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Chemometric Analysis of Multidimensional Fluorescence Data Recorded from Benzo[a]pyrene Metabolites in Shpol'skii Like Matrixes

Mohammadreza Chehelamirani
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CHEMOMETRIC ANALYSIS OF MULTIDIMENSIONAL FLUORESCENCE DATA RECORDED FROM BENZO[A]PYRENE METABOLITES IN SHPOL’SKII LIKE MATRIXES

by

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

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2020

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ABSTRACT

This dissertation extends the application of Shpol’skii spectroscopy to polar metabolites in polar organic solvents. For the first-time, we reported experimental evidence on the line-narrowing effect caused by primary alcohols on the spectral features of PAH metabolites at 77 K and 4.2 K. The effect of primary alcohols (RCH$_2$OH) on the 77 K and 4.2 K fluorescence characteristics of PAH metabolites showed the best spectral narrowing when matching the length of the alkyl group (R) of the primary alcohol to the length of the n-alkane that best fitted the dimensions of the parent PAH. The spectral narrowing and the fluorescence enhancements observed for 1-hydroxypyrene, 2-hydroxyfluorene, 9-hydroxyphenanthrene, 3-hydroxybenzo[a]pyrene, 4-hydroxybenzo[a]pyrene, 5-hydroxybenzo[a]pyrene, B[a]P-trans-7,8-dihydrodiol (±), B(a)P-trans-9,10-dihydrodiol (±), B[a]P-r-7,t-8-dihydrodiol-c-9,10-epoxide(±) (syn-BPDE), and B[a]P-r-7,t-8-dihydrodiol-t-9,10-epoxide(±) (anti-BPDE) demonstrated the potential for the qualitative and quantitative analysis of PAH metabolites at trace concentration levels. The obtained enhancements for B[a]P metabolites provided limits of detection varying from 0.1 ng.mL$^{-1}$ (anti-BPDE (±)) to 0.8ng.mL$^{-1}$. Since B[a]P is the most toxic PAH in EPA list, four B[a]P metabolites were selected for chemometric studies. These included 1-Hydroxybenzo[a]pyrene, Benzo[a]pyrene-cis-4,5-dihydrodiol, Benzo[a]pyrene-r-7, t-8, t-9, c-10-tetrahydrotetroli (±/-) and Benzo[a]pyrene-r-7, t-8, t-9, t-10-tetrahydrotetroli (+/-). In all cases, 1-octanol was used as the Shpol’skii matrix. MCR-ALS, UPLS/RBL, and PARAFAC were tested for multi-way calibration purposes. These algorithms carry with them the higher order advantage, which refers to their ability to perform a “mathematical” separation, identification, and quantitation of analytes in complex samples with chemically unknown composition. Three-way data formats consisted of 77
K EEMs recorded with the spectrofluorimeter. Four-way data formats were 4.2 K TREEMs recorded with the instrumental system built in-house. In both cases, the best prediction abilities were obtained with PARAFAC. This algorithm was able to provide accurate determinations at the parts-per-billion concentration levels.
To Elham, my amazing wife
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CHAPTER 1 INTRODUCTION

1.1 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are important environmental pollutants originating from a wide variety of natural and anthropogenic sources. PAH are generally formed during incomplete combustion of organic matter containing carbon and hydrogen\(^1\)\(^-\)\(^7\). Chemical analysis of PAH is of great environmental and toxicological importance. The US Environmental Protection Agency (EPA) lists sixteen PAH as “Consent Decree” priority pollutants. Benzo[a]pyrene (B[a]P) is the most carcinogenic PAH on the EPA list and its concentration alone is often used as a measure of risk\(^8\)\(^-\)\(^16\). Human exposure to PAHs derives from a variety of sources such as air pollution from automobile, diesel and industrial or incinerator emissions, which may modulate a spectrum of other combustion, dietary and occupational sources\(^17\)\(^-\)\(^18\). The primary source of PAHs exposure in humans is through food consumption\(^10\),\(^12\),\(^15\),\(^19\)-\(^24\). Cereal and vegetables are the major dietary sources of PAHs, except when there is a high consumption of meat cooked over an open flame\(^10\),\(^12\),\(^15\),\(^20\),\(^22\),\(^24\)-\(^25\). More than 150 PAHs have been found in tobacco smoke\(^26\), which also contributes significantly to exposure to PAHs and to adverse health effects\(^20\),\(^27\). A less pervasive source of air pollution leading to PAHs exposure involves biomass burning from forest fires and peat bogs resulting from incidental incineration, or from deliberate forest cleaning strategies that expand beyond planned boundaries\(^28\). Wood combustion as an energy source for heating and/or cooking also generates a source of indoor PAHs pollution leading to health problems\(^22\). Occupational exposures may result from daily contact with intensive combustion processes such as iron and steel production\(^29\), coke production\(^30\), waste incinerators\(^7\), transportation maintenance\(^31\), mining exhaust exposure\(^32\) and aluminum smelters\(^33\), or from tobacco smoke in commercial venues such as bars,
restaurants and nightclubs\textsuperscript{34}. More intensive PAHs exposures in occupational environments – relative to the general population – have resulted in reports of lung, genitourinary and skin cancers\textsuperscript{20, 35-36}. In addition to inhalation, dermal absorption in occupational environments significantly contributes to exposure to PAHs\textsuperscript{36}.

To prevent human exposure to PAHs contamination, the EPA recommends the routine monitoring of the sixteen priority pollutants in air, soil and water samples. The molecular structures of sixteen EPA-PAHs are shown in Figure 1. Environmental analysis of PAHs follows the classical pattern of sample collection, PAH extraction and chromatographic analysis. High-performance liquid chromatography (HPLC) and gas chromatography (GC) are the basis for standard PAHs identification and determination. HPLC is often coupled to ultraviolet absorption and room temperature fluorescence detection\textsuperscript{11, 14, 19, 21, 24, 37, 38}. When HPLC is used to analyze “unfamiliar” samples, it is recommended to using a supporting analytical technique for compound identification. Compound identification and peak-purity check of HPLC fractions is usually pursued via gas chromatography-mass spectrometry (GC-MS)\textsuperscript{39-41}.\textsuperscript{2}
<table>
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<th>MW</th>
<th>PAH name</th>
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</tbody>
</table>

**Figure 1.** Molecular structures of EPA-PAHs.
1.2 Metabolites of Polycyclic Aromatic Hydrocarbons

Although routine monitoring of PAHs is a critical step in controlling sources of environmental pollution, it provides limited information on human health risks associated to PAHs intake. Parent PAHs are relatively inert and need metabolic activation to express their toxicity. Covalent binding of PAH metabolites to DNA appears to be the first critical step in the initiation of the tumor formation process\(^{42-44}\). By virtue of the rich heterogeneous distribution of metabolic products they produce, PAHs provide a full spectrum of the complexity associated with understanding the initial phase of carcinogenesis. For instance, a given metabolite may bind to different bases or different nucleophilic centers of a base and exist in distinctly different adduct-DNA conformations\(^{45-47}\). Metabolism may also lead to a distribution of stereoisomeric metabolites and, therefore, stereospecific additions to DNA.

A classic example is benzo[a]pyrene (B[a]P). This PAH, which is the most toxic PAH in the EPA priority pollutants list, is often used a measure of human risk to environmental contamination. Due to B[a]P potent carcinogen effects, its metabolic pathways have been studied extensively. Biotransformation starts with B[a]P binding to the cytochrome P450 enzyme system, which then catalyzes the addition of an oxygen atom across a double bond of the molecule to form arene oxides and phenols \(^{48-51}\). The arene oxides may arrange spontaneously to phenols or undergo hydration catalyzed by epoxide hydrolase, leading dihydrodiols. 7,8-Dihydro-7,8 dihydroxybenzo[a]pyrene is further oxidized to anti- and syn-7,8-dihydroxy-7,8,9,10tetrahydrobenzo[a]pyrene (BPDE) in a reaction catalyzed by P450s and other enzymes\(^{52-54}\). Figure 2 summarizes the main steps and metabolic products of B[a]P biotransformation in mammals.
Figure 2. Metabolic activation of Benzo[a]pyrene.
Among the four BPDE enantiomers produced in the biotransformation of B[a]P, the (7R,8S,9S,10R)-enantiomer of anti-BPDE is usually formed to the greatest extent and shows the highest carcinogenic activity\textsuperscript{52-55}. This enantiomer reacts with DNA producing a major adduct in which the exocyclic amino group of deoxyguanosine undergoes trans addition to carbon 10 of BPDE. Apparently, the formation of this DNA adduct is the major metabolic activation pathway of B[a]P\textsuperscript{52-57}. Other pathways of B[a]P metabolism lead predominantly to detoxification.

Numerous analytical approaches have been developed to monitor PAH metabolites in physiological fluids. Similar to PAH methodology, the analysis of metabolites in urine and blood samples follows the general pattern of sample clean up and pre-concentration, and separation prior to qualitative and quantitative determination. Separation of metabolites has been accomplished via high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and gas chromatography (GC)\textsuperscript{58-67}. This is not a trivial task as many metabolites often present similar chromatographic and electrophoretic behaviors. The selectivity of the approach becomes particularly relevant when targeting a distribution of stereo-isomeric metabolites and, therefore, stereo-specific additions to DNA.

High-resolution mass spectrometry (MS) either coupled to gas chromatography (GC-MS) or HPLC (HPLC-MS) provides the analyst with the highest specificity. Unfortunately, the existence of chemically related metabolic products with virtually identical fragmentation patterns still challenges the specificity of these techniques. A combination of chromatographic steps is often necessary that lead to time-consuming procedures. The numerous separation steps open ample opportunity to metabolite loss and collection of inaccurate data\textsuperscript{68-74}. 
1.3 Our Proposition

The ability to directly determine the presence and the amounts of targeted metabolites without the need of extensive chromatographic separation is our goal. Our proposition combines unique elements from three areas in analytical chemistry, namely the multidimensionality of photoluminescence spectroscopy, Shpol’skii spectroscopy (SS) and multiway calibration methods. The multidimensionality of photoluminescence spectroscopy refers to combining spectral and lifetime information on data formats known as wavelength time matrices (WTMs) and time-resolved excitation emission matrices (TREEMs). SS is an established photoluminescence technique capable to determine parent PAHs in environmental samples without previous chromatographic separation. Multiway calibration methods enable one to achieve direct (no chromatographic separation) qualitative and quantitative analysis of chemicals of interest (analytes) in complex systems of unknown composition, i.e. in the presence of uncalibrated species. This property is known as the 2nd order advantage. Simply put, multiway calibration methods that carry with them the 2nd order advantage are capable to perform a “mathematical” separation, identification, and quantitation of complex samples with chemically unknown composition.

1.4 Photoluminescence Spectroscopy

Photoluminescence spectroscopy is the generic nomination given to a group of fluorescence and phosphorescence techniques widely used for the identification and
quantitation of organic compounds in samples with environmental, pharmaceutical, clinical, and forensic relevance. When an analyte is inherently fluorescent and/or phosphorescent, excitation of photoluminescence provides a straightforward approach for the determination of analytes in samples of unknown composition. Direct photoluminescence techniques include solution Room Temperature Fluorescence (RTF)\textsuperscript{75}, Low-Temperature Fluorescence (LTF)\textsuperscript{76}, and Solid-Surface RTF (SS-RTF)\textsuperscript{77}. Direct phosphorescence approaches include Low-Temperature Phosphorescence (LTP)\textsuperscript{78}, Solid-Surface RTP (SS-RTP)\textsuperscript{79}, Micelle-stabilized RTP (MS-RTP)\textsuperscript{80}, and Sensitized RTP in Liquid Solutions\textsuperscript{81}. When the analyte is not fluorescent or phosphorescent, the main indirect methods that are used in this case are the following: (a) Derivatization, i.e. reaction of the analyte with a reagent leading to a fluorescent compound; which is often used in conjunction with liquid chromatography with fluorescence detection\textsuperscript{82}; (b) Formation of a fluorescent complex; which is the basis of most methods for ion and molecule recognition\textsuperscript{83}; and (c) Fluorescence quenching; which results from the collision of the analyte with a fluorescent compound. This method is particularly well suited to the detection of gases such as oxygen, SO\textsubscript{2}, H\textsubscript{2}S, ammonia, HCl, Cl\textsubscript{2}, etc\textsuperscript{84}. This dissertation deals with RTF and LTF.

The Jablonski diagram shown in Figure 3 is broadly used to illustrate the energetic processes involving organic molecules with inherent fluorescence and/or phosphorescence emission. Upon absorption of a photon with appropriate energy,
excitation leads the organic molecule to a single excited state with higher energy (S₂, S₁) than the ground singlet state (S₀).

Figure 3. Deactivation processes for an excited molecule. a, absorption; b, vibrational relaxation; c, internal conversion; d, fluorescence; e, external conversion; f, intersystem crossing; g, phosphorescence\textsuperscript{85}.
Via a combination of vibrational relaxation (VR) and internal conversion (IC), or just VR, excited molecules reach the lowest vibrational level of the first singlet excited state ($S_1$). VR and IC are non-radiative processes that lead to the de-activation of excited molecules via energy transferring to the surrounding medium. Thru VR, the dissipated energy goes into thermal or vibrational motion of the solvent molecules in condensed phases. This process usually proceeds in a stepwise fashion ($\Delta \nu = 1$) in which one vibrational quantum is lost per collision. This typically takes $10^{-11}$ to $10^{-10}$ s. Since a typical vibrational period is $10^{-13}$s, many vibrations occur before the excess vibrational energy is lost. IC is a crossover between two states of the same multiplicity, i.e. singlet to singlet ($S_2$ to $S_1$ or $S_1$ to $S_0$) or triplet to triplet ($T_n$ to $T_{n-1}$). It occurs when the potential energy curves of two electronic states cross, i.e. when the lower vibrational levels of the higher electronic state are approximately of the same energy as the higher vibrational levels of the lower electronic state. IC ultimately results in the conversion of excess electronic energy to excess vibrational energy. After IC, the excess vibrational energy is rapidly dissipated through vibrational relaxation to the ground vibrational level of the lower electronic state. IC between excited electronic states is rapid ($10^{-12}$s).

IC between $S_1$ and $S_0$ is less efficient if there is a wide energy separation between the two states. This condition favors the emission of fluorescence (F), which involves the return of excited molecules in the lowest vibrational level of $S_1$ to a vibrational level of $S_0$. This process is the basis of all the fluorescence techniques that are widely applied to analytical chemistry. The intensity of fluorescence emission and, therefore, the limits of
detection of the analytical method, depend – to same extent – on the ability of the analyst to experimentally restrict processes that compete with fluorescence for the deactivation of S₁. In addition to IC, these include intersystem crossing (ISC). ISC is a process in which there is a crossover between electronic states of different multiplicity (Singlet to Triplet). It is favored by the presence of heavy atoms in the molecular structure of the fluorophore (internal heavy atom effect) or in the chemical environment of the fluorophore (external heavy atom effect). Heavy atoms favor spin-orbital coupling perturbing the electron spins and enhancing state mixing between Singlet and Triplet manifolds.

Once molecules have reached the lowest vibrational level of the first triplet excited state (T₁), return to the ground electronic state (S₀) might occur with the emission of phosphorescence. This process is the basis for all the phosphorescence techniques applied to analytical chemistry. The achievement of competitive phosphorescence limits of detection depends - to some extent - on the analyst ability to inhibit competing processes for the de-activation of T₁. Due to the relatively long lifetime of triplet excited states (see Table 1), avoiding collisional deactivation of excited molecules in T₁ is particularly relevant to observing intense phosphorescence emission.
Table 1. Time scales of photoluminescence phenomena

<table>
<thead>
<tr>
<th>Phenomenon</th>
<th>Time Scale (s)</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption</td>
<td>$10^{-15}$</td>
<td>Radiative</td>
</tr>
<tr>
<td>Internal Conversion</td>
<td>$10^{-14}$-$10^{-11}$</td>
<td>Non-Radiative</td>
</tr>
<tr>
<td>Vibrational Relaxation</td>
<td>$10^{-14}$-$10^{-11}$</td>
<td>Non-Radiative</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>$10^{-10}$-$10^{-6}$</td>
<td>Radiative</td>
</tr>
<tr>
<td>Intersystem Crossing</td>
<td>$10^{-8}$-$10^{-3}$</td>
<td>Non-Radiative</td>
</tr>
<tr>
<td>Phosphorescence</td>
<td>$10^{-3}$-$10^{3}$</td>
<td>Radiative</td>
</tr>
</tbody>
</table>

The intensity of fluorescence emission is best described by the fluorescence quantum yield ($\phi_F$). In the absence of an external quencher, the fluorescence quantum yield (or fluorescence quantum efficiency) is given by the following equation:

$$\phi_F = \frac{k_F}{k_F + k_{ic} + k_{isc}}$$  \hspace{1cm} (1)

Where:

- $k_F =$ first order rate constant for fluorescence (photons.s$^{-1}$ or s$^{-1}$)

- $k_{ic} =$ first order rate constant for internal conversion (photons.s$^{-1}$ or s$^{-1}$)

- $k_{isc} =$ first order rate constant for intersystem crossing (photons.s$^{-1}$ or s$^{-1}$)

The ideal quantum yield approaches unity which, according to equation (1), is attained when non-radiative transitions (IC and ISC) are kept to a minimum. The molecular structure of the
fluorophore is the main factor contributing to the magnitude of $k_F$. Most unsubstituted aromatic hydrocarbons fluoresce in solution, with increasing fluorescence as the number of rings and the degree of condensation increases\(^8\). Molecules with rigid, fused ring structures, such as PAHs and PAH metabolites, also exhibit strong fluorescence. The other factors rely less on structure than on experimental factors such as the presence of paramagnetic species (like dissolved oxygen), temperature, and solvent effects.

The presence of species such as oxygen can lead to quenching of the fluorescence signal. Quenching generally refers to nonradiative energy transfers from excited species to other molecules. There are two main types of quenching, static and dynamic. Static quenching occurs when the quencher forms a stable complex with the ground state analyte molecule\(^8\). Dynamic quenching is a primary mechanism of external conversion and requires contact between the excited molecule and the quencher. Temperature and viscosity of a solution have a direct effect on the rate of dynamic quenching, which is diffusion controlled. The concentration of the quenching species must also be high enough to ensure a probability of collisions during the lifetime of the excited species. In the presence of an external quencher ($Q$), the fluorescence quantum yield is given by the following expression:

$$\phi_F = \frac{k_F}{k_F + k_{ic} + k_{isc} + k_q[Q]}$$

(2)

Where $k_q$ is the second order rate constant for quenching in $L.mol^{-1}s^{-1}$ and $[Q]$ is the concentration of the quenching species. At short excited-state lifetimes – such as those usually observed for fluorescence emission - dynamic quenching is usually negligible at concentrations of dissolved oxygen typically found in many solvents\(^8\).
In compounds such as PAHs and PAH metabolites where electronic transitions are typically \( \pi-\pi^* \), molar absorptivities are relatively higher than rate constants for intersystem crossing due to larger energy differences between singlet and triplet states. As such, more energy is required to unpair the \( \pi^* \) excited state electrons, and the overlap of triplet state vibrational levels with those in the singlet state is decreased, reducing the chances of intersystem crossing. The presence of heavy atoms in the chemical environment of the fluorophore will also act to reduce fluorescence by intersystem crossing. On the other end, reducing the sample temperature usually enhances fluorescence as collisions between excited molecules that may cause quenching are minimized. Increasing solvent viscosity leads to similar enhancements of fluorescence emission.\(^8\)

For optically homogeneous and transparent solutions as those frequently encountered in Shpol’skii spectroscopy, the intensity of fluorescence emission \( I_F \) is related to the intensity of excitation light \( I_0 \) by the following equation:

\[
I_F = \phi_F (I_0 - I_T)
\]

Where \( I_T \) is the intensity of the transmitted light.

For transparent media, \( I_T \) can be obtained from the Lambert Law, which is given by:

\[
\frac{I_T}{I_0} = 10^{-\varepsilon bc}
\]

Where \( \varepsilon \) is the molar absorptivity, \( c \) is the concentration of the chromophore, and \( b \) is the optical path or thickness of the sample. The equation above can be re-written as follows:

\[
I_F = I_0 \phi_F (1 - 10^{-\varepsilon bc})
\]

This expression can be expanded in a Taylor series to yield
\[ I_F = I_0 \phi_F [2.303 \varepsilon_{bc} - (2.303 \varepsilon_{bc})^2 + (2.303 \varepsilon_{bc})^3 + \ldots] \]

\[ \frac{2!}{3!} \]

For diluted solutions, i.e. \(2.303 \varepsilon_{bc} < 0.05\), all the subsequent terms in the brackets become negligible with respect to the first, so:

\[ I_F = 2.303 I_0 \phi_F \varepsilon_{bc} \]

This equation shows that the slope of an analytical calibration curve – i.e. \(I_F\) versus \(c\) – obtained from a fluorophore in a transparent and homogeneous medium is proportional to the fluorescence quantum yield of the fluorophore.

1.5 **Fluorescence Data Formats with Commercial Spectrofluorimeters**

RTF spectroscopy is a simple analytical technique that provides competitive analytical figures of merit with relatively inexpensive instrumentation. Since deoxygenation of the sample is not strictly necessary to avoid excessive fluorescence quenching in liquid solutions, fluorescence measurements are simply performed by pouring the liquid solution into a cuvette and placing the cuvette into the sample compartment of the spectrofluorimeter.

Commercial spectrofluorimeters are typically equipped with an excitation source, an excitation monochromator, a sample compartment, an emission monochromator and a detection unit consisting of a photon detector and a read-out system. The excitation source usually consists of xenon arc lamp operated at high pressure for continuum excitation between 200 and 1100 nm. Excitation and emission spectra are recorded with excitation and emission monochromators respectively, which consist of single channel spectrometers equipped with holographic gratins.
blazed for maximum diffraction efficiency in the excitation and emission wavelength regions. Typical wavelength regions for excitation and emission in commercial spectrofluorimeters range from 200 - 400 nm and 400 - 800 nm, respectively. The photodetector is often a photomultiplier tube (PMT) operating in the photon counting mode that converts the luminescence photon flux to an electrical signal that is amplified by the read out system and displayed on a computer screen as intensity (counts per second) versus wavelength.

Spectrofluorimeters with dual sample-reference measurement capability are also available commercially and may be used to compensate for instrumental distortions caused by intensity variations and wavelength dependence from the excitation source, diffraction efficiency of excitation and emission monochromators and wavelength dependence of detector response. Several accessories are commercially available to facilitate the collection excitation and emission spectra from solid samples. These solid sample holders are widely used in SS-RTF and SS-RTP.

The popular format of fluorescence spectroscopic data is a two-dimensional (2D) spectrum – i.e., a signal intensity versus wavelength plot - recorded within the excitation and emission regions of the fluorophore. Examples of 2D spectra recorded with a commercial spectrofluorimeter are shown Figure 4. 2D spectra are recorded by setting the excitation or the emission monochromator at the maximum excitation or emission wavelength of the fluorophore and scanning the other monochromator within the excitation or emission region of the fluorophore.
Figure 4. Excitation and fluorescence spectra of methanol–water 30–70 (v/v) solid-line and 10–90 (v/v) (dotted line) of (A) 2-hydroxyfluorene; (B) 9-hydroxy-phenanthrene; (C) 1-hydroxypyrene; (D) 6-hydroxy-chrysene; (E) 4-hydroxybenzo[a]pyrene; and (F) 3-hydroxybenzo[a]pyrene recorded with a commercial spectrofluorimeter.

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The main limitation of RTF spectroscopy for the analysis of analytes in complex fluorophore samples is the spectral overlapping that results from the broad nature of excitation and fluorescence spectra. A relatively simple way to reduce spectral overlapping from fluorescence concomitants is known as the Energy Constant Synchronous Fluorescence Spectroscopy (EC-SFS) approach. SFS refers to varying simultaneously both the excitation ($\lambda_{\text{exc}}$) and the emission ($\lambda_{\text{em}}$) wavelengths while keeping a constant wavelength difference ($\Delta \lambda = \lambda_{\text{em}} - \lambda_{\text{exc}}$) between them. The advantages of EC-SFS include spectral simplification, reduction of the spectral range for any given fluorophore and narrowing of spectral bandwidth. Additional selectivity can be obtained with first-derivative SFS spectra, which are usually calculated from SFS spectra in the post-acquisition mode. Examples of CE SF spectra and first-derivative SF spectra are shown in Figure 5.
Figure 5. SF spectra of a monohydroxy-PAH mixture in methanol recorded with a $\Delta\lambda = 7$ nm.

(A) Excitation and emission band-pass = 1 nm (A) and 2 nm (B). (C) First-derivative of synchronous spectra in (B). Metabolite mixture contains 10 ng.mL$^{-1}$ 3-hydroxybenzo[a]pyrene, 5 ng.mL$^{-1}$ 1-hydroxybenzo[a]pyrene, 20 ng.mL$^{-1}$ 2-hydroxyfluorene, 4-hydroxybenzo[a]pyrene and 6-hydroxychrysene.$^{86}$
Recording 2D fluorescence spectra and SF spectra from a mixture with numerous fluorophores only provides partial information on the total fluorescence of the sample. At any given excitation wavelength, the resulting profile of the fluorescence spectrum correlates to the selected excitation wavelength and its proximity – or not - to the excitation maxima of the individual fluorophores in the mixture. The relative contribution of each fluorophore to the total fluorescence spectrum of the sample also depends on the fluorescence quantum yield of each fluorophore and possible quenching due to synergistic effects. Excitation-emission matrices (EEMs) convolute all this information in a single data format with the individual contributions of all the fluorophores in the sample. EEMs represent a true signature of the total fluorescence of the sample.

Figure 6 illustrates the process of recording EEMs with a scanning spectrofluorimeter. Fluorescence spectra are recorded at several excitation wavelengths within the emission range of the sample. The resulting I by J data matrix (EEM) is then compiled from an array of two-dimensional fluorescence spectra while the excitation wavelength is increased incrementally between each scan. The excitation step and the wavelength range of excitation determine the total number of fluorescence spectra in the data matrix. Each I row in the EEM corresponds to the emission spectrum at the \( i \)th excitation wavelength. Each J column in the EEM corresponds to the excitation spectrum at the \( j \)th emission wavelength. For a single emitting species in a sample, the elements of the EEM are given by:

\[
M_{ij} = 2.303 \Phi_F I_0(\lambda_i) \varepsilon(\lambda_i) \text{optical density of the sample, which results from the product of the analyte’s molar absorptivity } \varepsilon(\lambda_i), \text{ the optical path-length b, and the concentration of the emitting}
\]
species c; $\Phi_F$ is the quantum yield of fluorescence; $\gamma(\lambda_j)$ is the fraction of fluorescence photons emitted at wavelength $\lambda_j$; and $\kappa(\lambda_j)$ is an instrumental factor that represents the wavelength dependence of the spectrofluorimeter’s sensitivity.

The condensed version of equation 1 may be expressed as:

$$M_{ij} = \alpha x_i y_j$$

where $\alpha = 2.303 \Phi_F b c$ is a wavelength independent factor containing all of the concentration dependence, $x_i = I_0(\lambda_i) \varepsilon(\lambda_i)$ and $y_i = \gamma(\lambda_j) \kappa(\lambda_j)$. The observed relative fluorescence excitation spectrum may be represented by $\{x_i\}$, the wavelength sequenced set, and thought of as a column vector, $x$ in $\lambda_i$ space. The wavelength sequenced set, $\{y_i\}$, may be thought of as a row vector $y$ in $\lambda_i$ space, representing the observed fluorescence emission spectrum. Therefore, for a single component, $M$ is simply represented as:

$$M = \alpha x y$$

Where $M$ is the product of the vectors $x$ and $y$ multiplied by the compound specific parameter $\alpha$.

When data is taken from a sample containing multiple, $r$, different species, $M$ is given by the following expression:

$$M = \sum_{k=1}^{r} \alpha_k x^k y^k$$

where $k$ is used to detail the species. For a recorded $M$, the spectral characterization of single components then relies on finding $r$, $\alpha_k$, $x^k$, and $y^k$. 
1.6 Shpol’skii Spectroscopy

An inherent limitation of RTF approaches towards the selectivity of analysis is the broad nature of excitation and emission spectra. The diffuse character of such spectra considerably reduces the possibility to directly determine targeted fluorophores in complex matrixes without previous chromatographic separation. The nature of band broadening is well known. For amorphous matrixes in the condensed phase, characterized by their lack of long-range order, the microenvironments that affect the transition energies of the solute molecules (predominantly through electron-electron interactions) differ from one solute molecule to another. The resulting differences in electronic transition energies lead to a Gaussian broadening of excitation and emission spectra. This type of band broadening is called inhomogeneous band broadening.
Homogeneous broadening affects all molecules to the same extent. It arises from vibronic coupling to the rapidly fluctuating surrounding matrix and from the limited lifetimes of the states involved in the electronic transition. In an ideal situation, i.e. when all solute molecules experience the same microenvironment, the only remaining source of band broadening (apart from instrumental contributions) is homogeneous.

A well-known approach to reduce inhomogeneous band broadening is Shpol’skii spectroscopy. This technique deals with fluorescence measurements at cryogenic temperatures where samples are frozen to 77K or below in a Shpol’skii matrix. The term Shpol’skii matrix refers to a dilute solution of a guest molecule (analyte) in a solvent host (usually an n-alkane) where the solvent freezes into an ordered polycrystalline matrix. If the dimensions of the analyte and the solvent match up well enough, guest molecules occupy a small number of crystallographic sites in the host matrix. Matrix isolation of analyte molecules reduces inhomogeneous band broadening, which results in vibrationally resolved excitation and emission (fluorescence and/or phosphorescence) spectra with sharp line widths.

Although Shpol’skii spectroscopy has been widely employed for the analysis of parent PAHs, Publications describing the use of Shpol’skii spectroscopy for PAHs metabolites are relatively rare. The poor compatibility between guest (PAH metabolite) and host (n-alkane) molecules leads to rather broad spectral bands, rapid photo-degradation and poor limits of detection. Previous attempts to improve guest-host compatibility have been based on chemical derivatization strategies that form less polar derivatives. A methylation procedure was described to transform monohydroxybenz[a]anthracenes into methoxy derivatives, which could then be analyzed in n-octane at the parts-per-trillion concentration level. Based on a signal-to-noise ratio of 3 (S/N = 3), a limit of detection (LOD) equivalent to 0.6 parts-per-trillion was
The methylation procedure was extended to monohydroxy-benzo[a]pyrene (B[a]P) metabolites, B[a]P-dihydriodiol, B[a]P-dihydrioolepoxides and B[a]P-tetrahydrotetrols. For a 20 µL sample volume, an absolute LOD of 50 attomole (S/N = 3) was reported for 9,10-dimetoxy-B[a]P, which was the permethylation product of B[a]P-9,10-diol. This dissertation extends the application of Shpol’skii spectroscopy to polar metabolites in polar organic solvents.

1.7 Multiway Calibration Methods

The main motivation for using multivariate calibration algorithms in analytical chemistry results from their ability to extract information of analytes directly – i.e. no physical separation – from complex samples of unknown composition. Depending on the structure of the data format, chemometric algorithms fall into first (vector), second (matrix) third (cube) or higher order methods. To some extent, the progression of the orders reflects the ability of the algorithm to provide reliable data from samples of increasing complexity.

2D spectra and SF spectra recorded at a single Δλ fit into the category of first order data. Calibration methods that process first order data – i.e. first order algorithms – can identify and quantify analytes in the presence of sample concomitants with potential interference. However, their accurate prediction requires the laborious preparation of extensive calibration data sets with all the potential interference present in the analytical sample.

A more attractive approach to handle matrix interference is process second or higher order data with chemometric algorithms that carry with them the “second order advantage”. This property refers to calibration methods capable to provide accurate identification and quantitation of analytes in the presence of un-calibrated interference. The application of second order calibration methods requires second-order or tri-order linear data, i.e. data describing each analyte
with a triad of invariant pure profiles. EEMs are examples of second-order data. Multiway calibration methods relevant to this dissertation include parallel factor analysis (PARAFAC), multivariate curve resolution – alternating least squares (MCR-ALS) and unfolded-partial least squares with residual bi-linearization (U-PLS-RBL)\textsuperscript{94,95}.

1.7.1 Parallel Factor Analysis (PARAFAC)

PARAFAC is an algorithm able to model four-way data arrays (X) which are obtained by assembling third-order data such as TREECs of size J (excitation wavelength) × K (emission wavelength) × L (time) for a set of I calibration samples, whose dimensions are [(I+1) × J × K × L]. For a proper PARAFAC modeling, it is of extreme importance that X follows a quadrilinear structure, which can be represented by Eq. (12)\textsuperscript{96}:

\[
X_{ijkl} = \sum_{n=1}^{N} a_{in} b_{jn} c_{kn} d_{ln} + e_{ijkl}
\] (12)

Where \(X_{ijkl}\) is an element of four-way array, \(a_{in}\) is the score of component n in sample i, \(N\) is the total number of responsive components, \(b_{jn}\), \(c_{kn}\) and \(d_{ln}\) are the loading elements in the excitation, emission and time dimensions, respectively, and \(e_{ijkl}\) is an element of the array of errors not fitted by the model.

The model described by Eq. (12) defines a decomposition of X which provides access to excitation (B) and emission spectral profile (C), time profile (D) and relative concentrations (A) of individual components in the (I+1) samples. Decomposition is usually implemented with an alternating least-squares minimization scheme\textsuperscript{97}. Relative concentrations allow performing a pseudo-univariate calibration plot, regressing them vs. the normal concentrations of standards, in order to predict analyte concentrations in unknown samples by projecting their relative concentration in the mentioned plot.
Unfolded partial-least squares (U-PLS) is an extended version of the first order data PLS algorithm, which makes it possible to operate with higher order data. The cube-structured data for each sample are transformed into vectors by unfolding them. In the calibration step, the concentration information included in the vector $y$ ($I \times 1$) is employed excluding the data for the unknown sample. Thus, a set of loading $P$ and weight loading $W$ ($JKL \times A$, where $A$ is the number of latent variables) as well as regression coefficients $v$ (size $A \times 1$) are obtained. The parameter $A$ is usually selected by the well-known leave-one-out cross-validation procedure. Analyte concentrations in unknown samples are predicted with $v$ using the following equations:

$$y_u = t_{u}^T v$$

(13)

Where $t_u$ size ($A \times 1$) is the unknown sample score, obtained by projection of the (unfolded) data for the test sample $\text{vec}(X_u)$ of size ($JKL \times 1$) onto the space of the $A$ latent factors:

$$t_u = (w^T P)^{-1} W^T \text{vec}(X_u)$$

(14)

If the sample under evaluation contains unexpected components, the scores gived by Eq. (14) will generate abnormally large residuals compared with the typical instrumental noise when the prediction is performed using Eq. (13). Fortunately, the effect of unexpected components in samples can be modeled with the RTL procedure through the Tucker3 decomposition. The latter is performed by minimizing the norm of the residual vector $e_u$, i.e. $s_u$, computed while fitting the sample data to the sum of the significant contributions to the sample signal. For a single interference, the expression can be formulated:

$$\text{vec}(X_u) = Pt_u + g_{\text{int}} (d_{\text{int}} \otimes c_{\text{int}} \otimes b_{\text{int}}) + e_u$$

(15)
Where \( \mathbf{b}_{\text{int}}, \mathbf{c}_{\text{int}} \) and \( \mathbf{d}_{\text{int}} \) are normalized profiles in the three modes for the interference and \( g_{\text{int}} \) is the first core element obtained for Tucker3 analysis of \( \mathbf{E}_p \) in the following way:

\[
(g_{\text{int}}, \mathbf{b}_{\text{int}}, \mathbf{c}_{\text{int}}, \mathbf{d}_{\text{int}}) = \text{Tucker3} (\mathbf{E}_p)
\]

The number of interference \( N_i \) can be assessed by inspection of the final residuals \( s_u \) (obtained from \( e_u \) of Eq. (15)) as a function of \( N_i \) until \( s_u \) stabilizes at a value that matches the experimental noise.

### 1.7.2 Multivariate Curve Resolution – Alternating Least Squares (MCR-ALS)

MCR-ALS is a soft-modelling technique that accomplishes the decomposition of multicomponent information of a data matrix by providing a chemically meaningful bilinear model of pure contributions of each component involved in the system\(^{101}\). In spectroscopy, the basic assumption of MCR-ALS is the validity of multicomponent Beer-Lambert law for the studied system\(^{100}\). The MCR-ALS analysis can be expressed as a bilinear additive model formed by \( c_i s_i^T \) dyads, where \( s_i^T \) is the instrumental response of an individual component weighted by related abundance \( c_i \). Decomposition is achieved by iterative optimization of the spectroscopic multicomponent data set expressed as the bilinear model

\[
\mathbf{D} = \mathbf{C} \mathbf{S}^T + \mathbf{E}
\]

Where \( \mathbf{C} \) and \( \mathbf{S} \) comprise the concentration and spectral profile, respectively, of all components involved in the system, and \( \mathbf{E} \) represent the error of variance left unexplained by the bilinear model\(^{102}\). One of the most compelling characteristics of MCR-ALS resolution is its general applicability without prior information about the system under study. However, to achieve chemically meaningful component profiles additional knowledge can be allocated. Decomposition of \( \mathbf{D} \) is obtained by iterative optimization of the initial estimates of either \( \mathbf{C} \) or \( \mathbf{S} \) by using the
provided knowledge about the system. This information is introduced through the implementation of chemical or mathematical constraints, such as non-negativity, unimodality, normalization and closure, among others.

1.7.3 Unfolded-Partial Least Squares with Residual Bi-Linearization (UPLS/RBL)

The first calibration stage of this algorithm consists of concentration information only from the standards; i.e. it excludes data from the unknown sample. The I calibration data matrices $X_{c,i}$ (size $J \times K$, where $J$ and $K$ are the number of channels in each dimension) are vectorized (unfolded) and used to calibrate the U-PLS model and the vector of calibration concentration $y(N_c \times 1$, where $N_c$ is the number of calibrated analytes). This phase provides a collection of loadings $P$ and weight loadings $W$ (both of size $JK \times A$, where $A$ is the number of latent factors) as well as regression coefficients $v$ (size $A \times 1$). The parameter $A$ is obtained with the leave-one-out cross-validation technique. When no unexpected interferences occur in the samples, the concentration of the analyte is determined via the following equation.

$$y_u = t_u^T v$$

(13)

Where $t_u$ is the test sample value, obtained by projecting the (unfolded) data for the $X_u$ test sample to the space of the $A$ latent factors:

$$t_u = (W^TP)^{-1}W^Tvec(X_u)$$

(14)

When unexpected constituents occur in $X_u$, the sample scores are given by Eq. (14) are not appropriate for analyte prediction with Eq. (13). The residuals of the U-PLS prediction step will be abnormally high compared to the usual instrumental noise. This situation can be addressed by a different technique called residual bilinearization (RBL), which is based on a singular value decomposition (SVD) modeling of interference effects. The objective of RBL is to minimize the residual vector norm $e_u$, computed while fitting the sample data to the sum of the related contributions to the sample signal. For one interferent:
vec(X_u) = Pt_u + vec[g_int b_int (c_int)ᵀ] + e_u  \tag{18}

Where b_int and c_int are the left and right eigenvectors of the E_p (a J × K matrix containing the residual U-PLS prediction step) and g_int is a scaling factor:

( g_int, b_int, c_int ) = SVD₁(E_p)  \tag{19}

Where SVD₁ indicates the process of taking only the first principle component. During the RBL process, P is kept constant at the calibration values and t_u is modified until ||e_u|| is minimized. Minimization can be achieved using a Gauss-Newton method starting with t_u from Eq. (18). When ||e_u|| has been reduced in Eq. (18), the concentrations of the analyte are given by Eq. (13) by introducing the final t_u vector defined by the RBL method. The number of unforeseen components N_unx can be calculated by comparing the final residues s_u with the instrumental noise level.

s_u = ||e_u||/[JK - (N_c + N_unx)]^{1/2}  \tag{13}

Where e_u is the same as the one in Eq. (18). The right number of components is obtained from the s_u plot versus the trail number of components. It begins at s_p - for a number of components equal to A - and displays decreasing values until it stabilizes at a value compatible with experimental noise.
CHAPTER 2 INSTRUMENTAL DEVELOPMENTS TO GENERATE MULTIDIMENSIONAL DATA FORMATS AT LOW TEMPERATURE

2.1 Cryogenic Fiber Optic Probes

Established methodology for 77 K measurements consists of immersing a solution-filled small-diameter tube into an optical Dewar filled with liquid nitrogen. Disadvantages of this approach include the fragility of the Dewar flask, bubbling in the cryogen at irregular intervals from small ice particles that act as nucleation sites, condensation on the outside of the Dewar, and -more importantly - scattering at each of the interfaces encountered by the excitation light on its way to the sample. The latter is a much more severe problem because it sends stray light in the emission monochromator which degrades the reproducibility of measurements and the limits of detection.

As shown in Figure 7, three air/glass interfaces, two liquid nitrogen/glass interfaces, and one matrix/glass interface are involved when using Dewar Flasks. The number of scattering interfaces can be reduced with closed-cycle refrigerators or Joule-Thomson miniature refrigerators that employ contact cooling or cold vapor directed onto the sample cell. Their disadvantages are higher cost, reduced sample throughput, and slower cooling of the sample. Freezing times can take between 40 and 100 min per sample. Several attempts have been made to improve closed-cycle helium refrigerators, which include a user friendly closed-cycle refrigerator that enables the simultaneous cooling of four 10 μL samples placed in a gold-plated copper holder surrounded with sapphire windows. 71
Figure 7. Light scattering interfaces of typical Dewar flasks.

We use fiber optic probes for cryogenic measurements at both 77 K and 4.2 K\textsuperscript{106, 107}. Our probes, which retain the simplicity of “dunking” the sample into the liquid cryogen for fast and reproducible freezing, eliminate all interfaces that could scatter excitation light into the detection system. Figure 8 shows example of cryogenic probes for measurements at 77 K and 4.2 K. At the sample end, the fibers are epoxied in a 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. After the sample is introduced into the sample tube, the tip of the probe is positioned above the solution surface as the sample tube is lowered into a
container filled with liquid cryogen. Prior to luminescence measurements, the cell cools down for approximately 90 s in the liquid cryogen to ensure complete sample freezing. Since there is no physical contact between the fiber and the sample, fiber clean up between samples is not strictly necessary. Preparing frozen samples for luminescence measurements at 77 K and 4.2 K is now a routine technique. Samples are frozen in a matter of seconds.

Signal intensities from individual frozen aliquots of the same sample range between 5 and 10%. Depending on the spectrometer, wavelength reproducibility can reach down to 0.1 nm. Figure 9 shows typical results obtained in our lab. When frozen in n-heptane, benzo[a]pyrene occupies four crystallographic sites with maximum fluorescence wavelengths at 402.3, 402.7, 403.1 and 403.8 nm. Molecules occupying the same crystallographic site produce identical excitation and emission spectra. Molecules occupying different sites produce identical spectral profiles, which are slightly shifted by small wavelength increases.
Figure 8. Fiber optic probes for cryogenic measurements at 77 K and 7.2 K.
Figure 9. 4.2 K fluorescence spectrum of 2 ng. mL⁻¹ benzo[a]pyrene in n-heptane. Spectrum was recorded in our lab with the 4.2 K fiber optic probe.
2.2 Instrumentation

Previous reports on laser excited Shpol’skii spectrometry added time-resolution to fluorescence measurements by using laboratory-constructed gated integrators or commercial boxcar averagers. Later reports utilized multichannel detection systems consisting of commercially available delay generators and intensified linear photodiode arrays to acquire emission spectra at good signal-to-noise much faster than it 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 degrade analytical figures of merit.

The first laser system developed in our lab for time resolved fluorescence measurements used a pulsed tunable dye laser, a pulsed delay generator, a spectrograph, and an intensifier-charged coupled device (ICCD). When coupled to cryogenic fiber optic probes, our system is well suited for the rapid collection of wavelength time matrices (WTMs) and time-resolved excitation emission matrices (TREEMs). 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. Time resolution is achieved with the intensifier in front of the ICCD, which acts as a superfast shutter with a minimum gate of 2 ns (full width at half-maximum). 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 LABVIEW software made in-house.
2.3 Multidimensional Data Formats

2.3.1 Wavelength Time Matrices (WTMs)

Figure 10 shows an example of a WTM recorded in our lab. It consists of a series of fluorescence spectra recorded under one excitation wavelength and different time delays after the laser excitation pulse. By setting the ICCD gate step parameter, our system automatically increments the time interval between successive fluorescence scans so that a WTM is easily built up. The duration of the steps by which the gate delay is progressively increased during the sequence of acquisitions is entered on the control computer with the LABVIEW software.

Recording WTMs during the fluorescence decay of the sample provides an additional parameter (lifetime) for PAH identification. Unambiguous fluorophore determination is made possible based on spectral and lifetime information. Fluorescence lifetimes also report on spectral peak purity, i.e. an essential condition for the accurate quantitative determination of fluorophores in complex samples of unknown composition\textsuperscript{109 - 115}. Fluorescence lifetimes are recorded via a three-step procedure: (1) full sample and background WTM collection; (2) Background decay curve subtraction from the fluorescence decay curve at a wavelength of maximum emission for each fluorophore; and (3) fitting of the background corrected data to single-exponential decays. Commercial software is used for curve fitting of fluorescence lifetimes. Fitted decay curves \( y = y_0 + A_1 e^{-(x-x_0)^2/R_1} \) are obtained by fixing \( x_0 \) and \( y_0 \) at a value of zero.
Figure 10. Center Top: 4.2 K WTM of 10 ng mL-1 benzo[a]pyrene in n-heptane recorded in our lab. Bottom left: 2-D fluorescence spectrum extracted from the WTM at the maximum wavelengths of each crystallographic site. Excitation wavelength was 366 nm. Delay and gate times were 20 ns and 200 ns, respectively. Spectrograph entrance slit was 25 µm. Arrow shows the characteristic fluorescence peaks (0-0 transition) of the four crystallographic sites occupied by benzo[a]pyrene in the frozen matrix.

<table>
<thead>
<tr>
<th>Site (nm)</th>
<th>Fluorescence Lifetime (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>402.3 ± 0.1</td>
<td>52.1 ± 0.9</td>
</tr>
<tr>
<td>402.7 ± 0.1</td>
<td>46.9 ± 0.5</td>
</tr>
<tr>
<td>403.1 ± 0.1</td>
<td>42.8 ± 0.9</td>
</tr>
<tr>
<td>403.7 ± 0.1</td>
<td>41.3 ± 1.2</td>
</tr>
</tbody>
</table>
2.3.2 Time-Resolved Excitation – Emission Matrices (TREEMs)

Recall that an EEM can be viewed as either a series of emission spectra taken at different excitation wavelengths. When EEMs are recorded with a commercial spectrofluorimeter, the third data dimension, fluorescence decay time, does not enter conventional EEMs because the excitation source is continuous. Now imagine a series of emission WTMs that are acquired for different excitation wavelengths. The complete data set consists of fluorescence intensity as a function of excitation wavelength, emission wavelength, and delay time after the short duration pulsed excitation. We refer to the entire data array as a time-resolved excitation-emission matrix (TREEM).

Figure 11 shows a schematic representation of the processes involved in the collection of TREEMs. When TREEMs are recorded as series of WTMs acquired at different excitation wavelengths, the superposition of the WTMs produces a third order data that we name excitation-modulated WTM (EMWTM) \(^{116}\). Alternatively, TREEMs can be recorded as series of EEMs acquired at different delay and gate times after the excitation pulse. All the intensity values - as a function of excitation and emission wavelengths - for a decay time are assembled into an EEM format specific for that decay time. The superposition of EEMs produces a third-order data that we name time-resolved excitation-emission cube (TREEC) \(^{117}\).
Figure 11. Schematic representation of (A) an excitation-modulated WTM (EMWTM); (B) a time-resolved excitation-emission cube (TREEC).

In comparison to EEMs, chemical analysis with TREEMs is more selective because it adds the temporal dimension to the orthogonal spectral dimension of EEM. TREEMs provide the analyst with the possibility to choose a time window – during the fluorescence decay of the sample – that minimizes the fluorescence interference from concomitants. A visual example is provided in Figure 12 with a synthetic mixture of benzo[a]pyrene and benzo[k]fluoranthene in n-octane. Figure 12 A shows the effect of temperature in the EEMs of the binary mixture. At room-temperature, the spectral features of these two PAHs overlap throughout the entire excitation and emission ranges of the EEMs. By lowering the temperature to 77K, it is possible to differentiate the spectral features of the two PAHs. As shown in the 2D spectra, baseline resolution of the vibrational transitions of Benzo[a]pyrene is only obtained at 4.2K. Since these three EEMs were
recorded using the same delay (10 ns) and gate (150 ns), there is no contribution of the time domain toward selectivity. The effect of time on the selectivity of EEMs is clearly noted in Figure 12 B. TREEMs were recorded from the same mixture at 4.2 K using a constant gate (150 ns) at four different delays (10, 22, 34 and 46 ns). The contribution of benzo[k]fluoranthene to the fluorescence of the mixture is completely removed from the TREEM by using a delay of 46 ns. Since the fluorescence lifetime of benzo[k]fluoranthene (8.9 ns) is much shorter than 46 ns, its fluorescence emission is no longer observed.

As previously mentioned, processing TREEMs with second order multivariate calibration algorithms provides an accurate solution to unpredictable spectral interference, a ubiquitous problem in samples of unknown composition. Pioneering work in our lab combined parallel factor analysis (PARAFAC) to 4.2K phosphorescence EMWTMs for the direct determination of 2,3,7,8-tetrachloro-dibenzo-para-dioxin in a heavily contaminated water sample of the American Petroleum Institute \textsuperscript{117}; 4.2K fluorescence TREECs for the analysis of the 15 EPA-PAHs in soil samples \textsuperscript{116}; and 4.2K fluorescence EEMs for the analysis of the 5 dibenzopyrene isomers in a coal tar sample extract (SRM 1597a) \textsuperscript{118}. 
Figure 12. 4.2 K EEMs (A) and TREEMs (B) recorded in our lab from a synthetic mixture of benzo[a]pyrene (B[a]P) and benzo[k]fluoranthene (B[k]F) in n-octane. Both PAHs were at the concentration of 10 ng.mL\(^{-1}\). \(\tau\) = fluorescence lifetimes. TREEMs were recorded using a 150 ns gate and four different delays (10, 22, 34 and 46 ns). Spectrograph entrance slit was 25 \(\mu\)m.
CHAPTER 3 EXPERIMENTAL AND INSTRUMENTATION FOR THE ANALYSIS OF PAH METABOLITES IN SHOPL’SKII LIKE MATRICES

3.1 Chemicals and Materials

Nanopure water from a Milli-Q® ultra-pure water system was used throughout. All chemicals were used as received. HPLC grade methanol, 1-octanol and n-octane were from Sigma-Aldrich. 1-Hydroxybenzo[a]pyrene, benzo[a]pyrene-cis-4,5-dihydrodiol, benzo[a]pyrene-r-7, t-8, t-9, c-10-tetrahydrodetrol (+/-) and benzo[a]pyrene-r-7, t-8, t-9, t-10-tetrahydrodetrol (+/-) were purchased from the Midwest Research institute (Kansas City, MO) at their highest available purity (99%).

3.2 Sample Preparation

Stock solutions were prepared by dissolving B[a]P metabolites in the appropriate solvent. All stock solutions were kept in the dark at 4 °C. Working solutions were prepared daily by serial dilution with methanol, octane or 1-octanol. All stock solutions were monitored frequently for possible photo degradation via ultraviolet-visible absorption spectroscopy and room-temperature fluorescence spectroscopy.

3.3 Room-Temperature Measurements

Room temperature absorption and fluorescence measurements were made by pouring un-
degassed liquid solutions into micro-quartz cuvettes (1 cm path length x 2 mm width) that held a maximum volume of 400 μL. Fluorescence emission was collected at 90° from excitation using appropriate cutoff filters to reject straight-light and second order emission.

3.4 77 K and 4.2 K Measurements

Low-temperature fluorescence measurements were performed with the aid of two fiber optic probes (FOPs). The spectrofluorimeter FOP consisted of two excitation and eight emission fibers. As previously described, the laser FOP consisted of one excitation and six emission fibers. All fibers were 2 m long and 500 μm core diameter, silica-clad silica with polyimide buffer coating (Polymicro Technologies, Inc.). In both FOPs, the fibers were fed into 1.2 m long sections of copper tubing that provided mechanical support for lowering the probes into the liquid cryogen. At the sample ends, the fibers were arranged either in a six-around-one or an eight-around-one configuration with the excitation fiber(s) in the center. At the instrument end, the six or eight emission fibers were position in a “slit” (vertical line) configuration. Vacuum epoxy was used to hold the fibers in place, which were then fed into metal sleeves for mechanical support. At the sample ends, the copper tubing sections were flared stopping phenolic screw caps threaded for 0.75 mL propylene sample vials.

Sample procedure with the FOPs was as follows: after microliter volumes (100 – 750 μL) of un-degassed sample solution were pipetted into the sample vial, the tip of the FOP was positioned and held constant with the screw cap below the solution surface. Sample freezing was accomplished by lowering the copper tubing into the liquid nitrogen (77 K) of liquid helium (4.2 K). Liquid nitrogen and liquid helium were held in two separate Dewar containers with 5 L and 60 L storage capacity, respectively. The 60 L liquid helium volume would typically last three weeks of daily use, averaging 15-20 samples per day. Complete sample freezing took less than 90 s. The
~ 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 organic solvent, and drying it with warm air from the heat gun. The entire freeze, thaw, and clean up cycle took less than 5 min per sample.

3.5 Instrumentation

3.5.1 Room-Temperature Absorption Measurements

Absorption measurements were made with a single-beam spectrophotometer (model Cary 50, Varian) equipped with a 75W pulsed xenon lamp, 2 nm fixed band-pass and maximum scanning rate of 24,000 nm.min\(^{-1}\). All spectra were recorded using a 600 nm.min\(^{-1}\) scanning speed.

3.5.2 Steady-State Fluorescence Measurements

Steady-state fluorescence measurements were performed with a FluoroMax-P spectrofluorimeter (Horiba Jobin-Yvon) equipped with a continuous 100 W pulsed xenon lamp with broadband illumination in the ultraviolet and visible spectral regions. The excitation and emission monochromators had the same reciprocal linear dispersion (4.2 nm·mm\(^{-1}\)) and accuracy (±0.5 nm). Both monochromators were equipped with diffraction gratings containing the same number of grooves per unit length (1200 grooves·mm\(^{-1}\)). The excitation grating was blazed at 330nm and emission grating at 500nm. The photomultiplier tube (Hamamatsu, model R928) had a spectral response ranging from 185 to 650 nm. It was operated at room temperature operated in the photon-counting mode. Commercial software (DataMax version 2.20, Hriba-Jobin-Yvon) was used to computer-control the spectrofluorimeter.

The cryogenic probe was coupled to the sample compartment of the spectrofluorimeter with the aid of a commercial fiber optic mount (F-3000, Horiba Jobin-Yvon) that optimized the collection efficiency of photons via two concave mirrors. Alignment of each FOP end – i.e.
excitation fiber and emission bundle - with the respective focusing mirror was facilitated by commercially available adapters (Horiba Jobin-Yvon) 121.

3.5.3 Time-Resolved Fluorescence Measurements

Time-resolved fluorescence measurements were carried out with an instrumental set-up recently built in-house 122. With the previous system 108, excitation energy was generated by directing the output of a tunable dye laser through a frequency-doubling crystal. Its main drawback was the narrow range of excitation wavelengths that one could achieve with a single fluorescence dye. Each dye change involved flushing the previous dye, adding the new dye, and readjusting the doubling optics. In some cases, it was not possible to reach maximum excitation wavelengths because their values would fell between the ranges of dyes.

In these studies, instead of the frequency doubled tunable dye laser, we use an optical parametric oscillator (OPO)-based wavelength tuning laser that covers the whole excitation range of PAHs continuously and quickly. Additional modifications include the replacement of the previous multichannel detection system with a new spectrograph and ICCD. Figure 13 shows a schematic diagram of our new system. A Radiant 355 LD UV pulsed tunable laser system (OPOTek Inc.) provided sample excitation from 210 to 2500 nm. A half-wave plate and a polarizer cube controlled the intensity of the laser radiation sent to a neutral density filter that blocked the residuals of the laser output at 1064 nm, 532 nm and 355 nm. After passing through a digitally controlled shutter, the portion of laser light transmitted by the density filter entered the excitation fiber of the laser FOP placed on a translation stage that allowed fine movements to achieve horizontal (X) and vertical (Y) alignment with the laser beam. A focusing lens reduced the spot size of the laser beam to the inner diameter of the fiber and helped to maximize optical throughput into the sample. The six-emission bundle of the laser FOP was mounted in a XY translational stage
that had an additional mode of rotation to allow for alignment into the entrance slit of the
spectrograph (Shamrock; Andor). The spectrograph was equipped with a diffractive grating (1200
grooves.mm⁻¹) blazed at 500 nm and attached to an iStar ICCD (Andor) with an active area of 690
x 256 pixels, pixel size of 26 microns, and a well depth of 500,000 electrons. Data acquisition and
instrument control was made possible with the aid of LABVIEW software developed in-house.

Fluorescence lifetimes were obtained as previously described; i.e. from emission decays via a
three-step procedure that included (1) recording WTM from individual standards and n-octane
(background decay) at the maximum excitation wavelength of each PAH; (2) subtracting the
background decay from the fluorescence decay at the maximum fluorescence wavelength of the
PAH; and (3) fitting the background corrected data to single exponential decays.
**Figure 13.** Laser-based instrumentation for the analysis of PAH metabolites in frozen matrixes. 

ICCD = Intensified Charge-Coupled Device. FOP = fiber optic probe.  

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CHAPTER 4  RESULTS AND DISCUSSION

4.1 Initial Survey of Excitation and Fluorescence Spectra

Previous efforts in our group have investigated the RTF of mono-hydroxy PAH metabolites on solid-phase extraction membranes.\textsuperscript{123–127} Metabolites were extracted from urine samples with octadecyl silica membranes and determine directly on the surface of the membrane via RTF spectroscopy. Due to the strong fluorescence emitted by the metabolites on the extraction membranes, limits of detection (LODs) at the pg.mL\textsuperscript{-1} level were obtained for all the studied mono-hydroxy PAHs. The main limitation of solid-phase extraction – RTF (SPE-RTF) is its limited selectivity. The spectral overlapping of fluorophores co-extracted from urine samples prevent the accurate determination of targeted metabolites in samples of unknown composition. We have improved the selectivity of SPE-RTF by processing either EEMs or total synchronous fluorescence spectra with unfolded-partial least squares/residual bi-linearization (UPLS/RBL). EEMs and TSF spectra were recorded under steady-state conditions with the aid of a commercial spectrofluorimeter. In a later study, we added the temporal dimension to EEMs by recording TREEMs with the aid of the multidimensional luminescence system built in-house.\textsuperscript{128} TREEMs were recorded from the surface of extraction membranes at 77 K with a cryogenic fiber optic probe.

The research presented here extends the applicability of SS to the analysis of PAH metabolites in primary alcohols. As a way of improving the solubility of PAH metabolites in frozen matrixes, recent studies in our lab tested a series of primary alcohols (RCH\textsubscript{2}OH) as solvent hosts. By optimizing the length of the alkyl group (R), our expectation was to minimize the number of crystallographic sites occupied by metabolite molecules in the crystal lattice of the frozen alcohol. We thought that the combination of these two effects could lead to spectral narrowing and strong fluorescence emission for the trace determination of PAH metabolites.
Figure 14 depicts the molecular structures of the studied metabolites. The spectral narrowing and the fluorescence enhancements observed for 1-hydroxypyrene, 2-hydroxyfluorene, 9-hydroxyphenanthrene, 3-hydroxybenzo[a]pyrene (3-OHB[a]P), 4-hydroxybenzopyrene (4-OHB[a]P), 5-hydroxybenzopyrene (5-OHB[a]P), B[a]P-trans-7,8-dihydriodiol (±), B[a]P-trans-9,10-dihydriodiol (±), B[a]P-r-7,t-8-dihydriodiol-c-9,10-epoxide(±) (syn-BPDE), and B[a]P-r-7,t-8-dihydriodiol-t-9,10-epoxide(±) (anti-BPDE) showed potential for the qualitative and quantitative analysis of PAH metabolites via SS. The obtained enhancements for B[a]P metabolites provided 4.2K LODs varying from 0.1 ng.mL⁻¹ (anti-BPDE (±)) to 0.8 ng.mL⁻¹ (3-OHB[a]P).¹²⁹

For the purposes of this dissertation, which centers on the evaluation of chemometric approaches for the qualitative and quantitative analysis of PAH metabolites with strong spectral and/or lifetime overlapping, we selected the following B[a]P metabolites: 1-hydroxybenzo[a]pyrene (BaP-1-ol), Benzo[a]pyrene-cis-4,5-dihydriodiol (BaP-cis-4,5-diol), benzo[a]pyrene-r-7, t-8, t-9, c-10-tetrahydrotetrol (+/-) (BaP-rttc-tetrol) and benzo[a]pyrene-r-7, t-8, t-9, t-10-tetrahydrotetrol (+/-) (BaP-rttt-tetrol). Their molecular structures are highlighted in Figure 14.

The n-alkane that provides the best spectral narrowing for B[a]P at 77 K and 4.2 K is n-octane.¹³⁰ Therefore, all the studies with B[a]P metabolites were performed with standard solutions and synthetic mixtures prepared in 1-octanol. The effect of the position of the OH- group in the C8 alkyl chain of the alcohol – i.e. secondary alcohol, tertiary alcohol, and so forth – has not been investigated yet.
Figure 14. Molecular structures of PAH metabolites studied in our lab. Highlighted structures refer to the B[a]P metabolites selected for the chemometric studies in this dissertation.
Appendix A shows the room temperature, 77 K and 4.2 K excitation and fluorescence spectra of BaP-1-ol, BaP-cis-4,5-diol, BaP-rttc-tetrol and BaP-rttt-tetrol recorded with the commercial spectrofluorimeter using a 2nm excitation and emission band-pass. Narrower band-pass values did not improve spectral resolution of any of the studied metabolites.

Table 2 summarizes the effect of temperature on the full width at half maximum (FWHM) of the studied metabolites in 1-octanol recorded with the spectrofluorimeter. Lowering the temperature to 77 K or 4.2 K narrowed the excitation and fluorescence spectra of the four metabolites. In addition, when compared to room temperature spectra, the 77 K and 4.2 K spectra show additional vibrational features that add qualitative information for identification purposes. On the other end, not much improvement was observed when lowering the temperature of analysis from 77 K and 4.2 K. Based on the spectral narrowing observed at cryogenic temperatures, all further measurements were made at 77K and 4.2K.

Table 3 reports the effect of temperature in the fluorescence intensities of the studied metabolites. All measurements were made with the spectrofluorimeter under steady-state conditions. Fluorescence intensities were recorded at the maximum excitation and fluorescence wavelengths of each metabolite. Lowering the temperature to 4.2K enhanced the fluorescence emission of BaP-1-ol, BaP-cis-4,5-diol and BaP-rttc-tetrol. Their fluorescence enhancements ($I_F^{4.2K} / I_F^{77K}$) varied from ~1.56 (BaP-1-ol) to ~1.92 (BaP-rttc-tetrol). The $I_F^{4.2K} / I_F^{77K}$ of BaP-rttt-tetrol was ~ 1.01. Since the fluorescence enhancements were not significant (i.e., at least one order of magnitude) for any of the studied metabolites, we pursued the analytical figures of merit at both 77 K and 4.2 K.
Table 2. Effect of Temperature on the Full Width at Half-Maximum \(^a\) of PAH Metabolites

Measured with a Commercial Spectrofluorimeter

<table>
<thead>
<tr>
<th>Metabolites (^b)</th>
<th>Full-Width at Half Maximum (nm)</th>
<th>Room Temperature ((\lambda_{ex}/\lambda_{em})) (^c)</th>
<th>77K ((\lambda_{ex}/\lambda_{em})) (^c)</th>
<th>4.2K ((\lambda_{ex}/\lambda_{em})) (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP-1-ol</td>
<td></td>
<td>20.33 ± 1.11 (408/427)</td>
<td>3.93 ± 0.02 (409/420)</td>
<td>3.25 ± 0.04 (409/420)</td>
</tr>
<tr>
<td>BaP-CIS-4,5-diol</td>
<td></td>
<td>5.2 ± 0.43 (275/365)</td>
<td>4.96 ± 0.05 (276/365)</td>
<td>4.93 ± 0.05 (276/385)</td>
</tr>
<tr>
<td>BaP-r7-t8-t9-c10-tetrol</td>
<td></td>
<td>13.84 ± 0.59 (345/398)</td>
<td>2.80 ± 0.05 (347/396)</td>
<td>2.90 ±0.05 (347/396)</td>
</tr>
<tr>
<td>BaP-r7-t8-t9-t10-tetrol</td>
<td></td>
<td>14.40 ± 0.71 (346/399)</td>
<td>4.63 ± 0.12 (347/396)</td>
<td>4.0 ± 0.56 (347/396)</td>
</tr>
</tbody>
</table>

\(^a\) Full width at half maximum was measured at the fluorescence peak with maximum intensity from pure standard solutions at medium linear concentrations, namely 50 ng/ml BaP-1-ol, 50 ng/ml BaP-cis-4,5-diol, 50 ng/ml BaP-r7-t8-t9-c10, and 50 ng/ml BaP-r7-t8-t9-t10. Reported values correspond to the average of three excitation and emission spectra recorded from individual aliquots. Excitation and emission band-pass = 2nm.

\(^b\) Metabolite concentrations were as follows: 50 ng/ml BaP-1-ol, 50 ng/ml BaP-cis-4,5-diol, 50 ng/ml BaP-r7-t8-t9-c10, and 50 ng/ml BaP-r7-t8-t9-t10.

\(^c\) Excitation and emission maxima. All measurements were made at the maximum excitation wavelength of each metabolite.
Table 3. Effect of Temperature on the Fluorescence Intensity of PAH Metabolites Measured with a Commercial Spectrofluorimeter

<table>
<thead>
<tr>
<th>Metabolites b</th>
<th>Fluorescence Intensity a (cps)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>77K ( (\lambda_{ex}/\lambda_{em}) ) c</td>
</tr>
<tr>
<td>BaP-1-ol</td>
<td>( (6.16 \pm 0.03) \times 10^5 ) (409/420)</td>
</tr>
<tr>
<td>BaP-CIS-4,5-diol</td>
<td>( (2.28 \pm 0.09) \times 10^5 ) (276/385)</td>
</tr>
<tr>
<td>BaP-r7-t8-t9-c10-tetrol</td>
<td>( (1.35 \pm 0.05) \times 10^4 ) (347/396)</td>
</tr>
<tr>
<td>BaP-r7-t8-t9-t10-tetrol</td>
<td>( (4.78 \pm 0.04) \times 10^5 ) (347/396)</td>
</tr>
</tbody>
</table>

a Fluorescence intensity was measured from pure standard solutions at medium linear concentrations, namely 50 ng/ml BaP-1-ol, 50 ng/ml BaP-cis-4,5-ol, 50 ng/ml BaP-r7-t8-t9-c10, and 50 ng/ml BaP-r7-t8-t9-t10.

b Reported values are the average intensities recorded from three frozen aliquots of the same standard solution.

c Excitation and emission maxima in nm. All measurements were made at the maximum excitation and emission wavelength of the fluorophore.
4.2 Analytical Figures of Merit Obtained with the Spectrofluorimeter

Appendix B compiles the 77 K and 4.2 K calibration curves of the studied metabolites obtained from standard solutions in 1-octanol. Signal intensities were measured at the maximum excitation and fluorescence wavelength of each metabolite. All fluorescence intensities plotted in the calibration curves corresponded to the average of three independent measurements taken from three frozen aliquots. The best straight line within the experimental points was obtained via the least-squares method.

Table 4 and Table 5 summarize the analytical figures of merit of the four metabolites at the two cryogenic temperatures. The correlation coefficients ($R^2$) close in unity demonstrate the linear relationships that exist between fluorescence intensities and the metabolites concentrations. The limits of quantitation (LOQs) were calculated using the formula $10s_B / m$; where $s_B$ is the standard deviation of 16 blank (1-octanol) measurements and $m$ is the slope of the linear dynamic range (LDR) of the calibration curve. No efforts were made to obtain the upper concentration limits of the linear dynamic ranges. The limits of detection (LODs) were calculated with the formula $3s_B / m$. In all cases, it was possible to reach parts-per-billion concentration levels at both temperatures. The relative standard deviations (RSDs) of three fluorescence measurements at medium linear concentrations varied between 0.8 % (B[a]P-r7,t8,t9,t10-tetrol at 77 K) and 5.7% (B[a]P-r7,t8,t9,c10-tetrol at 4.2 K). Clearly, using 1-octanol as the Shpol’skii matrix makes the quantitative analysis of B[a]P metabolites at trace concentration levels possible.

Since lowering the temperature to 4.2 K slightly improved the LOQs and the LODs of B[a]P-1-ol, B[a]P-cis-4,5-diol and B[a]P-r7,t8,t9,t10-tetrol, LETRSS measurements with the MLS were only performed at 4.2K.
Table 4. 77 K Analytical Figures of Merit Obtained with the Commercial Spectrofluorimeter

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$\lambda_{\text{ex}}$/$\lambda_{\text{em}}$ (nm)</th>
<th>LDR$^b$ (ng/mL)</th>
<th>$R^2$ $^c$</th>
<th>LOQ$^d$ (ng/mL)</th>
<th>LOD$^e$ (ng/mL)</th>
<th>RSD$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]P-1-ol</td>
<td>409/420</td>
<td>0.21-50</td>
<td>0.998</td>
<td>0.21</td>
<td>0.06</td>
<td>1.6</td>
</tr>
<tr>
<td>B[a]P-cis-4,5-diol</td>
<td>276/385</td>
<td>1.48-50</td>
<td>0.996</td>
<td>1.48</td>
<td>0.44</td>
<td>1.2</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,c10-tetrol</td>
<td>347/397</td>
<td>0.21-50</td>
<td>0.999</td>
<td>0.21</td>
<td>0.07</td>
<td>1.7</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,t10-tetrol</td>
<td>347/397</td>
<td>1.48-50</td>
<td>0.992</td>
<td>1.48</td>
<td>0.44</td>
<td>0.8</td>
</tr>
</tbody>
</table>

$^a$ $\lambda_{\text{ex}}$/$\lambda_{\text{em}}$ = Excitation and emission maxima.

$^b$ LDR = Linear dynamic range.

$^c$ R = Correlation coefficient.

$^d$ LOQ = Limit of quantitation. See text for definition.

$^e$ LOD = Limit of detection. See text for definition.

$^f$ RSD = Relative standard deviation. RSD values calculated as $S/I_F \times 100$; where $S$ is the standard deviation of 3 signal intensities measured from independent frozen aliquots and $I_F$ is the average intensity of the 3 measurements at medium linear concentrations.
### Table 5. 4.2 K Analytical Figures of Merit Obtained with the Commercial Spectrofluorimeter

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$\lambda_{ex}$/$\lambda_{em}$</th>
<th>LDR $^b$ (ng/mL)</th>
<th>$R^2$ $^c$</th>
<th>LOQ $^d$ (ng/mL)</th>
<th>LOD $^e$ (ng/mL)</th>
<th>RSD $^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]P-1-ol</td>
<td>409/420</td>
<td>0.07-50</td>
<td>0.992</td>
<td>0.07</td>
<td>0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>B[a]P-cis-4,5-diol</td>
<td>276/385</td>
<td>0.77-50</td>
<td>0.996</td>
<td>0.77</td>
<td>0.23</td>
<td>2.1</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,c10-tetrol</td>
<td>347/396</td>
<td>0.93-50</td>
<td>0.970</td>
<td>0.93</td>
<td>0.28</td>
<td>5.7</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,t10-tetrol</td>
<td>347/397</td>
<td>0.77-50</td>
<td>0.996</td>
<td>0.77</td>
<td>0.23</td>
<td>1.2</td>
</tr>
</tbody>
</table>

$^a$ $\lambda_{exc}/\lambda_{em}$ = Excitation and emission maxima.

$^b$ LDR = Linear dynamic range.

$^c$ R = Correlation coefficient.

$^d$ LOQ = Limit of quantitation. See text for definition.

$^e$ LOD = Limit of detection. See text for definition.

$^f$ RSD = Relative standard deviation. RSD values calculated as $S/I_F \times 100$; where $S$ is the standard deviation of 3 signal intensities measured from independent frozen aliquots and $I_F$ is the average intensity of the 3 measurements at medium linear concentrations.
4.3 4.2K Analytical Figures of Merit of B[a]P Metabolites via Laser Excited-Time-Resolved Shopl’skii Spectroscopy

Appendix C compiles examples of WTM recorded from standard solutions of the studied metabolites. All WTM recorded with an initial delay of 5 ns and a gate width of 250 ns. The 5 ns delay was sufficient to avoid convolution of the laser pulse with the metabolite signal. The 250 ns gate width was long enough to collect most of the metabolite emission but still avoid the contribution of instrumental noise. Each WTM consisted of 50 fluorescence spectra recorded at 30 ns gate steps during a total decay of 1500 ns. Each spectrum in a WTM was averaged over 20 laser pulses.

Appendix D compiles the calibration curves obtained from the WTM. Signal intensities plotted in the calibration graphs correspond to the maximum intensities of the first spectra of the WTM. No efforts were made to experimentally measure the highest possible linear concentrations of the calibration curves. In all cases, the correlation coefficients were close to unity, demonstrating a linear relationship between fluorescence intensity and metabolite concentration. The relative standard deviations show excellent reproducibility of measurements at the parts per billion-concentration level.

Table 6 summarizes the 4.2 K LETRSS AFOMs of the studied metabolites. The LOQs and LODs were calculated with the formula k x s_{blank} /m; where s_{blank} is the standard deviation of 16 1-octanol measurements at the maximum excitation and fluorescence wavelength of each metabolite, m is the slope of the calibration curve and k was either 10 or 3, respectively. In all cases, the LOQs and the LODs were in the sub-parts-per-billion or parts-per-trillion concentration levels. The RSDs
at medium linear concentrations varied between 0.7 % (B[a]P-1-ol) and 6.1 % (B[a]P-r7,t8,t9,c10-tetrol). When compared to the typical reproducibility of fluorescence measurements (10 – 15 %), these RSD values are excellent. The high precision of measurements results from the use of cryogenic fiber optic probes.

Table 7 compares the LODs obtained with the spectrofluorimeter at 77 K (LOD_{SPF}^{77K}) and 4.2 K (LOD_{SPF}^{4.2K}) to those obtained with the MLS at 4.2 K (LOD_{MLS}^{4.2K}). When compared to the LOD_{SPF}^{77K}, the LOD improvements with the MLs varied between 1.38 times (B[a]P-cis-4,5-diol) and 5.5 times (B[a]P-r7,t8,t9,t10-tetrol). When compared to the LOD_{SPF}^{4.2K}, the MLS provided LOD improvements only for B[a]P-r7,t8,t9,c10-tetrol (14 times) and B[a]P-r7,t8,t9,t10-tetrol (2.88 times). B[a]P-1-ol and B[a]P-cis-4,5-diol showed better 4.2 K LODs with the spectrofluorimeter. Since LODs are only estimates of detectable concentrations that carry with them approximately 33% of statistical variation \(^{131}\), the only metabolite that showed a significant improvement at 4.2 K with the MLS was B[a]P-r7,t8,t9,c10-tetrol.
Table 6. 4.2 K Analytical Figures of Merit Obtained with the MLS

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$\lambda_{ex}/\lambda_{em}$ a (nm)</th>
<th>LDR b (ng/mL)</th>
<th>$R^2$ c</th>
<th>LOQ d (ng/mL)</th>
<th>LOD e (ng/mL)</th>
<th>RSD f (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]P-1-ol</td>
<td>409.0/421.7</td>
<td>0.11-50</td>
<td>0.997</td>
<td>0.11</td>
<td>0.03</td>
<td>0.7</td>
</tr>
<tr>
<td>B[a]P-cis-4,5-diol</td>
<td>326.5/384.7</td>
<td>0.99-50</td>
<td>0.996</td>
<td>0.99</td>
<td>0.32</td>
<td>5.1</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,c10-tetrol</td>
<td>347.5/375.9</td>
<td>0.07-50</td>
<td>0.993</td>
<td>0.07</td>
<td>0.02</td>
<td>6.1</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,t10-tetrol</td>
<td>346.0/376.2</td>
<td>0.28-50</td>
<td>0.998</td>
<td>0.28</td>
<td>0.08</td>
<td>1.0</td>
</tr>
</tbody>
</table>

a $\lambda_{ex}/\lambda_{em}$ = Excitation and emission maxima.
b LDR = Linear dynamic range.
c $R = $ Correlation coefficient.
d LOQ = Limit of quantitation. See text for definition.
e LOD = Limit of detection. See text for definition.
f RSD = Relative standard deviation. RSD values calculated as $S/I_F \times 100$; where $S$ is the standard deviation of 3 signal intensities measured from independent frozen aliquots and $I_F$ is the average intensity of the 3 measurements at medium linear concentrations.
Table 7. Comparison of LODs Obtained with the Spectrofluorimeter (LOD_{SPF}) and the MLS (LOD_{MLS})

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>LOD_{SPF}^{77K}</th>
<th>LOD_{SPF}^{4.2K}</th>
<th>LOD_{MLS}^{4.2K}</th>
<th>LOD_{SPF}^{77K} / LOD_{MLS}^{4.2K}</th>
<th>LOD_{SPF}^{4.2K} / LOD_{MLS}^{4.2K}</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]P-1-ol</td>
<td>0.06</td>
<td>0.02</td>
<td>0.03</td>
<td>2</td>
<td>0.67</td>
</tr>
<tr>
<td>B[a]P-cis-4,5-diol</td>
<td>0.44</td>
<td>0.23</td>
<td>0.32</td>
<td>1.38</td>
<td>0.72</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,c10-tetrol</td>
<td>0.07</td>
<td>0.28</td>
<td>0.02</td>
<td>3.5</td>
<td>14</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,t10-tetrol</td>
<td>0.44</td>
<td>0.23</td>
<td>0.08</td>
<td>5.5</td>
<td>2.88</td>
</tr>
</tbody>
</table>
4.4 Comparison of Spectral Features Obtained with the Commercial Spectrofluorimeter and the MLS

Table 8 compares the 4.2 K full width at half maximum of the studied metabolites recorded with the spectrofluorimeter and the MLS. Except for B[a]P-cis-4,5-diol, all the other metabolites showed narrower FWHM with the MLS. This outcome was somehow expected due to the spectral resolutions of the two instruments. Spectra with the spectrofluorimeter were recorded with a 2 nm excitation and emission bandpass. Spectra with the MLS were recorded with an excitation bandwidth of 0.2 nm (OPO laser) and the narrowest possible slit width of the spectrometer (10 μm). When measured with a mercury lamp at 435.8nm and considering the crosstalk of 4 pixels on the chip of the CCD, the 10 μm spectrometer slit width provides a limiting resolution of 0.23 nm.
Table 8. Comparison of the 4.2 K Full-Width at Half Maximum Obtained with the spectrofluorimeter (FWHM$_{\text{SPF}^{4.2K}}$) and the MLS (FWHM$_{\text{MLS}^{4.2K}}$)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>FWHM$_{\text{SPF}^{4.2K}}$ (nm)</th>
<th>FWHM$_{\text{MLS}^{4.2K}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]P-1-ol</td>
<td>3.25 ± 0.04</td>
<td>0.58 ± 0.04</td>
</tr>
<tr>
<td>B[a]P-cis-4,5-diol</td>
<td>4.93 ± 0.05</td>
<td>6.67 ± 0.8</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,c10-tetrol</td>
<td>2.90 ± 0.05</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,t10-tetrol</td>
<td>4.0 ± 0.56</td>
<td>1.85 ± 0.08</td>
</tr>
</tbody>
</table>

FWHM were measured from pure standard solutions at medium linear concentrations, namely 50 ng/ml BaP-1-ol, 50 ng/ml BaP-cis-4,5-ol, 50 ng/ml BaP-r7-t8-t9-c10, and 50 ng/ml BaP-r7-t8-t9-t10. Reported values are the average values recorded from three frozen aliquots of the same standard solution. All measurements were made at the maximum excitation ($\lambda_{\text{exc}}$) and emission ($\lambda_{\text{em}}$) wavelength of the fluorophore.
Since the FWHM of BaP-1-ol, BaP-rttc-tetrol and BaP-rttt-tetrol improved with the spectral resolution of the recording instrument, the natural bandwidth of these three metabolites might be narrower than the FWHM we observed. The same is not true for BaP-cis-4,5-diol, which appears to have a “poorer fitting” than the other three metabolites in the 1-octanol frozen lattice. Although the 0.2 nm excitation bandwidth is a good match for the narrow excitation spectra of the metabolites, site-selective excitation was not attempted. Based on Table 8, the best analyte guest (metabolite) – solvent host (1-octanol) fitting was obtained for B[a]P-r7,t8,t9,c10-tetrol (narrowest FWHM_{MLS}^{4.2K}) followed by B[a]P-1-ol, B[a]P-r7,t8,t9,t10-tetrol and B[a]P-cis-4,5-diol.

Figures 15 – 18 show the 4.2 K excitation and fluorescence spectra of the metabolites recorded with the MLS. The excitation spectra were recorded by stepping the laser at 0.5 nm steps and measuring the fluorescence emission of the metabolites with the spectrometer centered at the maximum emission wavelengths the metabolites. The fluorescence spectra correspond to the first 2D-emission spectra of the WTM recorded upon sample excitation at the maximum excitation wavelengths of the metabolites. The fluorescence spectra of B[a]P-1-ol and B[a]P-r7,t8,t9,c10-tetrol show characteristic quasi-line structures expected from Shpol'skii systems. Another peculiar feature of the observed spectra is the presence of featureless fluorescence bands underlying the quasi-line fluorescence spectra. Broad emission bands in the spectra of PAH/n-alkane systems have been attributed to the presence of disordered groups of solute molecules or pre-aggregates in the frozen matrix.
Figure 15. 4.2 K excitation and fluorescence spectra of B[a]P-1-ol (50 ng/ml) in 1-octanol. The spectra were recorded with the MLS using a 0.2 nm excitation bandwidth and a 0.2 nm emission band-pas. Only maximum excitation and emission peaks are assigned.
Figure 16. 4.2 K excitation and fluorescence spectra of B[a]P-cis-4,5-diol (50 ng/ml) in 1-octanol. The spectra were recorded with the MLS using a 0.2 nm excitation bandwidth and a 0.2 nm emission band-pass.
Figure 17. 4.2 K excitation and fluorescence spectra of B[a]P-r7,t8,t9,c10-tetrol (50 ng/ml) in 1-octanol. The spectra were recorded with the MLS using a 0.2 nm excitation bandwidth and a 0.2 nm emission band-pas.
Figure 18. 4.2 K excitation and fluorescence spectra of B[a]P-r7,t8,t9,t10-tetrol (50 ng/ml) in 1-octanol. The spectra were recorded with the MLS using a 0.2 nm excitation bandwidth and a 0.2 nm emission band-pas.
4.5 4.2 K Fluorescence Lifetimes

Appendix E compiles the 4.2 K fluorescence decays stripped from WTM. Single exponential decays were obtained in all cases. The agreement between the calculated and experimental points over the first two lifetimes of the decays agreed to within about 1% and the residuals showed no systematic trends. The mathematical equation that best describes the behavior of $I_F(t)$ as a function of time ($t$) is the following:

$$I_F(t) = I_F^0 \exp \left(-t/\tau\right)$$

where:

- $I_F^0$ is the fluorescence intensity at $t = 0$
- $\tau$ = the time it takes for the signal to reach an intensity equal to 1/e of its original value, i.e.:

$$t = \tau \Rightarrow I_F(t) = I_F^0 / e = I_F^0 / 2.7183.$$

In the absence of an external quencher, the fluorescence lifetime in terms of rate constants is given by:

$$\tau = 1 / k_F + k_{nr}$$

Where:

- $k_F$ = first order rate constant for fluorescence (photons.s$^{-1}$ or s$^{-1}$)
- $k_{nr}$ = first order rate constant for non-radiative deactivation (photons.s$^{-1}$ or s$^{-1}$)

In the absence of an external quencher, the rate constant for the non-radiative deactivation of S1 is given by:

$$k_{nr} = k_{ic} + k_{isc}$$
Where:

\[ k_{ic} = \text{first order rate constant for internal conversion from } S_1 \text{ to } S_0 \text{ (photons.s}^{-1} \text{ or s}^{-1}) \]

\[ k_{isc} = \text{first order rate constant for intersystem crossing from } S_1 \text{ to } T_n \text{ (photons.s}^{-1} \text{ or s}^{-1}) \]

Therefore, in the absence of an external quencher, the fluorescence lifetime of a fluorophore can be expressed as:

\[ \tau = \frac{1}{k_F + k_{ic} + k_{isc}} \] (23)

In the presence of an external quencher (Q), the fluorescence lifetime of a fluorophore is best described by the following equation:

\[ \tau_F = \frac{1}{k_F + k_{ic} + k_{isc} + k_q[Q]} \] (24)

Where \( k_q \) (Lmol\(^{-1}\)s\(^{-1}\)) is the second rate constant of the quenching reaction between the fluorophore in the \( S_1 \) excited state and the quencher.

Table 9 compares the 4.2 K fluorescence lifetimes of the studied metabolites recorded from standard solution in 1-octanol. Our data shows a direct correlation between the fluorescence lifetimes of the metabolites and the number of hydroxyl groups in their molecular structures. In addition, the remarkable difference in the fluorescence lifetimes of BaP-rttc-tetrol and BaP-rttt-tetrol show a strong correlation with the relative positions of the hydroxyl groups in the molecular structures of the metabolites.

At 4.2 K, the vibrational relaxation of fluorophore molecules in excited states is minimized by the frozen matrix. Based on this fact, it is often assumed that there is a negligible contribution from internal conversion to the deactivation of \( S_1 \) at cryogenic temperatures, i.e. \( k_F >> k_{ic} \). Considering that no heavy atom was present in the frozen matrix and no phosphorescence was
observed at 4.2 K, it is also possible to assume that $k_F \gg k_{ISC}$. By freezing the sample to 4.2 K, the presence of oxygen – which is a well-known and common quencher of fluorescence measurements at room-temperature - in the frozen matrix is minimized. All these considerations, lead us to approximate the observed fluorescence lifetime to the natural lifetime of the fluorophore ($\tau_0^F$); i.e.:

$$\tau_F \sim \tau_0^F = 1 / k_F$$  \hspace{1cm} (25)

Based on these assumptions and the lifetime values in Table 9, we predict the following trend for the fluorescence rate constant of the studied metabolites at 4.2 K: $k_F^{B[\text{a}]P-1\text{-ol}} < k_F^{B[\text{a}]P-\text{cis}-4,5\text{-dilol}} < k_F^{B[\text{a}]P-r7,t8,t9,t10\text{-tetrol}} < k_F^{B[\text{a}]P-r7,t8,t9,c10\text{-tetrol}}$. Although the validity of our prediction has to be tested with fluorescence quantum yield measurements, the four metabolites have distinctive fluorescence lifetimes than can be used as qualitative parameters for their identification.
Table 9. 4.2 K Fluorescence Lifetimes of B[a]P metabolites in 1-Octanol

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>τ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]P-1-ol</td>
<td>9.20 ± 0.1</td>
</tr>
<tr>
<td>B[a]P-cis-4,5-diol</td>
<td>33 ± 1.2</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,c10-tetrol</td>
<td>332 ± 1.6</td>
</tr>
<tr>
<td>B[a]P-r7,t8,t9,t10-tetrol</td>
<td>120 ± 0.1</td>
</tr>
</tbody>
</table>

Fluorescence lifetimes were measured from pure standard solutions at medium linear concentrations, namely 50 ng/ml BaP-1-ol, 50 ng/ml BaP-cis-4,5-ol, 50 ng/ml BaP-r7-t8-t9-c10, and 50 ng/ml BaP-r7-t8-t9-t10. Reported values are the average values recorded from three frozen aliquots of the same standard solution. All measurements were made at the maximum excitation ($\lambda_{exc}$) and emission ($\lambda_{em}$) wavelength of the fluorophore.

4.6 Direct Analysis of Metabolites with Strong Overlapping in the spectral and Lifetime Domains

Comparison of the excitation spectra of B[a]P-1-ol, B[a]P-cis-4,5-diol, B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol in Figures 15 to 18 show the possibility to selectively excite B[a]P-1-ol in the presence of the other three metabolites. The maximum excitation wavelength of B[a]P-1-ol (409 nm) is located at a wavelength region beyond the excitation range of B[a]P-cis-4,5-diol, B[a]P-r7,t8,t9,c10-tetrol, B[a]P-r7,t8,t9,t10-tetrol. Since the maximum excitation
wavelength of B[a]P-1-ol is located at a longer wavelength than the excitation range of the other three metabolites (300 – 350 nm), the relatively lower energy that is required to excite B[a]P-1-ol should not promote fluorescence emission from any of the other three metabolites.

The same is not true for any of the other three metabolites. As sown in Figure 19, the spectral features of B[a]P-cis-4,5-diol, B[a]P-r7,t8,t9,c10-tetrol, B[a]P-r7,t8,t9,t10-tetrol at 4.2 K strongly overlap in both the excitation and the emission ranges. Since the position of the vibrational features of the three metabolites do not change much with temperature and/or instrumental approach, similar overlapping – or even worst - should be expected at room – temperature and 77 K. Since the spectral features of B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol are nearly identical, the overlapping between their spectra is close to 100%. Comparison of their emission spectra to the fluorescence spectrum of B[a]P-cis-4,5-diol reveals the possibility to selectively detect B[a]P-cis-4,5-diol in the presence of the other two metabolites. However, the accuracy of such approach will depend on the relative concentrations of the three metabolites in the sample at hand. Excitation of B[a]P-cis-4,5-diol at its maximum (326.5 nm) will require relatively high energy that might promote the excitation of B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol to a higher singlet excited state (S2) than S1.
**Figure 19.** 4.2 K excitation and fluorescence spectra of BaP-cis-4,5-diol, BaP-r7,t8,t9,c10-tetrol and BaP-r7,t8,t9,t10-tetrol recorded from pure standard solutions in 1-octanol. Spectra were recorded with the MLS using a 0.2 nm excitation bandwidth and a 0.2 nm emission band-pass.
Figure 20 shows the mathematical fittings of the 4.2 K fluorescence decays of B[a]P-cis-4,5-diol, B[a]P-r7,t8,t9,c10-tetrol, B[a]P-r7,t8,t9,t10-tetrol recorded from pure standard solution in 1-octanol. The significantly shorter decay of B[a]P-cis-4,5-diol suggests the possibility to select a relatively long delay – such as 250 ns – to completely remove its fluorescence contribution from the total emission spectrum a hypothetically synthetic mixture with the three metabolites. Since the maximum excitation wavelengths of B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol are at longer wavelengths and, therefore, lower energies than the one required to excite B[a]P-cis-4,5-diol, the combination of a longer delay time and an appropriate excitation wavelength could prove useful for the accurate determination of B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol in the presence of B[a]P-cis-4,5-diol. On the other end, the strong overlapping that exists among the spectral features of B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol and their fluorescence decays illustrates the analytical challenge associated with the heterogeneous distribution of metabolic products of B[a]P.
**Figure 20.** Mathematical fittings of 4.2 K fluorescence decays of B[a]P-cis-4,5-diol, B[a]P-
r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol recorded from pure standard solutions in 1-octanol.
Fluorescence decays were recorded with the MLS using 118 ns gate delay and 250 ns gate width.
4.6.1 Qualitative and Quantitative Analysis via Multivariate Calibration Methods

In addition to spectral overlapping, the analyst needs to consider the potential for chemical interference and inner filter effects not only from the fluorophores of interest but from other sample concomitants. Chemical interaction among analytes or among analytes with sample concomitants may change the analytical response and deteriorate the accuracy of instrumental analysis. Inner filter effects are common phenomena in fluorescence spectroscopy. Primary inner effects usually occur with highly concentrated solutions and relate to the attenuation of the excitation beam by the analytical sample so that the surface of the cuvette facing the excitation beam fluoresces strongly in comparison to the center of the cuvette. Since the center of the cuvette is the unit volume imaged by the emission monochromator, the lower fluorescence results in a lower signal detection. Secondary inner effects usually occur when there is overlapping of excitation and emission spectra. On its way to the emission monochromator, the fluorescence emitted at the center of the cuvette is re-absorbed by the sample itself. Absorbing species can be the fluorophore itself and/or sample concomitants with overlapping excitation spectra.

Since the ability of univariate calibration methods to handle interference is limited, analytical chemists prefer to apply multiway calibration methods for qualitative and quantitative purposes. In a multivariate calibration method, it is often necessary to prepare a calibration set, a validation set, a prediction set and a blank set. The calibration set usually consists of synthetic mixtures with various analytes concentrations and the solvent of the analytical sample. The analytical sample refers to the sample matrix that is presented to the instrument. In addition to the chemicals of interest, the analytical sample contains concomitants of known and unknown chemical identity. The composition of the calibration set excludes the presence of concomitants and is used to determine whether a multiway calibration method can accurately predict the identity
and the concentration of all the analytes in the absence of interference. The purpose of the validation set is to find out if a multiway calibration method can accurately predict the identity and the concentrations of analytes in the presence of interference. It is prepared by spiking known amounts of analytes to the analytical sample. The validation set should be analyzed in the same manner as the prediction set. The prediction set represents the real sample. It is prepared by spiking known amounts of analytes to the real sample. The ideal blank set consist of samples containing all components of the real sample except the analytes. The real blank set is usually an approximation of the ideal blank set. It typically consists of the solvent used in the calibration validation and prediction sets and sample concomitants of known chemical identity. The blank set is used to subtract the response of the background signal – i.e. instrumental noise + solvent signal + concomitants response – from the signal of a real sample prior to predicting the concentration of analytes in the real sample.

Herein, we applied multiway calibration methods to three-way (EEMs) and four-way (TREEMs) data formats. EEMs were recorded at 77 K with the aid of a commercial spectrofluorimeter. TREEMs were recorded at 4.2K with the MLS. In both cases, a central composite design (CCD) \(^{132}\) was used to calculate the concentrations of B[a]P-cis-4,5-diol, B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol used for the preparation of the three-way and four-way calibration sets. Please, see Appendix F for CCD calculations.
4.6.1.1 Three-Way Data Experiments for the Collection of 77 K EEMs with the Spectrofluorimeter

Table 10 lists the concentrations of the three metabolites used for the calibration data set. 77 K EEMs were recorded over wavelength ranges common to the excitation (326 – 350 nm) and emission (360 – 450 nm) spectra of the three metabolites. These ranges included the maximum excitation and emission wavelengths of B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol (347/396 nm) and the maximum emission wavelength of B[a]P-cis-4,5-diol (385 nm). Since the maximum excitation wavelength of B[a]P-cis-4,5-diol (276 nm) was excluded from the EEMs, the concentration of B[a]P-cis-4,5-diol was rather large in comparison to the concentrations of the other two metabolites. All concentrations in Table 10 provided spectral features with strong signal intensities and away from the instrumental noise. The excitation and the emission monochromator steps were 3 and 1 nm, respectively. The resulting matrix for each calibration sample had the dimensions of 9 x 91; i.e. 9 excitation wavelengths and 91 emission wavelengths. The dimensions of the tensor for the 15-calibration set were 15 x 9 x 91. Appendix G compiles the 77 K EEMs recorded from the 15 calibrations samples.
Table 10. Concentrations of Metabolites\(^a\) in the Calibration Samples Used for Three-Way Data Formats (77 K EEMs) Recorded with a Commercial Spectrofluorimeter

<table>
<thead>
<tr>
<th>Calibration Sample</th>
<th>B[a]P-cis-4,5-diol</th>
<th>B[a]P-r7,t8,t9,c10-tetrol</th>
<th>B[a]P-r7,t8,t9,t10-tetrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>450</td>
<td>150</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>900</td>
<td>150</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>450</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>5</td>
<td>450</td>
<td>300</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>450</td>
<td>150</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>450</td>
<td>150</td>
<td>180</td>
</tr>
<tr>
<td>8</td>
<td>150</td>
<td>250</td>
<td>150</td>
</tr>
<tr>
<td>9</td>
<td>150</td>
<td>250</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>750</td>
<td>250</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>750</td>
<td>250</td>
<td>150</td>
</tr>
<tr>
<td>12</td>
<td>150</td>
<td>50</td>
<td>150</td>
</tr>
<tr>
<td>13</td>
<td>150</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>14</td>
<td>750</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>15</td>
<td>750</td>
<td>50</td>
<td>150</td>
</tr>
</tbody>
</table>

\(^a\) All concentrations are in ng.mL\(^{-1}\). All solutions were prepared in 1-octanol.
Prior to initiating validation experiments, we decided to evaluate the prediction ability of MCR-ALS, UPLS-RPL and PARAFAC with the simplest case possible, i.e. in the absence of sample concomitants. Table 11 summarizes the concentrations of the metabolites in the three test samples used for this evaluation.

**Table 11.** Concentrations of B[a]P Metabolites in Test Samples with the Spectrofluorimeter

<table>
<thead>
<tr>
<th>Test Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>B[a]P-cis-4,5-diol</th>
<th>B[a]P-r7,t8,t9,c10-tetrol</th>
<th>B[a]P-r7,t8,t9,t10-tetrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>450</td>
<td>150</td>
<td>120</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>50</td>
<td>180</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>60</td>
<td>30</td>
</tr>
</tbody>
</table>

<sup>a</sup> All concentrations are in ng.mL<sup>-1</sup>. All solutions were prepared in 1-octanol.

Appendices H, I and J compile the 77 K EEMs recorded from the three test samples and the chemometric results obtained with the three algorithms. The qualitative prediction ability of the algorithms was based on the visual comparison of excitation and emission spectra extracted from the EEMs of the test samples and those recorded from pure standard solutions of the studied metabolites in 1-octanol. Their quantitative prediction ability was based on the values of the relative error prediction (REP)<sup>132</sup>. REP values were calculated according to the following equation:

\[
REP = 100 * \left( \sqrt{\frac{\sum_{n=1}^{K}(C_{nom,n} - C_{pred,n})^2}{K}} \right) / C_{mean} 
\]

(26)

where \(K\) is the number of samples, \(C_{mean}\) is the mean concentration, and \(C_{nom}\) and \(C_{pred}\) are nominal and predicted concentrations, respectively. Acceptable prediction abilities require REP values equal or lower than 15%<sup>132</sup>.
Table 12 summarizes the results reported in Appendices H, I and J. In the case of B[a]P-cis-4,5-diol, the unacceptable predictions obtained with MCR-ALS and UPLS/RBL are probably related to the excitation range of the EEMs, which did not cover the maximum excitation wavelength of B[a]P-cis-4,5-diol. The rather low fluorescence intensities emitted by the metabolite resulted in relatively noise EEMs and unacceptable qualitative and quantitative predictions.

Under this assumption, it appears that PARAFAC is better equipped to handle spectral noise. Clearly, the best prediction abilities for the three metabolites were obtained with PARAFAC. Considering the virtually identical spectral features of B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol, the inability of PARAFAC to provide an accurate quantitative prediction for B[a]P-r7,t8,t9,t10-tetrol was rather intriguing. Interesting to note is that none of the algorithms were able to provide an accurate quantitation of B[a]P-r7,t8,t9,t10-tetrol. One explanation for the observed phenomenon relates to the possible photochemical degradation of B[a]P-r7,t8,t9,t10-tetrol during the collection of an EEM. It takes approximately 2 minutes to record an EEM. If the photochemical degradation of B[a]P-r7,t8,t9,t10-tetrol upon irradiation for 2 minutes reduces significantly its fluorescence emission, noise contributions to low signal levels might cause the inaccurate quantitative predictions.
Table 12. Prediction Ability\(^\text{a}\) of Chemometric Algorithms Based on Three-Way Data Formats (77 K EEMs) Recorded with a Commercial Spectrofluorimeter

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>B[a]P-cis-4,5-diol</th>
<th>B[a]P-r7,t8,t9,c10-tetrol</th>
<th>B[a]P-r7,t8,t9,t10-tetrol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Qualitative</td>
<td>Quantitative</td>
<td>Qualitative</td>
</tr>
<tr>
<td>MCR-ALS</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>UPLS/RBL</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>PARAFAC</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

\(^{a}\) Yes = acceptable; No = non-acceptable.
4.6.1.2 Four Way Data Experiments for the Collection of 4.2 K TREEMs with the MLS

Considering that B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol represent the ultimate overlapping challenge in both the wavelength and time domains, we performed all further studies with only these two metabolites. Since UPLS/RBL provided the worst results with 77K EEMs, the qualitative and quantitative prediction of the two metabolites was only attempted with MCR-ALS and PARAFAC. Table 13 lists the calibration data set used for B[a]P-r7,t8,t9,c10-tetrol and B[a]P-r7,t8,t9,t10-tetrol.

Each 4.2 K TREEM was obtained from six WTM recorded at six excitation wavelengths. As an attempt to minimize possible photodegradation effects, each WTM was recorded from a fresh sample aliquot. The excitation wavelength range and the excitation step were 345.0 nm - 347.5 nm and 0.5 nm, respectively. The emission wavelength range and the emission step were 369.1 nm – 410.8 nm and 0.065 nm, respectively. These wavelength ranges included the maximum excitation and emission wavelengths of the two metabolites (347/396 nm). The delay after the excitation pulse varied between 148 ns and 813 ns. Emission spectra were recorded at 35 ns time intervals and the number of laser pulses per emission spectrum was 100. The gate width was kept constant at 700 ns. Under these instrumental parameters, each WTM consisted of 22 emission spectra, it took approximately 2 minutes of recording time and required approximately 2,200 laser pulses.

The tensor for each calibration sample had the dimensions of 20 x 6 x 645; i.e. 20 time windows, 6 excitation wavelengths and 645 emission wavelengths. As a result, the dimensions of the tensor for the entire calibration set were 9×20x6x645. Appendix K compiles the 4.2 K TREEMs recorded from the 9 calibrations samples. Similar to our previous studies with the spectrofluorimeter, the prediction ability of MCR-ALS and PARAFAC was evaluated with the
simplest case possible, i.e. in the absence of sample concomitants. Table 14 summarizes the concentrations of the metabolites in the four test samples used for this evaluation.

**Table 13.** Concentrations of Metabolites\(^a\) in the Calibration Samples Used for Four-Way Data Formats (4.2 K TREEMs) Recorded with the MLS

<table>
<thead>
<tr>
<th>Calibration Sample</th>
<th>B[a]P-r7,t8,t9,c10-tetrol</th>
<th>B[a]P-r7,t8,t9,t10-tetrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>430</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>250</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>500</td>
</tr>
<tr>
<td>6</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>340</td>
<td>70</td>
</tr>
<tr>
<td>8</td>
<td>340</td>
<td>430</td>
</tr>
<tr>
<td>9</td>
<td>400</td>
<td>250</td>
</tr>
</tbody>
</table>

\(^a\) All concentrations are in ng.mL\(^{-1}\). All solutions were prepared in 1-octanol.
Table 14. Concentrations of B[a]P Metabolites in Test Samples with the MLS

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>B[a]P-r7,t8,t9,c10-tetrol</th>
<th>B[a]P-r7,t8,t9,t10-tetrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>280</td>
<td>225</td>
</tr>
<tr>
<td>2</td>
<td>350</td>
<td>225</td>
</tr>
<tr>
<td>3</td>
<td>350</td>
<td>450</td>
</tr>
<tr>
<td>4</td>
<td>300</td>
<td>350</td>
</tr>
</tbody>
</table>

*All concentrations are in ng.mL$^{-1}$. All solutions were prepared in 1-octanol.
Appendices L and M compile the 4.2 K TREEMs recorded from the four test samples and the chemometric results obtained with the two algorithms. As previously, the qualitative prediction ability of the algorithms was based on the visual comparison of excitation and emission spectra extracted from the TREEMs of the test samples and those recorded from pure standard solutions of the studied metabolites. Their quantitative prediction ability was based on the REP values calculated according to equation 26 using the 15 % cut off value.

Table 15 summarizes the results reported in L and M. Again, the best prediction abilities were obtained with PARAFAC. The same is true for its inability to provide an accurate quantitative prediction of B[a]P-r7,t8,t9,t10-tetrol. Figure 21 plots the intensity of fluorescence at 4.2 K measured from pure standard solutions of the two metabolites in 1-octanol as a function of laser pulses. Since all the intensities plotted in the graphs were recorded using the same delay (5 ns) and gate times (700 ns), the decreases in signal intensities are most likely due to the photochemical degradation of the two metabolites. Since the spectral profiles of the two metabolites did not change during the duration of our experiments, the intensities of the fluorescence signals correspond to the remaining fractions of the two metabolites in their original chemical forms. Considering that no additional contributions to the fluorescence spectra of the two metabolites were observed from the photodegraded samples, the photodegradation products were either non-fluorescent or did fluoresce but at excitation and emission wavelengths not covered by our experiments.

The main significant aspect of the plots in Figure 21 is the difference of the fittings of the experimental points to the mathematical decays. Although the fittings deteriorate for both metabolites as the number of laser pulses increases, the variations observed from B[a]P-r7,t8,t9,t10-tetrol are significantly worse than those observed from B[a]P-r7,t8,t9,c10-tetrol.
Apparently, PARAFAC is not able to handle the signal variations observed from \( \text{B}[\alpha]\text{P}-\text{r7,t8,t9,c10-tetrol} \).

**Table 15.** Prediction Ability\(^a\) of Chemometric Algorithms Based on Four-Way Data Formats (4.2 K TREEMs) Recorded with the MLS

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>B[\alpha]P-r7,t8,t9,c10-tetrol</th>
<th>B[\alpha]P-r7,t8,t9,t10-tetrol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Qualitative</td>
<td>Quantitative</td>
</tr>
<tr>
<td>MCR-ALS</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>PARAFAC</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

\(^a\)Yes = acceptable; No = non-acceptable.
**Figure 21.** Intensity of fluorescence ($I_F$) as a function of number of laser pulses (N). Fluorescence intensities were measured at 4.2 K from pure standard solutions (50 ng.mL$^{-1}$) in 1-octanol. All measurements were made at the maximum excitation and emission wavelength of each metabolite.
CONCLUSION

Numerous analytical approaches have been developed to monitor PAH metabolites in physiological fluids. Separation of metabolites has been accomplished via HPLC, CE and GC. This is not a trivial task as many metabolites often present similar chromatographic and electrophoretic behaviors. The selectivity of the approach becomes particularly relevant when targeting a distribution of stereo-isomeric metabolites with diverse toxicological effects on human health. HRGC-MS or HPLC-MS provides the analyst with the highest specificity. Unfortunately, the existence of chemically related metabolic products with virtually identical fragmentation patterns still challenges the specificity of these techniques. A combination of chromatographic steps is often necessary that lead to time-consuming procedures. The numerous separation steps open ample opportunity to metabolite loss and collection of inaccurate data.

The ability to directly determine the presence and the amounts of targeted metabolites without the need of extensive chromatographic and/or electrophoretic separation is our ultimate goal. This dissertation explored the multidimensionality of photoluminescence spectroscopy in Shpol’skii matrixes for the qualitative and quantitative determination of PAH metabolites via multiway calibration methods. Multiway calibration methods enable one to achieve direct (no chromatographic separation) qualitative and quantitative analysis of chemicals of interest (analytes) in complex systems of unknown composition, i.e. in the presence of uncalibrated species. This property is known as the 2nd order advantage. Simply put, multiway calibration methods that carry with them the 2nd order advantage are capable to perform a “mathematical” separation, identification, and quantitation of complex samples with chemically unknown composition.
The term Shpol’skii matrix refers to a dilute solution of a guest molecule - usually a non-polar analyte, such as parent a PAH - in a solvent host - usually a non-polar solvent, such as n-alkane - where the solvent freezes into an ordered polycrystalline matrix. If the dimensions of the analyte and the solvent match up well enough, guest molecules occupy a small number of crystallographic sites in the host matrix. Matrix isolation of analyte molecules reduces inhomogeneous band broadening, which results in vibrationally resolved excitation and emission (fluorescence and/or phosphorescence) spectra with sharp line widths. Although Shpol’skii spectroscopy has been widely employed for the analysis of parent PAHs, publications describing the use of Shpol’skii spectroscopy for PAH metabolites are rare. The poor compatibility between polar guest (PAH metabolite) and the non-polar host (n-alkane) leads to rather broad spectral bands, rapid photo-degradation, and poor limits of detection.

This dissertation extended the application of Shpol’skii spectroscopy to polar metabolites in polar organic solvents. We reported for the first-time experimental evidence on the line-narrowing effect caused by primary alcohols on the spectral features of PAH metabolites at 77 K and 4.2 K. The effect of primary alcohols (RCH₂OH) on the 77 K and 4.2 K fluorescence characteristics of PAH metabolites showed the best spectral narrowing when matching the length of the alkyl group (R) of the primary alcohol to the length of the n-alkane that best fitted the dimensions of the parent PAH. The spectral narrowing and the fluorescence enhancements observed for 1-hydroxypyrene, 2-hydroxyfluorene, 9-hydroxyphenanthrene, 3-hydroxybenzo[a]pyrene (3-OHB[a]P), 4-hydroxybenzopyrene (4-OHB[a]P), 5-hydroxybenzopyrene (5-OHB[a]P), B[a]P-trans-7,8-dihydrodiol (±), B[a]P-trans-9,10-dihydrodiol (±), B[a]P-r-7,t-8-dihydrodiol-c-9,10-epoxide(±) (syn-BPDE), and B[a]P-r-7,t-8-dihydrodiol-t-9,10-epoxide(±) (anti-BPDE) demonstrated the potential for the qualitative and
quantitative analysis of PAH metabolites at trace concentration levels. The obtained enhancements for B[a]P metabolites provided 4.2K LODs varying from 0.1 ng.mL\(^{-1}\) (anti-BPDE (±)) to 0.8ng.mL\(^{-1}\) (3-OHB[a]P)\textsuperscript{129}.

Since B[a]P is the most toxic PAH in the EPA priority pollutants list and it is often used as a measure of risk, four B[a]P metabolites - BaP-1-ol, BaP-cis-4,5-diol, BaP-rttc-tetrol and BaP-rttt-tetrol - were selected for chemometric studies. In all cases, 1-octanol was used as the Shpol’skii matrix. Two instruments were used to acquire experimental data. Steady-state measurements were performed with the aid of a commercial spectrofluorimeter. Time-resolved measurements were made with the MLS, an instrumental set up built in house for the collection of time-resolved excitation and emission spectra and photoluminescence decays.

When compared to room-temperature spectra, lowering the temperature to 77 K or 4.2 K narrowed the excitation and fluorescence spectra of the four metabolites. At cryogenic temperatures, the excitation and fluorescence spectra of the four metabolites showed additional vibrational features that added qualitative information for identification purposes. Not much improvement was observed when lowering the temperature of analysis from 77 K to 4.2 K. Except for B[a]P-cis-4,5-diol, all the other metabolites showed narrower spectra with the MLS. This outcome was somehow expected due to the spectral resolutions of the two instruments. Spectra with the spectrofluorimeter were recorded with a 2 nm excitation and emission bandpass. Spectra with the MLS were recorded with an excitation bandwidth of 0.2 nm (OPO laser) and the narrowest possible slit width of the spectrometer (10 μm). The 10 μm spectrometer slit width provides a limiting resolution of 0.23 nm.

Fluorescence lifetimes were measured with the MLS at 4.2 K. Our data shows a direct correlation between the fluorescence lifetimes of the metabolites and the number of hydroxyl
groups in their molecular structures. In addition, a remarkable difference in the fluorescence lifetimes of BaP-rrttc-tetrol and BaP-rrtt-tetrol was observed which showed a strong correlation with the relative positions of the hydroxyl groups in the molecular structures of the metabolites.

The LODs and LOQs were at the parts-per-billion or sub-parts-per-billion concentration levels. When compared to the LODs recorded with the spectrofluorimeter at 77 K (LOD$_{\text{SPF}77\text{K}}$), the LODs recorded with the MLS at 4.2 K (LOD$_{\text{MLS}4.2\text{K}}$) showed improvements varying from 1.38 times (B[a]P-cis-4,5-diol) to 5.5 times (B[a]P-r7,t8,t9,t10-tetrol). When compared to the LOD$_{\text{SPF}4.2\text{K}}$, the MLS provided LOD improvements only for B[a]P-r7,t8,t9,c10-tetrol (14 times) and B[a]P-r7,t8,t9,t10-tetrol (2.88 times). B[a]P-1-ol and B[a]P-cis-4,5-diol showed better 4.2 K LODs with the spectrofluorimeter. Since LODs are only estimates of detectable concentrations that carry with them approximately 33% of statistical variation, the only metabolite that showed a significant improvement at 4.2 K with the MLS was B[a]P-r7,t8,t9,c10-tetrol.

MCR-ALS, UPLS-RBL and PARAFAC were tested for the multi-way calibration of synthetic mixtures containing BaP-cis-4,5-diol, BaP-rrttc-tetrol and BaP-rrtt-tetrol (three-way data formats) or BaP-rrttc-tetrol and BaP-rrtt-tetrol (four-way data formats). Three-way data formats consisted of 77 K EEMs recorded with the spectrofluorimeter. Four-way data formats were 4.2 K TREEMs recorded with the MLS. In the case of 77 K EEMs, the best prediction abilities were obtained with PARAFAC. Except for the quantitative prediction of BaP-rrtt-tetrol, PARAFAC provided acceptable qualitative and quantitative predictions for all the other cases. Similar results were obtained with 4.2 K TREEMs. The inability of PARAFAC to provide an accurate quantitative prediction for B[a]P-r7,t8,t9,t10-tetrol was attributed to the photochemical degradation of this metabolite during the acquisition of EEMs or TREEMs.
Our assumption is based on the fluorescence intensities of BaP-rttc-tetrol and BaP-rttt-tetrol as a function of the number of laser pulses. Although the fittings of the experimental points to the mathematical decays deteriorate for both metabolites as the number of laser pulses increases, the variations observed from B[a]P-r7,t8,t9,t10-tetrol are significantly worse than those observed from B[a]P-r7,t8,t9,c10-tetrol. Apparently, PARAFAC is not able to handle the signal variations observed from B[a]P-r7,t8,t9,c10-tetrol.

Future studies will seek to minimize photochemical degradation with an optimization of excitation parameters for the collection of EEMs or TREEMs. It should be noted that the collection times of an EEM or a TREEM were approximately the same. In the case of 77 K EEMs with the spectrofluorimeter, we will attempt to minimize the number of excitation wavelengths for the acquisition of EEMs. In addition, we will invert the order of excitation wavelengths. In this dissertation, EEMs were recorded by increasing the excitation wavelengths, i.e. from higher to lower energy regions. Future experiments will record EEMs by decreasing the excitation wavelengths, i.e. from lower to higher energy regions. In this dissertation, TREEMs collection was based on 100 laser pulses per 2D spectrum of the WTM. In addition to reducing the number of laser pulses per 2D spectrum of the WTM, we will increase the delay between 2D spectra to further reduce the collection time of a WTM.

Additional experiments will attempt to reducing the excitation energy reaching the frozen sample. Since the EEMs in this dissertation were recorded using the narrowest excitation bandpass of the spectrofluorimeter (2 nm), we will reduce the excitation energy by placing density filters at the exit slit of the excitation monochromator. A similar approach can be used with the MLS by placing density filters at the instrumental end of the excitation fiber of the cryogenic probe. We can also reduce the energy output of the OPO laser.
Controlling the photochemical degradation of any given metabolite appears to be a key parameter for a successful prediction with PARAFAC. If we gain expertise in controlling photochemical degradation, we should be able to add an additional dimension to multiway calibration approaches.
APPENDIX A: EXCITATION AND FLUORESCENCE SPECTRA OF B[a]P METABOLITES IN 1-OCTANOL RECORDED WITH A COMMERCIAL SPECTROFLUORIMETER
**Figure A1:** Excitation and fluorescence spectra of BaP-1-ol recorded with the spectrofluorimeter at room temperature in 1-octanol. Spectra were recorded from a 50 ng.mL⁻¹ solution using a 2nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
Figure A2: Excitation and fluorescence spectra of BaP-1-ol recorded with the spectrofluorimeter at 77 K in 1-octanol. Spectra were recorded from a 50 ng.mL⁻¹ solution using a 2nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
**Figure A3:** Excitation and fluorescence spectra of BaP-1-ol recorded with the spectrofluorimeter at 4.2 K in 1-octanol. Spectra were recorded from a 50 ng.mL\(^{-1}\) solution using a 2nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
Figure A4: Excitation and fluorescence spectra of BaP-cis-4,5-diol recorded with the spectrofluorimeter at room-temperature in 1-octanol. Spectra were recorded from a 50 ng.mL⁻¹ solution using a 2 nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
Figure A5: Excitation and fluorescence spectra of BaP-cis-4,5-diol recorded with the spectrofluorimeter at 77 K in 1-octanol. Spectra were recorded from a 50 ng.mL^{-1} solution using a 2 nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
Figure A6: Excitation and fluorescence spectra of BaP-cis-4,5-diol recorded with the spectrofluorimeter at 4.2 K in 1-octanol. Spectra were recorded from a 50 ng.mL$^{-1}$ solution using a 2 nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
Figure A7: Excitation and fluorescence spectra of BaP-r-7,t-8,t-9,c-10-tetrahydrotetrol recorded at room temperature with the spectrofluorimeter at room-temperature in 1-octanol. Spectra were recorded from a 50 ng.mL$^{-1}$ solution using a 2 nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
**Figure A8:** Excitation and fluorescence spectra of BaP-r-7,t-8,t-9,c-10-tetrahydrotetrol recorded at 77 K with the spectrofluorimeter at room-temperature in 1-octanol. Spectra were recorded from a 50 ng.mL\(^{-1}\) solution using a 2 nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
Figure A9: Excitation and fluorescence spectra of BaP-r-7,t-8,t-9,c-10-tetrahydrotetrol recorded at 4.2 K with the spectrofluorimeter at 4.2 K in 1-octanol. Spectra were recorded from a 50 ng.mL$^{-1}$ solution using a 2 nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
**Figure A10:** Excitation and fluorescence spectra of BaP-r-7,t-8,t-9,7-10-tetrahydrotetrol recorded at room temperature with the spectrofluorimeter at room-temperature in 1-octanol. Spectra were recorded from a 50 ng.mL⁻¹ solution using a 2 nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
**Figure A11:** Excitation and fluorescence spectra of BaP-r-7,t-8,t-9,7-10-tetrahydrotetrol recorded at room temperature with the spectrofluorimeter at room-temperature in 1-octanol. Spectra were recorded from a 50 ng.mL$^{-1}$ solution using a 2 nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
Figure A12: Excitation and fluorescence spectra of BaP-r-7,t-8,t-9,7-10-tetrahydrotetrol recorded at room temperature with the spectrofluorimeter at room-temperature in 1-octanol. Spectra were recorded from a 50 ng.mL\(^{-1}\) solution using a 2 nm excitation and emission band pass. Each spectrum represents the average of three spectra recorded from three individual aliquots. Maximum excitation and fluorescence wavelengths are underlined.
APPENDIX B: 77K AND 4.2K CALIBRATION CURVES OF B[a]P METABOLITES IN 1-OCTANOL RECORDED WITH A COMMERCIAL SPECTROFLUORIMETER
Figure B1: 77 K Calibration curve of BaP-1-ol in 1-octanol.
Figure B2: 4.2 K Calibration curve of BaP-1-ol in 1-octanol.

The equation for the calibration curve is given by:

\[ y = 84425x + 38636 \]

with a coefficient of determination, \( R^2 = 0.9914 \).
Figure B3: 77 K Calibration curve of BaP-cis-4,5-diol in 1-octanol.
Figure B4: 4.2 K Calibration curve of BaP-cis-4,5-diol in 1-octanol.
Figure B5: 77 K Calibration curve of BaP-rttetra-tetrol in 1-octanol.
Figure B6: 4.2 K Calibration curve of BaP-rttct-tetrol in 1-octanol.
Figure B7: 77 K Calibration curve of BaP-rttt-tetrol in 1-octanol.

\[ y = 1152.9x + 5529 \]

\[ R^2 = 0.9925 \]
Figure B8: 4.2 K Calibration curve of BaP-rttt-tetrol in 1-octanol.
APPENDIX C: 4.2 K WAVELENGTH TIME MATRICES OF B[a]P METABOLITES IN 1-OCTANOL
Figure C1: 4.2 K WTM of BaP-1-ol in 1-octanol. Metabolite concentration = 50 ng.mL\(^{-1}\).

Spectrograph slit-width = 409.0 nm.
Figure C2: 4.2 K WTM of BaP-cis-4,5-diol in 1-octanol. Metabolite concentration = 50 ng.mL$^{-1}$.

Spectrograph slit-width = 326.5 nm.
Figure C3: 4.2 K WTM of BaP-rttct-tetrol in 1-octanol. Metabolite concentration = 50 ng.mL⁻¹.
Spectrograph slit-width = 345 nm.
Figure C4: 4.2 K WTM of BaP-rttt-tetrol in 1-octanol. Metabolite concentration = 50 ng.mL⁻¹.
Spectrograph slit-width = 345.5 nm.
APPENDIX D: 4.2 K LETRSS CALIBRATION CURVES OF B[a]P METABOLITES IN 1-OCTANOL
Figure D1: 4.2 K LETRSS calibration curve of BaP-1-ol in 1-octanol.
Figure D2: 4.2 K LETRSS calibration curve of BaP-cis-4,5-diol in 1-octanol.
Figure D3: 4.2 K LETRSS calibration curve of BaP-rttc-tetrol in 1-octanol.
Figure D4: 4.2 K LETRSS calibration curve of BaP-rttt-tetrol in 1-octanol.

\[
y = 12253x + 86412 \\
R^2 = 0.9987
\]
APPENDIX E: 4.2 K FLUORESCENCE DECAYS OF B[a]P METABOLITES IN 1-OCTANOL RECORDED WITH THE MLS
**Figure E1:** 4.2 K fluorescence decay of BaP-1-ol in 1-octanol.
Figure E2: 4.2 K fluorescence decay of BaP-cis-4,5-diol in 1-octanol.
Figure E3: 4.2 K fluorescence decay of BaP-rttc-tetrol in 1-octanol.
Figure E4: 4.2 K fluorescence decay of BaP-rttt-tetrol in 1-octanol.
APPENDIX F: CCD CALCULATIONS FOR THREE-WAY AND FOUR-WAY CALIBRATION SETS
**Figure F1**: CCD calculation of five levels design of two factors.
Figure F2: CCD calculation of five levels design of three factors.
APPENDIX G: 77 K EEMS RECORDED WITH THE COMMERCIAL SPECTROFLUORIMETER
Figure G1: EEMs Calibration1 recorded with the Commercial Spectrofluorimeter at 77K.
Figure G2: EEMs Calibration2 recorded with the Commercial Spectrofluorimeter at 77K.
**Figure G3:** EEMs Calibration3 recorded with the Commercial Spectrofluorimeter at 77K.
Figure G4: EEMs Calibration 4 recorded with the Commercial Spectrofluorimeter at 77K.
**Figure G5**: EEMs Calibration5 recorded with the Commercial Spectrofluorimeter at 77K.
Figure G6: EEMs Calibration6 recorded with the Commercial Spectrofluorimeter at 77K.
**Figure G7:** EEMs Calibration7 recorded with the Commercial Spectrofluorimeter at 77K.
**Figure G8**: EEMs Calibration8 recorded with the Commercial Spectrofluorimeter at 77K.
Figure G9: EEMs Calibration9 recorded with the Commercial Spectrofluorimeter at 77K.
Figure G10: EEMs Calibration10 recorded with the Commercial Spectrofluorimeter at 77K.
Figure G11: EEMs Calibration11 recorded with the Commercial Spectrofluorimeter at 77K.
Figure G12: EEMs Calibration12 recorded with the Commercial Spectrofluorimeter at 77K.
Figure G13: EEMs Calibration13 recorded with the Commercial Spectrofluorimeter at 77K.
Figure G14: EEMs Calibration14 recorded with the Commercial Spectrofluorimeter at 77K.
Figure G15: EEMs Calibration15 recorded with the Commercial Spectrofluorimeter at 77K.
APPENDIX H: MCR-ALS RESULTS FROM 77 K EEMS RECORDED
WITH THE COMMERCIAL SPECTROFLUORIMETER
Figure H1: Excitation spectral profiles of 3 metabolites and background in 1-octanol at 77K predicted by MCR-ALS for augmented mode.
Figure H2: Fluorescence spectral profiles of 3 metabolites and background in 1-octanol at 77K predicted by MCR-ALS for non-augmented mode.
Table H1. Nominal and predicted concentrations of 3 metabolites in test samples by MCR-ALS

<table>
<thead>
<tr>
<th>Test sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>[BaP-cis-4,5-diol]</th>
<th>[BaP-r7,t8,t9,c10-tetrol]</th>
<th>[BaP-r7,t8,t9,t10-tetrol]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nominal</td>
<td>Predicted</td>
<td>Nominal</td>
</tr>
<tr>
<td>Test 1</td>
<td>450</td>
<td>580</td>
<td>150</td>
</tr>
<tr>
<td>Test 2</td>
<td>400</td>
<td>679</td>
<td>50</td>
</tr>
<tr>
<td>Test 3</td>
<td>300</td>
<td>212</td>
<td>60</td>
</tr>
<tr>
<td>REP (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41</td>
<td>14.6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> All concentrations are in ng.mL<sup>-1</sup>. All solutions were prepared in 1-octanol.

<sup>b</sup> Relative error percentage (REP) were calculated according to the equation \( \text{REP} = 100 \times \sqrt{\frac{1}{K} \sum_{n=1}^{K} \left( C_{\text{nom},n} - C_{\text{pred},n} \right)^2} \), where \( K \) is the number of samples, \( C_{\text{mean}} \) is the mean concentration, and \( C_{\text{nom}} \) and \( C_{\text{pred}} \) are nominal and predicted concentrations respectively.
APPENDIX I: UPLS-RBL RESULTS FROM 77 K EEMS RECORDED
WITH THE COMMERCIAL SPECTROFLUORIMETER
**Figure I1:** Excitation spectral profiles of 3 metabolites in 1-octanol at 77K predicted by UPLS-RBL.
**Figure I2:** Fluorescence spectral profiles of 3 metabolites in 1-octanol at 77K predicted by UPLS-RBL.
Table I1. Nominal and predicted concentrations of 3 metabolites in test samples by UPLS-RBL

<table>
<thead>
<tr>
<th>Test sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BaP-cis-4,5-diol</th>
<th>BaP-r7,t8,t9,c10-tetrol</th>
<th>BaP-r7,t8,t9,t10-tetrol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nominal</td>
<td>Predicted</td>
<td>Nominal</td>
</tr>
<tr>
<td>Test 1</td>
<td>450</td>
<td>456</td>
<td>150</td>
</tr>
<tr>
<td>Test 2</td>
<td>400</td>
<td>320</td>
<td>50</td>
</tr>
<tr>
<td>Test 3</td>
<td>300</td>
<td>488</td>
<td>60</td>
</tr>
<tr>
<td>REP (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>118</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> All concentrations are in ng.mL<sup>-1</sup>. All solutions were prepared in 1-octanol.

<sup>b</sup> Relative error percentage (REP) were calculated according to the equation REP = 100 * √(Σ<sub>n=1</sub>^K (C<sub>nom,n</sub> - C<sub>pred,n</sub>)^2)/K, where K is the number of samples, C<sub>mean</sub> is the mean concentration, and C<sub>nom</sub> and C<sub>pred</sub> are nominal and predicted concentrations respectively.
APPENDIX J: PARAFAC RESULTS FROM 77 K EEMS RECORDED

WITH THE COMMERCIAL SPECTROFLUORIMETER
Figure J1: Excitation spectral profiles of 3 metabolites in 1-octanol at 77K predicted by PARAFAC.
Figure J2: Fluorescence spectral profiles of 3 metabolites and background in 1-octanol at 77K predicted by PARAFAC.
Table J1. Nominal and predicted concentrations of 3 metabolites in test samples by MCR-ALS

<table>
<thead>
<tr>
<th>Test sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BaP-cis-4,5-diol</th>
<th>BaP-r7,t8,t9,c10-tetrol</th>
<th>BaP-r7,t8,t9,t10-tetrol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nominal</td>
<td>Predicted</td>
<td>Nominal</td>
</tr>
<tr>
<td>Test 1</td>
<td>450</td>
<td>440</td>
<td>150</td>
</tr>
<tr>
<td>Test 2</td>
<td>400</td>
<td>428</td>
<td>50</td>
</tr>
<tr>
<td>Test 3</td>
<td>300</td>
<td>321</td>
<td>60</td>
</tr>
<tr>
<td>REP (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>4.1</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> All concentrations are in ng.mL<sup>-1</sup>. All solutions were prepared in 1-octanol.

<sup>b</sup> Relative error percentage (REP) were calculated according to the equation REP= 100 * \(\sqrt{\frac{\sum_{n=1}^{K}(C_{\text{nom}, n} - C_{\text{pred}, n})^2}{K}}\), where K is the number of samples, \(C_{\text{mean}}\) is the mean concentration, and \(C_{\text{nom}}\) and \(C_{\text{pred}}\) are nominal and predicted concentrations respectively.
APPENDIX K: 4.2 K TREEMS RECORDED WITH THE MLS
**Figure K1**: TREEM1 to TREEM4 Calibration recorded with MLS at 4.2K.
**Figure K2:** TREEM5 to TREEM8 Calibration recorded with MLS at 4.2K.
**Figure K3**: TREEM9 to TREEM12 Calibration recorded with MLS at 4.2K.
Figure K4: TREEM13 to TREEM16 Calibration recorded with MLS at 4.2K.
Figure K5: TREEM17 to TREEM20 Calibration recorded with MLS at 4.2K.
Figure K6: TREEM1 to TREEM4 Calibration recorded with MLS at 4.2K.
Figure K7: TREEM5 to TREEM8 Calibration recorded with MLS at 4.2K.
Figure K8: TREEM9 to TREEM12 Calibration recorded with MLS at 4.2K.
Figure K9: TREEM13 to TREEM16 Calibration recorded with MLS at 4.2K.
Figure K10: TREEM$^{17}$ to TREEM$^{20}$ Calibration$^{2}$ recorded with MLS at 4.2K.
Figure K11: TREEM1 to TREEM4 Calibration recorded with MLS at 4.2K.
Figure K12: TREEM5 to TREEM8 Calibration recorded with MLS at 4.2K.
Figure K13: TREEM9 to TREEM12 Calibration recorded with MLS at 4.2K.
Figure K14: TREEM13 to TREEM16 Calibration recorded with MLS at 4.2K.
**Figure K15:** TREEM17 to TREEM20 Calibration recorded with MLS at 4.2K.
Figure K16: TREEM1 to TREEM4 Calibration recorded with MLS at 4.2K.
Figure K17: TREEM5 to TREEM8 Calibration recorded with MLS at 4.2K.
Figure K18: TREEM9 to TREEM12 Calibration recorded with MLS at 4.2K.
Figure K19: TREEM13 to TREEM16 Calibration recorded with MLS at 4.2K.
Figure K20: TREEM17 to TREEM20 Calibration4 recorded with MLS at 4.2K.
Figure K21: TREEM1 to TREEM4 Calibration recorded with MLS at 4.2K.
Figure K22: TREEM5 to TREEM8 Calibration recorded with MLS at 4.2K.
Figure K23: TREEM<sup>9</sup> to TREEM<sup>12</sup> Calibration<sup>5</sup> recorded with MLS at 4.2K.
Figure K24: TREEM13 to TREEM16 Calibration recorded with MLS at 4.2K.
Figure K25: TREEM17 to TREEM20 Calibration recorded with MLS at 4.2K.
Figure K26: TREEM1 to TREEM4 Calibration recorded with MLS at 4.2K.
Figure K27: TREEM5 to TREEM8 Calibration recorded with MLS at 4.2K.
Figure K28: TREEM9 to TREEM12 Calibration recorded with MLS at 4.2K.
Figure K29: TREEM13 to TREEM16 Calibration recorded with MLS at 4.2K.
Figure K30: TREEM17 to TREEM20 Calibration recorded with MLS at 4.2K.
Figure K31: TREEM1 to TREEM4 Calibration recorded with MLS at 4.2K.
Figure K32: TREEM5 to TREEM8 Calibration recorded with MLS at 4.2K.
Figure K33: TREEM9 to TREEM12 Calibration recorded with MLS at 4.2K.
Figure K34: TREEM13 to TREEM16 Calibration recorded with MLS at 4.2K.
Figure K35: TREEM17 to TREEM20 Calibration recorded with MLS at 4.2K.
Figure K36: TREEM1 to TREEM4 Calibration recorded with MLS at 4.2K.
Figure K37: TREEM5 to TREEM8 Calibration recorded with MLS at 4.2K.
Figure K38: TREEM9 to TREEM12 Calibration8 recorded with MLS at 4.2K.
Figure K39: TREEM13 to TREEM16 Calibration8 recorded with MLS at 4.2K.
Figure K40: TREEM17 to TREEM20 Calibration recorded with MLS at 4.2K.
Figure K41: TREEM1 to TREEM4 Calibration recorded with MLS at 4.2K.
Figure K42: TREEM5 to TREEM8 Calibration recorded with MLS at 4.2K.
Figure K43: TREEM\textsuperscript{9} to TREEM\textsuperscript{12} Calibration\textsuperscript{9} recorded with MLS at 4.2K.
Figure K44: TREEM13 to TREEM16 Calibration recorded with MLS at 4.2K.
**Figure K45:** TREEM\textsuperscript{17} to TREEM\textsuperscript{20} Calibration\textsuperscript{9} recorded with MLS at 4.2K.
APPENDIX L: MCR-ALS RESULTS FROM 4.2K TREEMS RECORDED
WITH THE MLS
**Figure L1**: Excitation spectral profiles of 2 metabolites in 1-octanol at 4.2K predicted by MCR-ALS.
Figure L2: Fluorescence spectral profiles of 2 metabolites in 1-octanol at 4.2K predicted by MCR-ALS.
Figure L3: Fluorescence decay of 2 metabolites in 1-octanol at 4.2K predicted by MCR-ALS.
Table L1. Nominal and predicted concentrations of 2 metabolites in test samples by MCR-ALS

<table>
<thead>
<tr>
<th>Test Sample(^a)</th>
<th>[BaP-rttc-tetrol]</th>
<th>[BaP-rttt-tetrol]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nominal</td>
<td>Predicted</td>
</tr>
<tr>
<td>Test 1</td>
<td>280</td>
<td>272</td>
</tr>
<tr>
<td>Test 2</td>
<td>350</td>
<td>246</td>
</tr>
<tr>
<td>Test 3</td>
<td>350</td>
<td>289</td>
</tr>
<tr>
<td>Test 4</td>
<td>300</td>
<td>323</td>
</tr>
<tr>
<td>REP (%)(^b)</td>
<td></td>
<td>24</td>
</tr>
</tbody>
</table>

\(^a\) All concentrations are in ng.mL\(^-1\). All solutions were prepared in 1-octanol.

\(^b\) Relative error percentage (REP) were calculated according to the equation REP\(=100\times\sqrt{\frac{\sum_{n=1}^{K}(C_{nom,n} - C_{pred,n})^2}{K}}\), where \(K\) is the number of samples, \(C_{mean}\) is the mean concentration, and \(C_{nom}\) and \(C_{pred}\) are nominal and predicted concentrations respectively.
APPENDIX M: PARAFAC RESULTS FROM 4.2K TREEMS RECORDED WITH THE MLS
Figure M1: Excitation spectral profiles of 2 metabolites in 1-octanol at 4.2K predicted by PARAFAC.
**Figure M2:** Excitation spectral profiles of 2 metabolites in 1-octanol at 4.2K predicted by PARAFAC.
Figure M3: Fluorescence decay of 2 metabolites in 1-octanol at 4.2K predicted by PARAFAC.
Table M1. Nominal and predicted concentrations of 2 metabolites in test samples by PARAFAC.

<table>
<thead>
<tr>
<th>Test Sample(^a)</th>
<th>[BaP-rttc-tetrol]</th>
<th>[BaP-rttt-tetrol]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nominal</td>
<td>Predicted</td>
</tr>
<tr>
<td>Test 1</td>
<td>280</td>
<td>258</td>
</tr>
<tr>
<td>Test 2</td>
<td>350</td>
<td>308</td>
</tr>
<tr>
<td>Test 3</td>
<td>350</td>
<td>337</td>
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<tr>
<td>Test 4</td>
<td>300</td>
<td>285</td>
</tr>
<tr>
<td>REP (%)(^b)</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

\(^a\) All concentrations are in ng.mL\(^{-1}\). All solutions were prepared in 1-octanol.

\(^b\) Relative error percentage (REP) were calculated according to the equation \(\text{REP} = 100 \times \sqrt{\frac{\sum_{n=1}^{K}(C_{\text{nom,}\_n}-C_{\text{pred,}\_n})^2}{K}}\), where \(K\) is the number of samples, \(C_{\text{mean}}\) is the mean concentration, and \(C_{\text{nom}}\) and \(C_{\text{pred}}\) are nominal and predicted concentrations respectively.
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