of ethanol.

6.2.4 Recovery studies in Contaminated Water Samples

Recovery studies were performed with water collected from an animal feeding area of a Honey Bee Farm located at 10 miles east to Orlando, Florida. The water sample was collected in a glass bottle with a PTFE-lined cap and stored at 4 °C. Analytical samples were prepared by mixing 1mL of a 10μg.mL⁻¹ stock solution of FQ with 70mL of water sample, 10mL of buffer solution and 19mL methanol. All measurements were performed from extraction membranes previously spiked with 100μL of ethanol.

6.2.5 HPLC Analysis

HPLC analysis was performed using a Hitachi HPLC system equipped with a model L-7100 gradient pump, an L-7400 UV detector, an L-7485 fluorescence detector, and a D-7000 control interface. A Supelco Supercosil LC-PAH column (15 cm length, 4mm inner diameter and 5μm particle sizes) was used. All sample injections were at a volume of 20μL using a fixed-
volume injection loop. All solvents were Aldrich HPLC grade. Nanopure water was used throughout.

6.3 Results and Discussion

6.3.1 Initial survey of Excitation and Fluorescence Spectra at Room Temperature

The molecular structures of the studied FQ are shown in Figure 6.1. Their spectral characteristics were investigated in water, methanol and ethanol. Figure 6.2 depicts the excitation and emission spectra of the seven FQ in ethanol. Spectra were recorded with a commercial spectrofluorimeter using the narrowest excitation and emission slit widths. Clearly, the strong overlapping of the naturally broad excitation and fluorescence spectra prevents unambiguous direct determination of individual FQ. Similar FWHM were observed for each compound in the two other solvents but, because all the FQ showed the highest fluorescence intensity in ethanol, we chose this solvent for all further studies.
Figure 6.1 Molecular structure of enoxacin (ENO), ofloxacin (OFL), norfloxacin (NOR), oxolinic acid (OXO), cinoxacin (CIN), sarafloxacin (SAR) and ciprofloxacin (CIP).
Figure 6.2 RT excitation (---) and emission spectra (—) of QAs

ENO (red, $\lambda_{\text{exc}} = 293$ nm, $\lambda_{\text{em}} = 380$nm), NOR (purple, $\lambda_{\text{exc}} = 336$ nm, $\lambda_{\text{em}} = 413$ nm), OFL (black, $\lambda_{\text{exc}} = 292$ nm, $\lambda_{\text{em}} = 470$nm), OXL (blue, $\lambda_{\text{exc}} = 336$ nm, $\lambda_{\text{em}} = 430$nm), CIN (dark yellow, $\lambda_{\text{exc}} = 380$ nm, $\lambda_{\text{em}} = 431$nm), SAR (orange, $\lambda_{\text{exc}} = 331$ nm, $\lambda_{\text{em}} = 422$nm) and CIP (green, $\lambda_{\text{exc}} = 336$ nm, $\lambda_{\text{em}} = 416$nm). All solutions were prepared in pure water at 1 µg mL$^{-1}$. All spectra were recorded with a commercial spectrofluorimeter. $\lambda_{\text{exc}}$ and $\lambda_{\text{em}}$ refer to the excitation and the emission wavelengths used to record emission and excitation spectra, respectively. Excitation and emission band-pass were 0.18nm.

6.3.2 Achieving Spectral Narrowing of FQ under FLN Conditions

Figure 6.3 shows the effect of lowering the temperature to 4.2K on the fluorescence spectra of two FQ. These spectra were recorded with the cryogenic fiber optic probe and the laser system. Sample excitation was performed at the maximum excitation wavelength of each compound. Because thermal broadening at 4.2K is almost none, the broad bands still observed can be mainly attributed to inhomogeneous broadening. Broad bands were also observed for the other FQ. Figure 6.4A shows the drastic spectral narrowing observed in the 4.2K fluorescence.
spectrum of SAR as the excitation wavelength approximates to the maximum fluorescence wavelength (422nm) of the compound. The slit width of the spectrograph was kept to a minimum (40μm) to assure best spectral resolution. Because similar narrowing was observed for the other FQ (see Figure 6.4B), FLNS offers a direct way to spectrally resolve the seven compounds without previous separation.

**Figure 6.3** RT (---) and 4.2K (—) fluorescence spectrum of NOR and SAR

NOR (purple, $\lambda_{\text{exc}} = 336.2$ nm, $t_g/t_d = 0/20$ ns) and SAR (orange), $\lambda_{\text{exc}} = 331.3$ nm, $t_g/t_d = 0/20$ ns
Figure 6.4 (A) 4.2K fluorescence spectrum of SAR at various excitation wavelengths (B) 4.2K FLN spectra of FQ recorded from ethanol solutions. 331.2nm (black), 410.0nm (red), 418.0nm (blue) and 420.8nm (green). (B) 4.2K FLN spectra of FQ recorded from ethanol solutions: ENO (red, $\lambda_{exc} = 378.2$ nm, $t_g/t_d = 0/30$ ns), NOR (purple, $\lambda_{exc} = 411.0$ nm, $t_g/t_d = 0/20$ ns), OFL (black, $\lambda_{exc} = 466.9$ nm, $t_g/t_d = 0/20$ ns), OXL (blue, $\lambda_{exc} = 428.0$ nm, $t_g/t_d = 0/30$ ns), CIN (dark yellow, $\lambda_{exc} = 429.6$ nm, $t_g/t_d = 0/20$ ns), SAR (orange, $\lambda_{exc} = 420.8$ nm, $t_g/t_d = 0/20$ ns) and CIP (green, $\lambda_{exc} = 414.5$ nm, $t_g/t_d = 0/20$ ns). In all cases, spectrograph slits were 40 $\mu$m. All spectra correspond to the accumulation of 100 laser pulses.
Table 6.1 summaries the FLN analytical figures of merit (AFOM) of the seven FQ obtained in ethanol solutions. Their relatively short fluorescence lifetimes imposed a zero delay for fluorescence collection after the excitation pulse. Major contribution of laser scatter to fluorescence FLN spectra was then avoided by placing the collection window of the ICCD away from the excitation wavelength. The gate of the ICCD was sufficiently long to collect most of the fluorescence decay of the FQ and short enough to avoid instrumental noise. All the fluorescence decays were single exponential decays. The agreement between the calculated and observed points over the first four lifetimes of the decays was approximately 1% and the residuals showed no systematic trends. The fluorescence intensities plotted in the calibration graphs were the average of at least three individual measurements taken from three frozen aliquots. The linear dynamic ranges (LDR) of the calibration curves were based on at least five FQ concentrations. No efforts were made to experimentally determine the upper concentration limit of the LDR. All the correlation coefficients and the slopes of the log-log-plots (data not shown) were close in unity, demonstrating a linear relationship between FQ concentrations and FLN intensity. Similarly, the precision of measurements was excellent with relative standard deviations at medium linear concentrations not higher than 5% (N = 6). LODs were calculated as 3S_{B}/m, where S_{B} is the standard deviation from 16 blank (ethanol) determinations and m is the slope of the calibration curve. The LOD at the sub-parts-per-million level shows the need for a pre-concentration step to reach realistic concentration levels (ng.mL^{-1} to pg.mL^{-1}) of FQ in environmental waters.
Table 6.1: Analytical figures of merit of seven FQ in ethanol obtained via FLNS

<table>
<thead>
<tr>
<th>FQ</th>
<th>$\lambda_{\text{exc}}/\lambda_{\text{em}}$ (nm)</th>
<th>$t_{\text{g}}/t_{\text{d}}$ (ns)</th>
<th>$\tau$ (ns)</th>
<th>LDR (µg/ml)</th>
<th>$R_f$</th>
<th>LOD (µg/ml)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENO</td>
<td>378.2/380.1</td>
<td>0/30</td>
<td>7.24±0.18</td>
<td>0.35-20</td>
<td>0.9898</td>
<td>0.35</td>
<td>3.3</td>
</tr>
<tr>
<td>NOR</td>
<td>411.0/412.7</td>
<td>0/20</td>
<td>3.61±0.09</td>
<td>0.22-20</td>
<td>0.9958</td>
<td>0.22</td>
<td>4.1</td>
</tr>
<tr>
<td>OFL</td>
<td>466.9/469.0</td>
<td>0/20</td>
<td>2.75±0.04</td>
<td>0.18-20</td>
<td>0.9911</td>
<td>0.18</td>
<td>2.8</td>
</tr>
<tr>
<td>OXO</td>
<td>428.0/429.4</td>
<td>0/30</td>
<td>9.05±0.26</td>
<td>0.27-20</td>
<td>0.9988</td>
<td>0.27</td>
<td>3.2</td>
</tr>
<tr>
<td>CIN</td>
<td>429.6/431.3</td>
<td>0/20</td>
<td>3.60±0.14</td>
<td>0.15-20</td>
<td>0.9920</td>
<td>0.15</td>
<td>2.5</td>
</tr>
<tr>
<td>SAR</td>
<td>420.8/421.8</td>
<td>0/20</td>
<td>3.47±0.08</td>
<td>0.51-50</td>
<td>0.9915</td>
<td>0.51</td>
<td>5.0</td>
</tr>
<tr>
<td>CIP</td>
<td>414.5/416.0</td>
<td>0/20</td>
<td>3.60±0.04</td>
<td>0.98-50</td>
<td>0.9901</td>
<td>0.98</td>
<td>3.6</td>
</tr>
</tbody>
</table>

a Standard solutions were prepared in ethanol at 1 µg.mL⁻¹; b $\lambda_{\text{exc}}/\lambda_{\text{em}}$ = excitation and emission wavelengths; c $t_{\text{g}}/t_{\text{d}}$ = optimum delay and gate time; d $\tau$ = Average lifetime taken from three frozen aliquots; e LDR = linear dynamic range estimated from the limit of detection to the upper linear concentration; f $R_f$ = correlation coefficient of LDR; g LOD = limit of detection. See text for calculations; h Relative standard deviation based upon six replicate measurements at medium linear concentrations. RSD were calculated with the formula RSD = [x/S]100; where x is the average fluorescence intensity of six measurements and S is the standard deviation of the average intensity.

6.3.3 Solid-Liquid Extraction Fluorescence Line-Narrowing Spectroscopy of Fluoroquinolones

Previous work in our group has shown the practical advantages of combining SLE to high-resolution luminescence approach. The pollutants are extracted from the water sample with a small disk that fits into the vessel of the fiber optic probe. The membrane is spiked with microliters of organic solvent, the pollutants partition into the layer of spiked solvent providing highly resolved spectra and distinct lifetime values for unambiguous compound determination. The concentration of pollutant in the water sample ($C_{\text{H}_2\text{O}}$) is related to its concentration in the layer of organic solvent ($C_{\text{SOL}}$) through the following equation: 71

$$C_{\text{H}_2\text{O}} = (V_{\text{SOL}}/f_1V_{\text{H}_2\text{O}} + K_dV_{\text{SLE}}/f_1V_{\text{H}_2\text{O}})C_{\text{SOL}}$$ (6.1)
where $f_j$ is the analyte fraction extracted from the water simple, $K_d$ is the distribution coefficient of the analyte between the membrane and the spiked solvent, and $V_{SOL}$, $V_{SLE}$, and $V_{H2O}$ are the volumes of spiked solvent, extraction membrane and water, respectively.

Table 6.2 shows the extraction efficiencies of FQ obtained with octadecyl (C$_{18}$) silica membranes. C$_{18}$ was chosen as the bonded phase based on previous studies reporting adequate retention of FQ from water samples.\textsuperscript{97,98,99} In our case, the extraction efficiency of 5.5mm disks was calculated by comparing the RTF intensities of aqueous solutions before and after extraction. With the exception of CIN, which showed the best extraction efficiency at pH = 6, all the other FQ were better extracted at pH = 4. It is important to note that the extraction efficiencies did not change throughout the entire LDR of the RTF calibration curves (data not shown) and that the upper concentrations of the LDR did not exceed the breakthrough volume of the extraction system.

**Table 6.2 SLE efficiency of FQs.**

<table>
<thead>
<tr>
<th>FQ$^a$</th>
<th>pH Value$^b$</th>
<th>Retention$^c$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENO</td>
<td>4</td>
<td>88.5±1.4</td>
</tr>
<tr>
<td>NOR</td>
<td>4</td>
<td>94.3±0.6</td>
</tr>
<tr>
<td>OFL</td>
<td>4</td>
<td>82.8±0.2</td>
</tr>
<tr>
<td>OXO</td>
<td>4</td>
<td>92.1±1.9</td>
</tr>
<tr>
<td>CIN</td>
<td>6</td>
<td>74.0±1.5</td>
</tr>
<tr>
<td>SAR</td>
<td>4</td>
<td>84.2±2.8</td>
</tr>
<tr>
<td>CIP</td>
<td>4</td>
<td>82.0±1.5</td>
</tr>
</tbody>
</table>

$^a$10ml of 1 µg.mL$^{-1}$ standard solutions were employed for extraction; $^b$pH adjusted with appropriate buffer solution; $^c$Extraction efficiency represents the average of individual RTF measurements taken from six membranes at the maximum excitation and emission wavelength of each FQ.

Table 6.3 summaries the SLE-FLNS AFOM obtained for the seven FQ. Comparison of maximum excitation and emission wavelengths to those in ethanol (Table 1) shows no significant difference. The same is true for fluorescence lifetimes. Within a confidence interval
of 95% (α = 0.05), the experimental lifetime averages of three replicate measurements from each
type of sample (N₁ = N₂ = 3) were statistically equivalent (t_{exp} < critical value of t = 2.57).

The single exponential decays observed in the presence of the membranes also suggest that only one
type of microenvironment surrounds FQ molecules. These are strong indications that the
presence of the membrane has no measurable effect on the spectral and lifetime characteristics of
FQ. Apparently, most of the signal collected with the fiber optic probe belongs to the FQ
partitioning into the ethanol layer. These facts confirm previous observations with other organic
pollutants. Because the presence of the membrane does not deteriorate spectral resolution and
the fluorescence decays follow well-behaved single-exponential decays with distinct lifetime
values, the direct determination of the seven FQ on the bases of spectral and lifetime analysis is
feasible.

### Table 6.3 Analytical figures of merit of FQ obtained via SLE-FLNS

<table>
<thead>
<tr>
<th>FQ^a</th>
<th>λ_{exc}/λ_{em}^b (nm)</th>
<th>t_{d}/t_{g}^c (ns)</th>
<th>τ^d (ns)</th>
<th>LDR^e (µg/ml)</th>
<th>R^f</th>
<th>LOD^g (ng/ml)</th>
<th>RSD^h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENO</td>
<td>378.2/380.1</td>
<td>0/30</td>
<td>7.27±0.10</td>
<td>0.045-2</td>
<td>0.9910</td>
<td>45</td>
<td>4.0</td>
</tr>
<tr>
<td>NOR</td>
<td>411.0/412.7</td>
<td>0/20</td>
<td>3.52±0.07</td>
<td>0.033-1</td>
<td>0.9925</td>
<td>33</td>
<td>1.9</td>
</tr>
<tr>
<td>OFL</td>
<td>466.9/469.0</td>
<td>0/20</td>
<td>2.69±0.02</td>
<td>0.020-1</td>
<td>0.9898</td>
<td>20</td>
<td>2.2</td>
</tr>
<tr>
<td>OXO</td>
<td>428.0/429.4</td>
<td>0/30</td>
<td>9.16±0.12</td>
<td>0.027-1</td>
<td>0.9875</td>
<td>27</td>
<td>2.8</td>
</tr>
<tr>
<td>CIN</td>
<td>429.6/431.3</td>
<td>0/20</td>
<td>3.55±0.05</td>
<td>0.025-1</td>
<td>0.9955</td>
<td>25</td>
<td>3.2</td>
</tr>
<tr>
<td>SAR</td>
<td>420.8/421.8</td>
<td>0/20</td>
<td>3.51±0.10</td>
<td>0.040-2</td>
<td>0.9932</td>
<td>40</td>
<td>2.7</td>
</tr>
<tr>
<td>CIP</td>
<td>414.5/416.0</td>
<td>0/20</td>
<td>3.64±0.02</td>
<td>0.047-2</td>
<td>0.9908</td>
<td>47</td>
<td>5.2</td>
</tr>
</tbody>
</table>

^a FQ solutions were prepared in 10ml of methanol/water(v/v 80%/20%). pH was adjusted to optimum value
using appropriate buffer; ^b λ_{exc} / λ_{em} = excitation and emission wavelengths; ^c t_{d}, t_{g} = optimum delay and gate
time; ^d τ = Average lifetime taken from three frozen aliquots; ^e LDR = linear dynamic range estimated from the
limit of detection to the upper linear concentration; ^f R = correlation coefficient of LDR; ^g LOD = limit of
detection. See text for calculations; ^h Relative standard deviation based upon six replicate measurements at
medium linear concentrations.

The correlation coefficients in Table 6.3 demonstrate the existence of a linear relationship
between C\textsubscript{H\textsubscript{2}O} and the fluorescence signal of the probe. Comparison of LOD values to those in
Table 6.1 shows improvements varying from 6x (CIN) to 21x (CIP). In the case of SLE-FLNS, the standard deviations of blank signals for LOD calculations were measured from 16 disks extracted with 10mL of blank aqueous solution and spiked with 100μL of ethanol. The differences among LOD improvements can be mainly attributed to the partition ratios FQ have between the extraction membrane and the ethanol layer (see \( K_d \) in equation 6.1). In all cases, however, further LOD improvements would be possible by extracting larger water volumes \( (V_{H2O}) \). The pre-concentration step prior to FLNS makes parts-per-trillion detection a straightforward procedure.

6.3.4 Direct Analysis of FQ in Synthetic Mixtures

The feasibility to determine each FQ in the presence of the other six was tested with a synthetic mixture containing each FQ at the 0.1ppm concentration. Figure 6.5 shows the FLN spectra of the mixture recorded upon tuning the excitation wavelength from 375nm to 500nm. Sample excitation at 378.2nm provides a narrow peak at 380.1nm and a broad band of lower intensity from 390-500nm. The narrow peak belongs to ENO while the featureless fluorescence band sums up the contribution of the other FQ in the sample. As the tunable dye laser is scanned towards 411nm, the fluorescence spectrum of the mixture reveals a narrow maximum peak corresponding to NOR, i.e. the FQ with a 0-0 transition wavelength (412.7nm) slightly longer than the excitation wavelength. The broad fluorescence band disappears from the spectrum as a result of lower excitation energy. The peak with lower intensity corresponds to CIP, i.e. the FQ with 0-0 transition wavelength at 416nm. As the excitation wavelength surpasses 412.7nm, the NOR peak disappears from the spectrum. Excitation at 414.5nm provides three narrow peaks corresponding to the 0-0 transitions of CIP (416.0nm), SAR (421.8nm) and OXO (429.4nm).
The maximum intensity at 416.0nm reflects the proximity of the excitation wavelength to the 0-0 transition of CIP. The trend repeats itself in increasing wavelength order. In all cases, baseline resolution is obtained making quantitative analysis of components in the mixture a straightforward procedure.

![Spectra graph]

**Figure 6.5** 4.2K FLN spectra recorded from a synthetic mixture of seven FQ at the 0.1 μg mL⁻¹ concentration level.

Spectra were collected from an extraction membrane previously used to extract 10 mL of the synthetic mixture. The membrane was spike with 100 μL of ethanol. Spectra were collected at the following excitation wavelengths and delay and gate times, from bottom to top: (ENO) λ<sub>exc</sub> = 378.2 nm, t<sub>e</sub>/t<sub>d</sub> = 0/30 ns; (NOR) λ<sub>exc</sub> = 411.0 nm, t<sub>e</sub>/t<sub>d</sub> = 0/20 ns; (CIP) λ<sub>exc</sub> = 414.5 nm, t<sub>e</sub>/t<sub>d</sub> = 0/20 ns; (SAR) λ<sub>exc</sub> = 420.8 nm, t<sub>e</sub>/t<sub>d</sub> = 0/20 ns; (OXO) λ<sub>exc</sub> = 428.0 nm, t<sub>e</sub>/t<sub>d</sub> = 0/30 ns; (CIN) λ<sub>exc</sub> = 429.6 nm, t<sub>e</sub>/t<sub>d</sub> = 0/20 ns; and (OFL) λ<sub>exc</sub> = 466.9 nm, t<sub>e</sub>/t<sub>d</sub> = 0/20 ns; In all cases, spectrograph slit was 40 μm.
Table 6.4 compares the fluorescence lifetimes stripped from the mixture to those recorded from pure standard solutions. The lifetimes were stripped from the WTM at the target wavelengths of each FQ. Single exponential decays were observed in all cases demonstrating spectral purity at target wavelengths. The statistical equivalence of fluorescence lifetimes \(N_1 = N_2 = 6; \alpha = 6\) shows no synergistic effects and confirms unambiguous FQ determination without previous separation. Additional confirmation on spectral peak purity was obtained from intensity measurements. Intensity ratios of fluorescence signals recorded from standards and mixture were all close in unity showing that no inner filter effects deteriorate the accuracy of analysis at these concentration levels.

<table>
<thead>
<tr>
<th>FQ</th>
<th>(\tau_S^b) (ns)</th>
<th>(\tau_M^b) (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENO</td>
<td>7.27±0.10</td>
<td>7.41±0.12</td>
</tr>
<tr>
<td>NOR</td>
<td>3.52±0.07</td>
<td>3.60±0.05</td>
</tr>
<tr>
<td>OFL</td>
<td>2.69±0.02</td>
<td>2.60±0.04</td>
</tr>
<tr>
<td>OXO</td>
<td>9.16±0.12</td>
<td>9.03±0.15</td>
</tr>
<tr>
<td>CIN</td>
<td>3.55±0.05</td>
<td>3.53±0.07</td>
</tr>
<tr>
<td>SAR</td>
<td>3.51±0.10</td>
<td>3.50±0.03</td>
</tr>
<tr>
<td>CIP</td>
<td>3.64±0.02</td>
<td>3.59±0.05</td>
</tr>
</tbody>
</table>

\(a\) All samples for fluorescence lifetimes consisted of octadecyl membranes previously spiked with 100μL of ethanol. SLE was performed with 10ml of aqueous solution (standard or mixture) at the appropriate pH for best extraction efficiency; \(b\) \(\tau_S\) and \(\tau_M\) = fluorescence lifetime of FQ in the standard and the mixture, respectively. Each lifetime value corresponds to the average of six replicate measurements taken from six frozen aliquots. Measurements were done at the same excitation and emission wavelengths shown in Table 6.3.
6.3.5 Direct Analysis of FQ in Heavily Contaminated Water Samples

The feasibility to monitor FQ in heavily contaminated waters was tested with a sample of unknown matrix composition. The sample was collected from an animal feeding area of a Honey Bee Farm located at 10 miles east of Orlando, Florida. The complexity of the matrix is illustrated in Figure 6.6 and Figure 6.7. Although no attempts were made to separate and identify all the components in the unknown, its chromatogram Figure 6.6 shows 39 peaks corresponding to at least 39 compounds with absorption at 256nm. Some of those components have strong fluorescence emission. Figure 6.7A compares the RTF spectrum of the unknown to the one recorded from the same sample previously spiked with SAR. Sample excitation was performed at 336nm, the maximum excitation wavelength of SAR. The delay (0ns) and the gate (1000ns) were selected to collect a substantial portion of the total fluorescence of the sample. The broad fluorescence band results from the contribution of all fluorescence compounds in the sample. Their strong spectral overlapping masks the presence of SAR in the spiked sample. Figure 6.7B shows the spectra of the two samples under FLN conditions - i.e. analysis at 4.2K, sample excitation at 420.8nm, delay time = 0 ns and gate time = 20ns. The spectral interference is eliminated and the narrow peak characteristic to SAR’s FLN spectrum appears at 421.8nm. A single exponential decay with fluorescence lifetime equivalent to that in Table 4 confirms peak assignment and the spectral purity of the peak. Quantitative analysis is then possible via the calibration curve method. Equivalent results were obtained for the remaining FQ.

Table 6.5 summaries the recoveries obtained for the seven target compounds in the spiked sample. All the recoveries were statistically equivalent to 100% (N = 3, α = 3)\(^48\), which
demonstrates satisfactory accuracy of analysis. Relative standard deviations were lower than 4%, which shows excellent precision of measurements at the parts-per-billion concentration level.

Figure 6.6 HPLC chromatogram showing the complexity of a water sample of unknown composition. Peak identification was not attempted.
Figure 6.7 RTF and FLN spectra from extraction membranes of unknown sample and unknown sample spiked with SAR

(A) RTF spectra recorded from extraction membranes previously used to extract 10mL of unknown sample (- --) and 10mL of unknown sample spiked with a 1μg/mL-1 SAR aqueous solution (—). Sample excitation = 336nm; td/tg = 0/1000ns. (B) 4.2K FLN spectra recorded from extraction membranes previously used to extract 10mL of unknown sample (---) and 10mL of unknown sample spiked with a 1μg/mL-1 SAR aqueous solution (—). Sample excitation = 420.8nm; td/tg = 0/20ns. Spectrograph slit was 40μm.
Table 6.5 Recovery of FQ via SLE-FLNS.

<table>
<thead>
<tr>
<th>FQa</th>
<th>Recoveryb (%)</th>
<th>texpc</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENO</td>
<td>99.3±1.2</td>
<td>1.01</td>
</tr>
<tr>
<td>NOR</td>
<td>95.7±3.6</td>
<td>2.07</td>
</tr>
<tr>
<td>OFL</td>
<td>103.5±2.0</td>
<td>3.03</td>
</tr>
<tr>
<td>OXO</td>
<td>102.1±2.9</td>
<td>1.25</td>
</tr>
<tr>
<td>CIN</td>
<td>97.2±1.7</td>
<td>2.85</td>
</tr>
<tr>
<td>SAR</td>
<td>101.9±2.2</td>
<td>1.50</td>
</tr>
<tr>
<td>CIP</td>
<td>102.5±2.1</td>
<td>2.06</td>
</tr>
</tbody>
</table>

a All measurements were done using the instrumental parameters (λexc/λem; td/tg) shown in Table 3; b Recoveries were obtained through the calibration curve method. Standard deviations (sx0) include propagation of errors within the linear dynamic range of the calibration curve. The formulas employed for their calculation are as follows81. Sx0 = (sx0/ys) {1 + 1/n + (y0 - ybar) / b2Σ(xi - xbar)2}1/2; where b is the slope of the calibration curve, xbar is the mean value of the standard concentrations (xi), ybar is the mean value of the standard intensity (yi), y0 is the intensity of the unknown, n is the number of data points (x,y) used in the calibration graph, and sx0 = {Σ(yi - ybar)2 / (n - 2)}1/2; where yi are the intensity values corresponding to the xi concentrations calculated through the regression line. c texp was calculated as t = (x0 - μ) n1/2 / s; experimental recovery (x0) and the concentration of quinolones (μ) spiked in the water sample. s is the standard deviation of the Gaussian population. Critical t value is 3.18 (n = 3, α = 0.05).

6.4 Conclusions

We have developed an attractive method of analysis for seven FQ in water samples. The pollutants are directly determined on the surface of a SLE membrane with no further separation from the sample matrix. Once extracted on the membrane, FQ partition into the layer of spiked ethanol to provide characteristic FLN spectra and distinct lifetime values for unambiguous compound determination. Highly reproducible spectra and lifetime values are easily obtained with the aid of the cryogenic fiber optic probe, tunable dye laser, spectrograph and ICCD. Tuning the excitation wavelength to the 0-0 transition of each FQ provides base line resolution of FLN spectra for direct quantitative analysis via the calibration curve method.

The analytical figures of merit demonstrate the existence of a linear relationship between FQ concentration in the water sample and the FLN signal from the probe. The slope of the
calibration curve depends on experimental parameters easily adjusted to reach lower LOD. The reported LOD values were obtained with 10mL of water sample, but larger volumes of water are certainly possible with no significant cost in analysis time. The excellent recoveries obtained from spiked samples of unknown – but complex -composition demonstrate the accuracy of analysis for real-world samples. The entire procedure, from water extraction to FLNS, takes less than 15min/sample and it consumes only 100 μL of ethanol. This fact makes our approach cost effective and environmentally friendly.
7 CHAPTER: INSTRUMENTATION FOR MULTIDIMENSIONAL LUMINESCENCE SLECTROSCOPY

7.1 Introduction

Research in our group has focused on exploring the full potential of SS with a significant selectivity enhancement based on information-rich multidimensional data formats. We have shown that the combination of a pulsed tunable dye laser, a pulsed delay generator, a spectrograph, and an intensifier-charged coupled device (ICCD) is well suited for the rapid collection of wavelength-time matrixes (WTMs), excitation-emission matrixes (EEMs), and time-resolved excitation emission matrixes (TREEMs) in the fluorescence time domain (ns to µs). Adding the temporal dimension to the highly resolved Shpol'skii spectra provides an extremely selective tool for the determination of structural isomers in complex matrixes with numerous PAHs. Unambiguous isomer identification is made possible on the bases of spectral and lifetime analysis. Fluorescence decays report on spectral peak purity, an important parameter for accurate quantitative analysis.

During the course of our experiments, we noticed the significant contribution of phosphorescence to the steady-state emission spectra of numerous PAHs, polychlorinated biphenyls, polychlorinated dibenzofurans, and polychlorinated dioxins imbedded in Shpol'skii matrixes. New instrumentation was then developed to handle the relatively long phosphorescence lifetimes (ms-s) at 77 and 4.2 K. The rather large lifetime differences observed from compounds within the same polycyclic aromatic class and the excellent analytical
figures of merit provided a solid foundation for including phosphorescence data formats in the multidimensional analyses in complex samples.

In this chapter, we present a single instrument with the capability to collect multidimensional data formats in both the fluorescence and the phosphorescence time domains. We also demonstrate the ability to perform luminescence measurements in highly scattering media by comparing the precision of measurements in Shpol'skii solvents to those obtained in "snowlike" matrixes and solid samples. For decades, conventional low-temperature methodology has been restricted to optically transparent media. This restriction has limited its application to organic solvents that freeze into a glass. In this article, we remove this limitation with the use of cryogenic fiber-optic probes.

7.2 Experimental

7.2.1 Chemicals and Solution Preparation

All chemicals were analytical-reagent grade and used without further purification. Otherwise noted, nanopure water was used throughout. PAHs were purchased from Aldrich at their highest available purity. HPLC grade solvents (Sigma-Aldrich) were used to prepare stock PAHs solutions. Working solutions were prepared by diluting the stock solution with the appropriate solvent (HPLC grade) prior to luminescence measurements. Rhodamine 6G, DCM, LDS 698, and LDS 759 were purchased from Exciton and used with the tunable dye laser according to specifications. Their tuning ranges were 275-290 nm, 300-340 nm, 330-370 nm, and 350-395 nm, respectively.
7.2.2 Survey of Excitation and Emission Spectra at RT and 77K

(See chapter 3 section 3.22)

7.2.3 Instrumentation for Multidimensional Shpol’skii Spectroscopy

The instrumentation for multidimensional luminescence spectroscopy (MLS) is shown in Figure 7.1. The major difference to the fluorescence system previously reported\textsuperscript{26} is the integration of a mechanical shutter to facilitate phosphorescence spectra and lifetime acquisition. The mechanical shutter (Oriel) is positioned in the path of the dye laser excitation beam prior to launch into the FOP. The shutter has a rise time (open time) of 1.5 ms and fall time (close time) of 3.0 ms with a maximum pulse width of 6.5 ms. The shutter is controlled either manually (always open or always closed) via a switch on the controller front panel or programmatically through a transistor-transient logic (TTL) pulse. This TTL pulse is programmed through the A + B outputs of the digital delay generator (Model DG535, Stanford Research Systems, Inc.) via a GPIB (general purpose interface bus) interface from National Instruments.
7.2.4 **Collection of Fluorescence Spectra and WTM\(s\) with the MLS**

Fluorescence measurements are made on the nanosecond time scale with the mechanical shutter in the open position. Time-resolution is achieved with the intensifier in front of the ICCD, which acts as a super-fast shutter with a minimum gate of 2 ns (full width at half maximum). Figure 7.2 shows the sequence of events leading to the collection of fluorescence spectra. Once triggered by the laser, the pulse delay generator uses this information to determine when the image intensifier in the detector head is gated on (gate delay = \(D\)) and for how long it is gated on (gate width = \(G\)). These parameters are entered on the control computer with Andor software.
The CCD acquires data while the intensifier is gated on. While the intensifier is gated off, the acquired data is transferred from the detector head to the controller card (32-bit Intelligent Bus-Mastering PCI card) in the computer. The use of a faster computer will certainly decrease DTS time. Otherwise noted, fluorescence spectra result from the accumulation of emission of 100 laser pulses. This process takes approximately 10ms per 40nm spectrum. If a wavelength range larger than 40nm is of interest, the spectrograph is tuned to the new wavelength range and the process is repeated.

### 7.2.5 Collection of Phosphorescence Spectra with the MLS

Depending on the strength and the duration of the phosphorescence emission, spectra are collected by one of two procedures. For PAHs that generate enough phosphorescence photons per laser pulse and have relatively short lifetimes (less than 40 ms), the use of the mechanical shutter is not necessary. The sequence of events is the same as the one used for collecting fluorescence spectra, but the delay and gate times are set at a much longer time scale. In our studies, typical delay and gate times were 20 μs and 40 ms, respectively. The delay time was long enough to eliminate sample and background fluorescence and the gate time was the maximum gate allowed for each firing of the laser. Our YAG laser fired every 100 ms (10 Hz) and, because approximately 50 ms were needed between shots for data transfer and storage, gate times longer than 40 ms would result in convolution of the collected data with the next laser shot.

The mechanical shutter comes on handy for PAHs that exhibit weak phosphorescence intensity and relatively long phosphorescence lifetimes. The pulse delay generator controls the timing for both the shutter and the intensifier on the ICCD. During the excitation cycle, the pulse generator is triggered by the laser to set the shutter to the open position for a certain number of
pulses (typically 20 pulses). During the emission cycle, the shutter is closed and the phosphorescence decay is recorded. The closing of the shutter sets the "zero reference time" for the delay time and the gate time on the ICCD. This method allows one to build up the triplet-state population to an acceptable signal-to-noise ratio. It also allows for gate times in excess of 40 ms to be used without concern of overlapping laser pulses.

7.2.6 Collection of Fluorescence and Phosphorescence WTM

Figure 7.2 depicts the series of events leading to fluorescence WTM collection. The duration of the steps by which the gate delay is progressively increased in the course of the sequence of acquisitions (gate step = DI - DI-1, where I = 2, ..., N) is entered on the control computer with Andor software. For collecting phosphorescence WTM from PAHs with strong phosphorescence and relatively short phosphorescence lifetimes (less than 40 ms), the sequence of events is the same but D and G are set at much longer time scales (ms). Figure 7.3 shows the sequence of events for collecting WTM from PAHs with weak phosphorescence and relatively long phosphorescence lifetimes (longer than 40 ms). Instead of using the laser pulse as the "zero reference time", the series of phosphorescence spectra is taken at incremental delay times from the closing of the shutter. For relatively long phosphorescence lifetimes, this method results in very long acquisition times, typically 1 h or more per WTM. The alternative method is depicted in Figure 7.4. It involves exciting the PAH for several seconds to build up the triplet-state population, closing the shutter, and then collecting the time-resolve emission over several seconds. The resulting WTM consists of many spectra with a delay step equal to the firing rate of the laser (100 ms). Since each spectrum is an accumulation of only one laser shot, many WTM (typically 100) need to be accumulated to average out the effects of noise. This averaging is, in
effect, similar to accumulating 100 laser shots at each delay increment, as in fluorescence WTM collection. Because the delay is eliminated from each acquisition cycle, the time it takes to collect a full WTM is considerably reduced. Collecting 100 WTM s over a total period of 5 s each takes ~9 min.

Figure 7.2 Sequence of events leading to WTM collection of fluorescence

P. laser pulse; D, delay time; DTS, data transfer and storage; TBP. Time between pulses.
Figure 7.3 Sequence of events leading to WTM collection of weak phosphorescence and lifetimes shorter than 40 ms.

P. laser pulse; D, delay time; DTS, data transfer and storage; TBP. Time between pulses.
Figure 7.4 Sequence of events leading to WTM collection of weak phosphorescence and lifetimes longer than 40 ms.

P. laser pulse; D, delay time; DTS, data transfer and storage; TBP. Time between pulses.

7.2.7 Fluorescence and Phosphorescence Lifetimes.

Fluorescence and phosphorescence lifetimes were obtained via a three-step procedure: (1) full sample and background WTMs collection; (2) Background decay curve subtraction from the fluorescence or phosphorescence decay curve at a wavelength of maximum emission for each PAH; and (3) fitting of the background corrected data to single exponential decays. Origin software (version 5, Micronal Software, Inc.) was used for curve fitting of fluorescence and phosphorescence lifetimes. Fitted decay curves \( y = y_0 + A_1 \exp^{-\frac{(x-x_0)\gamma}{\gamma}} \) were obtained by fixing \( x_0 \) and \( y_0 \) at a value of zero.
7.2.8 **Software for Phosphorescence Measurements.**

The Andor software was unable to handle the very long delay and gate times required for data acquisition. We were able to collect individual spectra with relatively long delay and gate times, but the software failed when we attempted to collect phosphorescence WTM. We circumvented these problems by authoring custom acquisition software with LabView (National Instruments, version 6.0).

7.2.9 **Sample Procedures**

*77K Measurements of Liquid Solutions with the Spectrofluorimeter.* Measurements followed the classic procedure of immersing the sample solution in a quartz tube into a nitrogen-filled Dewar flask.

*77K and 4.2K Measurements of Liquid Solutions with the FOP.* After a known volume of solution was pipetted into the sample vial (typically 100 µL), the tip of the probe was positioned and held constant with the screw cap below the solution surface. The dimensions of the vial were as follows: 30mm length, 5.5mm inner diameter and 7mm outer diameter. Its maximum volume capacity was 750 µL. Sample freezing was accomplished by lowering the copper tubing into the liquid cryogen. The liquid nitrogen and liquid helium were held in two separate Dewars with 5L and 60 L storage capacity, respectively. The 60L liquid helium would typically last three weeks of daily use, averaging 15-20 samples per day. At both temperatures, complete sample freezing takes less than 90 s.

The approximately one-minute probe clean up procedure involved removing the sample vial from the cryogen container, melting the frozen matrix and warming the resulting solution to approximately room temperature with a heat gun, rinsing the probe with n-alkane, and drying it
with warm air from the heat gun. The entire freeze, thaw, and cleanup cycle took no longer than 5 minutes.

_77K and 4.2K Measurements from Extraction Membranes with the FOP._ The 47mm SLE membranes were dissected into many 5.6mm disks using a #3 cork borer. A 5.6 mm membrane was placed into the stainless steel filter syringe kit (Alltech, IL) and attached to a 10 mL syringe (Hamilton, NV). The extraction of aqueous samples was carried out following the previously described procedure.\textsuperscript{26,107} This procedure guarantees $\approx 100\%$ extraction efficiencies of PAHs from aqueous samples. The membrane was first conditioned with 1mL of methanol followed by 5mL of nanopure water. After conditioning, a 10mL volume of sample solution (80/20 water/methanol v/v) was passed through the disk to a flow rate of $\approx 30 \text{ mL.min}^{-1}$. Void water was removed from the membrane by forcing three 100mL volumes of air through the disk using a 100mL syringe. The membrane was then removed from the extraction apparatus and placed in the desiccator prior to analysis. The SLE membrane was placed on the bottom of the FOP sample vial with the extracting side facing up, followed by attachment of the probe. The fiber optic bundle was then arranged vertically to $\approx 1$cm above the membrane. This distance provided excitation of the entire disk surface. The FOP was then immersed in the liquid cryogen as previously described. Because there was no physical contact between the fiber and the membrane, fiber clean up between samples was not necessary.
7.3 Results and Discussion

7.3.1 Phosphorescence WTM and Lifetime Analysis in Shpol'skii Matrixes

The accuracy and precision of fluorescence WTM collection and fluorescence lifetime analysis in Shpol'skii matrixes have been previously demonstrated.25,55 Here, we concentrate our efforts on discussing phosphorescence lifetimes and phosphorescence WTM collection with the MLS. The accuracy and precision of measurements was investigated with phosphorescent PAHs previously studied in our laboratory.45,46, 71 The data obtained with the MLS were then compared to data obtained with the dedicated phosphorescence system.45

Phosphorescence WTM can be collected in either the same manner as fluorescence WTM or in a "real-time" fashion. Figure 7.3 shows the fitted decay curves obtained for benzo[e]pyrene in n-octane at 4.2 K using the sequence of events shown in Figure 7.3 and Figure 7.4. Benzo[e]pyrene in n-octane is a well-known example of a single-site Shpol'skii system. The decay in Figure 7.5A was stripped from a WTM consisting of 100 accumulations with a delay incremented of 200 ms and a gate time of 1000 ms. In Figure 7.4 ("real-time mode"), the delay increment of the WTM was equal to the laser duty cycle (100 ms) with a gate of 40 ms. Both decays fitted single-exponential decays with no significant trends in the residuals of the fit.
Figure 7.5 Fitted phosphorescence decay curves for 0.2 µg/mL benzo[e]pyrene in n-octane at 4.2K.

(A) Fitted decay using data acquisition mode. Gate step of 200ms and gate width of 2000ms (B) Fitted decay using real-time data acquisition mode with gate width of 40 ms and gate step of 100 ms.

Table 7.1 compares these lifetimes to those measured with dedicated phosphorescence instrumentation. Within 95% of confidence level ($\alpha = 0.05$), the lifetime averages from individual measurements of six frozen samples were statistically equivalent ($N_1 = N_2 = N_3 = 6$), demonstrating the ability of the new instrument to collect accurate phosphorescence lifetimes.
The relative standard deviations were lower than 3.4%, showing excellent precision of measurements as well.

Table 7.1 Comparison of benzo[e]pyrene phosphorescence lifetimes obtained with dedicated phosphorescence instrumentation (DPI) and the multidimensional luminescence system (MLS)

<table>
<thead>
<tr>
<th>Instrument/Method</th>
<th>4.2K (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPI</td>
<td>1787 ± 39 (2.20%)</td>
</tr>
<tr>
<td>MLS-normal mode</td>
<td>1777 ± 31 (1.74%)</td>
</tr>
<tr>
<td>MLS-real time mode</td>
<td>1780 ± 60 (3.37%)</td>
</tr>
</tbody>
</table>

*a Excitation and emission wavelengths for all measurements were 386.8 and 614.6 nm, respectively. Benzo[e]pyrene concentration was 0.2 µg mL⁻¹. \( b \) Normal mode = many 2D spectra (100 laser shots) collected at incremental delay times. Real time mode = 100 WTM collected and averaged where each 2D spectrum represents emission resulting from 1 laser shot. \( c \) Average phosphorescence lifetime of six separate frozen aliquots. Value in parenthesis represents the relative standard deviation of six measurements.

Table 7.2 summaries the 4.2 K fluorescence and phosphorescence lifetimes of a multisite Shpol'skii system. Chrysene in n-octane exhibits emission spectra with three major 0-0 peaks appearing at 359.2, 359.6, and 360.6 nm for fluorescence and 499.1, 500.3, and 500.8 nm for phosphorescence. As we previously reported with other PAHs/alkane Shpol'skii systems, the three fluorescence sites show statistically different lifetimes \( (\alpha = 0.05, N_1 = N_2 = N_3 = 6) \). The same is true for the phosphorescence lifetimes. The comparison of the relative standard deviations shows excellent precision of measurements within the entire luminescence time domain (ns-s).
Table 7.2. Fluorescence and phosphorescence lifetimes at different sites of chrysene in n-octane at 4.2K recorded with the MLS

<table>
<thead>
<tr>
<th>site</th>
<th>λ (nm)</th>
<th>τ (ns)</th>
<th>λ (nm)</th>
<th>τ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>359.2</td>
<td>58.3±1.5</td>
<td>499.1</td>
<td>2078±63</td>
</tr>
<tr>
<td>2</td>
<td>359.6</td>
<td>63.4±2.1</td>
<td>500.3</td>
<td>2369±83</td>
</tr>
<tr>
<td>3</td>
<td>360.0</td>
<td>60.4±0.8</td>
<td>500.8</td>
<td>2154±56</td>
</tr>
</tbody>
</table>

* Fluorescence and phosphorescence lifetimes resulting from measurement of six separate sample aliquots of a 0.2 µg.mL⁻¹ solution. Excitation wavelength = 321.4nm.

7.3.2 Luminescence Measurements in Highly Scattering Media

Conventional low-temperature spectroscopy has been restricted to optically transparent media. This restriction has limited its application to solvents that freeze into a glass. The FOP removes this limitation and allows one to measure luminescence from highly scattering media such as snowlike matrixes and solid samples.

Table 7.3 lists the 4.2 K luminescence intensities (peak heights) from three PAHs in the three sample matrixes. Although the signal intensities for all three PAHs were larger in n-octane than in methanol, the relative standard deviations are comparable. These demonstrate the ability of the FOP to perform low-temperature measurements in "snowy" matrixes with precision similar to that in Shpol'skii matrixes. A similar statement can be made for lifetime measurements (see Table 7.4). It is interesting to note the better precision of lifetime measurements. This is mostly due to the nature of lifetime measurements, where the ratio between signal intensities cancels out variations of instrumental response.

When adsorbed onto the octadecyl membrane, all three PAHs exhibit large intensity variations. This is not surprising because the background emission of extraction membranes varies considerably from disk to disk and measuring wavelengths.²⁹,³⁰ The phosphorescence of
benzo[ghi]perylene in methanol and on extraction membranes was extremely weak. In fact, at concentrations of 50 µg·mL⁻¹, its phosphorescence intensity did not exceed 2 times the noise. The collection of phosphorescence spectra and lifetimes for benzo[ghi]perylene in methanol and on membranes was, therefore, not possible at those experimental conditions.

Table 7.3 4.2K fluorescence and phosphorescence intensities of PAHs in Shpol’skii, methanol, and extraction membranes.

<table>
<thead>
<tr>
<th>PAH</th>
<th>λ (nm)</th>
<th>n-octane</th>
<th>methanol</th>
<th>Extraction membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysene</td>
<td>359.6c</td>
<td>499.5±20.3(4.1)</td>
<td>5.0±0.2(3.4)</td>
<td>42.8±3.3(7.7)</td>
</tr>
<tr>
<td></td>
<td>499.1d</td>
<td>105.3±4.53(4.3)</td>
<td>13.6±0.7(5.5)</td>
<td>1.3±0.8(5.8)</td>
</tr>
<tr>
<td>Benzo[e]pyrene</td>
<td>387.1c</td>
<td>85.8±3.63(4.2)</td>
<td>5.6±0.3(4.5)</td>
<td>706.8±38.8(5.5)</td>
</tr>
<tr>
<td></td>
<td>536.8d</td>
<td>20.2±0.6(3.1)</td>
<td>6.3±0.4(5.6)</td>
<td>217.4±11.6(5.4)</td>
</tr>
<tr>
<td>Benzo[ghi]perylene</td>
<td>406.5c</td>
<td>3723.7±153.5(4.1)</td>
<td>7.3±0.3(4.7)</td>
<td>267.0±17.9(6.7)</td>
</tr>
<tr>
<td></td>
<td>614.6d</td>
<td>284.4±9.7(3.4)</td>
<td>f</td>
<td>f</td>
</tr>
</tbody>
</table>

aIntensities in ×10³ counts. Values in parentheses represent the relative standard deviations (%) of six measurements taken from six separate sample aliquots. All concentrations were 0.2 µg·mL⁻¹. Excitation wavelength was 321.4 nm for chrysene, 320.4 nm for benzo[e]pyrene, and 386.8 nm for benzo[ghi]perylene. Maximum fluorescence wavelength. Maximum phosphorescence wavelength. No phosphorescence was observed for this PAH-matrix combination.

Table 7.4 4.2K fluorescence and phosphorescence lifetimes of PAHs in Shpol’skii, methanol, and extraction membranes.

<table>
<thead>
<tr>
<th>PAH</th>
<th>λ (nm)</th>
<th>n-octane</th>
<th>methanol</th>
<th>Extraction membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysene</td>
<td>387.1c</td>
<td>200±2ns(0.8)</td>
<td>145±2.8ns(3.4)</td>
<td>135.4±1.0ns(0.7)</td>
</tr>
<tr>
<td></td>
<td>536.8d</td>
<td>292±4ms(1.5)</td>
<td>e</td>
<td>e</td>
</tr>
<tr>
<td>Benzo[e]pyrene</td>
<td>359.6c</td>
<td>63.4±1.0ns(1.6)</td>
<td>59.1±1.9ns(4.5)</td>
<td>26.9±0.8ns(2.9)</td>
</tr>
<tr>
<td></td>
<td>499.1d</td>
<td>2078±32ms(1.5)</td>
<td>2643±51ms(5.6)</td>
<td>1980±56ms(2.8)</td>
</tr>
<tr>
<td>Benzo[ghi]perylene</td>
<td>406.5c</td>
<td>57.9±1.3ns(2.2)</td>
<td>66.9±1.9ns(2.8)</td>
<td>62.9±1.7ns(2.7)</td>
</tr>
<tr>
<td></td>
<td>614.6d</td>
<td>1780±30ms(1.7)</td>
<td>1899±2.5ms(0.1)</td>
<td>1824±31ms(1.7)</td>
</tr>
</tbody>
</table>

a Values in parentheses represent the relative standard deviations (%) of six measurements taken from six separate sample aliquots. All concentrations were 0.2 µg·mL⁻¹. Excitation wavelength was 321.4 nm for chrysene, 320.4 nm for benzo[e]pyrene, and 386.8 nm for benzo[ghi]perylene. Maximum fluorescence wavelength. Maximum phosphorescence wavelength. No phosphorescence was observed for this PAH-matrix combination.
7.4 Conclusions

An instrument with the capability to collect multidimensional fluorescence and phosphorescence data formats has been presented. The integration of a pulsed tunable dye laser, a mechanical shutter, a spectrograph, an ICCD, and custom data acquisition software has made possible the collection of WTM$s$, EEM$s$, and TREEM$s$ within the entire time domain of luminescence (ns-s). This capability makes our instrument the appropriate tool for those applications seeking the full dimensionality of luminescence spectroscopy, including approaches based on room-temperature luminescence techniques. It is now possible to collect numerous qualitatitive parameters with a single instrument. The extension of the fiber-optic probe beyond Shpol'skii matrixes has been demonstrated with accurate and precise measurements in optically scattering media at 77 and 4.2 K. Because sample freezing with the fiber-optic probe is rapid and straightforward, the benefits of lowering the temperature are now available without the traditional constrains associated to cumbersome experimental procedures.
8 CHAPTER: MEASURING SCATTER WITH A CRYOGENIC PROBE AND AN ICCD CAMERA: A NOVEL APPROACH FOR RECORDING ABSORPTION SPECTRA IN SHPOL’SKII MATRIXES AND FLUORESCENCE QUANTUM YIELDS IN GLASSY SOLVENTS

8.1 Introduction

In this chapter, we extend our approach to low-temperature absorption measurements. 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 comparable intensity 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 regards to high spectral resolution.

To the extent of our literature search, only one instrument has been reported for the purpose at hand.\textsuperscript{111} It consists of a double beam spectrometer using two Czerny-Turner monochromators and a continuous wave Xenon lamp as the excitation source. The optical path is dispersed by the first monochromator and directed to a double beam arrangement composed of two fixed mirrors and two rotating mirrors. After being alternatively directed toward the sample and the reference pathways, the beam is focused on the entrance slit of the second monochromator. Transmittance spectra are recorded by setting the two monochromators at the
same wavelength and synchronously scanning them. The sample ($I_{\text{Sample}}$) and reference ($I_{\text{Reference}}$) signals are detected with a photomultiplier tube, amplified and integrated over a certain number of mirror cycles. Accumulated $I_{\text{Sample}}$ and $I_{\text{Reference}}$ values are then analog-digital converted and sent to a computer that calculates the absorbance – $\log \left( \frac{I_{\text{Reference}}}{I_{\text{Sample}}} \right)$ – as a function of excitation wavelength. Positioning one monochromator before the sample and the other one between the sample and the detector reduces the interference of stray light and fluorescence to very low levels.

Our approach to low-temperature absorption measurements is fundamentally different as the sought-for-information is the intensity of laser excitation returning from the frozen sample to the ICCD. The feasibility of our approach is here demonstrated with single-site and multiple-site PAH/n-alkane systems. For all the model systems numerous references are available in the literature, which allow its critical comparison to conventional methodology. Fluorescence quantum yields were measured at room temperature and 77K. Their excellent agreement to literature values and experimental quantum yields obtained via classical methodology in our lab demonstrates the feasibility to perform accurate absorption measurements in frozen matrixes. Our approach was then extended to creating original data on fluorescence quantum yields at 4.2K.

8.2 Experimental

8.2.1 Chemicals and Solution Preparation

All chemicals were analytical reagent grade and used without further purification. Otherwise noted, Nanopure water was used throughout. PAH were purchased from Aldrich at their highest available purity. HPLC grade solvents (Sigma-Aldrich) were used to prepare stock PAH solutions. Working solutions were prepared by diluting the stock solution with the
appropriate solvent prior to absorption and/or fluorescence measurements. Rhodamine 6G, DCM and LDS 759 were purchased from Exciton and used with the tunable dye laser according to specifications. Their tuning ranges were 275 – 290, 300 – 340 and 355-406nm, respectively.

8.2.2  
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 band-pass, ± 0.1nm wavelength precision and 24,000 nm.min⁻¹ maximum scan rate. All measurements were made by pouring liquid solutions into 1cm x 1cm quartz cuvettes.

8.2.3  
Room-Temperature and 77K 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 200-2000nm. Detection was made with a photomultiplier tube (PMT, model 1527) with spectral response from 185 to 650nm. The excitation and emission monochromators had the same reciprocal linear dispersion (4nm.mm⁻¹) and accuracy (±1nm with 0.25nm resolution). Their 1200 grooves/nm gratings were blazed at 300 and 400nm, respectively. Wavelength reproducibility was ~ ±2nm. The instrument was computer controlled using commercial software (Felix32) specifically designed for the system. Correction of excitation and emission spectra was made in post-acquisition mode to compensate for wavelength dependence of excitation light source and detector sensitivity, respectively. Room-temperature measurements were made by pouring liquid solutions into 1cm x 1cm quartz
cuvettes. 77K measurements followed the classic procedure of immersing the sample solution in a quartz tube into a nitrogen-filled Dewar flask.

8.2.4 Measurements with the FOP

Measurements were carried out as follows: after a known volume (typically 100-200μL) of undegassed sample solution was pipetted into the sample vial, the tip of the fiber optic probe was positioned and held constant with the screw cap above the solution surface. The dimensions of the vial were the following: 30-mm length, 5.5-mm inner diameter, and 7-mm outer diameter. Its maximum volume capacity was 750μL. Sample freezing was accomplished by lowering the copper tubing into the liquid cryogen. The liquid nitrogen and liquid helium were held in two separate Dewars with 5L and 60 L storage capacity, respectively.

8.2.5 Multidimensional Luminescence System (MLS)

A complete description of the MLS and its capabilities with regards to fluorescence and phosphorescence measurements have been made in chapter 7.

8.2.6 Spectral Acquisition

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. Otherwise noted, each spectrum corresponds to the accumulation of 100 laser pulses. A 0ns delay and a 10ns gate allowed the CCD to acquire laser scatter during the duration of the pulse (full-width at half maximum ~ 5ns). The 10ns gate-width provided satisfactory signal-to-
noise ratios with minimum fluorescence interference. Fluorescence spectra were recorded using a minimum delay of 10ns, 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 ~ 4x or 5x the lifetime of the PAH were sufficient to collect most of the PAH fluorescence and still avoid instrumental noise.

8.2.7 Limiting Resolution of MLS

For absorption measurements, the limiting resolution of the instrumental system was determined by the laser scanning rate, i.e. 0.1nm/dapoint. For fluorescence measurements, the limiting resolution was determined by the multi-channel 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 x 256) in the array, and the dimensions (18 x 6.7 mm) of the active area of the CCD chip, the spectral range of one pixel should give a 0.08 nm limiting resolution. Because of the detector crosstalk and the intensifier, the factual limiting resolution corresponds to 4-5 pixels (5), i.e. 0.32 - 0.40 nm.
8.3 Results and Discussion

8.3.1 Theoretical Consideration

Figure 8.1 illustrates the basis of our approach. 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. If the monochromatic excitation beam contains photons with energy corresponding to the energy differences necessary for absorption, portions of incident radiant power will be absorbed ($I_A$), transmitted ($I_T$), scattered ($I_S$) and reflected ($I_{RFL}$). Assuming negligible photon losses, the intensity of the incident radiant power ($I_0$) can be approximated to:

$$I_0(\lambda_0) = I_A(\lambda_0) + I_T(\lambda_0) + I_S + I_{RFL}(\lambda_0) \quad (8.1)$$

where $\lambda_0$ represents the wavelength of the monochromatic excitation beam. Keeping in mind that only a fraction of scattered, reflected and photoluminescence radiations are collected with the probe and that its front face configuration practically eliminates the detection of transmitted light, the radiant power intensity of the returning radiation ($I_{RR}$) can then be approximated to:

$$I_{RR} = I_S' + I_{RFL}'(\lambda_0) \quad (8.2)$$

where $I_S'$ and $I_{RFL}'$ are the collected fractions of scattered, reflected and photoluminescence radiations, respectively. The contribution of inelastic scattering to $I_{RR}$ can be neglected with no detrimental effect on the accuracy of measurements. The same is true for photoluminescence. Both types of radiation are detected with different groups of CCD pixels and their contribution to $I_{RR}$ at $\lambda_0$ can be approximated to zero:

$$I_{RR}(\lambda_0) = I_S(\lambda_0) + I_{RFL}(\lambda_0) \quad (8.3)$$

where $I_S(\lambda_0)$ corresponds to elastic scattering of $I_0(\lambda_0)$. Considering only the CCD pixels that detect $I_{RR}(\lambda_0)$, equation (1) can be re-written as:
\[ I_0 (\lambda_0) = I_A (\lambda_0) + I_{RR} (\lambda_0) \]  \hspace{1cm} (8.4)

This equation is the basis for generating absorption spectra with a rather straightforward procedure. \( I_{RR} \) is monitored as a function of excitation wavelength \( (\lambda_0) \) for both blank \( [I_{RR}^B (\lambda_0)] \) and analyte \( [I_{RR}^{An+B} (\lambda_0)] \) solutions:

\[ I_0 (\lambda_0) = I_A^B (\lambda_0) + I_{RR}^B (\lambda_0) \]  \hspace{1cm} (8.5)
\[ I_0 (\lambda_0) = I_A^{An+B} (\lambda_0) + I_{RR}^{An+B} (\lambda_0) \]  \hspace{1cm} (8.6)

From (5) and (6) we know that:

\[ I_A^B (\lambda_0) + I_{RR}^B (\lambda_0) = I_A^{An+B} (\lambda_0) + I_{RR}^{An+B} (\lambda_0) \]  \hspace{1cm} (8.7)
\[ I_{RR}^B (\lambda_0) - I_{RR}^{An+B} (\lambda_0) = I_A^{An+B} (\lambda_0) - I_A^B (\lambda_0) \]  \hspace{1cm} (8.8)

Keeping in mind that:

\[ I_{RR}^{An} = I_{RR}^B (\lambda_0) - I_{RR}^{An+B} (\lambda_0) \]  \hspace{1cm} (8.9)

and

\[ I_A^{An} = I_A^{An+B} (\lambda_0) - I_A^B (\lambda_0) \]  \hspace{1cm} (8.10)

Because \( I_A^{An} \) is equivalent to \( I_{RR}^{An} \) - see Eq. (8.8), (8.9) and (8.10) - an “absorption spectrum” can be originated by plotting \( I_{RR}^{An} (\lambda_0) \) versus \( \lambda_0 \). One should notice that Eq (8.10) is only valid for frozen solutions with relatively small blank absorptions and strong light scattering.
Figure 8.1(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
8.3.2 Accuracy of Spectral Analysis.

Figure 8.2A and Figure 8.2B show the 4.2K 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\textsuperscript{19,112,113} and multi-site systems,\textsuperscript{19,114,115} respectively. Both spectra compare well to previously reported spectra at 5K.\textsuperscript{1} 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.\textsuperscript{19} Comparison of Figure 8.2A spectrum to the room-temperature absorption and 4.2K fluorescence spectra of naphthalene (see Figure 8.2C and Figure 8.2D) demonstrates our ability to accurately record weak absorption spectra at the proximities of strong fluorescence emission. However, it is important to note that none of our attempts included absorption measurements within the 0-0 transition range. The ability of back scatter absorption measurements to record absorption spectra within the 0-0 transition range is still an open question and subject for future studies.
Figure 8.2 4.2K spectra recorded with the FOP and MLS.

(A) 10 µg.mL⁻¹ naphthalene in n-pentane; (B) 10µg.mL⁻¹ benzo[a]pyrene in n-octane. Spectra were generated recording laser scatter at 0.1nm excitation steps. Signal intensity at each excitation wavelength results from 100 laser pulses. The ICCD was operated at 0 and 10ns delay and gate times, respectively. Entrance slit-width of spectrograph, 40µm. (C) Room-temperature absorption spectrum of 10µg.mL⁻¹ naphthalene in n-pentane recorded with commercial instrumentation using a 2nm band-pass. (D) 4.2K fluorescence spectrum of 10µg.mL⁻¹ naphthalene in n-pentane recorded with the FOP and the MLS. Spectrum corresponds to the accumulation of 100 laser shots using a delay of 10ns and a gate of 1000ns. Slit width of spectrograph, 40 µm.
8.3.3 Analytical Precision

The ability to reproduce absorption spectra from sample to sample was investigated with chrysene, pyrene and benzo[g,h,i]perylene in n-octane. Figure 8.3 illustrates the type of data generated with the cryogenic probe and the laser system. The tunable dye laser was operated either with Rhodamine B (306.0-308.4nm) or DCM (324.0 - 326.0nm). Each data point plotted in the graph represents the average of three intensities recorded from three frozen samples. Curves I and II represent the intensities of excitation energies returning from n-octane and PAH/n-octane, respectively. These intensities correspond to $I_{RR}^B (\lambda_0)$ (n-octane) in Equation 8.5 and $I_{RR}^{An+B} (\lambda_0)$ (PAH/n-octane) in Equation.8.6. As expected, the relatively lower absorption of n-octane provides a higher signal than the PAH. Curves III represent the net PAH signals $[I_{RR}^{An}(\lambda_0)]$ calculated according to Equation 8.7. Table 8.1 summaries the relative standard deviations of $I_{RR}^B (\lambda_0)$, $I_{RR}^{An+B} (\lambda_0)$ and $I_{RR}^{An} (\lambda_0)$ within the wavelength range of excitation. The excellent precision of measurements demonstrates the outstanding sample-to-sample spectral reproducibility of our approach.
Figure 8.3 Experimental data generated at 4.2K with the FOP and the MLS.
(A) 10μg.mL⁻¹ chrysene in n-octane; (B) 10μg.mL⁻¹ pyrene in n-octane. Curves I and II represent the intensities of the excitation energies monitored from the blank [IRRB (λ₀)] and analyte [IRRAn + B (λ₀)] solutions. Curve III represents the net PAH signal [IRRAn(λ₀)]. Each data point in the calibration graphs corresponds to the accumulation of 100 laser pulses. Delay and gate times were 0 and 10ns, respectively. Slit width of spectrograph, 40μm.
Table 8.1 Reproducibility of 4.2K absorption spectra recorded with the FOP and the MLS

<table>
<thead>
<tr>
<th>PAH(^a)</th>
<th>Peak under the area(^b)</th>
<th>Average</th>
<th>RSD (%)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysene</td>
<td>9.87×10(^5)</td>
<td>9.78×10(^5)</td>
<td>9.80 ± 0.06×10(^5)</td>
</tr>
<tr>
<td>Pyrene</td>
<td>7.28×10(^5)</td>
<td>7.14×10(^5)</td>
<td>7.26 ± 0.11×10(^5)</td>
</tr>
<tr>
<td>Benzo[g,h,i]pyrene</td>
<td>8.11×10(^5)</td>
<td>8.17×10(^5)</td>
<td>8.16 ± 0.06×10(^5)</td>
</tr>
</tbody>
</table>

\(^a\) PAH solutions were prepared in octane at the following concentrations: 1ppm

\(^b\) Area under the peak calculated with Origin software from the plot \(I_{RR}^{An}(\lambda_0)\) versus \(\lambda_0\).

\(^c\) Relative standard deviation calculated with the formula: \(RSD = \frac{\text{standard deviation} \times 100}{\text{average}}\)

8.3.4 Quantitative Measurements

Acenaphthene in n-pentane, naphthalene in n-pentane and benzo[\(a\)]pyrene in n-octane were selected to investigate the correlation between \(I_{RR}^{An}(\lambda_0)\) and PAH concentration. Table 8.2 presents the statistics of the fitting for the calibration curves of the three PAH. The correlation coefficients close to unity demonstrate excellent linear correlations between signal intensity and PAH concentration. Figure 8.4 shows the quasi-line 4.2K spectrum of acenaphthene in n-pentane. Its spectral features are an excellent matching to previously reported 5K spectra.\(^{26}\) Comparison of the peak intensities at 312.9 and 315.4nm to the slopes reported in Table 8.2 show a steeper slope at 312.94nm. This fact agrees well to the higher signal intensity at this excitation wavelength. These results provide sufficient evidence to propose a linear relationship of the type \(I_{RR}^{An}(\lambda_0) = ek\,[PAH]\), where \(k\) is a constant related to the optical path of the excitation radiation in the frozen matrix. Most probably, \(k\) value varies with the excitation wavelength and PAH concentration. Further studies are certainly needed to fully understand the experimental meaning of the \(k\) value.
Table 8.2 Correlation between $I_{RR}^{Aa}$ and PAH concentration.

<table>
<thead>
<tr>
<th>PAH/n-alkane</th>
<th>Wavelength (nm)</th>
<th>Concentration range (μg.mL$^{-1}$)$^a$</th>
<th>Best linear fitting$^b$</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene/</td>
<td>306.00</td>
<td>5-100</td>
<td>$Y=4098X + 285$</td>
<td>0.9970</td>
</tr>
<tr>
<td>pentane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzo[a]pyrene/</td>
<td>309.90</td>
<td>1-50</td>
<td>$Y=11052X - 889$</td>
<td>0.9899</td>
</tr>
<tr>
<td>octane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acenaphthene/</td>
<td>312.94</td>
<td>5-100</td>
<td>$Y=4627X + 96$</td>
<td>0.9991</td>
</tr>
<tr>
<td>pentane</td>
<td>315.03</td>
<td>5-100</td>
<td>$Y=3868X + 158$</td>
<td>0.9910</td>
</tr>
</tbody>
</table>

$^a$ No efforts were made to obtain the lowest and highest concentrations of the linear dynamic range. $^b$ All data points were graphically examined to ensure that the concentrations were within the linear dynamic range of the calibration curves. Best linear fittings were obtained via the least squares method.

Figure 8.4 4.2K absorption spectrum of acenaphthene in n-pentane.

Spectrum was generated by tuning the dye laser at 0.1nm excitation steps. Each data point in the IRRAn ($I_{RR}$) vs. $\lambda_0$ plot corresponds to the accumulation of 100 laser shots using a 0ns delay and a 10ns gate. Slit width of spectrograph, 40 μm.
8.3.5 Luminescence Quantum Yields.

A direct benefit of this research is the possibility to measuring low-temperature absorption, excitation, fluorescence and phosphorescence spectra and luminescence lifetimes with a single instrument via a simple experimental procedure. An application that takes full advantage of this benefit is the measurement of luminescence quantum yields. The most popular indirect method for determining fluorescence quantum yields (Q_F) is the Parker – Rees method.\(^{116}\) Q_F is evaluated comparing the emission spectra of the analyte (x) and a reference fluorescent species (r) of known fluorescence quantum yield (Q_F, r):

\[
Q_{F, x} = Q_{F, r} \left[ \frac{A_r}{A_x} \right] \left[ \frac{\Phi_{r,p}}{\Phi_{x,p}} \right] \left[ \frac{\int E_x \, d\lambda'}{\int E_r \, d\lambda'} \right]
\]  \hspace{1cm} (8.11)

where A is the absorbance at the excitation wavelength, \(\Phi_p\) is the relative incident power (photons.s\(^{-1}\)), and \(\int E \, d\lambda'\) is the integral of the corrected emission band (relative quanta per second). Additional factors may be included to account for other differences in excitation or emission variables if different excitation or emission wavelengths or different solvents (e.g. refractive index effects) are used.

All the quantum yield work reported in these studies was performed with glassy matrixes, which provide low-resolution spectra. The main reason for using glassy matrixes was to compare our results under similar experimental conditions to those previously reported. Table 8.3 compares literature values\(^{117}\) of room-temperature Q_F, x to those measured in our lab via classic methodology and the new approach (FOP/MLS). Classic methodology refers to absorption and fluorescence measurements carried out with standard quartz cuvettes (1 x 1 cm) and commercial instrumentation. For each PAH in Table III, more than one Q_F, x value is reported in the literature.\(^{16-18}\) The values we selected for comparison were obtained under similar experimental conditions to ours, including reference standard (perylene) and solvent (ethanol). The excellent
agreement among the tabulated averages and their standard deviations demonstrates the ability of
the FOP/MLS to measuring accurate and reproducible room-temperature $Q_{F,x}$.

Table 8.3 Comparison of room-temperature fluorescence quantum yields$^a$ measured with classic
methodology$^b$ and the FOP/MLS$^c$

<table>
<thead>
<tr>
<th>PAH$^d$</th>
<th>Literature Values$^e$</th>
<th>Classic Methodology$^f$</th>
<th>FOP /MLS$^g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>$0.205 \pm 0.014$; $\lambda_{exc} = 253.7$nm</td>
<td>$0.207 \pm 0.009$; $\lambda_{exc} = 285$nm (4.3%)</td>
<td>$0.206 \pm 0.009$; $\lambda_{exc} = 285.3$nm (4.4%)</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>$0.12 \pm 0.005$; $\lambda_{exc} = 253.7$nm</td>
<td>$0.128 \pm 0.007$; $\lambda_{exc} = 294$nm (5.5%)</td>
<td>$0.125 \pm 0.007$; $\lambda_{exc} = 294.0$nm (5.6%)</td>
</tr>
<tr>
<td>Chrysene</td>
<td>$0.16 \pm 0.06$; $\lambda_{exc} = 253.7$nm</td>
<td>$0.166 \pm 0.008$; $\lambda_{exc} = 321$nm (4.8%)</td>
<td>$0.177 \pm 0.009$; $\lambda_{exc} = 320.9$nm (5.1%)</td>
</tr>
<tr>
<td>Pyrene</td>
<td>$0.175 \pm 0.014$; $\lambda_{exc} = 253.7$nm</td>
<td>$0.518 \pm 0.027$; $\lambda_{exc} = 336$nm (5.2%)</td>
<td>$0.565 \pm 0.027$; $\lambda_{exc} = 336.0$nm (4.8%)</td>
</tr>
</tbody>
</table>

$^a$All quantum yields were calculated following the Parker-Rees method. See Equation. 8.10 and reference
$^b$Classic methodology refers to absorption and fluorescence measurements carried out with standard quartz
$^c$FOP/MLS refers to absorption and fluorescence
$^d$All PAH
$^e$Literature values obtained from reference 16.
$^f$Values between parentheses refer to the relative standard deviations of average values obtained from
$^g$Experimental

Table 8.4 compares the 77K fluorescence quantum yields of naphthalene, phenanthrene
and fluorene measured with our approach to those reported in the literature.$^{118, 119, 120, 121}$

Previously reported values were calculated using the room-temperature (RT) quantum yield of
perylene in cyclohexane ($Q_{F,r}^{RT} = 0.94$). This approximation, i.e. $Q_{F,r}^{77K} \sim Q_{F,r}^{RT} = 0.94$, was
probably made to circumvent the lack of previously reported data on quantum yields at 77K
($Q_{F,r}^{77K}$). Because of the high value of $Q_{F,r}^{RT}$, the approximation should provide accurate $Q_{F,x}^{77K}$.
We further examined this possibility by measuring the absorption and fluorescence of perylene at RT and low temperature (77K and 4.2K), and calculating its low temperature quantum yields via Equation. (8.11). All measurements were performed with the FOP/MLS and using $Q_{F,x}^{RT} = 0.94$ as the reference value. As expected, lowering the temperature does not significantly increase the quantum yield of perylene, as both values – 77K and 4.2K - were close to its RT yield ($Q_{F,x}^{77K} = 0.95 \pm 0.05$ and $Q_{F,x}^{4.2K} = 0.95 \pm 0.04$). The standard deviations of the experimental averages are based on three independent quantum yield measurements. Additionally, we examined the possibility of perylene phosphorescence emission at 77K and 4.2K. If present, this process would compete with fluorescence for the deactivation of the first singlet excited state and possibly reduce its quantum yield. No phosphorescence was observed, which correlates well to the approximation made.

Table 8.4 77K and 4.2K fluorescence quantum yields of PAH measured with the FOP/MLS

<table>
<thead>
<tr>
<th>PAH$^a$</th>
<th>$Q_{exp}^b$ at 77K</th>
<th>$Q_{lit}^c$ at 77K</th>
<th>$Q_{exp}^b$ at 4.2K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Napthalene</td>
<td>0.44 ± 0.03</td>
<td>0.39</td>
<td>0.51 ± 0.04</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>0.12 ± 0.01</td>
<td>0.14</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Fluorene</td>
<td>0.61 ± 0.05</td>
<td>0.54</td>
<td>0.67 ± 0.05</td>
</tr>
</tbody>
</table>

$^a$ 1ppm Standard solutions were prepared in EPA (ether/isopentane/ethanol 5/5/2) $^b$ Experimental quantum yields ($Q_{exp}$) were calculated based on Eq. (8.10) using quantum yields of perylene in cyclonehexane at 77K (0.95) or at 4.2K (0.95).$^c$ 77K Literature values obtained from references 116-121. No 4.2K values were found in the literature for comparison

Comparison of 77K values obtained with our approach to those reported in the literature$^{118,119,120,121}$ shows reasonable agreement, mainly if one considers that different experimental and instrumental set ups were used for their measurement. A direct comparison of $Q_{F,x}^{4.2K}$ to literature values is not possible because of the lack of previous reported data. To the extent of our literature search, this is the first time $Q_{F,x}^{4.2K}$ are reported for PAH/n-alkane systems. Interesting to note is that all $Q_{F,x}^{4.2K}$ values are higher than their 77K counterparts.
This observation agrees well with the known concept of fluorescence enhancement via low-temperature analysis.

### 8.4 Conclusions

This chapter introduces a unique approach to recording reproducible and accurate absorption spectra at 77K and 4.2K. Laser excitation returning from the frozen sample to the ICCD is collected with the FOP via a rapid and simple freezing sample procedure. Considering the limiting resolution of the multi-channel detection system (spectrograph/ICCD ~ 0.42nm), the scanning capability (0.1nm/step) and the narrow excitation bandwidth (~ 0.03nm) of the tunable dye laser are well suited to excite small wavelength intervals in PAH absorption spectra. Because the fluorescence is detected with a different group of CCD pixels and the intensity of scatter is stronger than the fluorescence intensity, the fluorescence does not introduce large errors in the scatter measurements.

The observed linear relationship between $I_{RR}^{An}(\lambda_0)$ and PAH concentration provides sufficient evidence to propose a correlation of the type $I_{RR}^{An}(\lambda_0) = \varepsilon k [\text{PAH}]$, where $k$ is a constant related to the optical path of the excitation radiation in the frozen matrix. Further studies are certainly needed to fully understand the experimental meaning of the $k$ value, particularly with regards to its expected variation with excitation wavelength and PAH concentration. However, because of the well-behaved linearity between $I_{RR}^{An}(\lambda_0)$ and [PAH], the $k$ value appears to be fairly constant within the excitation wavelength and concentration ranges of the studied PAHs.

A direct benefit of this research is the measurement of fluorescence quantum yields with a single sample procedure and instrument. Our approach provides the analyst with a unique tool
for the direct comparison of quantum yields at room-temperature, 77K and 4.2K. Considering the MLS capability to perform fluorescence lifetime measurements and phosphorescence measurements in the ms-s time domain, the researcher interested in the photo-physics of aromatic compounds has access now to experimental and instrumentation to generate outstanding spectroscopic information related to deactivation constants of excited states and intersystem crossing rate constants. The excellent accuracy and precision of measurements, associated to the simplicity of data collection can certainly impact a research field that currently lacks reference databases.
9 CHAPTER: GENERAL CONCLUSION

Applications of our methodology have centered on the direct analysis of PAHs in complex environmental samples. We have unfolded the full potential of SS with a significant selectivity enhancement based on information rich, multidimensional data formats. WTM and TREEM provide unambiguous determination of target PAH on the bases of spectral and lifetime analysis. We have shown the ability of luminescence decays to report on spectral peak purity for accurate quantitative determination in highly complex matrixes. Adding the temporal dimension to excitation and emission spectra gives the analyst the opportunity to select the best time window with minimum spectral overlapping. Initially, our attention was focused to the sixteen PAHs in the EPA priority pollutants list. The analytical potential of our approach was successfully compared to classical chromatographic techniques. The main advantages of LETRSS included shorter analysis time (~15min per sample), reduced solvent consumption (100μL per sample) and lower limits of detection (LOD). Depending on the PAH, LETRSS-LOD were one to two order of magnitude better than HPLC-LOD.

More recently, we directed our attention to PAH for which analytical techniques are completely lacking. Recently toxicological studies correlate a significant portion of the biological activity of contaminated samples to PAH not included in the EPA priority pollutants list. Dibenzo[a,l]pyrene (DB[a,l]P) is a crucial example. This compound is considered the most potent carcinogenic of all PAH ever tested. DB[a,l]P shows 1-2 orders of magnitude higher tumorigenicity than B[a]P in mouse experiments, the most toxic PAH in the EPA list. Its four isomers, which include DB[a,e]P, DB[a,h]P, DB[e,l]P and DB[a,i]P are also carcinogenic but
not to the extent DB[a,/]P is. Chromatographic methodology does not meet the challenge of specifically analyzing these structural isomers at the concentration ratios found in environmental samples. We have fulfilled this gap with an accurate method capable to unambiguously determine the presence of DB[a,/]P in environmental samples, even if present at 400 times lower concentration levels than its isomers. The same is true for the other four structural isomers. To the extent of our literature search, our approach is the only available tool fully capable of monitoring the five dibenzopyrene isomers in water samples.

In principle, any disordered matrix can be used for FLNS, as long as the vibronic coupling between analyte and solvent molecules is not too strong. In practice, however, its application has been restricted to organic solvents that freeze into a glass. We have removed this limitation extending the FOP to optically scattering media. A similar statement can be made for lifetime measurements. The instrumental ability to collect the entire spectrum at once avoids problems associated with pulse-to-pulse fluctuation, laser intensity drift, and sample photodecomposition. These phenomena usually degrade the quality of Shpol’skii and FLN spectra. We have also shown that time-resolution of pulsed excitation reduces broadband fluorescence background. Moreover, we can eliminate the contamination of excitation stray light on FLN spectra with very short delay times. This is an important advantage for FLNS of compounds with short fluorescence lifetimes. While single channel detection systems typically need delays longer than ~20ns, our system is able to time-resolve stray light with delays as short as ~5ns. This advantage results from tuning the excitation wavelength away from the CCD chip and the relatively low signal amplification of the edges of the intensifier. Their combination considerably reduces the intensity of stray light reaching the active pixel area of the CCD.
A unique approach to recording reproducible and accurate absorption spectra at 77K and 4.2K has been presented. Our approach provides the analyst with a unique tool for the direct comparison of quantum yields at room-temperature, 77K and 4.2K. Considering the MLS capability to perform fluorescence lifetime measurements and phosphorescence measurements in the ms-s time domain, the researcher interested in the photo-physics of aromatic compounds has access now to outstanding spectroscopic information related to deactivation constants of excited states and intersystem crossing rate constants. The excellent accuracy and precision of measurements, associated to the simplicity of data collection can certainly impact a research field that currently lacks reference databases.
APPENDIX A: ULTRAVIOLET/VISIBLE SPECTRA OF PAH AT ROOM TEMPERATURE
Figure A-1 Napthalene in n-petane 1ppm
Figure A-2 Acenaphthene in $n$-petane 1ppm

Figure A-3 Pyrene in $n$-hexane 1ppm
Figure A-4 Phenanthrene in $n$-hexane 1ppm

Figure A-5 Fluorene in $n$-heptane 1ppm
Figure A-6 Fluoranthene in $n$-heptane 1ppm

Figure A-7 Benzo[b]fluoranthene in $n$-heptane 1ppm
Figure A-8 Anthracene in $n$-heptane 1ppm

Figure A-9 Benzo[ghil]perylene in $n$-heptane 1ppm
Figure A-10 Indeno[1,2,3-cd]pyrene in n-octane 1ppm

Figure A-11 Chrysene in n-octane 1ppm
Figure A-12 Benzo[k]fluoranthene in n-octane 1ppm

Figure A-13 Benzo[a]pyrene in n-octane 1ppm
Figure A-14 Benzo[a]anthracene in n-octane 1ppm

Figure A-15 Dibenzo[a,h]anthracene in n-octane 1ppm
APPENDIX B: EXCITATION AND FLUORESCENCE SPECTRA OF PAH AT ROOM TEMPERATURE AND 77K
Figure B-1 Naphthalene in $n$-pentane 1ppm at RT

Figure B-2 Naphthalene in $n$-pentane 1ppm at 77K
Figure B-3 Acenaphthene in $n$-pentane 1ppm at RT

Figure B-4 Acenaphthene in $n$-pentane 1ppm at 77K
Figure B-5 Pyrene in \( n \)-hexane 1ppm at RT

Figure B-6 Pyrene in \( n \)-hexane 1ppm at 77K
Figure B-7 Phenanthrene in $n$-hexane 1ppm at RT

Figure B-8 Phenanthrene in $n$-hexane 1ppm at 77K
Figure B-9 Fluorene in $n$-heptane 1ppn at RT

Figure B-10 Fluorene in $n$-heptane 1ppn at 77K
Figure B-11 Fluoranthene in $n$-heptane 1ppm at RT

Figure B-12 Fluoranthene in $n$-heptane 1ppm at 77K
Figure B-13 Benzo[b]fluoranthene in n-heptane 1ppm RT

Figure B-14 Benzo[b]fluoranthene in n-heptane 1ppm 77K
Figure B-15 Anthracene in n-octane RT 1ppm

Figure B-16 Anthracene in n-octane 77K 1ppm
Figure B-17 Benzo[g,h,i] perylene in \textit{n}-heptane 1ppm RT

Figure B-18 Benzo[g,h,i] perylene in \textit{n}-heptane 1ppm 77K
Figure B-19 Indeno[1,2,3-cd] pyrene in n-octane 1ppm RT

Figure B-20 Indeno[1,2,3-cd] pyrene in n-octane 1ppm 77K
Figure B-21 Chrysene in \textit{n}-octane 1 ppm RT

Figure B-22 Chrysene in \textit{n}-octane 1 ppm 77K
Figure B-23 Benzo[â]fluoranthene in n-octane 1ppm RT

Figure B-24 Benzo[k]fluoranthene in n-octane 1ppm 77K
Figure B-25 Benzo[a]pyrene in n-octane 1ppm RT

Figure B-26 Benzo[a]pyrene in n-octane 1ppm 77K
Figure B-27 Benzo[a]anthracene in n-octane 1ppm RT

Figure B-28 Benzo[a]anthracene in n-octane at 1ppm 77K
Figure B-29 Dibenzo[\textit{a,h}]anthracene in \textit{n}-octane 1ppm RT

Figure B-30 Dibenzo[\textit{a,h}]anthracene in \textit{n}-octane 1ppm 77K
LIST OF REFERENCES


70. Hurley, S “An Investigation of the Biproduct Formation and Removal at the Moorhead


