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On The Capillary Electrophoresis Of Monohydroxy Metabolites Of Polycyclic Aromatic Hydrocarbons And Its Application To The Analysis Of Biological Matrices

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ON THE CAPILLARY ELECTROPHORESIS OF MONOHYDROXY METABOLITES OF POLYCYCLIC AROMATIC HYDROCARBONS AND ITS APPLICATION TO THE ANALYSIS OF BIOLOGICAL MATRICES

by

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B.S. National University of Rosario, Argentina 2006

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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2013

Major Professor: Andres D. Campiglia
ABSTRACT

Polycyclic aromatic hydrocarbons (PAH) are a class of environmental pollutants consisting of a minimum of two fused aromatics rings originating from the incomplete combustion of organic matter and/or anthropogenic sources. Numerous possible anthropogenic and natural sources make the presence of PAH ubiquitous in the environment. The carcinogenic nature of some PAH and their ubiquitous presence makes their chemical analysis a topic of environmental and toxicological importance. Although environmental monitoring of PAH is an important step to prevent exposure to contaminated sites, it provides little information on the actual uptake and subsequent risks. Parent PAH are relatively inert and need metabolic activation to express their carcinogenicity. Covalent binding to DNA appears to be the first critical step in the initiation of the tumor formation process.

To this end, the determination of short term biomarkers – such as monohydroxy-PAH metabolites (OH-PAH) - fills an important niche to interpret actual PAH exposure levels, prevent extreme body burdens and minimize cancer risk. One would certainly prefer an early warning parameter over a toxicological endpoint – such as DNA-adducts – indicating that extensive damage has already been done. Several methods have been developed to determine OH-PAH in specific tissue or excreta and food samples. The general trend for the analysis of OH-PAH follows the pattern of sample collection, sample clean-up and pre-concentration, chromatographic separation and quantification. Popular approaches for sample clean-up and pre-concentration include liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Chromatographic separation and quantification has been based on high-performance liquid
Although chromatographic techniques provide reliable results in the analysis of OH-PAH, their experimental procedures are time consuming and expensive. Elution times of 30-60 minutes are typical and standards must be run periodically to verify retention times. If the concentrations of target species are found to lie outside the detector’s response range, the sample must be diluted and the process repeated. On the other end of the concentration range, many samples are “zeroes,” i.e. the concentrations are below detection limits. Additional problems arise when laboratory procedures are scaled up to handle thousands of samples under mass screening conditions. Under the prospective of a sustainable environment, the large usage of organic solvents is one of the main limitations of the current chromatographic methodology.

This dissertation focuses on the development of a screening methodology for the analysis of OH-PAH in urine and milk samples. Screening techniques capable of providing a “yes or no” answer to OH-PAH contamination prevent unnecessary scrutiny of un-contaminated samples via conventional methods, reduce analysis cost and expedite the turnaround time for decision making purposes. The proposed methodology is based on capillary zone electrophoresis (CZE) and synchronous fluorescence spectroscopy (SFS). Metabolites extraction and pre-concentration is achieved with optimized SPE, LLE and/or QuEChERS (quick, easy, cheap, effective, rugged and safe) procedures. The small sample and extracting solvent volumes facilitate the simultaneous extraction of numerous samples via an environmentally friendly procedure, which is well-suited for routine monitoring of numerous samples. Sample stacking is successfully implemented to improve CZE limits of detection by two orders of magnitude. The unique electrophoretic pattern of positional isomers of OH-PAH demonstrates the potential of CZE for
the unambiguous determination of metabolites with similar chromatographic behaviors and virtually similar fragmentation patterns. The direct determination of OH-PAH without chromatographic separation is demonstrated via SFS. The non-destructive nature of SFS provides ample opportunity for further metabolite confirmation via chromatographic techniques.
A mi esposa Paula, gracias por la paciencia. A mis hijas. A mi familia por su apoyo constante.
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A special thanks to:

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CHAPTER 1. INTRODUCTION

1.1 Relevance of Polycyclic Aromatic Hydrocarbons and their Metabolites

Polycyclic aromatic hydrocarbons (PAH) are a class of environmental pollutants consisting of a minimum of two fused aromatics rings originating from the incomplete combustion of organic matter and/or anthropogenic sources. Numerous possible anthropogenic and natural sources make the presence of PAH ubiquitous in the environment. The carcinogenic nature of some PAH and their ubiquitous presence make their chemical analysis a topic of environmental and toxicological importance.¹⁻³

As a tentative means of reducing human exposure to contaminated samples, the US Environmental Protection Agency (EPA) includes sixteen PAH in its priority pollutants list and recommends their routine monitoring in environmental samples.⁴ The list includes the following PAH: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, fluoranthene, pyrene, chrysene, anthracene, benzo[ghi]perylene, dibenz[a,h]anthracene, benzo[a]anthracene, benzo[k]fluoranthene, benzo[b]fluoranthene, benzo[a]pyrene and indeno[1,2,3-cd]-pyrene. Their molecular structures are shown in Figure 1.1.
Figure 1.1 Molecular structures of the 16 PAH in the EPA priority list.
Although environmental monitoring of PAH is an important step to prevent exposure to contaminated sites, it provides little information on the actual uptake and subsequent risks. PAH are introduced in the body by adsorption through the skin, ingestion or inhalation and can be subsequently metabolized to their monohydroxylated (OH-PAH) form. Due to the short average lifetime of OH-PAH elimination from the body, their quantitative determination in biological fluids such as urine, milk and blood samples provides accurate information on recent exposure to environmental PAH.5

Parent PAH present different degrees of toxicity but they need metabolic activation to express their carcinogenicity. The biotransformation process consists of two phases. In phase I metabolism, PAH are oxidized by the cytochrome P450 enzymes to form reactive epoxide intermediates, followed by reduction or hydrolysis to hydroxylated derivatives. In phase II metabolism, hydroxyl-PAH form glucoronate and sulfate conjugates to facilitate their excretion through urine, bile and feces.6 These biological processes lead to the formation of multiple metabolites including epoxide, dihydrodiols, OH-PAH and polyhydroxy-PAH. Covalent binding to DNA is believed to be the first critical step in the initiation of the tumor formation process.7,8 This is depicted in Figure 1.2 which shows the metabolic pathways of benzo[a]pyrene (B[a]P), which is the most carcinogenic compound in the EPA priority pollutants list.9 The metabolic pathways of B[a]P include the formation of diol epoxides and their subsequent binding to DNA to form the PAH-DNA adducts. These adducts can then be repaired, restoring the original healthy DNA, or they can originate mutant DNA leading to the formation and proliferation of tumors.10,11
Figure 1.2 Overview of B[a]P metabolism
CYP: Cytochrome P450. Adapted from [6].

In the environment, PAH can be transformed in OH-PAH by a number of oxidation pathways such as photo-oxidation or chemical oxidation. Biological transformations can also generate OH-PAH as a result of incomplete or unsuccessful remediation of PAH contaminated sites by various microorganisms such as bacteria and fungi. These microorganisms can partially degrade PAH into different oxygenated forms with higher polarity, such as OH-PAH. The greater mobility of polar PAH derivatives facilitates their migration and accumulation in soil and other surroundings via surface water and ground water.
Human exposure to PAH can originate from a wide range of occupational and non-occupational activities. Air pollution appears to be higher in urban areas compared to rural areas due to automobile exhaust and industrial activity. Cigarette smoking and environmental tobacco smoke, such as side-stream and mainstream smokes are the other main causes of air exposure to PAH.\textsuperscript{15, 16} Soil contamination originates from PAH adsorbed onto particles which fall to the ground and become accumulated.\textsuperscript{17} Water contamination can be observed after PAH leaching from soil into water and from industrial wastewater. The ingestion of PAH contaminated food is considered the primary source of non-occupational human exposure.\textsuperscript{18} The risk of PAH ingestion is even higher after cooking meat and fish with wood or charcoal. Some cereals, crops and other vegetables also contain PAH.\textsuperscript{19, 20} Occupationally related exposure to PAH is higher among workers in specific industries such as aluminum smelting;\textsuperscript{21} coke-oven workers;\textsuperscript{22} diesel engine mechanics;\textsuperscript{23} taxi, bus and truck drivers;\textsuperscript{23-25} painters;\textsuperscript{26} tool-booth operators\textsuperscript{27} and traffic police officers.\textsuperscript{28}

Not only humans can suffer the consequences of exposure to PAH in the environment, animals can also be affected by them. Water contamination can lead to PAH ingestion and adsorption by various aquatic creatures.\textsuperscript{29} Terrestrial animals can also be at risk of air-borne and aquatic PAH pollution. In addition, these animals can feed from grass or vegetables which grow in contaminated soil, especially if they are in heavily industrialized areas or in the proximity of highways.\textsuperscript{30} Since humans often feed on the exposed animals or consume their products and by-products such as eggs or milk, dairy products can also pose a health hazard and constitute an important additional reason to monitoring animal exposure to PAH.
1.2 Chromatographic Analysis of PAH Metabolites

Early methods focused on the analysis of a few OH-PAH, with particular emphasis on 1-hydroxypyrene. Considering that human exposure often occurs to complex mixtures with numerous PAH, recent methods have expanded their scope to a larger number of metabolites. Particular attention has been paid to metabolites resulting from exposure to EPA-PAH, i.e. PAH included in the EPA priority pollutants list (see Figure 1.1).

The general trend for the analysis of PAH metabolites in urine and milk samples follows the pattern of sample collection, sample clean-up and pre-concentration, chromatographic separation and quantification. Urine analysis of OH-PAH includes an additional hydrolysis step – which can be either enzymatic or acidic – to dissociate OH-PAH from their glucuronide and/or sulfate conjugates. Popular approaches for sample clean-up and pre-concentration include liquid-liquid extraction (LLE) and SPE. Both methodologies are well suited to automation, which increases sample throughput and reduces variability due to manual sample handling. Chromatographic separation and quantification has been based on high-performance liquid chromatography-room temperature fluorescence detection (HPLC) and gas chromatography-mass spectrometry (GC-MS).

Table 1 summarizes several features of previously reported methods for the chromatographic analysis of OH-PAH in urine samples. The tabulated information only includes the metabolites investigated in this dissertation, namely 2-hydroxyfluorene (2OH-Flu), 2-hydroxynaphthalene (2OH-Naph), 1-hydroxypyrene (1OH-Pyr), 9-hydroxyphenanthrene (2OH-Phen), 3-hydroxybenzo[a]pyrene (3OH-B[a]P), 4-hydroxybenzo[a]pyrene (4OH-B[a]P) and 5-
hydroxybenzo[a]pyrene (5OH-B[a]P). No literature reports were found on the analysis of 4-hydroxybenzo[a]pyrene and 5-hydroxybenzo[a]pyrene in urine samples.

Numerous HPLC and GC-MS methods have been published for the analysis of OH-PAH metabolites in urine samples. Interesting to note is the wide range of recoveries reported for the metabolites under investigation. Unfortunately, the standard deviations of the analytical recoveries were not reported, which make difficult the statistical comparison of the observed differences in their average values. Comparison of the analytical recoveries for those metabolites with reported values via the two methods reveals better recoveries via GC-MS for 2-hydroxynaphthalene and 2-hydroxyfluorene. 9-hydroxyphenanthrene and 1-hydroxypyrrene presented approximately the same recovery via HPLC and GC analysis. The best limits of detection (LOD) were clearly obtained via GCMS. Unfortunately GC-MS procedures are more complicated than HPLC methodology. GC-MS requires a chemical derivatization step prior to metabolite separation to avoid peak tailing in the chromatographic column.
Table 1.1 Analytical figures of merit reported for OH-PAH analysis in urine samples by HPLC and GC-MS

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>LOD (ng/L)</th>
<th>Recovery (%)</th>
<th>Sample Preparation</th>
<th>Instrumental method</th>
<th>Analysis time (min)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-hydroxynaphthalene</td>
<td>730</td>
<td>82</td>
<td>SPE, evap.</td>
<td>HPLC</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>2-hydroxynaphthalene</td>
<td>10.5</td>
<td>99</td>
<td>SPE, evap., derivatization</td>
<td>GC-MS</td>
<td>24</td>
<td>33</td>
</tr>
<tr>
<td>2-hydroxyfluorene</td>
<td>727</td>
<td>62</td>
<td>SPE, evap.</td>
<td>HPLC</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>2-hydroxyfluorene</td>
<td>3.6</td>
<td>82</td>
<td>SPE, evap., derivatization</td>
<td>GC-MS</td>
<td>27</td>
<td>34</td>
</tr>
<tr>
<td>9-hydroxyphenanthrene</td>
<td>165</td>
<td>57</td>
<td>SPE, evap.</td>
<td>HPLC</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>9-hydroxyphenanthrene</td>
<td>200</td>
<td>50</td>
<td>SPE, evap., derivatization</td>
<td>GC-MS</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td>1-hydroxypyrene</td>
<td>40</td>
<td>83</td>
<td>SPE, evap.</td>
<td>HPLC</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>1-hydroxypyrene</td>
<td>44</td>
<td>68</td>
<td>SPE, evap.</td>
<td>HPLC</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>1-hydroxypyrene</td>
<td>1.6</td>
<td>99</td>
<td>SPE, evap., derivatization</td>
<td>GC-MS</td>
<td>24</td>
<td>53</td>
</tr>
<tr>
<td>1-hydroxypyrene</td>
<td>3.3</td>
<td>80</td>
<td>SPE, evap., derivatization</td>
<td>GC-MS</td>
<td>27</td>
<td>34</td>
</tr>
<tr>
<td>3-hydroxybenzo[a]pyrene</td>
<td>1615</td>
<td>40</td>
<td>SPE, evap.</td>
<td>HPLC</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>3-hydroxybenzo[a]pyrene</td>
<td>40</td>
<td>48</td>
<td>SPE, evap.</td>
<td>HPLC</td>
<td>45</td>
<td>23</td>
</tr>
<tr>
<td>3-hydroxybenzo[a]pyrene</td>
<td>10.0</td>
<td>NA</td>
<td>SPE, evap., derivatization</td>
<td>GC-MS</td>
<td>27</td>
<td>34</td>
</tr>
</tbody>
</table>

a) Instrumental analysis only; does not include sample preparation procedures
Table 1.2 summarizes the pertinent features of HPLC and GC-MS methods previously reported for the analysis of OH-PAH in milk samples. Chromatographic analysis of milk samples have focused on the determination of 1-hydroxypyrene (1OH-PAH), 3-hydroxybenzo[a]pyrene (3OH-B[a]P), 2-hydroxyfluorene (2OH-Flu) and 1-, 2-, 3-, 4-, and 9-hydroxyphenanthrene (9OH-Phen). Sample volumes varying from 5 to 50 mL have provided LODs ranging from 0.04 ng.mL\(^{-1}\) (1OH-Pyr)\(^{46}\) to 5.1 ng.mL\(^{-1}\) (2OH-Flu).\(^{47}\) The main disadvantages of the reported methodology are the low recoveries (32 – 43%) and relatively long analyses times. Excluding the sample preparation steps, reported instrumental times, i.e. chromatographic elution times have varied from 26 to 70 min per sample. No literature reports were found on the analysis of 1-hydroxynaphthalene and 2-hydroxynaphthalene in milk samples. The information on the recoveries of OH-PAH from milk samples is scarce and incomplete.
Table 1.2 Reported methods for the analysis of OH-PAH in milk samples by HPLC and GC-MS

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>LOD (ppb)</th>
<th>Sample Volume (ml)</th>
<th>Recovery %</th>
<th>Sample Preparation</th>
<th>Instrumental method</th>
<th>Analysis time (min)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1OH-Pyr</td>
<td>NA</td>
<td>50</td>
<td>NA</td>
<td>LLE, SPE, derivatization</td>
<td>GC-MS</td>
<td>26</td>
<td>49</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>NA</td>
<td>50</td>
<td>NA</td>
<td>LLE, SPE, derivatization</td>
<td>GC-MS</td>
<td>26</td>
<td>49</td>
</tr>
<tr>
<td>3OH-Phen</td>
<td>NA</td>
<td>50</td>
<td>NA</td>
<td>LLE, SPE, derivatization</td>
<td>GC-MS</td>
<td>26</td>
<td>49</td>
</tr>
<tr>
<td>2OH-Flu</td>
<td>2.3-5.1</td>
<td>10</td>
<td>43</td>
<td>LLE, SPE, derivatization</td>
<td>GC-MS</td>
<td>26</td>
<td>47</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>2.3-5.1</td>
<td>10</td>
<td>43</td>
<td>LLE, SPE, derivatization</td>
<td>GC-MS</td>
<td>26</td>
<td>47</td>
</tr>
<tr>
<td>1OH-Phen</td>
<td>2.3-5.1</td>
<td>10</td>
<td>43</td>
<td>LLE, SPE, derivatization</td>
<td>GC-MS</td>
<td>26</td>
<td>47</td>
</tr>
<tr>
<td>2OH-Phen</td>
<td>2.3-5.1</td>
<td>10</td>
<td>43</td>
<td>LLE, SPE, derivatization</td>
<td>GC-MS</td>
<td>26</td>
<td>47</td>
</tr>
<tr>
<td>3OH-Phen</td>
<td>2.3-5.1</td>
<td>10</td>
<td>43</td>
<td>LLE, SPE, derivatization</td>
<td>GC-MS</td>
<td>26</td>
<td>47</td>
</tr>
<tr>
<td>4OH-Phen</td>
<td>2.3-5.1</td>
<td>10</td>
<td>43</td>
<td>LLE, SPE, derivatization</td>
<td>GC-MS</td>
<td>26</td>
<td>47</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>2.3-5.1</td>
<td>10</td>
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<td>LLE, SPE, derivatization</td>
<td>GC-MS</td>
<td>30</td>
<td>46</td>
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</table>

a) Volume of milk b) LLE: liquid-liquid extraction c) Instrumental analysis only; does not include sample preparation procedures.
Although chromatographic techniques provide reliable results in the analysis of OH-PAH, their experimental procedures are time consuming and expensive. Elution times of 30-60 minutes are typical and standards must be run periodically to verify retention times. If the concentrations of target species are found to lie outside the detector’s response range, the sample must be diluted and the process repeated. On the other end of the concentration range, many samples are “zeroes,” i.e. the concentrations are below detection limits. Additional problems arise when laboratory procedures are scaled up to handle thousands of samples under mass screening conditions. Under the prospective of a sustainable environment, the large usage of organic solvents is one of the main limitations of the current chromatographic methodology.

Research in our group has focused on the development of a screening methodology for OH-PAH. Screening techniques capable of providing a “yes or no” answer to OH-PAH contamination prevent unnecessary scrutiny of un-contaminated samples via conventional methods, reduce analysis cost and expedite the turnaround time for decision making purposes. The remaining of this chapter is then devoted to cover the basic principles of the techniques we chose for the development of screening methodology, namely capillary electrophoresis (CE) and synchronous fluorescence spectroscopy (SFS).

1.3 Capillary Electrophoresis (CE)

CE is an analytical technique used for the separation of a variety of analytes such as small molecules, peptides and proteins. Unlike chromatography, in which separation is the result of the different affinities of the sample components with the stationary and mobile phases, separation in CE is based on the size and on the amount of charge of the analytes. Analyte migration takes
place on the inside of a small bore capillary filled with a solution of a buffer electrolyte. When an electric field is applied across the capillary, species migrate with a mobility that is directly proportional to their charge and inversely proportional to their size. This means that if two ions have the same size but one has a greater charge, it will move faster; and in the same way if two ions have equal charges but one has a larger size, it will exhibit more friction with respect to the solvent and therefore will migrate slower than the smaller ion.

CE analyses provide high resolution separations since the two main sources of band broadening that take place in a separation column, i.e. eddy diffusion and mass transfer are eliminated due to the lack of packing in the capillary tube. The only source of band broadening in CE is longitudinal diffusion, which can result in a plate number one order of magnitude higher than those obtained with chromatographic methods, especially for large molecules with low diffusion coefficients such as proteins. Another advantage of CE is the low sample (\( \sim 10^{-9} \) L) and solvent consumption, making it less expensive to run and maintain than HPLC. It also means that it is a more environmentally friendly technique since it minimizes the use of potentially toxic organic solvents.

The simplest form of CE is capillary zone electrophoresis (CZE), in which analytes are separated, in a capillary previously filled with a buffer, into different zones. This technique works well for cationic and anionic analytes, neutrals will not separate under such conditions. To expand the application of CE to neutral and charged molecules, a type of CE called micellar electro-kinetic chromatography (MEKC) can be used. In this technique, a surfactant is added to the buffer to form micelles, which will serve as a pseudo-stationary phase similar to HPLC. Analytes will interact in a variety of ways with the micelle and they will show a certain partitioning between the aqueous phase and the micelle, which will contribute to separation. This
partitioning can be regulated by changing experimental conditions such as salt content, temperature, pH, and addition of ion pairing and complexing agents. Another additive that can be added to the buffer are cyclodextrins (CD), which are cyclic oligosaccharides possessing a cavity that can interact with analytes, improving separations based on partitioning of the analyte between the solution and the CD. Given the amount of variables to optimize in MEKC, a simpler approach such as CZE is preferred.

1.3.1 Electrophoretic mobility and electro-osmotic flow

Electrophoresis is the movement of charges under an applied electric field. In CE, the movement of charged species will have an electrophoretic component that will exert a force towards the appropriate electrode, i.e. a positively charge particle will tend to migrate towards the cathode (negative electrode) and a negatively charged particle will be attracted by the anode (positive electrode). The electrophoretic mobility ($\mu_E$) will depend on the size of the solute ion and the charge it bears, i.e. the charge/mass ratio:

$$\mu_E = \frac{q}{f} = \frac{q}{(6\pi \eta r)} \quad (1.1)$$

where $q$ is the solute charge, $f$ is a frictional coefficient given by the Stokes’ law (assuming spherical particle), $\eta$ is the viscosity of the liquid and $r$ is the radius of the particle. The resulting electrophoretic velocity ($V$) will be dependent on $\mu_E$ and the applied electric field ($E$):

$$V = \mu_E \times E \quad (1.2)$$

When a potential is applied across a capillary filled with an electrolyte solution, this electrolyte solution will move from one end of the capillary to the other. The explanation of this
phenomenon is related to the chemical nature of the inside wall of fused silica capillaries. Silanol groups (Si-OH) are an integral part of fused silica capillaries and are exposed to the electrolyte solution. At pH values higher than four, these silanol groups will begin to deprotonate and become ionized to negatively charged silanolate (Si-O'). Positive ions in solutions will become attracted to the newly formed negative charges on the capillary wall forming a double layer. The first layer or “fixed layer” will be tightly held to the negatively charged silanols and the second layer (diffuse layer) will form at a greater distance from the capillary wall and will have freedom to move under the influence of an external electric field. When the ions from the diffuse layer move so does the water molecules solvating them, therefore the bulk of the electrolyte solution experiences a net flow towards the cathodic end of the capillary referred to as the electro-osmotic flow (EOF):

\[ \mu_{EOF} = \frac{\varepsilon \zeta}{\eta} \]  

(1.3)

where \( \mu_{EOF} \) is the EOF mobility, \( \varepsilon \) is the dielectric constant of the electrolyte solution and \( \zeta \) is the zeta potential. Since the zeta potential depends on the charge of the capillary wall and on the level of ionization of silanol groups, the buffer pH usually plays a very important role in determining EOF velocity.

### 1.3.2 Instrumental set-up

CE instrument main components are a capillary tube, two buffer reservoirs, two electrodes, a high power voltage supply, a sample introduction system (or autosampler) and a
detector connected to a data acquisition device. A schematic representation of a typical CE system is illustrated in Figure 1.3.

Capillaries are constituted by fused silica and have an outer diameter of ~ 0.4 mm. Since this material can be fragile and can break easily upon slight bending, a polyimide coating is used to provide the capillary with physical support. A small portion of a few mm of this coating is usually burned-off to expose the silica and act as a window to perform detection on the capillary, since fused silica is transparent to UV and visible light. The capillary is filled with an electrolyte solution and is connected to two buffer reservoirs. A high voltage power supply is also connected to the buffer vials by means of two electrodes which help produce an electric field across the
capillary to generate the electrophoretic phenomena and also the EOF. Sample injection can be done in two ways: hydrodynamically or electrokinetically. In hydrodynamic injection the capillary is placed in the sample vial and a certain volume of sample is pressure driven into the capillary. In electrokinetic injection, upon immersion of the capillary in the sample vial a voltage is applied causing two effects: movement of the buffer inside of the capillary, which will push a volume of sample via siphoning, and electrophoretic migration of sample ions. These two methods of sample injection usually last only a few seconds and result in injections of nano-liter volumes of sample solution. The detector is typically placed at the cathodic end of the capillary and it can be UV-Vis, fluorescence, electrochemical or refractive index.

1.3.3 Sample Stacking

Absorption spectroscopy is one of the most popular detection modes in CE but its sensitivity is quite modest. Several strategies exist to improve ultraviolet-visible (UV-Vis) LODs. These include increasing optical path lengths with the aid of bubble, Z- or U-shaped detection cells, solid-phase extraction (SPE) and in-capillary micro-extraction. Another alternative for improving the sensitivity of CE is an on-line pre-concentration technique called sample stacking. Sample stacking is based on the different conductivities of the sample solution and the CE running buffer solution. The only requirement for its successful application is to operate with a sample solvent of lower conductivity than the running buffer solution. This requirement is usually met with either a diluted background electrolyte or a pure solvent such as methanol. When an electric potential is applied across the separation capillary, an amplified electric field is created in the sample zone. Under the influence of the electric field, sample ions
migrate from the high to low drift velocity region. This migration leads to a local accumulation or ‘stacking’ of sample ions near the interface between regions of high and low conductivity. After crossing this boundary the thin zone of ions moves through the running buffer and separates into individual zones under normal electrophoretic conditions. The stacking mechanism occurs for both positively and negatively charged species. The positive species stack up in front of the sample plug while the negative species stack up in the back of the sample plug. The neutral compounds are left in the sample plug and co-elute. In comparison to conventional CZE, sample stacking allows the analyst to increase sample loading (up to a certain threshold value) at no cost of separation efficiency. The narrow zones of concentrated ions often enhance signal intensities and improve the sensitivity of CZE measurements.\textsuperscript{57, 58}

1.4 Fluorescence Spectroscopy

1.4.1 Basic Principles

Fluorescence spectroscopy is based on the detection of radiation emitted during the deactivation of electronically excited molecules.\textsuperscript{59-61} Under normal conditions, the orbital of lowest energy of an organic molecule are occupied by pairs of electrons with spin in opposite directions.\textsuperscript{62} Since most of the organic molecules have an even number of valence electrons, the resulting electron spin is zero. Such a state, with no net spin, is called a singlet state. The singlet state of lowest energy is known as the ground state, and it is represented in the Jablonski diagram of Figure 1.4 by \( S_0 \). Through the absorption of electromagnetic radiation (\( A \)), a molecule can pass from the ground state to an excited state of higher energy. This transition occurs in
approximately $10^{-15}$ s and entails the promotion of an electron from the highest occupied orbital to a previously unoccupied one. If the transition occurs with no change in the spin of the promoted electron, the excited state will have two unpaired electrons with anti-parallel spins and, therefore, net spin equal to zero. An electronic state with these characteristics is known as a singlet excited state. In Figure 1.4 the first and second singlet-excited states are represented by $S_1$ and $S_2$, respectively. If the transition involves a change in the electronic spin, the excited state will be characterized by two unpaired electrons with parallel spins. In this case, the resultant spin is one, and the excited state receives the name of triplet state. In Figure 1.4, the triplet state of the lowest energy is symbolized by $T_1$ while any other triplet state of higher energy is represented by $T_n$. 
Figure 1.4 Jablonski diagram. A is the absorption, F is the fluorescence, P is the phosphorescence, VR is the vibrational relaxation, IC is internal conversion, and ISC is the intersystem crossing.

When the energy of an absorbed photon is enough to excite a molecule to a state such as $S_2$, it usually releases the extra vibrational energy to reach the lowest vibrational level of the state. This radiationless deactivation mechanism is known as vibrational relaxation (VR), and it is the consequence of the energy transfer from the excited molecule to the surrounding medium in the form of thermal energy. Through another radiationless process called internal conversion (IC), the molecule passes from the lowest vibrational level of $S_2$, to a vibrational level of $S_1$. This process results in the transformation of excitation energy into vibrational-rotational energy\(^{63}\) and occurs between electronic states of the same multiplicity.\(^{64}\) The lowest vibrational level of $S_1$ is then reached through VR. If the molecule was initially excited to a higher excited state than $S_2$,
the lowest vibrational level of \( S_1 \) would be reached by a succession of IC and VR processes. From the lowest vibrational level of \( S_1 \), the molecule has two ways of directly returning to the ground state: through IC, without the emission of radiation, or by the emission of a photon with no change in the electronic spin. The latter process is responsible for the emission of fluorescence and occurs in a time period of \( 10^{-10} \) to \( 10^{-7} \) s. The energy of the emitted photon corresponds to the energy gap between the lowest vibrational level of \( S_1 \) and the ground state. When the lowest vibrational level of the \( S_1 \) state overlaps with \( S_0 \), the excited state is deactivated by nonradiative relaxation, and the emission of fluorescence does not occur.

When the emitted photon has the same energy as the one initially absorbed, the process is termed resonance fluorescence. Most times, however, the energy loss in VR and IC results in the emission of fluorescence at longer wavelengths than the excitation wavelength, and resonance fluorescence is not observed.

The remaining possibility to return to \( S_0 \) from \( S_1 \) begins with a process called intersystem crossing (ISC). ISC is radiationless mechanism involving systems of different multiplicity which requires a change in the electronic spin.\(^{62}\) Although this kind of transition has a much lower probability to occur than spin-allowed transition,\(^{61, 62}\) the time scale of ISC is similar to the one for fluorescence (\( 10^{-10} \) – \( 10^{-7} \) s); therefore, it competes with fluorescence for the deactivation of \( S_1 \). From \( S_1 \), the molecule can then pass to the excited triplet state manifold (\( T_n \)) and reach, by a succession of VR an IC processes, the lowest vibrational level of \( T_1 \). From \( T_1 \), and through ISC, the molecule can revert back to the excited singlet state manifold. Since the triplet states have lower energy than the corresponding singlet states, the transition from \( T_1 \) to \( S_1 \) requires some additional activation energy. This activation energy can be obtained either by a thermal process or by the interaction of two molecules in the triplet state to produce one
molecule in the excited singlet state. The emission of radiation from the excited S\(_1\) to S\(_0\) receives the name of delayed fluorescence. If reverse ISC does not occur, the molecule has two additional possibilities to return from T\(_1\) to S\(_0\): through ISC followed by VR or through the emission of radiation in a process called phosphorescence (P). The emission of phosphorescence involves states of different multiplicity and, as a consequence, has a longer lifetime than fluorescence (between 10\(^{-3}\) and 10 s). Since the energy gap between T\(_1\) and S\(_0\) is usually smaller than the one between S\(_1\) and S\(_0\), phosphorescence occurs in a region of larger wavelength than fluorescence.

1.4.2 Synchronous Fluorescence Spectroscopy

Conventional fluorescence spectra - in which either the excitation or the emission wavelength is set at its maximum position while the other is scanned – present limited selectivity. The similarity and/or overlapping of broad fluorescence bands at room temperature usually interfere in the characterization of targeted compounds without previous separation. Several strategies exist to improve the selectivity of room temperature fluorescence measurements. The one employed here is known as synchronous fluorescence spectroscopy (SFS). The synchronous excitation approach consists of varying simultaneously both the excitation (\(\lambda_0\)) and emission (\(\lambda\)) wavelengths while keeping constant a wavelength interval (\(\Delta\lambda = \lambda - \lambda_0\)) between them. The judicious choice of the \(\Delta\lambda\) parameter often leads to spectral simplification and resolution of spectral overlapping. An example of spectral simplification due to the judicious choice of wavelength interval is shown in Figure 1.5, which compares the excitation and fluorescence spectra of perylene (top) to synchronous fluorescence spectra recorded
at two $\Delta \lambda$ values (bottom). The synchronous fluorescence spectrum obtained with a $\Delta \lambda = 10$nm consists of a single emission peak. The 30nm wavelength difference results in two emission peaks. In the analysis of a complex mixture without previous chromatographic separation, the direct determination of perylene with a $\Delta \lambda = 10$nm should be less prone to spectral overlapping than its determination with a $\Delta \lambda = 30$nm.

Figure 1.5. Excitation and fluorescence spectra (top) and SFS (bottom) of perylene
CHAPTER 2. INSTRUMENTATION

2.1 Absorption Spectroscopy

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

2.2 Fluorescence Spectroscopy

Fluorescence spectra were recorded with a spectrofluorimeter (Photon Technology International) equipped with a continuous 75 W xenon lamp with broadband illumination (200 – 1000 nm). The excitation and the emission monochromators had the same reciprocal linear dispersion (4 nm mm\(^{-1}\)) and accuracy (±1 nm with 0.25 nm resolution). Their gratings had 1200 grooves mm\(^{-1}\) and were blazed at 300 nm (excitation) and 400 nm (emission). The detector was a photomultiplier tube with spectral response from 185 to 650 nm. Instrument computer control was performed with commercial software (Felix32) specifically designed for the system. All fluorescence measurements were made at 90° geometry with 1 cm x 1 cm standard quartz cuvettes.
2.3 **pH Meter**

pH measurements were made with an AR-15 pH/mV/°C meter and a glass body standard size combination electrode (Fischer Scientific).

2.4 **Solution Shaking and Centrifugation**

Sample shaking was carried out with a Maxi Mix III Rotary Shaker (Thermolyne). Centrifugation was performed with a MiniSpin centrifuge (Eppendorf) with 13,400 RPM maximum speed and a twelve-position rotor.

2.5 **Capillary Electrophoresis**

CZE was carried out with a GPA100 system purchased from Groton Biosystems. Its detection unit consisted of a variable, single wavelength absorption spectrometer (Model VUV 9022-0000; JMST Systems) equipped with a deuterium lamp and a silicon photodiode detector. Instrument control was performed with a PC and customized software (WinPrinceCE, PrinCE CE systems). Fused-silica capillaries with 50 mm internal diameter and 375 mm outer diameter were purchased from Polymicro. Their polyamide coating was removed with a window maker (MicroSolv-CE) to provide an UV transparent detection window with an approximate length of 4 mm. The total length of the capillaries was either 61 or 82 cm, depending on the specific application, with a corresponding center of their optical window located at 38 or 58 cm from the injection port of the CE instrument, respectively.
CHAPTER 3. SOLID-PHASE EXTRACTION, SAMPLE STACKING AND CAPILLARY ELECTROPHORESIS FOR THE ANALYSIS OF URINARY POLYCYCLIC AROMATIC HYDROCARBON METABOLITES


3.1 Introduction

Despite its recognized capability to separate charged species with relevance in biochemical, biological and biomedical research, only a few articles exist on the separation of PAH metabolites via CE. Baseline resolution of positional isomers has been reported via γ-cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) and capillary zone electrophoresis (CZE). The investigated OH-PAH were 1-hydroxynaphthalene (1OH-Naph), 2-hydroxynaphthalene (2OH-Naph) and 1-hydroxybenzo[a]pyrene (1OH-B[a]P), 3-hydroxybenzo[a]pyrene (3OH-B[a]P), 7-hydroxybenzo[a]pyrene (7OH-B[a]P), 9-hydroxybenzo[a]pyrene (9OH-B[a]P) and 12-hydroxybenzo[a]pyrene (12OHB-[a]P). Metabolite detection was carried out via laser-induced fluorescence (LIF) or UV-Vis absorption spectroscopy. LIF limits of detection (LODs) were reported at the parts per billion (ppb) concentration levels. UV-Vis LODs were not reported.

The lack of reported data on UV-Vis LODs is not surprising. As previously mentioned, the sensitivity of absorption detection in CE is quite modest. Z- or U-shaped detection cells, solid-phase extraction (SPE) and in-capillary micro-extraction. Previous work by our group reported the first application of in-capillary micro-extraction capillary zone electrophoresis (CZE) for the urine analysis of OH-PAH. Hydrolyzed urine samples were
submitted to a manual SPE procedure optimized in our lab.\textsuperscript{35} In-capillary micro-extraction of OH-PAH was carried out from methanol SPE extracts with gold nanoparticle deposited capillaries made in-house. Gold nanoparticles were coated on the inner walls of fused silica capillaries (50 mm internal diameter) following previously published methodology.\textsuperscript{74, 75} Baseline resolution of 1-hydroxypyrene (1OH-Pyr), 9-hydroxyphenanthrene (9OH-Phen), 3OH-B[a]P, 4-hydroxybenzo[a]pyrene (4OH-B[a]P) and 5-hydroxybenzo[a]pyrene (5OH-B[a]P) was achieved with a 100 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer solution prepared in 40% methanol–water v/v. Complete separation was accomplished in approximately 40 min of migration time with metabolite recoveries varying from 69.8 ± 5.5 (9OH-Phen) to 80.6 ± 5.3% (3OH-B[a]P). UV-Vis LODs varied between 8.8 (9OH-Phen) and 14.4 ng.mL$^{-1}$ (4OH-B[a]P).\textsuperscript{73}

In this chapter, we present significant advances in all fronts. Baseline resolution of 2-hydroxyfluorene (2OH-Flu), 2OH-Naph, 1OH-Pyr, 9OH-Phen, 3OH-B[a]P, 4OH-B[a]P and 5OH-B[a]P was achieved in approximately 17 min with a 20 mM borate buffer prepared in 50% methanol–water v/v. The SPE procedure was accomplished with the aid of a twelve-port vacuum manifold. On-line pre-concentration was performed via sample stacking.\textsuperscript{56, 76} To the extent of our literature search, this is the first application of sample stacking to the analysis of OH-PAH. Metabolite recoveries varied from 93.2 ± 7.7% (5OH-B[a]P) to 108.7 ± 7.8% (2OH-Naph). LODs were at the ppb level ranging from 0.99ppb (3OH-B[a]P) to 8.54ppb (2OH-Naph). The new method was found to be free of interference from four pharmacological drugs – naproxen, ibuprofen, diclofenac and amoxicillin – that might be found in urine samples of unhealthy individuals.
3.2 Materials and Methods

3.2.1 Instrumentation

Instrumentation for pH measurements, absorption and fluorescence spectroscopy, and CZE measurements was previously described in chapter 2. Sample injection for CZE analysis was made via hydrodynamic pressure. Prior to CZE data collection, the silica capillary was rinsed for 10 minutes with 0.1 M NaOH and 10 minutes with the running buffer. At the end of data collection, the capillary was rinsed for 10 minutes with methanol and 10 minutes with Nanopure water to remove residual impurities. The optimized procedure for sample stacking consisted of the following steps: (1) the separation capillary was pressure-filled with the running buffer; (2) the sample was injected during the optimized time (60 s) at 45 mbar pressure; and (3) the buffer vial was re-positioned at the inlet of the separation capillary to carry out metabolite separation at a voltage of 30 kV. Fused-silica capillaries with 50 mm internal diameter and 375 mm outer diameter were purchased from Polymicro. Their polyamide coating was removed with a window maker (MicroSolv-CE) to provide an UV transparent detection window with an approximate length of 4 mm. The total length of the capillaries was 82 cm and the center of the optical window was located at 58 cm from the injection port of the CE instrument.

The optimized procedure for sample stacking consisted of the following steps: (1) the separation capillary was pressure-filled with the running buffer; (2) the sample was injected during the optimized time (60 s) at 45 mbar pressure; and (3) the buffer vial was re-positioned at the inlet of the separation capillary to carry out metabolite separation at a voltage of 30 kV. The total length of the capillaries was 82 cm and the center of the optical window was located at 58 cm from the injection port of the CE instrument.
3.2.2 Reagents

All solvents were Aldrich HPLC grade. All chemicals were analytical-reagent grade and utilized without further purification. Unless otherwise noted, Nanopure water was used throughout. 2OH-Flu, 2OH-Naph, 1OH-Pyr, 9OH-Phen, creatinine, naproxen, ibuprofen, diclofenac and amoxicillin were purchased from Sigma-Aldrich. 3OH-B[a]P, 4OH-B[a]P and 5OH-B[a]P were from Midwest Research Institute. The synthetic urine solution was manufactured by RICCA Chemical Company (Arlington, TX) and purchased from Fischer Scientific. Its chemical composition mimicked main components of human urine at the concentrations found in healthy urine samples. Borate buffer was acquired from Fisher Scientific. All other chemicals were purchased from Fisher Chemical. Sep-Pak Plus cartridges were acquired from Waters.

3.2.3 Preparation of stock solutions

Stock solutions of OH-PAH were prepared by dissolving pure standards in methanol. Creatinine, naproxen, ibuprofen, diclofenac and amoxicillin stock solutions were prepared in methanol. All stock solutions were kept in the dark at 4 °C. Prior to use, stock solutions of OH-PAH were monitored via fluorescence spectroscopy for possible photo-degradation of metabolites. Spectral profiles and fluorescence intensities of stock solutions remained the same for a period of six months. Working solutions were prepared daily by serial dilution with methanol.
3.2.4 Sample preparation

Urine donations from an anonymous volunteer group were pooled, frozen and stored at -20 °C until further analysis. Urine samples were spiked with micro-liters of stock solutions of appropriate concentrations and equilibrated for 30 min to allow for the interaction of metabolites and naproxen with urine components such as urea and various salts. Then 2 mL of 0.1 M HCl was added to the sample and the mixture was buffered with 10 mL of a 0.05 M potassium phthalate sodium hydroxide buffer (pH 5.0). The buffered sample was stirred for 30 minutes to allow for urine hydrolysis.

3.2.5 Solid-phase extraction of urine samples

SPE was carried out with a Visiprep 12 port vacuum manifold (Supelco). Urine samples were processed through a Sep-Pak Plus SPE cartridge pre-conditioned with 10 mL of methanol and 10 mL of buffered water (pH = 5). The cartridges were then washed with 10 mL of buffered water (pH = 5) and air dried for 10 seconds at 15 mm Hg. OH-PAH were eluted with 2 aliquots of 1.5 mL of pure methanol.

3.3 Results and Discussion

3.3.1 Separation of OH-PAH

Table 3.1 summarizes the migration times, peak resolutions and peak efficiencies of the studied metabolites. Baseline resolution of the seven studied metabolites in the shortest possible
time was achieved upon optimization of buffer concentration (20 mM), methanol percentage (50% v/v), pH (9.7) and temperature (21 °C). Buffer selection was based on the reported range of pKa values for the studied metabolites (8.6 ≤ pKa ≤ 10.8).\textsuperscript{77-81} Borate, whose working pH ranges from 8.2 and 10.2, promotes the partial dissociation of metabolites in the separation capillary and forces some degree of negative charge on each OH-PAH. Because our detector was placed on the negative end (cathode) of the separation capillary, the detection of OH-PAH results from the predominance of the electro-osmotic flow (\(\mu_{\text{EOF}}\)) over the electrophoretic mobility (\(\mu_{\text{OH-PAH}}\)). The separation of 4OH-B[a]P from 5OH-B[a]P and 3OH-B[a]P from 1OH-Pyr required the use of methanol (see Figure 3.1), i.e. an organic modifier capable of reducing \(\mu_{\text{EOF}}\). Methanol percentages higher than 50% v/v caused peak distortions that compromised separation efficiency.
Table 3.1 Migration time, peak resolution and peak efficiency of OH-PAH

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<th>Rs (^b)</th>
<th>Efficiency (^c)</th>
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<td>2OH-Naph</td>
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<tr>
<td>4OH-B[a]P</td>
<td>14.59 ± 0.03</td>
<td>1.80</td>
<td>2.48 x 10(^5)</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>16.32 ± 0.04</td>
<td>13.66</td>
<td>2.30 x 10(^5)</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>16.62 ± 0.04</td>
<td>2.39</td>
<td>3.04 x 10(^5)</td>
</tr>
</tbody>
</table>

\(^a\) Values represent the average of 3 independent electrophoretic runs.

\(^b\) Resolution between peaks calculated from \(2(t_2-t_1)/w_2-w_1\). \(t_1\) and \(t_2\) are migration time of peaks 1 and 2, respectively. \(w_1\) and \(w_2\) are the widths at the base of each peak.

\(^c\) Calculated from \(5.54(t_m/w_{0.5})^2\). \(t_m\): migration time. \(w_{0.5}\): width at half height of the peak.

The separation occurs due to the apparent electrophoretic mobility (\(\mu_{\text{app}}\)) of each metabolite. Because \(\mu_{\text{OH-PAH}}\) correlates with \(\mu_{\text{app}}\) according to the equation:

\[
\mu_{\text{app}} = \mu_{\text{EOF}} - \mu_{\text{OH-PAH}}
\]

(3.1)

our experiments indicate the following trend for the electrophoretic mobility: \(\mu_{\text{2OH-Flu}} < \mu_{\text{2OH-Naph}} < \mu_{\text{9OH-Phen}} < \mu_{\text{5OH-B[a]P}} < \mu_{\text{4OH-B[a]P}} < \mu_{\text{1OH-Pyr}} < \mu_{\text{3OH-B[a]P}}\). \(\mu_{\text{OH-PAH}}\) is directly proportional to the metabolite’s charge and inversely proportional to its solvation radius. The magnitude of the negative charge depends on the fraction of the dissociated metabolite (\(\alpha\)) at the experimental pH of the separation buffer, i.e. \(\alpha = 10^{pK_a}/10^{pK_a} + 10^{pH}\). The migration times of the three benzo[a]pyrene metabolites – which probably have similar solvation radii – are in good
agreement with the relative values of the reported pKa [81]; i.e. pKa_{3OH-B[a]P} < pKa_{4OH-B[a]P} < pKa_{5OH-B[a]P}. Assuming no significant changes of the acid dissociation constants with the composition of the separation medium, prediction of the α values at the pH of the separation (9.7) leads to the following trend: α_{3OH-B[a]P} > α_{4OH-B[a]P} > α_{5OH-B[a]P}. This trend is in good agreement with the relative values of the electrophoretic mobilities of the three benzo[a]pyrene metabolites.

Figure 3.1 Electropherograms of a synthetic mixture of the studied metabolites separated with 20mM borate buffer prepared in methanol-water mixtures.

Peak identification: (1) EOF marker (methanol); (2) 2OH-Flu; (3) 2OH-Naph; (4) 9OH-Phen; (5) 5OH-B[a]P; (6) 4OH-B[a]P; (7) 1OH-Pyr; (8) 3OH-B[a]P. Buffer pH = 9.7; voltage 30kV; temperature 21°C.
3.3.2 **SPE optimization**

Numerous SPE methods exist for the analysis of OH-PAH in urine samples. Typically, metabolites are extracted via reverse-phase interactions with commercial cartridges containing C-18 alkyl chains chemically bound to silica particles. OH-PAH elution is carried out predominantly with methanol. Using the same type of solid sorbent and eluting solvent, our optimization studies focused on obtaining both high pre-concentration factors (CFs) and overall metabolite recoveries. CFs in SPE correlate with the ratio between volume of the sample ($V_S$) and volume of the eluting solvent ($V_E$); i.e. $CF = V_S / V_E$. For any volume of the eluting solvent, the best CF values are obtained when the sample volume matches the breakthrough volume of the solid sorbent. Sample volumes larger than the breakthrough volume are prone to analyte losses. Within the context of urine analysis, where the volume of the sample is sometimes limited, a compromise was made ($V_S = 60$ mL) based on the literature values (10–250 mL) considered to be appropriate for urine analysis.$^{38, 84, 85}$ $V_E$ was reduced to its minimum possible value (3 mL) at no cost of metabolite recoveries.

The overall recoveries were optimized by monitoring the concentrations of OH-PAH via room-temperature fluorescence (RTF) spectroscopy, i.e. a technique with a well-known ability to monitor trace concentration levels of metabolites in liquid solutions.$^{86}$ Excitation and fluorescence spectra of the studied metabolites are provided in Appendix A of this dissertation. Because the adsorption of OH-PAH onto the solid sorbent occurs from an aqueous-based matrix (urine), the initial survey of excitation and emission spectra was carried out in methanol–water (0.05% v/v) and methanol. All spectra were recorded using the same excitation and emission band-pass (2 nm). This band-pass provided appropriate signal-to-background ratios (S/B = 3) for
analytical use at the parts-per-billion (ng.mL$^{-1}$) concentration level. No attempts were made to adjust slit widths to optimize spectral resolution, nor were the spectra corrected for instrumental response. The spectral features of the studied metabolites were virtually the same in the two types of solvents. Apparently, the solvation effect of methanol in the aqueous solution provides a similar micro-environment to the one in pure methanol.

Table 3.2 summarizes the RTF analytical figures of merit (AFOM) of the studied metabolites in the two types of solvents. Fluorescence measurements were made at the maximum excitation wavelength ($\lambda_{ex}$) and maximum emission wavelength ($\lambda_{em}$) of each metabolite. The correlation coefficients of the calibration curves were close to unity, indicating a linear relationship between OH-PAH concentration and fluorescence intensity. The lowest concentrations of the linear dynamic ranges (LDR) correspond to the limit of quantitation (LOQ) of the calibration method. The LOQ was calculated according to the formula $\text{LOQ} = 10S_B/m$; where $S_B$ is the standard deviation of the average blank signal from a minimum of sixteen measurements ($N = 16$) at the $\lambda_{ex}$ and $\lambda_{em}$ of each metabolite and $m$ is the slope of the calibration curve. The LOD was calculated according to the formula $\text{LOD} = 3S_B/m$. No efforts were made to experimentally obtain the upper concentration limits of the calibration curves. The highest concentrations in Table 3.2 already surpass the concentration thresholds of OH-PAH often found in urine samples. It is important to note, however, that the experimental concentrations did not surpass the breakthrough volume of the SPE device for a sample volume of 60 mL. At LOQ concentrations, the relative standard deviations (RSD) of the average RTF signals ($N = 6$) varied between 5 and 7%. These RSD values demonstrate the ability to precisely monitor OH-PAH at the parts-per-billion concentration level.
### Table 3.2 RTF analytical figures of merit of OH-PAH in methanol/water and methanol

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>$\lambda_{\text{ex/em}}$ a</th>
<th>Methanol/water (0.05% v/v)</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDR b</td>
<td>$R^2$ c</td>
<td>LOD d</td>
</tr>
<tr>
<td>2OH-Naph</td>
<td>331/357</td>
<td>12.2 - 100</td>
<td>0.9999</td>
</tr>
<tr>
<td>2OH-Flu</td>
<td>278/328</td>
<td>1.58 - 50</td>
<td>0.9999</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>305/367</td>
<td>2.38 - 100</td>
<td>0.9989</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>348/387</td>
<td>0.23 - 50</td>
<td>0.9997</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>382/432</td>
<td>0.31 - 50</td>
<td>0.9933</td>
</tr>
<tr>
<td>4OH-B[a]P</td>
<td>372/421</td>
<td>4.85 - 50</td>
<td>0.9911</td>
</tr>
<tr>
<td>5OH-B[a]P</td>
<td>301/430</td>
<td>5.49 - 50</td>
<td>0.9953</td>
</tr>
</tbody>
</table>

a Maximum excitation and emission wavelength in nm. b Linear dynamic range in ng/mL. c Correlation coefficient of calibration curve. d Limit of detection (ng/mL) is calculated from $3 \times$ standard deviation ($S_b$) of 16 blank measurements divided by slope (m) of the calibration curve. e Limit of quantification (ng/mL) is calculated from $10 \times S_b / m$. 
During the course of our experiments, we noted a strong dependence between the elution of OH-PAH and the duration of the air drying step, which consisted of aspirating air through the cartridge with the aid of the vacuum manifold. 5–10 minutes of air drying caused the recoveries of some OH-PAH to fall below 30%. 10 s of drying time at 15 mm Hg removed most of the water from the cartridge with no loss in metabolite recoveries. The main reason for removing the excess water prior to metabolite elution was to minimize its presence in the methanol solutions used for CE injection and sample stacking.

The percentage of extraction (%EX) was calculated with the formula:

\[
%EX = (I_B - I_A) \times 100
\]

where \(I_B\) and \(I_A\) refer to the fluorescence intensity of the solution before and after extraction, respectively. The eluting efficiency (%EL) of methanol was obtained from the following equation:

\[
%EL = \left( \frac{M_E}{M_R} \right) \times 100
\]

where \(M_E\) and \(M_R\) correspond to the eluted and retained mass of OH-PAH, respectively. The mass of eluted metabolite was calculated from the product \(M_E = V_E \times C_E\), where \(V_E\) is the volume of eluted methanol and \(C_E\) is the metabolite concentration in the eluted methanol. \(C_E\) values were obtained from the calibration curves in Table 3.2. The mass of retained metabolite was calculated from the product \(M_R = C_{ST} \times V_{ST} \times \%R\), where \(V_{ST}\) is the volume of the standard solution processed through the cartridge, \(C_{ST}\) is the metabolite concentration in the standard solution, and \(\%R\) is the percentage of retention which is equivalent to %EX from equation (3.2).

Table 3.3 summarizes the figures of merit for the optimized SPE procedure in aqueous and urine samples. Metabolites were spiked into the urine matrix 24 h prior to analysis. All metabolite concentrations were at the parts-per-billion level. Spiked samples were submitted to
acidic hydrolysis and then extracted via SPE. The overall recoveries (%OR) were calculated using the following equation:

\[
\text{%OR} = \text{%EX} \cdot \text{%EL}
\] (3.4)

The standard deviations of the overall recoveries (S\text{OR}) were based on three repetitions of the entire SPE procedure and were calculated as 
\[
\text{S}_{\text{OR}} / \text{%OR} = \left( \left( \text{S}_{\text{EX}} / \text{%EX} \right)^2 + \left( \text{S}_{\text{EL}} / \text{%EL} \right)^2 \right)^{1/2},
\]
where S\text{EX} and S\text{EL} are the standard deviations of %EX and %EL, respectively.48 Within a confidence interval of 95\% (N = 3), the overall recoveries were statistically the same in water and urine samples. This agreement demonstrates that the matrix composition of the urine sample does not interfere with the recoveries of OH-PAH.
<table>
<thead>
<tr>
<th>OH-PAH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Extraction (EE)</th>
<th>% Elution (EL)</th>
<th>% Overall Efficiency (OE)</th>
<th>t&lt;sub&gt;exp&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EE ± S&lt;sub&gt;ext&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>EL ± S&lt;sub&gt;elu&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>OE ± S&lt;sub&gt;OE&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂O/CH₃OH</td>
<td>Urine</td>
<td>H₂O/CH₃OH</td>
<td>Urine</td>
</tr>
<tr>
<td>2OH-Naph</td>
<td>95.7 ± 0.4</td>
<td>96.7 ± 2.1</td>
<td>96.9 ± 0.8</td>
<td>99.9 ± 2.6</td>
</tr>
<tr>
<td>2OH-Flu</td>
<td>99.9 ± 0.1</td>
<td>99.8 ± 0.3</td>
<td>99.3 ± 1.8</td>
<td>95.7 ± 2.9</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>98.8 ± 0.1</td>
<td>99.3 ± 0.3</td>
<td>91.4 ± 1.8</td>
<td>88.6 ± 1.3</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>99.3 ± 0.2</td>
<td>99.6 ± 0.4</td>
<td>89.1 ± 1.3</td>
<td>88.5 ± 1.0</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>99.0 ± 0.3</td>
<td>99.6 ± 0.1</td>
<td>99.2 ± 1.4</td>
<td>99.8 ± 1.0</td>
</tr>
<tr>
<td>4OH-B[a]P</td>
<td>96.7 ± 0.2</td>
<td>94.2 ± 1.9</td>
<td>86.2 ± 1.4</td>
<td>89.0 ± 1.9</td>
</tr>
<tr>
<td>5OH-B[a]P</td>
<td>99.1 ± 0.4</td>
<td>99.4 ± 0.2</td>
<td>93.7 ± 1.3</td>
<td>91.3 ± 2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> The final concentrations of spiked samples are 50 ng/mL for 2OH-Naph, 2OH-Flu, and 9OH-Phen and 20 ng/mL for 1OH-Pyr, 3OH-B[a]P, 4OH-B[a]P and 5OH-B[a]P.  
<sup>b</sup> % Standard deviation of % retention efficiency.  
<sup>c</sup> % Standard deviation of % elution efficiency.  
<sup>d</sup> % Standard deviation of % overall efficiency.  
<sup>e</sup> t<sub>exp</sub> – t value calculated for experimental measurements according to<sup>87</sup>. t<sub>tab</sub> = 2.78 (α = 0.05; N₁ = N₂ = 3).
3.3.3 Optimization of sample stacking

Sample stacking is based on the different conductivities of the sample solution and the CE running buffer solution. The only requirement for its successful application is to operate with a sample solvent of lower conductivity than the running buffer solution. This requirement is usually met with the use of a diluted background electrolyte or a pure solvent such as methanol. In comparison to conventional CZE, sample stacking allows the analyst to increase sample loading at no cost of separation efficiency. The general optimization strategy is to monitor the amount of sample loading as a function of both peak height and peak broadening. Figure 3.2 (A) correlates the peak heights of the studied metabolites with injection times made at 45 mbar of hydrostatic pressure. Intensity values are averages of three measurements. In comparison to 60 s, 90 s of injection time reduced the peak heights of 9OH-Phen and 3OH-B[a]P. The peak heights of the remaining metabolites increased with injection time. Figure 3.2 (B) compares the electropherograms recorded at 60 and 90 s of injection times. Clearly, 90 s of injection time caused deterioration in peak shape and compromised metabolite separation. The stacking effect under optimized conditions is shown in Figure 3.3, which compares the electropherograms of two synthetic standard mixtures prepared in either 100% methanol or the running buffer (20 mM borate; 50% methanol; pH = 9.7) of the CZE separation. The separation conditions were the same in both cases. Based on these observations, we chose 60 s as the optimal injection time for all further studies.
Figure 3.2 (A) Peak heights of OH-PAH as a function of injection time; (B) electropherograms of a synthetic mixture with the studied metabolites using injection times of 90 seconds (top) and 60 seconds (bottom).

Metabolites were separated using 20mM borate buffer prepared in methanol-water 50% v/v. Peak identification as in Figure 3.1 Buffer pH = 9.7; voltage = 30 kV; temperature = 21 °C.
Figure 3.3 Electropherograms of two synthetic mixtures of the studied metabolites prepared in pure methanol (top) and in the separation buffer (bottom).

OH-PAH concentrations and hydrodynamic injection times (60 seconds) were the same in both cases. Buffer: 20mM borate, 50% v/v methanol; pH = 9.7; voltage = 30 kV; temperature = 21 °C. Peak identification: (1) EOF marker (methanol); (2) 2OH-Flu; (3) 2OH-Naph; (4) 9OH-Phen; (5) 5OH-B[a]P; (6) 4OH-B[a]P; (7) 1OH-Pyr; (8) 3OH-B[a]P.

3.3.4 Analytical figures of merit

Table 3.4 summarizes the AFOM obtained via sample stacking and CZE analysis. Calibration curves were built with standard mixtures containing known concentrations of the seven metabolites in methanol. All LDR were based on the average peak heights of at least five OH-PAH concentrations. The average peak heights plotted in the calibration graphs correspond
to a minimum of three measurements made from triplicate aliquots submitted to three complete experimental trials. The correlation coefficients (data not shown), the slopes (data not shown) and the intercepts (data not shown) of the linear fittings were calculated with the least squares method.\(^89\) The \(R^2\) values close to unity confirm the existence of linear relationships in all cases. The lowest concentrations of the LDR correspond to the LOQ, which were calculated according to the formula \(\text{LOD} = 10S_B/m\); where \(S_B\) is the standard deviation of the average blank signal estimated from one-fifth of the peak-to-peak noise \((N_{p-p}/5)\) and \(m\) is the slope of the calibration curve.\(^90\) The \(N_{p-p}\) was measured at the base peak of each OH-PAH over a sufficiently wide region of the electropherogram. No efforts were made to reach the experimental values of the upper concentration limits of the calibration curves. The RSD values at medium linear concentrations show excellent reproducibility of measurements. On the other end, the LOD values stress the need for SPE prior to sample stacking–CZE analysis. LOD values in Table 3.4 are at the higher end of typical OH-PAH concentration ranges in urine samples.\(^{22,34,38,40-42,91,92}\)
Table 3.4 AFOM of CZE analysis of OH-PAH

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>LDR (ppm)(^a)</th>
<th>(R^2)</th>
<th>LOQ (ppb)(^b)</th>
<th>LOD (ppb)(^b)</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OH-Flu</td>
<td>0.091 – 5</td>
<td>0.999</td>
<td>91</td>
<td>28</td>
<td>2.34</td>
</tr>
<tr>
<td>2OH-Naph</td>
<td>0.582 – 5</td>
<td>0.999</td>
<td>582</td>
<td>176</td>
<td>1.04</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>0.103 – 5</td>
<td>0.996</td>
<td>103</td>
<td>31</td>
<td>1.36</td>
</tr>
<tr>
<td>5OH-B[a]P</td>
<td>0.056 – 5</td>
<td>0.999</td>
<td>56</td>
<td>17</td>
<td>5.75</td>
</tr>
<tr>
<td>4OH-B[a]P</td>
<td>0.081 – 5</td>
<td>0.999</td>
<td>81</td>
<td>24</td>
<td>6.16</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>0.066 – 5</td>
<td>0.998</td>
<td>66</td>
<td>20</td>
<td>2.16</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>0.165 – 5</td>
<td>0.988</td>
<td>165</td>
<td>50</td>
<td>6.56</td>
</tr>
</tbody>
</table>

\(^a\) Linear dynamic range. \(^b\) Limit of detection and limit of quantitation calculated as 3 S\(_B\)/m and 10 S\(_B\)/m, respectively; where S\(_B\) is the standard deviation of the average blank signal and m is the slope of the calibration curve.

3.3.5 Analysis of synthetic urine samples

Table 3.5 summarizes the AFOM obtained via SPE, sample stacking and CZE analysis of synthetic urine samples. Calibration curves were built with standard mixtures containing known concentrations of the seven metabolites in urine. Synthetic urine spiking and hydrolysis, SPE and sample stacking were carried out as previously described in this chapter. The \(R^2\), LOD and LDR were calculated as those reported in Table 3.4. LDR were based on the average peak heights of at least five OH-PAH concentrations. The average peak heights plotted in the calibration graphs correspond to a minimum of three measurements made from triplicate aliquots submitted to three
complete experimental trials. No efforts were made to reach the experimental values of the upper concentration limits of the calibration curves. Metabolite recoveries were calculated from three urine aliquots (N = 3) submitted to the entire experimental procedure. Within a confidence interval of 95% (N = 3), all the recoveries were equivalent to 100%.\textsuperscript{87} Comparison of LODs in Table 3.4 and Table 3.5 shows the advantage of using SPE prior to sample stacking. LOD improvements varied from ~ 14x (3OH-B[a]P) to ~23x (1OH-Pyr). Their values in Table 3.5 demonstrate the ability of the new method to determine OH-PAH at the low-ppb concentration level.

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>LDR (ppb)\textsuperscript{a}</th>
<th>(R^2)</th>
<th>LOQ (ppb)\textsuperscript{b}</th>
<th>LOD (ppb)\textsuperscript{b}</th>
<th>Recovery (%)\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OH-Flu</td>
<td>3.9 – 20</td>
<td>0.983</td>
<td>3.9</td>
<td>1.2</td>
<td>108.4 ± 9.1</td>
</tr>
<tr>
<td>2OH-Naph</td>
<td>30 – 100</td>
<td>0.993</td>
<td>30.2</td>
<td>9.2</td>
<td>92.9 ± 6.2</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>4.5 – 20</td>
<td>0.992</td>
<td>4.5</td>
<td>1.4</td>
<td>108.2 ± 7.5</td>
</tr>
<tr>
<td>5OH-B[a]P</td>
<td>2.7 – 20</td>
<td>0.983</td>
<td>2.7</td>
<td>0.8</td>
<td>97.9 ± 8.0</td>
</tr>
<tr>
<td>4OH-B[a]P</td>
<td>4.2 – 20</td>
<td>0.992</td>
<td>4.2</td>
<td>1.3</td>
<td>93.6 ± 5.2</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>4.8 – 20</td>
<td>0.990</td>
<td>4.8</td>
<td>1.4</td>
<td>96.8 ± 5.2</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>7.2 – 50</td>
<td>0.992</td>
<td>7.2</td>
<td>2.2</td>
<td>105.5 ± 7.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Linear dynamic range. \textsuperscript{b} Limit of detection and limit of quantitation calculated as 3 \(S_B/m\) and 10 \(S_B/m\), respectively; where \(S_B\) is the standard deviation of the average blank signal and \(m\) is the slope of the calibration curve. \textsuperscript{c} Recoveries calculated from three urine aliquots (N=3) submitted to the entire experimental procedure.
Figure 3.4 displays an electropherogram of a synthetic urine sample previously spiked with the seven metabolites and five potential concomitants. Creatinine is a metabolite that appears in urine at a constant rate and is often monitored to check possible sample manipulation in standard drug tests. Naproxen, ibuprofen and diclofenac are non-steroidal anti-inflammatory drugs and amoxicillin is an antibiotic. Because these pharmacological drugs are frequently used in our society, it is possible to find them in urine samples of unhealthy individuals. The final concentrations of the five concomitants in the spiked samples were within the range of concentrations usually found in human urine samples. Although the SPE procedure does not remove any of these concomitants from the analytical sample, their presence in the electropherogram does not overlap with the migration of the studied metabolites.
Figure 3.4 Electropherogram of a synthetic mixture with the studied metabolites and four possible interferents.

Separation was achieved using 20 mM borate buffer prepared in methanol-water 50% v/v. Buffer pH = 9.7; voltage = 30 kV; temperature = 21 °C. Peak identification: (1) EOF marker (methanol) and Creatinine; (2) 2OH-Flu; (3) 2OH-Naph; (4) 9OH-Phen; (5) 5OH-B[a]P; (6) 4OH-B[a]P; (7) 1OH-Pyr; (8) 3OH-B[a]P; (I) Ibuprofen; (II) Diclofenac; (III) Amoxicillin; (IV) Naproxen.

3.3.6 Analysis of urine samples

AFOM obtained via SPE, sample stacking and CZE analysis of urine samples are summarized in Table 3.6. Calibration curves were built by spiking urine samples with standard mixtures of 7 OH-PAH. Urine spiking and hydrolysis, SPE and sample stacking were carried out as previously described. The R2, LOD and LDR were calculated as those reported in the
previous section. The average peak heights plotted in the calibration graphs correspond to a minimum of three measurements made from triplicate aliquots submitted to three complete experimental trials. No efforts were made to reach the experimental values of the upper concentration limits of the calibration curves. Metabolite recoveries were calculated from three urine aliquots (N = 3) submitted to the entire experimental procedure. Within a confidence interval of 95% (N = 3), recoveries were statistically equivalent to those obtained with synthetic urine.

Table 3.6 SPE-CZE analytical figures of merit of OH-PAH in urine samples

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>LDR (ppb)(^a)</th>
<th>R(^2)</th>
<th>LOQ (ppb)(^b)</th>
<th>LOD (ppb)(^b)</th>
<th>Recovery (%)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OH-Flu</td>
<td>4.3 – 20</td>
<td>0.987</td>
<td>4.3</td>
<td>1.3</td>
<td>103.8 ± 8.4</td>
</tr>
<tr>
<td>2OH-Naph</td>
<td>28 – 100</td>
<td>0.990</td>
<td>28</td>
<td>8.5</td>
<td>108.7 ± 7.8</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>6.2 – 20</td>
<td>0.995</td>
<td>6.2</td>
<td>1.8</td>
<td>104.3 ± 6.1</td>
</tr>
<tr>
<td>5OH-B[a]P</td>
<td>3.8 – 20</td>
<td>0.985</td>
<td>3.8</td>
<td>1.1</td>
<td>93.2 ± 7.7</td>
</tr>
<tr>
<td>4OH-B[a]P</td>
<td>5.7 – 20</td>
<td>0.990</td>
<td>5.7</td>
<td>1.7</td>
<td>94.5 ± 6.4</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>3.3 – 20</td>
<td>0.987</td>
<td>3.3</td>
<td>1.0</td>
<td>106.3 ± 9.0</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>6.8 – 50</td>
<td>0.993</td>
<td>6.8</td>
<td>2.0</td>
<td>93.8 ± 8.1</td>
</tr>
</tbody>
</table>

\(^a\) Linear dynamic range. \(^b\) Limit of detection and limit of quantitation calculated as 3 S\(_B\)/m and 10 S\(_B\)/m, respectively; where S\(_B\) is the standard deviation of the average blank signal and m is the slope of the calibration curve. \(^c\) Recoveries calculated from three urine aliquots (N=3) submitted to the entire experimental procedure.
Figure 3.5 shows an electropherogram of the analysis of urine previously spiked with seven OH-PAH and four pharmacological drugs. Although some of the urine concomitants are removed in the washing steps of the SPE process, the ones remaining appear to have larger migration times than the PAH metabolites, with the exception of neutral molecules which co-migrate with the EOF. HPLC experiments in our lab are challenged with the separation of 4OH-B[a]P and 5OH-B[a]P under a single set of chromatographic conditions. In addition to similar chromatographic behaviors, the strong overlapping of excitation and fluorescence spectra – see Figure 3.6 – makes their selective determination in co-eluted HPLC fractions impossible. The same is true for Solid-Phase Extraction and Room- Temperature Fluorescence spectroscopy (SPE-RTF), an analytical approach currently investigated in our lab for the direct determination of OH-PAH without previous chromatographic separation. The unique electrophoretic pattern of 4OH-B[a]P and 5OH-B[a]P demonstrates the potential of CZE for the determination of positional isomers in urine samples.
Figure 3.5 Electropherogram of a human urine sample spiked with the studied metabolites and four possible interferents

Separation was achieved using 20 mM borate buffer prepared in methanol-water 50% v/v. Buffer pH = 9.7; voltage = 30 kV; temperature = 21 °C. Peak identification: (1) EOF; (2) 2OH-Flu; (3) 2OH-Naph; (4) 9OH-Phen; (5) 5OH-B[a]P; (6) 4OH-B[a]P; (7) 1OH-Pyr; (8) 3OH-B[a]P; (I) Ibuprofen; (II) Diclofenac; (III) Amoxicillin; (IV) Naproxen.
Figure 3.6 Room temperature excitation and fluorescence spectra of 50 μg L$^{-1}$ 4OH-B[a]P (A) and 100 μg L$^{-1}$ 5OH-B[a]P (B) in methanol.

Excitation and emission band pass = 2 nm/2 nm. Excitation and emission spectra were recorded at the maximum excitation ($\lambda_{\text{exc}}$) and fluorescence ($\lambda_{\text{em}}$) wavelengths $\lambda_{\text{exc}}$/$\lambda_{\text{em}} = 376$ nm/422 nm (4OH-B[a]P) and $\lambda_{\text{exc}}$/$\lambda_{\text{em}} = 388$ nm/445 nm (5OH-B[a]P).

3.4 Conclusion

We have developed a new method for the analysis of 2OH-Flu, 2OH-Naph, 1OH-Pyr, 9OH-Phen, 3OH-B[a]P, 4OH-B[a]P and 5OH-B[a]P in urine samples. Baseline resolution of the
seven metabolites was achieved in less than 17 minutes of electrophoretic separation. The lack of competitive LOD – a main limitation of CZE and UV-Vis absorption detection with commercial instrumentation – was overcome with the combination of SPE and sample stacking. Their hyphenation was facilitated with the use of a single organic solvent (methanol) for metabolite elution and electrophoretic stacking. The obtained LODs were up to two orders of magnitude better than reported CZE-LIF LODs, one order of magnitude better than in-capillary micro-extraction CZE LODs, and within the LOD range of chromatographic LOD. All CZE recoveries were statistically equivalent to 100% (P = 95%; N = 3). To the extent of our literature search, our values compare well to the most frequently reported recoveries via GC-MS (≤80%) and HPLC (≤75%).

In comparison to GC-MS, CZE presents the additional advantage of not requiring chemical derivatization prior to metabolite separation. The unique electrophoretic pattern of 3OH-B[a]P, 4OH-B[a]P and 5OH-B[a]P demonstrates the potential of CZE for the unambiguous determination of positional isomers with similar chromatographic behaviors and virtually similar fragmentation patterns. Our approach might prove useful for the analysis of rather large and structurally similar polar metabolites with difficult chromatographic behavior and lengthy GC-MS derivatization procedures.
 CHAPTER 4. ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBON METABOLITES IN COW’S MILK BY LIQUID-LIQUID EXTRACTION AND SYNCHRONOUS ROOM-TEMPERATURE FLUORESCENCE SPECTROSCOPY


4.1 Introduction

As shown in Table 1.2, only a few articles exist on the analysis of PAH metabolites in cow milks, with emphasis on monohydroxylated PAH (OH-PAH).45-50 Most articles have focused on the analysis of 1-hydroxypyrene (1OH-Pyr), a metabolite proven to provide a direct correlation between its concentration in milk samples and the ruminant ingestion of pyrene.48, 50 Previous work in our group has demonstrated the advantages of combining SPE to room-temperature fluorescence (RTF) spectroscopy for the analysis of OH-PAH in urine samples.35, 51. Quantitative determination of OH-PAH was carried out either in the eluent extract35, 51 or on the surface of the extraction membrane.52 The strong fluorescence resulting from the rigid and delocalized π-electron system of OH-PAH provided competitive LODs with minimum sample pre-concentration. Spectral overlapping and matrix interference were eliminated with the aid of excitation-emission matrices and chemometrics.35, 51

In this chapter, we present the first application of synchronous RTF spectroscopy to the analysis of OH-PAH in liquid-liquid extracts of milk samples. Although synchronous RTF spectroscopy has been reported for the analysis of OH-PAH in urine97-100 and fish samples,101-104 the extent of our literature search revealed no applications toward the analysis of metabolites in...
milk samples. Herein, we combine synchronous RTF spectroscopy to a new LLE procedure consisting of three simple experimental steps, namely sample hydrolysis, mixing of methanol with the hydrolyzed sample and centrifugation of the supernatant. 2-hydroxyfluorene (2OH-Flu), 6-hydroxychrysene (6OH-Chry), 1-hydroxypyrene (1OH-Pyr) and 3-hydroxybenzo[a]pyrene (3OH-B[a]P) are directly determined in the supernatant with no need of chromatographic separation. The non-destructive nature of LLE-synchronous RTF spectroscopy provides ample opportunity for OH-PAH confirmation via chromatographic techniques.

4.2 Materials and Methods

4.2.1 Chemicals

All solvents were HPLC grade. All chemicals were analytical-reagent grade and utilized without further purification. Unless otherwise noted, Nanopure water was used throughout. 2OH-Flu, 1OH-Pyr and 6-OHCChry were purchased from Sigma-Aldrich. 3OH-B[a]P was from Midwest Research Institute. All other chemicals were purchased from Fisher Scientific. Stock solutions of OH-PAH were prepared by dissolving pure standards in methanol and were stored in the dark at 4 °C. Possible photo-degradation of metabolites was monitored weekly via fluorescence spectroscopy. Working solutions were prepared daily by serial dilution with methanol.

Note: Use extreme caution when handling OH-PAH known to be extremely toxic.
4.2.2 Liquid-liquid extraction

400μL of commercially available bovine whole milk samples were spiked with micro liters of OH-PAH stock solutions of appropriate concentrations. The spiked sample was allowed to equilibrate at room temperature and in the dark for 24 hours. Enzymatic hydrolysis was carried out following previously established protocol. After adjusting the pH to 5 with HCl, 1 μL β-glucuronidase type H-2 from Helix pomatia (MP Biomedicals) with a volume activity of 65 unit/μl was added to the spiked sample. The mixture was incubated at 37 °C overnight to convert glucuronide and sulfate conjugates into free OH-PAH. Following hydrolysis, 1400 μL of methanol were added to the sample and the mixture was shaken for 30 minutes at 1,400 RPM. Sample centrifugation was carried out in a 2 mL polypropylene tube for 1 min at 13,400 RPM. The supernatant was then removed and analyzed with a spectrofluorimeter.

4.2.3 Instrumentation and measurements

Instrumentation for pH measurements, sample shaking and centrifugation, absorption and fluorescence spectroscopy was previously described in chapter 2.

Unless otherwise noted, synchronous fluorescence spectra were recorded using a wavelength off-set equal to 5 nm and an excitation/emission band-pass of 1 nm.
4.3 Results and Discussion

The four metabolites we chose for this study - 2OH-Flu, 6OH-Chry, 1OH-Pyr and 3OH-B[a]P - are part of the “EPA-PAH group”, which provide us with ample opportunity to compare our analytical figures of merit (AFOM) with previously reported data.  

4.3.1 Optimization of the LLE procedure

Literature procedures for the analysis of OH-PAH in milk samples report their extraction with a mixture of ethyl acetate/cyclohexane (50/50; v/v), supernatant evaporation, SPE, evaporation of the eluent and LLE with cyclohexane and methanol–water (80/20; v/v). The LLE procedure presented here consists of sample mixing and centrifugation with methanol. The duration of the mixing and centrifugation steps were optimized for short analysis time with no compromise of extraction efficiency. The main consideration with the optimization of the extracting volume was to avoid unnecessary metabolite dilution in the supernatant. All optimization studies were carried out with hydrolyzed milk samples previously spiked with pure OH-PAH standards at the parts-per-billion concentration level. Visual monitoring of the centrifugation step revealed that one minute was sufficient for residue precipitation and transparent supernatants well-suited for optical spectroscopy.

Optimization of sample mixing time and volume of extracting solvent was carried out via RTF spectroscopy. Figure 4.1 compares the excitation and fluorescence spectra of 2OH-Flu, 6OH-Chry, 1OH-Pyr and 3OH-B[a]P recorded from standard solutions in pure methanol and 5/95 v/v methanol/water mixtures. All fluorescence spectra were recorded using the same
excitation and emission band-pass (2 nm). No attempts were made to adjust slit-widths to optimize spectral resolution, nor were the spectra corrected for instrumental response. Because the spectral profiles of the metabolites did not vary with the proportion of methanol added to the aqueous mixture, we monitored the concentration of each OH-PAH with a single set of excitation and fluorescence wavelengths. All measurements were made at the maximum excitation and emission wavelengths of the metabolite.

Figure 4.1 Excitation (darker) and fluorescence (lighter) spectra recorded from 100% methanol (solid line) and 5-95(v/v) methanol/water (dotted line) of A: 2OH-Flu; B: 6OH-Chry; C: 1OH-Pyr; D: 3OH-B[a]P.

All spectra recorded with the same excitation (2nm) and emission (2nm) band-pass.
The fluorescence intensities of the four metabolites in the supernatants increased with the volume of extracting solvent. This trend was common to any given period of shaking time. The shortest shaking time that provided the highest fluorescence intensities was 30 min. The highest fluorescence intensities were obtained with 1400 and 1600 μL of methanol. These two volumes provided statistically equivalent fluorescence intensities \((\alpha = 0.05; N_1 = N_2 = 3)\). Figure 4.2 correlates the percentages of recovered metabolites to the volume of extracting solvent. Each recover plotted in the graph corresponds to the average of three determinations taken from three samples submitted to the entire LLE procedure. The recovery values \((%R)\) were obtained with the formula:

\[
%R = \left( \frac{[\text{OH-PAH}] \times V_S}{C_{\text{OH-PAH}} \times V_{\text{OH-PAH}}} \right) \times 100
\]  

where \(C_{\text{OH-PAH}}\) and \(V_{\text{OH-PAH}}\) are the concentration and the volume of spiked standard solution, respectively; and \([\text{OH-PAH}]\) is the metabolite concentration in the volume of supernatant \((V_S)\).

Supernatant concentrations were determined with the aid of calibration curves prepared in methanol-water mixtures of appropriate volume/volume compositions. The best extraction efficiencies were obtained with 1400 and 1600 μL of methanol. These two volumes provided statistically equivalent extractions \((\alpha = 0.05; N_1 = N_2 = 3)\). Considering the dilution of metabolites in the supernatants, we opted to conduct all further studies with 1400 μL of methanol.
Figure 4.2 Effect of methanol volume on the percentage of recovery (R%) of OH-PAH.

Each recovery corresponds to the average of three determinations taken from three samples submitted to the entire LLE procedure. All measurements were made at the maximum excitation and emission wavelength of the metabolite using an excitation/emission band-pass of 2 nm.

Table 4.1 reports the RTF analytical figures of merit (AFOM) obtained with 1400 μL of extracting solvent. Calibration curves of pure standards were prepared in methanol-water 78/22 v/v, i.e. the methanol-water proportion corresponding to 1400 μL of methanol in the supernatant. Each calibration curve was built with a minimum of five metabolite concentrations. For each
concentration plotted in the calibration graph, the RTF intensity was the average of at least three determinations taken from three sample aliquots. No efforts were made to experimentally obtain the upper concentration limit of the calibration curve. The correlation coefficients of the calibration curves were close to unity, indicating a linear relationship between OH-PAH concentration and fluorescence intensity. Within the linear dynamic range (LDR) of the calibration curves, the relative standard deviations (RSD) at medium concentrations were lower than 2%. The limits of detection (LOD) were calculated using the equation \( \text{LOD} = 3 \times \text{S}_B / m \); where \( \text{S}_B \) is the standard deviation of sixteen blank determinations and \( m \) is the slope of the calibration curve. The limits of quantitation (LOQ) were calculated according to the formula \( \text{LOQ} = 10 \times \text{S}_B / m \).\(^{87}\) The \( m \) values were calculated via the least squares method.\(^{87}\) At the LOQ concentration levels, the RSD of fluorescence measurements varied between 5 and 7%, allowing us to make precise measurements at low parts-per-billion concentration levels. Care was taken to rule out possible metabolite adhesion to the walls of the centrifugation vessels or metabolite precipitation in the absence of methanol, i.e. solely due to the centrifugation step.
Table 4.1 RTF Analytical figures of merit of OH-PAH in methanol/water (78/22 v/v)

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>$\lambda_{ex/em}^{a}$</th>
<th>LDR (ppb)$^{b}$</th>
<th>$R^2 c$</th>
<th>LOD (ppb)$^{d}$</th>
<th>LOQ (ppb)$^{e}$</th>
<th>RSD (%)$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OH-Flu</td>
<td>281/328</td>
<td>1.44 – 50</td>
<td>0.9986</td>
<td>0.436</td>
<td>1.448</td>
<td>1.31</td>
</tr>
<tr>
<td>6OH-Chry</td>
<td>269/378</td>
<td>0.41 - 50</td>
<td>0.9924</td>
<td>0.125</td>
<td>0.413</td>
<td>0.71</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>347/387</td>
<td>0.24 - 50</td>
<td>0.9971</td>
<td>0.073</td>
<td>0.240</td>
<td>1.34</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>381/432</td>
<td>0.21 - 50</td>
<td>0.9982</td>
<td>0.063</td>
<td>0.207</td>
<td>0.58</td>
</tr>
</tbody>
</table>

$^{a}$ Maximum excitation and emission wavelength in nm. $^{b}$ Linear dynamic range. $^{c}$ Correlation coefficient of the calibration curve. $^{d}$ Limit of detection calculated as 3 $S_B$/$m$; where $S_B$ is the standard deviation of 16 blank measurements and $m$ is the slope of the calibration curve. $^{e}$ Limit of quantification calculated as 10 $S_B$ /m. $^{f}$ Relative standard deviation (RSD) = $S_F$/I$_F$ x 100, where $S_F$ is the standard deviation of the average calculated from three RTF measurements at medium linear OH-PAH concentrations.

4.3.2 **Synchronous RTF spectroscopy of OH-PAH**

The synchronous excitation approach consists of varying simultaneously both the excitation ($\lambda'$) and emission ($\lambda$) wavelengths while keeping constant a wavelength interval $\Delta \lambda$ ($= \lambda - \lambda'$) between them. The judicious choice of the $\Delta \lambda$ parameter should introduce a new degree of selectivity for resolving the spectral overlapping of 2OH-Flu, 6OH-Chry, 1OH-Pyr and 3OH-B[a]P. Spectral discrimination was first attempted with wavelength offsets varying from 5 to 120 nm, i.e. within the $S_0$-$S_1$ and $S_0$-$S_2$ absorption ranges of the studied metabolites. The best spectral resolution was obtained upon synchronous excitation with relatively small wavelength offsets ($\Delta \lambda < 10$ nm). The smaller $\Delta \lambda$ values led to spectral simplification with narrower full-
width at half maxima. This trend is clearly shown in Figure 4.3, which compares the synchronous spectra of a synthetic mixture of the four metabolites recorded with $\Delta \lambda$s equal to 9 and 40 nm. Both spectra were recorded with the same excitation and emission band-pass (3 nm). Figure 4.4 demonstrates the effect of decreasing the $\Delta \lambda$ value and the instrumental band-pass on the spectral resolution of 6OH-Chry and 1OH-Pyr. The two peaks used for their discrimination appear in a different spectral region than those obtained with a $\Delta \lambda$ value of 9nm. Base line resolution of the two metabolites was only achieved under synchronous excitation with a $\Delta \lambda = 5$ nm and an excitation/emission band-pass of 1 nm. It should be noted that 2OH-Flu and 3OH-B[a]P pose no interference to 6OH-Chry and 1OH-Pyr because their synchronous excitation peaks appear at 325 (2OH-Flu) and 433 nm (3OH-B[a]P).
Figure 4.3 Synchronous fluorescence spectra of a synthetic mixture of PAH metabolites recorded with $\Delta \lambda$s equal to 9 and 40 nm.

Both spectra were recorded with excitation and emission band-pass equal to 3 nm. I: 15 ng.ml$^{-1}$ 2OH-Flu; II: 25 ng.ml$^{-1}$ 6OH-Chry; III: 10 ng.ml$^{-1}$1OH-Pyr; IV: 6 ng.ml$^{-1}$3OH-B[a]P.
Figure 4.4 Normalized Synchronous Fluorescence Spectra of 6 ng.ml\(^{-1}\) 6OH-Chry (II) and 2 ng.ml\(^{-1}\) 1OH-Pyr (III).

1: \(\Delta \lambda = 7\) nm, band-pass = 2 nm/2 nm; 2: \(\Delta \lambda = 5\) nm, band-pass = 1 nm/1 nm.

4.3.3 Analysis of milk samples via synchronous RTF spectroscopy

The feasibility to monitor 2OH-Flu, 6OH-Chry, 1OH-Pyr and 3OH-B[a]P in real world samples was investigated with different brands of commercially available cow milk. These included whole milk, reduced-fat milk and fat-free milk. Possible spectral interference from
matrix concomitants of unknown composition was ruled out with the analysis of milk samples previously submitted to hydrolysis and LLE. Representative examples of synchronous spectra from spiked and un-spiked milk samples are provided in Figure 4.5. Potential matrix effects that could cause variations of signal intensities were investigated with milk samples previously spiked with synthetic mixtures of the four metabolites. The peak intensities recorded from the milk extracts were compared to those from synthetic mixtures of equivalent metabolite concentrations. All synthetic mixtures were prepared in methanol/water 78/22 v/v. There was no statistical difference in any of the analyzed samples ($\alpha = 0.05\%$; $N_1 = N_2 = 3$).
Figure 4.5 Synchronous RTF spectra of OH-PAH extracted from milk samples of different fat content.

Solid line: spiked sample; dotted line: un-spiked sample. I: 7 ng.ml-1 2OH-Flu; II: 13 ng.ml-1 6OH-Chry; III: 5 ng.ml-1 1OH-Pyr; IV: 4 ng.ml-1 3OH-B[a]P.

Table 4.2 reports the AFOM obtained from the analysis of milk samples via LLE-synchronous RTF spectroscopy. Calibration curves were built with spiked milk samples submitted to the entire experimental procedure. Signal intensities plotted in the graph corresponded to the averages of three individual determinations taken from three analyzed samples. LDR, RSD, LOQ and LOD values were calculated as those reported in Table 4.1.
The LOQs and LODs for 6OH-Chry and 1OH-Pyr were calculated under two sets of $\Delta \lambda$/excitation/emission band-pass values, i.e. 9/3/3 and 5/1/1 nm. Metabolite recoveries (%R) were calculated with the help of equation (4.1)

Table 4.2 LLE-synchronous RTF spectroscopy AFOM of OH-PAH in milk samples

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>Maximum peak $^a$</th>
<th>LDR (ppb) $^b$</th>
<th>$R^2$ $^c$</th>
<th>LOQ (ppb) $^d$</th>
<th>LOD (ppb) $^e$</th>
<th>RSD (%) $^f$</th>
<th>R (%) $^g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OH-Flu</td>
<td>325</td>
<td>2.25 – 50</td>
<td>0.995</td>
<td>6.75</td>
<td>2.25</td>
<td>3.7</td>
<td>91.9 ± 3.4</td>
</tr>
<tr>
<td>6OH-Chry</td>
<td>379</td>
<td>8.17 - 50</td>
<td>0.999</td>
<td>24.51</td>
<td>8.17</td>
<td>1.2</td>
<td>93.3 ± 1.1</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>392</td>
<td>0.91 - 50</td>
<td>0.997</td>
<td>2.73</td>
<td>0.91</td>
<td>3.0</td>
<td>94.0 ± 2.8</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>433</td>
<td>0.76 - 50</td>
<td>0.996</td>
<td>2.28</td>
<td>0.76</td>
<td>5.8</td>
<td>86.0 ± 5.0</td>
</tr>
</tbody>
</table>

$^a$ Wavelength of maximum emission intensity, in nm. $^b$ Linear dynamic range lower concentration = limit of detection. $^c$ Correlation coefficient of the calibration curve. $^d$ Limit of quantification calculated as 10 $S_B/m$; where $S_B$ is the standard deviation of 16 blank measurements and $m$ is the slope of the calibration curve. $^e$ Limit of detection calculated as 3 $S_B/m$. $^f$ Relative standard deviation based on average values obtained three independent extractions. $^g$ Recoveries calculated from three milk aliquots (N=3) submitted to the entire experimental procedure. $^h$ $\Delta \lambda$=5 nm; bandpass 1 nm /1 nm.
4.4 Conclusion

Table 1.2 summarizes several features of previously reported methods for the analysis of OH-PAH in milk samples. No literature reports were found on the analysis of 6OH-Chry in milk samples. Comparison of literature values to the LODs in Table 4.2 places LLE-synchronous RTF spectroscopy at the higher end of the reported range. However, it should be noted that the LODs in Table 1.2 were obtained with larger volumes of milk than those in Table 4.2. As previously mentioned, the examination of Table 1.2 reveals scarce information on the analytical recoveries of OH-PAH. In comparison to the recoveries in Table 4.2, the reported recoveries are rather low. An additional advantage of LLE-synchronous RTF spectroscopy appears to be the relatively short analysis time. It should be noticed that analysis times in Table 1.2 only include instrumental times. The new LLE procedure reported here takes less than 35 min. Its implementation to the simultaneous extraction of numerous samples is straightforward due to the simplicity of its experimental procedure, small sample (400 μL) and methanol (1.4 mL) volumes. Considering the additional 3 minutes per sample that it takes to perform synchronous RTF measurements of the LLE supernatants, we were able to analyze 10 samples of milk in a total of 65 min. This is equivalent to less than 7 min per sample. The non-destructive nature of LLE-synchronous RTF spectroscopy provides ample opportunity for OH-PAH confirmation via chromatographic techniques. Under this prospective, the determination of OH-PAH via LLE-synchronous RTF spectroscopy appears to be a useful approach to monitor cow exposure to PAH contamination.
CHAPTER 5. DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBON METABOLITES IN MILK BY QUECHERS EXTRACTION AND CAPILLARY ELECTROPHORESIS

5.1 Introduction

Although RTF and SFS provides fast and sensitive OH-PAH determination with simple experimental procedures\(^{35,51,52,105}\), the unambiguous identification of positional isomers is often challenged by almost identical excitation and fluorescence spectra. A valuable alternative for the analysis of positional isomers with overlapping spectra is CZE.\(^{73,106}\) Research in our group has focused on the CZE analysis of 2OH-Flu, 2OH-Naph, 1OH-Pyr, 9OH-Phen, 3OH-B[a]P, 4-hydroxybenzo[a]pyrene (4OH-B[a]P) and 5- hydroxybenzo[a]pyrene (5OH-B[a]P) in urine samples.\(^{73,106}\) The fastest separation time – 17 min – was achieved with a 20mM borate buffer in methanol-water 50% volume/volume (v/v).\(^{106}\) Metabolites determination was carried out via ultraviolet-visible (UV-VIS) spectroscopy. The combination of SPE and on-line sample stacking lead to LODs ranging from 0.99 ng.mL\(^{-1}\) (3OH-B[a]P) to 8,54 ng.mL\(^{-1}\) (2OH-Naph).\(^{106}\)

Herein, we present a rapid method for the CZE determination of OH-PAH in milk samples. To the extent of our literature search, this is the first application of CZE to the analysis of OH-PAH in milk samples. The same is true for the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction technique, which had been applied to the analysis of antibiotics, pesticides and PAH\(^{107-114}\) but not to the extraction of OH-PAH. The separation of 2OH-Flu, 1-hydroxynaphthalene (1OH-Naph), 2OH-Naph, 3-hydroxyphenanthrene (3OH-Phen) and 9OH-Phen was accomplished in approximately 4 min with a 20mM borate buffer in
methanol-water 15% v/v. On-line sample stacking made possible to achieve UV-VIS LODs at the low parts-per-billion level. The simplicity of the extraction procedure and the speed of the electrophoretic separation make this method a valuable alternative for the routine analysis of OH-PAH in milk samples.

5.2 Materials and Methods

5.2.1 Instrumentation

Instrumentation for pH measurements, sample shaking and centrifugation, and CZE measurements was previously described in chapter 2. Electrophoretic runs were generated with the aid of a 61 cm × 50 μm I.D. (375 μm O.D.) fused-silica capillary (Polymicro). Its polyamide coating was removed with a window maker (MicroSolv-CE) to provide an UV transparent detection window with an approximate length of 4 mm. The center of the optical window was located at approximately 38 cm from the injection port.

5.2.2 Reagents

Solvents were HPLC grade and chemicals were analytical-reagent grade. Unless otherwise noted, Nanopure water (18 MΩ) was used throughout. OH-PAH were from Sigma-Aldrich except 3OH-Phen which was from Toronto Research Chemicals. Supelclean™ primary secondary amine (PSA) and Discovery® DSC-18 octadecyl (C18) dispersive solid-phase extraction (SPE) sorbents were purchased from Sigma-Aldrich. All other chemicals were purchased from Fisher Scientific.
Stock solutions of OH-PAH were prepared by dissolving pure standards in methanol and were stored in the dark at 4°C. Their stability was monitored weekly via fluorescence spectroscopy for possible photo-degradation of metabolites. Spectral profiles and fluorescence intensities of stock solutions remained the same for a period of six months. Working solutions were prepared daily by serial dilution with methanol. Buffer solutions (BGE) were prepared daily and sonicated for 10 minutes to degas before use.

*Note: Use extreme caution when handling OH-PAH known to be extremely toxic.*

### 5.2.3 OH-PAH extraction and sample clean-up

1200μL of commercially available bovine milk were spiked with micro-liters of OH-PAH stock solutions and allowed to equilibrate in the dark for 24 hours at room temperature. After adjusting the sample pH to 5 with HCL, 3 μL of β-glucuronidase type H-2 from Helix pomatia (MP Biomedicals, volume activity = 65 unit/μl) were added to the sample. The mixture was incubated at 37 °C overnight to convert glucuronide and sulfate conjugates into free OH-PAH. After hydrolysis, 300 μL of acetonitrile was added and the sample was shaken for 5 min at 1,400 RPM. A mixture of 120 mg sodium chloride (NaCl) and 480 mg magnesium sulfate (MgSO₄) was added; the sample was shaken for 1 min and subsequently centrifuged for 2 minutes at 13,400 RPM. The upper acetonitrile phase was removed and transferred to a clean 2 mL polypropylene centrifuge tube containing 30 mg of PSA and 30 mg of C18. The mixture was shaken by vortex for 1 minute and centrifuged at 13,400 RPM for an equal period of time. An
aliquot of the supernatant (50 μL) was then transferred into a 300 μL vial insert for CZE analysis.

5.3 Results and Discussion

The five metabolites we chose for this study - 2OH-Flu, 1OH-Naph, 2OH-Naph, 3OH-Phen and 9OH-Phen –belong to the “EPA-PAH group”, which facilitates the comparison of the analytical figures of merit (AFOM) of the new method to those previously reported and listed in Table 1.2

5.3.1 Electrophoretic separation of OH-PAH

As previously mentioned, the electrophoretic separation of OH-PAH is based on the difference among the individual values of their apparent electrophoretic mobility (μ_{app}). The apparent electrophoretic mobility depends on the electro-osmotic flow (μ_{EOF}) and the electrophoretic mobility of each metabolite (μ_{OH-PAH}). The electrophoretic mobility of each metabolite follows the equation μ_{OH-PAH} α q/r, where q is the metabolite’s charge and r its solvation radius. The negative charge on each metabolite depends on the fraction of dissociated metabolite (α = [O-PAH]/[OH-PAH]) at the experimental pH of the separation buffer. The α value is given by the equation α = 10^{pK_a}/10^{pK_a} + 10^{pH} 83, where K_a is the metabolite’s acid dissociation constant at the experimental conditions of the separation.

The choice of borate as the separation buffer was based on previous work in our lab. Based on its working pH range (8.2 – 10.2) and the reported values of the dissociation constants
of 2OH-Flu, 1OH-Naph, 2OH-Naph, 3OH-Phen and 9OH-Phen (9.1 ≤ pK_a ≤ 9.6), the presence of borate should promote the partial dissociation of the metabolites in the separation capillary and force some degree of negative charge on each OH-PAH. Considering the position of the detector at the negative end (cathode) of the separation capillary, the detection of 2OH-Flu, 1OH-Naph, 2OH-Naph, 3OH-Phen and 9OH-Phen should result from the predominance of the electro-osmotic flow over the electrophoretic mobility, i.e. \( \mu_{\text{app}} = \mu_{\text{EOF}} - \mu_{\text{OH-PAH}} \).

Since our previous work showed that the separation of positional isomers of OH-B[a]P was best accomplished with the use of methanol, an organic modifier capable to reduce \( \mu_{\text{EOF}} \), we first attempted the optimization of this parameter for the separation of 2OH-Flu, 1OH-Naph, 2OH-Naph, 3OH-Phen and 9OH-Phen. The final concentration of methanol in the separation buffer was varied from 0% to 20% v/v. Buffer pH was kept at 9.5, i.e. a pH value within the pK_a range of the studied metabolites. The obtained results are shown in Figure 5.1.
Figure 5.1 Effect of MeOH% in the running buffer on the CE separation of a mixture of OH-PAH.

Buffer: 20 mM borate; pH = 9.5; voltage = 30kV; temperature = 23 °C. Peaks are identified as follows: (1) 2OH-Flu; (2) 2OH-Naph; (3) 3OH-Phen; (4) 1OH-Naph; (5) 9OH-Phen.

As the percentage of methanol increases, peak resolution improves, especially in the cases of 1OH-Naph and 9OH-Phen. Complete resolution of the five metabolites is achieved with 15 and 20% v/v methanol. Based on the shorter separation time, 15% v/v methanol was used in all further studies. The effect of buffer pH on the $\mu_{app}$ of the studied metabolites was investigated within the 9.5-10.2 pH range. The best results are shown in Figure 5.2. The fastest
separation was obtained with pH = 9.5, which also provided the best metabolites resolution. A summary of migration times, peak resolutions and peak efficiencies of the five metabolites with 20mM borate, pH = 9.5 and 15% v/v methanol is presented in Table 5.1.

![Image of chromatograms showing effect of buffer pH on OH-PAH separation](image)

**Figure 5.2** Effect of buffer pH on the separation of the studied OH-PAH.

Buffer: 20 mM borate; methanol: 15% v/v. Peak identification as in Figure 5.1.
Table 5.1 Migration time, peak resolution and peak efficiency of studied OH-PAH

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>Migration time (min) (^{a)})</th>
<th>Rs (^{b)})</th>
<th>Efficiency (^{c)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OH-Flu</td>
<td>3.31 ± 0.01</td>
<td>-</td>
<td>1.68 x 10^5</td>
</tr>
<tr>
<td>2OH-Naph</td>
<td>3.50 ± 0.01</td>
<td>3.00</td>
<td>1.86 x 10^5</td>
</tr>
<tr>
<td>3OH-Phen</td>
<td>3.59 ± 0.01</td>
<td>1.43</td>
<td>1.97 x 10^5</td>
</tr>
<tr>
<td>1OH-Naph</td>
<td>3.68 ± 0.01</td>
<td>1.60</td>
<td>2.31 x 10^5</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>3.73 ± 0.01</td>
<td>1.30</td>
<td>2.13 x 10^5</td>
</tr>
</tbody>
</table>

\(^{a)}\) Values represent the average of 3 independent electrophoretic runs.

\(^{b)}\) Resolution between peaks calculated from \(2(t_2-t_1)/w_2+w_1\). \(t_1\) and \(t_2\) are migration time of peaks 1 and 2, respectively. \(w_1\) and \(w_2\) are the widths at the base of each peak.

\(^{c)}\) Calculated from \(5.54(t_m/w_{0.5})^2\). \(t_m\): migration time. \(w_{0.5}\): width at half height of the peak.

5.3.2 Sample stacking

A usual approach for the improvement of LODs in instrumental methods is to increase the amount of sample. In the particular case of CZE, this approach is known as sample stacking.\(^ {57}\) The one condition for its successful application to CZE is the use of a sample solvent with lower conductivity than the running buffer. Typical solvents for sample stacking include diluted buffers, water or pure organic solvents. The preferential accumulation of analyte ions at the narrow boundary that is formed between sample solvent and separation buffer minimizes analyte zone broadening.\(^ {56, 58}\) Simply put, sample stacking allows the analyst to increase sample loading at no cost of separation efficiency.
Considering the straightforward procedure that would result from matching the sample injection solvent to the QuEChERS extraction solvent, we selected acetonitrile as the sample stacking solvent. Sample injection volume was optimized by monitoring peak intensity and shape as a function sample injection time. Figure 5.3 (A) shows typical plots obtained at 45 mbar of hydrostatic pressure. Intensity values are the averages of three independent electrophoretic runs. The relative standard deviations of the average peak intensities were within 1.87 (9OH-Phen) and 4.71% (2OH-Naph). The signal intensities of the five metabolites reach maximum values at 60 s of sample injection time. In addition to lower signal intensities, injection times longer than 60 s produced substantial peak distortion. Figure 5.3 (B) compares the electropherograms recorded after 45 and 60 s of sample injection time. Because baseline resolution of 1OH-Naph and 3OH-Phen was only obtained with 45 s, this sample injection time was selected for all further studies.
Figure 5.3 (A) Peak heights of OH-PAH standards as a function of injection time; (B) Electropherograms of a mixture of OH-PAH using injection times of 45 (top) and 60 seconds (bottom).

Buffer: 20 mM borate; methanol: 15% v/v; pH = 9.5. Peak identification as in Figure 5.1.
The advantage of sample stacking for 45 s of sample injection time is presented in Figure 5.4, which compares the electropherogram of a synthetic mixture of OH-PAH prepared in 100% acetonitrile to the one from a synthetic mixture with the same metabolites concentrations prepared in the separation buffer, i.e. 20 mM borate, 15% v/v methanol, pH = 9.5. The CZE AFOM under optimum sample stacking conditions are summarized in Table 5.2. Calibration curves were built with standard mixtures containing known concentrations of the five metabolites in acetonitrile. Each linear dynamic range (LDR) is based on the average peak heights of at least five OH-PAH concentrations. The average peak heights plotted in the calibration graphs correspond to a minimum of three measurements made from triplicate aliquots submitted to three independent electrophoretic runs (N=9). Correlation coefficients (R), slopes (data not shown) and intercepts (data not shown) of the linear fittings were calculated with the least squares method. The R² values close to unity confirm the existence of linear relationships in all cases. The lowest concentrations of the LDRs correspond to the LODs, which were calculated with the formula LOD = 3 S_B/m; where S_B is the standard deviation of the average blank signal estimated from one-fifth of the peak-to-peak noise (N_{p-p}/5) and m is the slope of the calibration curve. The N_{p-p} was measured at the base of each peak over a sufficiently wide region of the electropherogram. No efforts were made to reach the experimental values of the upper concentration limits of the calibration curves. The RSD values at medium linear concentrations show great reproducibility of measurements.
Figure 5.4 Electropherograms of two synthetic mixtures of OH-PAH prepared in acetonitrile (top) and in the separation buffer (bottom).

Table 5.2 Analytical figures of merit of CZE analysis of OH-PAH

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>$R^2$</th>
<th>RSD%</th>
<th>LDR $^3$ (ppb)</th>
<th>LOD (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OH-Flu</td>
<td>0.990</td>
<td>3.45</td>
<td>11.62 – 1000</td>
<td>11.62</td>
</tr>
<tr>
<td>2OH-Naph</td>
<td>0.992</td>
<td>2.18</td>
<td>17.02 – 1000</td>
<td>17.02</td>
</tr>
<tr>
<td>3OH-Phen</td>
<td>0.995</td>
<td>4.45</td>
<td>3.25 – 1000</td>
<td>3.25</td>
</tr>
<tr>
<td>1OH-Naph</td>
<td>0.992</td>
<td>1.79</td>
<td>15.82 – 1000</td>
<td>15.82</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>0.992</td>
<td>3.77</td>
<td>3.80 – 1000</td>
<td>3.80</td>
</tr>
</tbody>
</table>

$^a$ Linear dynamic range.
5.3.3 **Optimization of QuEChERS extraction**

Figure 5.5 shows the schematic diagram of the optimized QuEChERS procedure for the extraction of OH-PAH from milk samples. All chemicals we used in the procedure followed previous literature reports on the application of QuEChERS to milk samples.\textsuperscript{110, 116} The addition of NaCl and MgSO\textsubscript{4} to the milk/acetonitrile mixture is supposed to reduce the aqueous phase and facilitate the partitioning of metabolites into the organic phase. NaCl and MgSO\textsubscript{4} were added to the sample in the proportion stated by Anastassiades.\textsuperscript{117}

![Schematic diagram of the optimized QuEChERS procedure](image)

**Figure 5.5** Schematic diagram of the optimized QuEChERS procedure.
The volume of milk was arbitrarily set at 1.2 mL. The volume of acetonitrile was optimized for best metabolite pre-concentration at no cost of extraction efficiency. The initial volume of acetonitrile we used in the optimization studies was 1.2 mL. This 1:1 acetonitrile/milk volume/volume ratio corresponded to the recommended weigh/weight proportion of acetonitrile and milk previously reported in the literature. Figure 5.6 compares the overall recoveries (%R) of the studied metabolites as a function of acetonitrile volume. Recovery values (%R) were obtained with the formula: 

\[ \%R = \left( \frac{[\text{OH-PAH}] \times V_S}{C_{\text{OH-PAH}} \times V_{\text{OH-PAH}}} \right) \times 100, \]

where \( C_{\text{OH-PAH}} \) and \( V_{\text{OH-PAH}} \) were the concentration and the volume of spiked standard solution, respectively; and \([\text{OH-PAH}]\) was the metabolite concentration in the volume of extracting solvent (\( V_S \)).

Figure 5.6 OH-PAH recoveries versus milk/acetonitrile ratios.
Concentrations of OH-PAH in the acetonitrile supernatant were determined via CZE with the aid of the calibration curves in Table 5.2. Considering the statistically equivalent recoveries ($\alpha = 0.05; N_1 = N_2 = 3$) of the five metabolites at the four investigated ratios, we adopted a 0.3mL as the volume of extracting solvent. Further sample clean-up was achieved with a combination of C18 and PSA. C18 has been used to remove non-polar compounds such as lipids. PSA has shown to be effective for removing impurities such as fatty acids and sugars. The advantage of using a combination of the two is illustrated in Figure 5.7.

Figure 5.7 Electropherograms of dispersive SPE clean-up optimization using PSA (top), C18 (middle) and C18+PSA (bottom)
5.3.4 QuEChERS – CZE analysis of milk samples

The new method was tested with several brands of commercially available whole milk. The AFOM are summarized in Table 5.3. Calibration curves were built by spiking milk samples with standards mixtures of OH-PAH. Spiked samples were submitted to the entire experimental procedures previously described. Calibration curves consisted of a minimum of five data points. Signal intensities plotted in the calibration graphs correspond to the averages of three individual determinations taken from three analyzed samples. No efforts were made to obtain the upper concentration limits of the calibration curves. LODs, LDRs and RSDs were calculated as those reported in Table 5.2. LODs in Table 5.3 are considerably higher than those obtained via chromatographic methods (see Table 1.2). The poorer LODs probably result from the smaller volume of milk sample and the lack of a substantial pre-concentration step such as SPE and/or LLE. The theoretical value for the pre-concentration factor due to the QuEChERS procedure is equal to 4 (see Figure 5.6). Interesting to note is the good agreement of the theoretical value to the experimental LOD improvements due to the QuEChERS procedure. Comparison of LODs in Table 5.3 to those in Table 5.2 reveal LOD ratios - i.e. LOD\text{Table 5.2} / LOD\text{Table 5.3} - varying from ~ 3 (9OH-Phen) to ~ 4.6 (2OH-Naph). The RSDs via QuEChERS - CZE (see Table 5.3) are higher than those via CZE (see Table 5.2) but still acceptable for analytical use. The poorer reproducibility of measurements is probably due to the additional steps of the QuEChERS procedure. Metabolite recoveries were calculated as those in Figure 5.6. Unfortunately, the comparison of recoveries to those obtained with chromatographic methods is not possible due to the incomplete nature of previously reported data (see Table 1.2). The reason for the lack of reported data is unknown.
Table 5.3 QuChERS-CZE analytical figures of merit of OH-PAH in milk samples

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>R²</th>
<th>LDR (ppb) a)</th>
<th>LOD (ppb)</th>
<th>RSD(%)</th>
<th>Recovery (%) b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OH-Flu</td>
<td>0.994</td>
<td>2.82 – 200</td>
<td>2.82</td>
<td>5.8</td>
<td>95.6 ± 4.8</td>
</tr>
<tr>
<td>2OH-Naph</td>
<td>0.995</td>
<td>3.72 – 200</td>
<td>3.72</td>
<td>4.5</td>
<td>105.0 ± 4.4</td>
</tr>
<tr>
<td>3OH-Phen</td>
<td>0.992</td>
<td>0.98 – 200</td>
<td>0.98</td>
<td>7.4</td>
<td>83.0 ± 3.5</td>
</tr>
<tr>
<td>1OH-Naph</td>
<td>0.992</td>
<td>3.60 – 200</td>
<td>3.60</td>
<td>4.2</td>
<td>104.3 ± 2.8</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>0.999</td>
<td>1.29 – 200</td>
<td>1.29</td>
<td>4.3</td>
<td>80.4 ± 4.1</td>
</tr>
</tbody>
</table>

a) Linear dynamic range. b) Recoveries calculated from three milk aliquots (N = 3) submitted to the entire experimental procedure.

5.4 Conclusions

We have presented the first application of QuEChERS – CZE to the analysis of OH-PAH in milk samples. Complete resolution of 2OH-Flu, 1OH-Naph, 2OH-Naph, 3OH-Phen and 9OH-Phen was accomplished in approximately 4 min of electrophoretic run. UV-VIS LODs at the parts-per-billion concentration level were obtained with the aid of sample stacking. CZE analysis of QuEChERS extracts was facilitated with the use of a single solvent (acetonitrile) for both metabolite extraction and sample stacking. Although the LODs of the new method are considerably higher than those from chromatographic methods, the LODs of 2OH-Flu, 3OH-Phen and 9OH-Phen are between one and two orders of magnitude lower than their concentrations in milk samples of ruminants exposed to fluorene and phenanthrene. There is no previous data on milk concentration levels of 1OH-Naph and 2OH-Naph.
In comparison to chromatographic methods, an attractive feature of QuEChERS – CZE for screening purposes appears to be the relatively short analysis time. The small sample volume and the conservative usage of chemical reagents make QuEChERS extraction possible in a 2mL centrifuge vial. This fact facilitates the implementation of the QuEChERS procedure to the simultaneous extraction of numerous samples. Using a centrifuge with maximum capacity of ten sample vials, it is possible to process ten samples in 10 min of extraction time. Adding 4 min of electrophoretic run per sample, it should be possible to screen ten samples in approximately one hour of analysis time. The small extract volume (~ $10^{-9}$ L) required for CZE injection provides ample opportunity for further chromatographic usage and confirmation of positive samples. The unique electrophoretic pattern of 1OH-Naph and 2OH-Naph as well as 3OH-Phen and 9OH-Phen demonstrates the potential of CZE for the unambiguous determination of positional isomers with very similar chromatographic behaviors and undistinguishable mass fragmentation patterns. Considering the plethora of metabolites originating from EPA-PAH exposure, and the possible formation of rather large and structurally similar polar metabolites, the new method could be a valuable alternative for the analysis of PAH metabolites with difficult CG behavior and lengthy derivatization procedures.
CHAPTER 6. DETERMINATION OF ACID DISSOCIATION CONSTANTS FOR MONOHYDROXY METABOLITES OF POLYCYCLIC AROMATIC HYDROCARBONS BY CAPILLARY ELECTROPHORESIS

6.1 Introduction

Attempts to separate 1OH-Naph from 2OH-Naph in a synthetic mixture with 2OH-Flu, 9OH-Phen, 3OH-B[a]P, 4OH-B[a]P, 5OH-B[a]P and 1OH-Pyr using the same conditions as those in chapter 3 (borate buffer, 50% MeOH, pH = 9.7) were unsuccessful due to the severe overlapping between the two naphthalene isomers. In this chapter, we present the resolution of the eight metabolites under a new set of separation conditions, namely N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer in 40% MeOH. The migration times of the studied metabolites were then used to calculate their apparent (μapp) and effective (μOH-PAH) electrophoretic mobilities under the conditions of the separation. An investigation of the dissociation constants was then undertaken to better understand the electrophoretic behavior of the studied metabolites. Previous articles 77-81, 83, 115, 119-121 provide useful information on the dissociation constants of PAH metabolites but their values do not reflect the actual degrees of metabolites dissociations under the separation conditions of our studies. The majority of the reports present dissociation constants obtained from aqueous mixtures of organic solvents using potentiometric 120 and spectrophotometric 77-81, 83, 115, 119, 121 data. The few articles that exist on CZE dissociation constants provide pKa values for only three monohydroxy-PAH, i.e. 1OH-Naph, 1OH-Pyr and 3OH-B[a]P. 80, 83, 115
6.2 Materials and Methods

6.2.1 Instrumentation

pH measurements were made with an AR-15 pH/mV/C meter and a combined electrode from Fischer Scientific.

CZE measurements were carried out as described in section 2.3. The total length of the capillary was 82 cm (Polymicro). Its polyamide coating was removed with a window maker (MicroSolv-CE) to provide an UV transparent detection window with an approximate length of 2 mm. The center of the optical window was located at approximately 58 cm from the injection port. Sample injection was accomplished via hydrodynamic pressure applying 50mbar for 6 s. At the beginning of the day, the capillary was rinsed for 10 minutes with 0.1M NaOH and 10 minutes with the BGE. Between runs, the capillary was rinsed with 0.1M NaOH (1 min) and BGE (2 min). At the end of each working day, the capillary was rinsed for 10 minutes with methanol and 10 minutes with nanopure water to remove residual impurities.

6.2.2 Reagents

All reagents were of analytical grade and were used without further purification. Buffer solutions were prepared with Nanopure water (18 MΩ) and, when needed, HPLC grade methanol acquired from Fisher Scientific. CAPS and 3-Cyclohexylamino-2-hydroxy-1-propane sulfonic acid (CAPSO) buffers were purchased from Sigma. 3OH-B[a]P, 4OH-B[a]P and 5OH-B[a]P were acquired from MRI (Kansas City, MO). The remaining OH-PAH were acquired from Aldrich.
6.2.3 **Solution preparation**

Stock solutions of OH-PAH were prepared in HPLC grade methanol with a concentration of 100 mg/l. Working solutions were prepared by serial dilution of stock solutions with HPLC grade methanol. All solutions were kept stored in the dark at 4°C. Buffer solutions (BGE) were prepared daily and sonicated for 10 minutes before use.

6.3 **Results and Discussion**

6.3.1 **Resolution of OH-PAH using CAPS buffer in 40% MeOH**

The separation of the eight metabolites was first attempted in 100% water CAPS. No separation was observed from any of the metabolites in the mixture. The excessively fast electro-osmotic flow did not provide enough time for the separation to occur. Figure 6.1 shows an electropherogram with the separation of the eight metabolites using a 100mM CAPS buffer solution in 40% MeOH. Lower contents of methanol in the CAPS buffer (50 v/v) were also attempted but the resolution of 4OH-B[a]P and 5OH-B[a]P was not possible.
Figure 6.1 CZE separation of a mixture of eight OH-PAH.


6.3.2 Calculation of apparent and electrophoretic mobility based on migration times

Table 6.1 correlates the migration times of OH-PAH to their apparent ($\mu_{\text{app}}$) and electrophoretic ($\mu_{\text{OH-PAH}}$) mobilities. The $\mu_{\text{app}}$ values were calculated using equation (6.1) \(^{82}\):

$$\mu_{\text{app}} = \frac{[L \times L_D]}{V \times [1/t]}$$  \hspace{1cm} (6.1)
where \( L \) refers to the length of the capillary (82 cm), \( L_D \) is the distance from the injection port to the detection window (58 cm), \( V \) is the applied voltage (26,000 volts) and \( t \) is the migration time of OH-PAH in seconds. The \( \mu_{\text{OH-PAH}} \) values were calculated with the following equation:

\[
\mu_{\text{OH-PAH}} = \mu_{\text{EOF}} - \mu_{\text{app}} \tag{6.2}
\]

The electro-osmotic flow mobility (\( \mu_{\text{EOF}} \)) was considered constant over the entire time of the separation. Its value \((1.69 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1})\) was calculated with equation 6.1 using the migration time of methanol (18.03 min).

Table 6.1 Migration time and electrophoretic mobility of OH-PAH in CAPS 40% MeOH

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>Migration time (min)a)</th>
<th>( \mu_{\text{app}} ) ((10^{-4} \text{cm}^2 \text{ V}^{-1} \text{ s}^{-1}))b)</th>
<th>( \mu_{\text{OH-PAH}} ) ((10^{-5} \text{cm}^2 \text{ V}^{-1} \text{ s}^{-1}))c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OH-Flu</td>
<td>22.75 ± 0.05</td>
<td>1.340 ± 0.003</td>
<td>3.51 ± 0.10</td>
</tr>
<tr>
<td>2OH-Naph</td>
<td>25.11 ± 0.24</td>
<td>1.214 ± 0.012</td>
<td>4.77 ± 0.15</td>
</tr>
<tr>
<td>1OH-Naph</td>
<td>26.79 ± 0.02</td>
<td>1.138 ± 0.001</td>
<td>5.53 ± 0.10</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>27.69 ± 0.29</td>
<td>1.101 ± 0.012</td>
<td>5.90 ± 0.15</td>
</tr>
<tr>
<td>5OH-B[a]P</td>
<td>29.79 ± 0.02</td>
<td>1.023 ± 0.001</td>
<td>6.68 ± 0.10</td>
</tr>
<tr>
<td>4OH-B[a]P</td>
<td>30.33 ± 0.04</td>
<td>1.005 ± 0.001</td>
<td>6.86 ± 0.10</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>34.43 ± 0.09</td>
<td>0.885 ± 0.002</td>
<td>8.05 ± 0.10</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>35.41 ± 0.07</td>
<td>0.861 ± 0.002</td>
<td>8.30 ± 0.15</td>
</tr>
</tbody>
</table>

a) Migration time of EOF marker: 18.03 min.

b) Apparent mobility.

c) Effective electrophoretic mobility.
6.3.3 Calculation of dissociation constant values

Determination of pKa values via CE is based on the assumption that an acid will achieve its maximum electrophoretic mobility when it is fully ionized. The ionized (O-PAH\(^-\)) and protonated forms (HO-PAH\(^+\)) of PAH metabolites co-exist in the electrophoretic buffer according to the equilibrium \( \text{OH-PAH} \Leftrightarrow \text{H}^+ + \text{O-PAH}^- \), which is described by the following equation:

\[
K_a = \gamma_{\text{O-PAH}^-} \gamma_{\text{H}^+} \frac{[\text{H}^+][\text{O-PAH}^-]}{[\text{HO-PAH}]} \tag{6.3}
\]

where \( \gamma_{\text{O-PAH}^-} \) and \( \gamma_{\text{H}^+} \) are the activity coefficients of the ionized forms of the acid and the proton respectively. The activity coefficient of the neutral form OH-PAH is considered to be equal to 1. Keeping in mind that the effective electrophoretic mobility of the PAH metabolite can be calculated as follows:

\[
\mu_{\text{OH-PAH}} = \frac{L \times L_D}{V} \left( \frac{1}{t} - \frac{1}{t_{\text{eo}}} \right) \tag{6.4}
\]

where \( L \) refers to the length of the capillary, \( L_D \) is the distance from the injection port to the detection window, \( V \) is the applied voltage, \( t \) is the migration time of the metabolite, and \( t_{\text{eo}} \) is the migration time of a neutral marker,\(^80\) in our case methanol, the above equations lead to the following derived linear equation\(^83\):

\[
\frac{1}{\mu_{\text{OH-PAH}}} = \frac{[\text{H}^+]}{K_a \mu_{\text{O-PAH}^-}} + \frac{1}{\mu_{\text{O-PAH}^-}} \tag{6.5}
\]

where \( \mu_{\text{O-PAH}^-} \) is the mobility of the fully ionized species. \( K_a \) can then be determined calculating the slope of the plot \( 1/\mu_{\text{OH-PAH}} \) versus \([\text{H}^+]\).

\( \mu_{\text{OH-PAH}} \) were calculated with equation (6.4) by monitoring OH-PAH migration times at different pH values using the buffer conditions of the separation (100mM CAPS, 40%MeOH).
Mobilities were evaluated every 0.15 – 0.2 pH units in the pH range 8.9<pH<10.6 keeping a constant ionic strength of 0.03M with NaCl. OH-PAH pKa values were then obtained from the slope of the plot 1/μOH-PAH versus [H⁺] as described in equation (6.5).

Figure 6.2 shows an example of a typical set of experimental results. The migration times of all the studied metabolites increased with the pH of the separation buffer. All the 1/μOH-PAH versus [H⁺] plots showed a linear behavior with correlation coefficients close to unity.

Figure 6.2 Electropherograms of 2OH-Flu at three different pH (left) and Plot of 1/μOH-PAH versus [H⁺] for 2OH-Flu (right).

EOF marker: MeOH, buffer: 100mM CAPS; temperature: 25°C; voltage: 26 kV (EOF markers are intentionally matched).
Table 6.2 lists pKa, α values, and mobilities in 40% MeOH CAPS buffer at the experimental pH (10.1) of our separation (see Section 6.3.1). The α values were calculated with the use of equation 83:

\[
\alpha = \frac{10^{-pK_a}}{10^{-pK_a} + 10^{-pH}} \tag{6.6}
\]

\(\mu_{OH-PAH}\) and mobilities of the fully ionized species (\(\mu_{OH-PAH^-}\)) were calculated from the plot of equation (6.5) at pH=10.1. Standard deviations of \(\mu_{OH-PAH}\) (\(S_{\mu_{OH-PAH}}\)) and \(\mu_{O-PAH^-}\) (\(S_{\mu_{O-PAH^-}}\)) were calculated from the standard deviations of the slope (Sm) and intercept (Sb) of the plot of \(1/\mu_{OH-PAH}\) versus \([H^+]\) (N=3). Standard deviations of α values (\(S_\alpha\)) were calculated as

\[
S_\alpha / \alpha = [((S_{\mu_{OH-PAH}} / \mu_{OH-PAH})^2 + (S_{\mu_{O-PAH^-}} / \mu_{O-PAH^-}))^2]^{1/2}.
\]
Table 6.2 pKa, α values and mobilities of OH-PAH determined in 40% MeOH CAPS buffer

<table>
<thead>
<tr>
<th>OH-PAH</th>
<th>pKa</th>
<th>$\mu_{\text{OPAH}}$ (10^{-4} \text{cm}^2 \text{V}^{-1} \text{s}^{-1})</th>
<th>α$^a$</th>
<th>$\mu_{\text{OH-PAH}}$ $^b$ (10^{-5} \text{cm}^2 \text{V}^{-1} \text{s}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OH-Flu</td>
<td>10.32 ± 0.08</td>
<td>0.975 ± 0.164</td>
<td>0.376 ± 0.069</td>
<td>3.67 ± 0.27</td>
</tr>
<tr>
<td>2OH-Naph</td>
<td>10.29 ± 0.06</td>
<td>1.278 ± 0.190</td>
<td>0.391 ± 0.064</td>
<td>5.00 ± 0.34</td>
</tr>
<tr>
<td>1OH-Naph</td>
<td>10.23 ± 0.07</td>
<td>1.370 ± 0.188</td>
<td>0.424 ± 0.065</td>
<td>5.81 ± 0.39</td>
</tr>
<tr>
<td>9OH-Phen</td>
<td>10.06 ± 0.05</td>
<td>1.192 ± 0.111</td>
<td>0.522 ± 0.057</td>
<td>6.22 ± 0.35</td>
</tr>
<tr>
<td>5OH-B[a]P</td>
<td>9.82 ± 0.04</td>
<td>1.077 ± 0.063</td>
<td>0.657 ± 0.048</td>
<td>7.07 ± 0.31</td>
</tr>
<tr>
<td>4OH-B[a]P</td>
<td>9.78 ± 0.04</td>
<td>1.074 ± 0.056</td>
<td>0.676 ± 0.045</td>
<td>7.26 ± 0.29</td>
</tr>
<tr>
<td>3OH-B[a]P</td>
<td>9.55 ± 0.03</td>
<td>1.099 ± 0.037</td>
<td>0.781 ± 0.035</td>
<td>8.59 ± 0.26</td>
</tr>
<tr>
<td>1OH-Pyr</td>
<td>9.66 ± 0.03</td>
<td>1.207 ± 0.050</td>
<td>0.733 ± 0.040</td>
<td>8.84 ± 0.31</td>
</tr>
</tbody>
</table>

$^a$ Alpha values calculated with equation (6.6) at pH=10.1; $^b$ $\mu_{\text{OH-PAH}}$ values calculated with equation (6.5) at pH=10.1

Comparison of $\mu_{\text{OH-PAH}}$ values obtained with equation (6.5) (Table 6.2) to those calculated from migration times in Table 6.1 shows statistical equivalence ($\alpha = 0.05; N_1 = N_2 = 3$). This is not surprising since the range of pH that was used to obtain $\mu_{\text{OH-PAH}}$ values from Table 6.2 includes the pH (10.1) at which $\mu_{\text{OH-PAH}}$ values in Table 6.1 were calculated.
6.4 Conclusion

The complete separation of 8 OH-PAH, namely 2OH-Flu, 1OH-Naph, 2OH-Naph, 9OH-Phen, 1OH-Pyr, 3OH-B[a]P, 4OH-B[a]P and 5OH-B[a]P is achieved via CZE using a 40% methanol CAPS buffer. This OH-PAH separation could not be completed using a buffer system like the one described in chapter 3 due to significant overlapping of peaks belonging to 1OH-Naph and 2OH-Naph. Electrophoretic parameters describing the migration of OH-PAH in the conditions of the separation were determined. Using the obtained values, it was possible to better understand the behavior of some PAH metabolites in the separation.

Upon close examination of Table 6.2 several observations can be made regarding specific cases of OH-PAH migration in the separation presented in section 6.3.1. 2OH-Flu and 2OH-Naph have very similar pKa and alpha values. Despite this fact, their peaks are very well resolved in the electropherograms. This is explained by the rather large difference between their $\mu_{OH-PAH}$ that causes separation. Keeping in mind equation 1.1, this variation in their $\mu_{OH-PAH}$ is a direct consequence of their difference in mass. Difference in migration times of 1OH-Naph and 2OH-Naph was surprisingly large even though their alpha values are similar and they have equivalent molecular weights. Considering that the mass of naphthols is relatively small compared with the rest of OH-PAH, the effect of a small change in ionization seems to cause a significant variation of their $\mu_{OH-PAH}$ facilitating their separation. Another case of metabolites with similar alpha values is the one of 4OH-B[a]P and 5OH-B[a]P. In the case of the OH-B[a]P isomers, given their larger mass, a small $\Delta \alpha$ does not cause such a significant difference in their $\mu_{OH-PAH}$, but it is enough as to have baseline resolution of the analyte peaks (see Figure 6.1).
CHAPTER 7. OVERALL CONCLUSION

The development of screening methods for the analysis of OH-PAH in urine and milk samples has been accomplished with the aid of CZE and SFS. Optimized procedures for the extraction and pre-concentration of metabolites have been developed to interface SPE, LLE and/or QuEChERS with CZE and/or SFS. A CZE method was first developed for the analysis of urine samples achieving baseline resolution of 2OH-Flu, 2OH-Naph, 9OH-Phen, 1OH-Pyr, 3OH-B[a]P, 4OH-B[a]P and 5OH-B[a]P in approximately 17 min. The separation buffer consisted of 20mM borate in 50% methanol-water (volume/volume). Competitive limits of detection were obtained for all the studied metabolites using commercial instrumentation equipped with an UV-VIS absorption detector. Detection at the sub-parts-per-billion concentration levels was made possible via sample pre-concentration based on SPE and sample stacking. SPE was performed with the aid of a twelve port vacuum manifold. Sample stacking was made in methanol, i.e. the eluting solvent from the SPE procedure. Metabolite recoveries varied from 93.2 ± 7.7% (5OH-B[a]P) to 108.7 ± 7.8% (2OH-Naph). LODs were at the trace level ranging from 0.99 ng.mL$^{-1}$ (3OH-B[a]P) to 8.54 ng.mL$^{-1}$ (2OH-Naph). The new method was found to be free of interference from four pharmacological drugs - naproxen, ibuprofen, diclofenac and amoxicillin – that might be found in urine samples of unhealthy individuals. The analysis of OH-PAH in milk samples was first attempted via SFS. Metabolites were extracted from milk samples with a two-step LLE procedure. Quantitative analysis was carried out in the sample extract without the need of previous chromatographic separation. Excellent recoveries and LODs at the parts-per-billion level were obtained for 2OH-Flu, 6OH-Chry, 1OH-
Pyr and 3OH-B[a]P. The small sample (400 μL) and extracting solvent (1.4 mL) volumes facilitates the simultaneous extraction of numerous samples via an environmentally friendly procedure, which is well-suited for routine monitoring of numerous samples. The non-destructive nature of the new method provides ample opportunity for metabolite confirmation via chromatographic techniques.

The main drawback of SFS is its inability to differentiate among positional isomers with almost identical excitation and fluorescence spectra. A CZE method was then developed for the complete resolution of 2OH-Flu, 1OH-Naph, 2OH-Naph, 3OH-Phen and 9OH-Phen in approximately 4 min of electrophoretic run. Metabolites extraction and pre-concentration was accomplished with an optimized QuEChERS procedure. LODs at the parts-per-billion level were obtained using a single solvent (acetonitrile) for metabolite extraction and sample stacking. The small sample volume (1.2 mL) and the conservative usage of chemicals provided a simple and rapid procedure for the simultaneous extraction of numerous samples. Adding 4 min of electrophoretic run per sample, it should be possible to screen ten samples in approximately one hour of analysis time. The nanoliter extract volume required for sample injection allows for further chromatographic usage and confirmation of positive samples.

Attempts to separate 1OH-Naph from 2OH-Naph in a synthetic mixture with 2OH-Flu, 9OH-Phen, 3OH-B[a]P, 4OH-B[a]P, 5OH-B[a]P and 1OH-Pyr using borate buffer, 50% MeOH, pH = 9.7 were unsuccessful due to the severe overlapping between the two naphthalene isomers. The complete resolution of the eight metabolites was then obtained using CAPS buffer in 40% MeOH. An investigation of their dissociation constants was then undertaken to better understand the electrophoretic behavior of the studied metabolites. This was the first step to future work in our lab, which will focus on the rational development of CZE methodology for
the analysis of positional isomers with very similar chromatographic behaviors and undistinguishable mass fragmentation patterns.
APPENDIX A: EXCITATION AND FLUORESCENCE SPECTRA OF THE STUDIED METABOLITES IN PERTINENT SOLVENTS
Figure A-1 Room temperature excitation and fluorescence spectra of 2OH-Flu 100 ppb in methanol

Figure A-2 Room temperature excitation and fluorescence spectra of 2OH-Flu 100 ppb in methanol/water 0.05% v/v
Figure A-3 Room temperature excitation and fluorescence spectra of 2OH-Naph 100 ppb in methanol

Figure A-4 Room temperature excitation and fluorescence spectra of 2OH-Naph 200 ppb in methanol/water 0.05% v/v
Figure A-5 Room temperature excitation and fluorescence spectra of 9OH-Phen 100 ppb in methanol

Figure A-6 Room temperature excitation and fluorescence spectra of 9OH-Phen 200 ppb in methanol/water 0.05% v/v
Figure A-7 Room temperature excitation and fluorescence spectra of 1OH-Pyr 50 ppb in methanol

Figure A-8 Room temperature excitation and fluorescence spectra of 1OH-Pyr 50 ppb in methanol/water 0.05% v/v
Figure A-9 Room temperature excitation and fluorescence spectra of 3OH-B[a]P 50 ppb in methanol

Figure A-10 Room temperature excitation and fluorescence spectra of 3OH-B[a]P 50 ppb in methanol/water 0.05% v/v
Figure A-11 Room temperature excitation and fluorescence spectra of 4OH-B[a]P 100 ppb in methanol

Figure A-12 Room temperature excitation and fluorescence spectra of 4OH-B[a]P 100 ppb in methanol/water 0.05% v/v
Figure A-13 Room temperature excitation and fluorescence spectra of 5OH-B[a]P 100 ppb in methanol

Figure A-14 Room temperature excitation and fluorescence spectra of 5OH-B[a]P 100 ppb in methanol/water 0.05% v/v
Figure A-15 Room temperature excitation and fluorescence spectra of 2OH-Flu 150 ppb in methanol/water 78/22% v/v

Figure A-16 Room temperature excitation and fluorescence spectra of 6OH-Chry 50 ppb in methanol/water 78/22% v/v
Figure A-17 Room temperature excitation and fluorescence spectra of 1OH-Pyr 50 ppb in methanol/water 78/22% v/v

Figure A-18 Room temperature excitation and fluorescence spectra of 3OH-B[a]P 50 ppb in methanol/water 78/22% v/v
APPENDIX B: ABSORPTION SPECTRA OF OH-PAH IN THE SEPARATION BUFFERS
Figure B-1 UV-vis absorption spectra of 2OH-Flu 3ppm in 20mM borate buffer, 50% v/v methanol, pH=9.7

Figure B-2 UV-vis absorption spectra of 2OH-Naph 6ppm in 20mM borate buffer, 50% v/v methanol, pH=9.7
Figure B-3 UV-vis absorption spectra of 9OH-Phen 3ppm in 20mM borate buffer, 50% v/v methanol, pH=9.7

Figure B-4 UV-vis absorption spectra of 1OH-Pyr 3ppm in 20mM borate buffer, 50% v/v methanol, pH=9.7
Figure B-5 UV-vis absorption spectra of 3OH-B[a]P 3ppm in 20mM borate buffer, 50% v/v methanol, pH=9.7

Figure B-6 UV-vis absorption spectra of 4OH-B[a]P 3ppm in 20mM borate buffer, 50% v/v methanol, pH=9.7
Figure B-7 UV-vis absorption spectra of 5OH-B[a]P 3ppm in 20mM borate buffer, 50% v/v methanol, pH=9.7

Figure B-8 UV-vis absorption spectra of 2OH-Flu 3ppm in 20mM borate buffer, 15% v/v methanol, pH=9.5
Figure B-9 UV-vis absorption spectra of 2OH-Naph 6ppm in 20mM borate buffer, 15% v/v methanol, pH=9.5

Figure B-10 UV-vis absorption spectra of 1OH-Naph 3ppm in 20mM borate buffer, 15% v/v methanol, pH=9.5
Figure B-11 UV-vis absorption spectra of 3OH-Phen 3ppm in 20mM borate buffer, 15% v/v methanol, pH=9.5

Figure B-12 UV-vis absorption spectra of 9OH-Phen 3ppm in 20mM borate buffer, 15% v/v methanol, pH=9.5
Figure B-13 UV-vis absorption spectra of 2OH-Flu 3ppm in 100mM CAPS buffer, 40% v/v methanol, pH=10.1

Figure B-14 UV-vis absorption spectra of 2OH-Naph 6ppm in 100mM CAPS buffer, 40% v/v methanol, pH=10.1
Figure B-14 UV-vis absorption spectra of 1OH-Naph 3ppm in 100mM CAPS buffer, 40% v/v methanol, pH=10.1

Figure B-15 UV-vis absorption spectra of 9OH-Phen 3ppm in 100mM CAPS buffer, 40% v/v methanol, pH=10.1
Figure B-16 UV-vis absorption spectra of 1OH-Pyr 3ppm in 100mM CAPS buffer, 40% v/v methanol, pH=10.1

Figure B-17 UV-vis absorption spectra of 3OH-B[a]P 3ppm in 100mM CAPS buffer, 40% v/v methanol, pH=10.1
Figure B-18 UV-vis absorption spectra of 4OH-B[a]P 3ppm in 100mM CAPS buffer, 40% v/v methanol, pH=10.1

Figure B-19 UV-vis absorption spectra of 5OH-B[a]P 3ppm in 100mM CAPS buffer, 40% v/v methanol, pH=10.1
APPENDIX C: PLOTS OF $1/\mu_{\text{OH-PAH}}$ VERSUS $[\text{H}^+]$ USED FOR DETERMINATION OF PKA VALUES
Figure C-1 Plot of $1/\mu_{\text{OH-PAH}}$ versus $[\text{H}^+]$ for 2OH-Naph in 100mM CAPS buffer, 40% v/v methanol, pH=10.1

Figure C-2 Plot of $1/\mu_{\text{OH-PAH}}$ versus $[\text{H}^+]$ for 1OH-Naph in 100mM CAPS buffer, 40% v/v methanol, pH=10.1
Figure C-3 Plot of $1/\mu_{\text{OH-PAH}}$ versus $[\text{H}^+]$ for 9OH-Phen in 100mM CAPS buffer, 40% v/v methanol, pH=10.1

Figure C-4 Plot of $1/\mu_{\text{OH-PAH}}$ versus $[\text{H}^+]$ for 1OH-Pyr in 100mM CAPS buffer, 40% v/v methanol, pH=10.1
Figure C-5 Plot of $1/\mu_{\text{OH-PAH}}$ versus $[\text{H}^+]$ for 3OH-B[a]P in 100mM CAPS buffer, 40% v/v methanol, pH=10.1

Figure C-6 Plot of $1/\mu_{\text{OH-PAH}}$ versus $[\text{H}^+]$ for 4OH-B[a]P in 100mM CAPS buffer, 40% v/v methanol, pH=10.1
Figure C-7 Plot of $1/\mu_{\text{OH-PAH}}$ versus $[\text{H}^+]$ for 5OH-B[a]P in 100mM CAPS buffer, 40% v/v methanol, pH=10.1
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